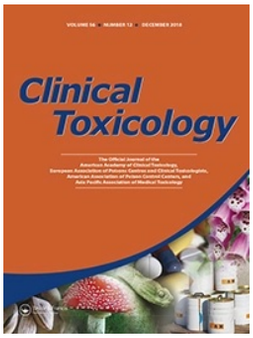

Clinical Toxicology

ISSN: 1556-3650 (Print) 1556-9519 (Online) Journal homepage: https://www.tandfonline.com/loi/ictx20

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To cite this article: Julia Herbert, Horst Thiermann, Franz Worek & Timo Wille (2019): COPD and asthma therapeutics for supportive treatment in organophosphate poisoning, Clinical Toxicology, DOI: 10.1080/15563650.2018.1540785

To link to this article: https://doi.org/10.1080/15563650.2018.1540785

Published online: 30 Jan 2019.

CLINICAL TOXICOLOGY
https://doi.org/10.1080/15563650.2018.1540785

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BASIC RESEARCH

COPD and asthma therapeutics for supportive treatment in organophosphate poisoning

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ABSTRACT

Context: Nerve agents like sarin or VX have repeatedly been used in military conflicts or homicidal attacks, as seen in Syria or Malaysia 2017. Together with pesticides, nerve agents assort as organophosphorus compounds (OP), which inhibit the enzyme acetylcholinesterase. To counteract subsequent fatal symptoms due to acetylcholine (ACh) accumulation, oximes plus atropine are administered, a regimen that lacks efficacy in several cases of OP poisoning. New therapeutics are in development, but still need evaluation before clinical employment. Supportive treatment with already approved drugs presents an alternative, whereby compounds from COPD and asthma therapy are likely options. A recent pilot study by Chowdhury et al. included β2-agonist salbutamol in the treatment of OP-pesticide poisoned patients, yielding ambiguous results concerning the addition. Here, we provide experimental data for further investigations regarding the value of these drugs in OP poisoning.

Methods: By video-microscopy, changes in airway area were analyzed in VX-poisoned rat precision cut lung slices (PCLS) after ACh-induced airway contraction and subsequent application of selected anticholinergics/β2-agonists.

Results: Glycopyrrolate and ipratropium efficiently antagonized an ACh-induced airway contraction in VX-poisoned PCLS (EC_{50} glycopyrrolate 15.8 nmol/L, EC_{50} ipratropium 2.3 nmol/L). β2-agonists formoterol and salbutamol had only negligible effects when solely applied in the same setting. However, combination of formoterol or salbutamol with low dosed glycopyrrolate or atropine led to an additive effect compared to the sole application [50.6 ± 8.8% airway area increase after 10 nmol/L formoterol +1 nmol/L atropine versus 11.7 ± 9.2% (10 nmol/L formoterol) or 8.6 ± 5.9% (1 nmol/L atropine)].

Discussion: We showed antagonizing effects of anticholinergics and β2-agonists on ACh-induced airway contractions in VX-poisoned PCLS, thus providing experimental data to support a prospective comprehensive clinical study.

Conclusions: Our results indicate that COPD and asthma therapeutics could be a valuable addition to the treatment of OP poisoning.

ARTICLE HISTORY
Received 16 August 2018
Revised 18 October 2018
Accepted 21 October 2018
Published online 28 January 2019

KEYWORDS
Organophosphate poisoning; nerve agents; PCLS; COPD; asthma

Introduction

Lately, repetitive use of organophosphorus compounds (OP), which include nerve agents such as sarin or VX were reported, either as part of assassination attempts or as weapons in military conflicts (e.g. Syria 2013 and 2017 [1–3]). In addition, self-poisoning and accidental exposure with OP-pesticides account for 100,000 – 200,000 deaths per year worldwide [4]. Clearly, there is a need for readily obtainable and efficient countermeasures in the field and in emergency departments. In the body, OP lead to inhibition of the enzyme acetylcholinesterase (AChE, [5]), which physiologically cleaves the neurotransmitter acetylcholine (ACh, [6]). Due to AChE inhibition, cholinergic overstimulation induces distinct symptoms [7], whereby the impairment of respiratory functions, such as airway contraction, hypersecretion, disturbance of respiratory drive in the central nervous system and paralysis of respiratory muscles prove to be the most challenging and may ultimately lead to death [8,9]. As standard therapy, an oxime (to reactivate inhibited AChE) combined with atropine (to counteract muscarinic overstimulation) is administered [8, 10]. There are different scenarios of OP-poisoning however, in which this therapeutic approach is not efficient enough. Oxime effectiveness in particular depends on factors like affinity for the OP-AChE-binding complex, chemical characteristics of the OP or aging reactions [11,12]. In soman poisoning for example, an aging reaction within minutes leads to an irreversible bond between AChE and the nerve agent, thus preventing oxime-induced reactivation [13,14]. Also, reduced efficacy of oxime therapy has been described in cases of tabun and cyclosarin poisoning [15,16] and the value of oxime therapy is still under debate in case of intentional OP pesticide poisoning [16]. Additional challenges lie in the sufficient availability of medication at sites of OP dissemination as well as the need for rapid and specific antidote administration in all cases of OP poisoning [10,17]. In the same context, atropine-connected adverse effects have to be considered, because determination of the appropriate dosing regimen can be difficult [18].

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Ongoing research is directed to improve oxime therapy or to identify new compounds to address the flaws of the current treatment. Although there are novel candidates in development (e.g. allosteric modulators of muscarinic ACh-receptors [19], bioscavengers [20,21], bispyridinium-non-oximes [22,23]), an employment of these compounds in cases of OP poisoning is not yet in prospect. An alternative approach is to test the efficacy of already approved therapeutics as adjunct element to the existing treatment regimen. β2-agonists and anticholinergics from COPD and asthma therapy are interesting candidates, especially because respiratory symptoms in these lung diseases compare to those occurring in OP poisoning [24,25]. In a recent pilot study featuring a small cohort of OP pesticide poisoned patients in a Bangladeshi hospital, Chowdhury et al. added nebulized β2-agonist salbutamol to the standard therapy for evaluation of possible beneficial effects. The acquired data is limited and therefore, a more comprehensive study was suggested by the authors [26]. In nerve agent poisoning, improvement of therapy also is necessary, especially in mass casualty scenarios. However, human *in vivo* experiments are not possible due to ethical reasons, therefore only experimental procedures can be applied to address specific questions. In our study, employing our established test system utilizing rat precision cut lung slices (PCLS, [27]), we analyzed whether anticholinergics and β2-agonists from COPD and asthma therapy have beneficial effects on airway contractions induced by VX poisoning and ACh-overstimulation.

Materials and methods

Chemicals

A stock solution of the nerve agent VX (O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate, provided by the German Ministry of Defence, 0.1% v/v in acetonitrile) was prepared and stored at room temperature. At the day of the experiment, a working solution (100 µmol/L in cell culture medium (Dulbecco's Modified Eagle Media/Nutrient mixture F-12 Ham, Sigma-Aldrich, Taufkirchen, Germany), final concentration 1 µmol/L) was prepared.

Also, stock solutions of acetylcholine chloride (ACh), glycopyrrolate, ipratropium bromide monohydrate and atropine sulfate (all Sigma-Aldrich) were prepared in cell culture medium (0.01 mol/L or 0.1 mol/L for ACh) and stored at −80 °C. Prior to experiments, working solutions were prepared in cell culture medium, to yield final concentrations of 0.5 µmol/L (ACh), 1, 2.5, 7.5, 10, 100 and 1000 nmol/L (glycopyrrolate and ipratropium) or 1, 5, 50, 500 or 5000 nmol/L (atropine). The β2-agonists formoterol and salbutamol, free base were diluted in dimethylsulfoxide (DMSO; all Sigma-Aldrich) to a stock concentration of 0.1 mol/L and stored at −80 °C. Working solutions were prepared in cell culture medium to yield final concentrations of 10, 100 and 1000 nmol/L for both substances. In control group experiments, cell culture medium containing only DMSO was applied (max. solvent concentration in all experiments 0.01%).

Animals

For experimental procedures, male Wistar rats weighing approximately 250-300 g were bought from Charles River, Sulzfeld, Germany. The animals were kept under standard housing conditions (ambient temperature of 21 °C, 12 h light/dark cycle, air-condition, food and water *ad libitum*). The acclimatization period prior to experiments was at least seven days. All experiments conducted were in agreement with the German Animal Welfare Act of 18th May 2006 (BGBl. I S. 1206, 1313) and the European Parliament and Council Directive of 22nd September 2010 (2010/63/EU).

Preparation of PCLS

PCLS were prepared as described previously [27]. In short, anaesthetized rats (75 mg/kg ketamine, Ketavet 100 mg/mL, zoetis Deutschland GmbH, Berlin, Germany, mixed with 10 mg/kg xylazine, Xylasel 20 mg/mL, Selectavet Dr. Otto Fischer GmbH, Weyarn-Holzolling, Germany) were euthanized by exsanguination. Subsequently, the lungs were filled via the trachea with about 15 mL of a 1.5% and 37 °C warm agarose-solution (low melting point agarose, Sigma-Aldrich), carefully removed from the thoracic cavity and cooled at 4 °C to congeal the agarose. After 30 min, lung lobes were separated and tissue cores were prepared with an 8 mm biopsy punch (pfm medical ag, Cologne, Germany). With a microtome (VT-1000 S, Leica Biosystems, Nussloch, Germany), slices of 250 µm thickness were cut from the tissue cores and subsequently transferred to a 24-well plate containing 500 µl of cell culture medium (Dulbecco's Modified Eagle Media/Nutrient mixture F-12 Ham, Sigma-Aldrich, supplemented with 100 U penicillin and 100 µg/mL streptomycin) per well. Slices were incubated at 37 °C, 5% CO_2, under humid conditions. The cell culture medium was exchanged following the slicing process three times every 30 min, followed by two times every 60 min (5 times in total, over 3.5 h).

Substance evaluation

Experiments to evaluate the efficacy of the selected test-substances were conducted approximately 24 h after the slicing process via microscopic analysis, as described previously [27]. Briefly, in PCLS mounted with steel wires, visibly vital airways (displaying vigorously beating cilia and spontaneous tonus) cut in cross section were put into focus of an inverted, camera-connected microscope (Axio Observer D1, Zeiss, Jena, Germany). Substances were administered directly into the cell culture medium, whereby final compound concentrations were calculated accordingly (see above). As main parameter of analysis, substance-induced changes of the airway area were documented by image acquisition. For dose-effect-experiments with glycopyrrolate and ipratropium, four to five PCLS were treated in parallel first with 1 µmol/L VX, followed by 0.5 µmol/L ACh 5 min afterwards. 30 min after that, glycopyrrolate or ipratropium were applied (1, 2.5, 7.5, 10, 100 or 1000 nmol/L, respectively). The airway area was pictured prior to VX, 30 min after ACh and 15 min after application of glycopyrrolate or ipratropium.

For formoterol, salbutamol and combined substance-application experiments, firstly, VX 1 μmol/L was applied. 5 min afterwards, 0.5 μmol/L ACh was administered and set as starting point for the image-acquisition of the airway area, whereby one picture was taken every 60 s. 30 min after ACh application, formoterol, salbutamol or a combination of formoterol and atropine/glycopyrrolate, or salbutamol and atropine were administered in the respective concentrations. Experiments were terminated 25 min afterwards.

Data analysis

Airway area analysis from the acquired images was performed computer-based with the software StrataQuest Version 5.0.1.306 (TissueGnostics GmbH, Vienna, Austria). Substance-induced airway area changes were then calculated as percent of the initial area (set as 100%) and calculation of the % net effect of a treatment was performed for all respective substances or combinations. Here, airway area values from the different concentrations and time-points (TP) were related to the airway area values at TP 30 min after ACh-application using the following equation:

% net effect = [(Airway area Treatment TPx – Airway area ACh TP 30)/(100 – Airway area ACh TP 30)]*100. Statistical analyses (sigmoidal dose-response analysis, Mann-Whitney-*U*-Test) were performed with GraphPad Prism (Version 5.04, GraphPad Software, Inc., California, USA).

p-values $\leq$0.05 were regarded as statistically significant. Data are presented as mean ± standard error of the mean (SEM).

Results

Effect of VX-poisoning

As described in our previous publication [27], effects of VX-poisoning can be quantified by analysis of airway area changes after ACh-application. The mean initial airway area was 48737 ± 2775 μm^2 (mean ± SEM) and the mean airway area 30 min after ACh-application was 14450 ± 1540 μm^2. The mean decrease of the airway area was from 100% (initial airway area) down to 28.8 ± 2.1% 30 min after ACh-application. After this time-point, treatment was applied to antagonize the airway contraction. For better visualization of the drugs' effects, see Figure 1.

Ipratropium and glycopyrrolate efficiently antagonize ACh-induced airway contractions in VX-poisoned PCLS

In VX-poisoned PCLS, airway area changes were analyzed by video-microscopy after ACh-induced airway contraction and treatment with the respective test substances. The treatment was expected to antagonize the ACh-induced airway contraction. For the anticholinergics glycopyrrolate and ipratropium, dose response relationships were examined. For both compounds, concentrations $\geq$1 nmol/L were sufficient to result in an increase in airway area (net effect for 1 nmol/L: 0.8 ± 0.7% (mean ± SEM) for glycopyrrolate and 11.1 ± 7.3% for ipratropium, Figure 2(A)). The EC_{50} for glycopyrrolate was 15.8 nmol/L, the EC_{50} for ipratropium 2.3 nmol/L. The most effective concentration was 1000 nmol/L for glycopyrrolate (airway area increase net effect 77.1 ± 3.9%) and 100 nmol/L for ipratropium (net effect 80.1 ± 8.4%, Figure 2(A)). For comparison, we included raw atropine data from our previous study [27] and analyzed it according to the glycopyrrolate and ipratropium data. The experimental setting in the atropine-experiments was the same as described for glycopyrrolate/ipratropium. However, different concentrations were examined, which were 1, 5, 50, 500 and 5000 nmol/L for atropine. The EC_{50} for atropine was 1.3 nmol/L. Concentrations from 5 to 5000 nmol/L effectively antagonized the ACh-induced airway contractions in VX-poisoned PCLS, with a maximum airway area increase net effect of 82.5 ± 6.15% (Figure 2(B)).

β2-agonists formoterol and salbutamol have only negligible antagonizing effects on ACh-induced airway contractions in VX-poisoned PCLS

Potential effects of the β2-agonists formoterol and salbutamol on ACh-induced airway contractions were examined in VX poisoned PCLS. Interestingly, both substances only marginally antagonized the ACh-induced airway contraction. 25 min after application, mean net effects for formoterol were 11.7 ± 9.2%, 0.4 ± 0.6% and 1.6 ± 0.9% for 10, 100 and 1000 nmol/L, respectively (Figure 3(A)). For salbutamol, detected airway area increases were equally as low or even non-existent, shown by net effects of 1.4 ± 2.1%, −2 ± 0.4% and 2.1 ± 1.3% for 10, 100 and 1000 nmol/L, respectively (Figure 3(B)). In control groups, the solvent DMSO was

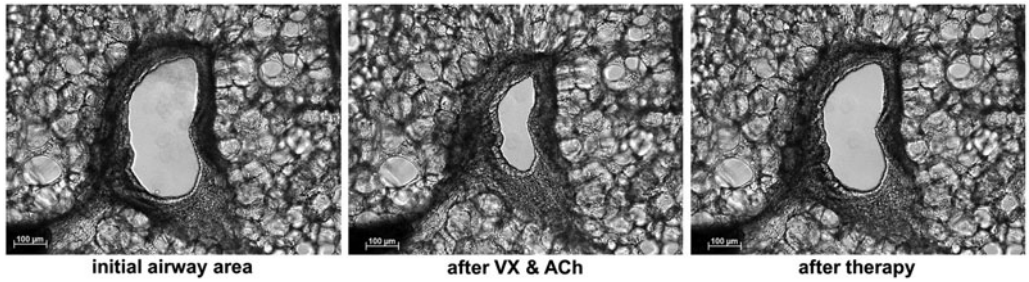

Figure 1. Exemplary picture-series of substance-effects in precision cut lung slices. By video-microscopic analysis, the area of cross-section cut airways was captured in precision cut lung slices before administration of any compound (= initial airway area), after VX poisoning (1 μmol/L) and acetylcholine (ACh, 0.5 μmol/L)-induced airway contraction and after application of different therapeutic drugs. Notably, the airway area increase after therapy was variable between the different therapeutics, as stated in the respective results section. The picture shown here was taken from a combination formoterol + atropine experiment for better visualization.

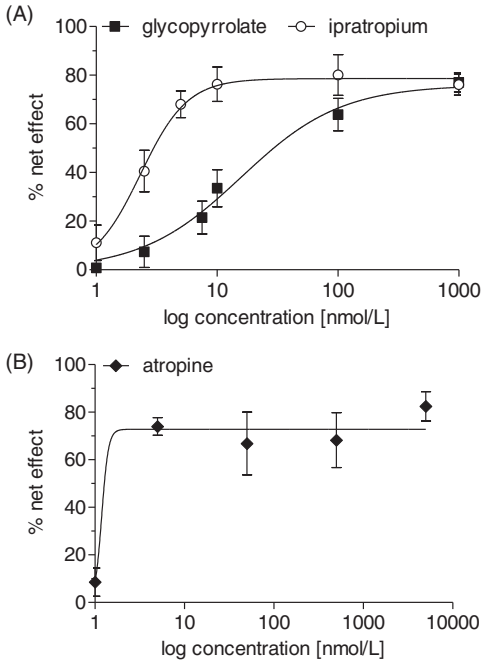

Figure 2. Effects of different glycopyrrolate, ipratropium and atropine concentrations on acetylcholine-induced airway contractions in VX-poisoned precision cut lung slices. By video-microscopic analysis, the area of cross-section cut airways was captured in precision cut lung slices after VX poisoning (1 µmol/L), acetylcholine (ACh, 0.5 µmol/L)-induced airway contraction and treatment with the respective concentrations of glycopyrrolate, ipratropium (1, 2.5, 7.5, 10, 100, 1000 nmol/L, (A)) or atropine (1, 5, 50, 500, 5000 nmol/L, (B)). Snapshots of airways were taken before VX application (= initial airway area), 30 min after ACh and 15 min after treatment. Airway area values were related to the initial area (set as 100%) and the % net effect after 15 min of treatment was calculated for each concentration. Data are given as sigmoidal dose response with variable slope (bottom =0), mean ± SEM, n = 6–13, from at least 3 different animals. EC_{50} = 1.3 nmol/L for atropine, EC_{50} = 2.3 nmol/L for ipratropium, EC_{50} = 15.8 nmol/L for glycopyrrolate.

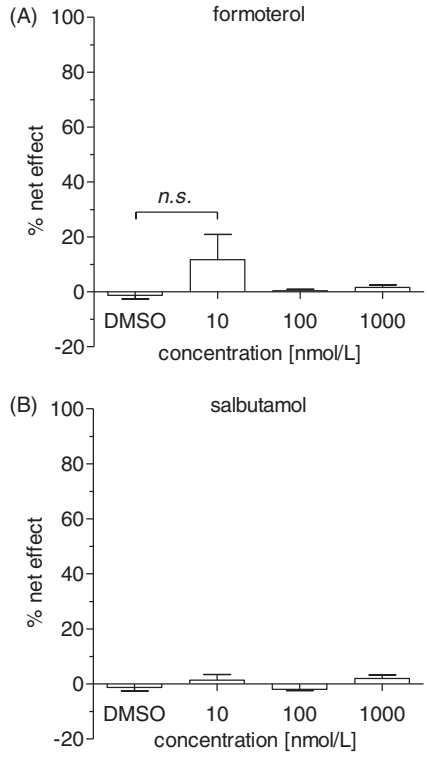

Figure 3. Effects of different formoterol and salbutamol concentrations on acetylcholine-induced airway contractions in VX poisoned precision cut lung slices. By video-microscopic analysis, the area of cross-section cut airways was captured in precision cut lung slices after VX poisoning (1 µmol/L), acetylcholine (ACh, 0.5 µmol/L)-induced airway contraction and treatment with the respective concentrations of formoterol (A), or salbutamol (B). In control groups, the solvent dimethylsulfoxide (DMSO) was applied in equal amount to the 1000 nmol/L concentration of formoterol and salbutamol (max. solvent concentration 0.01%). Snapshots of airways were taken before VX poisoning (= initial area) and every 60 s starting with the ACh-application. Treatment application started 30 min after ACh administration. Airway areas were related to the initial area (set as 100%) and the % net effect of the treatment was calculated for each concentration and time-point. The bar graphs show the % net effect (mean + SEM) of formoterol or salbutamol 25 min after treatment-application. n = 6–7, from at least 3 different animals. *n.s.* = not significant.

administered in amounts equal to the 1000 nmol/L concentration of formoterol or salbutamol (max. solvent concentration 0.01%). DMSO had no effect at all on airway responsiveness (net effect −1.3 ± 1.3%, Figure 3(A,B)).

Combinations of anticholinergics and β2-agonists efficiently antagonize ACh-induced airway contractions in VX poisoned PCLS

Combined applications of formoterol and atropine (F + A), formoterol and glycopyrrolate (F + G) and salbutamol and atropine (S + A) were examined. Notably, combined anticholinergics and β2-agonists proved to be very efficient in comparison to the sole administration of the respective substances and concentrations. 10 nmol/L formoterol applied in combination with 1 nmol/L atropine (F + A) induced a significant increase of the airway area in VX-poisoned PCLS (net effect 50.6 ± 8.8%, Figure 4(A)). The sole application of 10 nmol/L formoterol had only negligible effects (Figure 3(A)). 1 nmol/L atropine, also only marginally antagonized the airway contraction (airway area increase net effect 8.6 ± 5.9%, Figure 2(B)). Comparable to F + A, combination of F + G (airway area increase net effect of 27 ± 11.9%, Figure 4(B)) and S + A (net effect 29 ± 9.5%, Figure 4(C)) also efficiently antagonized the airway contraction.

Discussion

Optimization of a standard treatment by development of new therapeutics can be challenging, especially with regards to mandatory time-consuming and cost-intensive pharmacological testing. Therefore, investigation of already approved therapeutics can provide a valuable alternative. Several respiratory symptoms due to OP poisoning are comparable to those connected to COPD or acute asthma exacerbations. Hence, in this study, anticholinergics and β2-agonists usually employed in COPD or asthma therapy were evaluated with regards to counteracting an ACh-induced airway contraction in VX-poisoned rat PCLS. Different concentrations of the anticholinergics glycopyrrolate and ipratropium proved to be very effective in this setting. The β2-agonists formoterol and salbutamol however, had only negligible effects at all tested concentrations when applied solely. Notably, the combined administration of formoterol or salbutamol with low-dose anticholinergics efficiently antagonized the ACh-induced airway contraction in VX-poisoned PCLS, as compared to the sole application of the respective compounds.

With regards to a possible supportive use of COPD and asthma medications in treatment of OP poisoning, routes of

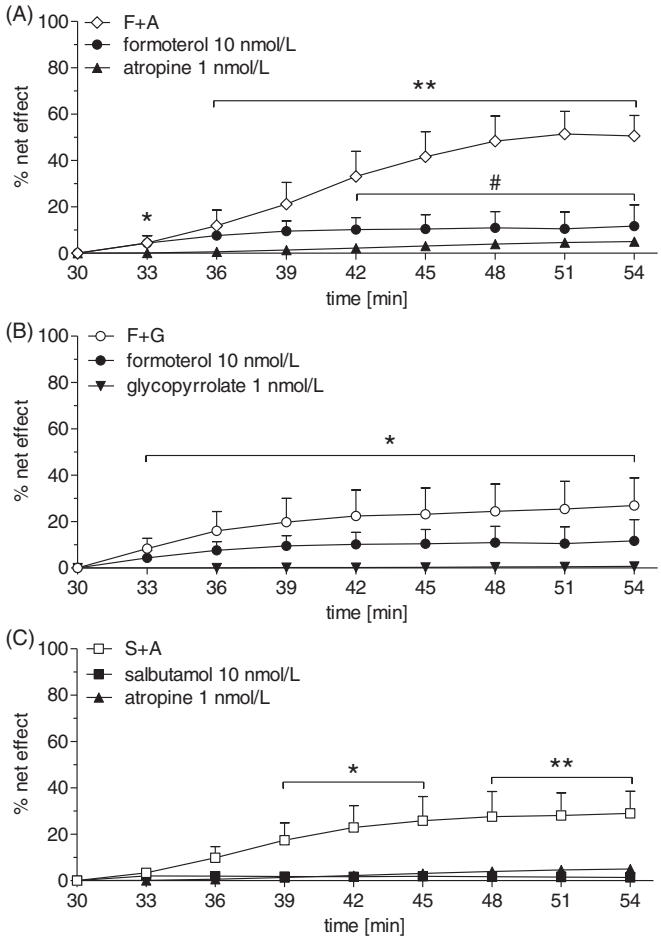

Figure 4. Effects of formoterol, salbutamol, glycopyrrolate, atropine and combinations on acetylcholine-induced airway contractions in VX poisoned precision cut lung slices. By video-microscopic analysis, the area of cross-section cut airways was captured in precision cut lung slices after VX poisoning (1 µmol/L), acetylcholine (ACh, 0.5 µmol/L)-induced airway contraction and treatment with (A) formoterol (10 nmol/L), atropine (1 nmol/L) or the combination of both (F + A), (B) formoterol, glycopyrrolate (1 nmol/L) or combination (F + G) and (C) salbutamol (10 nmol/L), atropine (1 nmol/L) or combination (S + A). Snapshots of airways were taken before VX poisoning (= initial area) and every 60 s beginning with the ACh application. Treatment application started 30 min after ACh administration. Airway areas were related to the initial area (set as 100%) and the % net effect of the treatment was calculated for each concentration and time-point. Data are given as mean + SEM, n = 5–7, from at least 3 different animals. * $p < .05$ for F + G versus glycopyrrolate, S + A versus salbutamol and F + A versus atropine, respectively; # $p < .05$ for F + A versus formoterol; ** $p < .01$ for F + A versus atropine and S + A versus salbutamol.

application and dosing need to be considered. In our study, drug effects were evaluated in PCLS. We opted for this *ex vivo* technique because here, concentrations of test substances can be kept on a level comparable to dosages applied to humans, whereas in *in vivo* experiments, disproportionate amounts can become necessary to achieve analogous effects. Hence, we tested in a nano-molar concentration range for anticholinergics (1–5000 nmol/L) and β2-agonists (10, 100, 1000 nmol/L). The chosen concentrations were based on published data about effects of formoterol, salbutamol, glycopyrrolate and ipratropium in human- or animal-derived PCLS [28–31] and our own work in the case of atropine [27]. We also regarded pharmacokinetic studies for each substance, to further ensure that the tested concentrations did not exceed the usual therapeutic doses applied to patients [32–38]. The main route of administration for COPD or asthma therapeutics is inhalation, which might not always be applicable in patients with OP poisoning. Therefore, intramuscular or intravenous substance application should be as effective, which has been described for glycopyrrolate and β2-agonists [39,40]. In our study, drugs were applied directly into the culture medium and most likely distributed in the lung tissue by diffusion. An inhalative application also ensures drug deposition in the target tissue, but naturally, this route still differs from the direct application conducted in our study. In this context, Bäckström et al. investigated the lung retention of formoterol and salbutamol in rat PCLS by LC-MS/MS analysis to determine the rate of drug uptake and release [41]. Their data was comparable to results from pharmacokinetic *in vivo* studies, which highlights that drug-associated effects observed in PCLS can be related to the *in vivo* situation.

In OP poisoning, airflow in the lungs is severely obstructed because of airway contraction in combination with bronchial hypersecretion [8]. Comparable conditions can be found in patients suffering from asthma (triggered airway contraction [24]) or COPD (bronchial hypersecretion [25]). Therefore, β2-agonists and anticholinergics applied in COPD or asthma treatment might be of value as supportive therapy in OP poisoning. These compounds induce airway dilation [24,25,42], and β2-agonists might also contribute to reduction of airway hypersecretion by drug-induced enhancement of fluid removal [4,43]. With atropine, anticholinergics are already a pivotal part of the current antidotal therapy [10]. It is highly effective in counteracting the muscarinic symptoms linked to OP poisoning, and several authors described atropine-induced reductions of centrally mediated symptoms of OP poisoning, presumably because of penetration through the blood brain barrier [4,18]. Correct atropine-dosing presents a challenge, as application of high doses can be necessary in treatment of OP poisoning, which can induce side-effects like confusion, delirium, psychotic reactions and seizures [4,18,44]. Current application regimens propose atropine dose titration against response and toxicity and individualized atropine dosing, which effectively reduced the occurrence of these side-effects in RCTs [45–47]. The anticholinergic glycopyrrolate has been described to be twice as potent as atropine for peripheral effects [44,48], but it barely crosses the blood-brain barrier [44,49], which has also been described for ipratropium [42]. A case study by Choi et al. reported beneficial effects of glycopyrrolate therapy in a patient unresponsive to atropine therapy [50]. In a case study from 2008, ipratropium treatment improved symptoms in a patient poisoned with diazinon, whereas atropine and glycopyrrolate induced unwanted tachycardia and agitation [51]. In our experiments, we determined the effects of different concentrations of both compounds on ACh-induced airway contractions in VX poisoned PCLS. The airway area was the main parameter of analysis, whereby drug application was expected to antagonize the airway contraction. Both substances showed distinguishable effects already at very low concentrations, with ipratropium being slightly more potent (Figure 2(A)). Best results were observed with concentrations of 100 or 1000 nmol/L (ipratropium or glycopyrrolate, respectively) which led to a significant increase in airway

area 15 min after application (Figure 2(A)). Both compounds were therefore equally as effective as atropine, which was analyzed in our previous study in the same setting and depicted here again for better comparison (Figure 2(B)).

β2-agonists are well established assets in COPD and asthma therapy for reduction of symptoms (airway relaxation in particular) and exacerbations [24,25]. Therefore, β2-agonists might also have beneficial effects in OP poisoning. In our study, effects of β2-agonists salbutamol and formoterol on ACh-induced airway contractions in VX poisoned PCLS were evaluated solely and in combination with anticholinergics. Surprisingly, salbutamol and formoterol alone had only negligible antagonizing effects on airway contraction (Figure 3(A,B)). This is in line with observations by Chávez and Segura et al., who analyzed effects of salbutamol on bronchoconstriction in parathion-poisoned guinea-pigs and paraoxon or physostigmine treated guinea pig tracheal ring preparations. Administration of salbutamol led to a short initial decrease of the bronchoconstriction or tracheal ring contraction, but was followed by a paradoxical re-contraction, which sustained until the end of the experiments and occurred also after increasing salbutamol concentrations [52,53]. The authors hypothesized that β2-adrenoceptor stimulation led to the release of ACh from cholinergic nerves, which in the case of an inhibited AChE enhances the contracting stimulus [53,54]. This phenomenon could be an explanation for the low effectiveness of both β2-agonists in our study.

Combined anticholinergic/β2-agonist treatment is standard in COPD therapy and a second line treatment option for some asthma patients [24,25]. In a previous *in vivo* study, Perkins et al. reported that ipratropium plus salbutamol improved respiratory parameters in wake rats poisoned with soman vapor (higher minute volume, reduced lung lobe edema), whereby mortality was the same as in non-treated animals [55]. However, in a pilot study with patients suffering from OP pesticide poisoning recently conducted by Chowdhury et al., no benefit could be detected after addition of nebulized salbutamol to the i.v. standard treatment (oxime + atropine) on parameters like oxygen saturation, speeding of atropinization or resuscitation in general [26]. Notably, in these studies, different physiological parameters were analyzed and naturally, there are great differences in toxicity between nerve agents and OP pesticides. Chowdhury et al. proposed that a more comprehensive clinical study in a larger cohort under better controlled parameters (e.g. type and amount of particular pesticide, uptake route, time until admission/start of therapy) is needed, to further elucidate the current data. With our work, we provide experimental data to support this proposition and include nerve agent poisoning in this consideration. We analyzed effects of anticholinergic/β2-agonist combinations on ACh-induced airway contractions in VX poisoned PCLS. With regards to a potential supportive use of the combined treatment in OP poisoning, a concentration of 1 nmol/L for glycopyrrolate and atropine was chosen to prevent overdosing. In this concentration, glycopyrrolate as well as atropine alone only marginally antagonize the ACh-induced airway contraction (Figure 2(A,B)), which we also observed for concentrations of 10 nmol/L salbutamol or formoterol (Figure 3(A,B)). Interestingly however, combination of F + A, F + G and S + A antagonized the ACh-induced airway contraction efficiently (Figure 4(A–C)), thereby demonstrating a beneficial effect of the combined treatment in OP nerve agent poisoning. From a clinical perspective this might be of great value in a mass casualty scenario, e.g. after a terrorist attack with nerve agents, when available respirators will be a limiting factor and administration of anticholinergic/β2-agonist combinations could decrease the number of patients requiring artificial ventilation. As for the mechanism behind the additive effect of anticholinergic/β2-agonist combinations on airway contractions observed in our study, it is hypothesized that both components bind to G-protein coupled receptors (β2-adrenergic G-protein receptors or G-protein-coupled muscarinic receptors, predominantly subtype M3) and activate comparable intracellular post-receptor signaling pathways [42,56], which is presumed to induce additive dilatory effects on constricted airways [42]. This synergy could explain the observed effects on airway contraction in our study.

Conclusions

We could show that combinations of anticholinergics and β2-agonists used clinically as COPD and asthma therapeutics have beneficial effects on ACh-induced airway contractions in VX-poisoned PCLS. Our results provide conclusive experimental data to support a comprehensive future clinical study, e.g. in patients suffering from OP-pesticide poisoning, like suggested by Chowdhury et al. The anticholinergics glycopyrrolate and ipratropium are readily obtainable alternative options for patients e.g. unresponsive to atropine treatment. Application in combination with β2-agonists could prevent anticholinergic over-dosing for both substances as well as for atropine administration. Concluding, COPD and asthma therapeutics could be potent assets in the supportive treatment of OP poisoning.

Acknowledgements

The study design, execution of experiments, data analyses and manuscript writing were conducted by the authors alone and have never been influenced.

Disclosure statement

The authors declare that there are no conflicts of interest.

Funding

The presented study was supported by the German Ministry of Defense and designed in the framework of the DFG-funded Research Training Group GRK2388, P02.

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