Aus dem Walther-Straub-Institut für Pharmakologie und Toxikologie der Ludwig-Maximilians-Universität München

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Isolierte Organmodelle zur Entwicklung neuer therapeutischer Ansätze bei Organophosphatvergiftungen

Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus

Aichach

2018

Mit Genehmigung der Medizinischen Fakultät der Universität München

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Tag der mündlichen Prüfung: 11.12.2018

Zusammenfassung

Jährlich sterben mehrere hunderttausend Menschen durch Organophosphat (OP)-Vergiftungen, sei es in suizidaler Absicht mit Pestiziden oder im Rahmen militärischer Einsätze, wie 2013 und 2017 durch den Einsatz von Sarin in Syrien. Bei diesen Vergiftungen kommt es zu einer irreversiblen Hemmung der Acetylcholinesterase. Die Patienten leiden aufgrund der daraus resultierenden Übererregung der cholinergen Rezeptoren an Miosis, Salivation, Bronchorrhoe, Faszikulationen, Konvulsionen und einer peripheren, bzw. zentralen Atemlähmung, an der sie auch versterben können. Da die bisherige Standardtherapie bei einigen OP-Vergiftungen unzureichend ist, bedarf es neuer Therapeutika. Aus ethischen Gründen können Untersuchungen von potentiellen Antidoten nicht am Menschen erfolgen. Deswegen stellen isolierte Organmodelle, die schon seit Jahrzehnten zur Pharmaka-Testung verwendet werden, sinnvolle und notwendige *in vitro* Methoden zur Eignungsprüfung dar.

Ein möglicher Therapieansatz im Rahmen einer OP-Vergiftung ist die direkte Modulation von nikotinergen, bzw. muskarinergen Rezeptoren durch Bispyridinium-(Non)-Oxime, z.B. HI-6 und SAD-128. Die weiterentwickelte Leitsubstanz MB 327 zeigte in *in vitro* und *in vivo* Versuchen mit Soman vielversprechende Therapieerfolge, war jedoch in hohen Konzentrationen *in vivo* toxisch. Dies hat uns dazu veranlasst, erstmalig nikotinerge und muskarinerge rezeptoraktive Substanzen am Langendorff-Rattenherz und im Rattenjejunum, als Modell der glatten Muskulatur, zu untersuchen.

Keine der getesteten Substanzen war im isolierten Langendorff-Herz kardiotoxisch. Demgegenüber zeigten die getesteten Substanzen im isolierten

Dünndarm-Modell nach vorheriger Stimulation mit Carbamoylcholin eine konzentrationsabhängige Relaxation der glatten Muskulatur. Die Leitsubstanz MB 327 war hierbei am potentesten (EC_{50} = 0,7 x 10^{-5} M).

Aufgrund der Relevanz von Speziesunterschieden im Rahmen von OP-Vergiftungen und der entsprechenden Therapeutika-Entwicklung, wurden anschließend humane Dünndarmpräparate mit den Ergebnissen der Ratte verglichen. MB 327 zeigte einen zur Ratte vergleichbaren Effekt (EC_{50} = 0,7 x 10^{-5} M) bzw. entsprechende AChE-Aktivitäten.

Isolierte Organmodelle stellen eine praktikable Untersuchungsmethode potentieller Therapeutika im Rahmen einer OP- Vergiftung als Zwischenschritt von *in vitro* und *in vivo* Versuchen dar. Kardiologische Effekte lassen sich am isolierten Langendorff-Herz und glattmuskuläre Relaxation an Darmgewebe untersuchen, wobei für die relaxierende Wirkung eine Übertragbarkeit auf den Menschen gewährleistet ist.

Summary

Intoxications with organophosphorous compounds (OP) still pose a major threat. This is demonstrated by annual several hundred thousand deaths annually due to pesticide (self-) poisoning and the recent attacks with sarin in Syria 2013 and 2017. Here a partly irreversible inhibition of the enzyme acetylcholinesterase results in a subsequent overstimulation of cholinergic receptors. Patients suffer from miosis, salivation, bronchorrhoe, fasciculation, convulsions and a peripheral, as well as central respiratory paralysis possibly resulting in death. As the current standard therapy is insufficient for some OP compounds, new therapeutics have to be developed. Since these potential antidotes cannot be examined in humans due to ethical reasons and isolated organ models are used in pharmacological research since decades, these *in vitro* methods pose a sensible and necessary suitability test.

One possible therapeutic approach in case of OP poisoning is the direct modulation of nicotinergic and muscarinergic receptors with bispyridinium-(non)-oximes, e.g. HI-6 or SAD-128. The refined lead substance MB 327 showed promising results with soman *in vitro* and *in vivo*. However, it was toxic in high dosages *in vivo*. Therefore, nicotinic and muscarinic receptoractive substances were examined for the first time in the isolated Langendorff-heart, as well as the isolated rat jejunum, being a model for smooth musculature. None of the tested compounds showed a cardiotoxicity in the isolated Langendorff-heart. All substances displayed a concentration dependent smooth muscle relaxing effect in the isolated small bowel model; MB 327 being the most potent (EC_{50} = 0.7 x 10^{-5} M).

As species differences play a major role for OP poisoning as well as for their drug development, we compared human small bowel samples with the results gained with rat tissue in a follow-up study. MB 327 showed a comparable relaxing effect (EC_{50} = 0.7 x 10^{-5} M), and respective AChE-activity.

Summarizing, isolated organ models are useful tools to assess potential therapeutics for OP poisoning, as intermediate stage between *in vitro* and *in vivo* experiments. Cardiotoxic effects can be examined with isolated Langendorff-hearts. On the other hand the spasmolytic effect of different compounds can be investigated with small bowel samples, whereby transferability from rat to humans is warranted.

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema "Isolierte Organmodelle zur Entwicklung neuer therapeutischer Ansätze bei Organophosphatvergiftungen" selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Dr. Olga Prokopchuk (Chirurgische Klinik des Klinikum rechts der Isar, TU München) hat an der Durchführung dieser Arbeit mitgewirkt. Sie nahm die Patientenaufklärung, Probenentnahme und Probenübergabe des humanen Darmgewebes vor.

München, den 19.12.2018,

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(Katharina Marquart)

Inhalt

Abbildungsverzeichnis

Abkürzungsverzeichnis

ACh	Acetylcholin
AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
GA	Tabun
GB	Sarin
GD	Soman
GF	Cyclosarin
mAChR	muskarinerge ACh Rezeptoren
nAChR	nikotinerge ACh Rezeptoren
OP	phosphororganische Verbindungen
PAM	positiver allosterischer Modulator
NKS	Nervenkampfstoff(e)

Publikationsliste

Publikationen

1. **Neumaier, K.**, Worek, F., Thiermann, H., Wille, T., 2016. Bispyridinium non-oximes: An evaluation of cardiac effects in isolated hearts and smooth muscle relaxing effects in jejunum. **Toxicol. In Vitro** 35, 11–16. 10.1016/j.tiv.2016.05.005.
2. **Marquart, K.**, Prokopchuk, O., Worek, F., Thiermann, H., Martignoni, M.E., Wille, T., 2017. Human small bowel as a useful tool to investigate smooth muscle effects of potential therapeutics in organophosphate poisoning. **Toxicol. Lett.** 293, 235–240. 10.1016/j.toxlet.2017.11.012.

Zusätzliche Publikationen

1. Wille, T., **Neumaier, K.**, Koller, M., Ehinger, C., Aggarwal, N., Ashani, Y., Goldsmith, M., Sussman, J.L., Tawfik, D.S., Thiermann, H., Worek, F., 2016. Single treatment of VX poisoned guinea pigs with the phosphotriesterase mutant C23AL: Intraosseous versus intravenous injection. **Toxicol. Lett.** 258, 198–206. 10.1016/j.toxlet.2016.07.004.
2. Worek, F., Schilha, M., **Neumaier, K.**, Aurbek, N., Wille, T., Thiermann, H., Kehe, K., 2016. On-site analysis of acetylcholinesterase and butyrylcholinesterase activity with the ChE check mobile test kit-Determination of reference values and their relevance for diagnosis of exposure to organophosphorus compounds. **Toxicol. Lett.** 249, 22–28. 10.1016/j.toxlet.2016.03.007.

3. Worek, F., Seeger, T., **Neumaier, K.**, Wille, T., Thiermann, H., 2016. Blaptica dubia as sentinels for exposure to chemical warfare agents - a pilot study. **Toxicol. Lett.** 262, 12–16. 10.1016/j.toxlet.2016.09.006.

4. **Marquart, K.**, Herbert, J., Amend, N., Thiermann, H., Worek, F., Wille, T., 2018. Effect of cholinergic crisis on the potency of different emergency anaesthesia protocols in soman-poisoned rats. **Clin. Toxicol.** 11, 1–7. 10.1080/15563650.2018.1520241.

1. Einleitung

Der Einsatz von Sarin in Syrien 2013 und 2017 sowie mehrere hunderttausend Tote jedes Jahr durch Vergiftungen mit Organophosphatpestiziden zeigen die hohe toxikologische Relevanz von phosphororganischen Verbindungen (OP) [1–4]. Im Nachfolgenden werden zunächst die Unterschiede von verschiedenen OP und deren Wirkmechanismus aufgezeigt sowie Diagnostik- und Therapiemöglichkeiten erklärt. Anschließend wird auf nikotinerge bzw. muskarinerge rezeptormodulierende Substanzen eingegangen und deren Untersuchung in verschiedenen Organmodellen und entsprechende Ergebnisse in beiden Veröffentlichungen diskutiert.

1.1 Phosphororganische Verbindungen

Zu den OP zählen neben einer Vielzahl von Pestiziden auch Nervenkampfstoffe (NKS). Hierbei wird zwischen der G-Reihe, bestehend aus Tabun (GA), Sarin (GB), Soman (GD), Cyclosarin (GF), und der V-Reihe (VX, VR, CVX) unterschieden (siehe Abb. 1-1). Diese Phosphon- bzw. Phosphorsäurederivate mit zwei Alkylgruppen und einer Abgangsgruppe weisen Unterschiede hinsichtlich ihrer Toxizität und ihrer physikochemischen Eigenschaften auf [5,6]. Kampfstoffe der G-Reihe sind hydrolyselabilere und volatilere Flüssigkeiten als die Substanzen der V-Reihe und ihre Aufnahme erfolgt meist inhalativ. Bei den hydrolysestabileren NKS der V-Reihe hingegen kommt es meist zu einer perkutanen Vergiftung. Dies kann in einer kontinuierlichen Resorption des NKS resultieren und zu einer verzögerten, jedoch länger andauernden Vergiftung führen [5,7,8].

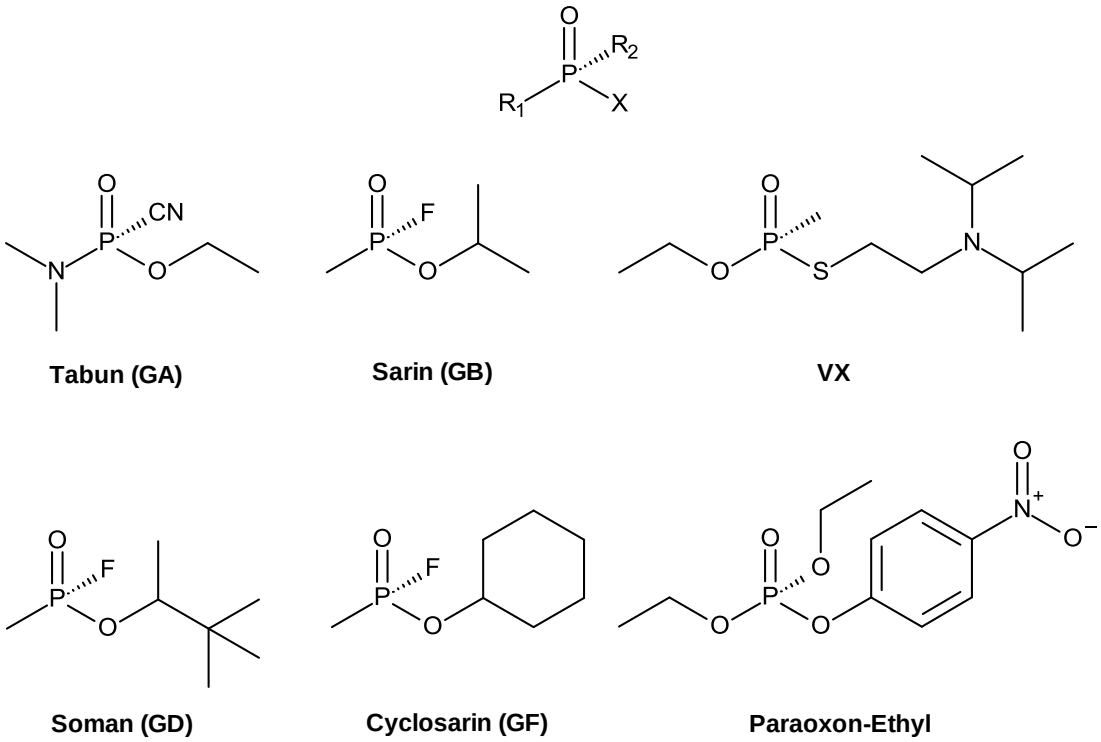

Abb. 1. 1 Strukturformeln verschiedener OP-Verbindungen. Dargestellt ist die Grund-Strukturformel aller OP, verschiedene NKS der G-Reihe bzw. VX und Paraoxon-Ethyl als Vertreter der Pestizide.

1.2 Wirkmechanismus

Bei einer Vergiftung mit OP kommt es zur irreversiblen Hemmung von Esterasen, vor allem der Acetylcholinesterase (AChE). Hierbei wird die Serin-Hydroxyl-Gruppe im aktiven Zentrum des Enzyms phosphoryliert bzw. phosphonyliert [5]. Durch diese Hemmung der lebensnotwendigen AChE kommt es zu einer Akkumulation von Acetylcholin (ACh) im synaptischen Spalt, was zu einer Übererregung der cholinergen Rezeptoren mit cholinerger Krise (siehe 1.3.1) führt [3,9–11]. Die phosphorylierte bzw. phosphonylierte AChE kann in einigen Fällen durch spontane Hydrolyse reaktiviert werden (Spontanreaktiverung; Abb. 1-2). Bei einigen NKS, wie z.B. Soman, kommt es innerhalb weniger Minuten durch Dealkylierung zu einer schnellen „Alterung“

der AChE. Der so entstandene stabile Komplex kann anschließend nicht mehr durch Nukleophile reaktiviert werden (siehe Abb. 1-2) [5,6,8,12,13]. Bei einigen OP, wie z.B. Tabun, ist eine Reaktivierung aufgrund stabilisierender Konformationsänderungen des Enzym-NKS-Komplexes schwer möglich [5,14]. Im besten Fall kann durch Nukleophile, wie z.B. Oxime, eine Ablösung des OP-Restes von der AChE erreicht werden und so die Enzymfunktion reaktiviert werden (siehe Abb. 1.4).

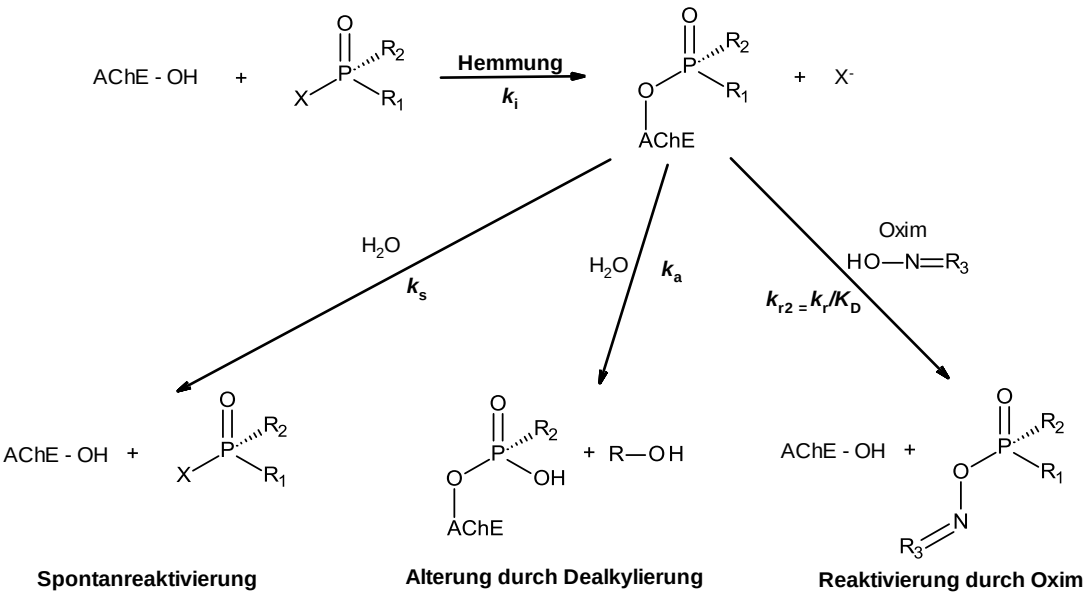

Abb. 1. 2 Reaktion eines Organophosphats (OP) mit dem Enzym Acetylcholinesterase (AChE) und die daraus resultierende Hemmung der AChE. Die Hemmkonstante k_i ist abhängig von den chemischen Eigenschaften des OPs. In den folgenden Schritten sind die Spontanreaktivierung (Spontanreaktivierungskonstante k_s), Alterung durch Dealkylierung (Alterungskonstante k_a) und die Reaktivierung durch ein Oxim (Reaktivierungskonstante k_{r2}) dargestellt. Modifiziert nach [15].

1.3 Diagnostik

1.3.1 Symptomatik

Durch die Hemmung der AChE im Rahmen einer OP Vergiftung kommt es zu einer Akkumulation von ACh im synpatischen Spalt. Infolge dessen werden

muskarinerge bzw. nikotinerge ACh Rezeptoren (mAChR, bzw. nAChR) überstimuliert (vgl. Kapitel 1.2). MAChR kommen im Gehirn und in parasympathischen Erfolgsorganen vor, wobei bisher fünf Subtypen (M1 bis M5) mit Signaltransduktion durch heptahelikale Rezeptoren mit Aktivierung eines G-Proteins beschrieben sind [5,16,17]. NAChR hingegen sind Ionenkanäle, die aus fünf Untereinheiten bestehen und im Gehirn, in vegetativen Ganglien und an neuromuskulären Endplatten vorkommen [5]. Je nach Ort der Übererregung wird deswegen zwischen drei unterschiedlichen Symptomengruppen unterschieden: muskarinerge (Miosis, Bradycardie Bronchorrhoe, Bronchospasmus, Hyperhidrose, Salivation, Defäkation, Urinabgang), nikotinerge (Tachykardie, Hypertension, Faszikulationen, Plegien) und zentrale Symptome (Atemdepression, Angstzustände, Unruhe, Schwindel, Kopfschmerzen, Tremor, Konvulsionen). Die Patienten versterben i.d.R. an einer Kombination aus peripherer bzw. zentraler Atemlähmung [5,6,13]. Bei inhalativen Intoxikationen, wie z.B. durch Sarin, zeigen die Patienten erst lokale Veränderungen an den Schleimhäuten und Augen (z.B. Miosis, Rötungen, Lakrimation), anschließend kommt es rasch zu einer Generalisierung. Bei perkutaner Intoxikation hingegen, z.B. durch VX, tritt die Symptomatik verzögert auf [6].

1.3.2 Labordiagnostik

Da die Symptomatik bei inhalativen Intoxikationen schnell nach der Exposition auftritt und umgehend therapeutische Maßnahmen ergriffen werden müssen, kommt der Labordiagnostik nur ein konfirmatorischer Nachweis zu. Bei V-Stoffen hingegen, bei denen es zu einer langsamen Resorption kommt, stellt sie

die einzige Möglichkeit dar, um ggf. schon vor dem Auftreten der Symptome mit therapeutischen Maßnahmen zu beginnen. Die Ermittlung der Cholinesteraseaktivitäten des Patienten ist hier die einzige einsatzrelevante Diagnostikmethode (ChE check mobile bzw. ChE status monitor, Securetec Detektions-Systeme AG, Neubiberg, Deutschland). Hierbei wird die AChE-Aktivität im Vollblut und die Butyrylcholinesterase (BChE)-Aktivität im Vollblut bzw. Plasma mittels kolorimetrischen Ellman Assays gemessen [6,18–20]. Des Weiteren können NKS, deren Metabolite und Proteinaddukte im Vollblut, Plasma und Urin durch aufwendige Verfahren im Labor nachgewiesen werden. Diese Methoden besitzen jedoch keine Relevanz für die Einleitung und die Steuerung der Therapie [6,21–25].

1.4 Standardtherapie

Die bisherige Standardtherapie im Rahmen einer OP-Vergiftung besteht aus Atropin und einem Oxim.

Atropin wirkt hierbei als kompetitiver muskarinerger Rezeptorantagonist, verhindert somit eine Übererregung und die Ausbildung lebensbedrohlicher Symptomatik, wie z.B. Bronchokonstriktion und zentrale Atemdepression [12,13,17,26–29]. In hohen Konzentrationen wirkt es sogar krampflösend [17]. Initial sollten dem Patienten 2 mg Atropin intravenös oder intramuskulär verabreicht werden. Diese Dosis wird bis zum Sistieren der Symptomatik verdoppelt (4 – 8 – 16 – 32 mg, etc.) [17,26]. Dies kann dazu führen, dass Atropinkonzentrationen erreicht werden, die in unvergifteten Patienten zu schwerwiegenden Nebenwirkungen führen können [30].

Oxime hingegen reaktivieren die AChE indem sie die kovalente Bindung zwischen dem OP und dem Enzym in zwei Schritten brechen (siehe Abb. 1-2) [27,29,31]: Zuerst bindet das Oxim an die kovalent gebundene Phosphylgruppe, wodurch ein Oxim-Phosphyl-AChE-Konjugat entsteht [13,29,32]. Anschließend wird die reaktivierte AChE freigesetzt (Abb. 1-2) [12,27,29,32]. Die Reaktivierung wird durch mehrere Faktoren, wie z.B. Speziesunterschiede, spontane Reaktivierung und die "Alterung" der AChE beeinflusst (siehe Abb. 1-2) [33–40].

In Deutschland und weiteren Ländern wird das 1964 entwickelte Obidoxim verwendet [41,42]. Die vorgeschlagene Dosierung liegt hier initial bei einem Bolus von 250 mg und anschließender kontinuierlicher intravenöser Gabe von 750 mg über 24 Stunden [27,43,44]. Der ATOX II Combopen® (Meridian Medical Technologies Ltd, Belfast, Großbritannien) Autoinjektor enthält neben 2 mg Atropinsulfat 220 mg Obidoximchlorid. Das in den 1950er Jahren synthetisierte Pralidoxim (2-PAM) hingegen wird in Großbritannien und USA, aber auch in asiatischen Ländern eingesetzt [42,45–47]. Autoinjektoren enthalten 600 mg Pralidoximchlorid [48,49]. Zusätzlich gibt es noch TMB-4, das in Israel und HI-6, welches in Kanada und in Schweden in Autoinjektoren zum Einsatz kommt [13]. Jedoch sind alle bisher entwickelten Oxime nur gegen ein begrenztes OP-Spektrum wirksam. Obidoxim z.B. ist gegen Paraoxon, Sarin und VX effektiv wirksam. Es ist jedoch nur ein schwacher Reaktivator gehemmter AChE bei Cyclosarin- und Tabun-Vergiftungen [13,29].

Die im Rahmen der Vergiftung auftretenden Krampfanfälle sollten mit Benzodiazepinen behandelt werden. Hierbei wird je nach Schweregrad initial eine Dosis von 5 – 20 mg intravenös verabreicht [50,51].

1.5 Alternativer Therapieansatz: Bispyridinium-Non-Oxime

Da die bisherige Standardtherapie bestehend aus Atropin und einem Oxim (z.B. Obidoxim) bei einigen Nervenkampfstoffen, wie z.B. bei Soman, Tabun oder Cyclosarin, und Pestiziden, wie z.B. Fenamiphos und Profenofos, unzureichend ist, bedarf es neuer Therapeutika (vgl. Kapitel 1.1 und 1.4). Eine vielversprechende Möglichkeit stellt dabei die Modulation nikotinerger Rezeptoren dar.

Schon in den 1970er bzw. 1980er Jahren zeigten Oxime (z.B. HI-6) sowohl *in vitro* als auch *in vivo* neben der Reaktivierung der AChE einen therapeutischen Effekt [30,52–55]. Für SAD-128, eine weiterentwickelte Substanz ohne Oximgruppe, konnte eine Interaktion mit nikotinergen und muskarinergen Rezeptoren nachgewiesen werden [55,57–62]. Aufgrund dieser Rezeptormodulation war SAD-128 in *in vivo* Versuchen mit Soman-vergifteten Mäusen wirksam, ohne die gehemmte AChE zu reaktiveren [63–65]. Neben SAD-128 konnte eine Vielzahl von weiteren Bispyridinum-(Non)-Oximen die muskuläre Übertragung nach Somanvergiftung am isolierten Rattendiaphragma wiederherstellen [66]. MB 327 (1,1'-(propan-1,3-diyl)bis(4-tert-butylpyridinium)), eine weiterentwickelte Variante von SAD-128, wurde zunächst im Defence Science & Technology Laboratory, Porton Down, UK, untersucht und zählt zu den Bispyridinium-Non-Oximen. Sie zeichnet sich durch zwei Tert-Butyl-Gruppen und einen Propyl-Linker, der die beiden Pyrdiniumringe verbindet, aus (siehe Abb. 1-3) [30]. MB 327 zeigte ebenfalls eine rezeptormodulierende Wirkung an nikotinergen und muskarinergen Rezeptoren [67–69] sowie einen anti-nikotinergen Effekt in Rhabdomyosarkom-Zellen [69]. Neueste Studien belegen, dass Bispyridinium-Non-Oxime an muskarinergen Rezeptoren als

kompetitiver Antagonist über eine orthosterische Bindungsstelle und an nikotinergen Rezeptoren als positiv allosterische Modulatoren (PAM) wirken [67,70,71]. Zusätzlich war MB 327 in der Lage, die neuromuskuläre Übertragung in der quergestreiften Muskulatur nach Somanvergiftung wiederherzustellen [69,72]. Darüber hinaus hatte es *in vivo* einen protektiven Effekt bei Soman-vergifteten Meerschweinchen. Kontrolltiere, die kein Soman injiziert bekommen hatten, zeigten jedoch ab 30 mg/kg MB 327 Vergiftungserscheinungen in Form von schlaffer Lähmung und respiratorischer Insuffizienz. Ab 100 mg/kg wirkte MB 327 sogar tödlich, wobei der genaue Mechanismus der Toxizität nicht geklärt wurde [73].

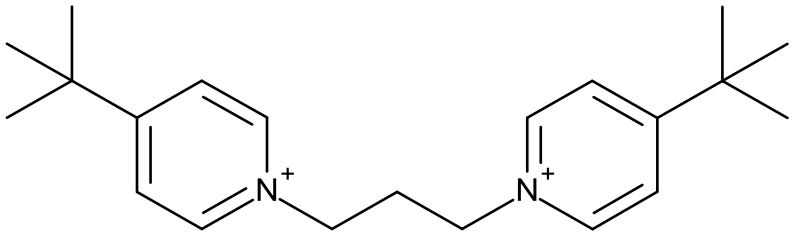

Abb. 1. 3 Strukturformel von MB 327. MB 327 (1,1'-(propan-1,3-diyl)bis(4-tert-butylpyridinium)) besitzt zwei Tert-Butylgruppen und einen Propyl-Linker.

Bispyrdinium-Non-Oxime wirken somit sowohl an mAChR als auch an nAChR. Voraussetzungen für eine mögliche Zulassung von cholinergen Antagonisten sind jedoch eine gute Verträglichkeit in unvergifteten Patienten, die Wirksamkeit selbst bei hohen ACh-Konzentrationen und eine erhaltene, kontrollierte Erregungsweiterleitung [30].

1.6 Speziesunterschiede

Ratten wurden bis in die späten 1970er Jahre als das Standardversuchstier für Versuche mit Nervenkampfstoffen verwendet [8]. Jedoch hatten einige (potentielle) Therapeutika, die in anderen Spezies wirksam waren, in diesem Tiermodell eine viel schwächere Wirkung [8].

Zusätzlich weist diese Tierart im Gegensatz z.B. zum Meerschweinchen, hohe Aktivitäten der Plasma-Carboxylesterasen auf. Diese funktionieren als natürliche Bioscavenger und können somit die Toxizität von OP um bis zu 90 % reduzieren [74–77]. Obwohl die AChE nur von einem Gen kodiert wird und somit in jedem Gewebe einer Spezies hoch konserviert vorliegt [9,78], gibt es deutliche Unterschiede zwischen verschiedenen Tierarten hinsichtlich der Struktur und somit der Kinetik dieses Enzyms [13].

Diese Diskrepanzen können die Übertragbarkeit für die in der Ratte gewonnenen Daten beeinflussen. So war in Soman-vergifteter, quergestreifter Muskulatur die Wiederherstellung der neuromuskulären Aktivität im humanen Gewebe nur mit MB 327, nicht jedoch mit HI-6 möglich. In der Ratte hingegen zeigten beide Substanzen einen therapeutischen Effekt [53,72,79,80].

1.7 Ziel der Dissertation

Die Therapielücken bei Vergiftungen mit OP-Pestiziden und NKS, die trotz jahrzehntelanger, intensiver Forschung nach einem Breitspektrum-Therapeutikum weiterhin bestehen, unterstreichen den Forschungsbedarf für die Entwicklung neuartiger Therapieansätze bei OP-Vergiftungen. Da humane Studien aus ethischen Gründen nicht möglich sind, stellen *in vitro* Versuche mit

tierischem bzw. humanem Gewebe eine unerlässliche Untersuchungsmethode dar. In der pharmakologischen und toxikologischen Forschung werden isolierte Organmodelle eingesetzt, um potentielle Therapeutika im komplexen Zellverband zu untersuchen [81]. So wurden diese bisher u.a. in der quergestreiften Muskulatur, in Precision Cut Lung Slices, aber auch in der glatten Muskulatur getestet [71,72,79,82–84]. Speziesspezifische Unterschiede in der Toxizität von OP aufgrund verschiedener Cholinesterasen erschweren die Übertragbarkeit von tierischen Daten auf die humane Situation [39,85].

Ein vielversprechender Therapieansatz im Rahmen einer OP-Vergiftung ist die Modulation von nikotinergen bzw. muskarinergen Rezeptoren. Bispyridinium-Non-Oxime sind in der Lage, an nikotinergen Rezeptoren als PAM und an muskarinergen Rezeptoren als kompetitive Antagonisten zu binden [67,70,71]. Diese Wirkstoffgruppe, vor allem die Leitsubstanz MB 327, zeigte vielversprechende Therapieerfolge im Rahmen einer OP-Vergiftung *in vitro* und *in vivo*. Jedoch führte MB 327 im Tierversuch mit Meerschweinchen in Dosen knapp oberhalb des therapeutischen Bereichs zum Tod der Tiere [73]. Daher wurde in der ersten Studie dieser Arbeit der Effekt von Bispyridinium-Non-Oximen an zwei unterschiedlichen Modellen getestet: 1) dem isolierten Langendorff-Herz, um eine mögliche Kardiotoxizität der Substanzen zu untersuchen und 2) dem Modell der glattmuskulären Relaxation, um die anticholinerge Wirksamkeit am Rattendarm nachzuweisen. (siehe Kapitel 2.1). Keine der getesteten Substanzen war hierbei kardiotoxisch. In der glatten Muskulatur hingegen, zeigten alle Bispyridinium-Non-Oxime nach vorheriger Stimulation mit Carbamoylcholin einen deutlichen spasmolytischen Effekt.

Da speziesspezifische Unterschiede bei der Toxizität von OP und der Wirksamkeit von Antidoten vorliegen, die ggf. die Übertragbarkeit der in der

Ratte gewonnenen Daten auf den Menschen verhindern, wurde ein humanes Darmmodell vergleichbar zu dem Modell der glatten Muskulatur mit Rattengewebe aus der ersten Veröffentlichung etabliert. Hierbei wurden verschiedene Bispyridinium-Verbindungen untersucht, um die Ergebnisse mit den zuvor gewonnenen Rattendaten zu validieren. Zusätzlich wurden die Cholinesteraseaktivität im humanen und im Ratten-Dünndarmgewebe (Jejunum, Ileum) gemessen. Die Untersuchungen weisen auf eine Übertragbarkeit der für aus Rattengewebe mit Bispyridinium-(Non)-Oxim-Verbindungen gewonnenen Daten auf den Menschen hin (siehe Kapitel 2.2).

2. Veröffentlichungen

2.1 Veröffentlichung I

Toxicology in Vitro 35 (2016) 11–16

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Bispyridinium non-oximes: An evaluation of cardiac effects in isolated hearts and smooth muscle relaxing effects in jejunum

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ARTICLE INFO

Article history:
Received 28 January 2016
Received in revised form 10 May 2016
Accepted 11 May 2016
Available online 13 May 2016

Keywords:
Bispyridinium non-oximes
Cardiac
Isolated heart
Oximes
Smooth muscle function

ABSTRACT

Bispyridinium non-oximes seem to be promising candidates for the generic treatment of nerve agent poisoning as they interact with nicotinic and muscarinic acetylcholine receptors. The lead compound MB327 showed therapeutic effectiveness in vitro and in vivo but was toxic at higher doses. In the present study, the effect of various bispyridinium non-oximes on isolated heart and small intestine function was investigated. Bispyridinium non-oximes and oximes were tested in at least seven different concentrations in rat jejunum preparations pre-treated with carbachol. All bispyridinium non-oximes showed classical dose response curves with MB327 being the most effective (EC_{50} = 6.6 μM) and MB782 being slightly less effective (EC_{50} = 10.4 μM). Neither the bispyridinium non-oximes nor the oximes showed cardiotoxic effects in the isolated Langendorff heart. The tested bispyridinum compounds showed no direct cardiac effect but had variable smooth muscle relaxing effects. Further in vivo studies are required to get more insight into potential toxic mechanisms of these promising nerve agent antidotes.

1. Introduction

Poisoning by organophosphorus (OP) pesticides in suicidal intention or by accident leads up to 300,000 deaths worldwide every year, primarily in southeast Asia (Gunnell et al., 2007). In addition, the recent homicidal use of the OP nerve agent sarin in Syria 2013, exerting a substantial higher toxicity than OP pesticides resulted in an enormous number of poisoned civilians and in thousands of deaths (Rosman et al., 2014). The toxic mechanism of OP compounds is mediated by the inhibition of the pivotal enzyme acetylcholinesterase (AChE) with subsequent accumulation of acetylcholine (ACh) in the synaptic cleft. This ACh-overflow finally leads to cholinergic crisis and death by central and peripheral respiratory failure (Marrs, 1993; Thiermann et al., 2013). Currently, a pre-treatment is only feasible in a military environment, e.g. with the reversible AChE inhibitor pyridostigmine (Lundy, 1999). However, its use is discussed controversially and a relation to the Gulf War syndrome has been postulated (Binns, 2008). Current standard post-exposure therapy consists of an enzyme reactivator, mostly obidoxime or pralidoxime (2-PAM) in combination with atropine (Eyer, 2003). Oximes restore the AChE function by removing the phosphyl group bound to the active site of the enzyme (Worek and Thiermann, 2013) and atropine acts as competitive muscarinic receptor antagonist. Unfortunately, 60 years of research for a multipotent broad spectrum oxime covering all OPs were not successful (Eyer and Worek, 2007; Worek et al., 2004; Worek and Thiermann, 2013).

An alternative therapeutic approach counteracts the toxic effects of OP compounds at nicotinic receptors. Previous work with the bispyridinium non-oxime SAD128 and the bispyridinium oxime HI-6 indicate interaction of such compounds with nicotinic and muscarinic receptors (Alkondon et al., 1988; Alkondon and Albuquerque, 1989; Clement, 1981; Kuhnen-Clausen, 1972; Lundy and Tremblay, 1979). In fact, SAD-128 showed a therapeutic effect in soman-poisoned mice and HI-6 could restore OP-blocked neurotransmission in isolated rat diaphragms without reactivating inhibited AChE (Clement, 1981; Oldiges and Schoene, 1970; Schoene et al., 1976; van Helden et al., 1991). A variety of bispyridinium non-oximes were able to partially restore soman-blocked neurotransmission in isolated guinea pig diaphragms (Tattersall, 1993). MB327 (Table 1) is at present the most promising compound. It restored neurotransmission in soman-blocked rat, guinea pig and human respiratory muscles and showed therapeutic effectiveness in nerve agent-poisoned guinea pigs in vivo (Price et al., 2015; Seeger et al., 2012; Turner et al., 2011). However, a recent guinea pig study demonstrated a toxic effect at high MB327 doses (Price et al., 2015). This prompted us to investigate potential effects of MB327 and related bispyridinium compounds in isolated rat heart and jejunum models.

2. Materials and methods

2.1. Chemicals

Obidoxime was supplied by Merck KG (Darmstadt, Germany) and HI-6 dichloride monohydrate was kindly donated by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada).

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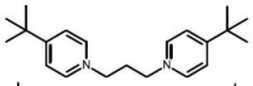
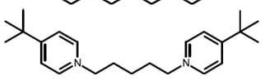
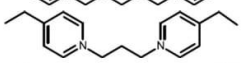
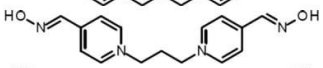
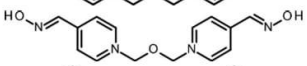
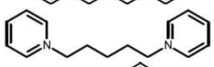
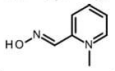
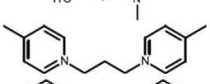
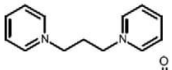
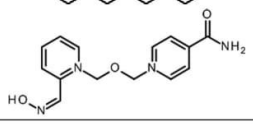
http://dx.doi.org/10.1016/j.tiv.2016.05.005
0887-2333/

Table 1
Smooth muscle relaxing effects and chemical structure of different compounds. The EC_{50} is given as mean and the corresponding confidence interval is presented (n ≥ 10 segments of different parts of the jejunum of at least three rats per concentration).

Compound	Structure	EC_{50} (μM)	Confidence interval (μM)
MB327		6.6	6.3–7.0
MB782		10.4	9.7–11.2
MB454		35.4	32.7–38.2
TMB-4		35.8	34.4–37.2
obidoxime		126.3	118.7–134.3
MB442		232.2	209.1–257.8
2-PAM		261.8	246.0–278.7
MB414		263.8	240.9–289.0
MB408		491.9	456.5–430.0
HI-6		963.0	910.6–1018.0

Carbachol, pyridine-2-aldoxime-methochloride (2-PAM) and TMB-4 were purchased from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany) and NaCl, $CaCl_2$, $NaHCO_3$, NaH_2PO_4, Glucose and KCl were delivered by Carl Roth GmbH + Co. KG (Karlsruhe, Germany). MB327, MB 408, MB 414, MB442, MB454 and MB782 were kindly supplied by Dr. C. M. Timperley (DSTL, Porton Down, UK; see Table 1). All tested compounds showed 98% purity based on the analysis of LC–MS, $^1H/^{13}C$ NMR.

2.2. Rat heart preparation

All experiments were in accordance with the German Animal Welfare Act of 18 May 2006 (BGBl. I S. 1206, 1313) and the European Parliament and Council Directive of 22 September 2010 (2010/63/EU).

Male Wistar rats (300 ± 50 g; Charles River, Sulzfeld, Germany) were held in small groups of 6 animals with a 12/12 h light-dark cycle, standard laboratory diet (Altromin, Lage, Germany) and water ad libitum. The rats were given an at least 7 day adapting phase. At the time of the experiment the animals did not show any signs of disorder or disease.

After the heart was dissected it was immediately placed in ice cold buffer and fixed at a modified Langendorff apparatus (Bell et al., 2011; Langendorff, 1895; Skrzypiec-Spring et al., 2007; Sutherland and Hearse, 2000). The heart was perfused via the aorta and coronary arteries with gassed (95% O_2 and 5% CO_2), modified Krebs Henseleit buffer (NaCl 118.0 mM, $NaHCO_3$ 24.88 mM, glucose 5.55 mM, KCl 4.5 mM, sodium pyruvate 2.0 mM, $MgSO_4$ 1.66 mM, $CaCl_2$ 1.6 mM, KH_2PO_4 1.2 mM; pH = 7.4, T = 38.0 °C) at constant pressure (60 mm Hg). The Langendorff heart setup consisted of a gassed buffer reservoir (Radnoti, Monrovia, CA, USA) with a downstream pump (IPC High Precision Multichannel Dispenser, Ismatec, IDEX, Wertheim, Germany), which could either work with constant pressure or constant flow (Fig. 1). The buffer was then pumped in a smaller non-gassed reservoir with a bubble-trap, after which an inline flow probe (ME3PXN,

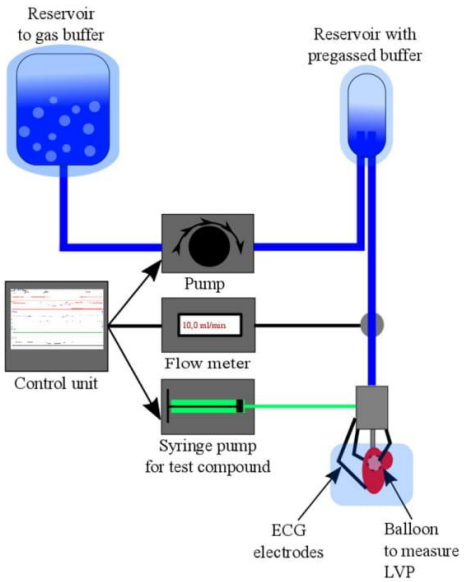


Fig. 1. Experimental setup of the isolated heart model with feedback mechanism for the buffer pump and the substance pump. The pump transported buffer from a gas-flushed reservoir to a smaller reservoir with a bubble trap (not shown). Then the flow probe measured the actual buffer flow through the aorta and coronary arteries and the flow meter transmitted either a positive or a negative feedback to the buffer and substance pumps. The heart and reservoirs were water-jacketed (39 °C) to maintain a constant temperature.

Transonic Systems Inc., Ithaca, NY, USA) was installed. This sent a feedback signal to the buffer pump and the syringe pump (PH 2000 Infusion, Harvard Apparatus, March-Hugstetten, Germany) via the flowmeter (Transit-time tubing flowmeter TS410, Transonic Systems Inc., Ithaca, NY, USA) and the computer software Haemodyn W (Hugo Sachs Electronics, March-Hugstetten, Germany) for autoregulation. The setup enabled to adjust the flow of the syringe pump proportionally to the buffer pump resulting in constant concentrations of the test compounds in the heart even during arrhythmias and pressure changes. A balloon, made from cling film, was filled with distilled water and was placed in the left ventricle with a diastolic pressure of 4–10 mm Hg. Two electrodes were positioned at the left atrium and the left ventricle for ECG-monitoring. The reservoirs, the tubing and the heart were water-jacketed to maintain a constant temperature which was 36.5 ± 0.4 °C (mean ± SD for all experiments) in the left ventricle.

2.3. Experimental protocol heart

The heart was equilibrated for up to 30 min with a constant perfusion of 60 mm Hg. Then, 0.5 ml buffer was injected via the syringe pump to quantify possible turbulence due to the injection, to check the feedback loop and to record the baseline values. Thereafter, five different test compound concentrations were injected in a cumulative manner. Due to the perfusion with constant pressure, the flow rate was variable, but on an average the substance affected the heart for 5 min. After injecting 0.05 ml of test compound or buffer data were evaluated for 3 min. After the fifth dose of test compound the heart was washed with buffer solution for 5 min and the baseline was again evaluated. Control experiments were performed with injections of modified Krebs Henseleit buffer.

2.4. Data analysis heart

Data were recorded for each test compound concentration and were calculated as % of baseline: heart rate (HR, bpm), systolic left ventricular pressure (LVP_{sys}, mmHg), diastolic left ventricular pressure (LVP_{dias}, mmHg), delta left ventricular pressure (ΔLVP, mmHg), coronary flow (CFlow, ml/min), maximum relaxation velocity ($dLVPt_{min}$) and maximum contraction velocity ($dLVPt_{max}$). All data are means ± SD for n ≥ 4 individual hearts. A Kruskal-Wallis-Test with Dunns-post-test was employed to test for significant differences ($p < 0.05$).

Haemodyn W (Hugo Sachs Electronics, March-Hugstetten, Germany), GraphPad Prism 5.04 (San Diego, CA, USA) and Microsoft-Excel were used for data recording and analysis.

2.5. Rat jejunum preparation

In accordance with the 3 Rs (Replacement, Reduction and Refinement) the animals served also as jejunum donators. We applied the previously described model (Königer et al., 2013) with slight modifications. Approximately 15 cm of the aboral part of the jejunum were excised and cut in 10 pieces (each ~1 cm, with discharging the first centimetre). Than the pieces were placed into ten gassed (95% O_2 and 5% CO_2) 15 ml water-jacketed single organ baths (Experimetria Ltd., Budapest, Hungary) containing Tyrode buffer (NaCl 137.0 mM, $NaHCO_3$ 22.0 mM, glucose 5.5 mM, KCl 2.68 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.05 mM, NaH_2PO_4 0.42 mM; pH = 7.4; T = 38.0 °C). They were fixed with hooks on one side to a glass retainer (Michael Murner, Waging, Germany) and on the other side to a force transducer which was connected to an amplifier (Hugo Sachs, March-Hugstetten, Germany). Five organ baths were connected to one gassed (95% O_2 and 5% CO_2) reservoir and one vacuum flask (Radnoti, Monrovia, CA, USA) to allow rapid buffer changes.

2.6. Experimental protocol jejunum

The segments were allowed to equilibrate at approximately 1 Nm basic tension for 30 min during which the buffer was changed twice in a 15 min interval. The pretension was adjusted if necessary. After 38 min the baseline was evaluated for 2 min and $\overline{x}_{basal\ activity}$ was calculated for each channel. Then 2 μM carbachol were added and channels were recorded for 5 min. The last 2 min of this interval were used to calculate the Area Under Curve ($AUC_{carbachol}$), followed by washing the jejunum twice with buffer and an adjusting phase of 10 min during which the last 2 min served as a baseline ($\overline{x}_{basal\ activity}$) for each dose of carbachol plus test compound. Subsequently, carbachol (2 μM) and then 2 min later the test compound in increasing concentrations were added and the alteration of smooth muscle force was recorded. The last 2 min were used to calculate $AUC_{substance}$.

2.7. Data analysis jejunum

The effect of the oxime on the small muscle relaxation was calculated with following formulas:

$$AUC_{carbachol} = \sum (y - baseline)$$

$$AUC_{substance} = \sum (y - baseline)$$

y represented the recorded value.

Finally, the isometric force was calculated as % of maximal carbachol stimulation by the equation:

$$\%\ \text{isometric force} = (AUC_{substance}/AUC_{carbachol}) \times 100$$

The EC_{50} values were calculated by non-linear normalized regression analysis from semi-logarithmic plots of the test compound concentration versus % of isometric force. A normalized curve was used because data should range between 0 and 100%. Values out of the range were probably due to an overreaction on the second carbachol dose or a relaxation in the end of the experiment. All data are shown as means and the corresponding confidence intervals in μM of n ≥ 10 segments of different parts of the jejunum of at least three rats per concentration of one compound. Due to the high sample size a one-way analysis of variance (ANOVA) with Bonferroni post-test was employed to test for significant differences ($p < 0.05$).

Table 2
Sham administration of test compounds (buffer) in the isolated heart indicates a stable system for different cardiac parameters during the experiment duration. Data are given as mean values ± SD (n ≥ 4).

Parameter	1. Sham administration	2. Sham administration	3. Sham administration	4. Sham administration	5. Sham administration	6. Sham administration
Heart rate	100.4 ± 4.6	98.7 ± 3.9	100.6 ± 6.8	101.6 ± 8.1	101.4 ± 8.7	101.0 ± 9.2
LVP_{sys}	103.1 ± 1.9	100.8 ± 4.3	98.5 ± 4.4	99.0 ± 3.8	100.7 ± 4.9	99.3 ± 5.8
LVP_{dias}	98.6 ± 4.1	97.9 ± 3.4	100.4 ± 2.4	99.8 ± 2.2	98.7 ± 3.5	89.9 ± 3.8
Cflow	99.2 ± 1.4	96.5 ± 3.5	96.6 ± 3.3	95.9 ± 3.7	94.5 ± 4.7	93.1 ± 6.3
$dLVPdt_{max}$	107.3 ± 14.2	105.3 ± 13.3	96.7 ± 7.3	98.5 ± 10.7	104.1 ± 11.8	100.9 ± 9.0
$dLVPdt_{min}$	111.1 ± 12.9	110.7 ± 22.7	98.5 ± 2.5	98.5 ± 3.4	102.2 ± 6.7	99.3 ± 6.3

Table 3
Bispyridinium non-oximes and oximes do not show cardiac effects in the isolated Langendorff heart model. Concentrations used in the experiments with bispyridinium non-oximes and TMB-4 were 50 μM, 100 μM, 200 μM, 300 μM and 500 μM. With obidoxime, HI-6 and 2-PAM 1 μM, 5 μM, 10 μM, 50 μM and 100 μM were applied. Data are shown as mean ± SD % of baseline ($n \geq 4$).

	Heart rate					LVP_{sys}					LVP_{dias}				
Compound	1. Dose	2. Dose	3. Dose	4. Dose	5. Dose	1. Dose	2. Dose	3. Dose	4. Dose	5. Dose	1. Dose	2. Dose	3. Dose	4. Dose	5. Dose
MB 327	102.0 ± 3.3	101.9 ± 4.4	99.4 ± 6.4	97.2 ± 11.5	96.6 ± 9.0	100.8 ± 1.7	99.8 ± 1.7	103.4 ± 9.3	105.6 ± 9.7	106.6 ± 11.0	99.4 ± 3.3	98.8 ± 5.9	98.3 ± 9.5	98.5 ± 11.1	98.3 ± 11.8
MB 782	100.2 ± 6.2	101.5 ± 10.1	102.6 ± 12.6	101.2 ± 10.8	103.4 ± 14.0	105.0 ± 6.5	108.4 ± 5.5	110.4 ± 7.7	116.2 ± 8.4	103.4 ± 15.8	98.5 ± 1.7	99.4 ± 6.7	97.9 ± 6.0	97.5 ± 4.6	96.3 ± 7.1
MB 454	97.2 ± 0.8	102.2 ± 11.3	99.3 ± 9.7	95.4 ± 10.7	94.4 ± 11.7	99.9 ± 0.9	97.1 ± 9.3	100.8 ± 9.0	101.4 ± 11.7	98.3 ± 13.7	98.5 ± 1.2	98.0 ± 1.5	95.7 ± 0.5	95.7 ± 1.3	97.3 ± 4.1
MB 414	99.1 ± 4.2	100.0 ± 5.4	100.7 ± 6.9	102.1 ± 5.5	103.4 ± 8.1	101.6 ± 3.2	104.9 ± 4.6	105.2 ± 2.4	107.7 ± 4.2	110.4 ± 6.5	100.8 ± 9.1	98.9 ± 10.0	97.7 ± 11.3	93.9 ± 13.6	94.1 ± 15.6
MB 442	98.2 ± 2.8	101.3 ± 5.9	99.8 ± 5.1	100.9 ± 5.4	101.6 ± 4.2	103.0 ± 3.1	101.2 ± 1.8	106.0 ± 5.9	109.0 ± 8.2	108.1 ± 6.4	96.0 ± 4.5	91.8 ± 5.5	90.9 ± 6.8	89.2 ± 7.8	88.4 ± 8.2
MB 408	98.3 ± 2.6	98.0 ± 2.8	96.7 ± 3.6	96.3 ± 3.6	97.7 ± 3.2	98.3 ± 5.8	101.0 ± 4.4	103.7 ± 8.3	104.7 ± 7.4	102.5 ± 1.4	96.4 ± 4.0	93.3 ± 6.6	91.2 ± 8.5	89.5 ± 8.3	90.0 ± 7.4
TMB-4	99.6 ± 0.5	96.0 ± 2.7	93.6 ± 2.6	89.3 ± 2.7	89.3 ± 2.3	101.8 ± 5.6	103.7 ± 5.8	102.2 ± 8.0	105.6 ± 9.4	100.3 ± 5.3	101.1 ± 3.9	99.4 ± 2.9	99.8 ± 7.7	97.5 ± 9.4	98.7 ± 11.9
Obidoxime	99.2 ± 2.2	96.7 ± 3.8	95.9 ± 6.4	95.5 ± 5.9	94.3 ± 7.4	101.1 ± 1.6	103.6 ± 5.2	103.7 ± 4.0	100.6 ± 3.9	102.3 ± 2.8	98.7 ± 2.5	96.8 ± 3.0	96.1 ± 4.0	94.1 ± 5.2	94.8 ± 5.7
HI-6	100.9 ± 1.8	101.1 + 1.2	101.5 ± 4.6	99.7 ± 5.8	99.4 ± 4.7	102.5 ± 1.9	100.5 ± 5.5	102.2 ± 7.4	102.8 ± 7.7	107.5 ± 15.1	101.3 ± 1.5	104.3 ± 3.9	102.4 ± 3.5	103.7 ± 4.4	105.0 ± 5.3
2-PAM	99.2 ± 4.5	99.5 ± 4.5	99.5 ± 6.7	96.3 ± 7.4	93.2 ± 10.4	106.2 ± 10.2	97.4 ± 2.6	99.3 ± 4.5	98.7 ± 4.2	99.4 ± 4.1	96.4 ± 2.6	97.8 ± 4.2	97.6 ± 3.8	96.8 ± 3.2	95.1 ± 3.5

	Cflow					$dLVPdt_{max}$					$dLVPdt_{min}$				
	1. Dose	2. Dose	3. Dose	4. Dose	5. Dose	1. Dose	2. Dose	3. Dose	4. Dose	5. Dose	1. Dose	2. Dose	3. Dose	4. Dose	5. Dose
MB 327	99.4 ± 2.5	96.7 ± 3.4	94.3 ± 4.7	91.6 ± 6.7	93.6 ± 2.6	104.4 ± 2.2	104.2 ± 2.2	107.1 ± 10.1	108.0 ± 5.4	108.9 ± 13.2	103.5 ± 4.1	103.0 ± 3.7	105.3 ± 6.5	108.3 ± 7.6	107.4 ± 10.7
MB 782	99.3 ± 2.8	98.9 ± 4.0	99.3 ± 6.2	97.4 ± 6.1	85.9 ± 17.7	112.1 ± 13.8	113.9 ± 11.5	120.3 ± 13.6	123.0 ± 12.4	119.7 ± 16.9	106.9 ± 8.6	111.3 ± 9.2	116.7 ± 11.7	120.9 ± 10.5	108.4 ± 21.6
MB 454	97.9 ± 1.3	97.0 ± 2.7	97.0 ± 5.8	94.0 ± 6.7	91.1 ± 5.5	100.8 ± 0.4	102.5 ± 5.0	104.2 ± 5.0	102.7 ± 7.4	98.7 ± 10.1	98.7 ± 1.0	96.6 ± 9.1	99.9 ± 8.2	101.5 ± 12.8	96.2 ± 17.9
MB 414	98.2 ± 2.9	98.9 ± 4.1	98.8 ± 4.3	101.3 ± 5.0	105.8 ± 9.8	102.5 ± 5.3	107.0 ± 6.0	108.5 ± 5.8	112.8 ± 6.4	116.8 ± 8.8	101.0 ± 4.4	105.1 ± 4.9	106.1 ± 4.4	109.9 ± 4.7	113.3 ± 7.8
MB 442	98.6 ± 1.4	98.9 ± 3.8	98.5 ± 3.5	99.2 ± 3.7	100.5 ± 6.1	104.5 ± 1.1	107.4 ± 4.3	111.4 ± 5.3	117.8 ± 9.2	115.7 ± 5.8	103.3 ± 3.6	105.5 ± 7.3	108.4 ± 7.6	113.4 ± 9.5	112.5 ± 9.1
MB 408	96.5 ± 3.6	95.5 ± 4.7	94.4 ± 4.3	93.6 ± 2.4	94.9 ± 2.5	97.1 ± 5.5	101.5 ± 5.2	102.4 ± 6.2	102.7 ± 3.3	103.3 ± 3.0	97.0 ± 5.1	101.1 ± 5.7	102.9 ± 7.0	104.9 ± 6.0	104.9 ± 1.7
TMB-4	99.7 ± 1.5	96.5 ± 3.3	94.4 ± 5.9	92.3 ± 6.7	92.9 ± 7.5	103.7 ± 8.0	102.7 ± 7.0	100.4 ± 8.2	100.2 ± 9.3	97.1 ±8.2	103.0 ± 4.5	104.2 ± 4.2	100.3 ± 7.7	101.4 ± 8.7	98.3 ± 5.6
Obidoxime	97.4 ± 2.0	95.0 ± 1.6	93.4 ± 2.4	92.7 ± 3.2	90.7 ± 2.2	102.0 ± 3.6	104.5 ± 5.6	104.5 ± 3.1	102.4 ± 2.7	103.9 ± 2.5	103.9 ± 1.3	104.7 ± 4.0	105.5 ± 4.4	102.2 ± 1.7	104.1 ± 6.6
HI-6	100.1 ± 1.0	99.5 + 3.0	98.9 ± 4.3	97.1 ± 5.1	100.0 ± 4.7	102.2 ± 10.4	98.0 ± 12.8	101.5 ± 16.1	100.9 ± 16.8	105.9 ± 26.1	104.8 ± 3.4	102.7 ± 6.0	106.3 ± 8.5	104.4 ± 6.8	111.9 ± 17.3
2-PAM	100.0 ± 1.3	95.6 ± 3.7	95.3 ± 4.2	91.1 ± 7.0	88.9 ± 10.2	105.2 ± 8.8	98.5 ± 5.1	101.4 ± 3.8	96.7 ± 7.4	94.8 ± 11.2	106.8 ± 6.7	95.6 ± 4.5	100.1 ± 2.5	97.9 ± 8.3	98.2 ± 4.9

HSE Acad W (Hugo Sachs Electronics, March-Hugstetten, Germany), GraphPad Prism 5.04 (San Diego, CA, USA) and Microsoft-Excel were used for data recording and analysis.

3. Results

3.1. Effect of different test compounds on the Langendorff heart

The setup with the feedback mechanism to adjust the test compound flow rate (Fig. 1) resulted in a stable system after sham administration during the whole experimental period (Table 2). None of the tested compounds showed an effect on the isolated heart (Table 3). All test results did not differ significantly from the others.

3.2. Smooth muscle relaxation of MB327 and related substances

The same bispyridinium non-oximes, which were tested on the isolated heart, were used with the jejunum preparation and the smooth muscle relaxing effect after carbachol stimulation was evaluated. All compounds showed a concentration-dependent smooth muscle relaxing effect following a dose response curve which allowed the determination of the concentration showing 50% of the maximal relaxing effect ($EC_{50;}$ Fig. 2).

Atropine had the most pronounced smooth muscle relaxing effect (EC_{50} = 0.01 μM). The normalized does response curves are shown in Fig. 2 and the EC_{50} for each substance can be seen in Table 1. This results in the following sequence of potency: Atropine ≫ MB327 > MB 782 ≫ MB454 > TMB-4 ≫ obidoxime > MB442 > 2-PAM > MB414 > MB408 ≫ HI-6. All EC_{50} of the various compounds differed significantly from the others ($p < 0.05$), except for TMB-4 vs MB454; and MB442 vs MB414 vs 2-PAM. The latter dose response curves were very close to each other or even superimposable (Fig. 2).

4. Discussion

Modulation of nicotinic receptors by bispyridinium non-oximes has been proposed as a new concept for the treatment of OP-poisoning (Niessen et al., 2011; Tattersall, 1993). Recently, the bispyridinium compound MB327 showed toxicity at higher doses in vivo, the mechanism still being unknown (Price et al., 2015). Hence, we investigated the effect of MB327 and related compounds in isolated heart and jejunum as model for smooth muscles.

The effect of all tested bispyridinium non-oximes on isolated hearts was low even in supratherapeutical concentrations (c.f. Table 3) which were up to 25 times higher than the plasma C_{max} (22 μM) in in vivo experiments with guinea pigs (Price et al., 2015). The well-established obidoxime did not show any adverse effects in the current study (c.f. Table 1). In earlier studies from Will (Will, 2008) and Ben-Haim (Ben-Haim et al., 1992) the LVP increased with the concentration of obidoxime. However, Will used a Langendorff apparatus without the feedback loop to the syringe pump for the test compound (Fig. 1) and Ben-Haim and colleagues used a working heart model with up to millimolar concentrations. HI-6 is an oxime in advanced development by several European countries and Canada. In humans up to 500 mg/person HI-6 (C_{max} = 38.9 ± 3.4 μM) in combination with atropine did not result in any changes of blood pressure, heart rate or the ECG (Clement et al., 1995). Here, 100 μM did not result in any cardiac effects. Although we used the improved Langendorff apparatus with feedback loop, this model lacks neural innervation and neurohumoral regulation and we were not able to simulate the complex regulation as in animal experiments (Barnes et al., 1972; Klimmek and Eyer, 1986; Worek and Szinicz, 1993). Further in vivo research with anaesthetized or conscious animals is needed to investigate the mechanisms of toxicity of bispyridinium non-oximes.

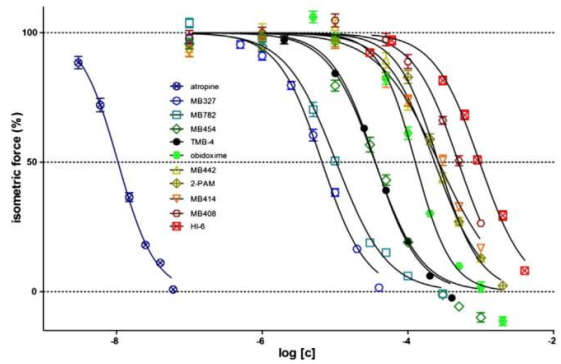


Fig. 2. Smooth muscle relaxation of MB327 and related substances. Normalized dose response curves of all tested compounds. Data are given as % isometric force (Mean ± SEM) after pre-treatment with 2 μM carbachol (n ≥ 10 segments of different parts of the jejunum of at least three rats per concentration).

MB327 is one of the lead substances of the nicotinic receptor active compounds and was the most potent bispyridinium non-oxime modulating muscarinic receptors after pre-constriction with carbachol (EC_{50} = 6.6 μM; Table 2). This is in line with previous experiments from our institute (MB327 EC_{50} = 6 μM) (Königer et al., 2013) suggesting that the effect is most probably due to binding at muscarinic receptors (Hobbiger et al., 1969; Königer et al., 2013; Niessen et al., 2012). Pharmakokinetic studies revealed a plasma concentration of MB327 in guinea pigs of 22 μM. This concentration, which is three times higher than the EC_{50} might cause a distinct antimuscarinic effect (Price et al., 2015). MB782 showed a similar efficacy (EC_{50} = 10.4 μM). MB327 and MB782 were 3 to 100-fold more potent than all of the bispyridinium non-oximes tested (Table 1). This gives a hint that the *tert*-butylgroups of MB327 and MB782 are the most important characteristics for structure-activity relationships. Compounds without side chains (MB442, MB408), or only a methyl (MB414), an ethyl (MB454) or oxime group at the bispyridinium rings were less effective. This is in line with previous studies, where a *tert*-butylgroup showed a higher receptor activity than an oxime group (Amitai et al., 1980; Kloog et al., 1986; Kloog and Sokolovsky, 1985; Königer et al., 2013).With the tert-butylgroups the pentylene linker was less effective than the propylene linker (EC_{50}: MB327 = 6.6 μM < MB782 = 10.4 μM). In the absence of side chains at the pyridinium rings a longer linker resulted in a higher receptor affinity and thus smooth muscle relaxation (MB442 > MB408) (Wille et al., 2010).

In conclusion, no cardiotoxic effects of the tested compounds were observed in an isolated Langendorff model. A pronounced smooth muscle relaxing effect may be considered as a therapeutic action of bispyridinium non-oximes in OP poisoning. To further evaluate the bispyridinum non-oxime compounds in vivo experiments providing cardiovascular parameters are mandatory.

Conflict of interest statement

The authors declare that there are no conflicts of interest. The design, performance, data interpretation and manuscript writing was under the control of the authors and has not been influenced by the German Ministry of Defence.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

This study was funded by the German Ministry of Defence. The authors are grateful to Dr. C.M. Timperley, DSTL (Porton Down, UK) for donating the bispyrdinium-non-oxime compound and to Mrs. Brosig for her excellent expert technical assistance.

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2.2 Veröffentlichung II

Toxicology Letters 293 (2018) 235–240

Contents lists available at ScienceDirect

Toxicology Letters

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

Human small bowel as a useful tool to investigate smooth muscle effects of potential therapeutics in organophosphate poisoning

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ARTICLE INFO

Keywords:
Acetylcholinesterase
Butyrylcholinesterase
Bispyridinium non-oxime
Isolated organ
Human
Oxime
Smooth muscle function
Small bowel

ABSTRACT

Isolated organs proofed to be a robust tool to study effects of (potential) therapeutics in organophosphate poisoning. Small bowel samples have been successfully used to reveal smooth muscle relaxing effects. In the present study, the effects of obidoxime, TMB-4, HI-6 and MB 327 were investigated on human small bowel tissue and compared with rat data. Hereby, the substances were tested in at least seven different concentrations in the jejunum or ileum both pre-contracted with carbamoylcholine. Additionally, the cholinesterase activity of native tissue was determined. Human small intestine specimens showed classical dose response-curves, similar to rat tissue, with MB 327 exerting the most potent smooth muscle relaxant effect in both species (human EC_{50} = 0.7 × 10^{-5} M and rat EC_{50} = 0.7 × 10^{-5} M). The AChE activity for human and rat samples did not differ significantly (rat jejunum = 1351 ± 166 mU/mg wet weight; rat ileum = 1078 ± 123 mU/mg wet weight; human jejunum = 1030 ± 258 mU/mg wet weight; human ileum = 1293 ± 243 mU/mg wet weight). Summarizing, our isolated small bowel setup seems to be a solid tool to investigate the effects of (potential) therapeutics on pre-contracted smooth muscle, with data being transferable between rat and humans.

1. Introduction

Organophosphorus (OP) compounds still pose a major threat in military conflicts, as the recent homicidal dissemination of sarin in Syria 2013 and 2017 prove (UN Secretary-General, 2013; Rosman et al., 2014; Pinheiro et al., 2017). Additionally, a recent publication estimates up to 168,000 fatal cases due to self-poisoning with pesticides every year with a focus on the Western Pacific region (Mew et al., 2017). The life-threatening toxicity of OP pesticides and nerve agents is mediated by irreversible inhibition of the pivotal enzyme acetylcholinesterase (AChE) which results in an accumulation of acetylcholine (ACh) in the synaptic cleft. This results in cholinergic overstimulation, eventually leading to death due to peripheral and/or central respiratory failure (Marrs, 1993; Lee, 2003; Thiermann et al., 2013; Worek and Thiermann, 2013). Therefore, effective medical countermeasures are urgently needed. At present, the standard treatment protocol comprises the muscarinic receptor antagonist atropine, an oxime, e.g. pralidoxime or obidoxime, to reactivate the inhibited AChE and benzodiazepines to suppress seizures and to reduce brain damage (Eyer, 2003; Eddleston et al., 2004; Eyer and Worek, 2007; Thiermann et al., 2011, 2013; Worek and Thiermann, 2013). The inhibited enzyme is reactivated by sequestering the phosphyl group (Worek and Thiermann, 2013). However, due to the broad spectrum of existing OPs including analogs treatment is challenging (Holmstedt, 1959; Marrs, 2007; Worek et al., 2016b). Unfortunately, a therapy covering all or most OPs does not exist and necessitates research on new therapeutics. (Worek et al., 2004; Eyer and Worek, 2007; Lundy et al., 2011; Worek and Thiermann, 2013) Further hurdles for the development of new medical countermeasures are the lack of studies in humans. As human *in vivo* studies due to obvious ethical reasons are not possible *in vitro* experiments with human material are of great value. Isolated organ baths have been utilized in pharmacological and toxicological research for decades, allowing the examination of living tissue in its complex 3D environment (Jespersen et al., 2015). With special regard to the development of new therapeutics in OP poisoning typical target tissues have been developed as models e.g. striated muscles as model for nicotinic ACh receptors (Seeger et al., 2007, 2011, 2012). Additionally, the lung with precision cut lung slices (PCLS) as typical port de entrée of OP into the body causing life-threatening signs in this particular tissue (Herbert et al., 2017) and small bowel

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https://doi.org/10.1016/j.toxlet.2017.11.012
Received 29 September 2017; Received in revised form 7 November 2017; Accepted 12 November 2017
Available online 14 November 2017
0378-4274/

preparations, both used as model for smooth muscle contraction mediated by ACh receptors, were applied (Lehman, 1962; Königer et al., 2013; Neumaier et al., 2016). As species-specific differences in the toxicity of different OP compounds are a major confounder in medical countermeasure research for OP poisoning due to variations in the cholinesterase and serum carboxylesterase activity (Worek et al., 2002, 2011; Duysen et al., 2011), we here established a model for human small bowel preparations and compared rat and human tissue. Bispyridinium compounds are modulators of cholinergic receptors. As their target is downstream AChE inhibition they are promising candidates for a generic therapeutic approach covering all types of OP poisoning (Tattersall, 1993; Turner et al., 2011; Niessen et al., 2013; Price et al., 2015). We previously analyzed the spasmolytic effect of these substances in rat jejunum samples (Tattersall, 1993; Turner et al., 2011; Königer et al., 2013; Price et al., 2015; Neumaier et al., 2016). Thus, it was tempting to investigate the spasmolytic effect of the bispyridinium non-oxime MB 327 and clinically approved oximes TMB-4, HI-6 and obidoxime in a modified setup with human specimens.

2. Materials & methods

2.1. Ethics statement

This study was approved by the Ethical Committee of the TUM School of Medicine (46/16 S), according to the German legislation and the statement of the Central Ethics Commission (ZEKO) on the further use of human tissue for medical research (http://www.zentrale-ethikkommission.de/). The study participants gave their written informed consent and the samples were coded pseudonymously.

2.2. Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) were housed in small groups of three to six animals with a 12/12 h light–dark cycle with water *ad libitum* and a standard laboratory diet (Altromin, Lage, Germany). After a seven-day resting phase the animals were euthanized by isoflurane overdose and cervical dislocation or bleeding. Afterwards the jejunum was removed. At this time the animals did not show any signs of infection. The study protocol was approved by the competent authority (Az 42-34-30/C).

2.3. Chemicals

HI-6 dichloride monohydrate was kindly donated by Dr. J. G. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada) and obidoxime was supplied by Merck KG (Darmstadt, Germany). NaCl, $CaCl_2$, $NaHCO_3$, NaH_2PO_4, Glucose and KCl were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and TMB-4 was delivered by Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). MB 327 was kindly supplied by Dr. C. M. Timperley (DSTL, Porton Down, UK; see Table 1). MB 327 was 98% pure based on LC–MS, $^{1}H/^{13}C$ NMR analysis.

2.4. Rat jejunum preparation

We applied the previously described model (Königer et al., 2013; Neumaier et al., 2016). In brief, about 11 cm of the aboral part of the jejunum were removed and dissected in 10 pieces (each ~ 1 cm). The first centimeter was discharged. The tissue samples were subsequently placed into ten gassed (95% O_2 and 5% CO_2) 15 ml water-jacketed single organ baths (Experimetria Ltd, Budapest, Hungary) containing Tyrode buffer (NaCl 137.0 mM, $NaHCO_3$ 22.0 mM, glucose 5.5 mM, KCl 2.68 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.05 mM, NaH_2PO_4 0.42 mM; pH = 7.4; T = 38.0 °C). The preparations were fixed with hooks between a glass retainer (Michael Murner, Waging, Germany) and a force transducer which was connected to an amplifier (HSE PLUGSYS ® TAM-A, type 705/1, Hugo Sachs, March-Hugstetten, Germany). Five organ baths were connected to one gassed (95% O_2 and 5% CO_2) reservoir and one vacuum flask (Radnoti, Monrovia, CA, USA) to allow rapid buffer changes.

2.5. Human small bowel preparation

Tissue collected during surgery, which would have been discarded otherwise, was directly transported in saline soaked gauze to our laboratory within one hour at ambient temperature. First, different preparation methods were examined. Samples were dissected in longitudinal or circular direction or in rings and were placed into organ baths (see above). For all conditions different sizes were tested with and without mucosa. Longitudinal stripes of ~ 1.5 cm x 0.5 cm with mucosa proved to be the best approach for the experimental setup as they had comparable sample size like rat tissue and displayed reproducible force generation.

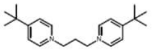
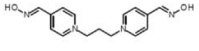
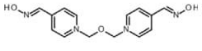
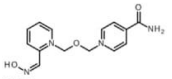
2.6. Experimental protocol rat jejunum

The segments were equilibrated at approximately 1 g basic tension for 30 min during which the buffer was changed twice in a 15-min interval followed by a short resting phase of 8 min. Then, the baseline was evaluated for 2 min and $\bar{x}_{basalactivity}$ was calculated for each preparation. This was followed by adding 2 µM carbamoylcholine and the last 2 min of this 5-min interval were used to calculate the Area Under Curve ($AUC_{carbamoylcholine}$), followed by washing the jejunum twice with buffer and a resting phase of 10 min during which the last 2 min served as a baseline ($\bar{x}_{basalactivity}$) for each dose of carbamoylcholine plus test substance. Subsequently, carbamoylcholine (2 µM) and then 2 min later the test compound in increasing concentrations were added and the effect on smooth muscle force was recorded. The last 2 min were used to compute $AUC_{substance}$.

2.7. Experimental protocol human small bowel

For the human small bowel preparations only 1 µM carbamoylcholine (dose obtained maximum reaction without signs of fatigue in preliminary tests, see Fig. 1) was applied. Preparations were recorded

Table 1
Smooth muscle relaxing effects and chemical structure of the tested compounds. The EC_{50} is given as mean and the corresponding confidence interval (CI) is depicted (10^{-5} M; n ≥ 5 segments of different parts of the small bowel of at least three patients or rats per concentration).[a] From Neumaier et al. (2016).

	MB 327	TMB-4	Obidoxime	HI-6
Structure				
Human EC_{50}	0.7	4.7	9.3	69.8
Human CI	0.6–0.8	4.0–5.5	8.4–10.3	57.7–84.4
Rat EC_{50}[a]	0.7	3.6	12.6	96.3
Rat CI[a]	0.6–0.7	3.4–3.7	11.9–13.4	91.1–101.8

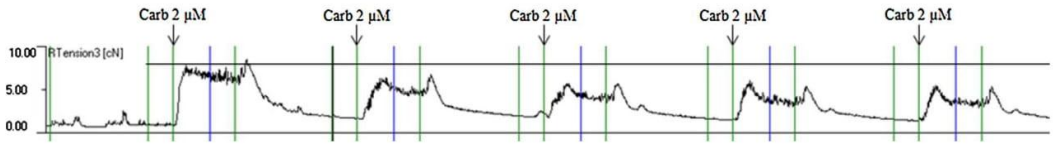


Fig. 1. Screenshot of an experiment with human small bowel and 2 µM carbamoylcholine. The interval between the first and second green line describes the baseline. The second green line is the marker for the carbamoylcholine administration. The interval between the blue line and third green line describes the AUC of the carbamoylcholine-mediated effect used for calculation. The third green line is the wash event to remove test compounds. The maximum pre-contraction with 2 µM carbamoylcholine was not consistent. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

slightly longer (6 vs. 5 min), as the human tissue is thicker and diffusion of test compounds may take longer. The last 2 min of this interval were used to calculate the Area Under Curve ($AUC_{carbamoylcholine}$), followed by washing the jejunum three times with buffer and a resting phase of 10 min. Subsequently, carbamoylcholine (1 µM) and then 2.5 min later the test compound in increasing concentrations were added and the alteration of smooth muscle force was recorded. The last 2 min were used to compute $AUC_{substance}$.

2.8. Data analysis jejunum

The effect of the tested compounds was calculated as follows:

$$AUC_{carbamoylcholine} = \sum (y - baseline)$$

$$AUC_{substance} = \sum (y - baseline)$$

y represents the recorded value.

Finally, the isometric force was computed as % of maximal carbamoylcholine stimulation by the equation:

$$\%\ \text{isometric force} = (AUC_{substance}/AUC_{carbamoylcholine}) \times 100$$

The EC_{50} values were calculated by non-linear normalized regression analysis from semi-logarithmic plots of the test compound concentration versus % of isometric force. As values should range between 0 and 100% a normalized curve was used. Data out of the range were due to relaxation at the end of the experiment or overreaction on the second carbamoylcholine dose. All data are shown as means and the corresponding confidence intervals in 10^{-5} M of $n \geq 5$ segments of different parts of the jejunum, respectively ileum of at least three patients or rats per concentration of one compound. A one-way analysis of variance (ANOVA) with Bonferroni post-test was employed to test for significant differences due to the high sample size ($p < 0.05$).

HSE Acad W (Hugo Sachs Electronics, March-Hugstetten, Germany), GraphPad Prism 5.04 (San Diego, CA, USA) and Microsoft-Excel (Redmond, WA, USA) were used for data recording and analysis.

2.9. Cholinesterase assay

Rat and human small bowel samples were shock frozen in liquid nitrogen and stored at − 80 °C until analysis of cholinesterase assay. The specimens were mixed with a fivefold volume of ice-cold phosphate buffer (100 mmol L^{-1}, pH = 7.4) containing 1% Triton X-100. Subsequently, the tissue was homogenized on ice eight times for 15 s with an Ultra-Turrax (T25 IKA-Werke, Staufen, Germany; S25N-10G-ST IKA-Werke, Staufen, Germany) and additionally 30 times with a glass-Teflon potter (Braun, Darmstadt, Germany). Then, the homogenates were centrifuged (Rotina 420 R, Hettich Zentrifugen, Tuttlingen, Germany) at maximum speed for 10 min at 4 °C and the supernatant was used for cholinesterase assays. The enzyme activities were measured with a modified Ellman assay (Worek et al., 1999) at 412 nm and 37 °C (Cary 50, Varian, Darmstadt, Germany) using polystyrol cuvettes with 0.45 mmol L^{-1} ATCh or 1.00 mmol L^{-1} BTCh as substrate, 0.02 mmol L^{-1} ethopropazine as selective butyrylcholinesterase (BChE) inhibitor and 0.3 mmol L^{-1} DTNB as a chromogen. Small bowel enzyme activities were calculated as mU/mg wet weight.

Due to the high sample size, a one-way analysis of variance (ANOVA) with Bonferroni post-test was employed to test for significant differences ($p < 0.05$).

3. Results

3.1. Patient demographics

During routine surgery, small bowel samples were collected from 18 female and 18 male patients. Only small bowel samples that were removed during routine procedures, which were not needed for further purposes (e.g. pathology) and would have been discarded otherwise, were used. The age distribution was between 34 and 87 years. 11 jejunum and 25 ileum samples were taken. Patients underwent surgery due to restoration of bowel continuity (n = 16), hepatobiliary-pancreatic and colon malignancies (n = 14), inflammatory bowel disease (n = 3), bowel perforation, bowel ischemia or chronic bile duct stenosis (each n = 1). Patient demographics are depicted in Table 2.

3.2. Method adaptation for human samples

As repetitive pre-contraction with 2 µM carbamoylcholine resulted in muscle fatigue (see Fig. 1) a dose response curve for carbamoylcholine was performed, which resulted in a concentration-dependent contraction of the small bowel preparations (see Fig. 2). Maximum contraction was achieved with 1 µM and therefore subsequent experiments were performed with this concentration.

Table 2
Patient demographics (n = 36).

Gender	
Female	18 (50.0%)
Male	18 (50.0%)
Age (y)	
Mean ± SD	60.8 ± 13.8
Minimum	34
Maximum	87
Small bowel segment	
Jejunum	11 (30.6%)
Ileum	25 (69.4%)
Indication for surgery	
Restoration of bowel continuity	16 (44.4%)
Hepatobiliary-pancreatic and colon malignancies	14 (38.9%)
Chronic inflammatory bowel disease	3 (8.3%)
Bowel perforation	1 (2.8%)
Mesenteric ischemia	1 (2.8%)
Chronic bile duct stenosis	1 (2.8%)

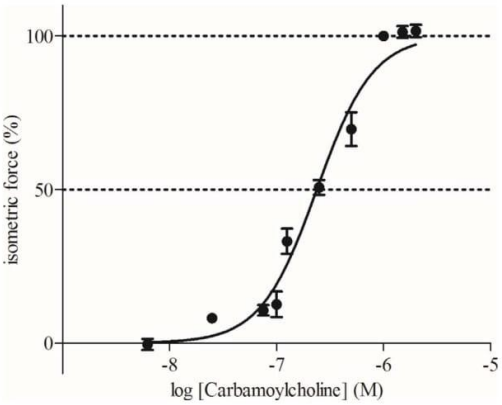


Fig. 2. Contractility of different carbamoylcholine concentrations on human small bowel referred to 1 µM carbamoylcholine as 100%. Data are given as % isometric force (Mean ± SEM) (n ≥ 9 segments of different parts of the jejunum of four patients per concentration).

3.3. Comparison of smooth muscle relaxing effect of the tested compounds in human and rat small bowel preparations

All tested substances showed a concentration-dependent smooth muscle relaxing effect following a classical sigmoidal-shaped dose response curve which allowed the determination of EC_{50} (concentration showing 50% of the maximal relaxing effect; Fig. 3).

MB 327 had the most pronounced smooth muscle relaxing effect (human $EC_{50} = 0.7 \times 10^{-5}$ M, respectively rat $EC_{50} = 0.7 \times 10^{-5}$ M). The EC_{50} for all tested compounds are displayed in Table 1. The EC_{50} values of the tested compounds were significantly different ($p < 0.05$) with the potency being as follows: MB 327 > TMB–4 > obidoxime > HI-6. However, the results for human and rat tissue were comparable with regard to the potency and the EC_{50}s (Table 1).

3.4. Cholinesterase activities in rat and human small bowel samples

The mean rat jejunum total cholinesterase (ChE) activity was 7.8 times higher than the human activity (10952 ± 1351 mU/mg wet weight, respectively 1398 ± 306 mU/mg wet weight), the ileum ChE activity of the rat, on the other hand, was only 4.3 times higher (7355 ± 1024 mU/mg wet weight, respectively 1729 ± 325 mU/mg wet weight). All samples showed a lower BChE than ChE activity (rat jejunum = 7428 ± 1126 mU/mg wet weight; rat ileum = 4242 ± 1766 mU/mg wet weight; human jejunum = 1296 ± 290 mU/mg wet weight), except for the human ileum (1753 ± 375 mU/mg wet weight). The species-specific total cholinesterase and BChE activity differed significantly ($p < 0.05$). The rat AChE activity was distinctly lower and comparable to the human AChE activity (rat jejunum = 1351 ± 166 mU/mg wet weight; rat ileum = 1078 ± 123 mU/mg wet weight; human jejunum = 1030 ± 258 mU/mg wet weight; human ileum = 1293 ± 243 mU/mg wet weight). The difference for the AChE was not significant (see Fig. 4).

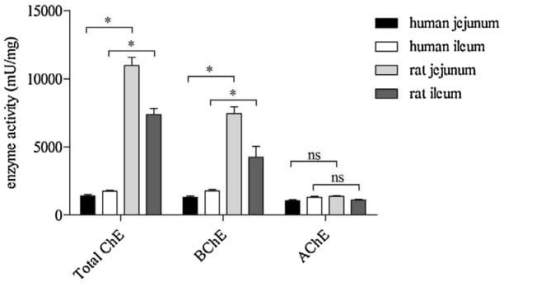


Fig. 4. Cholinesterase activity by comparison of human jejunum and ileum, respectively rat jejunum and ileum. Data of small bowel enzyme activity are given as enzyme activity (mU/mg wet weight) (n ≥ 5; Mean ± SEM; asterisk indicate significance $p < 0.05$; ns = not significant).

4. Discussion

Isolated animal organs are regarded as a useful tool for the examination of potential therapeutics against OP poisoning in complex tissues. Nevertheless, inevitable species differences may play an important role when the results have to be translated to humans. Hence, we here investigated the spasmolytic effect of the candidate therapeutics in human. We used carbamoylcholine to allow comparability of human data with previously gained rat data (Neumaier et al., 2016) and to allow wash out of carbamoylcholine. Additionally, with this substance analysis of the receptor modulating activity without overlay of AChE reactivation is possible. To our best knowledge effects of potential therapeutics for OP poisoning have not been examined in human small bowel preparations until now. Additionally, cholinesterase assays were performed for both species and showed comparable results for AChE in rat and human tissue.

Cholinergic crisis results in increased motility *in vivo* which was also

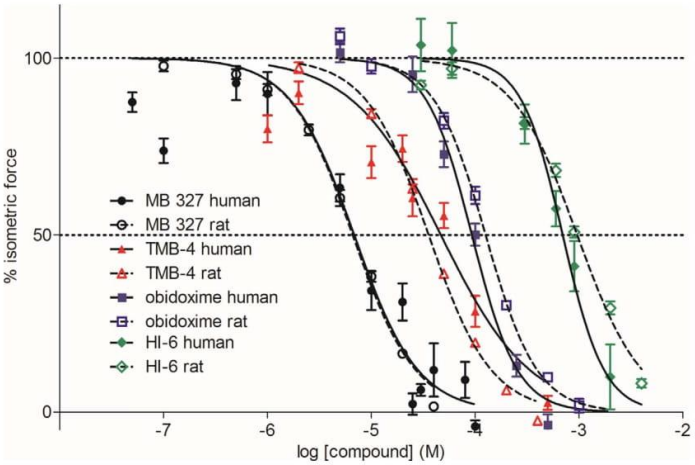


Fig. 3. Smooth muscle relaxation of the tested compounds as normalized dose response curves of all tested substances. Data are given as % isometric force (Mean ± SEM) after pre-treatment with 1 µM carbamoylcholine in humans, respectively 2 µM in rats (n ≥ 5 segments of different parts of small bowel of at least three patients, respectively rats per concentration). Rat data from Neumaier et al. (2016).

seen in our experiments (cf. Fig. 1). We here observed massive force generation after stimulation with carbamoylcholine which might have resulted in intussusceptions *in vivo* as previously observed after soman poisoning in a guinea pig model (Wetherell et al., 2007). The current model seems to be quite robust, as despite variances in transfer time from the operating room, age, gender, diet, and morbidity, results were comparable (cf. Table 2). Slight variations are probably due to species-specific differences of AChE activity, distribution of ACh and differences of cholinesterase activity in the small bowel because of an age-dependent decrease in the distribution pattern of neurons (Feldberg and Lin, 1950; Ambache et al., 1971; Mandic et al., 2015).

MB 327 is the most promising bispyridinium compound resulting in an antinicotinic effect and subsequent restoration of neuromuscular transmission (Seeger et al., 2012; Niessen et al., 2013; Tattersall, 2016). It was also a potent smooth muscle relaxing compound after pre-constriction with carbamoylcholine in human tissue ($EC_{50} = 0.7 \times 10^{-5}$ M; Table 1), probably partly due to a modulation of the cholinergic receptors as it was shown in previous radioligand binding assays (Niessen et al., 2012, 2013). The results of the tested compounds in human tissue are very similar to previous findings in rats (e.g. MB 327 $EC_{50} = 0.7 \times 10^{-5}$ M, respectively 0.6×10^{-5} M; Table 1, Fig. 3) (Königer et al., 2013; Neumaier et al., 2016), suggesting that the data obtained in rats are transferable to humans (see Fig. 3). An appropriate translation of data for smooth muscle relaxants obtained in rats to humans has been shown previously for other compounds in an isolated ileum model (Borrelli et al., 2009). Comparable results were also gained for striated muscles with recovery of muscle force after administration of MB 327 in soman poisoned human intercostal muscle and rat diaphragm (Seeger et al., 2012). This is an important finding as major species-specific differences occurred for inhibition constants with various OP and oxime-mediated reactivation kinetics hampering a direct extrapolation from animal data to the human situation, which is probably due to differences in the erythrocyte esterase activity (Worek et al., 2002, 2011).

Human intestine, despite being more massive than rat intestine, seems to be more sensitive to the ACh analog carbamoylcholine. Maximum pre-contraction in human small bowel preparations was achieved with 1 μM whereas rat jejunum specimens showed maximum force after 2 μM carbamoylcholine (see Fig. 1). An explanation could be differences in the cholinesterase activity, especially the AChE activity. Therefore, we investigated the enzyme activity for human tissue and compared data to rat samples. The total ChE and BChE activities in the rat were several times higher compared to human activity, whereby the differences for the jejunum (highly significant) were more pronounced (7.8 times for ChE, 5.7 times for BChE) than for the ileum (4.3 times for ChE, 2.4 times for BChE) (see Fig. 4). However, as BChE has no clearly defined physiological relevance (apart of being a stoichiometric scavenger in the nanomolar range) and the AChE activities did not differ significantly, a transferability of rat to human data is emphasized (Lenz et al., 2007; Worek et al., 2016a). Therefore, we assume that species-specific differences are due to minor sequence changes in the otherwise highly conserved cholinergic receptors (Caulfield and Birdsall, 1998). Human ileum showed higher AChE activity than jejunum, which is in line with other studies, where human, as well as rat ileum, showed a higher enzyme activity (Heitkemper and Marotta, 1983; Sine et al., 1991). However, the AChE activity was higher than the BChE activity in previous studies (Varela and Mandel, 1976; Sine et al., 1991). This distinction could be due to examination of only epithelial cells and different experimental protocols. The variation in the total ChE activity for human and rat tissue is probably due to the higher activity of BChE in rat samples. It was shown, that there exist species-specific differences in the concentration of the different enzymes (Rudakova et al., 2011).

In conclusion, we here established a human small bowel model and for the first time investigated the spasmolytic impact of different model substances in human tissue and compared the results with rat data. The tested compounds resulted in a similar relaxant effect in human compared to rat tissue. This allows translation of rat data to humans and *vice versa*. Additionally, human and rat AChE activity were in the same range, emphasizing the comparability of rat and human data.

Conflict of interest statement

The authors declare that there are no conflicts of interest. The design, performance, data interpretation and manuscript writing was under the control of the authors and has not been influenced by the German Ministry of Defence.

Acknowledgments

This study was funded by the German Ministry of Defence.

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4. Danksagung

Den Personen, die zum Gelingen dieser Arbeit direkt oder indirekt beigetragen haben, möchte ich hier meinen Dank aussprechen.

Besonders möchte ich meinem Doktorvater Oberstarzt Prof. Dr. Worek und meinem Betreuer Oberfeldarzt PD Dr. Wille danken, die mich während meiner wissenschaftlichen Tätigkeit und dem Schreiben der Publikationen, als auch der Promotion stets begleitet, unterstützt und motiviert haben, als auch für die persönliche Unterstützung und ihre geduldigen Hilfestellungen, Ratschläge und Korrekturen. Joanna Brosig, die mich eingearbeitet und mit ihrer Erfahrung unterstützt hat, gilt ebenfalls mein Dank. Bei allen Mitarbeitern, Doktoranden, Post-Docs und Arbeitsgruppenleitern des Institutes bedanke ich mich für ihre Hilfsbereitschaft und die angenehme Atmosphäre, besonderer Dank gilt hier Fr. Oberstabsveterinär Dr. Herbert. Herrn Oberstarzt Prof. Dr. Thiermann, Leiter des Instituts für Pharmakologie und Toxikologie der Bundeswehr, danke ich für die Möglichkeit an diesem Institut wissenschaftlich tätig zu sein und meine Dissertation anfertigen zu können.

Meinen Dank möchte ich auch Prof. Dr. Martignoni und Dr. Prokopchuk für die gute und produktive Kooperation aussprechen.

Meinen Geschwistern Christopher und Teresa bin ich dankbar, für ihr Vorbild, die Motivation und ihre Hilfestellung. Besonderer Dank gilt meiner Mutter Ulrike, die mich in all meinen Interessen unterstützt und gefördert hat. Die es aber auch geschafft hat, mir in einer sehr schweren Zeit Anker und Stütze zu sein und maßgeblich zum Gelingen meines Studiums, als auch der Promotion beigetragen hat.

Meinem Ehemann Stefan, der mir nicht nur während des Studiums und meiner Promotion immer mit aufbauenden Worten, Wissen und Ermutigungen zur Seite gestanden und somit besonders zum Gelingen dieser Arbeit beigesteuert hat.

Mein letzter Dank gilt meinem verstorbenen Vater Albert.