Regulatory Toxicology and Pharmacology 119 (2021) 104823

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Pharmacokinetics and efficacy of atropine sulfate/obidoxime chloride co-formulation against VX in a guinea pig model

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ARTICLE INFO

Keywords:
Atropine sulfate
Obidoxime chloride
Pharmacokinetics
Guinea pig
Nerve agents

1. ABSTRACT

Nerve agent exposure is generally treated by an antidote formulation composed of a muscarinic antagonist, atropine sulfate (ATR), and a reactivator of acetylcholinesterase (AChE) such as pralidoxime, obidoxime (OBI), methoxime, trimedoxime or HI-6 and an anticonvulsant. Organophosphates (OPs) irreversibly inhibit AChE, the enzyme responsible for termination of acetylcholine signal transduction. Inhibition of AChE leads to over-stimulation of the central and peripheral nervous system with convulsive seizures, respiratory distress and death as result. The present study evaluated the efficacy and pharmacokinetics (PK) of ATR/OBI following exposure to two different VX dose levels. The PK of ATR and OBI administered either as a single drug, combined treatment but separately injected, or administered as the ATR/OBI co-formulation, was determined in plasma of naïve guinea pigs and found to be similar for all formulations. Following subcutaneous VX exposure, ATR/OBI-treated animals showed significant improvement in survival rate and progression of clinical signs compared to untreated animals. Moreover, AChE activity after VX exposure in both blood and brain tissue was significantly higher in ATR/OBI-treated animals compared to vehicle-treated control. In conclusion, ATR/OBI has been proven to be efficacious against exposure to VX and there were no PK interactions between ATR and OBI when administered as a co-formulation.

1. Introduction

Organophosphates (OPs) are a class of chemicals that are commonly used as pesticides, industrial solvents and, in weaponized form, as chemical warfare agents (nerve agents). Due to their highly toxic nature, exposure to OPs has led to many casualties, both accidental and intentional (Gunnell et al., 2007; OPCW, 2019). OPs irreversibly inhibit acetylcholinesterase (AChE), causing accumulation of acetylcholine (ACh) in neuronal synapses and neuromuscular junctions. This results in overstimulation of synapses, termination of signal transduction and symptoms of cholinergic syndrome (McDonough and Shih, 1997; Rice, 2016). Current treatment for OP poisoning consists of a combination of an anticholinergic, such as atropine, for the relief of cholinergic symptoms and a cholinesterase reactivator, typically an oxime, such as pralidoxime, obidoxime (OBI), methoxime, trimedoxime or HI-6 as a causal antidote to restore AChE activity. Depending on the type of OP poisoning and progression of symptoms, treatment can be supplemented with an anticonvulsant in an attempt to attenuate brain damage as a result of seizures (Hulse et al., 2019). Emergency treatment is generally provided by self- or buddy aid in a military standards using autoinjector devices containing combinations of drugs. Depending on the severity of poisoning as indicated by progression of clinical signs and symptoms, up to 3 devices can be injected, followed by emergency medical treatment and advanced medical care (Rice et al., 2016). In spite of many years of research towards improved reactivators, clinical use is limited to a few oximes and efficacy depends on the accompanying therapeutic regimen (Worek et al., 2016). One of the oximes that made it into the clinic and several national regimens as a standard reactivator, next to pralidoxime, is OBI.

Despite the current treatment standards, experimental data on *in vivo* pharmacokinetics (PK) of OP poisoning and medical countermeasures is scarce. In our previous study (Joosen et al., 2018) the efficacy of

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https://doi.org/10.1016/j.yrtph.2020.104823
Received 25 October 2020; Accepted 11 November 2020
Available online 16 November 2020

atropine sulfate (ATR) and obidoxime chloride (OBI) co-formulated an autoinjector (ATR/OBI), was studied in guinea pigs exposed to sarin (GB). Treatment with a three human equivalent dose (3HED) of ATR/OBI, led to a significant increase in survival rate over 24 h. ATR/OBI co-formulation treatment led to higher levels of cholinesterase activity, which could be associated with a decrease in symptoms of sarin toxicity. The effects were most evident in the initial period after treatment, coinciding with pharmacologically relevant plasma levels. *In vivo* and *in vitro* experiments have shown that protection and enzyme reactivation differ between various oximes and nerve agents (Dawson, 1994; Maxwell et al., 2008; Wilhelm et al., 2014; Worek et al., 2016). Therefore, the primary aim of this study was to complement previous work on the efficacy of ATR/OBI co-formulation against a G-series agent (Joosen et al., 2018), with an *in vivo* study addressing the efficacy and PK of ATR/OBI co-formulation following exposure to a V-series agent, VX, with differing properties of effect. Although exposure to the nerve agent VX is presumed to be dermally, due to its low volatility, we choose to use the subcutaneous (SC) route in the present model to minimize the variability of exposure, yet allow evaluation of efficacy in relation to PK. This will allow adjustment of plasma levels to those required in dermal exposure scenarios, in which signs and symptoms appear more gradual. Without decontamination, VX will lead to progressive poisoning from the skin depot (Joosen et al, 2010, 2013, 2017). Efficacy and PK of the ATR/OBI co-formulation following SC exposure to VX at two levels was assessed using 24-h survival outcome, evaluation of clinical signs of cholinergic syndrome and cholinesterase activity in red blood cells and brain tissue. The two levels reflect ~1.5 and ~2.5 LD_{50} when left untreated in the guinea pig (Fawcett et al., 2009), at a similar range of dose levels as used in the efficacy studies for sarin exposure (Joosen et al., 2018). Although theoretically extrapolated from previous research, there is currently no publicly available scientific evidence that supports the assumption that the active pharmaceutical ingredients (APIs) ATR and OBI administered as one co-formulation show similar PK *in vivo* as compared to separately administered components. Previous investigations mainly focused on cholinesterase reactivation and efficacy of various oximes in conjunction with atropine treatment, while PK was not deeply studied. Hence, a second aim of this study was to obtain *in vivo* evidence if any PK interaction between ATR and OBI when administered as co-formulation, both in naïve and VX exposed animals. PK of ATR and OBI, administered as single APIs, combined treatment but separately injected (ATR + OBI), or as the ATR/OBI co-formulation, was determined in plasma of naïve guinea pigs. The data presented here fills parts of the gaps in data underlying emergency use autoinjector recommendations and approval for use available in open literature, supporting the (operational) needs for such data for new medicinal products and their approval by regulatory health agencies.

2. Material and methods

2.1. Animals

Male Dunkin – Hartley albino guinea pigs were obtained from Envigo (Horst, the Netherlands), with a starting weight of approximately 200–250 g (~350 g at time of VX challenge or PK studies). Prior to the experiments, animals were pair-housed in Macrolon polycarbonate type IV cages and allowed to acclimatize to the housing conditions for one week. Room temperature was maintained at 19–22 °C, relative humidity was maintained at 55–65%, and lights remained on from 7 a.m. to 7 p.m. Acidified water (pH ranging between 3.5 and 4.5) and guinea pig chow (Teklad global diet 2040, Envigo, Horst, the Netherlands) were available *ad libitum*. All experiments were carried out according to the European Union (EU) legislation for testing on experimental animals (EU Directive, 2010/63/EU) including approval by the TNO Animal Welfare Board, and in line with International Guiding Principles for Biomedical Research Involving Animals. Unnecessary suffering was minimized in all cases.

2.2. Chemicals

VX was obtained from internal TNO stocks (purity >98%) and diluted aliquots in isopropyl alcohol (IPA) were stored at −20 °C. VX injection solutions were freshly prepared in saline. Atropine sulfate and obidoxime chloride were provided by Streuli Pharma AG (Uznach, Switzerland) and dissolved in sterile filtered 0.4% phenol solution in water for injection. All solutions were stored at 4–6 °C until use.

2.3. Study design and animal preparation

The study consisted of two phases: 1) determination of PK of ATR/OBI and its individual components in naïve guinea pigs and 2) determination of efficacy and PK of ATR/OBI and its individual components in guinea pigs exposed to a SC challenge with two dose levels of VX.

For all groups, animal preparation was similar and as follows. Before surgery, animals received the analgesic carprofen (5.0 mg/kg, 1.0 mL/kg SC) and Borgal antibiotic (20.0 mg/kg sulfadoxine and 4.0 mg/kg trimethoprim SC). Animals were anesthetized with 4–5% isoflurane (IsoFlo, 100% isoflurane, Abbott) in oxygen and maintained at 2–3% while they were kept on a 37 °C warmth plate. Animals were equipped with an indwelling jugular vein catheter (4 cm) that was subcutaneously tunneled to an opening on the skull roof and fixed with dental cement (GC Fuji PLUS®) to a screw in the skull. Local analgesia was applied to the periosteum. The catheter was filled with 500 international units (IU) heparin in glycerol and capped. Animals were allowed to recover for five days with postoperative analgesia with carprofen (5 mg/kg, SC) two days after surgery.

2.3.1. Phase 1: pharmacokinetic interactions in naïve Guinea pigs

The aim of this phase was to determine and compare the PK profiles of ATR and OBI administered via the intramuscular (IM) route as single components or as the ATR/OBI co-formulation in naïve animals (Table 1). Four experimental groups were evaluated: A) a single injection of the ATR/OBI co-formulation (ATR/OBI; 0.4 mg/kg ATR and 44.0 mg/kg OBI); B) a single injection of 0.4 mg/kg ATR; C) a single injection of 44.0 mg/kg OBI; and D) ATR and OBI administered as two separate injections (ATR + OBI; one injection with 0.4 mg/kg ATR and one injection in the other hindleg with 44.0 mg/kg OBI). Doses, reflecting three human autoinjector equivalents ($3 \times 220 = 660$ mg OBI and $3 \times 2 = 6$ mg ATR) were calculated using a BSA correction from human to guinea pig (70 kg body weight and correction coefficient 4.6) (Reagan-Shaw et al., 2008). To minimize the total number of animals, a counterbalanced repeated dosing schedule was used with 4 days of washout period between injections (PK day 1 and PK day 2). On experimental days, animals were treated at $t = 0$ with IM injections (0.4 mL/kg) of one of four treatments as described in Table 1. For the combined ATR and OBI treatment via separate injections, the second injection was always given within 20 s of the first injection. Half of the animals received ATR first and the other half was first injected with OBI. Blood samples (250 μL per sample) were collected before treatment and at 2, 5, 10, 20, 40, 80, 120, and 240 min after treatment for PK assessment. As there were no differences between data obtained from PK day 1 or day 2, the data was pooled.

Table 1
Phase 1 animal group assignment.

Group	Treatment	Treatment dose (mg/kg IM)	No. of animals
A	ATR/OBI (co-formulation)	ATR: 0.4 mg/kg; OBI: 44.0 mg/kg	9
B	ATR	ATR: 0.4 mg/kg	11
C	OBI	OBI: 44.0 mg/kg	8
D	ATR + OBI (separate injections)	ATR: 0.4 mg/kg; OBI: 44.0 mg/kg	9

2.3.2. Phase 2: efficacy and pharmacokinetics of atropine sulfate and obidoxime chloride in VX-exposed Guinea pigs

In phase 2, the efficacy of the ATR/OBI co-formulation and its single components at two SC VX challenge doses were assessed in a guinea pig model. One half of the animals was treated with a 12 μg/kg of VX (~1.5 LD_{50}) and the others with a dose of 20 μg/kg VX (~2.5 LD_{50}) (Table 2). One minute after VX exposure (diluted in PBS prior to injection, 1.0 mL/kg), animals were IM-treated (0.4 mL/kg) with one of the following four treatments: 1) vehicle (VEH); 2) ATR: 1 injection of 0.4 mg/kg; 3) OBI: 1 injection of 44.0 mg/kg; or 4) ATR/OBI co-formulation: 1 injection of 0.4 mg/kg ATR and 44.0 mg/kg OBI (outlined in Table 2).

Blood samples (250 μL per sample) were collected for PK assessment and determination of AChE and BuChE levels before treatment and at t = 2, 5, 10, 20, 40, 80, 120, 240, and 1440 min after VX exposure. Animals were video-recorded for 24 h using Axis M1144-L cameras (Axis Communications, Lund, Sweden) and Ponemah Software (version 5.32, DataScience International, USA). During the experiment, animals were monitored for onset of cholinergic signs, e.g., chewing, shivering, stretched posture, tremors, convulsions, salivation, lacrimation, rasping breathing, and gasping. Moreover, posture of the animals was scored on a scale of 0–6 over a 24-h period after VX exposure to capture the stages of organophosphate poisoning (Table 3).

Scoring was performed both live and from video recordings by two researchers, blinded from treatment information. Posture was scored at timepoints 20, 60, 120, 240, 480, 720, 1080, and 1440 min after VX exposure through behavioral observations for 30–60 s per timepoint. Brain tissue of the animals was collected for determination of brain AChE and BuChE levels after death of the animal, which occurred either as a consequence of VX poisoning or after 24 h after sacrifice by pentobarbital (>100 mg/kg Euthasol, AST Farma, the Netherlands).

2.4. Sample workup for biochemistry

Blood and brain tissue were collected for AChE activity assessment. Two aliquots of 20 μL blood (from each sample) were diluted tenfold in 1% saponin solution, frozen immediately in dry ice and stored at −70 °C until AChE activity analysis was performed as described below. Blood samples were centrifuged for 5 min at 14,000 RPM) to obtain plasma for determination of atropine sulfate and obidoxime chloride levels. The plasma was divided into two fractions of 50 μL and 30 μL and to each fraction 10 μL of internal standard (1 μM Atropine-D3 and 5 μM 2-PAM) was added before storage at −70 °C.

Brain hemispheres were homogenized with a SilentCrusher (1500 RPM, 10% w/v homogenate) in ice cold TENT buffer (1 M NaCl, 50 mM Tris, 5 mM EDTA and 1% v/v Triton X-100, pH 7.4). Homogenates were snap frozen in dry ice and stored at −70 °C until determination of enzyme activity in supernatant using the Ellman method as described below.

Table 2
Phase 2 animal group assignment.

Group	VX challenge dose (μg/kg SC)	Treatment	Treatment dose (mg/kg IM)	No. of animals
1	12*	VEH	n/a	5
2	12	ATR	0.4 mg/kg	6
3	12	OBI	44.0 mg/kg	7
4	12	ATR/OBI	ATR: 0.4 mg/kg; OBI: 44.0 mg/kg	8
5	20**	VEH	n/a	5
6	20	ATR	0.4 mg/kg	6
7	20	OBI	44.0 mg/kg	7
8	20	ATR/OBI	ATR: 0.4 mg/kg; OBI: 44.0 mg/kg	8

* ~1.5 LD50 significant effect of ATR alone.
** (~2.5 LD50) no effect of ATR alone.

Table 3
Scores assigned to stages of organophosphate poisoning.

Score	Signs Observed
0	No signs; normal posture
1	Upright posture with full mobility, but could be a bit unstable; first signs of shivering; the distance between front and hind limbs is less than approximately half of the total length of the animal
2	Upright posture with mobility, but unstable; the distance between front and hind limbs is more than approximately half of the total length of the animal (i.e., stretched posture)
3	Animal is lying down, is not able to walk around; head is held up and seems stable
4	Animal is lying down with limbs spread out; control over body, including the head, is unstable (i.e., the head could be on the ground or could be held up but unstable)
5	Animal is lying flat on its belly or on its side; no control over body parts; no muscle tone; often coinciding with muscle spasms
6	Dead

2.4.1. Acetylcholinesterase assay

Samples were analyzed for AChE and butyrylcholinesterase (BuChE) activity using a modification of the method by Ellman (Ellman et al., 1961). AChE and BuChE activity were determined simultaneously on a 96-well plate. Plasma samples were thawed and diluted in 0.8 mM 5, 5′-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich Inc.). To 100 μL of diluted sample, 100 μL of 0.8 mM β-methylacetylthiocholine iodide was added, in quadruple, for determination of AChE activity. For assessment of BuChE activity in the samples, 100 μL of 0.8 mM of butyrylthiocholine was added. The change in extinction per minute at 412 nm at ambient temperature served as a measure for cholinesterase activity. AChE and BuChE activities in blood were normalized versus the baseline sample. For brain homogenate supernatant, only AChE activity was determined and activity in VX-exposed animals was normalized versus activity of a subset of unchallenged animals from phase 2.

2.5. Atropine sulfate and obidoxime chloride analysis in plasma

For quantification of atropine sulfate, the plasma sample was precipitated with acetonitrile +0.1% formic acid and centrifuged for 10 min at 14,000 RPM. The supernatant was analyzed with liquid chromatography with tandem LC-MS/MS.

For quantification of obidoxime chloride, the plasma sample was diluted 1:10 with 0.1% formic acid in MQ (Millipore water, SimPak® 1). This mixture was filtered by centrifugation for 10 min at 14,000 RPM using AmiconUltra 10 kDa filter and the filtrate was used for LC-MS/MS analysis.

2.5.1. LC MS/MS conditions

For analysis of atropine sulfate, 5 μL of the sample was injected (full loop). For analysis of obidoxime chloride, 2 μL of the sample was injected. Chromatographic separation was achieved on an Acquity HSS T3 column, 2.1 × 100 mm, 1.8 μm particles at 0.1 mL/min, with water/acetonitrile gradient with 0.01% heptafluorobutyric acid (HFBA) as the mobile phase. Over 5 min, the mobile phase was switched linearly from 0% acetonitrile to 80% acetonitrile and was maintained at this ratio for another 3 min after which the mobile phase was switched to 100% water. Obidoxime, 2-PAM, and atropine were quantified in Multiple Reaction Monitoring (MRM) mode at a Waters Xevo TQ-S, Desolvation T 350°C; ESI Voltage 3.5 kV; desolvation gas 800 L/h and cone gas 150 L/h.

Based on the results, the lower limit of quantification (LLOQ) of the method for atropine sulfate was determined at 2 nM and for obidoxime chloride at 5 μM in guinea pig plasma.

2.6. Data analysis

Data is presented as an average ± SEM and checked for normal

distribution using built-in analyses in GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). Survival (Kaplan-Meier plots) was analyzed using a Log-rank (Mantel Cox) test and percentages of surviving animals at 4 and 24 h after exposure were analyzed using a Fisher's exact test, with Holm-Sidak correction for multiple comparisons. Posture score data was analyzed using a Two-Way Repeated Measures ANOVA with *post hoc* comparisons of autoinjector formulation against other groups with Holm-Sidak correction for multiple testing. Remaining data was analyzed using One- or Two-Way ANOVAs followed by *post hoc* tests with Holm-Sidak correction for multiple comparisons where applicable. Results were considered significantly different for $p < 0.05$.

PK parameters were determined using Phoenix WinNonLin version 8.1 (Certara). All data could be fit to a one compartment model, first-order elimination. Data was weighted 1/y2 and Gauss-Newton (Levenberg and Hartley) minimization was applied. All groups fit to this model with a correlation >0.97.

3. Results

3.1. Efficacy of atropine sulfate and obidoxime chloride against VX challenge in Guinea pigs

3.1.1. Survival

At both VX challenge doses, ATR/OBI treatment led to a significantly improved survival rate (Fig. 1). Thus, treatment with 3HED of ATR/OBI co-formulation IM 1 min after VX poisoning significantly improved 24-h survival compared to VEH for both 12 μg/kg VX ($p = 0.002$, Figs. 1A) and 20μg/kg VX SC ($p = 0.006$; Fig. 1B). Though both ATR or OBI single treatment seem to improve survival rate to some extent, only ATR treatment after 12 μg/kg VX ($p = 0.002$) and OBI treatment after 20 μg/kg VX ($p = 0.008$) reached statistical significance compared to no intervention.

At both 4 and 24 h after exposure to 12 μg/kg VX, the survival proportion of ATR/OBI-treated animals was significantly higher than that of VEH-treated animals ($p = 0.005$ and $p = 0.004$, respectively, Table 4). After exposure to 20 μg/kg VX, survival as compared to VEH was significantly higher at 4 h ($p = 0.005$) but not 24 h ($p = 0.098$).

Additionally, survival of ATR/OBI -treated animals was compared to survival of animals treated with either ATR or OBI alone. At the lower VX dose of 12 μg/kg, ATR/OBI did not perform significantly better than either ATR or OBI alone ($p = 0.085$ and $p = 0.350$, respectively, Fig. 1A). At the highest VX challenge dose of 20 μg/kg VX, ATR/OBI treatment outperformed ATR alone ($p = 0.004$), but not OBI treatment ($p = 0.210$, Fig. 1B).

At 4 h after exposure to 20 μg/kg VX, the survival proportion of ATR/OBI-treated animals was higher than that of ATR-treated animals, but not OBI-treated animals (ATR/OBI vs ATR; $p = 0.005$, Table 4). At 24 h, this difference was not significant. There were no differences in survival ratios between ATR/OBI- and ATR- or OBI-treated animals at 4 or 24 h after exposure to 12 μg/kg VX.

Table 4
Survival of animals exposed to VX at 4 and 24 h and treated 1 min after exposure.

VX Dose	Treatment	4-h Survival (Surviving/Total)	24-h Survival (Surviving/Total)
12 μg/kg	VEH	0/5	0/5
	ATR	2/6	1/6
	OBI	4/7	3/7
	ATR/OBI	7/8**	5/8**
20 μg/kg	VEH	0/5	0/5
	ATR	0/6	0/6
	OBI	6/7	1/6
	ATR/OBI	7/8**/&&	4/8

** $p < 0.01$ ATR/OBI compared to VEH.
&& $p < 0.01$ ATR/OBI compared to ATR; Fisher's exact test with Holm-Sidak correction for multiple testing.

3.1.2. Clinical signs

Signs of the cholinergic syndrome were observed in animals of all groups. The first signs, reflected by mastication and shivering, or posture score 1, were observed around 13.4 ± 5.4 min or 10.4 ± 0.9 min for 12 and 20 μg/kg VX respectively (mean ± SD), irrespective of the 1 min treatment. Onset times of tremors and convulsions were similar for all animals, regardless of treatment (One-way ANOVA; $p = 0.303$ and $p = 0.201$ for 12 and 20 μg/kg VX, respectively, Table 5).

Posture of the animals was scored from video at predefined timepoints over a 24-h period after VX exposure to quantify clinical signs of cholinergic crisis as an indication of severity of OP poisoning and treatment efficacy. After exposure to 12 μg/kg VX, the severity of clinical signs was lower for ATR/OBI -treated animals than VEH-treated animals from timepoint t = 60 min onwards (Two-Way Repeated Measures ANOVA with Holm-Sidak correction for multiple comparisons; $p < 0.001$, Fig. 2A). Posture scores after 20 μg/kg VX were lower for ATR/OBI-treated animals from 60 to 480 min ($p < 0.001$, Fig. 2B). Additionally, ATR/OBI-treated animals were in better shape than animals treated with ATR alone at several timepoints.

3.1.3. Acetylcholinesterase activity

AChE activity measured in blood of ATR/OBI-treated animals was significantly higher than AChE activity of VEH-treated animals in the first hours after VX exposure, both after 12 and 20 μg/kg VX challenges (p values ranged from <0.0001 to 0.023, Fig. 3AB). AChE activity of ATR/OBI-treated animals was also significantly higher than that of ATR-treated, but not OBI-treated, animals between 2 and 40 min after exposure after both VX challenges (p values ranged from <0.0001 to 0.001).

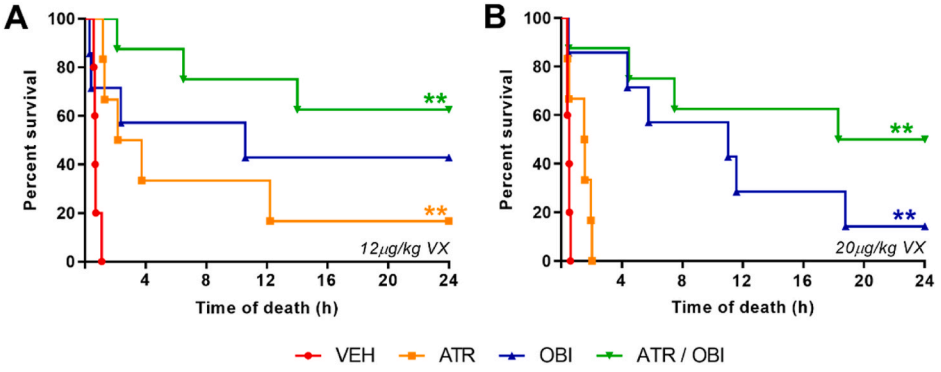

Fig. 1. Kaplan Meier plots of survival of guinea pigs challenged with VX at 12 μg/kg (A) or 20 μg/kg (B) SC, treated at 1 min with a single IM injection of VEH, ATR, OBI, or ATR/OBI. ATR and ATR/OBI treatment at 1 min significantly improved survival rate compared to VEH after exposure to 12 μg/kg (**$p < 0.01$, Panel A), whereas OBI and ATR/OBI treatment at 1 min improved survival rate compared to VEH after exposure to 20 μg/kg VX (**$p < 0.01$, Panel B).

Table 5
Descriptive statistics of onset (in minutes) of tremors and convulsions of guinea pigs exposed to VX.

VX Exposure		VX Dose (SC)							
		12 μg/kg				20 μg/kg			
Group		VEH	ATR	OBI	ATR/OBI	VEH	ATR	OBI	ATR/OBI
No. of Animals		5	6	6*	8	5	6	7	8
Tremors	Mean	22.00	24.00	17.00	17.00	12.00	15.00	12.00	12.00
	SD	6.20	7.10	8.60	8.20	1.20	4.00	2.20	2.70
Convulsions	Mean	26.76	30.80	22.40	22.16	13.99	16.85	14.25	14.93
	SD	4.27	5.03	5.66	7.62	1.46	4.30	1.92	2.63

* In the OBI-treated group exposed to 12 μg/kg VX one animal did not experience tremors and convulsions.

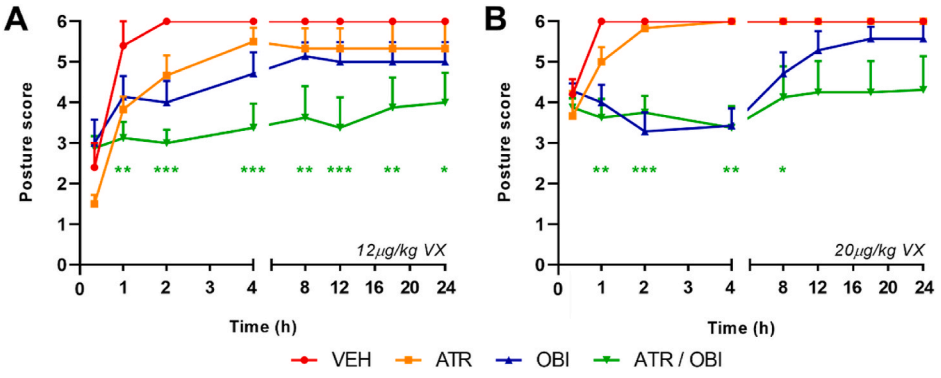

Fig. 2. Averaged posture score over 24 h at predefined timepoints in guinea pigs challenged with VX at 12 μg/kg or 20 μg/kg, treated at 1 min with 3HED IM VEH, ATR, OBI, or ATR/OBI. At both VX challenge doses, ATR/OBI -treated animals were in better shape than VEH-treated animals as seen by significantly lower posture scores from 60 min onwards (*p < 0.05; **p < 0.01; ***p < 0.001, ATR/OBI compared to VEH, Repeated Measures ANOVA followed by Holm-Sidak correction for multiple testing).

Likewise, BuChE activity measured in blood was also inhibited (Fig. 3CD). BuChE activity of ATR/OBI-treated animals exposed to 12 μg/kg VX was significantly higher than BuChE activity of VEH-treated animals in the first hour after exposure (Fig. 3A), whereas BuChE activity in ATR/OBI-treated animals exposed to 20 μg/kg VX was higher than VEH-treated animals after the first 20 min after exposure (Fig. 3B). Regarding the single treatments, BuChE activity of ATR/OBI-treated animals was comparable to that of OBI-treated animals, but significantly higher than ATR-treated animals from 10 to 20 min after VX exposure (p values ranged from <0.001 to 0.015).

AChE activity in brain tissue of ATR/OBI-treated animals was higher than VEH-treated animals after both 12 and 20 μg/kg VX challenges (p = 0.014 and 0.002, respectively, Fig. 4). In addition, ATR/OBI animals showed higher AChE activity compared to ATR-treated animals after both 12 and 20 μg/kg VX challenges (p = 0.047 and 0.002, respectively). There was no difference between ATR/OBI and OBI treatment.

3.2. Pharmacokinetics of atropine sulfate and obidoxime chloride

3.2.1. Naïve Guinea pigs

Fig. 5 shows the PK of ATR (panel A) and OBI (panel B) after a single IM injection of either ATR or OBI, ATR and OBI injected as two separate injections in separate hindlegs, or a single injection of the ATR/OBI co-formulation in naïve guinea pigs.

Calculated first-order kinetic parameters are provided in Table 6 and Table 7. For OBI, curves were similar for all groups. For ATR in the ATR/OBI group, T_{max} was significantly shorter, also reflected by a significantly shorter absorption half-life ($t_{1/2,\ abs}$) compared to both other groups. The total bioavailability between groups was the same, reflected by comparable values for C_{max} and area under the curve (AUC).

3.2.2. VX-challenged Guinea pigs

Panels C and D of Fig. 1 show the PK of IM ATR and OBI in animals challenged with 12 and 20 μg/kg VX, respectively. Animals received either OBI alone, ATR alone, or the combination of drugs in one injection

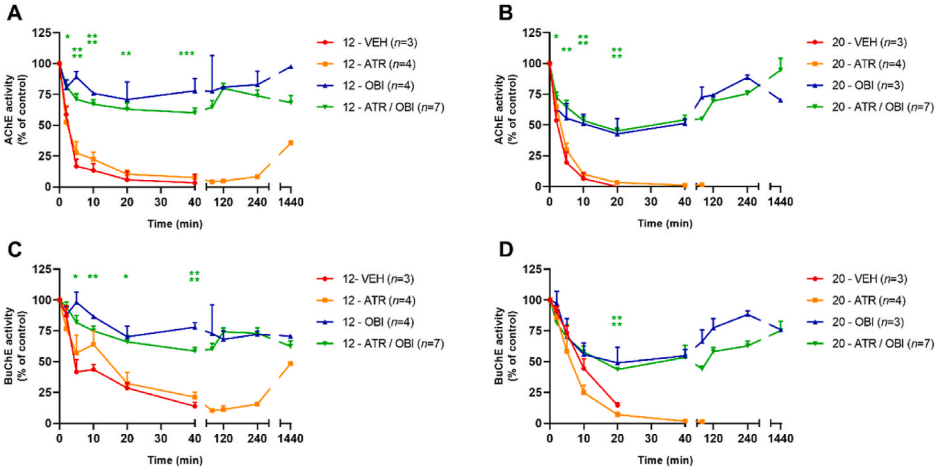

Fig. 3. Acetylcholinesterase activity in blood samples as compared to baseline activity before exposure to 12 μg/kg (A) or 20 μg/kg VX (B) SC and treated IM at t = 1 min with 3HED ATR/OBI, its separate components (ATR and OBI) or vehicle (VEH) (Average ± SEM). Unpaired one-sided t-tests were performed for each timepoint between ATR/OBI and VEH groups with Holm-Sidak correction for multiple testing. No other combinations were tested.*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 between ATR/OBI and VEH.

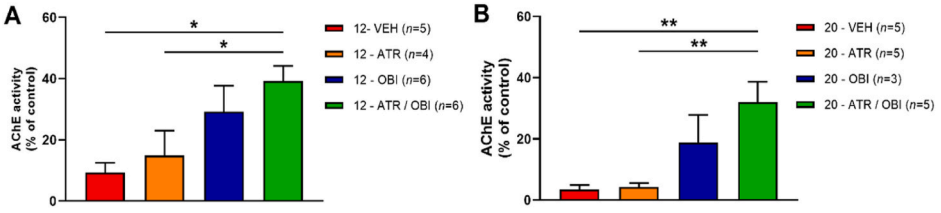

Fig. 4. Brain acetylcholinesterase activity in guinea pigs challenged with 12 μg/kg (A) or 20 μg/kg VX (B) SC and treated IM at t = 1 min with VEH, ATR, OBI, or ATR/OBI. At both challenge levels, significantly higher levels of activity were observed at 24 h after exposure or at time of death in ATR/OBI-treated animals compared to VEH and ATR, but not OBI-treated animals. *p < 0.05; **p < 0.01, One-way ANOVAs, corrected for multiple comparisons using Holm-Sidak.

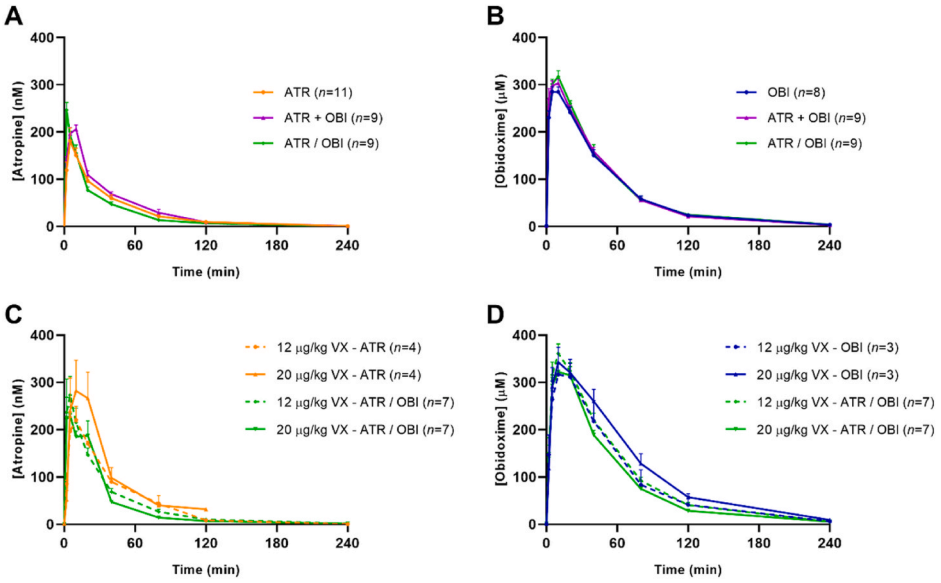

Fig. 5. ATR and OBI levels in plasma of naïve and VX-exposed guinea pigs. Panel A and B show plasma concentrations of ATR (panel A) and OBI (panel B) in naïve guinea pigs, injected at t = 0 with a single injection of ATR (orange) or OBI (blue), both ATR and OBI as two separate injections (purple), or a single injection of ATR/OBI IM (green). Doses were 0.4 mg/kg for ATR, 44.0 mg/kg for OBI, representing 3HED of the ATR/OBI co-formulation. Panels C and D show plasma concentrations of ATR (C) and OBI (D) in guinea pigs exposed to either 12 μg/kg or 20 μg/kg VX SC at t = 0 and treated IM at t = 1 min with 3HED ATR/OBI or its separate components (Average ± SEM).

Table 6
PK parameters for ATR in naïve guinea pigs.

Parameter	Unit	Treatment Group					
		ATR (0.4 mg/kg)		ATR (0.4 mg/kg) + OBI (44.0 mg/kg)		ATR/OBI (0.4 mg/kg ATR + 44.0 mg/kg OBI)	
		Mean	SD	Mean	SD	Mean	SD
C_{max}	nmol/L	139.4	32.4	165.7	15.0	153.9	34.7
T_{max}	min	5.10	2.78	3.66	1.53	1.10*	0.11
$T_{1/2}$	min	28.03	7.83	28.14	4.89	24.62	7.71
Cl	mL/min/kg	198.29	57.35	158.63	20.65	204.16	37.83
Vd	mL/kg	7856.0	2414.3	6327.6	792.0	7540.4	1379.5
$T_{1/2,\ abs}$	min	1.16	0.88	0.71	0.38	0.15*	0.01
AUC	min*nmol/L	6385.30	2253.62	7339.89	1017.4	5476.6	1545.0

* $p < 0.05$, statistically significant from other treatments, multiple *t*-test corrected by Holm-Sidak.

Table 7
PK parameters for OBI in naïve guinea pigs.

Parameter	Unit	Treatment Group					
		OBI (44.0 mg/kg)		ATR (0.4 mg/kg) + OBI (44.0 mg/kg)		ATR/OBI (0.4 mg/kg ATR + 44.0 mg/kg OBI)	
		Mean	SD	Mean	SD	Mean	SD
C_{max}	μmol/L	280.9	26.7	308.9	34.1	306.4	34.6
T_{max}	min	5.57	1.47	4.27	1.13	5.56	1.18
$T_{1/2}$	min	33.67	4.67	31.93	4.42	35.57	5.40
Cl	mL/min/kg	8.52	1.59	8.07	1.08	7.18	1.22
Vd	mL/kg	405.9	42.0	366.9	35.7	362.1	34.9
$T_{1/2,\ abs}$	min	1.12	0.41	0.79	0.25	1.08	0.30
AUC	min*μmol/L	15,360.2	2950.7	15,467.2	2189.4	16,892.3	2439.2

Table 8
PK parameters for ATR in plasma of guinea pigs exposed to 12 or 20 μg/kg VX SC treated with a single injection of ATR or ATR/OBI at t = 1 min after VX exposure.

VX Exposure		VX Dose							
		12 μg/kg				20 μg/kg			
Group		ATR (0.4 mg/kg)		ATR/OBI (0.4 mg/kg ATR + 44.0 mg/kg OBI)		ATR (0.4 mg/kg)		ATR/OBI (0.4 mg/kg ATR + 44.0 mg/kg OBI)	
Parameter	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C_{max}	nM	203.94	44.88	195.79	26.56	209.70	62.72	189.64	27.05
T_{max}	min	6.76	0.79	3.09	2.77	11.33	5.68	1.69	1.39
$T_{1/2}$	min	30.96	14.23	27.14	7.58	28.13	12.50	22.74	3.79
Cl	nmol/min/kg	117.32	19.47	146.03	27.19	108.22	15.21	179.31*	19.40
Vd	mL/kg	4977.68	1503.00	5539.95	938.56	4478.00	2285.51	5829.71	895.83
$T_{1/2.\ abs}$	Min	1.58	0.52	0.62	0.65	3.93	3.48	0.29	0.29
AUC	min*nmol/L	10,032.6	1732.2	8134.5	1668.0	10,791.3	1634.7	6480.2*	703.9

* $p < 0.05$; significantly different from atropine alone at highest VX dose.

Table 9
PK parameters for OBI in plasma of guinea pigs exposed VX and treated with a single injection of OBI or ATR/OBI at t = 1 min after exposure.

VX Exposure		VX Dose							
		12 μg/kg				20 μg/kg			
Group		OBI (44.0 mg/kg)		ATR/OBI (0.4 mg/kg ATR + 44.0 mg/kg OBI)		OBI (44.0 mg/kg)		ATR/OBI (0.4 mg/kg ATR + 44.0 mg/kg OBI)	
Parameter	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C_{max}	μM	301.99	87.09	340.68	44.54	358.05	62.10	307.10	45.90
T_{max}	Min	8.01	3.12	10.46	4.53	10.59	5.42	5.68	0.92
$T_{1/2}$	Min	38.68	6.82	34.96	2.11	42.00	3.35	36.10	1.97
Cl	mL/min/kg	6.67	2.51	5.91	1.03	4.85	0.85	7.01	1.29
Vd	mL/kg	359.64	73.77	298.22	55.09	295.42	70.01	363.12	50.52
$T_{1/2.\ abs}$	Min	1.79	1.04	2.75	1.71	2.58	1.83	1.10	0.23
AUC	min*μmol/L	19,752.3	7463.4	21,282.5	3921.8	25,771.7	4182.8	17,865.7	2961.6

as the ATR/OBI co-formulation. Calculated first-order kinetic parameters are provided in Table 8 and Table 9.

For OBI, curves were similar for all groups. For ATR, the bioavailability of ATR as part of the ATR/OBI treatment was significantly lower at the highest VX dose, indicated by a significantly lower AUC and higher clearance (Table 8). It must be noted, however, that this was not correlated to survival rate given the lower survival rate in the ATR-only group. Presumably, the lower clearance in the ATR-only group, leading to higher bioavailability, may be due to the declined clinical condition of the animals in this group, also indicated by a relatively worse survival rate.

4. Discussion

This study was conducted to evaluate the efficacy of the an autoinjector co-formulation containing atropine sulfate and obidoxime chloride following two different SC VX doses in a guinea pig model. In addition, PK profiles were established for ATR and OBI administered as a co-formulation and separately in both naïve and VX-exposed animals. ATR/OBI-treated animals showed significant improvement in survival rate and progression of clinical signs compared to untreated animals after SC exposure to 12 or 20 μg/kg VX. AChE activity in both blood and brain tissue was significantly higher in ATR/OBI-treated animals compared to VEH-treated animals exposed to VX.

To compare the PK profiles of ATR and OBI in different treatment compositions and to evaluate potential interactions, plasma levels of naïve animals injected IM with either one of the two components, both components administered as two separate injections (ATR+OBI), or both components administered as the ATR/OBI co-formulation were collected and quantified using LC-MS/MS. In ATR/OBI-injected naïve animals, ATR levels reached the maximum concentration significantly faster compared to injection with ATR alone or injected as a single API combined with OBI (separate hindlegs). It must be noted that the variation in the ATR/OBI-injected animals was much lower than in the other groups. The faster uptake demonstrated in this analysis slightly deviates from previous work on ATR and OBI PK in ATR/OBI-treated guinea pigs (Joosen et al., 2018), where values for T_{max} and $T_{1/2abs}$ of ATR in ATR/OBI-treated guinea pigs were comparable to the values reported for the ATR alone and ATR + OBI groups in the current dataset. Although a change in absorption rate of ATR when combined with oxime treatment has been debated by others (Holland et al., 1975; Holland and White, 1971), other parameters representing bioavailability, C_{max}, and AUC were the same for all groups, indicating that effects, if any, are minor. OBI PK parameters were found to be similar for all groups. The current dataset provides *in vivo* evidence that combining ATR and OBI in one co-formulation does not change the PK of the drugs as compared to administration of the pharmaceutical ingredients separately (ATR + OBI) or as single API treatment (ATR or OBI). Similar results were found for combinations of ATR with other oximes, including HI-6, 2-PAM and HLö-7 (Abbara et al., 2010; Clement et al., 1990; Thiermann et al., 1996), although changes in oxime PK have been reported as well (Jovanović et al., 1992).

The VX challenges did not affect the PK of individual APIs in the ATR/OBI-treated animals. After 20 μg/kg VX SC, the bioavailability of ATR was increased in the group treated with ATR alone as compared to ATR/OBI-treated animals. This can presumably be associated with the worse condition of the animals in that group, also reflected by a relatively low survival rate. OBI PK data of VX-exposed animals is in accordance with earlier work from our lab on G-series nerve agents (Bohnert et al., 2020; Joosen et al., 2018; van Helden et al., 1994). Literature on PK in humans after OP poisoning is also available (Bentur et al., 1993; Thiermann et al, 1997, 2009, 2010, 2011). However, PK data in these studies was obtained from continuous treatment regimens fitting a definitive care setting, which differs from the bolus treatment aimed for with an autoinjector in an emergency setting.

To evaluate the efficacy of a single bolus treatment of ATR/OBI, guinea pigs were SC exposed to one of two VX doses, treated after 1 min with 3HED of ATR or OBI alone or combined as the ATR/OBI co-formulation and monitored for survival and clinical signs of cholinergic crisis for 24 h. ATR/OBI-treated animals showed significant

improvement in survival rate at both exposure levels of VX compared to VEH-treated animals. Although treatment consisting of 3 equivalents was administered at 1 min after exposure, the onset of cholinergic symptoms (i.e., tremors and convulsions) was similar across all groups. The progression from tremors to convulsions was fast: within 2–5 min after tremor onset convulsions developed, which would have been an indication for rapid repetition of additional doses, not significantly affecting T_{max} and C_{max} given the SDs observed. The current paradigm allowed for an evaluation of the efficacy resulting from the plasma levels following a single administration of a triple autoinjector dose, yielding a lower experimental variability in the model. Eventually, the severity of clinical signs (i.e., posture score) was significantly lower, starting 60 min after VX exposure in ATR/OBI-treated animals exposed to 20 μg/kg VX SC, even though at that time plasma levels of both compounds had decreased substantially. This effect was most pronounced in the first 8 h after exposure, whereas at the lower VX dose the ATR/OBI-treated animals had clearly better (i.e., lower) posture scores over the full 24-h observation period. AChE activity in both blood and brain of animals treated with ATR/OBI was approximately 1.5 times higher than AChE activity in VEH-treated animals at both VX challenge doses. These findings are in accordance with previously reported preclinical studies on the efficacy of OBI against VX (Dawson, 1994; Joosen et al., 2010). The present results also show that the PK of both ATR and OBI are proportional to the dose, and the effects over time related to plasma levels in guinea pigs could support the prediction of efficacy in humans (Joosen et al., 2018).

ATR and OBI exert their therapeutic effects via distinct mechanisms, resulting in a synergism when administered as a combination. While ATR provides symptomatic relief of cholinergic crisis through competitive reversible antagonism of muscarinic acetylcholine receptors, OBI induces reactivation of inhibited AChE, causally preventing further build-up of excess ACh. Because efficacy of either component is dependent on the type and dose of nerve agent exposure, the added value of each separate component and the resulting synergism consequently varies across the nerve agent spectrum (Ligtenstein and Moes, 1991; Thiermann et al., 2016). At the lower VX dose, ATR as a single treatment reached significant improvements in survival, whereas at the higher VX dose, OBI alone reached statistical significance. The ATR/OBI co-formulation outperformed these single treatments with regard to survival of animals at both VX challenge levels, confirming the necessity of the synergistic use of these drugs. At both challenge doses, clinical signs of ATR/OBI-treated animals were less severe than ATR-treated animals at several timepoints. AChE activity in blood and brain tissue of ATR/OBI-treated animals was similar to AChE activity of animals treated with OBI alone, but significantly higher than that of ATR-treated animals. As observed in previous studies, brain AChE activity can be associated with an improved outcome with regard to clinical symptoms and survival (Joosen et al., 2017). Taken together, these results indicate that particularly at higher challenge doses, the contribution of the oxime is greater.

The current study follows on a previous study (with a comparable design) on the efficacy of ATR/OBI against sarin (GB), another highly toxic OP compound with the same mechanism of toxicity (Joosen et al., 2018). Compared to sarin, 3HED ATR/OBI treatment against VX seems equally efficacious at both VX challenge levels with regard to 24-h survivability, yet mitigation of clinical signs was more successful, particularly after 20 μg/kg VX SC. Also, AChE reactivation in ATR/OBI-treated animals was higher in VX-exposed animals as compared to sarin-exposed animals. Whereas the maximal AChE inhibition in ATR/OBI-treated animals exposed to both doses of sarin was ~60–70%, maximal AChE inhibition after VX exposure was markedly less after both VX doses; ~40% inhibition after 12 μg/kg, and ~55% after 20 μg/kg VX. Though VX is a more potent inhibitor of AChE (with less affinity for BuChE as compared to sarin), VX-inhibited AChE ages slower than sarin-inhibited AChE, which enhances the potential for reactivation by OBI and might explain why AChE activity does not decline as much (Voicu et al., 2010). Besides VX and sarin, OBI has been shown to be a successful reactivator of AChE after exposure to paraoxon, but not soman (Snider et al, 2015, 2016; Thiermann et al., 2016; Wilhelm et al., 2014). In case of severe poisoning, in addition to initially lifesaving oxime and atropine treatment, an anticonvulsant should be included as soon as possible to prevent brain injury and advanced follow up medical care is required to support potential full recovery (Rice et al., 2016), and decontamination is to be included in case of dermal exposure and consequently possibly progressive poisoning (Joosen et al., 2017).

5. Conclusion

Taken together, the ATR/OBI co-formulation significantly improved survivability in VX-exposed animals over VEH treatment, whereas the efficacy of the single components was limited. The PK profiles of the co-formulated pharmaceutical ingredients ATR and OBI were similar to the PK profiles of the ingredients when administered separately, indicating no PK interactions when administered as a co-formulation. Together with a previous study by our lab it can be concluded that ATR/OBI is an effective countermeasure against exposure to both sarin and VX in guinea pigs. As the guinea pig is considered a valuable small animal model for translation to humans with regard to both the cholinergic enzyme balance and OP sensitivity, these results are a key addition in the development of effective medical countermeasures against chemical warfare agents.

6. Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors and was solely funded by Emergent BioSolutions Inc.

7. Funding body information

This work was solely funded by Emergent BioSolutions Inc. No external funding was received. The decision to publish the work was a joint decision of the funding organization (Emergent BioSolutions Inc.) and the performing organization (TNO, independent not for profit research organization).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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