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In vitro and *in vivo* toxicological studies of V nerve agents: Molecular and stereoselective aspects

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HIGHLIGHTS

- VX and VR enantiomers were preparatively separated with high purity.
- Stereospecific kinetics of AChE and BChE inhibition and reactivation were examined.
- *In vivo* p.c. toxicokinetics of VX and VR enantiomers were investigated in swine.
- Mechanisms of penetration/absorption of V agents through skin are presented.

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ABSTRACT

In vitro inhibition data of cholinesterases (ChEs) and reactivation with HI 6 are presented for separated VX and VR enantiomers with high purity (enantiomer excess >99.999%). Inhibition rate constants for (−)-VR were fourfold higher than for (−)-VX. Marked higher stereoselectivity of ChEs inhibition was observed for VR compared with VX enantiomers. Low/no reactivation was determined for respective (+)-enantiomers. Results were related to orientation of (−)- and (+)-enantiomers in ChEs active sites.

In vivo in swine, absorption rate constants were practically identical for VX and VR enantiomers after percutaneous application of 3xLD_{50} underlining relevance of amine group and postulated equilibria shifts between charged, uncharged, open and cyclic form (skin depot). *In vivo* toxicokinetics of VX and VR enantiomers differed markedly after 4 h. Elimination of VX was much slower compared with VR.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition *in vivo* differed for VX and VR. *In vivo* spontaneous reactivation was not observed for VX-inhibited AChE while VR-inhibited AChE was much faster spontaneously reactivated than expected and AChE inhibition by VR was slower than expected. Progredient BChE inhibition was detected after VX application while VR inhibited BChE weakly. Possible explanation may be impact of the agents on hemodynamics and different metabolisms. Thus, due to increase of the V agents' blood concentration after atropine administration (depot release) the present standard therapy should be thoroughly reconsidered.

Abbreviations: GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; VX, O-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothiate; VR, *O*-isobutyl *S*-[2-(diethylamino) ethyl] methylphosphonothiate; EA-2192, S-[2(diisopropylamino)ethyl] methylphosphonothioic acid; OP, organophosphorus compounds; ChE, cholinesterase; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CaEs, carboxylesterases; PTEs, phosphotriesterases; s.c., subcutaneous; i.m., intramuscular; i.v., intravenous; ACh, acetylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; ATCh, acetylthiocholine iodide; BTCh, S-butyrylthiocholine iodide; PEI, positive electrospray ionization; MRM, multiple reaction monitoring; IS, internal standard; ee, enantiomeric excess; k_i, inhibition rate constant; k_{obs}, first-order reactivation rate constant; K_D, dissociation constant; k_r, the reactivity constant; K_r, second order reactivation rate constant; FiO_2, isoflurane; SSA, steady-state anaesthesia.

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1. Introduction

Molecular mechanisms of *in vitro* and *in vivo* toxicology of V agents (VX, VR, CVX) that belong to most toxic synthetic compounds are of highest interest. Both V and G nerve agents act as highly specific acetylcholinesterase (AChE) inhibitors that cause increase of acetylcholine (ACh) in the synaptic cleft resulting in bronchorrhoe, bronchoconstriction, seizures, respiratory failure and death. In contrast to numerous literature concerning G agents (tabun (GA), sarin (GB), soman (GD), cyclosarin (GF) etc.), The number of available publications about V agents toxicology is much less. Thus, some aspects of the extraordinary high percutaneous toxicity of V agents are still not completely understood. Their long persistence in blood – compared with G agents – strongly complicates therapeutic treatment of patients with acute V agents' intoxication (Reiter et al., 2008). The present study aims at experimental and theoretical elucidation of essential aspects of *in vitro*/*in vivo* toxicokinetics and toxicodynamics of VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl]methylphosphonothiate) and VR (*O*-isobutyl *S*-[2-(diethylamino)ethyl] methylphosphonothiate) enantiomers with respect to therapy.

As scientific basis, German researchers (headed by Schrader and Kuhn) introduced acetylcholine-like substituents in organophosphorus compounds (OP) like the selective AChE substrate acetyl-β-methylcholine leading to first synthesis of GD by Henkel in 1944 (Fig. 1, Schmaltz, 2005). Later, first publication of introducing an aminothiol residue ($-S-R_1-N(R)_2$) in the molecule of *O*,*O*-dialkyl phosphoric acid was made by Ghosh and Newman (Ghosh and Newman, 1955; Ghosh, 1955). The first synthesis of toxic thio-/choline derivatives of methyl phosphonic acids was realized by Tammelin discovering substantial higher toxicity of *O*-alkyl methylphosphonic acid derivatives with a thiocholine fragment ($-S-CH_2-CH_2-N(CH_3)_2$ or $-S-CH_2-CH_2-N^+(CH_3)_3$) compared to those derivatives containing a choline fragment ($-O-CH_2-CH_2-N(CH_3)_2$ or $O-CH_2-CH_2-N^+(CH_3)_3$, thiol effect) (Tammelin 1957a,b; Tammelin, 1958). Intensive research on this new class of nerve agents was started in the United States, Canada, Great Britain and the Soviet Union (Hulet et al., 2007; Radilov et al., 2009). By optimizing the aminothiol residue of *O*-alkyl methylphosphonic acid derivatives, synthesis and weaponization of VX and VR was realized (Fig. 1). The synthesis and chemical structure of VX in the open literature was first published in two British patents in 1970s (Ley and Sainsbury, 1974; Wardrop and Stratford, 1974). The structure of Russian VX without the abbreviation VR was first published in 1993 (Воронов and феДоров, 1993). Under *in vivo* conditions, VX forms *S*-[2(diisopropylamino)ethyl] methylphosphonothioic acid (EA-2192, Fig. 1), that represents the only known highly toxic metabolite of OP nerve agents (Reiter et al., 2011).

Already the first toxicological investigations with tertiary aminothiol derivatives of *O*-alkyl methylphosphonic acids and *O*-dialkyl phosphoric acids revealed delayed development of symptoms and death compared to G agents and distinct peripheral effects in case of quaternary derivatives, respectively (Aquilonius et al., 1964; Koelle and Steiner, 1956). Crossing of blood–brain barrier of charged quaternary derivatives is very limited. After intraperitoneal and intravenous injection they have lower LD_{50} values causing stronger AChE inhibition. Compared with G agents, AChE inhibition in central nervous system is generally weaker and slower for V agents (Bajgar et al., 2007; Shih et al., 2005). An overview and analysis of toxicological data concerning the effect of VX on animals, including man, is presented (Maynard and Beswick, 1992; Munro et al., 1994; Opresko et al., 1998).

In contrast, only few data concerning VR toxicology are available in open literature. For instance, distinct peripheral cardiorespiratory symptoms after VR intoxication were described (Chang et al., 1998; Radilov et al., 2009). In addition, after subcutaneous (s.c.) or intramuscular (i.m.) administration of VX or VR, LD_{50} values in rodents (rat, guinea pig) are similar for both compounds although the *in vitro* AChE inhibition rate constant of VR racemate is about threefold higher than that of VX racemate (Bajgar et al., 2007; Chang et al., 1998; Chang et al., 2002; Maxwell et al., 1997).

Depending on the conditions of V agent administration, the peripheral and central symptomatology of acute intoxication may strongly vary. This underlines the essential impact of toxicokinetics. Commonly, V agents are more toxic than G agents, especially after percutaneous application. This is due to marked higher stability and higher selectivity of V agents, *i.e.*, distinct lower unspecific elimination of V agents by carboxylesterases (CaEs) and phosphotriesterases (PTEs) of mammalians (Reiter et al., 2014). Interestingly, V agents parallelely exist in different dynamic equilibria: cyclic and open forms with protonated and unprotonated amino function. Under complex *in vivo* conditions (*i.e.*, circulation and distribution including crossing of biological barriers), these shifts of equilibria are highly relevant for the profound understanding of penetration/absorption processes of these agents (Reiter et al., 2011) (Fig. 2).

As in case of G agents, V agents exist as stereoisomers. For comparative investigation of the molecular toxicity of V agents, data of at least two structurally different V agents should be considered. Additionally, isolation and characterization of VR isomers including cholinesterase (ChE) inhibition data were not described until now. The complete separation and quantification of VX enantiomers including stereospecific toxicokinetic data were previously published (Reiter et al., 2008).

Comprehensive understanding of *in vivo* toxicokinetics of V agents including stereoselective differences represents the

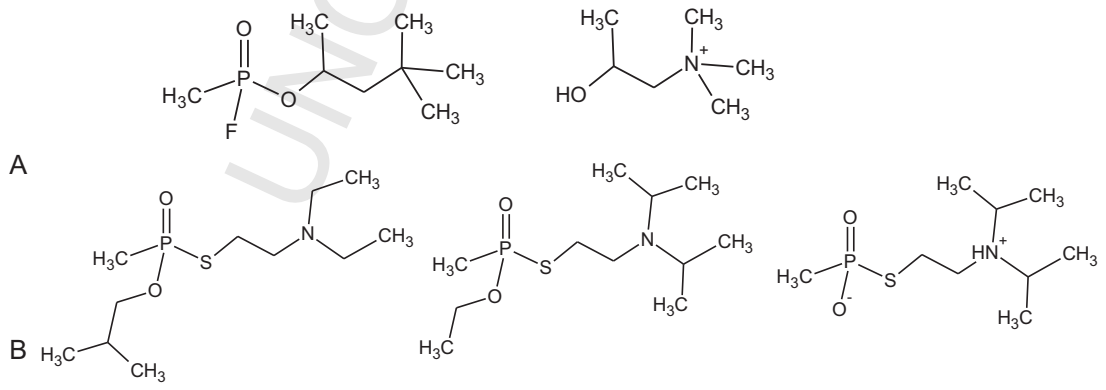

Fig. 1. Chemical structures of soman (GD, left) and β-methylcholine (right, A) and B: VR (left), VX (middle) and toxic VX-metabolite EA-2192 (right, B).

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