



Research paper

Optimization of substituted imidazobenzodiazepines as novel asthma treatments



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ABSTRACT

We describe the synthesis of analogs of XHE-III-74, a selective $\alpha 4\beta 3\gamma 2$ GABA_AR ligand, shown to relax airway smooth muscle *ex vivo* and reduce airway hyperresponsiveness in a murine asthma model. To improve properties of this compound as an asthma therapeutic, a series of analogs with a deuterated methoxy group in place of methoxy group at C-8 position was evaluated for isotope effects in preclinical assays; including microsomal stability, cytotoxicity, and sensorimotor impairment. The deuterated compounds were equally or more metabolically stable than the corresponding non-deuterated analogs and increased sensorimotor impairment was observed for some deuterated compounds. Thioesters were more cytotoxic in comparison to other carboxylic acid derivatives of this compound series. The most promising compound **16** identified from the *in vitro* screens also strongly inhibited smooth muscle constriction in *ex vivo* guinea pig tracheal rings. Smooth muscle relaxation, determined by reduction of airway hyperresponsiveness with a murine ovalbumin sensitized and challenged model, showed that **16** was efficacious at low methacholine concentrations. However, this effect was limited due to suboptimal pharmacokinetics of **16**. Based on these findings, further analogs of XHE-III-74 will be investigated to improve *in vivo* metabolic stability while retaining the efficacy at lung tissues involved in asthma pathology.

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1. Introduction

Asthma is a major healthcare challenge, effecting an estimated 300 million people globally [1]. Over \$56 billion in asthma-related healthcare expenses occur in the United States annually [2]. Moreover, asthma accounts for the majority of missed school/work days, doctor and emergency room visits, and patient hospitalizations in young persons [1–3]. Consequently, asthma continues to be a significant healthcare burden in terms of morbidity, productivity, and medical costs. Beta 2-adrenergic agonists and inhaled

corticosteroids (ICs) are the most often prescribed treatments for the acute and chronic management of asthma. Both agents present efficacy, compliance, and adverse side effect concerns [4–8]. Hence, there is an unmet need for asthma therapies with novel mechanisms of action to better control diseases with decreased adverse side effects.

The GABA_A receptor (GABA_AR) is a ligand-gated chloride ion channel best known for its role in central nervous system (CNS) inhibitory neurotransmission. GABA_ARs are heteropentameric receptors mainly comprised of combinations of 19 different subunits (α_{1-6} , β_{1-3} , γ_{1-3} , δ , ϵ , π , θ , ρ_{1-3}). Classical GABA_AR consist of two α , two β and one “tertiary” subunit (γ , δ , ϵ , θ , or π) [9,10]. The receptor subunits have been identified in airway smooth muscle, airway epithelium, and inflammatory cells, and their ligand-mediated activation has been shown to reduce immune response measures and reduce airway hyperresponsiveness (*ex vivo* and *in vivo*)

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[11–16]. In these studies, GABA dose-dependently reduced IL-12 and IL-6 production in LPS stimulated macrophages [15]. GABA and muscimol also inhibited anti-CD3 and antigen specific T cell proliferation [17]. Honokiol, a GABA_AR agonist, reduced cardinal features of the asthma-like phenotype including inflammation (reduced airway eosinophilia), mucous cell metaplasia, collagen deposition, and airway hyperresponsiveness in an acute and chronic ovalbumin-induced murine asthma models [18]. However, nonselective GABA_AR activation is associated with unwanted CNS effects [19] and increased mucous production [16,20,21]. To preclude these side effects in the treatment of asthma, subtype-selective GABA_AR ligands were identified, especially those exhibiting preferential efficacy at $\alpha 4/\alpha 5\beta 3\gamma 2$ GABA_AR [22–25]. In these studies, $\alpha 4\beta 3\gamma 2$ GABA_AR selective compounds CMD-45 and XHE-III-74 relaxed pre-contracted airway smooth muscle. In addition, XHE-III-74 reduced airway resistance in a murine house dust mite model of asthma. The ethyl ester of XHE-III-74 reduced airway hyperresponsiveness in a murine ovalbumin sensitized and challenged (S/C) asthma model, whereas XHE-III-74A, the corresponding carboxylic acid, significantly reduced airway eosinophilia [26] and reduced IL-2 production following PMA/PHA activation of Jurkat cells. Furthermore, XHE-III-74A exhibited no CNS effects because its negative charge precluded brain penetration.

Our previous studies showed that the C-8 methoxy group of XHE-II-74 (herein indicated as C-7) is essential for $\alpha 4\beta 3\gamma 2$ GABA_AR selectivity [27]. To improve the metabolic stability of this group, we herein describe the synthesis of compounds bearing a deuterated form (–OCD₃) because the C–D bond is stronger than the C–H bond. We further report the synthesis and characterization of a series XHE-III-74 analogs additionally bearing different ester, thioester, and amide functionalities at the C-3 position to identify compounds with high metabolic stability, absence of CNS effects, low toxicity, and efficacy in a murine asthma model.

2. Results

In agreement with the earlier route of Fryer and Gu [27] [28] the synthesis of XHE-III-74 (**7**) started with the synthesis of 5-methoxyanthranilic acid **2** (Scheme 1) from 5-methoxy-2-nitrobenzoic acid **1** by hydrogenation.

The aniline **2** was converted into isotoc anhydride **3** with triphosgene. Anhydride **3** was heated with L-proline in DMSO to generate the corresponding benzodiazepine **4**. This compound was converted into the imidazobenzodiazepine, XHE-III-74 ethyl ester **5**. The introduction of the OCD₃ group was achieved using the

demethylation-alkylation sequence as depicted in Scheme 2.

As shown in Scheme 2, ester **5** was demethylated at C-8 using aluminum chloride and ethanethiol in methylene chloride at room temperature to furnish the corresponding phenol **6**. The phenol was re-alkylated with deuterated iodomethane (CD₃I) using Cs₂CO₃ in methylene chloride at room temperature in excellent yield producing the OCD₃ analog **5a**.

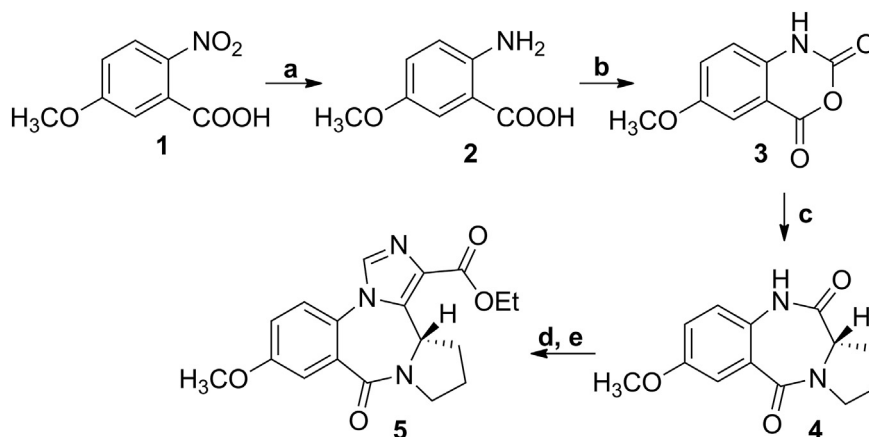
The esters **5**, **5a** and **6** were converted into the corresponding *tert*-butyl analogs **7** (XHE-III-74), **7a** and **8**, respectively, via *trans*-esterification with lithium in the presence of *tert*-butanol in THF at 50 °C (Scheme 3).

For generation of the other ester and amide analogs of XHE-III-74 (**7**), a three step protocol was used (Scheme 4).

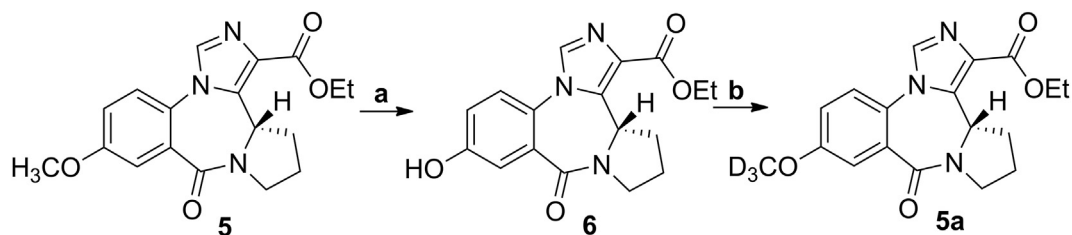
The ethyl esters **5** and **5a** were saponified to give the corresponding acids **9** and **9a**. The subsequent reactions with thionyl chloride in methylene chloride yielded the corresponding acid chlorides, which in turn were converted to esters, thioesters, and amides (**11**, **11a–19**, and **19a**). The methyl esters **10** and **10a** were formed in the presence of NaOMe in methanol.

Initially, human and mouse liver microsomal stability was investigated to identify metabolically labile compounds and deuterated compounds that are more stable than their non-deuterated counterparts. The results are summarized in Table 1.

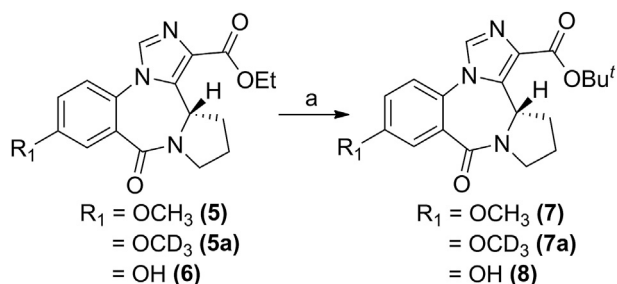
The parent compound XHE-III-74 (**7**) was metabolized rapidly by mouse liver microsomes with 16.2% remaining after 1 h (Table 1, Entry 4). The corresponding half-life was less than 24 min. However, **7** was stable in the presence of human liver microsomes, similar to the majority of compounds investigated. Less than 80% of the parent was observed after 1 h for compounds **9**, **11**, **11a**, **12a**, and **19** (Table 1, Entries 7, 11, 12, 14 and 27). For **9** and **19**, stability of the deuterated analog (**9a** and **19a**) is significantly increased (Table 1, Entries 7, 8, 27, and 28). A smaller number of compounds were stable in the presence of mouse liver microsomes for 1 h. The most stable compounds (as judged by less than 20% loss at 1 h) were **5**, **5a**, **6**, **9a**, **10a**, **16**, **16a**, **17a**, **19**, and **19a** (Table 1, Entries 1, 2, 3, 8, 10, 21, 22, 24, 27, and 28). All compounds that exhibited good stability in mouse microsomes were also stable in the presence of human liver microsomes. Importantly, different metabolic rates for deuterated and non-deuterated compounds in the presence of mouse liver microsomes were observed for **9**, **10**, **11**, and **17** and their corresponding deuterated analogs (Table 1, Entries 7–12, 23, 24). Further characterization of these compounds included the determination of their cytotoxicity using three different cell lines; HEK293 kidney cells, HepG2 liver cells, and BEAS2B lung epithelial cells (Table 2).



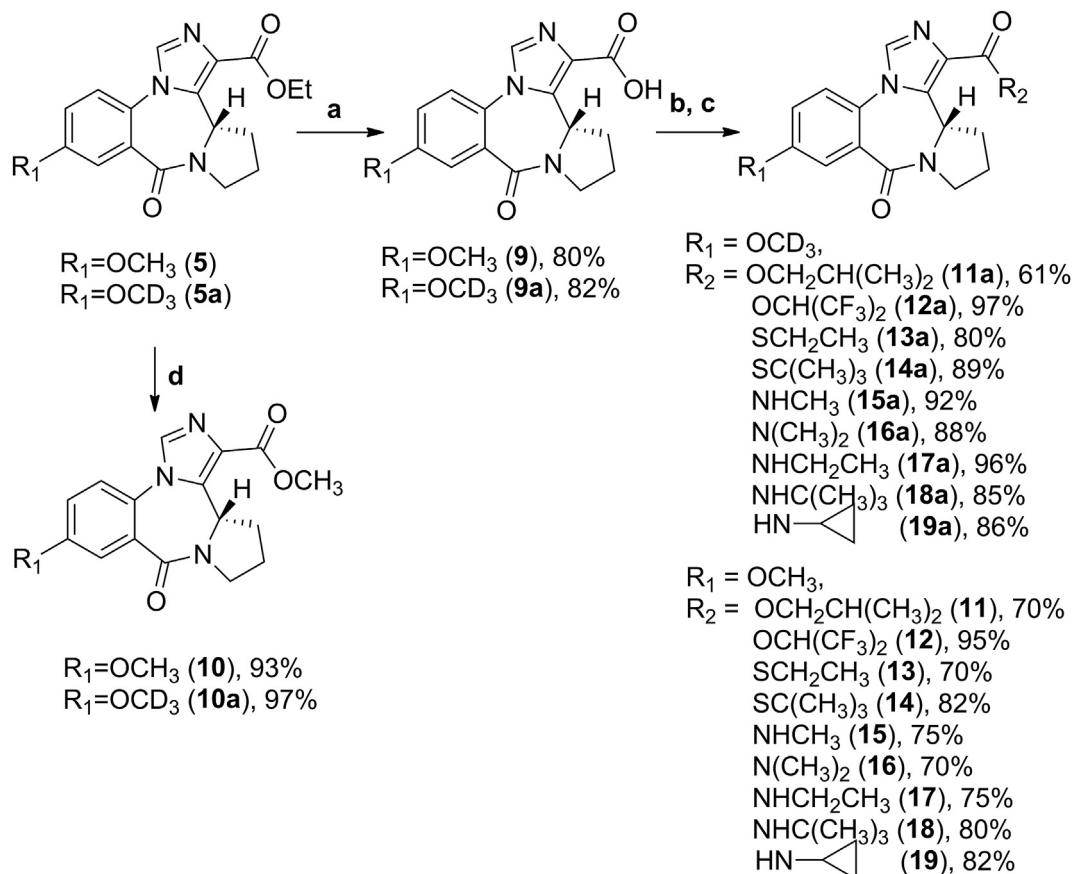
Scheme 1. Synthesis of XHE-III-74 EE. a. H₂, Pd/C, EtOAc, rt, 8 h, 97%; b. triphosgene, HCl/H₂O, rt, 4 h, 89%; c. L-proline, DMSO, 160 °C, 2 h, 96%; d. *t*-BuOK, (EtO)₂POCl, THF, –20 °C to rt, 4 h; e. *t*-BuOK, CNCH₂CO₂Et, –20 °C to rt, 8 h, 60%.



Scheme 2. a. AlCl_3 , ethanethiol, CH_2Cl_2 , rt, 24 h, 84%; b. Cs_2CO_3 , CD_3I , CH_2Cl_2 , rt, 18 h, 86%.



Scheme 3. a. Li rod pieces, *t*-BuOH, THF, 50 °C, 30 min, 65–67%.



Scheme 4. a. NaOH, H_2O , EtOH, 50 °C, 15 min; 1N HCl; b. CH_2Cl_2 , SOCl_2 , 52 °C, 1 h; c. CH_2Cl_2 , corresponding amine, thiol, or alcohol, Et_3N ; d. CH_3OH , NaOMe, reflux, 1 h.

Most of the compounds exhibited no major toxicity at the concentrations tested. The compounds with the most pronounced cytotoxicity were thioesters **13**, **13a**, **14**, and **14a** (Table 2, Entries 15–18). Among the compounds identified as stable in human and

mouse liver microsomes, only **19** and **19a** showed some toxicity in HEK293 cells (Table 2, Entries 27 and 28).

Next, possible adverse CNS sensorimotor effects were evaluated using a rotarod apparatus (Fig. 1).

Swiss Webster mice were injected i.p. with 40 mg/kg of the indicated compound. The sensorimotor test was carried out after 10, 30 and 60 min. All compounds, including the diazepam positive control, showed the greatest impairment of sensorimotor steadiness at 10 min, followed by 30 and 60 min. The compounds that caused the most severe motor impairment were **10a**, **11a**, **15** and **18a**. In addition, some of the compounds **11**, **12**, **12a**, **13**, **13a**, **14** and

14a were not soluble in the vehicle (50% PBS, 40% propylene glycol, 10% DMSO) and could not be tested. Similar rotarod data were published previously for **5** and **9**, showing that **5** weakly induced sensorimotor impairment whereas **9** did not, likely due to the

Table 1
In vitro liver microsomal stability of XHE-III-74 analogs.

Entry	Compound	Microsomal stability (human) % remaining after 1 h ^a	Microsomal stability (mouse) % remaining after 1 h ^a
1	5	99.1 ± 0.1	85.4 ± 0.3
2	5a	92.8 ± 0.3	85.5 ± 0.3
3	6	91.7 ± 0.2	91.1 ± 0.1
4	7	92.1 ± 1.0	16.2 ± 0.2
5	7a	91.9 ± 0.4	17.6 ± 0.2
6	8	90.6 ± 0.2	46.6 ± 0.3
7	9	56.1 ± 0.5	52.9 ± 0.5
8	9a	84.6 ± 0.3	84.6 ± 0.2
9	10	89.7 ± 0.2	70.3 ± 0.2
10	10a	92.9 ± 0.2	85.9 ± 0.2
11	11	77.8 ± 0.2	13.4 ± 0.9
12	11a	78.7 ± 0.2	22.4 ± 0.3
13	12	- _b	- _b
14	12a	77.1 ± 0.2	62.5 ± 0.3
15	13	- _b	- _b
16	13a	- _b	- _b
17	14	- _b	- _b
18	14a	- _b	- _b
19	15	93.8 ± 0.3	54.3 ± 0.2
20	15a	94.9 ± 0.2	56.8 ± 0.3
21	16	92.4 ± 0.2	82.5 ± 0.3
22	16a	96.3 ± 0.3	83.1 ± 0.2
23	17	90.7 ± 0.3	50.1 ± 0.2
24	17a	93.5 ± 0.2	94.8 ± 0.2
25	18	92.8 ± 0.5	4.2 ± 0.2
26	18a	95.6 ± 0.2	9.4 ± 0.4
27	19	79.9 ± 0.2	91.3 ± 0.2
28	19a	88.1 ± 0.2	94.1 ± 0.2

^a Half-life, intrinsic clearance and metabolic rates calculated for each experiment are shown in [Supporting Information](#).^b Compound was not soluble at 10 μM in PBS with 1% DMSO. Data were acquired by two independent experiments carried out in triplet.**Table 2**
In vitro cytotoxicity of XHE-III-74 analogs.

Entry	Compound	Toxicity in HEK293 (Kidney) LD ₅₀ (μM) ^a	Toxicity in HEPG2 (Liver) LD ₅₀ (μM) ^a	Toxicity in BEAS 2B (Lung) LD ₅₀ (μM) ^a
1	5	>400	>400	>400
2	5a	>400	>400	>400
3	6	>400	>400	>400
4	7	>100	>400	>200
5	7a	>100	>400	>200
6	8	>400	>400	>400
7	9	>400	>400	>400
8	9a	>400	>400	>400
9	10	>400	>400	>400
10	10a	>400	>400	>400
11	11	>100	>200	>200
12	11a	>100	>200	>200
13	12	>200	>400	>400
14	12a	>200	>400	>400
15	13	>100	>100	>100
16	13a	>100	>100	>100
17	14	18.8 ± 2.4	>100	>100
18	14a	16.8 ± 2.0	>100	>100
19	15	>100	>400	>400
20	15a	>100	>400	>400
21	16	>400	>400	>400
22	16a	>400	>400	>400
23	17	>400	>400	>400
24	17a	>400	>400	>400
25	18	>200	>400	>400
26	18a	>200	>400	>400
27	19	>200	>400	>400
28	19a	>200	>400	>400

^a Compounds were incubated at different concentrations with specified cells for 48 h, followed by detection of viability using Cell-Titer Glo (Promega). The results were normalized using DMSO (negative) and 3-dibutylamino-1-(4-hexyl-phenyl)-propan-1-one (150 μM in DMSO final concentration, positive). Data were acquired by three independent experiments carried out in quadruplet.

inability of the acid to penetrate the blood brain barrier [26]. Compound **7** (XHE-III-74) induced sensorimotor impairment in rats

at 15 mg/kg (unpublished results). Taken together, compounds in this series that are stable in the presence of human and mouse liver

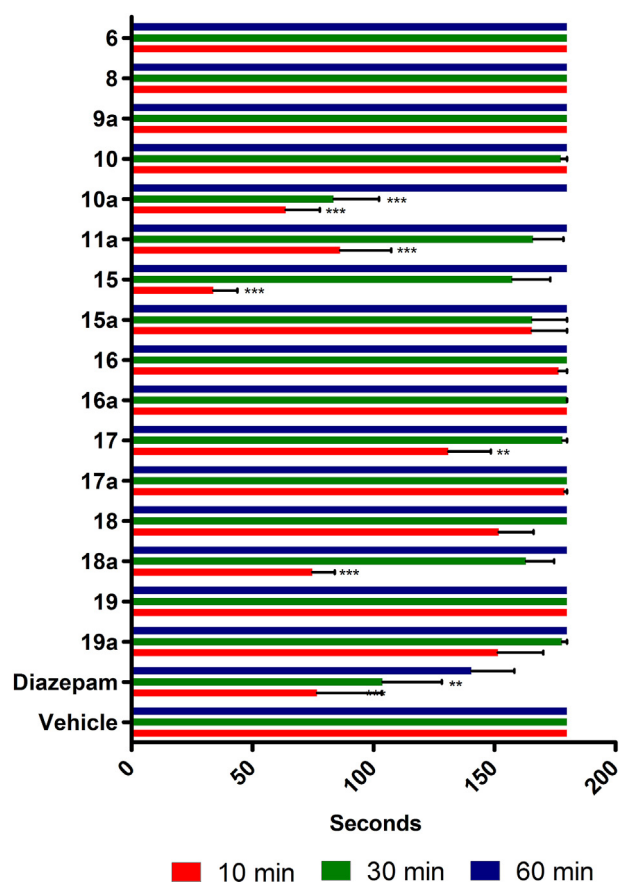


Fig. 1. Effect of compounds on sensorimotor coordination. Swiss Webster mice were tested on a rotarod at 15 rpm for 3 min at 10, 30, and 60 min following compound exposure. Mice ($N = 10$) received a single i.p. injection of test compound (40 mg/kg), diazepam (5 mg/kg), or vehicle (50% PBS, 40% propylene glycol, 10% DMSO). The time of fall was recorded if it occurred prior to 3 min. Data are expressed as mean \pm SEM ($N = 10$). ** ($p < 0.01$) or *** ($p < 0.001$) significance compared to vehicle-treated mice. Compounds 11, 12, 12a, 13, 13a, 14, 14a did not dissolve in the vehicle.

microsomes and exhibited neither cytotoxicity nor sensorimotor impairment are **6**, **9a**, **16**, **16a**, and **17a**.

At this point **16/16a** showed the most promising performance of the nondeuterated/deuterated pairs of compounds. We confirmed $\alpha 4\beta 3\gamma 2$ GABA_AR subtype selectivity of **16** by comparing its GABA induced current potentiation with the $\alpha 1\beta 3\gamma 2$ GABA_AR (see supporting information). At a concentration of 1 μ M **16**, 161% potentiation was observed for the $\alpha 1\beta 3\gamma 2$ GABA_AR in contrast to 319% potentiation for the $\alpha 4\beta 3\gamma 2$ GABA_AR in the presence of a GABA EC₂₀ concentration. To investigate if **16**, like other analogs of XHE-III-74, exhibits the ability to relax airway smooth muscle, we carried out an *ex vivo* assay employing tracheal rings pre-contracted with substance P (Fig. 2).

Results show that **16** reduced the contractile force of substance P in guinea pig tracheal rings over a period of 1 h. The highest significance was observed after 30 min with a $p < 0.001$. Thus, **16** is able to relax airway smooth muscle consistent with other analogs of XHE-III-74 [25,26].

We then investigated if **16** could reduce airway hyper-responsiveness in a mouse asthma model. To establish asthma-like disease, male BALB/c mice were sensitized with three i.p. injections of ovalbumin (2 mg/kg/d emulsified in 2 mg of alum on days 0, 7 and 14, in a total volume of 100 μ L), followed by intra-nasal challenge (1.6 mg/kg/d ovalbumin for 5 days on days 23–27). Control mice were sensitized with ovalbumin but challenged with saline

[26]. Separate groups of ovalbumin (S/C) BALB/c mice received a single 40 mg/kg i.p. dose of **16** 40 min before the measurement or twice daily 40 mg/kg of **16** i.p. for a duration of five days during the ovalbumin challenge period (Fig. 3A and B).

For animals treated with a single dose of **16**, no statistical significant specific airway resistance (sRAW) differences in comparison to the vehicle-treated group were observed at any of the methacholine concentrations tested. Importantly, significant reduction in sRAW ($p < 0.05$) was observed at the 12.5 mg/mL methacholine challenge for animals given **16** over a 5 day treatment course. In light of the observed partial *in vivo* efficacy of **16** in reducing airway hyperresponsiveness, we were prompted to carry out a pharmacokinetic study to investigate its stability *in vivo* (Fig. 4).

Following an i.p. dose of 5 mg/kg, **16** was quantified in blood, brain, and lung at different time points (Fig. 4, A). Compound **16** reached maximum absorption within minutes ($t_{max} = 3$ min) but also cleared very rapidly, with a half-life of 8.8 min. The overall exposure of **16** ($AUC = 6205$ ng*min/ml) was not very high. Markedly less distribution of **16** was observed in brain and lung. Compound **16** did not penetrate the blood brain barrier but reached only a very low C_{max} of 123 ng/g. The distribution in the lung was even less pronounced with an AUC of 764 ng*min/ml in comparison to $AUC = 6205$ ng*min/ml in blood. We investigated the presence of two likely metabolites of **16**, namely **15** (formed by *N*-demethylation) and **9** (formed by saponification). Compound **15** was observed in substantial amounts in blood after only 3 min after injection (Fig. 4, B). The peak blood concentration was higher than the parent compound and occurred later, as expected for a metabolite. Overall, the distribution of **15** was superior to **16** with an AUC of 64675 ng*min/ml. Compound **9** however, which was shown previously to reduce airway hyperresponsiveness, was not detected in any blood samples.

3. Discussion

Esters and amides are common functional groups in many FDA-approved drugs, although a number of such compounds are pro-drugs that rely on endogenous enzymes such as peptidases to be activated. XHE-III-74 (**7**), has a *tert*-butyl ester function shown to be labile in the presence of mouse liver microsomes, while relatively stable in human liver microsomes. Nevertheless, XHE-III-74 reduces airway hyperresponsiveness when given by aerosol delivery in mice [25]. Thus, the stability of XHE-III-74 is sufficient in mice when administered directly to the target organ. Other compounds with limited half-lives in the presence of mouse liver microsomes are **18** and **18a** bearing a *tert*-butyl amide, **8** bearing a *tert*-butyl ester, and **11**, **11a** bearing a isobutyl ester. Thus, we observed that esters and amides with longer and more branched carbon chains are metabolically less stable than their short carbon chain analogs. Another aspect of this SAR is the comparison of deuterated and non-deuterated compounds. Overall, we found a trend that deuterated compounds are equally or more stable than their corresponding non-deuterated analogs. This effect was very pronounced for acids **9** and **9a**, probably because a hydrolysable carboxylic ester or amide group inherent to other analogs in this series is missing. Thus, for **9a** and **9**, oxidation of the methoxy group (deuterated or non-deuterated) to the corresponding hemiacetal followed by hydrolysis might be the rate-determining step of metabolism. This specific route of metabolism can be species dependent. For instance, **17** and **17a** have similar stability in human liver microsomes, however in the presence of mouse liver microsomes non-deuterated compound **17** is notably less stable. Therefore, we confirmed that small molecule metabolism is highly species dependent and involves many different metabolic reactions

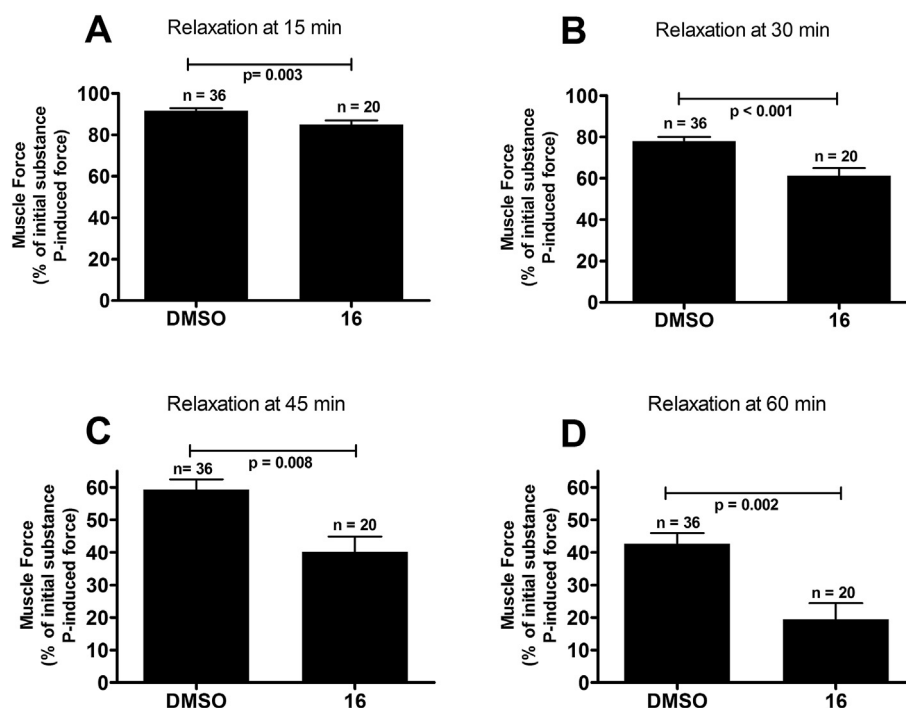


Fig. 2. Airway smooth muscle contractile force in guinea pig tracheal rings. Tracheal rings were contracted with 1 μ M substance P and then treated with 50 μ M of **16** (or the vehicle control 0.1% DMSO). The percent of remaining contractile force was measured at various time points and expressed as a percent of the initial substance P induced contractile force. N and p-values are given for each condition.

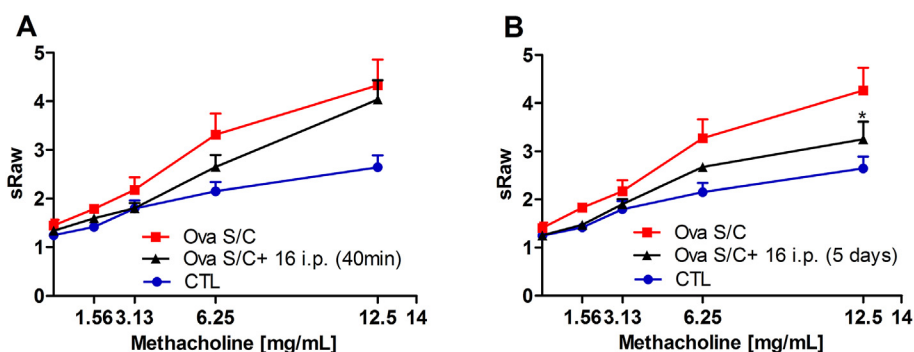


Fig. 3. Effect of **16** on airway hyperresponsiveness: Specific airway resistance (sRAW) to increasing doses of methacholine measured by DSI's Buxco® FinePointe non-invasive airway mechanics instrument. (A) Balb/c mice were administered 40 mg/kg of **16** single i.p. injection 40 min prior to analysis; (B) administration of **16** at 40 mg/kg i.p. injections daily for 5 days. Data represent mean \pm SEM from 4 to 7 mice in each group. * indicates p < 0.05 significance compared to vehicle-treated mice.

that are structure dependent.

The cytotoxicity of XHE-III-74 analogs was not very pronounced. The analysis showed that 50% of all compounds have LD₅₀ values greater than 400 μ M for the sensitive HEK293 kidney cells. For the BEAS2B lung cells, 71% of the compounds were not toxic. The compounds with the highest toxicity were thioesters **14** and **14a**. Interestingly, the deuterium effect was less pronounced for cytotoxicity, although small differences were observed (Supporting Information). Sensorimotor impairment is another important screen for unwanted side effects in a possible asthma drug. Among the different GABA_AR in the CNS, the α 1 β 3 γ 2 GABA_AR subtype is known to mediate sedation, which in turn compromises sensorimotor skills [29]. Thus, compounds with high efficacy toward this receptor subtype are expected to induce sedation *in vivo*, as seen for diazepam [30]. However, due to the unique scaffold of XHE-III-74 and its analogs, low efficacy towards α 1 β 3 γ 2 GABA_AR and high efficacy towards α 4 β 3 γ 2 GABA_AR was shown, e.g. XHE-III-74 (**7**)

[25], XHE-III-74EE (**5**) [30], and XHE-III-74A (**9**) [26]. Only a few of the compounds described herein induced sensorimotor effects comparable to diazepam, however at concentrations that were eight times greater. At 40 mg/kg deuterated compounds in comparison to non-deuterated compounds are more likely to impair sensorimotor abilities. This effect correlated with their better metabolic stability. For instance, 30% of **10** was metabolically converted after 1 h, whereas only 15% of deuterated **10a** was converted during the same time. Accordingly, **10** did not induce any sensorimotor impairment in contrast to **10a**. Thus, increasing the half-life of a drug candidate using deuterium amplified not only the therapeutic effect but also potentiated possible side effects. The only compounds investigated herein that were stable in mouse and human liver microsomes and did not induce sensorimotor impairment were compounds **6**, **9a**, **16**, **16a**, and **17a**.

Compound **16**, like other compounds in this series e.g. **5**, **7**, and **9** [26], significantly reduced airway muscle constriction caused by

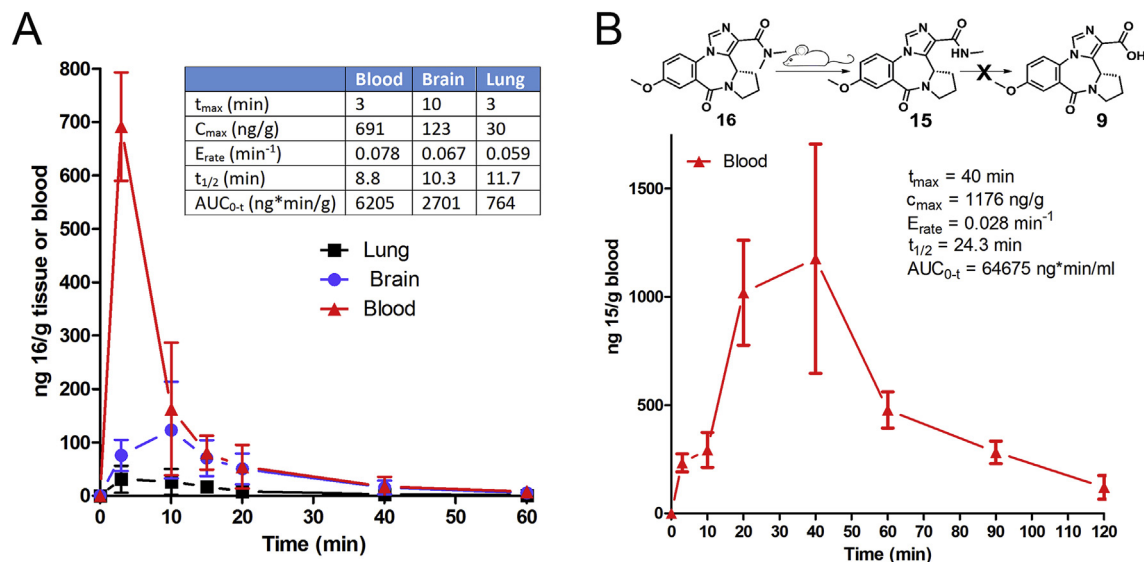


Fig. 4. Pharmacokinetic analysis. A) Concentration changes of compound **16** in mouse brain, lung, and blood over time when given as a 5 mg/kg, i.p. injection. B) Quantification of metabolite **15** in mouse blood at indicated time points.

substance P. Airway smooth muscle relaxation is an important hallmark of an efficacious asthma treatment and compound **16** mediated this effect within 15 min. *In vivo*, using ovalbumin S/C mice, **16** reduced airway hyperresponsiveness at high methacholine challenge when given chronically. Similar effects were reported for **5** when given repeatedly over five days.²⁶ Both compounds (**5** and **16**) are metabolically stable *in vitro* and non-toxic, however, their effect was less pronounced *in vivo* probably due to sub-pharmacological concentrations. Ethyl ester **5** was shown to be absorbed and cleared slower with a $t_{1/2}$ of 16.3 min instead of 8.8 min in comparison to dimethyl amide **16**. Using the same dose, the AUC for lung exposure decreased from 4516 ng*min/ml for **5** to 764 ng*min/ml for **16**. Finally, it was shown that **5** metabolized to **9** probably by mouse esterases and compound **9** decreased airway eosinophilia [26]. In contrast, compound **16** metabolized to **15** (demethylation) but no hydrolysis yielding **9** was detected. Thus, the incremental weaker pharmacodynamic effects of **16** is in part mediated by its short half-life and the inability to form the anti-inflammatory compound **9**.

It can be concluded that the unique scaffold of GABA_AR subtype-selective XHE-III-74 analogs is responsible for negligible inhibition of sensorimotor skills in mice in contrast to the non-selective GABA_AR modulator diazepam. Similarities of compounds derived from both scaffolds include the absence of cytotoxicity. We confirmed that deuterated compounds were equally or more resistant to metabolism when compared with their non-deuterated counterparts. However, metabolic stability in the presence of liver microsomes *in vitro* did not predict rapid clearance of lead compound **16** *in vivo*. Therefore, significant relaxation of airway smooth muscle induced by **16** *ex vivo* was only partially recapitulated *in vivo* by measuring airway hyperresponsiveness. To overcome the shortcomings of **16**, carboxylic ester and amide bioisosteres will be investigated in the future to identify new compounds with improved pharmacokinetics and better activity in murine models of asthma.

4. Experimental section

4.1. Chemistry

4.1.1. 5-Methoxyanthranilic acid (**2**)

A solution of 2-nitro-5-methoxybenzoic acid **1** (60.3 g,

306 mmol) in EtOAc (1.5 L) was degassed under reduced pressure and refilled with argon (repeated 3 times). Palladium (10% w/w on carbon, 3.9 g, 1.2 mol%) was added to the above solution. The reaction flask was evacuated under reduced pressure and refilled with H₂ from a balloon (repeated 3–4 times to make sure that the solution was saturated with H₂). The reaction mixture was stirred at rt for 6 h. After the completion of the reaction (TLC, silica gel), the solution was filtered over a bed of celite to remove the Pd. The solids were washed with ethyl acetate. The solvent was removed under reduced pressure to yield acid **2** as a yellow solid in 97% (49.7 g) yield: M.p = 147–149 °C; ¹H NMR (300 MHz, CDCl₃) δ 3.66 (s, 3H), 6.71 (d, 1H, *J* = 9.0 Hz), 6.94 (dd, 1H, *J* = 9.0 Hz, 3.0 Hz), 7.19 (d, 1H, *J* = 3.0 Hz), 8.40 (bs, 2H). The spectral data matched the reported values [27]. This material was employed directly in the next step.

4.1.2. 5-Methoxyisotoic anhydride (**3**)

The 5-methoxyanthranilic acid **2** (30 g, 179 mmol) was dissolved in a mixture of H₂O (1.2 L) and conc. HCl (15 mL), and this was followed by the addition of triphosgene (63.8 g, 215 mmol). The contents were stirred at rt for 3–4 h until the completion of the reaction (TLC, silica gel). A white solid precipitated from the solution after completion. The solids were collected by filtration and washed with H₂O (4 L). The solids were dried under vacuum to give pure anhydride **3** in 89% (30.7 g) yield: M.p = 237–239 °C; ¹H NMR (300 MHz, CDCl₃) δ 3.65 (s, 3H), 7.11 (d, 1H, *J* = 8.9 Hz), 7.35 (dd, 1H, *J* = 8.9 Hz, 2.7 Hz), 7.19 (d, 1H, *J* = 2.7 Hz), 11.61 (bs, 1H). The spectral data were identical with the reported values [27]. This material was employed directly in the next step. **Triphosgene is toxic; care must be exercised.**

4.1.3. (S)-2,3-Dihydro-7-methoxy-1H-pyrrolo[2,1-c][1,4]benzodiazepine-5,11(10H,11aH)-dione (**4**)

A mixture of 5-methoxyisotoic anhydride **3** (30.7 g, 158.9 mmol) and L-proline (20.2 g, 174.8 mmol) in dry DMSO (300 mL) was heated with stirring at 160 °C for 2 h. The white turbid reaction mixture which resulted, became a clear brown solution as the temperature was increased above 80 °C. After the completion of the reaction on examination by TLC (silica gel), the solution was cooled to rt. The mixture was poured into 250 mL of ice water to yield benzodiazepine **4** as a white solid. The solids were collected by

vacuum filtration and washed with ice cold water (2 × 50 mL). The filtrate was extracted with ethyl acetate and the solvent was removed under reduced pressure to yield solid diazepine **4**. The combined solids were dried in a vacuum oven at 80 °C for 4 h. The yield of **4** was 96% (37.9 g): M.p = 214–216 °C; $[\alpha]_D^{25} = +444.40$ (c 1%, in CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 1.82–2.01 (m, 3H), 2.50–2.51 (m, 1H), 3.40–3.49 (m, 1H), 3.56–3.63 (m, 1H), 3.78 (s, 3H), 4.07–4.10 (m, 1H), 7.07 (d, 1H, *J* = 8.7 Hz), 7.13 (dd, 1H, *J* = 8.7 Hz, 3.0 Hz), 7.26 (d, 1H, *J* = 3.0 Hz), 10.30 (bs, 1H). The spectral data were identical to the reported values [27]. This material was employed directly in the next step.

4.1.4. (*S*)-Ethyl-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate (**5**)

A well dried reaction flask was evacuated completely and flushed with argon. The flask was then charged with diazepine **4** (37 g, 150.2 mmol) in dry THF (800 mL). The turbid solution was cooled to –20 °C. Potassium *tert*-butoxide (33.7 g, 300.4 mmol) was added to the flask and the solution was stirred at rt for 1 h. Diethyl chlorophosphate (43.4 mL, 300.4 mmol) was then slowly added to the reaction mixture at –20 °C and it was allowed to stir at rt over a period of 2–3 h. The cloudy reaction mixture became a clear golden brown solution. After complete consumption of the starting material **4** (TLC), the reaction was cooled to –20 °C, after which ethyl isocyanoacetate (32.8 mL, 300.4 mmol) and potassium *tert*-butoxide (33.7 g, 300.4 mmol) were added. The reaction mixture was stirred at rt for 8 h. The reaction was quenched with a saturated aq solution of NaHCO₃ (80 mL). The THF was removed under reduced pressure and the aq layer was extracted with CH₂Cl₂ (300 mL × 3). The combined organic layer was separated and washed with brine (400 mL) and dried (Na₂SO₄). The CH₂Cl₂ was removed under reduced pressure and the dark brown pasty liquid residue, which resulted, was washed with ether to yield the crude ethyl ester **5**. This crude solid was recrystallized from CH₂Cl₂ to yield ethyl ester **5** as a pure white solid in 60% (30.85 g) yield: M.p = 195–197 °C; (Lit. report: 175–176 °C) [27]; $[\alpha]_D^{25} = +18.00$ (c 0.5%, in CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 1.44 (t, 3H, *J* = 7.2 Hz), 2.15–2.31 (m, 3H), 3.49–3.61 (m, 2H), 3.75–3.85 (m, 1H), 3.91 (s, 3H), 4.41 (q, 2H, *J* = 7.2 Hz), 4.75 (d, 1H, *J* = 6.9 Hz), 7.15 (dd, 1H, *J* = 8.9 Hz, 3.0 Hz), 7.32 (d, 1H, *J* = 8.9 Hz), 7.59 (d, 1H, *J* = 3.0 Hz), 7.82 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 14.3, 24.3, 28.4, 46.6, 53.4, 55.8, 61.2, 114.5, 119.7, 124.6, 126.0, 127.3, 130.6, 135.8, 137.6, 159.4, 162.6, 163.7; HRMS (ESI) (M + Na)⁺, calcd. for C₁₈H₁₉N₃O₄Na 364.1273; Found 364.1279. The spectral data were identical to the reported values [27]. This material was employed directly in the next step.

4.1.5. (*S*)-Ethyl-7-hydroxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate (**6**)

In an oven dried round bottom flask, dry CH₂Cl₂ (50 mL) was added and cooled to 0 °C. Then AlCl₃ (3 g, 22.8 mmol) and ethanethiol (4.5 mL, 60.8 mmol) were added to the above flask slowly at 0 °C. The ice bath was removed and the reaction was allowed to warm up to rt. After the AlCl₃ dissolved completely, ester **5** (2.6 g, 7.62 mmol) was added to the mixture at rt and it was stirred for 24 h under Ar. The reaction time will vary with the scale of the reaction. After completion of the reaction (TLC, silica gel), the solution was poured onto ice and was acidified using an aq 2N HCl solution. The solution was extracted 7 times with CH₂Cl₂ and 4 times with EtOAc separately. Since the product was soluble in water, the extraction procedure was carried out until there was no more product observed in the aqueous layer (TLC, silica gel). The combined organic layer was washed with brine and dried (Na₂SO₄). The solvent was removed under reduced pressure and the residue was purified by flash column chromatography on [silica gel, 4%

MeOH in CH₂Cl₂] to furnish the phenolic ethyl ester **6** as a solid (2.1 g) in 84% yield: M.p = >260 °C (decomp.); ¹H NMR (300 MHz, CDCl₃) δ 1.44 (t, 3H, *J* = 7.1 Hz), 2.19–2.42 (m, 3H), 3.55–3.64 (m, 2H), 3.81–3.89 (m, 1H), 4.42 (q, 2H, *J* = 7.1 Hz), 4.82 (d, 1H, *J* = 7.3 Hz), 7.13 (dd, 1H, *J* = 8.7 Hz, 2.6 Hz), 7.27–7.31 (m, 1H), 7.85 (s, 1H), 7.91 (d, 1H, *J* = 2.6 Hz), 9.22 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 14.3, 24.4, 28.4, 46.9, 53.8, 61.2, 117.5, 120.8, 124.9, 125.2, 127.7, 129.5, 136.0, 137.2, 157.6, 162.8, 164.6; HRMS (ESI) (M + H)⁺, calcd. for C₁₇H₁₈N₃O₄ 328.1292; Found 328.1293.

4.1.6. (*S*)-Ethyl-7-(²H₃)-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo-[1,2-a][1,4]diazepine-1-carboxylate (**5a**)

To a solution of phenol **6** (1.5 g, 4.6 mmol) in CH₂Cl₂ (30 mL), Cs₂CO₃ (3 g, 9.2 mmol) was added and the mixture stirred at rt for 30 min. Then CD₃I (2.3 mL, 36.8 mmol) was added slowly and the reaction mixture was stirred at rt for 24 h. After completion of the reaction the mixture was quenched with cold water and extracted with CH₂Cl₂. The combined organic layer was washed with brine and dried (Na₂SO₄). The solvent was removed under reduced pressure and the residue was purified by flash column chromatography [silica gel, 2% CH₃OH in CH₂Cl₂] to furnish ester **5a** as a solid (1.36 g) in 86% yield: M.p = 195–196 °C; $[\alpha]_D^{25} = +20.00$ (c 0.5%, in CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 1.46 (t, 3H, *J* = 7.1 Hz), 2.17–2.36 (m, 3H), 3.54–3.63 (m, 2H), 3.77–3.84 (m, 1H), 4.43 (q, 2H, *J* = 7.1 Hz), 4.77 (d, 1H, *J* = 7.3 Hz), 7.16 (dd, 1H, *J* = 8.8 Hz, 2.8 Hz), 7.33 (d, 1H, *J* = 8.8 Hz), 7.60 (d, 1H, *J* = 2.8 Hz), 7.82 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 14.3, 24.3, 28.3, 46.5, 53.4, 61.1, 114.4, 119.7, 124.5, 126.0, 127.6, 130.5, 135.8, 137.6, 159.3, 162.8, 163.7; HRMS (ESI) (M + H)⁺, calcd. for C₁₈H₁₇H₃N₃O₄ 345.1637; Found 345.1635. The ¹³C-D signal was not observed due to long relaxation time, line broadening, reduced NOE effect and spin-spin coupling.

4.1.7. (*S*)-*tert*-Butyl-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate (**7**)

A flame dried round bottom flask was charged with dry THF (30 mL) and lithium rod (excess, cut into small pieces) was added. Dry *tert*-butanol (2.6 mL, 27.1 mmol) was added to the above flask at rt and the mixture which resulted was heated to 45–50 °C under Ar until the *tert*-butanol reacted completely. This freshly prepared lithium *tert*-butoxide solution was transferred carefully with a cannula to another flame dried round bottom flask charged with ester **5** (1.0 g, 2.71 mmol) and stirred at 50 °C under Ar for 30 min. After the completion of the reaction (TLC, silica gel), the flask was cooled to rt and the THF removed under reduced pressure. Ice water (10 mL) was added to the residue and it was then extracted with EtOAc. The organic layer was washed with water (2 × 10 mL) and brine (15 mL). The solvent was removed under reduced pressure and the residue was purified by flash column chromatography [silica gel, EtOAc/hexane (7:3)] to yield *tert*-butyl ester (XHE-III-74) **7** as a solid (0.72 g) in 67% yield: M.p = 115–117 °C (119–121 °C) [27]; $[\alpha]_D^{25} = +36.00$ (c 0.5%, in CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 1.63 (s, 9H), 2.14–2.28 (m, 3H), 3.47–3.61 (m, 2H), 3.74–3.81 (m, 1H), 3.90 (s, 3H), 4.73 (d, 1H, *J* = 6.9 Hz), 7.14 (dd, 1H, *J* = 8.8 Hz, 3.0 Hz), 7.30 (d, 1H, *J* = 8.8 Hz), 7.57 (d, 1H, *J* = 3.0 Hz), 7.83 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 24.3, 28.2, 28.3, 46.7, 53.4, 55.9, 81.9, 114.5, 119.7, 124.6, 126.1, 128.9, 130.5, 135.6, 136.5, 159.4, 162.2, 163.8; HRMS (ESI) (M + Na)⁺, calcd. for C₂₀H₂₃N₃O₄Na 392.1586; Found 392.1574. The spectral data were identical to the reported values [27].

4.1.8. (*S*)-7-Methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylic acid (**9**)

The ester **5** (2.12 g, 6.21 mmol) was dissolved in a mixture of

EtOH (4 mL) and H₂O (3 mL) after which solid NaOH (1.2 g, 31.0 mmol) was added to the solution. This reaction mixture was heated to 50 °C for 15 min and the EtOH was removed under reduced pressure. The remaining aq solution was stirred at 0 °C for 10 min and then conc HCl was added dropwise to the solution until the pH was 3–4 (pH paper). A pale yellow precipitate which formed was left in the solution and the mixture was allowed to stir at rt for 2 h. The precipitate was collected by filtration, washed with cold water (2 × 5 mL) and the aq layer also was allowed to stand at rt for 10 h to obtain additional solid **9**. The combined solids were dried in a vacuum oven at 80 °C for 7 h to get pure acid **9** in 80% yield: M.p = 210–211 °C; [α]_D²⁵ = +8.00 (c 0.25%, in CH₃OH); ¹H NMR (300 MHz, DMSO) δ 2.03–2.16 (m, 3H), 3.50–3.63 (m, 3H), 3.87 (s, 3H), 4.84 (d, 1H, *J* = 7.5 Hz), 7.31 (dd, 1H, *J* = 8.9 Hz, 3.0 Hz), 7.41 (d, 1H, *J* = 3.0 Hz), 7.63 (d, 1H, *J* = 8.9 Hz), 8.21 (s, 1H); ¹³C NMR (75 MHz, DMSO) δ 24.9, 28.7, 47.3, 53.6, 56.8, 115.3, 119.9, 126.7, 127.0, 128.6, 131.0, 137.4, 137.5, 159.5, 164.0, 165.3; HRMS (ESI) (M + H)⁺, calcd. for C₁₆H₁₆N₃O₄ 314.1141; Found 314.1141. The spectral data were identical to the reported values [27]. This material was employed directly in the next step.

4.1.9. (*S*)-Methyl-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate (**10**)

To an oven dried two neck round bottom flask, ester **5** (0.30 g, 0.87 mmol) was added in dry methanol (10 mL) and then NaOMe (0.2 g, 3.48 mmol) was added to the solution. The mixture was heated to reflux until the reaction was complete (~1 h, confirmed by TLC, silica gel). Then the reaction mixture was quenched with an ice cold aq NaHCO₃ solution. The solvent was removed under reduced pressure and the residue was dissolved in water and extracted with EtOAc (2 × 20 mL). The combined organic layer was then washed with brine and dried (Na₂SO₄). The solvent was removed under reduced pressure and the residue was purified by flash column chromatography [silica gel, EtOAc/hexane (1:1)] to yield pure methyl ester **10** as a solid in (0.286 g) 93% yield: M.p = 180–182 °C; [α]_D²⁵ = +8.00 (c 0.25%, in CH₂Cl₂); ¹H NMR (300 MHz, CDCl₃) δ 2.16–2.35 (m, 3H), 3.52–3.61 (m, 2H), 3.75–3.81 (m, 1H), 3.91 (s, 3H), 3.94 (s, 3H), 4.75 (d, 1H, *J* = 6.9 Hz), 7.16 (dd, 1H, *J* = 8.7 Hz, 2.7 Hz), 7.34 (d, 1H, *J* = 8.7 Hz), 7.59 (d, 1H, *J* = 2.7 Hz), 7.88 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 24.4, 28.4, 46.6, 52.2, 53.4, 55.9, 114.5, 119.8, 124.7, 125.9, 126.9, 130.6, 135.9, 137.9, 159.5, 162.9, 163.7. HRMS (ESI) (M + Na)⁺, calcd. for C₁₇H₁₇N₃O₄Na 350.1117; Found 350.1125.

4.1.10. General method for the synthesis of esters and amides (**11**, **11a–19**, and **19a**)

A mixture of acid (**9** or **9a**, 0.32 mmol) individually, thionyl chloride (5.12 mmol) and dry CH₂Cl₂ (8 mL) was added to an oven dried round bottomed flask under argon. This suspension was allowed to reflux at 52 °C (the outside oil bath temperature was at 60 °C) for 1 h under an atmosphere of argon. The solution became a clear yellow color. The absence of the starting material was confirmed by the examination by TLC (silica gel). The organic solvent and excess thionyl chloride were removed under reduced pressure. This evaporation was repeated a couple of times with dry CH₂Cl₂ (5 mL) to remove excess thionyl chloride and any HCl. The yellow residue, which was obtained, was dissolved in dry CH₂Cl₂ (10 mL) and cooled to 0 °C for 10 min under argon. The appropriate nucleophile (alcohol/thiol/amine, 3.2 mmol), followed by Et₃N (1.6 mmol) was added to the reaction mixture at 0 °C and the mixture was then allowed to warm to rt and stirred for 2–7 h. After the completion of the reaction (TLC, silica gel), the solvent was removed under reduced pressure. The residue was quenched with ice cold water (5 mL) and extracted with CH₂Cl₂ (8 mL × 2). The

combined organic layer was washed with brine (5 mL). The solvent was removed under reduced pressure and the residue was purified by flash column chromatography (silica gel) to yield the corresponding pure esters, thioesters and amides whose properties are depicted below and as well as in the SI. Note: We observed in a control experiment that the mixture of dichloromethane plus thionyl chloride boils at 52 °C.

4.1.11. (*S*)-Isobutyl-7-methoxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate (**11**)

The isobutyl ester **11** was prepared from acid **9** following the general procedure with dry isobutanol as the nucleophile. The crude residue was purified by flash column chromatography [silica gel, EtOAc/hexane (7:3)] to yield pure isobutyl ester **11** as a solid in 70% yield: M.p = 126–127 °C (104–106 °C) [27]; ¹H NMR (300 MHz, CDCl₃) δ 1.04 (d, 6H, *J* = 6.7 Hz), 2.13–2.37 (m, 4H), 3.49–3.63 (m, 2H), 3.76–3.84 (m, 1H), 3.92 (s, 3H), 4.14 (d, 2H, *J* = 6.9 Hz), 4.76 (d, 1H, *J* = 6.9 Hz), 7.17 (dd, 1H, *J* = 8.8 Hz, 2.9 Hz), 7.33 (d, 1H, *J* = 8.8 Hz), 7.60 (d, 1H, *J* = 2.9 Hz), 7.82 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 19.3, 24.4, 27.8, 28.4, 46.6, 53.5, 55.9, 71.2, 114.5, 119.8, 124.6, 126.2, 127.8, 130.6, 135.9, 137.6, 159.4, 163.1, 163.8. HRMS (ESI) (M + Na)⁺, calcd. for C₂₀H₂₃N₃O₄Na 392.1586; Found 392.1594. The spectral data were identical to the reported values [27].

4.2. Biology

4.2.1. Experimental animals

5–10 week old male BALB/c and Swiss Webster mice (Charles River Laboratory, WIL, MA) and adult (425–450 g) male Hartley guinea pigs (Charles River Laboratory, WIL, MA) were used for the experiments. The animals were housed under specific pathogen-free conditions, under standard conditions of humidity, temperature and a controlled 12 h light and dark cycle and had free access to food and water. All animal experiments were in compliance with the University of Wisconsin, Milwaukee or Columbia University Institutional Animal Care and Use Committees (IACUC).

Microsomal stability assay and cytotoxicity assay. See [Supporting Information](#).

4.2.2. Rotarod assay

Swiss Webster mice were trained to maintain balance at a constant speed of 15 rpm on the rotarod apparatus (Omnitech Electronics Inc. Nova Scotia, Canada) until mice could perform for 3 min at three consecutive time points. Separate groups of mice received intraperitoneal (i.p.) injections of vehicle (10% DMSO, 40% propylene glycol and 50% PBS) or test compounds. Diazepam was used as a positive control compound (5 mg/kg) in an approximate volume of 100 μ l. Ten minutes after each injection, mice were placed on the rotarod for 3 min. A fail was assigned for each mouse that fell from the rotarod prior to 3 min. Mice were rested two to three days before administration of another dose or a different compound.

4.2.3. Guinea pig airway smooth muscle organ bath

Guinea pigs were anesthetized with intraperitoneal pentobarbital (100 mg/kg). Their tracheas were then surgically removed and transected into cross-sectional sections containing two cartilaginous rings as described previously.²⁶ The epithelium was removed with cotton swabs and the rings were suspended by two silk threads in 4 mL jacketed organ baths (Radnoti Glass Technology). One thread was attached to a Grass FT03 force transducer (Grass-Telefactor) coupled *via* Biopac hardware to a computer with Acknowledge 7.3.3 software (Biopac Systems) for continuous digital recording of muscle tension. The rings were bathed in 4 mL of KH

buffer solution (composition in mM: 118 NaCl, 5.6 KCl, 0.5 CaCl₂, 0.2 MgSO₄, 25 NaHCO₃, 1.3 NaH₂PO₄, 5.6 D-glucose) with 10 μM indomethacin (DMSO vehicle final concentration of 0.01%), which was continuously bubbled with 95% O₂ and 5% CO₂ at pH 7.4, 37 °C. The rings were equilibrated at 1 g of isotonic tension for 1 h with new KH buffer added every 15 min. All rings were precontracted with 10 μM *N*-vanillylnonanamide (capsaicin analog) and then two cycles of cumulatively increasing concentrations of acetylcholine (0.1–100 μM) with extensive buffer washes between and after those two cycles with resetting of the resting tension to 1.0 g. To eliminate the confounding effects of airway nerves and histamine receptors, tetrodotoxin (1 μM) and pyrilamine (10 μM) were added to the buffer. After a stable baseline tension of 1.0 g was established, tracheal rings were contracted with 1 μM substance P. After the peak contraction was reached, 50 μM of compound **16** (or the vehicle control 0.1% DMSO) was added to each bath. The percentage of initial contraction remaining at 15, 30, 45, and 60 min after compound exposure was compared between groups.

4.2.4. Assessment of airway hyper-responsiveness

Airway hyper-responsiveness to methacholine in conscious, spontaneously breathing animals was measured by DSI's Buxco® FinePointe Non-Invasive Airway Mechanics (NAM) instrument [26]. Before measurements were taken, mice were acclimated to the chamber for 15 min daily for 5 days. The chambers were also calibrated each time before data collection. Briefly, the nasal chamber in combination with the thoracic chamber permits computation of Specific Airway Resistance (sRaw). The FinePointe software computes sRaw with all other ventilatory parameters derived by the NAM analyzer. Mice were exposed to aerosolized PBS (for the baseline measurement) or methacholine (1.56–12.5 mg/mL) for 1 min and readings were taken and averaged for 3 min after each nebulization. Data obtained were presented as sRaw versus aerosolized methacholine concentration (mg/ml). Data analysis was carried out using 2way ANOVA repeated measures with Bonferroni post-test. Overall p-value was 0.0937 and $F = 1.763$. The Bonferroni post-test gave significance for 12.5 mg methacholine as indicated in Fig. 2.

Pharmacokinetic study: See Supporting Information.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2016.11.045>.

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