REPORTS
Arh Hig Rada Toksikol 2018;69:A44-A48

X. Stručni seminar: Određivanje onečišćujućih tvari u zraku, vodi i tlu specifičnim analitičkim tehnikama – upoznavanje s europskim standardima
Krakov, Varšava, Poljska, 3.-9. lipnja 2018.

Hrvatsko udruženje za zaštitu zraka (HUZZ) svake druge godine organizira stručne seminare u inozemstvu. Seminari se redovito održavaju od 2000. godine, a svrha im je upoznati članove Udruženja s europskim standardima i omogućiti stjecanje novih znanja o specifičnim analitičkim tehnikama za određivanje onečišćujućih tvari u okolišu. Suorganizator X. stručnog seminara pod naslovom "Određivanje onečišćujućih tvari u zraku, vodi i tlu specifičnim analitičkim tehnikama – upoznavanje s europskim standardima" kao i prethodnih godina bio Institut za medicinska istraživanja i medicinu rada. Organizator seminara od strane HUZZ-a bila je dr. sc. Gordana Pehnec. Ove godine seminar je po prvi puta održan u suradnji s Europskom federacijom udruženja za čisti zrak i okoliš (European Federation of Clean Air and Environmental Protection Associations, EFCA). Predsjednik EFCA-e Dr. Andrzej Jagusiewicz organizirao je posjet ključnim institucijama odgovornim za praćenje kvalitete zraka u Poljskoj. Za sustav praćenja kvalitete zraka zaduženi su Inspektorati zaštite okoliša pojedinih vojvodstava (Poljska ima 16 upravnih područja, vojvodstava), a vodi ih i koordinira Glavni inspektorat zaštite okoliša. Seminar je održan u razdoblju od 3. do 9. lipnja 2018. u Varšavi i Krakovu, a prisustvovalo mu je 22 sudionika iz sedam hrvatskih institucija (Institut za medicinska istraživanja i medicinu rada, Ekonerg, Medicinski fakultet Sveučilišta u Zagrebu, Agronomski fakultet Sveučilišta u Zagrebu, Metroalfa, HEP Proizvodnja, Agroproteinka). Dr. Andrzej Jagusiewicz bio je sa sudionicima tijekom svih dana trajanja seminara.

Tijekom seminara sudionici su prvo posjetili Inspektorat zaštite okoliša za regiju Mazowieckie u Varšavi. Nakon oralnih prezentacija poljskih i hrvatskih stručnjaka i provedene rasprave, članovi Udruženja posjetili su laboratorije i dvije postaje za praćenje kvalitete zraka (urbana pozadinska postaja Kondratowicza - Targówek i pozadinska postaja u Nacionalnom parku Kampinos). Dana 6. lipnja održan je sastanak s dr. Marekom Haliniakom, glavnim inspektorom i ministrom zaštite okoliša Poljske. Domaćini u Glavnom inspektoratu priredili su prezentacije o organizaciji sustava praćenja kvalitete zraka u Poljskoj, dok je aktivnosti HUZZ-a i sustav praćenja kvalitete zraka u Hrvatskoj ukratko predstavila predsjednica HUZZ-a Gordana Pehnec. U poslijepodnevnim satima organiziran je posjet Poljskoj gospodarskoj komori za održivi razvoj (PIGE), koja je članica EFCA-e. Sudionici seminara dana 8. lipnja posjetili su Inspektorat zaštite okoliša regije Małopolskie i Nacionalni referentni laboratorij Glavnog inspektorata u Krakovu.

Ovaj seminar omogućio je hrvatskim i poljskim stručnjacima i znanstvenicima razmjenu iskustava iz područja praćenja kvalitete zraka. Uspostavljeni su novi kontakti te su stečena nova saznanja koja će sudionicima seminara koristiti u budućem radu na sprječavanju utjecaja onečišćenog zraka na ljudsko zdravlje i okoliš.

Gordana Pehnec

EUROTOX 2018.
Bruxelles, Belgija, 2.-5. rujan 2018.

Održan je 54. kongres Udruženja europskih toksikoloških društava (EUROTOX) kojeg je ove godine organiziralo Belgijsko društvo za toksikologiju i ekotoksikologiju (BelTOX). Na 54. EUROTOX-u sudjelovalo je više od 1400 znanstvenika iz 61 zemlje. Tema ovogodišnjeg kongresa je bila *Toxicology Out-of-the-Box*, a vizija organizatora bila je predstaviti inovativna istraživanja iz područja toksikologije.

Ovogodišnji dobitnik Nagrade za zasluge (Merit Award) je prof. dr. sc. Corrado Ludovico Galli, toksikolog i farmakolog sa Sveučilišta u Milanu, autor više od 200 publikacija koji je dugi niz godina radio u području procjene rizika.

Na kongresu su predstavljena četiri plenarna predavanja, jedan simpozij, više od 100 predavanja tijekom 28 sekcija, više od 700 posterskih priopćenja te, prvi put, kratka predavanja (short oral communications) u tri sekcije. Kongres je otvoren predavanjem prof. dr. sc. Michaela Siegrista iz Švicarske s temom *Consumer Toxicology: Natural is good, synthetic is bad*. Prikazao je rezultate velikog istraživanja o utjecaju reklama o „prirodnim" proizvodima na privlačnost tih proizvoda za korištenje u svakodnevnom životu na laike i stručnjake.

Održana je tradicionalna rasprava između predstavnika Američkog udruženja toksikologa (SOT) i EUROTOX-a na temu *Adverse Outcome Pathways are the future for regulatory toxicology*. Sudionici kongresa jačim su pljeskom odlučili da je svoje argumente bolje branila dr. sc. Brigitte Landesmann, predstavnica Europske komisije iz Italije koja je branila afirmativno stajalište na strani EUROTOX-a. Osim tradicionalne rasprave, poboljšana je suradnja SOT-a i EUROTOX-a te je dogovoreno da dobitnici Nagrade za zasluge SOT-a održe predavanje na kongresu EUROTOX-a i obrnuto. Ove godine je prvi put predavanje održao dobitnik te nagrade SOT-a prof. dr. sc. Sam Cohen s temom *Cell proliferation and carcinogenesis: bad luck and environment*.

Na sastanku Poslovnoga vijeća izabran je novi predsjednik EUROTOX-a prof. dr. sc. Felix Carvalho iz Portugala, novi generalni tajnik prof. dr. sc. Martin Wilks iz Švicarske te novi član Izvršnog odbora prof. dr. sc. Mattias Öberg iz Švedske. Počasnim članovima su proglašeni prof. dr. sc. Bas Blaauboer iz Nizozemske i prof. dr. sc. Aristidis Tsatsakis iz Grčke.

Vizija organizatora o inovativnosti predstavljenih istraživanja najvećim je dijelom ostvarena tijekom posljednjeg i, vjerojatno, najboljeg predavanja na kongresu kojeg je održala dr. sc. Manasi Nandi iz Engleske na temu *Big data for biologists: A maths in medicine case study* u kojem je prikazala kako matematika može pomoći u analizi velikog broja podataka te pridonijeti dijagnostici.

Na kraju kongresa najavljen je 55. EUROTOX koji će se od 8. do 11. rujna 2019. godine održati u Helsinkiju. http://www.eurotox-congress.com/2019/

Dubravka Rašić

13th International Meeting on Cholinesterases and the 7th International Conference on Paraoxonases
Hradec Králové, Czech Republic, 9-14 September 2018

The role of acetylcholinesterase (AChE) as an acetylcholine disruptor in the central nervous system is essential. Organophosphates (OPs) such as nerve warfare agents (tabun, sarin, cyclosarin, soman, VX, etc.) and pesticides (parathion, chlorpyrifos, dichlorvos, etc.) permanently inhibit AChE therefore obstructing its physiological role which is manifested through mild (confusion, blurry vision, abnormal blood pressure and heart rate, vomiting, diarrhea, etc.) to severe (convulsions, loss of conciousness, breathing failure, and paralysis) symptoms that can lead to death from suffocation. Nowadays, when the threat of OP exposure is omnipresent (sarin attacks in Syria in 2013 and 2017, assassination of Kim Jong-nam by VX in 2017, Novichok incident in England in 2018, etc.), the search for oxime reactivators of inhibited AChE, and – or, bioscavengers [butyrylcholinesterase (BChE), AChE mutants, paraoxonases, phosphotriesterases, etc.] for human protection in the case of OP exposure has been placed in the focus of attention. Of course, the structure and molecular dynamics of AChE and the related enzyme BChE are of great importance not just in cases of OP poisoning, but also in studying neurodegenerative diseases like Alzheimer's and counteracting them, which is why they are intensively studied.

Researchers around the globe have been meeting every three years since 1975 to share their discoveries in the field. With time, the cholinesterase meeting was combined with paraoxonases and this year, from 9 to 14 September, the 13th International Meeting on Cholinesterases and 7th International Conference on Paraoxonases took place in Hradec Králové, Czech Republic. The Meeting was organized by the University of Hradec Králové under the auspices of the Minister of Health of the Czech Republic, the Mayor of the City of Hradec Králové and the President of the Hradec Králové Region, and under the supervision of Prof Kamil Kuča, the Rector of the University. The members of the International Advisory Board were eminent scientists from the field; Patric Masson, Florian Nachon, Oksana Lockridge, Shani Shenar-Tsarfaty, Israel Silman, Zoran Radić, Jonah Cheung, Terrone Rosenberry, Palmer Taylor, Eugenio Vilanova Gisbert, Franz Worek, Yuan-Ping Pang, Joel Sussman, Maria Laura Bolognesi, and Ana Martinez. The Meeting gathered 123 participants from Europe, America and Asia. Its six day program included 79 lectures divided in 12 oral sessions:

- Plenary lectures;
- Structure and dynamics of cholinesterases and OP hydrolases;
- Interaction of cholinesterases with substrates, inhibitors, and reactivators;
- Reactivators of AChE, OP inhibitors - mechanism of toxicity, detection and analytical methods, diagnosis of exposure, detoxification, and therapy;
- Enzymes and proteins other than ChEs interacting with OPs;
- Stoichiometric bioscavangers, biotechnology, and therapeutical aspects;
- Catalytical Bioscavangers - PON and PTE I;
- 3D section - structure and dynamics of α/β hydrolases and OP hydrolases, *in silico* methods for designing modulators;
- Biological functions, development, and non-cholinergic function of cholinesterases for counter-terrorism strategies;
- Alzheimer's disease and diseases related to cholinesterases;
- Multi-target-directed ligands in Alzheimer's disease primarily targeting cholinesterases;
- Others.

It is hard to single out or summarize the importance and excellence of the scientific achievements reported at this Meeting especially because of the great variety within the fields of cholinesterases and paraoxonases, but it has to be mentioned that for the first time, two separate teams, Israel Sillman's and Konstantin M. Boyko's group, reported the 3D structure of the natural tetrameric form of human BChE obtained by the Cryo-EM technique. BChE was very much in the center of attention, among other topics, and the participants were informed about the design of specific ligands of human BChE that would help develop inhibitors or reactivators. The mechanism of BChE activators was also discussed. X. Brazzolotto reported on the prokaryotic expression of active human BChE as a tool for catalytic bioscavenger development. Also, much was done on studying mosquito AChE in order to develop a species-specific AChE inhibitor, a cysteine targeting insecticide. There were a lot of lectures about counteracting nerve agent toxicity; Z. Kovarik talked about oxime reactivators of tabun-inhibited AChE and its mutants, P. Taylor and Y. Rosenberg reported about zwitterionic oximes prone to crossing the blood-brain barrier and reactivating phosphylated synaptic AChE, F. Ekström presented his discoveries about developing broad spectrum reactivators. The resurrection of the aged form of AChE was discussed, as were many other relevant topics. *In silico* methods for designing cholinesterase modulators were also a highly represented topic, G. Šinko talked about molecular docking and scoring functions, Z. Radić presented a 2D, 3D, and virtual reality (VR) visualization of cholinesterase structure modifications and it was for the first time that participants could actually see and "feel" VR being presented this way. Also, a VR corner was established where visitors could try it for themselves. A lot was said about Alzheimer's and other cholinesterase-related diseases, catalytic bioscavengers like paraoxonases or phosphotriesterases, and with lectures being so exquisite this review could have gone on and on. Some of the sessions started with speeches that paid tribute to eminent researchers - John Casida, Douglas Cerasoli, and Eric Barnard - who passed away recently.

The poster section was held for 4 days and 49 posters, divided in 11 sessions similarly to the oral presentation sessions, were presented. The prize for the best presented poster was won by T. Zorbaz for the poster titled *Chlorinated pyridinium oximes are potent reactivators of acetylcholinesterase inhibited by nerve agents* (T. Zorbaz, N. Maraković, K. Musilek, Z. Kovarik) (Figure 1).

Besides the excellent scientific programme, the participants enjoyed a very eventful social program which included a welcome reception, a gala dinner, as well as a walking tour around the city and concert in the Cathedral of the Holy Spirit. Moreover, participants could choose one of the excursion trips to Kuks Hospital, National Stud at Kladruby nad Labem or Hrádek u Nechanic Chateau.

As tradition suggests, the venues for the next congress were proposed, and among Russia, USA, Canada, and Italy, Croatia was also nominated. One could only report that the Meeting was very successful and everyone is looking forward with joy to the next one.

Nikolina Maček Hrvat

Chlorinated pyridinium oximes are potent reactivators of acetylcholinesterase inhibited by nerve agents

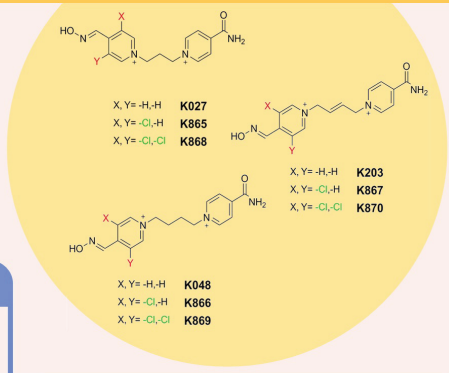
Tamara Zorbaz[1], Nikola Maraković[1], Kamil Musilek[2], Zrinka Kovarik[1]

[1] Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10001 Zagreb, Croatia
[2] University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62,500 03 Hradec Kralove, Czech Republic

AIMS

Chlorinated analogues of potent reactivators of AChE inhibited by different organophosphorus compounds (K027, K048, K203)[1,2] were synthesized with an aim to increase their lipophilicity and therefore possibly increase their propensity to cross the blood-brain barrier. We tested the influence of the chlorination on:

- acid dissociation constant (pK_a) of the oxime group
- affinity of AChE and BChE towards the oxime
- oxime's reactivation potential for AChE inhibited by nerve agents
- predicted interactions in a conjugated enzyme-oxime complex



MATERIAL AND METHODS

Oximes: K027, K048, K203, K865, K866, K867, K868, K869, K870 were synthesized at the University of Hradec Králové, faculty of Science, Department of Chemistry, Hradec Kralove, Czech Republic.

Enzymes: Human recombinant acetylcholinesterase (AChE) and purified plasma butyrylcholinesterase (BChE) were a generous gift of Dr Florian Nachon (Département de Toxicologie et Risques Chimiques, Institut de Recherche Biomédicale des Armées, 91220 Brétigny-sur-Orge, France).

pK_a determination

pK_a values of the oxime groups were determined spectrophotometrically from absorption spectra scanned at 200-500 nm in 0.1 M phosphate buffer, pH 4.6–11.1 (Cary 300, Varian Inc., Australia) at 25° C as described in Zorbaz et al. 2018.[3]

Reversible inhibition

Reversible inhibiton of AChE and BChE with an oxime was evaluated from dissociation constant (K_i; reciprocal value of enzyme's affinity) determined by measuring enzyme's activity in the presence of oxime and substrate acetylthiocholine (ATCh) of different concentrations using Ellman assay.[4] Activity was measured at 412 nm, 25° C on a microplate reader (Infinite M200PRO, Tecan Austria GmbH, Austria). Detailed procedure can be found in Zorbaz et al. 2018.[3]

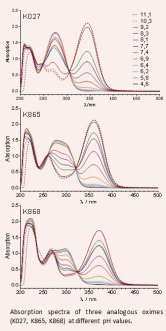
Reactivation

After the incubation with a nerve agent, inhibited enzyme was isolated and used to evaluate oxime's reactivation efficiency (first order reactivation rate, k_{obs} and maximal reactivated enzyme activity percentage, $React_{max}$) by measuring enzyme activity with the Ellman assay[4] at different time points after the start of reactivation, i.e., the addition of inhibited enzyme in the reactivation mixture containing an oxime. By testing wider oxime concentration range detailed reactivation parameters were determined: maximal first-order reactivation rate (k_{r2}), inhibited enzyme-oxime dissociation constant (K_{OX}), and total reactivation rate (k_r). Activity was measured at 412 nm, 25° C on Cary 300 spectrophotometer. Detailed procedure can be found in Zorbaz et al. 2018.[3]

Molecular modeling

Minimalized oxime structures (MMFF94 force field; ChemBio3D Ultra 12.0, PerkinElmer, Inc., USA) were docked (CDOCKER protocol) in crystal structures of cyclosarin-AChE conjugate (PDB ID: 5FPI) and tabun-AChE conjugate (PDB IDs: 2JEZ, 3DLK, and 2IPD) and analysed using Biovia Discovery Studio Client v17.2 (Accelrys, USA). Detailed procedure can be found in Zorbaz et al. 2018.[3]

pK_a values of the oxime group



Absorption spectra of three analogous oximes (K027, K865, K868) at different pH values.

Oxime	pK_a of oxime group	Oximate % at pH 7.4
K027	8.32	10
K048	8.44	10
K203	8.33[5]	10
K865	7.57	40
K866	7.68	35
K867	7.60	40
K868	7.53	45
K869	7.66	35
K870	7.55	40

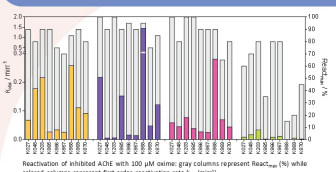
Chlorinated oximes had > 0.7 lower pK_a values of the oxime group than their non-chlorinated analogues.

Affinity of AChE and BChE for oximes

Oxime	Enzyme-oxime dissociation constant, K_i (μM)	
	AChE	BChE
K027	73 ± 10^2	660 ± 110^7
K048	110 ± 10^2	420 ± 60^7
K203	90 ± 12^6	910 ± 16^2
K865	42 ± 4	273 ± 42
K866	36 ± 5	265 ± 52
K867	24 ± 3	294 ± 41
K868	21 ± 2	195 ± 18
K869	12 ± 2	140 ± 12
K870	22 ± 2	187 ± 9

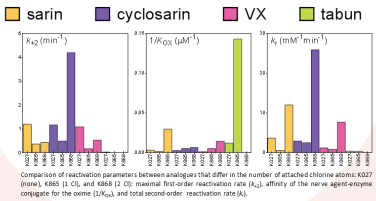
In the case of both AChE and BChE, the K_i values for the enzyme-oxime complexes decreased with the number of chlorine atoms in the oxime's structure. For all oximes, K_i values of AChE-oxime complexes were 7-12 times lower than for analogous BChE-oxime complexes.

Rectivation of nerve agent inhibited AChE



Reactivation of inhibited AChE with 100 μM oxime: gray columns represent $React_{max}$ (%) while colored columns represent first-order reactivation rate k_{obs} (min^{-1}).

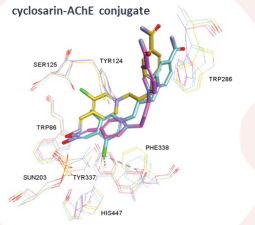
Chlorinated oximes were efficient reactivators of AChE inhibited by cyclosarin > sarin > VX, respectively. K868 was the most efficient reactivator. In the case of tabun, only non- and monochlorinated oximes were effective.

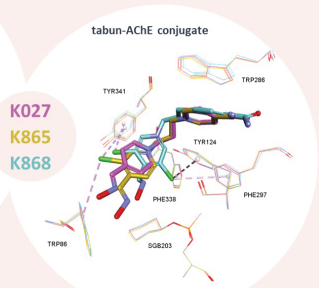


Comparison of reactivation parameters between analogues that differ in the number of attached chlorine atoms: K027 (none), K865 (1 Cl), and K868 (2 Cl): maximal first-order reactivation rate (k_{r2}), affinity of the nerve agent-enzyme conjugate for the oxime ($1/K_{OX}$), and total second-order reactivation rate (k_r).

Each nerve agent-AChE conjugate had the highest affinity for dichlorinated oxime K868, except for the tabun-AChE conjugate where the highest affinity was observed for monochlorinated oxime K865. The highest total reactivation rate was achieved in the reactivation of cyclosarin-AChE with K868 due to a high first-order reactivation rate and moderate affinity of the conjugate.

Molecular modeling





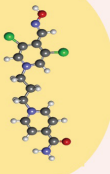
K027
K865
K868

No. of non-bonding interactions between oxime and amino acid residues increased in the following order: K027 < K865 < K868.

Three hydrophobic-π-alkyl interactions between chlorine atoms of K868 and aromatic amino acid residues.

CONCLUSION

- Chlorination caused lower pK_a values of the oxime group and resulted with oximes that are possibly stronger nucleophiles.
- Affinity of the AChE and BChE was higher for chlorinated oximes than for nonchlorianted analogues.
- Dichlorinated oxime with a propane linker (K868) showed the most efficient reactivation potential for AChE inhibited with sarin, cyclosarin, and VX, while it was completely ineffective for tabun-AChE conjugate.
- Molecular docking studies predicted that chlorinated oximes form non-bonding interactions between chlorine atoms and amino acid residues, and according to the reactivation data, these interactions resulted in both productive (e.g. cyclosarin-AChE conjugate) and non-productive conformation (e.g. tabun-AChE conjugate).



REFERENCES:
[1] Wintor *et al.* (2016) *Toxicology Letters*, 244, 136-142.
[2] Čalić *et al.* (2006) *Toxicology*, 219 (1-3), 85-96.
[3] Zorbaz *et al.* (2018) *Chemistry – A European Journal*, 24 (38), 9675-9691.
[4] Ellman *et al.* (1961) *Biochemical Pharmacology*, 7, 88-95.
[5] Katalinić *et al.* (2018). *Toxicology*, 406-407, 104-113.
[6] Kovarik *et al.* (2009) *Arhiv za higijenu rada i toksikologiju*, 60 (1), 19-26.
[7] Lucić Vrdoljak *et al.* (2006) *Toxicology*, 228 (1), 41-50.

Acknowledgement: This work was supported by the Croatian Science Foundation (no. 4307) and the Grant Agency of the Czech Republic (no. 18-01734S).

Figure 1 *The recipient of the Best Poster Award at the 13th International Meeting on Cholinesterases and the 7th International Conference on Paraoxonases, Hradec Králové, Czech Republic*