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Badania wybranych parametrów toksykologicznych dla związków fosforoorganicznych z grupy Novichok przy użyciu metod toksykologii *in silico*

Rozprawa doktorska w dziedzinie nauk medycznych i nauk o zdrowiu,
w dyscyplinie nauki medyczne

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Rzeszów 2023

Spis treści

1. Wykaz stosowanych skrótów

ACh – acetylocholina (ang. acetylcholine)

AChE – acetylocholinoesteraza (ang. acetylcholinesterase)

BChE – butyrylocholinoesteraza (ang. butyrylcholinesterase)

BŚT – bojowe środki trujące

CBRN – incydent chemiczny, biologiczny, radiologiczny oraz jądrowy (ang. chemical, biological, radiological and nuclear incident)

CWC - Konwencji o zakazie broni chemicznej (ang. Chemical Weapons Convention)

ECHA - Europejska Agencja ds. Chemikaliów (ang. European Chemicals Agency)

GD - Soman

GOSNIIOKhT - Instytut Chemii i Technologii Organicznej w Moskwie (ang. Institute for Organic Chemistry and Technology in Moscow)

HAZMAT - incydent z materiałami niebezpiecznymi (ang. hazardous materials incident)

LD_{50} - medialna dawka śmiertelna (ang. median lethal dose)

mc. - masa ciała (ang. body weight)

NA - środek paraliżujący (ang. nerve agent)

OECD - Organizacja Współpracy Gospodarczej i Rozwoju (ang. Organisation for Economic Cooperation and Development)

OP – związek fosforoorganiczny (ang. organophosphorus compound)

(Q)SAR - ilościowa zależność struktura-aktywność (ang. quantitative structure–activity relationship)

US EPA - Agencja Ochrony Środowiska Stanów Zjednoczonych (ang. United States Environmental Protection Agency)

2. Wykaz publikacji stanowiących rozprawę doktorską

Prace przeglądowe:

1. **M. Noga**, K. Jurowski. *What do we currently know about Novichoks? The state of the art*. Archives of Toxicology, 97, 651–661 (2023). https://doi.org/10.1007/s00204-022-03437-5; (140 pkt MEiN, IF 6.168).
2. **M. Noga**, A. Michalska, K. Jurowski. *Review of Possible Therapies in Treatment of Novichoks Poisoning and HAZMAT/CBRNE Approaches: State of the Art*. Journal of Clinical Medicine, 12(6):2221 (2023). https://doi.org/10.3390/jcm12062221; (140 pkt MEiN, IF = 4.964).

Prace oryginalne:

1. **M. Noga**, A. Michalska, K. Jurowski, *The prediction of hydrolysis and biodegradation of Novichoks using in silico toxicology methods*, Science of the Total Environment (2023). https://doi.org/10.1016/j.scitotenv.2023.164241; (200 pkt MEiN, IF 10.754).
2. **M. Noga**, A. Michalska, K. Jurowski, *Application of toxicology in silico methods for prediction of acute toxicity (LD_{50}) for Novichoks*, Archives of Toxicology, 97, 1691–1700 (2023). https://doi.org/10.1007/s00204-023-03507-2; (140 pkt MEiN, IF 6.168).

3. Zestawienie publikacji doktoranta

Rodzaj publikacji	Liczba	Impact Factor	Punktacja MEiN
Prace pełnotekstowe włączone do rozprawy doktorskiej	4	28.054	620
Rozdziały w monografii włączone do rozprawy doktorskiej	-	-	-
Prace pełnotekstowe, które nie zostały włączone do rozprawy doktorskiej	5	33.298	760
Rozdziały w monografii, które nie zostały włączone do rozprawy doktorskiej	-	-	-
Razem	**9**	**61.352**	**1380**

Liczba cytowań: 24 (według Scopus).

4. Wstęp

Novichok (ros. Новичóк, ‘nowicjusz’), grupa związków stanowiąca czwartą generację chemicznych bojowych środków trujących (BŚT) o działaniu paralityczno-drgawkowym[1]. Określane również mianem związków serii A, zostały potajemnie wyprodukowane podczas zimnej wojny przez Związek Radziecki[2]. Novichok to unikalne związki fosforoorganiczne (OP), których mechanizm toksycznego działania polega na nieodwracalnym wiązaniu się z acetylocholinoesterazą (AChE) oraz hamowaniu hydrolizy neuroprzekaźnika, tj. acetylocholiny (ACh) do octanu i choliny[3]. Związki typu Novichok działają na układ nerwowy poprzez miejsce aktywne AChE (triada Ser–His–Glu)[4]. Na poziomie molekularnym tworzenie wiązania kowalencyjnego między AChE i Novichok nie tylko spowalnia, ale całkowicie zatrzymuje hydrolizę ACh[5]. Nadmierna stymulacja receptorów cholinergicznych spowodowana nagromadzeniem ACh w szczelinie synaptycznej w wyniku hamowania AChE prowadzi, w zależności od drogi, dawki i czasu ekspozycji, do manifestacji objawów toksycznych[5] - tzw. toksydrom/zespół cholinergiczny. Odbywa się to poprzez trzy rodzaje reakcji:

• Reakcja muskarynowa zachodzi w przywspółczulnym układzie nerwowym powodując: zapalenie spojówek, zwężenie źrenic, utratę akomodacji oka, skurcz oskrzeli, szybkie wydzielanie śluzu oskrzelowego, utratę apetytu, wymioty, skurcze jelit, pocenie się, biegunkę, ślinotok, bradykardie i spadek ciśnienia krwi[6].

• Reakcja nikotynowa zachodzi w współczulnym oraz somatycznym układzie nerwowym powodując drżenie i porażenie mięśni[6].

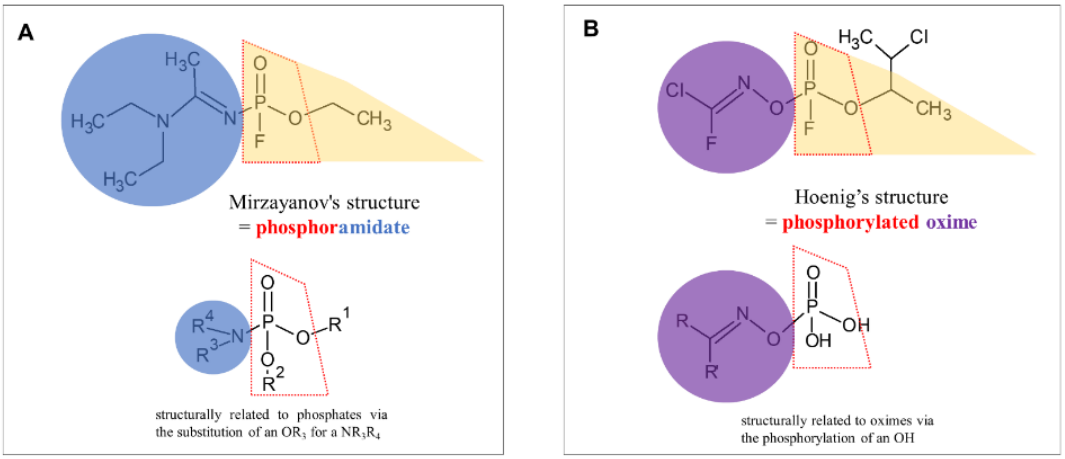
• Odpowiedź ośrodkowego układu nerwowego charakteryzuje się stanem splątania, utratą odruchów, zaburzeniami mowy, drażliwością, zmęczeniem, bezsennością oraz zatrzymaniem oddechu[3].

Pierwotnym zamiarem przy projektowaniu tych środków paraliżujących (NA; ‘nerve agent’) było obejście listy Konwencji o zakazie broni chemicznej (CWC; ‘Chemical Weapons Convention’) oraz niewykrywalność przy użyciu standardowego sprzętu do detekcji chemikaliów w ramach Organizacji Traktatu Północnoatlantyckiego (NATO)[7]. Niezwykle cenna przy identyfikacji związku typu Novichok (A-232) okazała się metoda ultraczułej spektroskopii wibracyjnej[8]. W 2023 roku opracowano specjalnie

zaprojektowane do wykrywania Novichok urządzenie kolorymetryczne, oparte na pochodnych hydrazonu z 2,4-dinitrofenylohydrazyny (2,4-DNPH) impregnowanych na podłożu z włókna szklanego[9]. Test ten umożliwia wykrycie związków fosforoorganicznych z grupy Novichok, natomiast wykazuje problemy przy identyfikacji ich prekursorów. Jest to o tyle istotna wada, ponieważ niektóre związki serii A można zaklasyfikować jako tzw. binarną broń chemiczną (dwa prekursory przed połączeniem są nietoksyczne lub małotoksyczne, dopiero po zmieszaniu oraz przereagowaniu tworzą aktywny środek paraliżujący.

Nadrzędnym problemem związków fosforoorganicznych z grupy Novichok jest znikoma ilość danych zawartych w literaturze naukowej. Obecnie istnieje wiele niewiadomych i luk w danych, które wymagają wyjaśnienia oraz uzupełnienia. Dodatkowo dochodzi kwestia niepewności dotychczas opublikowanych informacji, których wiarygodność należy niezwłocznie zweryfikować.

Pierwszą, a zarazem najważniejszą wątpliwą informację stanowią zgłoszone różne wersje **struktur związków typu Novichok**. Dotychczas trwa publiczny spór, co do słuszności chemicznej budowy związków serii A. Po jednej stronie polemiki stoi Vil S. Mirzayanov, dyrektor Departamentu Przeciwdziałania Zagranicznemu Wywiadowi Technicznemu w Instytucie Badań Naukowych Chemii i Technologii Organicznej Rosyjskiego Państwowego Związku (GosNIIOKhT), który jako pierwszy ujawnił informacje o zainicjowaniu tajnej radzieckiej inicjatywy broni chemicznej w celu opracowania struktur Novichok. Pierwsze związki serii A, zostały prawdopodobnie wyprodukowane przy użyciu szkieletu strukturalnego fosforoorganicznego, tj.: [R_1–P(=O)(R_2)–OR_3; R_1 = amid/oksym; R_2 = fluor; R_3 = alkil, alkoksyl, alkiloamino][10]. Z chemicznego punktu widzenia postuluje się, że struktury Novichok są związkami fosforoorganicznymi zawierającymi ugrupowanie dihaloformaldoksymu[11]. Mirzayanov[12] zaproponował budowę chemiczną Novichok jako fosforamidy (Ryc. 1A). Jednak w tym samym czasie S. Hoenig[13] i D. H. Ellison[14] zaproponowali kilkadziesiąt możliwych alternatywnych struktur Novichok oraz ich prekursorów jako fosforylowane oksymy (Ryc. 1B). Dodatkowo istnieje wzmianka o strukturze opisanej przez Jiri Bajgar'a, w której do centralnego atomu fosforu przyłączona jest podstawiona grupa imidoamidowa[15].



Rycina 1. Proponowane struktury chemiczne związków typu Novichok: (A) struktura Mirzayanova jako fosforoamid, (B) struktura Hoeniga jako ufosforylowany oksym[1].

Pan dr hab. inż. Leszek Konopski wskazywał również inną możliwą strukturę, w której atom fluoru związany z atomem fosforu zastąpiony jest ugrupowaniem dialkiloamidowym (analogicznym do obecnego w tabunie)[16]. Grupa irańskich naukowców opublikowała pracę opisującą widma mas i ścieżki fragmentacji analogu związku określanego przez Mirzayanov'a jako Novichok A-242 oraz kilku jego pochodnych[17]. Dlatego, pomimo posiadanych informacji dotyczących struktur związków serii A nadal pozostają one niewyjaśnione i okryte tajemnicą a jedyne dostępne dane (badania pionierskie) pochodzą z wywiadów i publikacji Mirzayanov'a[12] oraz Karev'a[18]. W opinii większości naukowców nie jest to do końca rzetelne źródło informacji, wersja Hoenig'a oraz Ellison'a wydaję się najbardziej wiarygodną[19,20]. Jednak Chai[3] oraz Harvey[21] zauważyli, że istnieje pewien konsensus odnośnie fosforoamidanowej natury środków paraliżujących serii A. Ustalenie rzeczywistych struktur związków typu Novichok jest kluczowe, stworzy fundament do przyszłych badań teoretycznych oraz eksperymentalnych, które są niezbędne, aby zrozumieć prawdziwą naturę, a zarazem zagrożenia, jakie mogą powodować związki fosforoorganiczne z serii A. Obecnie jakiekolwiek badania dotyczące BŚT z grupy Novichok są nad wyraz rzadkie oraz dopiero niedawno zaczęło pojawiać się zwiększone zainteresowanie społeczeństwa tematem tych bezsprzecznie niebezpiecznych związków[22].

Informacje dotyczące **właściwości fizycznych i chemicznych związków typu Novichok** są znikome i skupiają się w głównie na trzech strukturach upublicznionych przez Mirzayanov'a[12]: A-230, A-232 oraz A-234. Dla powyższych związków

w literaturze naukowej można uzyskać dane o ich gęstości (1,414–1,612 $g \cdot mL^{-1}$), stanie skupienia (brak danych dla A-232), ogólnej lotności bez wskazania dokładnych wartości[18,23,24]. Związki A-230, A-232 i A-234 w niskich temperaturach nie zestalają się[20]. Niskie prężności par (1.48-2.13 Pa) podane dla A-230, A-232, A-234 sugerują, że utrzymują się one niezmienne w środowisku[14,25]. Według Mirzayanov'a, A-242 posiada stały stan skupienia w temperaturze pokojowej[12]. Oprócz czterech wyżej wymienionych związków Novichok opisanych przez Mirzayanov'a istnieje jeszcze piąta struktura: A-262 oraz Novichok zsyntetyzowany przez grupę irańskich naukowców[17]. Dodatkowo Ellison[14] wskazał kilkanaście prawdopodobnych struktur (n = 11) C01-A035- C01-A045. W literaturze naukowej można zauważyć, że skupiono się jedynie na trzech strukturach (A-230, A-232 i A-234) przy ustalaniu ich właściwości fizycznych i chemicznych, natomiast dla reszty opisanych Novichok informacji w literaturze naukowej brak.

Równie niesłychanie istotną kwestią (o ile nie najważniejszą, na jakiej należy się skupić), jest **toksyczność związków typu Novichok**. Te środki paraliżujące stanowią potencjalne i ogromne zagrożenie ze względu na ich ekstremalną toksyczność. Jako społeczeństwo, trzykrotnie już doświadczyliśmy działania środków Novichok. Pierwszym przykładem ilustrującym zagrożenie ze strony związków tego typu był atak chemiczny z 4 marca 2018 r. w Salisbury (Wielka Brytania). Przypadek ostrego zatrucia Siergieja Skripala i jego córki Julii był wynikiem działania środka paralityczno-drgawkowego A-234[26]. Podobny przypadek miał miejsce 30 czerwca 2018 roku w Amesbury (Wielka Brytania), gdzie dwóch obywateli Wielkiej Brytanii zostało zatrutych tym samym związkiem, co w Salisbury[27]. Najprawdopodobniej pozostałościami znalezionymi po poprzednim ataku. Trzeci przypadek zatrucia, w wyniku którego stwierdzono zastosowanie związku typu Novichok, do którego doszło 20 sierpnia 2020 r. podczas lotu krajowego w Rosji. Wcześniej zdrowy 44-letni mężczyzna, około 10 minut po wyjściu, nagle poczuł się zdezorientowany i zaczął się obficie pocić, ślinić, łzawić, wymiotował, zasłabł, a następnie stracił przytomność. Na podstawie wyników badań klinicznych oraz laboratoryjnych, stwierdzono całkowite zahamowanie acetylocholinoesterazy w krwinkach czerwonych, co potwierdziło ekspozycję na inhibitor cholinoesterazy[28]. Przypadek Alexeia Navalnego jest jedynym opublikowanym badaniem klinicznym dotyczącym leczenia zatrucia środkiem paralitycznym typu Novichok, w którym udowodniono skuteczność terapii butyrylocholinoesterazy (BChE) oraz wykazano brak skuteczności reaktywacji

po podaniu obidoksymu[28]. W związku z tym należy rozważyć różne podejścia do leczenia zatruć związkami tego typu. Niewiele wiadomo w tej kwestii, lecz rozsądnym podejściem wydaję się ekstrapolacja sposobów leczenia klinicznego w przypadku ekspozycji na związki z serii -G oraz -V wprost na związki Novichok[29]. Powyższe przykłady ataków chemicznych potwierdziły skutki ostrego zatrucia związkami fosforoorganicznymi typu Novichok, wskazując na ich obecność w przestrzeni publicznej. Dlatego też, z punktu widzenia bezpieczeństwa społecznego istotne jest zbadanie aspektów toksykologicznych związków serii A, takich jak toksyczność ostra (wyznaczenie medialnej dawki śmiertelnej - LD_{50}), przewlekła oraz dermalna. Mirzayanov jako pierwszy upublicznił dane, w których określił toksyczność środków paraliżujących typu Novichok jako kilkukrotnie wyższą niż konwencjonalne środki paraliżujące[12]. Novichok A-230 był szacunkowo 5-8 razy bardziej toksyczny niż związek VX. Ponadto twierdził, że A-232 jest 10 razy bardziej niebezpieczny niż Soman (GD). Środki paraliżujące A-242 i A-262, toksyczne pochodne A-230 i A-232, zostały sklasyfikowane jako ultratoksyczne[12]. Zgodnie z danymi opublikowanymi w referacie seminaryjnym Karev'a, powyższe doniesienia Mirzayanov'a o toksyczności związków Novichok były nieprawdziwe[18]. A-232 wykazał toksyczność ostrą o rząd wielkości niższą od VX, natomiast A-234 dwukrotnie niższą od VX. Niższą ostrą toksyczność związków serii A w porównaniu z konwencjonalnymi środkami paraliżującymi w pewnym stopniu potwierdził Franca na podstawie danych z badań toksykologicznych typy *in silico*, tj. estymowanych[19]. Wartość LD_{50} (doustnie, szczury) dla A-230 była o rząd wielkości niższa, podczas gdy związki A-232 oraz A-234 były około trzykrotnie mniej toksyczne niż VX. Próbę weryfikacji danych dla Novichok (n = 6) podjął również Carlsen[30], który za pomocą ilościowych modeli zależności struktura-aktywność ((Q)SAR) estymował medianę dawki śmiertelną (szczury, podanie doustne), które zostały przeliczone na medialną dawkę śmiertelną dla ludzi. Carlsen przedstawił zupełnie odmienne dane dotyczące względnej toksyczności środków paraliżujących serii A, wbrew twierdzeniom Mirzayanov'a. Szacunkowe dane wykazały, że związki Novichok (A-230, A-232, A-234, A-242 i A-262) są 5-75 razy mniej niebezpieczne niż VX, a w przypadku irańskiego "Novichok"[17] prawie 1000 razy mniej toksyczny. Źródła literaturowe podają wartości LD_{50} dla maksymalnie sześciu związków fosforoorganicznych z grupy Novichok (szczury, podanie doustne). Natomiast dla pozostałych związków serii A (opisanych przez Mirzayanov'a, Ellison'a, Hoeing'a oraz Hosseini) występują luki w danych dotyczących toksyczności tych niebezpiecznych związków.

Los środowiskowy związków typu Novichok (w kontekście toksykologii środowiska) jako hipotetycznej grupy środków paraliżujących powinien stanowić kluczową kwestię do wyjaśnienia, niestety taka sytuacja nie ma obecnie miejsca (brak danych literaturowych). Po drugim ataku chemicznym w Amesbury wszczęto dyskusję na temat losów środowiskowych środków paraliżujących Novichok[31]. Po tym zdarzeniu narodziło się wiele wątpliwości o bezpieczeństwo otoczenia zewnętrznego po kontakcie z niebezpiecznymi związkami serii A (zagadnienia istotne z punktu widzenia medycyny katastrof – incydenty CBRN). W celu wyjaśnienia losów związków typu Novichok w środowisku, a co za tym idzie - oceny bezpieczeństwa w odniesieniu do ludzi przypadkowo narażonych, jest ocena ich trwałości w środowisku. W tym celu należy rozpatrzyć dwa parametry - hydrolizę oraz biodegradację, które są wymagane w celu określenia stabilności związków w środowisku oraz metod dekontaminacji w celu zmniejszenia szkód wtórnych. Kwestie te są jednak traktowane marginalnie oraz nigdy nie zostały odpowiednio rozwiązane. Wiąże się to również z tym, że temat hydrolizy oraz biodegradacji kojarzy się wyłącznie z domeną ekotoksykologii, a tymczasem ma to krytyczne znaczenie w odniesieniu do incydentów typu CBRN/HAZMAT. Badania pilotażowe przeprowadzone przez Carlsen'a dotyczyły estymacji okresu półtrwania hydrolizy struktur Novichok (n = 6)[30]. Umiarkowaną przemianę w wyniku hydrolizy, od 10 do 30 dni, wykazały: A-232, A-234 i A-262. Krótkie okresy półtrwania hydrolizy, poniżej jednego dnia, wykazały: A-230 i A-242. Szybkość hydrolizy związków typu Novichok została zmierzona eksperymentalnie przy pH = 7,2 (25°C) przez Harvey'a[21]. Dane ukazały, że hydroliza związków serii A była znacznie wolniejsza o kilka rzędów wielkości niż środków z serii G i V. Szybkość hydrolizy: GB = 6,68 μM/min, VX = 0,246 μM/min, A-230 = 0,17 μM/min, A-232 = 0,061 μM/min, A-234 = 0,0032 μM/min. Nie ma wątpliwości, że los tych niebezpiecznych substancji w środowisku jest znaczący, ale także trudny do określenia ze względu na ich toksyczność, a ma to niezaprzeczalne znaczenie dla bezpieczeństwa człowieka. Powyżej wskazane badania wstępne zostały wykonywane różnymi metodami *in silico* oraz eksperymentalnymi w różnych kontekstach, ale nigdy odnosząc się do wpływu na środowisko lub toksykologiczną ocenę ryzyka zdrowotnego. Uzasadnieniem estymacji parametru biodegradacji jest kompletny brak podstawowych danych na temat BŚT z grupy Novichok w literaturze naukowej. Natomiast w przypadku hydrolizy literatura zawiera jedynie dane o okresie półtrwania hydrolizy dla sześciu Novichok oraz szybkości hydrolizy dla trzech z nich.

Brak zatem danych dla kilkunastu związków Novichok oraz parametru stałej szybkości (Kn) hydrolizy.

Uznając wrażliwość na zagrożenie działalnością terrorystyczną, najbardziej pożądanym i uzasadnionym podejściem w tej sytuacji wydaje się zastosowanie metod toksykologicznych *in silico*. Aby uzupełnić luki w brakujących danych, fundamentalną kwestią jest określenie podstawowych parametrów fizycznych i chemicznych związków typu Novichok, określenie trwałości środowiskowej oraz wyznaczenie toksyczności.

5. Cele pracy

Ogólny plan badawczy pracy doktorskiej zakłada określenie wybranych parametrów toksykologicznych dla związków fosforoorganicznych z grupy Novichok (n = 17) przy wykorzystaniu metod toksykologii *in silico*. Pierwszy etap badań to przegląd literatury naukowej z wykorzystaniem wyszukiwarek naukowych (PubMed, Google Scholar, Scopus) oraz baz toksykologicznych (PubChem, CompTox Chemicals Dashboard oraz ECHA) w celu pozyskania danych z przeprowadzonych estymacji oraz znalezienia odpowiednich luk danych. Wyznaczenie parametrów toksykologicznych stanowi kolejny etap badań, tj. toksyczność ostra (medialna dawka śmiertelna, LD_{50}). Ostatni etap zakłada wyznaczenie parametrów istotnych z punktu widzenia toksykologii środowiska, tj. hydroliza (czasu półtrwania hydrolizy, stałej szybkości hydrolizy) oraz biodegradacji, co pozwoli określić, przez jaki czas związki te będą stanowiły zagrożenie dla ludzi po bezpośrednim kontakcie ze środowiska.

Pytania badawcze:

- Czy z powodzeniem można estymować wybrane parametry toksykologiczne dla środków paraliżujących typu Novichok przy użyciu metod *in silico*?
- Jakie zagrożenie stwarzają związki typu Novichok w kontakcie z człowiekiem?
- Jakie wartości przyjmuje medialna dawka śmiertelna (szczur, podanie doustne) dla każdego z hipotetycznych związków typu Novichok (n = 17)?
- Czy bojowe środki trujące z grupy Novichok przewyższają toksycznością ostrą (LD_{50}) poprzednie generacje środków paraliżujących (-V i -G)?
- Jak długo po zastosowaniu związki typu Novichok będą zagrażać człowiekowi w kontekście toksykologii środowiska (hydroliza oraz biodegradacja)?
- Jakiego rzędu wielkości można się spodziewać dla szybkości hydrolizy związków fosforoorganicznych z grupy Novichok?
- Czy związki serii A ulegają skutecznej biodegradacji tlenowej oraz beztlenowej?
- Które metody toksykologii *in silico* będą charakteryzowały się najwyższą wiarygodnością uzyskiwanych wyników w porównaniu do innych użytych?

6. Materiały i metody

Wyszukiwanie publikacji zawierających dane dotyczące związków typu Novichok. Do krytycznego przeglądu danych wykorzystano wyszukiwarki naukowe: PubMed, Google Scholar oraz Scopus (jako podstawowe repozytoria do wyszukiwania opublikowanych odniesień). Proces zbierania danych polegał na przeszukiwaniu wspomnianych źródeł naukowych, ale uwzględniał także tzw. „szarą" literaturę (V. S. Mirzayanov, State Secrets: An Insider's Chronicle of the Russian Chemical Program[12]), korzystając z danych teoretycznych i empirycznych. Zastosowano różne kombinacje następujących terminów w języku angielskim: Novichok; środki paraliżujące; związki serii A; Mirzayanov; Siergei Skripal; Alexei Navalny; związki fosforoorganiczne; A-230; A-232; A-234; *N*-[etoksy(-fluoro)fosforylo]-*N*,*N*-dietyloetanoimidamid; HAZMAT; CBRN. Tylko artykuły/prace związane ze strukturami typu Novichok brano pod uwagę przy filtrowaniu wyszukanych źródeł.

Badania toksykologiczne *in silico*

Wszelkie badania zrealizowano w oparciu o metody *in silico*. Etymologicznie wyrażenie to znaczy „w krzemie", natomiast technicznie oznacza badanie wykonywane za pomocą komputera. Teoretyczna metoda badawcza obejmująca modelowanie oraz symulacje komputerowe, służy do przewidywania prawdopodobnych działań toksycznych danej substancji. W dziedzinie toksykologii, jeśli uznamy jakąkolwiek działalność komputerową za „metodykę *in silico*", wówczas prawie wszystkie badania mieszczą się w tej kategorii, ponieważ obejmują komputerowe planowanie oraz analizę[32]. Podstawowym podejściem *in silico* wykorzystanym w tych badaniach jest (Q)SAR (ang. Quantitative Structure-Activity Relationship), którego celem jest opisanie struktur chemicznych za pomocą deskryptorów w celu skorelowania efektów lub właściwości.

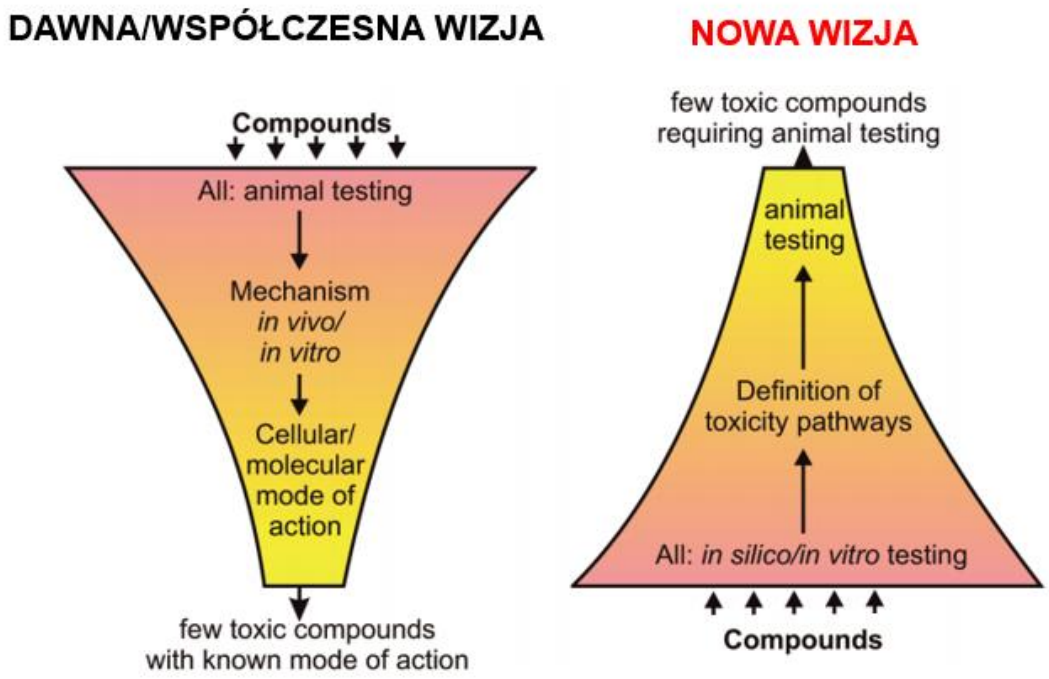
Badania eksperymentalne nad związkami Novichok są znikome, a dopiero niedawno zaczęły pojawiać się jakiekolwiek badania teoretyczne (*in silico*). Biorąc pod uwagę trzy przypadki ataków chemicznych z udziałem nowych środków paraliżujących, nie ma wątpliwości, że określenie aspektów toksykologicznych związków Novichok jest wymagające ze względu na ich wysoką reaktywność i toksyczność. Dodatkowo, ponieważ środki paraliżujące typu Novichok są niedostępne a wyłącznym sposobem ich otrzymania jest synteza, do której wymagane są nadzwyczajne środki ostrożności.

Jedynym nie stwarzającym zagrożenia dla zdrowia sposobem określenia parametrów związków Novichok jest wykorzystanie narzędzi *in silico*. Co więcej, aby sprostać współczesnym wymaganiom badań toksykologicznych oraz rozważyć ocenę ryzyka następnej generacji (NGRA, ang. next generation risk assessment)[33] z nowym podejściem do badań toksykologicznych (uwzględniając w pierwszej kolejności przewidywanie parametrów toksykologicznych), konieczne jest zastosowanie w pierwszej kolejności metod toksykologicznych *in silico* w celu wyeliminowania niepotrzebnych badań z wykorzystaniem zwierząt. Metody *in silico* oparte na ustalaniu ilościowej zależności struktura-aktywność (QSAR) zaliczamy do metod alternatywnych, których idea powstania całkowicie opiera się na zasadzie „3R"[34]. Zaproponowana przez Williama Russella oraz Rexa Brucha strategia odnosi się do trzech pojęć:

- Reduction (ograniczenie) – maksymalna redukcja liczby wykorzystywanych żywych zwierząt przy jednoczesnym zachowaniu dokładności wyników;
- Refinement (doskonalenie) – udoskonalanie metod w celu ciągłego zmniejszania bólu i cierpienia wykorzystywanych do badań zwierząt;
- Replacement (zastąpienie) – całkowite wykluczenie z badań modeli zwierzęcych lub zastąpienie ich modelami *in vitro* lub *in silico*.

Dotychczas, klasyczne procedury wyznaczania toksyczności ostrej, wyrażonej ilościowo poprzez parametr LD_{50} (medialna dawka śmiertelna), wiązały się z użyciem dużej liczby zwierząt oraz wysoką śmiertelnością. Testy alternatywne opracowano w celu istotnej poprawy dobrostanu zwierząt, a także dostarczenia wyników umożliwiających określenie klasy toksyczności oraz oszacowanie ryzyka dla zdrowia ludzi i środowiska. Zgodnie z zasadą 3R badania z zastosowaniem testów alternatywnych są wykonywane na mniejszej liczbie zwierząt i tylko jednej płci (w przeciwieństwie do testu 401, usuniętego z wytycznych OECD[35]). Przykładowo, procedura większej-mniejszej dawki[36] (test 425), umożliwiająca wyznaczenie krzywej zależności dawka-efekt, czyli oszacowanie LD_{50} wraz z przedziałem ufności. Procedura ta polega na podawaniu jednemu zwierzęciu (gryzonie, najlepiej szczury) jednej dawki, niższej od oczekiwanej wartości LD_{50}. Zależnie od uzyskanych efektów następnemu zwierzęciu podaje się dawkę większą (jeśli pierwsze zwierzę przeżyje) lub mniejszą (jeśli pierwsze zwierzę umrze). Jeśli dane nie są dostępne, badanie rozpoczyna się od dawki 1,75 mg/kg mc. zwiększanej o stały współczynnik równy 3,2 (wartość 0,5 na skali logarytmicznej). Badanie prowadzi

się do chwili ustalenia dawki, której zwiększenie powoduje zgon, a obniżenie – przeżycie zwierzęcia. Niemniej jednak, pomimo redukcji liczby wykorzystywanych żywych zwierząt oraz zmniejszenia ich cierpień podczas procedur alternatywnych, współczesne badania toksykologiczne dążą do zminimalizowania badań na modelach zwierzęcych metodami wykorzystującymi modele *in vitro* lub *in silico*. Nowa koncepcja badań toksyczności sugeruje radykalną zmianę paradygmatu (Ryc. 2). Rozpoczęcie oceny bezpieczeństwa od screeningu właściwości chemicznych związku metodami *in silico*, a następnie przejście do charakterystyki biologicznej poprzez systemy *in vitro*. Procedury bioinformatyczne przekształciłyby te informacje definiując drogi toksyczności. Nadałoby to priorytet tylko kilku związkom do dalszych badań na zwierzętach, a procedura sama w sobie byłaby wystarczająca do wyeliminowania wielu związków oraz mieszanin.



Rycina 2. Porównanie dotychczasowej a nowej koncepcji badań toksykologicznych[37].

W badaniach *in silico* dotyczących estymacji wybranych parametrów związków typu Novichok zastosowano serię komercyjnie dostępnych oprogramowań specjalistycznych zalecanych przez organy regulacyjne. Poniżej przedstawiono zwięzły zarys stosowanych metod badawczych.

QSAR Toolbox. Badania *in silico* przeprowadzono przy użyciu pakietu programów QSAR Toolbox w wersji 4.5, które organizacja OECD zaleca do uzupełniania luk w danych dotyczących (eko)toksyczności chemikaliów[38]. Zestaw narzędzi QSAR służący do oceny potencjalnych zagrożeń chemikaliów za pomocą modeli *in silico* w celu ułatwienia praktycznego stosowania podejść (Q)SAR w kontekście regulacyjnym[38,39]. Luki w danych są wypełniane za pomocą następującego elastycznego przepływu pracy, w ramach którego tworzone są złożone kategorie, a niekompletne dane są szacowane poprzez podejście przekrojowe lub zastosowanie lokalnych QSAR. Oprócz podejścia przekrojowego i analizy trendów zestaw narzędzi zawiera liczne bazy danych wyników eksperymentów.

Estimation Program Interface (EPI) Suite. Pakiet oprogramowania (wersja 4.11) do prognozy właściwości fizycznych, chemicznych i losów środowiskowych opracowany przez amerykańską Agencję Ochrony Środowiska (US EPA). Estymatory EPI Suite opierają się przede wszystkim na metodzie stałych fragmentów (wkładów grupowych), zweryfikowanej przy użyciu niezależnego zestawu substancji chemicznych[40]. Poszczególne modele zastosowane w pakiecie EPI Suite:

- KOWWIN: Szacuje logarytm współczynnika podziału oktanol-woda.
- HENRYWIN: Oblicza współczynnik podziału powietrze/woda.
- MPBPWIN: Szacuje temperaturę topnienia, temperaturę wrzenia i prężność par.
- BIOWIN: Oblicza podatność na biodegradację tlenową i beztlenową.
- WATERNT: Oszacowuje rozpuszczalność w wodzie.
- HYDROWIN: Estymuje stałe szybkości hydrolizy wodnej i okresy półtrwania.

TEST (Toxicity Estimation Software Tool). Specjalistyczne oprogramowanie naukowe opracowane przez US EPA[41]. Narzędzie do predykcji toksyczności (wersja 4.2.1 oraz 5.1.2) obejmuje kilka modeli, które oceniają progi toksyczności ostrej poprzez podejście przekrojowe między analogami strukturalnymi lub regresję wielowymiarową. Modele są zbudowane na setkach deskryptorów właściwości strukturalnych, konstytucyjnych, połączeń, kształtu, topologii, odległości molekularnych oraz fragmentów. Każdy model podejścia przekrojowego (tzw. read across) lub model regresji ma określoną dziedzinę zastosowania. Oprogramowanie oferuje szacunkowy próg LD_{50} na podstawie przewidywań każdego modelu i średniej konsensusu modeli składowych. TEST ocenia toksyczność ostrą przy użyciu czterech metodologii QSAR[42]:

- Metoda hierarchiczna – toksyczność dla konkretnego badanego związku jest szacowana przy użyciu średniej ważonej prognoz z różnych modeli.
- Metoda FDA (tylko wersja 4.2.1) – prognoza dla każdej badanej substancji chemicznej jest dokonywana przy użyciu nowego modelu, który pasuje do substancji chemicznych najbardziej podobnych do badanego związku.
- Metoda najbliższego sąsiada – toksyczność szacuje się przez uśrednienie trzech substancji chemicznych w zbiorze treningowym o największym podobieństwie do badanego związku.
- Metoda konsensusu – przewidywaną toksyczność szacuje się, biorąc średnią przewidywanych toksyczności z każdej z powyższych metodologii (Q)SAR.

7. Wyniki

Toksyczność związków typu Novichok

Ostra toksyczność, wyrażona jako medialna dawka śmiertelna (LD_{50}) badanych BŚT z grupy Novichok (n = 17), została oszacowana przy użyciu oprogramowania: QSAR Toolbox oraz TEST. Estymowano wartości LD_{50} dla doustnego podania szczurom. Ekstrapolacje toksyczności ostrej ze zwierzęcia na człowieka oparto na wartościach toksyczności zgodnie z wytycznymi dotyczącymi przeliczania dawek między zwierzętami i ludźmi[43]. Podejście allometryczne uwzględnia różnice w powierzchni ciała, które są związane z masą zwierząt, jednocześnie ekstrapolując dawki środków między gatunkami. Wartości LD_{50} dla szczurów ekstrapolowano w odniesieniu do ludzi (przeliczono na równoważne dawki dla ludzi), dzieląc dawkę dla szczura przez 6,2[43]. Obliczone wartości LD_{50} dla doustnego podania związków typu Novichok przedstawiono w **Tabeli 1**.

Tabela 1. Wartości LD_{50} po podaniu doustnym dla szczura i człowieka.

Związek	Nazwa	LD_{50} dla szczura (mg/kg mc.)			LD_{50} dla człowieka (mg/kg mc.)		
		TEST Consensus	TEST FDA	QSAR Toolbox	TEST Consensus	TEST FDA	QSAR Toolbox
1	A-230	2,14	9,59	3,09	0,35	1,55	0,50
2	A-232	1,31	3,52	2,53	0,21	0,57	0,41
3	A-234	3,57	4,43	3,91	0,58	0,71	0,63
4	A-242 (Novichok-5)	92,81	3,04	55,9	14,97	0,49	9,02
5	A-262 (Novichok-7)	276,26	45,56	141,7	44,56	7,35	22,85
6	Irański "Novichok"	1109,56	596,17	810,3	178,96	96,16	130,69
7	C01-A035	206,51	1082,59	263,8	33,31	174,61	42,55
8	C01-A036	124,61	427,72	193,1	20,10	68,99	31,15
9	C01-A037	266,37	813,51	324	42,96	131,21	52,26
10	C01-A038	1922,26	2099,13	1030	310,04	338,57	166,13
11	C01-A039	35,39	34,44	48,6	5,71	5,55	7,84
12	C01-A040	26,05	4,19	15,4	4,20	0,68	2,48
13	C01-A041	49,11	95,16	58,5	7,92	15,35	9,44
14	C01-A042	206,76	27,17	129,9	33,35	4,38	20,95
15	C01-A043	16,47	6,21	12,5	2,66	1,00	2,02
16	C01-A044	39,72	31,23	45,7	6,41	5,04	7,37
17	C01-A045	243,78	96,01	191,8	39,32	15,49	30,94

Ponieważ zastosowane modele Consensus oraz FDA wykazały liczne nieścisłości w wartościach LD_{50} dla badanych związków Novichok, zdecydowano dodatkowo użyć oprogramowania QSAR Toolbox w celu oszacowania parametru ostrej toksyczności. Wyniki uzyskane przez kolejne oprogramowanie umożliwiły weryfikację wiarygodności obliczeń uzyskanych z modeli Consensus i FDA zaimplementowanych w TEST poprzez porównanie danych. Wartości LD_{50} estymowane przy użyciu zestawu narzędzi QSAR dla większości związków korelowały z metodą Consensus oprogramowania TEST. Tylko dwie struktury Novichok (**2** i **6**) były bardziej zgodne z metodą FDA. Jednak w przypadku związków (**10**–**11**), dla których wartości obu metod TEST są porównywalne, nie można jednoznacznie stwierdzić większego stopnia korelacji. Oszacowana wartość dla struktury (**12**) w QSAR Toolbox różniła się zarówno od metody Consensus i FDA. Wspólną cechą wszystkich estymacji jest najwyższa wartość uzyskana przez Novichok C01-A038 (**10**). Najwyższe wartości ostrej toksyczności, czyli najmniej toksyczne, osiągnęły związki serii A: irański "Novichok" (**6**) oraz C01-A038 (**10**). Analogicznie do metody Consensus, pierwszych pięć związków o najniższych wartościach LD_{50}, zatem stwarzających największe zagrożenie to odpowiednio: A-232 (**2**), A-234 (**3**), A-230 (**1**), C01- A043 (**15**) i C01-A040 (**12**).

Stosując analogiczną metodę FDA zawartą w programie TEST (wersja 4.2.1) uzyskano identyczne wartości ostrej toksyczności dla struktur typu Novichok jak w pracy Carlsen'a[30]. Ponadto do oszacowania parametru LD_{50} wykorzystano również metodę Consensus zawartą w TEST (wersja 5.1.2) oraz pakiet programów QSAR Toolbox. Ponieważ wyniki metod Consensus i FDA różniły się, postawiono pytanie, które podejście jest najbardziej wiarygodne? Za pierwszym przemawiał fakt, iż wykorzystuje tu wszystkie metody QSAR zawarte w TEST do oceny toksyczności, dodatkowo jest rekomendowany przez US EPA[41]. Ponadto zgłoszono, że metoda Consensus stanowi najbardziej rzetelną estymację dostarczonym przez oprogramowanie TEST[44]. Interesującym faktem jest to, że najnowsza wersja oprogramowania TEST nie zawiera zaimplementowanej metody FDA. Prócz tego, wartości LD_{50} uzyskane przy użyciu zestawu narzędzi QSAR w przeważającej mierze korelują z metodą Consensus; jedynie dwa związki typu Novichok wykazały wartości w lepszej korelacji z metodą FDA. Biorąc pod uwagę powyższe argumenty, uznano za bardziej wiarygodne wyniki uzyskane metodą Consensus, wsparte ewaluacją przy wykorzystaniu QSAR Toolbox.

Na podstawie literatury naukowej[18,19,30], zebrano oraz zestawiono w **Tabeli 2** dostępne, przeliczone wartości LD_{50} dla związków typu Novichok (A-230, A-232, A-234, A-242, A-262, irański "Novichok" oraz związku VX).

Tabela 2. Dostępne dane literaturowe – toksyczność związków typu Novichok[18,19,30].

Związek	LD_{50} (mg/kg mc.)		
	(Karev, 2009)	(Franca, 2019)	(Carlsen, 2019)
VX	0,143	0,086-0,143	0,10
A-230	n/d	0,011-0,029	1,55
A-232	0,014	0,5	0,57
A-234	0,071	0,5	0,71
A-242	n/d	n/d	0,49
A-262	n/d	n/d	7,35
Irański "Novichok"	n/d	n/d	96,16

* n/d = nie dotyczy

Wartości LD_{50} dla środków paraliżujących Novichok oszacowane za pomocą oprogramowania TEST (metoda Consensus) oraz QSAR Toolbox różnią się znacznie od danych literaturowych zawartych w Tabeli 2. Jedynymi związkami, których wartości LD_{50} jakkolwiek wykazały podobieństwo, są A-230, A-232 i irański "Novichok". Według wykonanych estymacji najniebezpieczniejszym badanym związkiem typu Novichok jest A-232, w przeciwieństwie do wartości obliczonych przez Carlsen'a[30], gdzie środek paraliżujący A-242 miałby być najbardziej toksyczny. Natomiast, Franca[19] zasugerował A-230 jako Novichok o najniższej wartość LD_{50}. Żadna z estymowanych wartości ostrej toksyczności BŚT grupy Novichok nie przewyższa konwencjonalnych bojowych środków trujących – związku VX oraz GD. Niestety źródła literaturowe podają LD_{50} jedynie dla maksymalnie sześciu związków fosforoorganicznych z grupy Novichok. Uzupełniono lukę w danych poprzez estymację siedemnastu struktur Novichok z zastosowaniem różnych podejść *in silico*.

Biodegradacja związków typu Novichok

Modele Biowin1-2 w oparciu o bazę danych BIODEG szacują prawdopodobieństwo szybkiej biodegradacji. Każdemu z tych modeli przypisano oznaczenie zgodnie z zastosowanym w tym celu kryterium klasyfikacyjnym, a oszacowane prawdopodobieństwo to: „biodegraduje szybko" (⩾0,5) lub „nie ulega szybkiej biodegradacji" (< 0,5). Każda struktura zaproponowana przez Mirzayanov'a[12] (**1**-**5**) osiągnęła wartości powyżej 0,62, co według kryterium klasyfikuje je jako szybko degradowalne. Irański "Novichok" (**6**) Hosseiniego[17] osiąga wysoką wartość (0,747) modelu Biowin1. Spośród struktur wskazanych przez Ellisona[14] cztery wykazywały łatwą biodegradowalność (**8**-**9**, **12**-**13**). Model Biowin1 oszacował biodegradacje jako szybką dla 10 z 17 badanych związków Novichok (58,82%). Model Biowin2 obliczył szybką biodegradację środków paraliżujących Novichok dla 6/17 (35,29%). Zastosowany model ujawnił mniejszą ilość szybko ulegających biodegradacji środków paraliżujących (**1**-**2**, **4**, **6**, **9** oraz **13**) w porównaniu z Biowin1, co wynika z zastosowania modelu nieliniowego zamiast liniowego do ilościowego przewidywania prawdopodobieństwa.

Modele eksperckie (Biowin3 i Biowin4) szacują czas niezbędny do pierwotnej i ostatecznej biodegradacji tlenowej w wodzie. Pierwotna biodegradacja modyfikuje tożsamość związku macierzystego w pierwszym etapie, podczas gdy ostateczna biodegradacja całkowicie rozkłada substancję chemiczną w wodzie[46,47]. Ostateczne ramy czasowe biodegradacji dla Biowin3 wykazały, że wszystkie badane związki Novichok potrzebowały okresu tygodni do miesięcy, aby całkowicie zdegradować. Wyjątek stanowiły struktury **1**, **4**, **9** i **13**, które do całkowitej biodegradacji wymagały tygodni. Model Biowin4 dla związków serii A oszacował ramy czasowe pierwotnej biodegradacji jako dni do tygodni. Ostateczna biodegradacja (model Biowin3) A-230 (**1**) oraz A-242 (**4**) obliczono w tygodniach, co może być związane z obecnością w strukturze pochodnej kwasu fosfonowego zamiast kwasu fosforowego. Ponadto określono całkowity czas degradacji w tygodniach dla związków C01-A037 (**9**) oraz C01-A041 (**13**). Wspólną cechą obu jest niska masa molowa, która nie przekracza 191 $g \cdot mol^{-1}$ oraz obecność w strukturze dwóch zewnętrznych atomów fluoru. Jedyne znane badania literaturowe[30] dotyczące biodegradacji struktur typu Novichok nawiązuje jedynie do związków **1**-**6**. Wartości biodegradacji pierwotnej (model Biowin4) są zgodne z wynikami. Jednakże, ostateczna biodegradacja (model Biowin3) Carlsen'a[30], miała miejsce w ciągu tygodni do miesięcy dla każdego związku badanego. Co jest niezgodnie

z wykonanymi estymacjami, które kwalifikują związki A-230 (**1**) i A-242 (**4**) jako ulegające degradacji w tydzień. Prognoza za pomocą modeli Biowin 3 i Biowin 4 jest najbardziej odpowiednią[40], ponieważ odzwierciedla środowisko wodne pożądane zarówno z punktu widzenia toksykologii środowiskowej, jak i klinicznej (fizjologiczna wartość pH dla krwi obwodowej mieści się w przedziale około: 7,35 – 7,45).

Estymacje przy użyciu modeli Biowin5-6 oparte na liniowym/nieliniowym modelu prawdopodobieństwa wykorzystują bazę danych MITI. Oszacowane prawdopodobieństwo jest „łatwo biodegradowalne" ($\geqslant$ 0,5) lub „niełatwo biodegradowalne" (< 0,5). Spośród wszystkich zbadanych struktur Novichok (n = 17) tylko A-230 (**1**) oraz A-242 (**4**) przekroczyły wartości modelu Biowin5 $\geqslant$ 0,5, podczas gdy dla modelu Biowin6 żaden związek nie został sklasyfikowany jako łatwo biodegradowalny.

Model Biowin7, klasyfikując wszystkie prognozy degradacji w analogiczny sposób jak w modelach Biowin1-2 oraz 5-6, stosowany jest do przewidywania biodegradacji beztlenowej. Metoda ta wykazała pozytywny wynik testu szybkiej biodegradacji beztlenowej dla 76,47% (13/17) badanych związków Novichok. Struktury **6**, **10**, **14** i **17**, których model Biowin7 nie kwalifikuje się jako szybko biodegradowalne, charakteryzują się wszechstronną strukturą rozgałęzień oraz dużą masą molową (254 – 270 $g \cdot mol^{-1}$).

Kryteria przewidywania łatwej biodegradowalności mają miejsce, jeśli model Biowin3 wynosi „tygodnie" lub szybciej, a prawdopodobieństwo modelu Biowin5 wynosi $\geqslant$ 0,5; wtedy prognoza jest spełniona. Biorąc pod uwagę kryterium łatwej biodegradowalności, dwa analizowane związki typu Novichok spełniają przewidywanie: A-230 (**1**), A-242 (**4**). Podsumowane wyniki, w tym wartości dla każdego modelu Biowin uzyskane z oprogramowania EPI Suite, przedstawiono w **Tabeli 4**.

Tabela 4. Przewidywane wartości biodegradacji przy pomocą modeli Biowin1-7.

Związek	Biowin1	Biowin2	Biowin3	Biowin4	Biowin5	Biowin6	Biowin7
1	BF (0,655)	BF (0,562)	Tygodnie (2,77)	Dni-Tygodnie (3,57)	BS (0,514)	BW (0,0039)	BF (0,57)
2	BF (0,647)	BF (0,506)	Tygodnie-Miesiące (2,73)	Dni-Tygodnie (3,54)	BW (0,488)	BW (0,0029)	BF (0,57)
3	BF (0,641)	BT (0,456)	Tygodnie-Miesiące (2,70)	Dni-Tygodnie (3,52)	BW (0,492)	BW (0,0029)	BF (0,596)
4	BF (0,628)	BF (0,524)	Tygodnie (2,76)	Dni-Tygodnie (3,49)	BS (0,515)	BW (0,0029)	BF (0,542)
5	BF (0,620)	BT (0,313)	Tygodnie-Miesiące (2,61)	Dni-Tygodnie (3,46)	BW (0,489)	BW (0,0022)	BF (0,542)
6	BF (0,747)	BF (0,728)	Tygodnie-Miesiące (2,63)	Dni-Tygodnie (3,46)	BW (0,331)	BW (0,161)	BT (0,179)
7	BT (0,425)	BT (0,025)	Tygodnie-Miesiące (2,39)	Dni-Tygodnie (3,34)	BW (0,353)	BW (0,0003)	BF (0,727)
8	BF (0,544)	BT (0,169)	Tygodnie-Miesiące (2,60)	Dni-Tygodnie (3,47)	BW (0,416)	BW (0,0002)	BF (0,742)
9	BF (0,663)	BF (0,621)	Tygodnie (2,81)	Dni-Tygodnie (3,59)	BW (0,479)	BW (0,0002)	BF (0,756)
10	BT (0,443)	BT (0,089)	Tygodnie-Miesiące (2,43)	Dni-Tygodnie (3,33)	BW (0,292)	BW (0,0003)	BT (0,422)
11	BT (0,418)	BT (0,020)	Tygodnie-Miesiące (2,36)	Dni-Tygodnie (3,32)	BW (0,356)	BW (0,0003)	BF (0,753)
12	BF (0,537)	BT (0,143)	Tygodnie-Miesiące (2,57)	Dni-Tygodnie (3,45)	BW (0,419)	BW (0,0001)	BF (0,768)
13	BF (0,657)	BF (0,573)	Tygodnie (2,78)	Dni-Tygodnie (3,57)	BW (0,483)	BW (0,0001)	BF (0,782)
14	BT (0,436)	BT (0,074)	Tygodnie-Miesiące (2,39)	Dni-Tygodnie (3,31)	BW (0,296)	BW (0,0001)	BT (0,448)
15	BT (0,410)	BT (0,016)	Tygodnie-Miesiące (2,32)	Dni-Tygodnie (3,30)	BW (0,368)	BW (0,0001)	BF (0,859)
16	BT (0,403)	BT (0,013)	Tygodnie-Miesiące (2,29)	Dni-Tygodnie (3,28)	BW (0,295)	BW (0,0001)	BF (0,587)
17	BT (0,396)	BT (0,011)	Tygodnie-Miesiące (2,26)	Dni-Tygodnie (3,26)	BW (0,223)	BW (0,0002)	BT (0,316)

* BF = biodegradowalne łatwo; BT = biodegradowalne trudno; BS = biodegradujące szybko; BW = biodegradujące wolno.

Hydroliza związków typu Novichok

Okres półtrwania hydrolizy dla badanych bojowych środków trujących Novichok (n = 17) przy pH w zakresie: 6,5 - 7,4 oszacowano za pomocą modułu profilowania oprogramowania QSAR Toolbox. Zgodnie z klasyfikacją wykazywały one zmienność, od bardzo szybkiej (< 1 dzień) do bardzo wolnej (⩾ 100 dni). Najszybciej hydrolizującymi związkami typu Novichok okazały się A-230 (**1**), A-242 (**4**) oraz irański (**6**). Okres półtrwania hydrolizy liczony w godzinach dla dwóch pierwszych związków (**1**, **4**) wynosi około 5 h, natomiast dla (**6**) nieco ponad 9 h. Taka szybkość hydrolizy związana jest najprawdopodobniej z obecnością pochodnej kwasu fosfonowego w rdzeniu w porównaniu z resztą badanych związków Novichok zawierających pochodną kwasu fosforanowego. Okres półtrwania hydrolizy oszacowany przez Carlsen'a[30] został określony dla związków **2**, **3** i **5** jako umiarkowany (10-30 dni). Wyniki nie korelują; dla związków serii A: **2**, **3**, **5, 15-17**, okres półtrwania zakwalifikowano jako wolny (30-100 dni). Szczegółowe wyniki dla środków paraliżujących **2**, **3** i **5** to 38 dni (różnica 8 dni), podczas gdy dla **15-16** to 32,1 dni, a dla **17** to 56,8 dni. Chociaż trend między wynikami jest porównywalny, istnieją różnice w oszacowanej liczbie dni okresu półtrwania hydrolizy. Najwolniej hydrolizujące, sklasyfikowane jako bardzo wolne (⩾ 100 dni) i trwające nawet do 2310 dni, są związki typu Novichok **7-14**. Incydent zatrucia w Salisbury z 2018 roku sugerował, że A-234 (**3**) może być stosunkowo stabilny w środowisku[31]. Okres półtrwania hydrolizy tego związku z oszacowania wyniósł 38 dni, co potwierdza przypuszczenie o jego względnej stabilności w środowisku. Zdecydowano się na szacowanie przy pH 6,5-7,4; wynika to z ważności równania stałej szybkości hydrolizy w środowisku wodnym, w których aniony wodorotlenkowe nie są dominującymi czynnikami nukleofilowymi w środowisku[45].

Korzystając z metody profilowania w modelu HYDROWIN (oprogramowanie EPI Suite) oszacowano stałą szybkość hydrolizy tylko dla związków **7-17**. Najwolniej hydrolizującymi strukturami zaproponowanymi przez Ellison'a[14] okazały się związki **7-10**, dla których wartość stałej szybkości hydrolizy (Kn) wyniosła 0,7528/dni. Nieco szybciej hydrolizujące (osiągające wartość 0,7688/dni) wykazują związki **11-14**. Jedyną różnicą w stosunku do wcześniej opisanych środków paraliżujących jest dodatkowa grupa metylowa w strukturze, która wydaje się wpływać na przyspieszenie hydrolizy. Z drugiej strony, Novichok C01-A043 (**15**) ma najniższą stałą szybkość hydrolizy wynoszącą 0,6074/dni. C01-A043 (**15**) różni się od C01-A040 (**12**)

dodatkowym atomem fluorowca (Cl-) przyłączonym do zewnętrznej grupy alkilowej. Dwa ostatnie związki, różniące się ze strukturą **15** odpowiednio, dla **16** jedną dodatkową grupą metylową i dla **17** dwiema dodatkowymi grupami metylowymi, okazały się najszybsze z profilowanych i osiągnęły wartość stałej szybkości hydrolizy 0,8538/dni. Ta różnica w szybkości hydrolizy może wynikać z efektów elektronowych związanych z dodatkowymi grupami metylowymi. Dane dotyczące okresu półtrwania oraz stałej szybkości hydrolizy (Kn) przedstawiono zbiorczo w **Tabeli 3**.

Tabela 3. Przewidywane wartości okresu półtrwania i stałej szybkości hydrolizy (Kn) oszacowane dla badanych związków typu Novichok.

Związek	Okres półtrwania dla pH 6,5-7,4 (dni)	Stała szybkość, Kn (/dni)	
	QSAR Toolbox: Profilowanie	EPI Suite: Profilowanie	QSAR Toolbox: Estymacja
1	Ekstremalnie szybki (0,0207)	n/d	6,24 (-16,7÷28,5)
2	Wolny (38)	n/d	2,79 (-16,1÷21,6)
3	Wolny (38)	n/d	2,04 (-6,62÷10,7)
4	Ekstremalnie szybki (0,0207)	n/d	5,83 (-15,8÷27,5)
5	Wolny (38)	n/d	2,79 (-16,1÷21,6)
6	Ekstremalnie szybki (0,387)	n/d	4,22 (-18,3÷26,7)
7	Bardzo wolny (2310)	0,7528	0,711 (-2,21÷3,64)
8	Bardzo wolny (2310)	0,7528	0,711 (-2,21÷3,64)
9	Bardzo wolny (2310)	0,7528	0,711 (-2,21÷3,64)
10	Bardzo wolny (2310)	0,7528	0,711 (-2,21÷3,64)
11	Bardzo wolny (2310)	0,7688	0,834 (-2,59÷4,26)
12	Bardzo wolny (2310)	0,7688	0,834 (-2,59÷4,26)
13	Bardzo wolny (2310)	0,7688	0,834 (-2,59÷4,26)
14	Bardzo wolny (2310)	0,7688	0,834 (-2,59÷4,26)
15	Wolny (32,1)	0,6074	0,654 (-2,06÷3,37)
16	Wolny (32,1)	0,8538	0,961 (-1,96÷3,88)
17	Wolny (56,8)	0,8538	0,961 (-1,96÷3,88)

* n/d = nie dotyczy.

Obecnie dostępne dane były niewystarczające do uzyskania odpowiednich estymacji dla struktur **1-6** typu fosforanowego (O=P(-C/-O-)(-F/-O-)); oprogramowanie wymagało więcej danych eksperymentalnych. Pomimo jedynej znanej pracy eksperymentalnej dotyczącej stałej szybkości hydrolizy związków Novichok

(A-230, A-232 oraz A-243)[21], informacje nie zostały zaimplementowane do baz danych oprogramowania EPI Suite. Dlatego zdecydowano się na wykorzystanie QSAR Toolbox w celu autorskiej kategoryzacji i uzupełniania luk danych. W przypadku analizowanych wcześniej struktur **7-17** trend został utrzymany. Wartości nieznacznie odbiegają od profilowanych przez moduł HYDROWIN, potwierdzając poprawność obranej drogi estymacji stałej szybkości hydrolizy. Pomyślnie estymowano wartości dla związków Novichok **1-6**. Najłatwiej ulegający hydrolizie związek fosforoorganiczny z grupy Novichok A-230 (**1**), którego stała szybkości wynosiła 6,24/dni. A-242 (**4**) oraz irański "Novichok" (**6**) również osiągnęły wysokie wartości odpowiednio 5,83/dni i 4,22/dni. Związki **1**, **4** i **6** są związane z grupą metylową na atomie fosforu. Inne związki o ponad dwukrotnie większej stałej szybkości hydrolizy, 2,79/dni, to A-232 (**2**) oraz A-262 (**5**). Z kolei A-234 (**3**), różnił się od A-232 (**2**) dodatkiem grupy etylowej związanej z atomem tlenu bezpośrednio związanym z atomem fosforu, okazał się ulegać wolniejszej hydrolizie (2,04/dni). W przypadku związków **7-10** i **11-14**, dodatkowa grupa metylowa ułatwiała hydrolizę, tak tutaj, pomiędzy strukturami Mirzayanov'a, grupa etylowa spowalniała hydrolizę związku. Na podstawie uzyskanych wyników stwierdzono, że pochodne kwasu fosfonowego łatwiej hydrolizują niż pochodne kwasu fosforowego. Struktury zaproponowane przez Ellison'a[14] charakteryzują się wartościami stałej szybkości hydrolizy < 1,0/dni. W przeważającej większości wyniki stałej szybkości hydrolizy dla badanych Novichok są zgodne z wynikami okresu półtrwania hydrolizy, z wyjątkiem struktury **15**, której okres półtrwania określono jako wolno hydrolizująca. Estymowana szybkości reakcji hydrolizy, gdzie A-230 jest łatwiej hydrolizujący niż A-232 oraz A234, był zgodny z wynikami uzyskanymi eksperymentalnie w warunkach obojętnych (0,17 μM/min dla A-230, 0,061 μM/min dla A-232 oraz 0,0032 μM/min dla A-234)[21].

8. Wnioski

Na podstawie przeprowadzonych badań można wyciągnąć następujące wnioski:

- Z powodzeniem oszacowano toksyczność ostrą poprzez estymację medialnej dawki śmiertelnej (szczur, podanie doustne) różnymi metodami toksykologii *in silico*.
- Wyestymowano z pełnym powodzeniem parametry hydrolizy (okres półtrwania, stała szybkość) oraz dokonano prognozy biodegradacji dla związków Novichok stosując różnorodne metody *in silico* bazujące na podejściu (Q)SAR (ilościowa zależność struktura-aktywność).
- Najniższą medianę dawki śmiertelnej (LD_{50}; podanie doustne, człowiek) wyestymowano dla związków Novichok: A-232 (0,50 mg/kg mc.), A-230 (0,41 mg/kg mc.) oraz A-234 (0,63 mg/kg mc.), przy założeniu, że statystycznie człowiek waży około 70 kg [48,49].
- Środki paraliżujące Novichok nie przewyższają toksycznością ostrą znanych dotychczas konwencjonalnych bojowych środków trujących serii -V oraz -G. A-232 osiągnął najniższą wartość LD_{50} (0,41 mg/kg mc.) ze wszystkich badanych struktur Novichok; nie przewyższa jednak toksycznością związków takich jak, VX (0,1 mg/kg mc.) czy Soman (0,06 mg/kg mc.).
- Stabilność związków Novichok jest różnorodna w zależności od zastosowanego związku fosforoorganicznego; mogą być obecne w środowisku zewnętrznym od kilku godzin (A-230, A-242 i irański "Novichok"), przez ponad miesiąc (A-232, A-234, A-262 i C01A043- C01A045), nawet kilka lat (C01A035-C01A042).
- Związki serii A ulegają biodegradacji tlenowej oraz beztlenowej; dla wszystkich badanych struktur Novichok ramy czasowe ostatecznej biodegradacji wahały się od tygodni dla struktur A-230, A-242, C01-A037 i C01-A041, a także okresu tygodni do miesięcy dla reszty analizowanych związków fosforoorganicznych.
- Nie można jednoznacznie stwierdzić, który z zastosowanych modeli *in silico* cechował się najwyższą dokładnością uzyskanych wartości; wszelkie użyte oprogramowania specjalistyczne były zalecane przez organy regulujące i uzupełniały się wzajemnie pod względem różnych funkcji estymacyjnych oraz weryfikowały wyniki obustronnie.

9. Kopie publikacji wchodzących w skład rozprawy doktorskiej

Archives of Toxicology (2023) 97:651–661
https://doi.org/10.1007/s00204-022-03437-5

REVIEW ARTICLE

What do we currently know about Novichoks? The state of the art

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Received: 18 November 2022 / Accepted: 20 December 2022 / Published online: 30 December 2022

Abstract

Novichok is the name given to the group of nerve agents created stealthily in the later phases of the Cold War by the Soviet Union. Constitute the fourth generation of chemical warfare agents; like other nerve agents, they are organophosphorus compounds designed to be incurable and undetectable. The mechanism of action is based on the non-competitive and irreversible inhibition of acetylcholinesterase. Due to their enormous toxicity, Novichoks have become attractive targets for terrorists. However, little information is known about the identity of nerve agents. Furthermore, these compounds have never been submitted to the Chemical Weapons Convention. Our article aspires to provide a general overview of Novichoks knowledge. As part of this, we reviewed the available literature data to answer the question, what are Novichoks? In addition to the physical and chemical properties of A-agents, synthesis, mechanism of action, and toxicity of nerve agents were also reviewed. We hope that this review will highlight the tremendous threat posed by nerve agents and will inspire further studies on the interdisciplinary aspects of these compounds.

Highlights

- Novichoks, an extremely life-threatening nerve agents.
- General overview of the information on Novichoks: physical and chemical properties, mechanism of action and toxicity data.
- Novichoks should be treated as a separate group of chemical warfare compounds due to their hazardous properties.

Keywords Novichoks · Toxicology · Organophosphate · Nerve agents · Chemical warfare agent

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
DCM	Dichloromethane
DF	Methylphosphonic difluoride
GB	Sarin
GD	Soman
GOSNIIOKhT	Institute for Organic Chemistry and Technology in Moscow
HuBuChE	Human butyrylcholinesterase
LD_{50}	Median lethal dose
NNDA	*N*,*N*-Diethyl-2-iminopropan-1-amine
OP	Organophosphate
OPCW	Organization for the prohibition of chemical weapons
QSAR	Quantitative structure–activity relationship
Ser–His–Glu	Serin–histidine–glutamine
T.E.S.T	Toxicity Estimation Software Tool
TEA	Triethylamine
TEG	1,1,3,3 Tetraethylguanidine
TMG	1,1,3,3 Tetramethylguanidine

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Introduction

A Novichok agent (Russian: Новичóк, which means 'newcomer' in Russian) (Hussain and Sharma 2019) can be defined as a hypothetical group of nerve agents; some of these can be classified as binary (two inert substances combined prior to delivery to create the active nerve agent) chemical weapons. It is assumed that the Novichok agents come from the testimony and memoirs of Vil S. Mirzayanov, the Director of the Department of Counteraction against Foreign Technical Intelligence at the Russian State Union Scientific Research Institute for Organic Chemistry and Technology (GosNIIOKhT) (Mirzayanov 2008). Mirzayanov disclosed information about initiating a secret Soviet chemical weapons initiative to develop Novichok agents. The first three of these, Substance-33, A-230 and A-232, were probably produced in a GosNIIOKhT facility in Russia using an organophosphate structural backbone [R_1–P(=O)(R_2)–OR_3; R_1 = amide/oxime; R_2 = fluorine; R_3 = alkyl, alkoxy, aklylamino] (Smithson et al. 1995). From a chemical point of view, Novichok compounds are postulated to be organophosphates containing a dihaloformaldoxime moiety (Gupta 2015). Mirzayanov proposed the first chemical structure proposition for Novichoks (including A-234 as a phosphoramidat)—Fig. 1A.

However, (at the same time) Hoenig (2007) and Ellison (2007) proposed alternative structures for Novichoks as phosphorylated oxime—Fig. 1B. Therefore, data on these hazardous materials are still buried in mystery (Nepovimova and Kuca 2020; Franca et al. 2019). The only available data come from the interviews and articles of Mirzayanov et al. (Mirzayanov 2008; Karev 2009), but in the opinion of most scientists, it is not an entirely reliable data source (it seems that Hoenig's and Ellison's version is the most realistic). Currently, studies about Novichoks are rare and have only recently started to emerge (Imrit et al. 2020). However, Chai et al. (2018) and Harvey et al. (2020) noted that there is some consensus on the phosphoramidate nature of A-series nerve agents. So, the question is 'What exactly are these dangerous substances?' Perhaps, because of the high reactivity of these substances, predictions would be appropriate using in silico toxicology tools like QSAR? Only a few studies are available in the scientific literature on this topic.

An interesting fact is that Novichoks were designed to be undetectable by standard North Atlantic Treaty Organization (NATO) chemical detection equipment (Nepovimova and Kuca 2018), so their identification is much more difficult. For this purpose, a beneficial methodology is based on ultrasensitive detection of the Novichok nerve agent A-232 using vibrational spectroscopy (Tan et al. 2019).

There is no doubt that the chemical attack in Salisbury, Wiltshire, United Kingdom, on 4 March 2018 exposed a case of acute poisoning (Sergei Skripal and his daughter, Yulia) by this type of compound (probably a Novichok nerve agent, A-234) (Bhakhoa et al. 2019; Haslam et al. 2022). However, it was not only an exceptional case, because on 30 June 2018 in Amesbury, Wiltshire, United Kingdom, the poisoning of two British nationals occurred by a Novichok nerve agent of the same kind used in Salisbury (13 km away) (Haslam et al. 2022). Two years later (on 20 August 2020), a previously healthy 44-year-old man suddenly became confused and began sweating heavily on a domestic flight to Russia approximately 10 min after departure (Steindl et al. 2021). Two weeks later, the German government announced that a laboratory

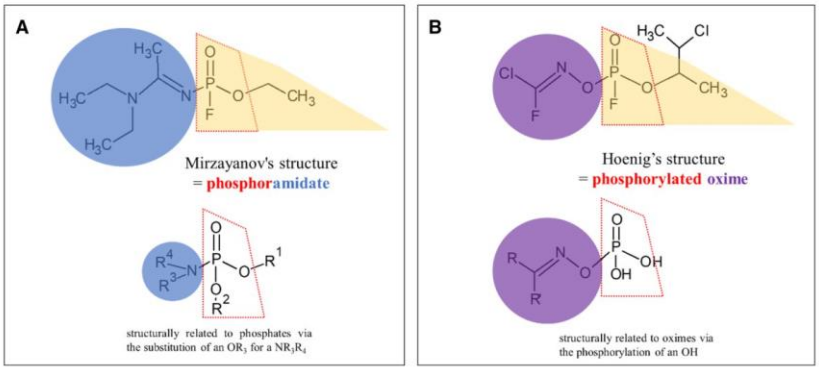


Fig. 1 Proposed chemical structures of the Novichok agent as A-234: **A** Mirzayanov's structure as a phosphoramidate, and **B** Hoenig's structure as a phosphorylated oxime

of German armed forces designated by the Organization for the Prohibition of Chemical Weapons (OPCW) had identified an organophosphorus nerve agent from the Novichok group in blood samples from this patient (Steindl et al. 2021). The examples confirmed the effects of acute poisoning by Novichok agents, indicating the probability of the presence of these substances.

As noted above, in the scientific literature, only a few articles are dedicated to the specific properties of the chosen Novichok agents; however, there is still a lack of a comprehensive review of what we know about Novichoks. To our knowledge, this is the first comprehensive and actual review of Novichoks that includes the current state of knowledge from an interdisciplinary perspective.

Materials and methods

Search for publications on Novichok data

For the critical review of Novichoks data: Scopus, Google Scholar, and Web of Science were applied as the primary repositories for finding published references on this topic. It should be noted that the data collection process involved searching mentioned scientific sources but also 'grey' literature (V. S. Mirzayanov, State Secrets: An Insider's Chronicle of the Russian Chemical Program, Outskirts Press, Inc., 2008; general commercial, trade body, and industrial collections), using theoretical and empirical data. Different combinations of the following principal terms were used: Novichok; nerve agents; A-series compounds; Mirzayanoy; Sergei Skripal; Alexei Navalny; organophosphate; A-234; A-230; A-232; *N*-[ethoxy(-fluoro)phosphoryl]-*N*,*N*-diethylethanimidamide; VX; CBRN. All available sources ($n = 52$ articles and related content) were analysed. Only articles/works related to Novichok agents were considered to filter the sources retrieved.

Classification and presentation of the results

A different Novichoks agent has been used as the target specimen, up to 14 species in the reviewed works. For better readability, data and characteristics of the described Novichoks were presented in appropriate sections of this work, i.e. physical and chemical properties, synthesis, mechanism of action, and toxicity.

Physical and chemical properties of Novichoks

There is no doubt that Novichok agents are extremely hazardous xenobiotics; therefore, the first step should be the physical and chemical characterisation of these substances. However, very little information is available (Karev 2009; Halámek and Kobliha 2011; Pitschmann 2014). In scientific articles (mainly described by Nepovimova and Kuca 2018, 2020), only a few properties according to three Novichok agents, that is, A-230, A-232, and A-234 (possible A-series nerve agents reported by Hoenig) and other possible Novichoks reported by Ellison (2007) and Patočka (2018) are published.

The first three A-agents reported by Hoenig (A230, A-232, and A-234) are volatile liquids with similar densities (1.414–1.612 $g \cdot mL^{-1}$) and similar boiling points. At low temperatures, A-232 and A-234 do not solidify. A-242 is solid at room temperature, according to Mirzayanov. However, these data are uncertain due to the lack of confirmation of this information in the literature. A-230 is resistant to moisture; however, A-232 is less stable to moisture than A-230. The low vapour pressures stated for A-230, A-232, A-234, and A-242 suggest that they persist indifferently in the environment. Other Novichoks reported by Ellison and Patocka can be considered to be a heterogeneous group because of differences in properties. A significant disadvantage of Novichoks is the lack of balance between volatility and persistence (Nepovimova and Kuca 2018). Table 1 represents available data on the physical and chemical properties of Novichoks reported by Hoenig (A-230, A232 and A-234) and other Novichoks reported by Ellison and Patocka. The volatility parameter for Novichoks (other than A-230, A-232 and A-234) was reported by Ellison and Patock using 'ppm' units (Ellison 2007; Patočka 2018). By comparing the volatility of compounds with a known parameter, such as G-agent and V-agent (GB = 2800 ppm, volatile; GD = 520 ppm, volatile; VX = 1.2 ppm, not volatile) with Novichoks listed in Table 1, we determined their volatility.

Synthesis of Novichoks

Most of the current available information about A-series nerve agents is speculative or comes from uncertain sources that have not yet been confirmed. This state of affairs creates a significant problem for which information may be considered reliable. Due to these divergences, we have included all known A-agent synthesis routes to show the differences between these sources.

As reported by Mirzayanov, the A-series compounds belong to the phosphoramidate group. Synthesis of compounds with code symbols: A-230, A-232, A-234, A-242, and A-262 (Fig. 2) is described in his book "State Secrets. An Insider's Chronicle of the Russian Chemical Weapons Program" (Mirzayanov 2008). The synthesis route of A-230 is based on the condensation of *N*,*N*-diethylethanimidamide (NNDA) with difluoride (DF). Replacement of DF with *O*-methylphosphonyl difluoride or

Table 1 Data available on the physical and chemical properties of chosen; based on (Ellison 2007; Mirzayanov 2008; Patočka 2018; Nepovimova and Kuca 2018, 2020)

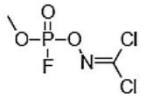
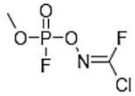
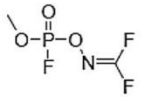
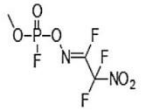
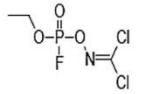
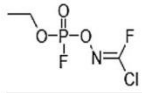
Novichok	State	Density, g·mL^{-1}	Volatility	Melting point, °C	Boiling point, °C	Vapour pressure, Pa	Solubility at 25 °C, g·L^{-1}	Log P	Behaviour at low temperature	Stability under moisture conditions
A-230	Liquid	1.612	Volatile	5.56	61–62 [5,10] 298 [X]	2.13	4.823	2.14	Solidifies at low temperature	Resistant to moisture
A-232	n.a	1.515	More volatile than A-230	5.65	70–71 [5,10] 259.92 [X]	1.48	1.775	2.55	Does not solidify at low temperature	less stable against moisture than A-230
A-234	Very viscous liquid	1.414	Poorly volatile	3.06	73–74 [5,10] 264.11 [X]	1.7	0.6512	2.97	n.a	Resistant to moisture
A-242	Solid	n.a	n.a	21.46	284.85 [X]	0.579	1000	0.45	n.a	n.a
	n.a	1.488	Volatile (600 ppm)	−12.33	218.93	18.5	16.74	1.76	n.a	n.a
	n.a	1.510	Volatile (2000 ppm)	−35.05	191.37	73.8	37.64	1.47	n.a	n.a
	n.a	1.424	Highly volatile (10,000 ppm)	−58.28	162.02	304	84.02	1.18	n.a	n.a
	n.a	1.639	Volatile (900 ppm)	48.34	256.81	1.53	9.940	1.34	n.a	n.a
	n.a	1.366	Poorly volatile (200 ppm)	−1.27	237.51	7.04	5.374	2.12	n.a	n.a
	n.a	1.450	Volatile (1000 ppm)	−23.2	211.21	27.4	12.15	1.83	n.a	n.a

Table 1 (continued)

Novichok	State	Density, g·mL^{-1}	Volatility	Melting point, °C	Boiling point, °C	Vapour pressure, Pa	Solubility at 25 °C, g·L^{-1}	Log P	Behaviour at low temperature	Stability under moisture conditions
	n.a	n.a	Volatile (4000 ppm)	−46.48	183.14	110	27.29	1.54	n.a	n.a
	n.a	1.506	Poorly volatile (200 ppm)	58.85	273.49	0.509	3.157	1.70	n.a	n.a

O-methylphosphonylfluorocyanide results in the formation of A-232. A-234 can be obtained using *O*-ethyl phosphonyldifluoride or *O*-ethyl phosphonylfluorocyanide instead of DF. Replacement of NNDA by 1,1,3,3-tetraethylguanidine (TEG) in condensation with DF resulted in the synthesis of A-242. A similar situation occurs when preparing A-262; replacing NNDA with TEG and condensation with *O*-methyl phosphonyldifluoride or *O*-methyl phosphonylfluoro cyanide produces A-262.

A different approach from what Mirzayanov claims is the approach in which A-agents belong to the group of phosphorylated oximes (Halámek and Kobliha 2011; Ellison 2016). In this case, their synthesis will take three steps (Fig. 3). The two initial steps in the synthesis are the preparation of A-agent precursors, referred to as: Novichok?, Novichok 5 and Novichok 7. It consists of the reaction between the phosphorus trichloride with the appropriate diol and the subsequent nucleophilic substitution, in which the chlorine atom is converted into a fluorine atom. The last step in the synthesis of compounds as phosphorylated oximes is the reaction between the 2-fluoro-1,3,2-dioxaphospholanes formed in the previous step and dichloro(fluoro)nitrosomethane. The resulting compound is stable at subzero temperatures (−40 °C). Heating the product facilitates the nucleophilic attack by chloride anion. Results in the opening of the phospholane ring with the transfer of the chlorine atom, creating the appropriate Novichok (Halámek and Kobliha 2011; Hoenig 2007). In addition to phosphorus chlorides or oxychlorides, many other intermediates in phosphorus chemistry used in the pesticide, plasticizer or detergent industry can be used to synthesize A-series compounds as substrates (Nepovimova and Kuca 2018).

A group of Iranian scientists published an article in which they presented a laboratory method for the synthesis of *O*-alkyl *N*-[bis(dimethylamino)methylidene]-*P*-methylphosphonamidates (Fig. 4) (Hosseini et al. 2016). The primary compound described was an analogue of Mirzayanov's A-242. The analogue had methyl substituents on nitrogen atoms instead of ethyl substituents. The compound was formed by mixing a solution of DF in dichloromethane (DCM) with a solution of 1,1,3,3-tetramethylguanidine (TMG) in triethylamine (TEA) and DCM. They also characterised several derivatives of this compound in which *O*-alkyl (methyl, ethyl, isopropyl) and *O*-aryl (phenyl and 2,5-dimethylphenyl) groups were substituted with the fluorine atom. They were obtained by dropping a solution of the compound previously described in DCM into a previously prepared solution of the appropriate alcohol (ROH) and sodium hydride (NaH) in the same solvent (Hosseini et al. 2016).

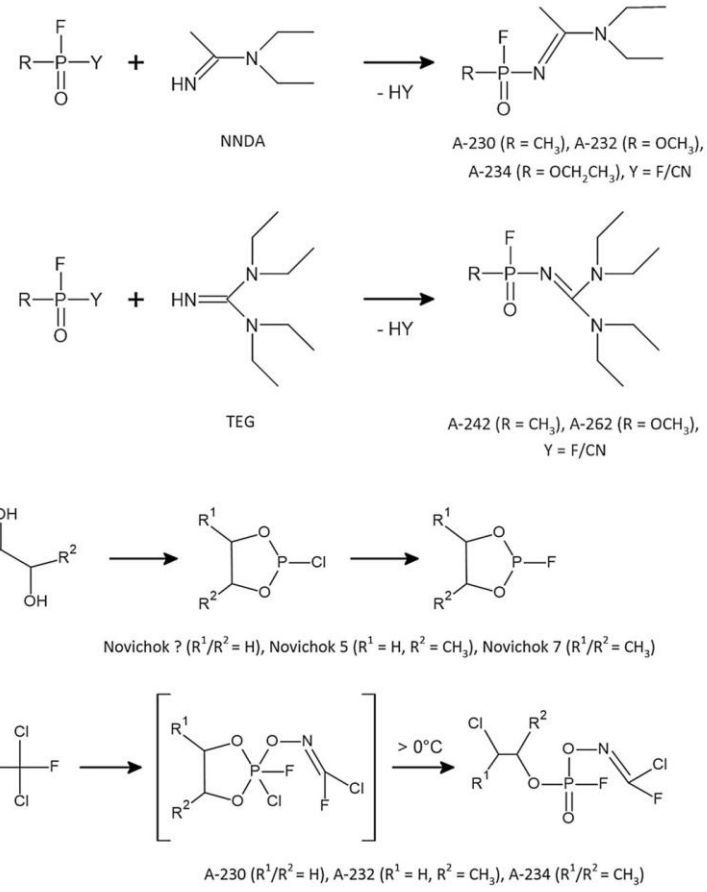


Fig. 2 Synthesis routes for the A-series compounds according to Mirzayanov. Group Y is either fluorine (F) or cyanide (CN)

Fig. 3 Synthetic pathway leading to phosphorylated oximes, according to Hoenig

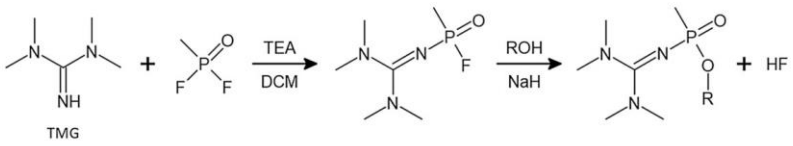


Fig. 4 Synthesis of *O*-Alkyl *N*-[bis(dimethylamino)methylidene]-*P*-methylphosphonamidates; based on Hosseini et al. (2016)

Mechanism of action of Novichoks

A-agents can bind to acetylcholinesterase (AChE) and inhibit acetylcholine (ACh) metabolism (Chai et al. 2018). AChE catalyses the hydrolysis of the ACh neurotransmitter to acetate and choline (Hoenig 2007). Hydrolysis of carboxylic esters occurs at the active site of AChE, specifically in the Ser–His–Glu triad (Chai et al. 2018; Nepovimova and Kuca 2018). Under physiological conditions, the hydrolysis of ACh is rapid, which reduces its concentration in neuronal cholinergic synapses and neuromuscular junctions (Dvir et al. 2010). The mechanism of the action of Novichok on the nervous system is through the active site of AChE (Ser–His–Glu triad) (Hoenig 2007). Based on the rapid attack of the hydroxyl groups in serine, which act as a nucleophile on the phosphate groups of the compound (Mercey et al. 2012). Thus, fluoride ions were released that form a phosphorylated enzyme complex. The effect of creating a covalent bond between phosphorus atoms and AChE serine is to slow the hydrolysis of ACh (from hours to days) (Korabecny et al. 2014) (Fig. 5).

There is a circumstance, such as ageing of the AChE enzyme, which causes its inactivation. Any therapy cannot restore it to an active state (Sharma et al. 2015). AChE inhibited by A agent is ageing rapidly. The ageing half-time of A-230 and soman is relatively similar (2–4 min) (Sirin et al. 2012). The aged form of the enzyme was created by rapid hydrolysis of the =N–O– bond in the Novichok-AChE adduct. The phosphonic oxyanion creates a salt bridge with protonated histidine, stabilising the conjugate (Nepovimova and Kuca 2018). Due to rapid ageing of AChE and the weak partial positive charge, reactivation of AChE inhibited by A-agents is a relatively thorny task. Therefore, symptomatic treatment or the dispensing of bioscavengers is an effective therapy (Nepovimova and Kuca 2020). The molecule

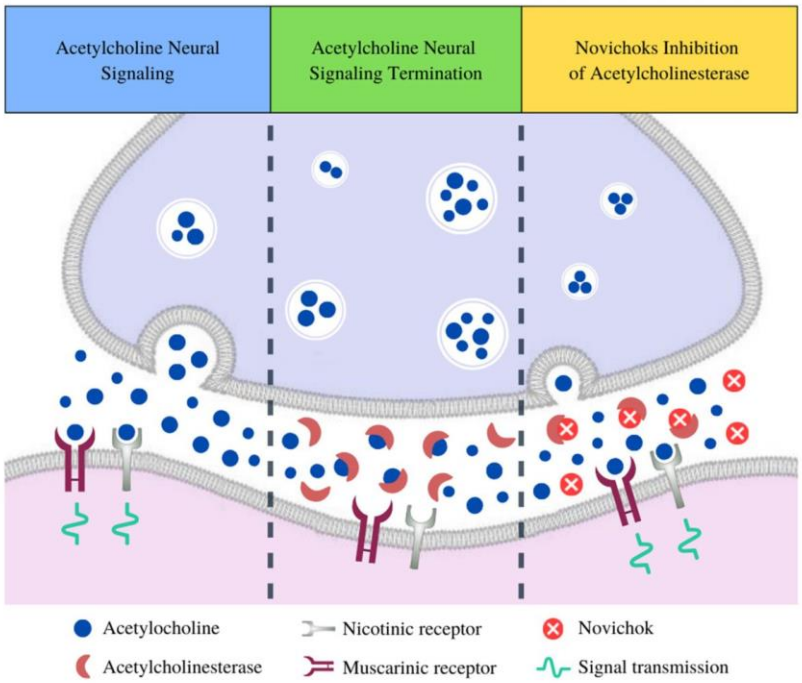


Fig. 5 The acetylcholine pathway in cholinergic synapses and interaction with the Novichok nerve agent. ACh neural signalling begins while presynaptic neurons release ACh vesicles that will bind to receptors (muscarinic and nicotinic) in postsynaptic neurons. The cascade ends when AChE cleaves free ACh into acetate and choline. Novichok competitively binds to AChE and inhibits ACh cleavage, thus maintaining constant signal transmission

dispensed in this way would bind to the A agent and thus it would not be able to reach the AChE tissue and cause symptoms of intoxication (Bajgar et al. 2009). An example of such a molecule is butyrylcholinesterases (BChE; E. C. 3.1.1.8). BChE can detoxify all types of A-agents, making it a universal treatment approach (Bajgar et al. 2009).

Toxicity of Novichoks

Information on the toxicity of A-series nerve agents is minimal. Regarding the exposure routes, depending on the structure of the A-agents: liquid or solid, they can be absorbed through the skin or inhaled (Korabecny et al. 2014). Exposure to A-series nerve agents depends directly on the dose absorbed into the body. These are three types of toxic reactions due to disturbance of AChE activity: muscarinic, nicotinic and central nervous system (Chai et al. 2018; Kloske and Witkiewicz 2019; Kloske 2020) (Fig. 6).

Furthermore, A-agents may be associated with peripheral sensory nerves, and in high doses and with sustained contact, they cause peripheral neuropathy (Gupta 2015). During the hydrolysis of ACh, AChE is also involved in haematopoiesis and the development of nerves and muscles (Colovic et al. 2013). The effect of A-series nerve agents other than those mentioned above is the induction of irreversible neuropathy, so treatment with traditional antidotes for paralytic convulsive agents may be ineffective (Kloske 2020).

According to Mirzayanov, A-230 is 5–8 times more toxic than VX, while A-232 is ten times more toxic than Soman. Moreover, A-242 and A-262 exceed even A-230 and A-232 in their toxicity, making them the most toxic of the A-series compounds (Mirzayanov 2008). However, there is a lack of adequate studies about this important topic. Carlsen presented completely different data on the relative toxicity of A-series nerve agents, contrary to Mirzayanov's claim (Carlsen 2019). Using quantitative structure–activity relationship (QSAR) models, precisely the Toxicity Estimation Software Tool (T.E.S.T), the median lethal dose—LD_{50} value was calculated for oral administration to rats. The dose value was then converted to human. The acute toxicity (LD_{50} value) of A-series nerve agents was 5–75 times lower than the VX compound. Data were confirmed on the basis of the LD_{50} value for VX. For Ellison, it was 10 mg/person weighing 70 kg. When converted to mg/kg, the value is 0.14 mg/kg, which is consistent with Carlsen's calculated LD_{50} value for humans: 0.1 mg/kg (Carlsen 2019). Assuming the weight of a "standard" person was 70 kg, the LD_{50} values for the Novichoks chosen based on different data sources (Table 2) were estimated.

The hydrolysis half-lives of the A-series nerve agents were estimated at pH 6.5–7.4 using the QSAR Toolbox (Carlsen 2019). Moderate transformation due to hydrolysis,

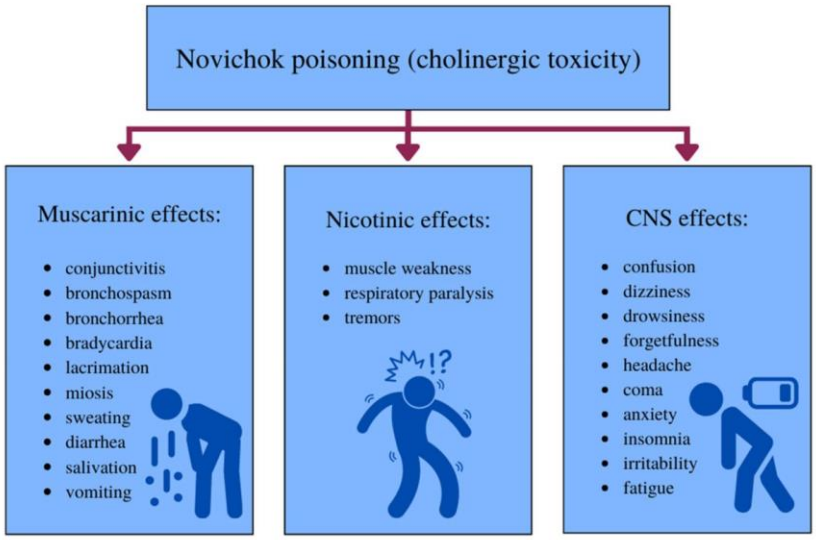


Fig. 6 Muscarinic, nicotinic, and central nervous system (CNS) effects of acute Novichok poisoning

Table 2 Available data on toxicity of the Novichoks; based on (Karev 2009; Ellison 2016; Carlsen 2019)

Compound	LD_{50} (mg/kg bw)		
	(Karev 2009)	(Ellison 2016)	(Carlsen 2019)
VX	0.143	0.086–0.143	0.10
A-230	n.a	0.011–0.029	1.55
A-232	0.014–0.029	0.5	0.57
A-234	0.071	0.5	0.71
A-242	n.a	n.a	0.49
A-262	n.a	n.a	7.35

from 10 to 30 days, was shown by: VX, A-232, A-234 and A-262. The short half-lives of hydrolysis, less than one day, were shown by the following: A-230 and A-242 (Carlsen 2019). Suggests that, except for A-230 and A-242, the rest of the A-agents are relatively stable in the environment. The hydrolysis rate of Novichoks has also been measured experimentally at pH 7.2 at 25 °C (Harvey et al. 2020). The data showed that the hydrolysis of A-series agents was much slower by several orders of magnitude than G-series and V-series agents. Hydrolysis rate: GB = 6.68 µM/min, VX = 0.246 µM/min, A230 = 0.17 µM/min, A232 = 0.061 µM/min, A234 = 0.0032 µM/min (Harvey et al. 2020). The hydrolysis rate confirms the stability of Novichok compounds in the environment or in the organism. An additional group (Otsuka and Miyaguchi 2021) used density functional theory to perform theoretical calculations for the hydrolysis reactions of nerve agents, including Novichok compounds. According to data, A-series agents were as resistant to hydrolysis as VX and more resistant to hydrolysis than GB under basic conditions. The activation energy of hydrolysis under basic conditions is lower for compound A-234 compared to that under neutral conditions. Therefore, decontamination will be more effective under basic conditions (Otsuka and Miyaguchi 2021).

Conclusions

Although Novichok is a relatively "hot topic", there is still a lack of accurate, reliable data, and many unknowns require explanation (Bolt and Hengstler 2022). Establishing the structures and properties of Novichoks using theoretical tools (Jeong and Choi 2019) such as QSAR methods and adductomics (Golime et al. 2019; Sabbioni and Day 2022) is a promising field of study and necessary to understand the dangers these compounds can generate and develop adequate protection. Understanding the appropriate structures will enable theoretical and experimental research to discover the appropriate antidote. The synthesis of A-series compounds would enable the assessment of their toxicological and physicochemical properties. It would also create the possibility of developing modern methods for detecting these extremely toxic xenobiotics, such as characterization and study of the fragmentation pathways of Novichoks in aqueous solution by LC–MS/MS (Lee et al. 2021). On the basis of collected data concluded that Novichoks, also called A-series compounds, should be treated by analogy as other nervous factors, distinguished into the groups of compounds: G, V, and A. Novichoks should not be treated as independent groups of chemical warfare compounds. The new types of nerve agents constitute a constant and enormous danger. Although until now they have been used for assassination, their extreme toxicity poses a severe threat. Therefore, the danger caused by the Novichoks must be urgently assessed to be able to deal with future terrorist attacks or the use of chemical weapons on the battlefield. Improving and modifying international regulations and verification is necessary to prevent a catastrophic scenario using the A-series compounds. A crucial phase was the modification of the CWC regulations after the poisoning of Sergei Skripal and his daughter, which resulted in the introduction of some Novichok compounds into the treaty. Unfortunately, it did not cover all known A-series compounds, including their precursors used to form the Novichok binary forms previously included in the CWC list. Another significant modification is proposed in the introduction of Novichok agents with guanidine branches, which CWC does not cover. Results from Alexei Navalny's poisoning incident in 2020, most likely using the A-series compounds containing guanidine branches. Given the global danger posed by the usage of Novichoks, we believe that it will stimulate future progress to improve our protection against toxic agents and develop an optimal diagnosis and new treatments for casualties poisoned with these compounds. Furthermore, improve the lawful aspects of the CWC to reduce the likelihood of an attack. We hope that this review will inspire scientists to conduct future research to fill in gaps in missing data and highlight the dangerous potential of using Novichoks as a chemical weapon.

Acknowledgements Not applicable.

Author contributions MN: Writing—original draft preparation, writing—review and editing, visualization; KJ: conceptualisation, data curation, supervision, writing—original draft preparation, writing—review and editing, visualization. All authors have read and approved the manuscript.

Funding This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials For this review, we based on existing data from scientific articles.

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethics approval The manuscript does not contain newly generated clinical studies or patient data.

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Journal of
Clinical Medicine

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Review

Review of Possible Therapies in Treatment of Novichoks Poisoning and HAZMAT/CBRNE Approaches: State of the Art

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Abstract: Novichoks-organophosphorus compounds belong to the nerve agents group, constituting the fourth generation of chemical warfare agents. The tremendous toxicity of Novichoks is assumed to be several times greater than that of VX, whereas no published experimental research supports this. They were surreptitiously created during the Cold War by the Soviet Union. Novichok's toxic action mechanism consists of the inhibition of acetylcholinesterase activity. The review includes data on treating poisoning caused by OPs which could be used as guidelines for the therapy in case of Novichok exposure and HAZMAT/CBRNE approaches. Novichoks pose a severe threat due to their toxicity; however, there is insufficient information about the identity of A-series nerve agents. Filling in the missing data gaps will accelerate progress in improving protection against Novichoks and developing optimal therapy for treating poisoning casualties. Furthermore, introducing solutions to protect medical personnel in contact with a hazardous substance increases the chances of saving casualties of HAZMAT/CBRNE incidents.

Keywords: Novichoks; organophosphate; toxicology; HAZMAT; CBRNE; nerve agents

Citation: Noga, M.; Michalska, A.; Jurowski, K. Review of Possible Therapies in Treatment of Novichoks Poisoning and HAZMAT/CBRNE Approaches: State of the Art. *J. Clin. Med.* **2023**, *12*, 2221. https://doi.org/10.3390/jcm12062221

Academic Editor: Alex Sparreboom

Received: 3 February 2023
Revised: 2 March 2023
Accepted: 11 March 2023
Published: 13 March 2023

1. Introduction

Novichoks (Russian: Новичóк, "newcomer") belong to the group of chemical warfare agents with a paralytic and convulsive effect, referred to as A-series nerve agents (NAs) [1]. First, Vil S. Mirzajanov revealed information about the secret Soviet initiative to develop Novichok agents (Figure 1) in his book "State Secrets: An inside chronicle of the Russian chemical weapons program" [2]. It is postulated that Novichoks are organophosphates (OPs) containing a dihaloformaldoxime moiety [3]. Two chemical structures of Novichoks have been proposed: the first as phosphoramides (Figure 2A) published by Mirzayanov, and the second structures as phosphorylated oximes (Figure 2B), proposed by Hoenig [4] and Ellison [5].

The primary assumption in the design of A-series compounds was the difficulty of their identification using standard equipment for chemical detection of the North Atlantic Treaty Organization (NATO) [6]. Novichok's mechanism of action consists of irreversible binding to acetylcholinesterase (AChE) and inhibiting the hydrolysis of the neurotransmitter acetylcholine (ACh) to acetate and choline (Figure 3) [7]. At the molecular level, the formation of a covalent bond between AChE and Novichok not only slows down but completely terminates ACh hydrolysis. In tissue exposed to Novichok, ACh hydrolysis is slowed down because many AChE molecules will be covalently inhibited by Novichok. The mechanism of ACh and OP hydrolysis is the same, although ACh hydrolysis is much faster than OP hydrolysis, which can take hours or even days [8].

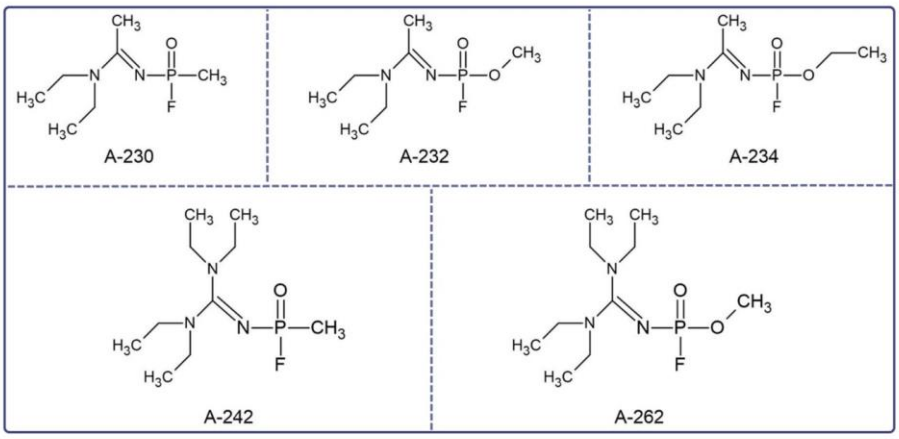

Figure 1. Novichoks' structures developed at the State Scientific Research Institute for Organic Chemistry and Technology (GOSNIIOKhT) and revealed by Mirzayanov [2].

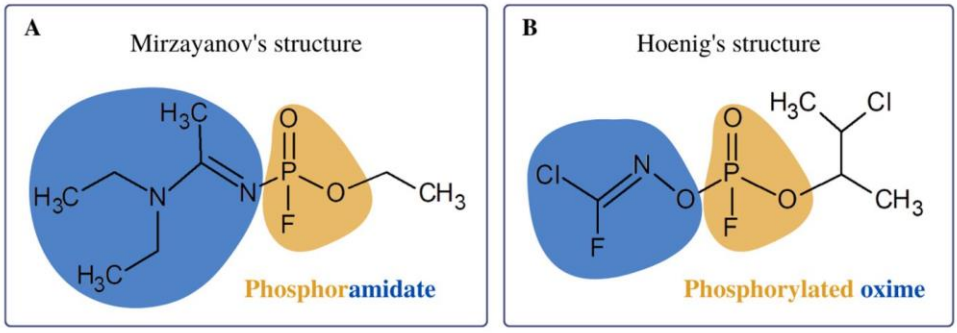

Figure 2. The chemical structures of Novichok A-234: (**A**) phosphoramidate and (**B**) phosphorylated oxime.

Overstimulation of cholinergic receptors caused by the accumulation of ACh in the synaptic cleft due to AChE inhibition leads to, depending on the route, dose and time of exposure, the manifestation of symptoms presented in Figure 3.

According to Mirzayanov, compound A-230 is 5–8 times more toxic than VX, while A-232 is 10 times more toxic than soman (GD) [2]. However, the calculations made by Carlsen assumed different data on the toxicity of Novichoks. The acute toxicity (LD_{50} value) of Novichok compounds are 5–75 times lower than compound VX [9]. According to the prediction data, the Novichoks' hydrolysis rate is slower (several orders of magnitude) than the series G- and V- series compounds. Hydrolysis data would be valuable for defining the tenacity of Novichoks in the environment or the body [10].

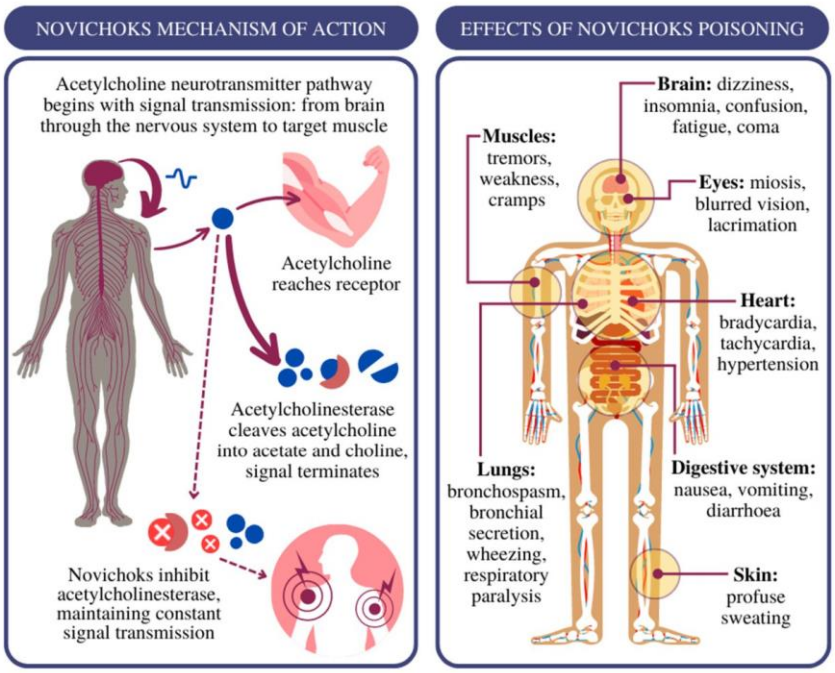

Figure 3. **Left** panel: the acetylcholine pathway in the cholinergic synapse and interaction with Novichok nerve agents; **Right** panel: effects of acute poisoning with nerve agents [7,11].

Novichoks pose a constant and enormous danger due to their extreme toxicity. An example illustrating the threat of Novichok was the chemical attack on 4 March 2018 in Salisbury (United Kingdom). The compound of series A revealed a case of acute poisoning (of Sergei Skripal and his daughter Yulia), probably with the nerve agent A-234 [12]. A similar case occurred on 30 June 2018 in Amesbury (United Kingdom), where two British citizens were poisoned with the same compound as in Salisbury [13]. The third case of poisoning, due to which the application of an OP-NA from the Novichoks group was identified, which occurred on 20 August 2020 during a domestic flight in Russia. The formerly healthful 44-year-old man, about 10 min after departure, suddenly felt disoriented and began to sweat profusely, vomited, collapsed and then lost consciousness. Following the results of clinical and laboratory studies, complete inhibition of acetylcholinesterase in red blood cells was identified, thus confirming exposure to the cholinesterase inhibitor and no proof of obidoxime reactivation. The case of Navalny is the only published clinical study on the treatment of Novichok poisoning, which has proven the effectiveness of butyrylcholinesterase therapy supported by the administration of fresh frozen plasma [14].

The above examples confirmed the effects of acute poisoning with Novichok compounds, indicating their presence in public spaces. For this reason, it is a crucial task to treat poisoning with these highly harmful substances. Clinical treatment of exposure to the G- and V- series of NAs and their extrapolation to Novichok seem reasonable. In addition, appropriate procedure specifics related to the decontamination of hazardous materials (HAZMAT) or during chemical, biological, radiological, nuclear or explosive (CBRNE) incidents should be prepared. Given the global threat posed by Novichok, accurate and reliable data must still be provided to explain the unknowns.

2. Treatment of Novichok Exposure: Where to Start?

In general, treatment of poisoning caused by NAs can be divided into nonpharmacological and pharmacological treatment. Decontamination is one of the non-pharmacological methods. The aim is to limit further exposure to the nerve factor. Different approaches are used according to national standards [15]. Decontaminating the accident scene is essential to prevent bystanders' poisoning. Vapors trapped in garments exposed to Novichok compounds can be released for up to 30 min [7]. Based on the similarity in the structure of the Novichok to other NAs, basic solutions might neutralize the A-series compounds. Increasing the pH of the solution could accelerate the reaction of Novichok hydrolysis [16]. The activation energy of hydrolysis for compound A-234 under basic conditions is lower compared to neutral conditions [17]. Theoretical calculations of the hydrolysis reactions of Novichoks confirm the greater effectiveness of decontamination under basic conditions. During decontamination, the dry bleach powder may hydrolyse Novichok compounds to hydrogen cyanide, hydrofluoric acid and hydrochloric acid. Therefore, it is recommended to avoid using these substances due to the formation of toxic metabolites [15]. Oxygen supply and resuscitation are non-pharmacological treatments in addition to decontamination [18].

The method of pharmacological treatment of NA poisoning, which could be applied to Novichoks poisoning as well, consists of the administration of (1) an anticholinergic agent (atropine), (2) an anticonvulsant agent (diazepam) and (3) the use of AChE reactivation agents (oxime) [19,20].

Immediately after poisoning with a Novichok group, clinicians should administer atropine, which will act as a competitive antagonist of central and peripheral muscarinic ACh receptors. Unfortunately, atropine is ineffective in neutralising the effects of overstimulation of neuromuscular nicotinic receptors overstimulation [21]. Atropine in doses of 2 mg to 6 mg with an interval of 5–10 min should be administered intravenously until bradycardia, bronchial bleeding and bronchospasm subside [7]. Atropine, in addition to intravenous administration, is also absorbed through the bronchial tree so that it can be administered by inhalation, for example, as an FDA-approved "Medical aerosolised nerve agent antidote" (MANAA) [22]. The impact of atropine has been shown to last from 3 to 5 h after 1–2 injections of 2 mg and 12–24 h after atroponisation [23]. A possible substitute for atropine, although tested in animal models, is the administration of the muscarinic acetylcholine receptor antagonist: diphenhydramine [24]. A drug called glycopyrrolate, in combination with atropine, was originally found to be suitable for controlling symptoms of OP poisoning and reducing central nervous toxicity [25]. Unfortunately, later studies contradicted these reports, claiming that the use of atropine alone causes fewer complications and results in lower mortality caused by organophosphorus compounds than the combination of atropine and glycopyrrolate [26,27]. On the contrary, atropine can be effectively replaced with glycopyrrolate in hypersensitivity; the recommended dose of the drug is 1 mg every 10–15 min until an antimuscarinic effect occurs [28]. Scopolamine may also serve as a substitute for atropine in hypersensitivity, used at a dose of 0.6–2 mg administered intramuscularly or intravenously. Scopolamine shows higher levels of CNS bioavailability compared to atropine [29]. In general, compounds that might block cholinergic effects should be detoxifying [30]. For example, benactizine, used as an antidepressant, is a fat-soluble anticholinergic drug, which allows it to penetrate the CNS more easily than atropine [31]. Moreover, it is additionally more efficient in preventing seizures than diazepam [32].

Diazepam prevents convulsions caused by Novichok poisoning, administered intravenously at 10–40 mg daily [33]. In addition to the anticonvulsant effect, diazepam has a specific effect on the cholinergic and GABAergic systems [34]. In animal models, diazepam has also been shown to reduce NA-induced brain damage [35]. Other benzodiazepines with effective anticonvulsant effects are lorazepam and midazolam. Because midazolam is more effective and faster than diazepam in stopping Novichok-induced seizures, it is recommended to replace the first with the second during urgent anticonvulsant treat-

ment [36,37]. The recommended initial dose of lorazepam is 0.1 to 0.2 mg/kg (2–4 mg), and midazolam is 2.2 mg/kg [23]. Barbiturates and phenytoin have proven to be ineffective anticonvulsants in preventing seizures caused by NA, hence similar findings can be related to Novichoks [38]. Possible supportive therapy includes intravenous administration of a lipid emulsion in critically ill patients [39].

Oximes (Figure 4) were created to supplement atropine, which is ineffective against nicotinic effects and does not restore AChE activity inhibited by Novichoks. These nucleophiles reactivate the phosphylated cholinesterase enzyme by breaking the bond between the enzyme and Novichoks. Standard oximes approved for clinical and military use include 2-PAM, obidoxime, HI-6, and TMB-4, although their effectiveness is not universal [40]. The difficulty encountered by pyridinium oximes is passing through the blood–brain barrier (BBB). Because they are hydrophilic and have a positively charged nitrogen atom, only 1% to 10% of the plasma concentration of oximes is present in the brain. Therefore, the effect is limited mainly to the PNS [41].

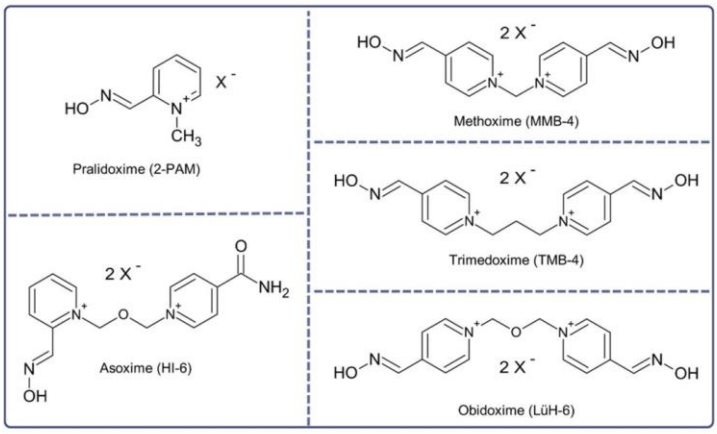

Figure 4. Chemical structures of oxime reactivators as an antidote against poisoning caused by nerve agents [42].

Pralidoxime (2-PAM) should be administered intravenously in a dose of 1–2 g every 3 to 6 h or as a continuous infusion for a minimum of 24 h after the last intake of atropine [43]. Side effects in humans, such as blurred vision, double vision, transient dizziness and increased diastolic blood pressure, are minimal at therapeutic doses of 2-PAM and can depend on the rate of administration [44]. It is crucial to monitor liver enzyme levels regularly in patients who receive 2-PAM at doses of 1200 to 1800 mg by auto-injectors; usually, enzyme levels return to normal within two weeks [45]. The preferred route of administration of 2-PAM in hospitals is intravenous; however, there is also a route of intramuscular administration via an auto-injector with a dose of 600 mg/2 mL [38]. For casualties with severe central nervous and respiratory symptoms, three DuoDote autoinjection kits containing 600 mg of pralidoxime chloride are recommended, together with 2.1 mg of atropine [46]. Obidoxime is another oxime administered intravenously at a dose of 250 mg to reactivate AChE [47]. Due to hepatotoxicity, an initial dose of 500 mg and a daily dose of 750 mg/day of obidoxime are not recommended. Furthermore, liver function tests should be regularly monitored during therapy [34]. Obidoxime has been shown to be unable to reactivate acetylcholinesterase in red blood cells in a patient exposed to Novichoks. Furthermore, signs of liver damage with increased levels of transaminases and γ-glutamyltransferases have been partly attributed to obidoxime. This resulted in the early

termination of obidoxime administration to the patient and recognition as an ineffective therapy [14]. In addition to the oximes mentioned above, asoxime chloride (HI-6) restores the activity of AChE inhibited by OP [21]. This double positive charged bis-pyridinium compound has been shown to be effective in the treatment of neural factors [48,49]. A bis-pyridinium oxime with superior therapeutic and pharmaceutical properties compared to both obidoxime and 2-PAM is a methoxime (MMB-4) [50]. An oxime with restricted use due to CNS and hepatotoxic side effects is thimodexime bromide (TMB-4) despite its effectiveness in reactivating AChE [51]. K203, a member of the new generation of oximes known as K-oximes, does not surpass the versatility of currently accessible oximes. On the contrary, its toxicity profile showed no perturbations compared to clinically used standards. K203 could be regarded as a backup or uncertain replacement for obidoxime and trimedoxime [52]. To our knowledge, the effectiveness of currently available oximes: 2-PAM, TMB-4, obidoxime and HI-6, in the reactivation of AChE inhibited by the Novichok A-242 surrogate, was evaluated. A combination of experimental and theoretical studies indicated that the most effective oxime for reactivating AChE inhibited by A-242 was trimedoxime (TMB), since they cannot adequately approach the substitute [53]. The newly synthesised phenoxyalkylpyridinium oximes managed to enter the brain and reverse the majority of NA-induced AChE inhibition. They were designed by adding an additional lipophilic chain to the pyridinium ring [54].

Another option in counteracting Ops as well as Novichock is the use of pretreatment like carbamate anticholinesterase, for example, pyridostigmine [55]. It is a reversible AChE inhibitor whose binding protects the enzyme from inhibition by NA [56]. The current pretreatment procedure is 30 mg every 8 h [55]. Pyridostigmine can be given orally (liquid or pill), intramuscularly or intravenously as injections to patients who cannot take oral drugs [57]. Pyridostigmine has been approved for military use by the FDA as a pretreatment to the GD [58]. The effectiveness of the prophylactic use of pyridostigmine depends on the quick use of atropine and pralidoxime (2-PAM) after exposure to a chemical warfare agent [55].

An alternative approach to poisoning treatment is the use of non-oxime compounds. Bispyridinium MB327 represents one of them, and the action is to reverse the neuromuscular blocking action of the OP compounds [59]. Animal studies have shown its protective efficacy against various NAs [60]. Unfortunately, there is no research on the effectiveness of MB327 on Novichoks, although the approach seems promising. Mannich phenol is a non-oxime that can reactivate an inhibited AChE enzyme, and 4-amino-2-[(diethylamino)methyl]phenol (ADOC) seems to be particularly interesting [61]. Based on the structure of Mannich phenols, more than a dozen non-oxime compounds capable of reactivating human AChE inhibited by NAs were synthesised [62,63]. At this point, we possess insufficient human studies on novel oximes, so currently, in the case of Novichok poisoning, commercially available oximes are still used.

Magnesium sulphate appears to be of note when treating patients with OP poisoning. This compound blocks calcium channels, which reduces the release of ACh. Moreover, it reduces excessive CNS stimulation resulting from activation of the N-methyl-D-aspartate receptor (NMDA) [64]. Intravenous administration of magnesium sulphate in a dose of 4 g on the first day shortens hospitalisation time and improves treatment results in patients with OP pesticide poisoning [65]. Furthermore, alkalisation of the blood with sodium bicarbonate and magnesium sulphate administration appears to be effective in recovering from moderate to severe poisoning. An infusion of 4 to 6 grammes of 20% $MgSO_4$ solution within 24 h may decrease atropine needs, count of intubations and number of days in the ICU in OP toxicity [66,67]. The use of magnesium sulphate in Novichoks poisoning still needs further research.

Anti-glutamate and anti-NMDA drugs could also be interesting for OP-poisoning treatment. The excitatory amino acid glutamate plays an essential role in the continuation of OP-induced seizures through excessive activation of NMDA [68]. One of the anti-NMDA compounds approved for human use in neurotraumatology is gacyclidine. It has been

shown in an animal model that gacyclidine administration can prevent neuropathology three weeks after exposure to OP, but there is no significant penetration into the CNS [22]. Gacyclidine provides optimal neuropathological protection when administered up to half an hour after poisoning with an organophosphorus compound [69]. Natural alkaloid huperzine A is an NMDA receptor antagonist and a reversible AChE inhibitor that can cross the BBB [70]. Huperzine A effectively protects peripheral and CNS AChE from OP compound poisoning [71]. Moreover, it prevents seizures and epilepticus after exposure by blocking NMDA-induced excitation [22]. Ketamine is another compound that can effectively stop epileptic seizures. This uncompetitive NMDA receptor antagonist is an appropriate epilepticus status prevention strategy after exposure [45]. In animal studies, ketamine and atropine have been shown to inhibit neutrophil infiltration and partially inhibit glial activity as significant neuroprotective effects [72].

The utilisation of antioxidants appears to be a reasonable approach to treating poisoning caused by organophosphorus compounds, which reduce total antioxidant capacity by generating nitric oxide and reactive oxygen radicals. Thus, they enhance the intensity of lipid peroxidation and increase the amount of reactive thiobarbituric substances. Vitamin E has been shown to have a therapeutic effect on OP-induced oxidative stress in rat erythrocytes [22,34]. Unfortunately, there is lack of information about application of vitamin E in Novichok's poisoning.

A new modern antidote based on hexahistidine-tagged organophosphorus hydrolase (His6-OPH, EC 3.1.8.1) proved to be effective in detoxifying OP in vivo [73].

Bioscavangers are worth considering as an alternative treatment for Novichok poisoning. An example of such bioscavengers is human butyrylcholinesterase (HuBuChE), whose detoxification mechanism of OP compounds is based on stoichiometric binding one mole of organophosphate neutralised by one mole of enzyme [74]. BChE clears the OP from the bloodstream before interacting with the AChE, protecting the native AChE from inhibition of OP. Using bioscavengers, the side effects of current antidotes can be avoided, and the need for prompt administration of antidotes will be reduced [75]. The study on soman-poisoned macaques showed that an intramuscularly administered dose of 13.1 mg/kg was protective [73]. It is the basis for research on the effect of this bioscavenger in the case of exposure to Novichoks. Studies in animal models suggest that therapeutic concentrations of HuBChE in the blood can be maintained for at least four days after a single dose. First, it is safe for humans and does not show tissue toxicity [74]. BChE activity tests are widely available in routine clinical practise, primarily used as liver function tests. However, they are usually the only laboratory parameter that confirms the clinical diagnosis of organophosphate poisoning. The administration of BChE in the case of Navalny's poisoning turned out to be the only effective treatment that saved the patient's life [14]. Another bioscavenger is fetal bovine serum AChE (FBSAChE), which protects mice from multiple LD_{50}'s of nerve agents [45]. Fresh frozen plasma (FFP) with albumin proved ineffective as a bioscavenger during a clinical trial in treating organophosphate-poisoned patients [76]. However, the data mentioned above are completely inconsistent with treating a human poisoned with Novichok, in which FFP was used with complete success. Administration of six units of FFP led to a marked increase in butyrylcholinesterase activity without a subsequent decrease, thus excluding the consumption of butyrylcholinesterase by an unbound inhibitory nerve agent in the bloodstream [14]. An interesting concept is the encapsulation of BChE, such an enzyme in a nanocarrier could more efficiently penetrate the BBB, which is associated with more effective treatment of acute neurotoxic effects of poisoning with organophosphorus compounds [77].

3. HAZMAT/CBRNE Approaches

There are two types of exposure to a hazardous material: HAZMAT (Hazardous Materials) and CBRNE (C—Chemical, B—Biological, R—Radiological, N—Nuclear, E—Explosives) [78]. The first type of event is unintentional and is randomly created, e.g., by an accident, breakdown or catastrophe [79]. On the other hand, the second type

of event consists of the intentional release of hazardous materials into the environment, e.g., by terrorist or military activities [80]. In contact with a hazardous substance such as Novichok, the most crucial action to take in the first place is to neutralise the hazardous factor. Next, a pyrotechnic inspection of the place and event participants is carried out. The general procedure of emergency medical services in the event of exposure to a hazardous substance consists of the following steps [81]: preliminary identification of the threat, securing the incident scene, evacuating casualties from the danger zone, triage and preparation for decontamination and patient supervision during it, securing patients at a medical point and preparing for transport, carrying out transport, and medical support for rescue operations. Personnel requires correct protective equipment to implement the above action plan. Appropriate personal protective equipment (PPE) is necessary to protect paramedics and enable them to operate under hazardous environmental conditions. PPE consists of respiratory protection equipment and protective suits (prevention against skin contamination). The ideal solution for rescuers is universal protective clothing that ensures safety in various events and allows the implementation of rescue activities necessary to save lives [82]. OSHA (Occupational Safety and Health Administration) compliant contamination protective equipment classification is as follows:

- Level A, a gas-tight protective suit with a self-contained breathing apparatus structure, prevents the implementation of medical procedures;
- Level B, a protective suit with thin but solid fabrics and special protective coatings, is equipped with a self-contained breathing apparatus;
- Level C, a protective suit with a filter mask or escape hood with a wide visor and a powered air respirator;
- Level D, essential protective clothing (goggles and mask with a P3 filter), provides adequate protection in daily practise [81].

The system of dividing the area of operation into three zones has been specified to improve the organisation of rescue operations [83]: first, a hot zone where a hazardous substance is present and casualties are exposed to its effects. Entering the hot zone requires the use of PPE. Life-support activities in the hot zone include haemorrhage control, airway management and securing the patient during evacuation. Second, the warm zone initially lacks a hazardous substance. The first clean zone is where victims are evacuated. In this area, initial triage, cardiopulmonary resuscitation, protection of the patient's basic vital functions and preparation for decontamination are performed. Third, the cold zone is free from hazardous substances. This is a safe area for paramedics who use essential PPE. This division of the area significantly facilitates the organisation of medical activities and the overall management of risk.

3.1. Personal Protection Equipment

Novichoks were designed as nerve agents that could not be stopped by the chemical protective gear available to NATO soldiers at that time [84]. Surprisingly, there is still a lack of relevant and appropriate review articles on HAZMAT/CBRNE approaches according to Novichok poisoning. Therefore, this is the first review of available data on this important and extraordinary topic [13,85,86].

First responders and medical personnel must ensure their own protection. Protection clothing and respiratory protection are the key to treating Novichok vapour or liquid exposure patients. Decontamination must occur outside the hospital, and many hospitals have a separate decontamination facility for self-tidy patients. HAZMAT personnel must establish a decontamination site for ambulatory and stretch cases. It should be noted that, since the first suspected Novichok poisoning (during the night on 4 March 2018), the attention has been focused on ensuring the safety not only of patients (decontaminated with thorough washing as part of our daily routine) but also of the staff who care for them. PPE has increased from standard precautions to different levels of protective clothing and equipment. Due to the new nature of Novichok and unknown physical properties, the necessary levels of protection were not initially well understood, and the recommended

levels of PPE varied over time. When Novichok's threats were better understood, the PPE required to care for patients was a single pair of disposable long sleeves, disposable long nylon gloves (to cover the sleeves), disposable surgical masks and eye protection for high-risk procedures. It is important to remember not only to treat patients but also to use PPE to handle potentially contaminated materials and equipment that should be appropriately contaminated. A lesson for any possible CBRNE incident is to start with high-level PPE and de-escalate only when the risk is clearer and more apparent. Fortunately, the agents involved seemed not volatile (as opposed to the 1995 Tokyo subway sarin attack, where the volatile agents threatened or affected first responders and health staff), and the level of contamination was limited but still significant. It should be emphasised that the strong persistence and hydrophobic nature of Novichoks, the removal of clothing from individuals and the decontamination before entering a screening area can prevent other patients and first responders from being exposed to the agent [87].

3.2. Supplies

The important issue is also supplies. The specific treatment regimen rapidly consumed available pharmaceutical countermeasures in the first accident mentioned. Interestingly, Salisbury District Hospital had a regional inventory of certain specific antidotes, so we immediately had sufficient pyridine for initial treatment (although we consumed a total supply of atropine in 24 h). The NHS blood transfusion service in the United Kingdom has a national reserve stock of pharmaceutical countermeasures for major events such as CBRN. The procurement also procured sufficient quantities of PPE while other teams coordinated the disposal of quarantined waste (urine, faeces, dirty linen, etc.) [88,89].

3.3. Decontamination

It should be emphasised that there is a lack of suggestions available about decontamination procedures due to the Novichok poisoning. However, it should be mentioned that the Treaty Manual of the Organisation for the Prevention of Chemical Weapons (OPCW) draws on previous human poisonings and the recent experience of sarin poisoning in Syria [90]. They stressed the importance of decontamination to prevent continued patient poisoning and continued patient poisoning protect other patients and personnel close to poisoned patients [91]. The type and level of decontamination depend on the physical properties of the Novichoks, if any, and the exposure route. Liquid Novichoks can be absorbed or evaporated through the skin, causing a danger of inhalation to those around them). On the contrary, Novichoks will be potentially systemically absorbed, metabolised or naturally expelled from the patient's respiratory system. Removing clothing (distinction) will provide at least 80% of the decontamination because the clothing fibre can capture and hold liquid Novichoks and steam. After decoupling, liquid agents must also be cleaned with materials such as Fuller's Earth (a fine adsorbent powder used by the army) or clinical paper towels (blue rolls) and then washed with soapy water at 35 °C.

4. Conclusions

Novichoks are the subject of considerable interest nowadays, although many unclear issues still need clarification. A full explanation of their structure and properties is necessary to better understand the threats these compounds pose and develop adequate protection against them. The potentially dangerous nature of the Novichoks used as chemical weapons poses a serious and global threat to the lives of casualties. The incidents in Salisbury and Amesbury only confirmed their extreme toxicity and the need to develop effective procedures to organise a medical response. In addition, introducing solutions to protect medical personnel when in contact with a hazardous substance increases the chances of saving lives. Therefore, the threat posed by Novichoks needs to be urgently assessed in order to be able to deal with future terrorist attacks or their use as chemical weapons. Developing efficient and effective HAZMAT/CBRNE approaches in contact with threatening A-series nerve agents is crucial. Despite the number of poisonings with these

OPs being negligible, physicians ought to be familiar with the management to protect the life of poisoning victims. We hope that this review will stimulate future research and help fill in missing data gaps, accelerate progress in improving our protection, and help develop an optimal treatment for casualties of Novichok poisoning.

Author Contributions: Conceptualisation, M.N. and K.J.; writing—original draft preparation, M.N. and K.J.; writing—review and editing, M.N., A.M. and K.J.; visualisation, M.N.; supervision, A.M. and K.J.; project administration, A.M. and K.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data are available for this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Archives of Toxicology (2023) 97:1691–1700
https://doi.org/10.1007/s00204-023-03507-2

IN SILICO

Application of toxicology in silico methods for prediction of acute toxicity (LD_{50}) for Novichoks

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Received: 3 April 2023 / Accepted: 24 April 2023 / Published online: 5 May 2023

Abstract
Novichoks represent the fourth generation of chemical warfare agents with paralytic and convulsive effects, produced clandestinely during the Cold War by the Soviet Union. This novel class of organophosphate compounds is characterised by severe toxicity, which, for example, we have already experienced three times (Salisbury, Amesbury, and Navalny's case) as a society. Then the public debate about the true nature of Novichoks began, realising the importance of examining the properties, especially the toxicological aspects of these compounds. The updated Chemical Warfare Agents list registers over 10,000 compounds as candidate structures for Novichoks. Consequently, conducting experimental research for each of them would be a huge challenge. Additionally, due to the enormous risk of contact with hazardous Novichoks, in silico assessments were applied to estimate their toxicity safely. In silico toxicology provides a means of identifying hazards of compounds before synthesis, helping to fill gaps and guide risk minimisation strategies. A new approach to toxicology testing first considers the prediction of toxicological parameters, eliminating unnecessary animal studies. This new generation risk assessment (NGRA) can meet the modern requirements of toxicological research. The present study explains, using QSAR models, the acute toxicity of the Novichoks studied ($n = 17$). The results indicate that the toxicity of Novichoks varies. The deadliest turned out to be A-232, followed by A-230 and A-234. On the other hand, the "Iranian" Novichok and C01-A038 compounds turned out to be the least toxic. Developing reliable in silico methods to predict various parameters is essential to prepare for the upcoming use of Novichoks.

Keywords Novichoks · Organophosphate · Nerve agents · Acute toxicity · Toxicology in silico

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
bw	Body weight
CWC	Chemical Weapons Convention
LD_{50}	Median lethal dose
NA	Nerve agent
NATO	North Atlantic Treaty Organization
OP	Organophosphate
OPCW	Organization for the Prohibition of Chemical Weapons
QSAR	Quantitative structure–activity relationship
SMILES	Simplified Molecular Line Input System
TEST	Toxicity Estimation Software Tool

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Introduction

Novichoks (Russian: овиок, 'newcomer'), referred to as nerve agents of series A (NA), pose a group of chemical warfare agents with a paralysing and convulsive effect (Noga and Jurowski 2023). Assume that Novichok compounds are unique organophosphates (OPs) containing a dihaloformaldoxime moiety (Watson et al. 2015). It is assumed that Vil S. Mirzayanov revealed the first information about A-series compounds in his book "State Secrets: An Inside Chronicle of Russia's Chemical Weapons Programme" (Mirzayanov 2008). Two possible Novichok structures have been

postulated, although it is unclear which one is more reliable. Mirzayanov published the first as phosphoramides (Fig. 1A). On the contrary, the second structure was proposed by Hoenig (2007) and Ellison (2007) as phosphorylated oximes (Fig. 1B). Furthermore, a group of Iranian scientists synthesised an additional Novichok structure in the laboratory (Hosseini et al. 2016). The probable chemical structures of the Novichoks and SMILES notation are presented in Table 1.

The original design intent of the Novichoks was to circumvent the Chemical Weapons Convention (CWC) list and be undetected using standard North Atlantic Treaty Organisation (NATO) chemical detection equipment (Nepovimova and Kuca 2020). One of the probable mechanisms of the toxic effect of Novichok compounds is irreversible binding to acetylcholinesterase (AChE) and inhibition of hydrolysis of the neurotransmitter acetylcholine (ACh) to acetate and choline (Chai et al. 2018). Overstimulation of cholinergic receptors caused by the accumulation of ACh in the synaptic cleft as a result of inhibition of AChE leads, depending on the route, dose and time of exposure, to the manifestation of several toxic symptoms through three types of reactions: muscarinic, nicotinic and central nervous system (CNS) (Korabecny et al. 2014; Kloske and Witkiewicz 2019; Kloske 2020).

So far, little is still known about Novichoks, and more data must be completed. One is knowledge about the threat level of these compounds, that is, their toxicity. We have already witnessed the "show" of the enormous toxic potential of Novichoks three times. The first two cases of use of these nerve agents took place in 2020 in Salisbury and Amesbury (UK) and started a public debate that made everyone aware of the dangerous nature of these compounds (Bhakhoa et al. 2019; Haslam et al. 2022). The third example of using A series nerve agents was the case of Navalny's acute poisoning in 2020 during a domestic flight in Russia. Following the results of clinical and laboratory studies, the use of a cholinesterase inhibitor was identified. This incident is critical because it is the only published clinical study on Novichok poisoning treatment, which proved the ineffectiveness of obidoxime reactivation and the effectiveness of butyrylcholinesterase therapy (Steindl et al. 2021).

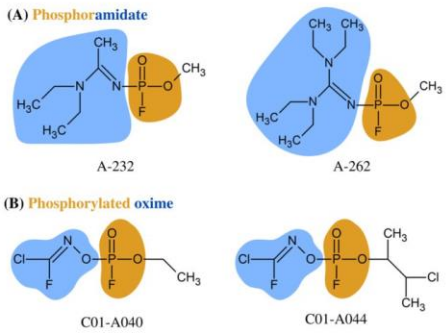
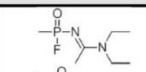
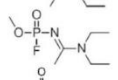
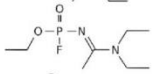
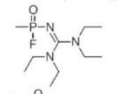
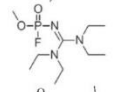
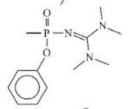
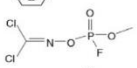
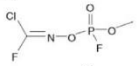
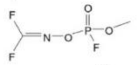
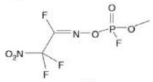
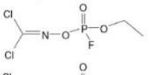
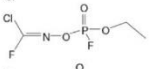
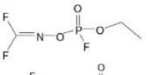
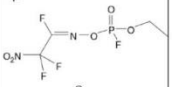
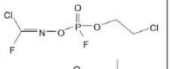
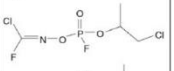
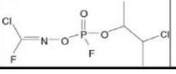


Fig. 1 Postulated chemical structures of Novichoks: **A** Mirzayanov's A-232 and A-262 as phosphoramidate, **B** Ellison's C01-A040 and C01-A045 as phosphorylated oxime

The above examples indicate the presence of Novichoks in public spaces and confirm the enormous threat and severe poisoning effects of these compounds in series A. Therefore, from the point of view of social security, it is crucial to study their properties, especially their toxicological aspects. The toxicity of Novichoks as a hypothetical group of nerve agents should be a critical national issue. There are many problematic issues, and the fundamental questions from a toxicological point of view are; What threat do these substances pose when in contact with humans? Exposure to what dose of these hazardous compounds is lethal? Do the A-series compounds exceed the toxicity of previous generations of NAs (-V and -G)? To answer these questions, it is essential to determine the toxicity of Novichoks. Therefore, estimating the acute toxicity of these chemicals (LD_{50}) in humans will be necessary to solve these problems. It should be noted that LD_{50} is based on crude endpoints (harmful effects) that estimate the average response (for statistical reasons) of a single exposure, in contrast to the value of the absence of the effect of multiple doses; it only documents when a compound causes death in animals. Although it appears to be a primary toxicological parameter, it is no longer usually experimentally determined in many situations (toxicological risk assessment usually requires other parameters such as the level of non-observed adverse effects, NOAEL). Furthermore, there is no correlation between LD_{50} and other compounds (e.g., biological activity, developmental, and reproductive toxicity (DART)). It differs significantly from a 'true' starting point for obtaining health values. Furthermore, LD_{50} is not generated based on the principles of replacement, reduction, and refinement of animal use and welfare (3R), which are principles aimed at minimising the use of animals in toxicity tests when applicable (Faria et al. 2016). However, given the specificity of this topic and the severe gap in determining such a fundamental toxicological parameter for Novichok (just a few works related to this crucial issue (Bolt and Hengstler 2022), there is a great need for this type of research. Therefore, to fulfil the modern requirements for toxicological research of toxicology of the twenty-first century and to consider the next-generation risk assessment (NGRA) (Pallocca et al. 2022) with a new approach to toxicity testing (Leist et al. 2008) (i.e., taking into account the prediction of toxicological parameters first), it is necessary to first apply in silico toxicology methods to eliminate unnecessary animal studies. Researching this

Table 1 Possible chemical structures of studied Novichok nerve agents

Compound	Name	SMILES	Potential structure	Reference
1	A-230	CCN(CC)C(=NP(=O)(C)F)C		(Mirzayanov, 2008)
2	A-232	CCN(CC)C(=NP(=O)(OC)F)C		
3	A-234	CCN(CC)C(=NP(=O)(OCC)F)C		
4	A-242 (Novichok-5)	CCN(CC)C(=NP(=O)(C)F)N(CC)CC		
5	A-262 (Novichok-7)	CCN(CC)C(=NP(=O)(OC)F)N(CC)CC		
6	Iranian "Novichok"	CN(C)C(=NP(C)(=O)Oc1ccccc1)N(C)C		(Hosseini et al., 2016)
7	C01-A035	COP(=O)(ON=C(Cl)Cl)F		(Ellison, 2007)
8	C01-A036	COP(F)(=O)ON=C(F)Cl		
9	C01-A037	COP(F)(=O)ON=C(F)F		
10	C01-A038	COP(F)(=O)ON=C(F)C(F)(F)[N+](=O)[O-]		
11	C01-A039	CCOP(F)(=O)ON=C(Cl)Cl		
12	C01-A040	CCOP(F)(=O)ON=C(F)Cl		
13	C01-A041	CCOP(F)(=O)ON=C(F)F		
14	C01-A042	CCOP(F)(=O)ON=C(F)C(F)(F)[N+](=O)[O-]		
15	C01-A043	FC(Cl)=NOP(F)(=O)OCCCl		
16	C01-A044	CC(CCl)OP(F)(=O)ON=C(F)Cl		
17	C01-A045	CC(Cl)C(C)OP(F)(=O)ON=C(F)Cl		

parameter is essential to determine the accurate level of risk that Novichoks may pose. However, surprisingly little attention has been paid to this fundamental issue (Hartung 2009; Krewski et al. 2020).

Studies on Novichoks are rare and have only recently begun to emerge (Imrit et al. 2020; Bolt and Hengstler 2022). Taking into account the three cases of chemical attacks involving novel nerve agents, it is no doubt that determining the toxicological aspects of these hazardous substances is essential, but also tricky because of their high reactivity and toxicity; as organophosphorus compounds they are treated differently in toxicology than other poisons, for example, in the Cramer classification (Kroes et al. 2004). Recognising the vulnerability to the threat of terrorist activity, the most desirable and justified approach in this situation seems to be the use of in silico toxicological tools. Furthermore, because such hazardous substances are not available, the only way to assess this possibility is to use in silico tools. Such methods are desirable and necessary to predict the acute toxicity (LD_{50}) of Novichoks.

Only a few studies on the application of computational/QSAR methods to study molecular aspects of Novichoks are available in the scientific literature (Nepovimova and Kuca 2018; Bhakhoa et al. 2019; Carlsen 2019; Franca et al. 2019; Jeong and Choi 2019; Harvey et al. 2020; Wang et al. 2021); however, they were never an exhaustive study of all known Novichoks. The rationale for conducting this study is the lack of primary data on Novichoks in the scientific literature (Bolt and Hengstler 2022). Reference is made to existing reports for only a few examples of these hazardous substances. To predict acute toxicity (LD_{50} for rats), we used models included in the software: QSAR Toolbox and Toxicity Estimation Software Tool (TEST) (Kleandrova et al. 2015). A general flow chart showing the acute toxicity parameter estimation process is presented in Fig. 2.

Methods

TEST

In silico studies were performed using the Toxicity Estimation Software Tool (TEST), an open-source application developed by the US EPA. Toxicity Estimation Software Tool (ver. 5.1.2 and ver. 4.2.1) comprises several models assessing acute toxicity thresholds by reading across structural analogues or multivariate regression. The models are built on hundreds of structural, constitutional, connectivity, shape, topological, molecular distance, fragments, and electrotopological property descriptors. The programme demands only SMILES (Simplified Molecular Line Input System) or CAS numbers, as inputs quickly evaluate chemical toxicity. The TEST software is trained on the endpoint from the EPA ECOTOX database (US EPA 2022). Every read-across or regression model has a specific applicability domain. The software offers an estimated LD_{50} threshold based on each model prediction and a Consensus average of the component models. TEST assesses acute toxicity (endpoint: oral rat LD_{50}) using four QSAR methodologies (Martin et al. 2008):

- Hierarchical method: The toxicity for a particular query compound is estimated using the weighted average of the predictions from various models. The different models are achieved using Ward's method to divide the training set into a series of similar structural clusters.

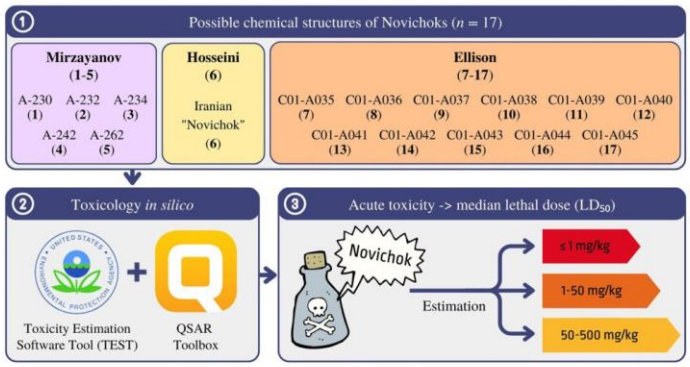


Fig. 2 Flow chart that displays the general concept of median lethal dose estimation process applying in silico toxicology tools

- FDA method (only ver. 4.2.1): The prediction for each test chemical is made using a new model that fits the chemicals most similar to the test compound. Each model is generated at runtime.
- Nearest-neighbour method: The predicted toxicity is estimated by averaging the three chemicals in training set with the closest similarity to the test compound.
- Consensus method: The predicted toxicity is estimated by taking an average of the predicted toxicities from each of the above QSAR methodologies (considering each method's applicability domain).

The Consensus result was reported as the most reliable estimate provided by the TEST software (Melnikov et al. 2016); therefore, we used it to estimate the acute toxicity of Novichok. In addition to the above method, the FDA method included in the TEST was also used to verify the reliability and compare the values with the results published by Carlsen (2019). Table 2 summarises the pros and cons of the available QSAR methodologies.

QSAR

We supported in silico analyses using the QSAR Toolbox ver. 4.5 standalone software application, which the OECD Organisation recommends for Economic Cooperation and Development. The QSAR Toolbox, developed by OASIS in collaboration with the OECD and the European Chemicals Agency (ECHA), is an application to evaluate the potential hazards of chemicals with in silico models to facilitate the practical application of (Q)SAR approaches in regulatory contexts by governments and industry, and to improve their regulatory acceptance (OECD and ECHA 2021). Data gaps are filled through the following flexible workflow in which compound categories are built, and incomplete data are estimated by read-across or applying local QSARs. In addition to read-across and trend analysis, the Toolbox includes numerous databases of experimental results. The calculated endpoint of this research was acute toxicity (LD_{50}).

Estimation QSAR

Acute toxicity was estimated using QSAR Toolbox software by manual categorisation and data gap-filling method. Additionally, using the TEST software, this estimation was used to verify the accuracy of the previously calculated value. The target endpoint was defined in the following order: Human health hazards, acute toxicity, LD_{50} (endpoint), Oral (Route of administration) and rats (test organisms/species). The categorisation was defined as Repeated Dose (Hess); only in this grouping option is the Organophosphate category (organophosphorus compounds are treated differently in toxicology than other poisons, i.e. Cramer classification (Kroes et al. 2004)). The read data were selected only for the initially targeted endpoint. The read-through method for "qualitative" endpoints was used to fill data gaps. The scale/unit used to estimate acute toxicity (LD_{50}) was chosen in (mg/kg). The rationale for selecting this scale/unit is that it offers the most considerable amount of chemicals and available converted data. Then, a subcategorisation was used to exclude structurally different prediction compounds from the investigated Novichoks. Individual subcategories were made for each chemical. The initial stage of subcategorisation for the targeted nerve agents had a particular common scheme. The option "Structure similarity" was used to remove dissimilar structures, and the option "US-EPA New Chemical Categories" and "Aquatic toxicity classification by ECOSAR" was used to remove selected analogues. The accepted predictions for Novichok compounds have been compiled in a table for the entire toxicity result section.

Table 2 Advantages and disadvantages of the QSAR methodology in TEST software (US EPA 2022)

Method	Advantages	Disadvantages
Hierarchical clustering	Produce more reliable predictions since predictions are made from multiple models	Cannot provide external estimates of toxicity for compounds in the training set
FDA	Generate a new model based on the closest analogues to test chemical Provides an external prediction of toxicity	Predictions sometimes take longer because it needs to generate a new model each time
Nearest neighbor	Provides a quick estimate of toxicity Allows to determine structural analogues for a given test compound Always provides an external prediction of toxicity	Does not use a QSAR model to correlate the differences between the test compound and the nearest neighbors Shown to achieve the worst prediction results during external validation
Consensus	Shown to achieve the best prediction results during external validation	Cannot provide external estimates of toxicity for compounds in the training set

Results

Acute toxicity, represented as the median lethal dose (LD_{50}) of the Novichoks investigated ($n = 17$), was estimated using two software: QSAR Toolbox (ver. 4.5) and TEST. In the case of the latter tool, two versions have been used; the older one (ver. 4.2.1) contains the FDA model, which is missing in the newer one (ver. 5.1.2), where the Consensus model was used. The software calculates the LD_{50} values for oral administration to rats. The extrapolation from animal to human (rat-to-human) was based on toxicity values conversed following the guidelines for converting doses between animals and humans based on body surface area. Rat doses were converted to equivalent human doses by dividing the rat dose by 6.2 (Nair and Jacob 2016). The calculated median lethal dose values for the oral administration of Novichoks and human-converted LD_{50} values are provided in Table 3.

In the case of estimated oral doses of LD_{50} for rats using the recommended Consensus method included in the TEST software and then converted doses for humans, the most perilous Novichok was A-232 (**2**), whose value was 0.21 mg/kg bw. Nerve agents indicated slightly higher median lethal doses: A-230 (**1**) 0.35 mg/kg bw and A-234 (**3**) 0.58 mg/kg bw. The remaining Novichok structures proposed by Mirzayanov (**4–5**) appeared to be weaker by almost two orders of magnitude than the compounds mentioned above (**1–3**). The 'Iranian' Novichok (**6**) and one of the structures proposed by Ellison C01-A038 (**10**) were the lowest potent of each compound studied, reaching the following values:

178.96 mg/kg bw and 310.04 mg/kg bw. The most lethal nerve agent among Ellison structures was C01-A043 (**15**), with a value of 2.66 mg/kg bw, although one order of magnitude weaker than the structure (**2**). The compounds (**11–13** and **16**) pose a slightly lower threat than the previously mentioned Novichok (**15**) and reach the following values: 5.71; 4.20; 7.92, and 6.41 mg/kg bw. Other Novichoks structures postulated by Ellison (**7–9**, **14** and **17**) showed values similar (from 20.10 to 42.96 mg/kg bw) to organophosphorus compounds (**4–5**).

The FDA model implemented in the TEST software calculated the LD_{50} values for each nerve agent studied. The results for only two compounds (**10–11**) were consistent compared to those estimated using the Consensus model. According to the FDA method, compound A-242 (**4**) had the lowest LD_{50} value, 0.49 mg/kg bw. Novichok A-232 (**1**) reached a slightly higher value of 0.57 mg/kg bw; with the Consensus method, it became the most hazardous compound. Interestingly, the next somewhat less toxic nerve agent appeared as C01-A040 (**12**) and the next A-234 (**3**): 0.68 and 0.71 mg/kg bw. Values between 1 and 1.55 mg/kg bw were achieved with compounds C01-A043 (**15**) and A-230 (**1**). Novichoks (**5, 11, 14** and **16**) pose a danger about 4–7 times lower than the structure mentioned above (**15**); they reach the following values: 7.35, 5.55, 4.38 and 5.04 mg/kg bw. Similar values were estimated for compounds (**13** and **17**); 15.35 and 15.49 mg/kg bw.

Table 3 Rat and human oral LD_{50} values calculated using the TEST and QSAR Toolbox software

Compound	Name	Rat oral LD50 (mg/kg bw)			Human oral LD50 (mg/kg bw)		
		TEST Consensus method	TEST FDA method	QSAR Toolbox	TEST Consensus method	TEST FDA method	QSAR Toolbox
1	A-230	2.14	9.59	3.09	0.35	1.55	0.50
2	A-232	1.31	3.52	2.53	0.21	0.57	0.41
3	A-234	3.57	4.43	3.91	0.58	0.71	0.63
4	A-242 (Novichok-5)	92.81	3.04	55.9	14.97	0.49	9.02
5	A-262 (Novichok-7)	276.26	45.56	141.7	44.56	7.35	22.85
6	Iranian "Novichok"	1109.56	596.17	810.3	178.96	96.16	130.69
7	C01-A035	206.51	1082.59	263.8	33.31	174.61	42.55
8	C01-A036	124.61	427.72	193.1	20.10	68.99	31.15
9	C01-A037	266.37	813.51	324	42.96	131.21	52.26
10	C01-A038	1922.26	2099.13	1030	310.04	338.57	166.13
11	C01-A039	35.39	34.44	48.6	5.71	5.55	7.84
12	C01-A040	26.05	4.19	15.4	4.20	0.68	2.48
13	C01-A041	49.11	95.16	58.5	7.92	15.35	9.44
14	C01-A042	206.76	27.17	129.9	33.35	4.38	20.95
15	C01-A043	16.47	6.21	12.5	2.66	1.00	2.02
16	C01-A044	39.72	31.23	45.7	6.41	5.04	7.37
17	C01-A045	243.78	96.01	191.8	39.32	15.49	30.94

'Iranian' Novichok (**6**) and the compounds (**7**–**9**) showed values from 68.99 to 174.61 mg/kg, classifying them as one of the least toxic nerve agents studied. Ellison postulated the structure C01-A038 (**10**) at the LD_{50} value of 338.57 mg/kg bw, the highest result, and thus, the weakest of the Novichoks investigated.

Because the Consensus and FDA models showed numerous inaccuracies in the LD_{50} values for the Novichoks tested, we decided to additionally use the QSAR Toolbox to estimate the acute toxicity parameter. The results obtained by the subsequent software made it possible to verify the reliability of the calculations obtained from the Consensus and FDA models implemented in TEST by comparing the data. Acute toxicity values were estimated using the QSAR Toolbox for most compounds correlated with the Consensus method of the TEST software. Only two Novichoks (**2** and **6**) were more consistent with the FDA method. However, in the case of compounds (**10**–**11**) where the values for both TEST methods are comparable, a greater degree of correlation cannot be unequivocally determined. The assessed value for structure (**12**) in the QSAR Toolbox varies between the Consensus and FDA methods. A common feature of all estimations is the highest value achieved by Novichok C01-A038 (**10**). By analogy to the Consensus method, the first five compounds with the lowest LD_{50} values and thus posing the most significant threat are, respectively: A-232 (**2**), A-234 (**3**), A-230 (**1**), C01-A043 (**15**) and C01-A040 (**12**).

Discussion

Based on sources from the available literature, the acute toxicity of Novichoks was perceived to be several times higher than that of conventional NAs (Ellison 2007; Mirzayanov 2008). Novichok A-230 was claimed to be 5–8 times more toxic than compound VX (a relative comparison of the LD_{50} values under the same conditions). Additionally, A-232 was said to be 10 times as harmful as Soman. Nerve agents A-242 and A-262, toxic derivatives of A-230 and A-232, were classified as ultra-highly toxic despite not specifying any value. However, these primary sources lack information on the acute toxicity of A-234 (Mirzayanov 2008). According to the data published in the seminar paper by Karev, the above reports on Novichok toxicological data were not valid (Karev 2009). In the case of A-232, it showed a value one order of magnitude lower than VX and A-234 two times lower than VX. The acute toxicity of the A series nerve agents, lower than conventional NAs, was somewhat confirmed by other estimated data (Franca et al. 2019). The LD_{50} value for Novichok A-230 was lower by an order of magnitude, while the compounds A-232 and A-234 were approximately three times less toxic than VX. It is worth mentioning that the similarity in toxicity between A-232 and A-234 was assumed here based on structural similarity. The results for these two Novichoks were highly different compared to the data published by Karev (2009).

Carlsen (2019) also attempted to verify these data for Novichoks ($n = 6$), using quantitative structure–activity relationship (QSAR) models for estimation and calculated the median lethal dose for oral administration rats that were converted to the human dose. Carlsen presented utterly different data on the relative toxicity of A-series nerve agents, contrary to Mirzayanov's claims. The estimated LD_{50} value for compound VX was used to indicate the reliability of the data. In the works of Karev (2009) and Franca et al. (2019), it reached the value of 10 mg/person (70 kg). Converted to mg/kg, this value is 0.14 mg/kg, which is very consistent with the calculated value of LD_{50} for humans: 0.1 mg/kg (Carlsen 2019). Estimated data proved that Novichoks (A-230, A-232, A-234, A-242, and A-262) are 5–75 times less dangerous than VX, and in the case of Iranian 'Novichok', almost 1000 times less toxic. While the values for A-232 and A-234 are similar to the data obtained by Franca et al. (2019), the median lethal dose for A-230 is very different. Assuming that a "standard" person weighs 70 kg, the LD_{50} values for Novichoks were recalculated based on the sources of the above literature and summarised in Table 4.

Using the analogous FDA method included in the TEST programme (ver. 4.2.1), we obtained precisely the same results for Novichoks as in the work of Carlsen (2019). Furthermore, to estimate the LD_{50} parameter, we also used the Consensus method included in TEST (ver. 5.1.2) and the second software, QSAR Toolbox. As the results between the Consensus and FDA methods differ, the question is which is more reliable? The first is supported by the fact that it applies all QSAR methods included in the TEST to assess toxicity and is additionally recommended by the US EPA (2022). Furthermore, the consensus method was reported to

Table 4 Available literature data about the toxicity of Novichoks; based on (Karev 2009; Gupta 2015; Nepovimova and Kuca 2018; Franca et al. 2019; Carlsen 2019)

Compound	Human LD_{50} (mg/kg bw)		
	(Karev 2009; Nepovimova and Kuca 2018)	(Gupta 2015; Franca et al. 2019)	(Carlsen 2019)
VX	0,143	0.086–0.143	0.10
A-230	n/a	0.011–0.029	1.55
A-232	0.014	0.5	0.57
A-234	0.071	0.5	0.71
A-242	n/a	n/a	0.49
A-262	n/a	n/a	7.35
Iranian	n/a	n/a	96.16

n/a not applicable

be the most reliable estimate provided by the TEST software (Melnikov et al. 2016). On the other hand, the FDA method is backed by the generation of new models based on the closest analogues of the test chemical. The latest TEST software version (ver. 5.1.2) does not include the implemented FDA method. Furthermore, the LD_{50} values obtained using the QSAR Toolbox overwhelmingly correlate with the consensus method; only two compounds from the A series nerve agents were the values more comparable to the FDA method. Taking into account the information above, we tend to evaluate the results obtained using the consensus method, primarily supported by verification using the QSAR Toolbox, as more trustworthy. The LD_{50} values for Novichoks estimated in our work using TEST (Consensus method) and QSAR Toolbox mainly differs from the data discussed earlier and are included in Table 4. The only compounds whose LD_{50} values were similar are A-230, A-232, and 'Iranian' Novichok. According to our estimates, the most dangerous Novichok was A-232, in contrast to the values calculated by Carlsen (2019), where the nerve agent A-242 would be the most toxic, and Franca et al. (2019) suggested A-230 as the most toxic Novichok. Unfortunately, sources from the literature only provide LD_{50} for 2–6 organophosphorus compounds from the Novichok group. Therefore, our work is unique because it includes up to 17 such A-series nerve agents.

Attention should also be paid to extrapolating doses between species, converting the rat to a human oral median lethal dose. The allometric scaling between species for dose conversion from animal to human studies is one of the most controversial areas of pharmacology and toxicology. The allometric approach considers differences in body surface area related to animal weight while extrapolating doses between species (Nair and Jacob 2016). This article's conversion to human toxicity followed the guidelines for animal-human dose conversion based on body surface area, i.e., rat doses were converted to human equivalent doses by dividing the rat dose by 6.2 (Nair and Jacob 2016; Carlsen 2019). Science changes in phases, experiencing anomalies that lead to a crisis and revolution, resulting in a new, immature scientific paradigm that, over time, becomes the new normal (Hartung 2021). Toxicology has encountered a series of such anomalies that have led to a crisis. One of them is the generally accepted guide for dose conversions between species, which is not necessarily the right one. As evidenced by the various studies, for example, many inflammatory mediators assume very different roles in different species; for example, TLR4 signaling differs in humans and mice (Schmidt et al. 2010). The above studies prove that animals are not particularly good predictors of humans in areas where we have comparative data across species. In toxicodynamics, a well-known example is that humans are 1000 times more responsive to inhibition of Na/K-ATPase by the cardiac glycoside ouabain than mice (Kent et al. 1987). Moreover, the difference in susceptibility to bacterial endotoxins can be up to a million times greater in range (Hasiwa et al. 2013). Thus, the above examples indicate that humans are not 70 kg mice in toxicology (Leist et al. 2008). A study based on a broad system approach confirmed the low predictability of animal responses to inflammation (Seok et al. 2013). The low-level predictability of animal studies in research areas, which allows direct comparison of data between species, raises serious doubts about the usefulness of animal data as crucial tools for predicting human safety. Perhaps this is the reason for the differences in prediction, or perhaps it is another proof of the validity of Hartung's concept? (Hartung 2009, 2021). Regardless, these studies were essential as an initial screening before undertaking acute toxicity studies on animals, concerning reactive substances such as Novichoks.

Conclusions

Undoubtedly, Novichoks pose a grave threat to human security. We have had the opportunity to experience examples of their excessive toxicity three times, including in Salisbury, Amesbury (UK) and the case of Navalny's poisoning. Some light was shed on the acute toxicity of Novichoks by estimating the median lethal dose (LD_{50}) of these hazardous nerve agents. The estimation has been made for organophosphorus compounds from the Novichok group using in silico tools: Toxicity Estimation Software Tool (TEST) and QSAR Toolbox. According to our evaluations, the deadliest Novichoks were compound A-232 (**2**), A-230 (1) and A-234 (**3**), whose LD_{50} values, when administered orally, did not exceed 0.65 mg/kg bw. On the other hand, the 'Iranian' Novichok (**6**) and C01-A038 (**10**) compounds, whose values exceeded 130 mg/kg bw, proved the least perilous. Unfortunately, despite the update of the CWC list, the exact structure of Novichoks is not known. It should be emphasised that the complete threat posed by Novichoks, in addition to the toxicity itself, also includes processes such as the vapour pressure, water solubility, skin permeability coefficients, the toxicokinetics, and the environmental fate of these compounds, which determine their durability in the external environment. Further in silico studies of different properties (chemical, physical, and toxicological) are required to deal with the inevitable utilisation of novel types of nerve agents in terrorist attacks. Our toxicology studies provide the first comprehensive insight into the acute toxicity of numerous Novichoks ($n = 17$). The TEST and QSAR Toolbox software can be successfully applied as tools to estimate the median lethal dose of organophosphorus compounds of the Novichok group preceding experimental laboratory tests.

Author contributions MN: Formal analysis and investigation, Writing—original draft preparation, Writing—review and editing, Visualization; AM: Data curation, Supervision; KJ: Conceptualisation, Data curation, Supervision, Writing—review and editing; All authors have read and approved the manuscript.

Funding This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethical approval The manuscript does not contain newly generated clinical studies or patient data.

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Science of the Total Environment 890 (2023) 164241

Contents lists available at ScienceDirect

Science of the Total Environment

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

The prediction of hydrolysis and biodegradation of Novichoks using *in silico* toxicology methods

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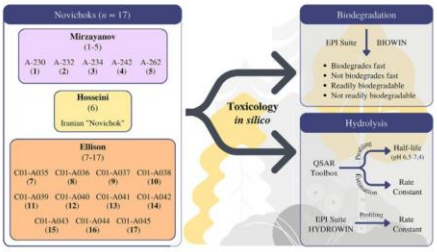
HIGHLIGHTS

- *In silico* estimations to evaluate dangerous Novichok compounds safely.
- Practical approach of QSAR models to predict hydrolysis and biodegradation.
- The destiny of studied nerve agents after release into the environment shows extremely fast to very slow transformation due to hydrolysis.
- Most of the analysed Novichoks completely biodegraded within weeks to months.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Jose Julio Ortega-Calvo

Keywords:
Novichoks
Hydrolysis
Biodegradation
Toxicology *in silico*
QSAR

ABSTRACT

Novichoks constitute a relatively new class of nerve agents of extreme toxicity that we have had the opportunity to experience three times already. After the first case (Salisbury, UK), a public debate about Novichoks began, which resulted in the realisation of the nature of these chemicals. From a social security point of view, examining their properties, especially toxicological and environmental aspects, are crucial. After the CWC (Chemical Warfare Agent) list update, the candidate structures for the Novichoks may be over 10,000 compounds. It would be extremely laborious to conduct experimental research for each. Understanding their environmental persistence and health hazards is an essential national issue. Moreover, due to the high risk posed by contact with hazardous Novichok substances, *in silico* research was applied to estimate hydrolysis and biodegradation safely. The present study elucidates, using QSAR models, the environmental fate of the Novichoks studied (n = 17). The results indicate that Novichoks released into the environment hydrolyse at various rates, from extremely fast (<1 day) to very slow (more than a year). Furthermore, ultimate biodegradation from weeks to months is expected for most compounds, which classifies them as relatively difficult biodegradable. Applying reliable *in silico* methods (QSAR Toolbox and EPI Suite) for predicting various parameters is crucial to prepare for the upcoming usage of Novichoks.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CWC, Chemical Weapon Convention; EPI, Estimation Program Interface; HAZMAT/CBRN incidents, Hazardous Materials/Chemical Biological Radiological Nuclear incidents; LD_{50}, median lethal dose; NA, nerve agent; NATO, North Atlantic Treaty Organisation; OP, organophosphorus compounds; QSAR, quantitative structure-activity relationship.

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http://dx.doi.org/10.1016/j.scitotenv.2023.164241
Received 6 April 2023; Received in revised form 26 April 2023; Accepted 14 May 2023
Available online 24 May 2023

1. Introduction

Novichoks (Russian: Новичо́к, "newcomer"), also known as A-series nerve agents (NAs), belong to a category of chemical warfare agents which are unique organophosphorus (OP) compounds containing a dihaloformaldoxime moiety (Noga and Jurowski, 2023). A paralysing and convulsive effect characterises these highly toxic compounds, and the mechanism is based on binding to acetylcholinesterase (AChE) and inhibiting the hydrolysis of the neurotransmitter acetylcholine (ACh) (Chai et al., 2018). According to Mirzayanov, the toxicity of Novichoks exceeds conventional nerve agents such as VX several times, but this statement has been questioned (Mirzayanov, 2008). Further research using quantitative structure-activity relationships (QSAR) models presented opposite data (Carlsen, 2019). The Novichoks' primary design goals were to be challenging to identify by the North Atlantic Treaty Organisation (NATO) and to circumvent the Chemical Weapons Convention (CWC) list (Nepovimova and Kuca, 2018). Two chemical structures have been postulated for Novichoks, although it is unclear which one is more reliable. The first was proposed by (Mirzayanov, 2008) as a phosphoramidate (Fig. A.1), and the second was put forward by (Hoenig, 2007) and (Ellison, 2007) as phosphorylated oximes (Fig. B.1). Therefore, data on these compounds remain still shrouded in mystery, and information remains being filled in. The probable chemical structures of Novichoks analysed with the appropriate SMILES notations are included in Table 1.

The fate of Novichok in the environment as a hypothetical group of nerve agents should pose a crucial national issue (Cavalcante et al., 2019). After the second apparently unintended Novichok poisoning in Amesbury (UK), the discussion of the fate of the environmental compounds was revealed, as one theory was that a couple of British citizens had accidentally contacted the remains of the Skripal poisoning left somewhere in the environment. Later, a small glass bottle containing the poison was recovered. There are many problematic issues and an essential question from a toxicological point of view. What is the impact of these hazardous materials on the environment? What type of environmental risk do these substances pose? Is there a genuine concern about their presence and persistence in the environment? If so, how long will they likely pose an environmental problem after the HAZMAT/CBRN incidents (Noga et al., 2023)? To answer these questions, the primary issue is determining the sustainability of the environment. Therefore, in addition to examining the physicochemical properties of Novichoks, two distinct processes are essential for these problems, hydrolysis and biodegradation. Studies of these parameters are required to determine environmental stability and decontamination approaches to reduce secondary damage.

Hydrolysis is the chemical decomposition of a substance by water, depending on the chemical composition, solubility, pH, and oxidation-reduction; it is assumed to be the most important reaction of organic molecules in aqueous environments (Speight, 2018). Investigating the fate of Novichoks in the environment is complicated because these compounds' hydrolysis rates (constant rate, Kn) exhibit a significant dependence on pH. Understanding the potential of this pH dependence to alter the fate of Novichok in various environments is essential to develop appropriate response measures to a potential release.

Biodegradation is a further essential parameter (Matula et al., 2020). Biodegradation refers to the conversion of chemicals through enzyme reactions in microorganisms. This process leads to ultimately decrease in toxicity without burdening the environment. However, biodegradation issues are treated marginally and have never been adequately addressed.

Novichok reactivity and toxicity make estimating the hydrolysis and biodegradation process difficult; therefore, the most desirable and justified approach is applying *in silico* methods. These kinds of pilot/preliminary studies have already been carried out (Carlsen, 2019; Cragan et al., 2009; Harvey et al., 2020; Imrit et al., 2020; Lyagin and Efremenko, 2019; Otsuka and Miyaguchi, 2021); however, they have never been a comprehensive study for all known/expected structures of Novichoks. In addition, they have been performed using various methods in various contexts but never with an impact on the environment or toxicological risk assessment. The rationale for conducting this study is that there is a lack of fundamental data on Novichoks in the scientific literature. The reference point is the existing reports for only a few examples of these hazardous substances. For this purpose, we applied the QSAR Toolbox (QSAR Toolbox, v.4.5) and the EPI Suite software (Estimation Program Interface, v.4.11).

2. Methods

2.1. QSAR models

In silico studies were performed using QSAR Toolbox version 4.5 software, which the OECD Organization recommends for filling gaps in (eco) toxicity data of chemicals (OECD, 2021). The QSAR Toolbox, developed by the Laboratory of Mathematical Chemistry (LMC) in Bourgas in collaboration with the OECD (The Organisation for Economic Co-operation and Development) and the European Chemicals Agency (ECHA), is a software application to evaluate the potential hazards of chemicals with *in silico* models to facilitate the practical application of (Q)SAR approaches in regulatory contexts by governments and industry, and to improve their regulatory acceptance (LMC, 2023; OECD and ECHA, 2021). The QSAR Toolbox explicitly detects the properties of the examined chemical structures using numerous databases containing the results of experimental studies for chemicals and profilers. The calculated endpoints of this study are the hydrolysis half-life and the hydrolysis rate constant.

We supported *in silico* analyses using HYDROWIN and BIOWIN models integrated into Estimation Program Interface (EPI) Suite (v4.11). This Windows-based physical, chemical, and environmental fate estimation software package was developed by the U.S. Environmental Protection Agency (US EPA) and Syracuse Research Corporation (SRC). The EPI Suite calculators have based primarily on the constant fragments (group contributions) method, validated using an independent set of chemicals. The EPI Suite runs all estimation programmes using a single input in the form of the canonical Simplified Molecular Line Input System (SMILES) (US EPA and OCSPP, 2017).

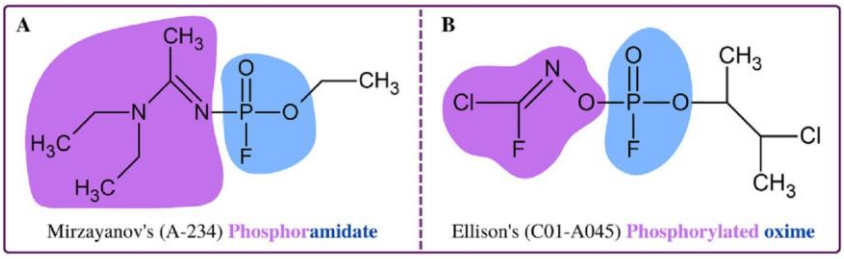
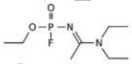
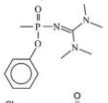
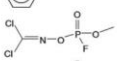
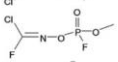
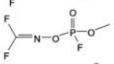
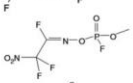
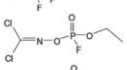
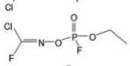
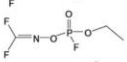
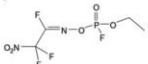
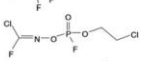
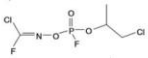
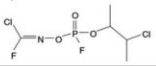


Fig. 1. Novichoks proposed chemical structures: phosphoramidate (A) and phosphorylated oxime (B).

Table 1
Chemical structures and SMILES notation of studied Novichok compounds.

Compound	Name	Potential structure	SMILES	Reference
1	A-230		CCN(*CC*)C(=NP(=O)(C)F)C	(Mirzayanov, 2008)
2	A-232		CCN(*CC*)C(=NP(=O)(OC)F)C	
3	A-234		CCN(*CC*)C(=NP(=O)(OCC)F)C	
4	A-242 (Novichok-5)		CCN(*CC*)C(=NP(=O)(C)F)N(*CC*)CC	
5	A-262 (Novichok-7)		CCN(*CC*)C(=NP(=O)(OC)F)N(*CC*)CC	
6	Iranian "Novichok"		CN(*C*)C(=NP(C)(=O)Oc1ccccc1)N(*C*)C	(Hosseini et al., 2016)
7	C01-A035		COP(=O)(ON=C(*Cl*)Cl)F	(Ellison, 2007)
8	C01-A036		COP(F)(=O)ON=C(*F*)Cl	
9	C01-A037		COP(F)(=O)ON=C(*F*)F	
10	C01-A038		COP(F)(=O)ON=C(*F*)C(F)(F)[N+](=O)[O-]	
11	C01-A039		CCOP(F)(=O)ON=C(*Cl*)Cl	
12	C01-A040		CCOP(F)(=O)ON=C(*F*)Cl	
13	C01-A041		CCOP(F)(=O)ON=C(*F*)F	
14	C01-A042		CCOP(F)(=O)ON=C(*F*)C(F)(F)[N+](=O)[O-]	
15	C01-A043		FC(Cl)=NOP(F)(=O)OCCCl	
16	C01-A044		CC(*CCl*)OP(F)(=O)ON=C(*F*)Cl	
17	C01-A045		CC(*Cl*)C(C)OP(F)(=O)ON=C(*F*)Cl	

2.2. Hydrolysis profiling

Hydrolysis half-lives (pH 6.5–7.4) were estimated using general mechanistic profiling schemes donated by LMC incorporated in QSAR Toolbox (LMC, 2023). The hydrolysis profiler classifies chemicals into categories depending on their half-lives. The hydrolysis grades according to calculated half-life values are as follows: very slow (>100 days), slow (30–100 days), moderate (10–30 days), fast (5–10 days), very fast (1–5 days) and extremely fast (<1 day). Hydrolysis rate constants were estimated using the profiling method through the Aqueous Hydrolysis Rate Program (HYDROWIN, v2.00) included in the EPI Suite (v4.11). HYDROWIN only requires a chemical structure to make these predictions (US EPA and OCSPP, 2017).

2.3. Biodegradation profiling

Possible biodegradation was studied by applying the Biodegradation Probability Program (Biowin, v4.10) module included in the EPI Suite. Biowin estimates the probability of rapid biodegradation of an organic chemical in the presence of various environmental microorganisms

(Boethling et al., 2003). Using the U.S. Environmental Protection Agency's tool Biowin v4.10 (EPIWIN), the aerobic and anaerobic biodegradability of testing xenobiotics can be estimated. The EPI Suite Biowin1–7 models were used in the study to estimate the biodegradation potential of Novichok compounds. A summary of Biowin models, including description, result classification (Gomis et al., 2015), endpoint, and prediction basis, is provided in Table 2. More details about the Biowin models are described in Supplementary Materials 1 (SM 1).

2.4. Hydrolysis estimation

The hydrolysis rate constant was also estimated using the QSAR Toolbox software by manual categorisation and data gap filling instead of using only the profiling method. This step was taken because the hydrolysis rate constant parameters were not obtained for all compounds studied by HYDROWIN profiling. Additionally, the validity of the values estimated by the EPI Suite HYDROWIN profiling method was checked. The target endpoint was defined in the following order: Environmental Fate and Transport, Stability in Water, Hydrolysis, and Hydrolysis Rate Constant. The categorisation was defined as Repeated Dose (Hess); only in this grouping option is the Organophosphates category. Data read selected only for the initially targeted endpoint. The read across method for "qualitative" endpoints was used to fill data gaps. The scale/unit used to estimate the hydrolysis constant was chosen in (1/days). The rationale for selecting this scale/unit is that it offers the most considerable quantity of chemicals and converted data available. Then, subcategorisation was used to exclude structurally different prediction compounds from the studied nerve agents. Individual subcategorisation was done for each Novichok. Specific schemes were used for all compounds, such as the option of removal of selected analogues: US-EPA New Chemical Categories (Anionic Surfactants), Aquatic toxicity classification by ECOSAR (Triazoles, Halopyrdines and Vinyl/Allyl Halids). The accepted predictions for Novichok nerve agents have been compiled in a table for the entire hydrolysis section.

3. Results

3.1. Hydrolysis

Half-life hydrolysis was revealed for studied Novichoks ($n = 17$) at pH 6.5–7.4 and estimated using the profiling module in the QSAR Toolbox. According to the classification described in the methods section, they showed variability, that is, from extremely fast (<1 day) to very slow (≥100 days). The fastest hydrolysing Novichoks turned out to be A-230 (**1**), A-242 (**4**), and "Iranian" (**6**). The hydrolysis half-life calculated to hours for the first two compounds (**1**, **4**) is approximately 5 h, while for (**6**), just over 9 h. The estimated half-life of hydrolysis was determined for nerve agents **2**, **3** and **5** to be 38 days, while for nerve agents **15**–**16** it was 32.1 days and for **17** is 56.8 days, qualifying them as slow (30–100 days). The slowest hydrolysing, classified as very slow (≥100 days) and amounting to even 2310 days, are the structures **7**–**14** of Novichoks. When converted, these compounds will remain in the environment for about 6 years and 3 months. Hydrolysis half-life and constant rate (Kn) data are collectively provided in Table 3.

Using the profiling method, the HYDROWIN model included in the EPI Suite software calculated the hydrolysis rate constant only for compounds **7**–**17**. One of the slowest structures proposed by (Ellison, 2007) turned out to be compounds **7**–**10**, for which the constant rate (Kn) value was 0.7528/days. Slightly faster hydrolysing, reaching the value of 0.7688/days, are nerve agents **11**–**14**. On the other hand, Novichok C01-A043 (**15**) has the lowest constant hydrolysis rate of 0.6074/days. The last two compounds, **16** and **17**, turned out to be the fastest of the profiled and reached the value of 0.8538/days. Currently, available data are insufficient to obtain suitable QSAR(s) for phosphor-type (O═P(—C/—O—)(—F/—O—)) structures **1**–**6**; the software requires more experimental data. Therefore, we decided to use the QSAR Toolbox for manual categorisation and data gap filling instead of using only the profiling method. In the case of the previously analysed structures **7**–**17**, the trend is maintained. The values differ slightly from those profiled by the HYDROWIN module, confirming the correctness of the chosen hydrolysis rate constant estimation route. Values for Novichoks 1–6 were successfully estimated. The most readily hydrolysed organophosphorus compound from the Novichok group A-230 (**1**), whose rate constant was 6.24/days. A-242 (**4**) and Iranian "Novichok" (**6**) also reached high values of 5.83/days and 4.22/days, respectively. Other compounds with a hydrolysis rate constant of 2.79/days are A-232 (**2**) and A-262 (**5**). On the contrary, A-234 (**3**) turned out to be slightly slower in hydrolysing (2.04/days).

3.2. Biodegradation

The summarised results, including the values for each Biowin model obtained from the EPI Suite software, are shown in Table 4.

The numerical output from Biowin1–2 models consists of the probability of fast biodegradation. For each of these models, a designation has been assigned according to the classification criterion used for this purpose, and the estimated probability is: "biodegrades fast" (≥0.5) or "does not biodegrade fast" (<0.5). Each structure proposed by Mirzayanov (Mirzayanov, 2008) (**1**–**5**) reached values above 0.62, which, according to the criterion, classifies them as fast degradable. The laboratory method synthesised Iranian 'Novichok' (**6**) by Hosseini (Hosseini et al., 2016) reached a high value (0.747) of the Biowin1 model. Of the structures indicated by Ellison (Ellison, 2007), four showed ready biodegradability (**8**–**9**, **12**–**13**).

Expert survey models (Biowin3 and Biowin4) estimate the time necessary for the primary and ultimate aerobic biodegradation in water. Primary biodegradation modifies the identity of the parent compound as the first

Table 2
Summary of the Biowin models.

Model number	Description	Result classification	Endpoint	Basis of prediction
Biowin1	Linear probability model based on the BIODEG database	Quantitative prediction of the probability that a chemical biodegrades 'fast' (≥0.500) or does not biodegrades 'fast' (<0.500)	Probability of rapid biodegradation	Multiple regression; fragments
Biowin2	Non-linear probability model based on the BIODEG database			
Biowin3	Ultimate biodegradation model based on expert survey	Semi-quantitative prediction based on the following subjective scoring of persistence: Recalcitrant ≤1.750; Months (1.750, 2.250]; Weeks to months (2.250, 2.750]; Weeks (2.750, 3.250]; Days to weeks (3.250, 3.750]; Days (3.750, 4.250]; Hours to days (4.250, 4.750]; Hours >4.750	Biodegradation rate (semi-quantitative)	
Biowin4	Primary biodegradation model based on expert survey			
Biowin5	Linear probability model based on the Japanese MITI database	Quantitative prediction of the probability that a chemical is readily biodegradable (≥0.500) or non-readily biodegradable (<0.500)	Probability of ready biodegradability in OECD 301C test	
Biowin6	Non-linear probability model based on the Japanese MITI database			
Biowin7	Linear probability model to predict anaerobic biodegradation, based on 'serum bottle' test conditions	Quantitative prediction of the probability that a chemical biodegrades 'fast' (≥0.500) or does not biodegrades 'fast' (<0.500)	Probability of rapid biodegradation	

Table 3
Predicted values of hydrolysis half-life and rate constant (Kn) estimated for studied Novichoks.

Compound	Half-life pH 6.5–7.4 (days)	Rate constant, Kn (/days)	
	QSAR Toolbox: Profiling	EPI Suite: Profiling	QSAR Toolbox: Estimation
1	Extremely fast (0.0207)	ND[a]	6.24 (−16.7 ÷ 28.5)
2	Slow (38)	ND	2.79 (−16.1 ÷ 21.6)
3	Slow (38)	ND	2.04 (−6.62 ÷ 10.7)
4	Extremely fast (0.0207)	ND	5.83 (−15.8 ÷ 27.5)
5	Slow (38)	ND	2.79 (−16.1 ÷ 21.6)
6	Extremely fast (0.387)	ND	4.22 (−18.3 ÷ 26.7)
7	Very slow (2310)	0.7528	0.711 (−2.21 ÷ 3.64)
8	Very slow (2310)	0.7528	0.711 (−2.21 ÷ 3.64)
9	Very slow (2310)	0.7528	0.711 (−2.21 ÷ 3.64)
10	Very slow (2310)	0.7528	0.711 (−2.21 ÷ 3.64)
11	Very slow (2310)	0.7688	0.834 (−2.59 ÷ 4.26)
12	Very slow (2310)	0.7688	0.834 (−2.59 ÷ 4.26)
13	Very slow (2310)	0.7688	0.834 (−2.59 ÷ 4.26)
14	Very slow (2310)	0.7688	0.834 (−2.59 ÷ 4.26)
15	Slow (32.1)	0.6074	0.654 (−2.06 ÷ 3.37)
16	Slow (32.1)	0.8538	0.961 (−1.96 ÷ 3.88)
17	Slow (56.8)	0.8538	0.961 (−1.96 ÷ 3.88)

[a] ND = not determined.

step, while ultimate biodegradation completely mineralises the chemical in water and carbon dioxide (Balakrishnan et al., 2020). Biowin3–4 models resulted in the biodegradation of recalcitrant compounds were not achieved. The ultimate biodegradation time frame for Biowin3 revealed that all investigated Novichoks required weeks to months to complete degradation, except for compounds **1**, **4**, **9** and **13**. For them, the value obtained from the Biowin3 model exceeds 2.750, which requires weeks for complete biodegradation. The Biowin4 model values for the Novichoks were within (3.250, 3.750], which estimated the primary biodegradation time frame as days to weeks.

For Biowin5–6 models, the numerical output of the probability of ready biodegradation. The estimated probability is 'readily biodegradable' (≥0.5) or 'not readily biodegradable' (<0.5). From all investigated structures of Novichoks (n = 17), only A-230 (**1**) and A-242 (**4**) exceeded Biowin5 model values ≥ of 0.5, while for the Biowin6 model, no compound was classified as readily biodegradable.

By classifying ≥0.5 as BF and all degradation predictions <0.5 as NBF, the Biowin7 model can be used to predict anaerobic biodegradation based on "serum bottle" test conditions. This method predicted a positive result of the fast biodegradation test for 76.47 % (13/17) of studied Novichok compounds (structures **6**, **10**, **14** and **17** not-readily biodegradable).

4. Discussion

Among the fastest hydrolysing Novichoks were A-230 (**1**), A-242 (**4**), and "Iranian" (**6**). The half-life of hydrolysis for the first two compounds (**1**, **4**) was about 5 h, while for the third one (**6**), it was just over 9 h. In the remaining compounds, the half-life of hydrolysis ranges from 32 to even 2310 days. A-230, A-242 and "Iranian" Novichok feature a phosphonic acid derivative in the core compared to the rest of the tested nerve agents that contain a phosphoric acid derivative, which may explain the much faster half-life of hydrolysis for these three compounds. We decided to profile the hydrolysis half-life at pH 6.5–7.4. An environment with a pH > 7 does not help estimate the hydrolysis of Novichoks under environmental conditions. This is because of the validity of the hydrolysis rate constant equation in aqueous media where the hydroxide ions are not the

Table 4
Predicted values of biodegradation estimated using EPI Suite Biowin1–7 models. Blue colour - correlation of applied models

Compound	Biowin1	Biowin2	Biowin3	Biowin4	Biowin5	Biowin6	Biowin7
1	BF (0.655)	BF (0.562)	Weeks (2.77)	Days to weeks (3.57)	RB (0.514)	NRB (0.0039)	BF (0.57)
2	BF (0.647)	BF (0.506)	Weeks to months (2.73)	Days to weeks (3.54)	NRB (0.488)	NRB (0.0029)	BF (0.57)
3	BF (0.641)	NBF (0.456)	Weeks to months (2.70)	Days to weeks (3.52)	NRB (0.492)	NRB (0.0029)	BF (0.596)
4	BF (0.628)	BF (0.524)	Weeks (2.76)	Days to weeks (3.49)	RB (0.515)	NRB (0.0029)	BF (0.542)
5	BF (0.620)	NBF (0.313)	Weeks to months (2.61)	Days to weeks (3.46)	NRB (0.489)	NRB (0.0022)	BF (0.542)
6	BF (0.747)	BF (0.728)	Weeks to months (2.63)	Days to weeks (3.46)	NRB (0.331)	NRB (0.161)	NBF (0.179)
7	NBF (0.425)	NBF (0.0247)	Weeks to months (2.39)	Days to weeks (3.34)	NRB (0.353)	NRB (0.0003)	BF (0.727)
8	BF (0.544)	NBF (0.169)	Weeks to months (2.60)	Days to weeks (3.47)	NRB (0.416)	NRB (0.0002)	BF (0.742)
9	BF (0.663)	BF (0.621)	Weeks (2.81)	Days to weeks (3.59)	NRB (0.479)	NRB (0.0002)	BF (0.756)
10	NBF (0.443)	NBF (0.0893)	Weeks to months (2.43)	Days to weeks (3.33)	NRB (0.292)	NRB (0.0003)	NBF (0.422)
11	NBF (0.418)	NBF (0.0203)	Weeks to months (2.36)	Days to weeks (3.32)	NRB (0.356)	NRB (0.0003)	BF (0.753)
12	BF (0.537)	NBF (0.143)	Weeks to months (2.57)	Days to weeks (3.45)	NRB (0.419)	NRB (0.0001)	BF (0.768)
13	BF (0.657)	BF (0.573)	Weeks (2.78)	Days to weeks (3.57)	NRB (0.483)	NRB (0.0001)	BF (0.782)
14	NBF (0.436)	NBF (0.0744)	Weeks to months (2.39)	Days to weeks (3.31)	NRB (0.296)	NRB (0.0001)	NBF (0.448)
15	NBF (0.410)	NBF (0.0158)	Weeks to months (2.32)	Days to weeks (3.30)	NRB (0.368)	NRB (0.0001)	BF (0.859)
16	NBF (0.403)	NBF (0.013)	Weeks to months (2.29)	Days to weeks (3.28)	NRB (0.295)	NRB (0.0001)	BF (0.587)
17	NBF (0.396)	NBF (0.0107)	Weeks to months (2.26)	Days to weeks (3.26)	NRB (0.223)	NRB (0.0002)	NBF (0.316)

*BF = biodegrades fast; NBF = not biodegrades fast; RB = readily biodegradable; NRB = not readily biodegradable.

dominant nucleophiles in an environment (it is challenging to establish) (Cragan et al., 2009). The Sergei Skripal poisoning incident in Salisbury (United Kingdom) in 2018 suggested that the A-234 compound used may be relatively stable in the environment and thus pose a threat to bystanders for a long time after usage (Peplow, 2018). The hydrolysis half-life of this Novichok obtained by profiling was over a month (38 days), supporting the assumptions about its relative stability in the environment. The only known theoretical study about estimating Novichok hydrolysis half-life is an article by Carlsen (Carlsen, 2019). This includes half-lives for six studied A-series nerve agents: A-230, A-232, A-234, A-242, A-262 and 'Iranian' Novichok. Compounds **2**, **3** and **5** were classified as moderate (10–30 days). Our results are inconsistent; for the Novichoks mentioned above, along with compounds **15–17,** the half-life is in the slow range (30–100 days). Detailed results for nerve agents **2**, **3** and **5** are 38 days (8 days difference), while for **15–16** are 32.1 days and for **17** are 56.8 days. Although the trend between the results is comparable, there are differences in the profiled number of days of the hydrolysis half-life. We assume that the most reasonable possible explanation for these discrepancies in the values obtained is the different versions of the QSAR Toolbox used (version 4.2 and version 4.5), and thus the databases implemented to a different extent.

For the hydrolysis rate constant estimation, we decided to apply, in addition to profiling with the HYDROWIN model included in the EPI Suite software, also the QSAR Toolbox for manual categorisation and filling data gaps. The rationale for this approach was the insufficient experimental data available to obtain appropriate QSAR(s) for phosphor-type (O═P(—C/—O—)(—F/—O—)) structures **1–6** by using the HYDROWIN model. Despite the only known experimental work on the hydrolysis rate constant of Novichoks (A-230, A-232 and A-243) (Harvey et al., 2020), this information has not yet been implemented into the EPI Suite database. The hydrolysis rate constant was estimated for structures **7–17** using HYDROWIN. In order to Novichoks **7–10**, the rate constant (Kn) value was 0.7528/days. More rapid hydrolysing, reaching the value of 0.7688/days, turned out to be nerve agents **11–14**. The only difference from the previously described compounds is an additional methyl group in the structure, which seems to impact the hydrolysis acceleration. On the other hand, the organophosphorus compound from the Novichok group (**15**) has a hydrolysis rate constant of 0.6074/days. This compound differs from C01-A040 (**12**) because it has an additional halogen group (Cl-) attached to the external alkyl group. The last two compounds, differing respectively for **16** with one additional methyl group and **17** with two extra methyl groups, compared to structure **15**, turned out to be the fastest of the profiled and reached the value of 0.8538/days. This difference in the rate of hydrolysis may be due to electronic effects associated with the additional methyl groups. Among the Novichoks **1–6**, estimated by the QSAR Toolbox, A-230 (**1**) was the most readily hydrolysed structure, with a rate constant of 6.24/days. Slightly slower hydrolysing compounds are A-242 (**4**) and Iranian "Novichok" (**6**), reaching values of 5.83/days and 4.22/days, respectively. This may be because the three Novichok compounds (**1**, **4** and **6**) mentioned above have a methyl group bonded to the phosphorus atom. In contrast, the other structures have oxygen instead of the methyl group. The value of the hydrolysis rate constant of 2.79/day was achieved by the A-series agents: A-232 (**2**) and A-262 (**5**). On the contrary, A-234 (**3**), which differed from A-232 (**2**) by the addition of an ethyl group bound to phosphoric oxygen, turned out to be slightly slower in hydrolysing (2.04/days). Interestingly, as in the case of compounds **7–10** and **10–14**, the additional methyl group facilitated the hydrolysis, so here, between the Mirzayanov structures, the ethyl group decelerated the hydrolysis of the compound. Based on the results, we consider phosphonic acid derivatives easier to hydrolyse than phosphoric acid derivatives. Structures proposed by (Ellison, 2007) are characterised by values of the hydrolysis rate constant <1.0, so slow hydrolysis concerning the remaining compounds, where the lowest value is >2.0. In an overwhelming majority, the results of the hydrolysis rate constant for the studied Novichoks are consistent with the results of the hydrolysis half-life, except for structure **15**, where the half-life was determined as slow (compounds **7–14** were classified as very slow). In contrast, according to the estimate of the rate constant, it is the slowest hydrolysing compound (most stable). The trend for the hydrolysis reaction rates estimated in this work, where A-230 is easier to hydrolyse than A232 and A234, was consistent with the results obtained experimentally under neutral conditions (0.17 μM/min for A-230, 0.061 μM/min for A-232 and 0.0032 μM/min for A-234) by (Harvey et al., 2020).

Besides the half-life and rate constant of hydrolysis, the possible biodegradability of the studied Novichoks was also estimated using seven distinct EPI Suite Biowin models. The Biowin1 model predicted fast biodegradation results for 10 of 17 compounds investigated Novichoks (58.82 %). However, the Biowin2 model predicted the fast biodegradation of Novichok compounds for 6/17 (35.29 %). The model revealed fewer rapid biodegradable nerve agents than Biowin1, resulting from using a non-linear model to predict the probability quantitatively instead of a linear one. Biowin 1 and 2 are intended to convey a general indication of biodegradability under aerobic conditions and not for any particular medium. The six structures of Novichoks (1, 2, 4, 6, 9 and 13) overlap between the two models used, both linear and non-linear, and we believe that only these compounds should be considered rapidly biodegradable. The ultimate biodegradation (Biowin3 model) of Novichoks A-230 (**1**) and A-242 (**4**) was calculated in weeks, which may be related to the presence of a phosphonic acid derivative in the structure compared to the rest of the compounds containing phosphoric acid derivatives. Furthermore, the complete degradation time in weeks was also determined for compounds C01-A037 (**9**) and C01-A041 (**13**). The common feature of both is the low molecular weight, which does not exceed 191 $g{\cdot}mol^{-1}$ and the presence of two external fluorine atoms in the structure. One or both of these characteristics may define the time required for the biodegradation of these compounds. The only known literature study on the biodegradation of Novichoks is Carlsen's theoretical work (Carlsen, 2019). This includes the estimation of primary and ultimate biodegradation for six A-series agents, which correspond only to certain compounds numbered **1–6** we examined. Primary biodegradation values (Biowin4 model) are in line with our results. However, the ultimate biodegradation (Biowin3 model), according to Carlsen's results, took place within weeks to months for every compound studied by the author. Following our estimate, compounds A-230 (**1**) and A-242 (**4**) will degrade in a week. We assume that the results differ from the most recent version of the model we used (Biowin, v4.10). Although there is no confirmation of this, information on the methodology is limited to indicating the model used and its purpose (Carlsen, 2019). It should be emphasised that Biowin 3 and Biowin 4 models yield estimates for the time required to achieve complete ultimate and primary biodegradation in a typical or "evaluative" aquatic environment (US EPA and OCSPP, 2017). Therefore, only the prediction with Biowin 3 and Biowin 4 models is most suitable for our needs, as they reflect an aqueous environment that is desirable from both environmental and clinical toxicology perspectives (the physiological value of pH for whole blood is in the range of about: 7.35–7.45). In the case of the Biowin5–6 models, out of all tested Novichoks (n = 17), only A-230 (**1**) and A-242 (**4**) were classified as rapidly biodegradable for the Biowin5 model. However, for Biowin6, no structure was determined as readily biodegradable. So why are there such differences between Biowin1/2 and Biowin5/6, since the latter models use an approach similar to that used in developing Biowin1/2? Note that the Biowin1/2 linear/non-linear probability model estimation uses the BIODEG database, while the Biowin5/6 model is based on the Japanese MITI database. The observed differences are, therefore, due to the different databases used. The Biowin7 model showed fast anaerobic biodegradation for each assessed Novichok except for **6**, **10**, **14** and **17**. Structures unqualified as rapidly biodegradable are characterised by high molecular weight (254–270 g·mol-1) and comprehensive branching structure. Criteria for the prediction of ready biodegradability take place if the Biowin3 model (ultimate survey model) is "weeks" or faster and the Biowin5 model (MITI linear model) probability is ≥0.5; then the prediction is achieved. A method based on the usage of Bayesian analysis to prepare biodegradation data. Biowin5 and 6 also predict ready biodegradability, although only for degradation in the OECD301C test,

using data from the Chemicals Evaluation and Research Institute Japan (CERIJ) database (US EPA and OCSPP, 2017). The data in the literature, an *in silico* study, show that none of the tested compounds corresponding to our **1–6** were readily biodegradable (Carlsen, 2019). Taking into account the criterion of ready degradability, two Novichoks we analysed fulfil the prediction: A-230 (**1**) and A-242 (**4**).

An intriguing issue is the possibility of a correlation between biodegradation and reactivation. A nucleophile attack is needed to break down the Novichok molecule in both cases. Two scientific papers examine commercial oximes' *in vitro* AChE reactivation results concerning a surrogate of Novichok A-242 action (Santos et al., 2022a, 2022b). The question is whether there is any correlation at this point? Generally, one can risk stating that hypothetically (due to the similarity of the mechanism), an analogy can be sought that the faster the biodegradation, the faster the reactivation in relation to the action of a given Novichok (the faster Novichok undergoes biodegradation, the less threat it poses, and consequently, enzyme reactivation may occur more quickly). Comparing our biodegradation predictions for A-242 (**4**), it can be concluded that this Novichok will undergo rapid biodegradation. This suggests that AChE could quickly reactivate (due to, for example, an enzyme ageing reaction or a specific antidote action). However, there is no possibility of a quantitative correlation of this interesting correlation (which at first glance does not seem to matter); hence this is another important research direction. Another limitation of the comparison is the fact that in the papers mentioned above on AChE reactivation, various antidotes were analysed in this context with a single Novichok compound (A-242), but not in terms of the substance itself, but its structural analogue that was supposed to imitate (NTMGMP - the first synthetic surrogate of a Novichok agent useful for experimental evaluation of antidotes (Santos et al., 2022a)) such action (AChE blocking). Therefore, hypothetically such a relationship exists (and our preliminary research confirms this compared to the available literature), but further research in this area is required. At this stage, such cause-and-effect conclusions can only be considered a contribution to further discussion rather than an absolute fact.

5. Conclusions

Clearly, Novichoks are a significant health threat and also environmental fate. We shed some light on the environmental fate of Novichoks by estimating the hydrolysis half-life, rate constant, and biodegradability of these extremely toxic OP. The destiny of three investigated Novichoks (A-230 (**1**), A-242 (**4**), and Iranian 'Novichok' (**6**)) after release into the environment shows an extremely fast transformation due to hydrolysis. In contrast, others display slow (A-232 (**2**), A-234 (**3**), A-262 (**5**), and C01A043-A045 (**15–17**)) or very slow (C01A035-A042) hydrolysis half-lives. Moreover, the rate constant of hydrolysis was estimated, and three compounds revealed high-level values of this parameter and thus readily hydrolysed, A-230 (**1**) (6.24/days), A-242 (**4**) (5.83/days) and Iranian "Novichok" (**6**) (4.22/days). Most of the investigated OP compounds appeared non-readily biodegradable. A-230 (**1**), A-242 (**4**), C01-A037 (**9**), and C01-A041 (**13**) were classified as biodegradable within weeks by the ultimate biodegradation module (Biowin3 model), while the rest of the studied compounds were classified as weeks to months. The prediction of Biowin4 revealed that for all the compounds studied, the primary biodegradation time frame varied from days to weeks. Environmental variability plays a significant role in this type of simulation; however, applying the hydrolysis half-life (pH 6.5–7.4) profiling method and hydrolysis rate estimations are crucial. It should be emphasised that other processes, such as vapour pressure and water solubility, also impact the environment's fate and require thorough examination. The hydrolysis data should be relevant to determining the persistence of Novichoks in the environment. Despite the update of the CWC list, the exact structure of OP compounds is not yet known. Further *in silico* studies of different chemical, physical and toxicological properties are required to deal with the impending usage of novel types of nerve agents as chemical weapons or in terrorist attacks. Our toxicological studies applying *in silico* tool estimation, for the first time, provide complex insight into the nature of Novichok in an environmental context. The QSAR Toolbox and EPI Suite software can be successfully used as tools to assess the hydrolysis and biodegradation of organophosphorus compounds from the Novichok group prior to experimental laboratory studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.164241.

CRediT authorship contribution statement

Maciej Noga: Methodology, Software, Writing - original draft preparation, Writing - review and editing, Visualization, Investigation. **Agata Michalska:** Conceptualization, Data curation, Supervision, Writing - review and editing. **Kamil Jurowski:** Conceptualization, Data curation, Supervision, Writing - original draft preparation, Writing - review and editing, Visualization.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

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

Acknowledgements

This research received no specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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10. Streszczenie w języku polskim

Niniejsza rozprawa doktorska została przygotowana w oparciu o cykl czterech powiązanych tematycznie publikacji pełnotekstowych poświęconych predykcji wybranych parametrów toksykologicznych dla związków fosforoorganicznych z grupy Novichok przy użyciu metod toksykologii *in silico*.

Novichok, związki serii A, to grupa środków paralityczno-drgawkowych stworzonych potajemnie podczas zimnej wojny przez Związek Radziecki. Stanowią czwartą generację chemicznych środków bojowych; podobnie jak inne środki paraliżujące, są związkami fosforoorganicznymi zaprojektowanymi tak, aby były nieuleczalne i niewykrywalne. Struktura chemiczna związków typu Novichok nadal pozostaje niepewna, postuluje się dwie wersje. Pierwszy wariant, według Vil S. Mirzayanov'a, jako fosforamidy. Druga alternatywna struktura zaproponowana przez Hoenig'a i Ellison'a opisuje związki Novichok jako fosforylowane oksymy. Mechanizm działania toksycznego związków serii A polega na nieodwracalnym wiązaniu się z acetylocholinoesterazą (AChE) i hamowaniu hydrolizy neuroprzekaźnika, tj. acetylocholiny (ACh) do octanu oraz choliny. Nadmierna stymulacja receptorów cholinergicznych w wyniku gromadzenia się ACh w szczelinie synaptycznej prowadzi, w zależności od drogi, dawki oraz czasu ekspozycji, do manifestacji szeregu objawów toksycznych poprzez trzy rodzaje reakcji: muskarynową, nikotynową i ośrodkowego układu nerwowego. Dotychczas trzykrotnie byliśmy świadkami „pokazu" ogromnego potencjału toksycznego środków paraliżujących Novichok. Pierwsze dwa przypadki użycia tych środków paraliżujących miały miejsce w 2018 roku w Salisbury i Amesbury (Wielka Brytania). Trzecim przykładem zastosowania związku Novichok było zatrucie Alexeia Navalnego podczas lotu krajowego w Rosji. Przypadek ten stanowi jedyne opublikowane badanie kliniczne dotyczące leczenia zatrucia związkiem Novichok, w którym udowodniono skuteczność terapii butyrylocholinoesterazą oraz wykazano brak skuteczności reaktywacji po podaniu obidoksymu.

Ze względu na swoją toksyczność, praca ze związkami serii A wymaga nadzwyczajnych środków ostrożności. Ponadto, badania eksperymentalne powinny zostać poprzedzone szacowaniem parametrów toksykologicznych. Co więcej, po zaktualizowaniu listy CWA (ang. Chemical Warfare Agent), ponad 10 000 struktur może kandydować na miano związku Novichok. Niemożliwe jest zatem zsyntetyzowanie

i oszacowanie docelowych parametrów dla każdej struktury z osobna. Biorąc pod uwagę globalne zagrożenie stwarzane przez związki serii A, wiele niewiadomych oraz luk w danych wymaga uzupełnienia. Stąd inspiracja do badań wykorzystujących metody toksykologiczne *in silico* w postaci predykcji wybranych parametrów Novichok.

Właściwości związków Novichok takie jak toksyczność oraz los środowiskowy określono stosując podejście *in silico*, ilościową zależność struktura-aktywność (QSAR). Do estymacji parametrów medialnej dawki śmiertelnej (LD_{50}), biodegradacji, okresu półtrwania hydrolizy oraz stałej szybkości hydrolizy wykorzystano oprogramowania takie jak: pakiet aplikacji QSAR Toolbox, Estimation Program Interface Suite oraz Toxicity Estimation Software Tool.

Dotychczas w literaturze twierdzono, że związki fosforoorganiczne z grupy Novichok przewyższają swoją toksycznością znane konwencjonalne związki serii -V czy -G. Związek A-230 miał być rzekomo 5-8 razy bardziej toksyczny niż VX, podczas gdy A-232 być 10 razy bardziej toksyczny niż Soman (GD). Wykonane predykcje zaprzeczyły tym doniesieniom, najgroźniejszą z badanych struktur Novichok A-232 (LD_{50} = 0,41 mg/kg mc.) oszacowano jako czterokrotnie mniej toksyczną niż związek VX (LD_{50} = 0,1 mg/kg mc.) oraz siedmiokrotnie słabszą od GD (LD_{50} = 0,06 mg/kg mc.). Estymowany okres półtrwania oraz stała szybkość hydrolizy wskazują, że użyte związki Novichok mogą utrzymywać się w środowisku zewnętrznym, w zależności od użytego związku fosforoorganicznego, od kilku godzin (A-230, A-242 i irańskiego "Novichok"), przez ponad miesiąc (A-232, A-234, A-262 i C01A043-A045), nawet do kilku lat (C01A035-A042). Dodatkowo, stosując modele *in silico* do predykcji szybkiej biodegradacji wykazano, że związki serii A są strukturami trudno ulegającymi degradacji tlenowej oraz beztlenowej. Nie uzyskano związków opornych, a ramy czasowe tego procesu oscylowały od tygodni (A-230, A-242, C01-A037 oraz C01-A041) do miesięcy.

Podsumowując, predykcja parametrów związków fosforoorganicznych jest procesem złożonym oraz wymagającym zastosowania różnorodnych podejść *in silico*. Dzięki badaniom toksykologicznym *in silico* w bezpieczny dla zdrowia sposób uzyskano kompleksowy wgląd w naturę środków paraliżujących Novichok. Choć nadal sporo informacji odnośnie związków serii A owianych jest tajemnicą, to pomyślna predykcja właściwości toksycznych oraz środowiskowych struktur Novichok (n = 17) uzupełniła część istotnych, brakujących w literaturze naukowej danych.

11. Streszczenie w języku angielskim

This doctoral dissertation was prepared based on four thematically related full-text publications devoted to predicting selected toxicological parameters for organophosphorus compounds from the Novichok group using *in silico* toxicology methods.

Novichoks, A-series compounds, are a group of nerve agents secretly created during the Cold War by the Soviet Union. They consist of the fourth generation of chemical warfare agents; like other nerve agents, they are organophosphate compounds designed to be incurable and undetectable. The chemical structure of organophosphorus compounds of the Novichok group still remains uncertain; two versions are postulated. The first variant, according to Vil S. Mirzayanov, is phosphoramides. A second alternative structure proposed by Hoenig and Ellison describes Novichoks as phosphorylated oximes. The mechanism of toxic action of A-series compounds consists of irreversible binding to acetylcholinesterase (AChE) and inhibition of the hydrolysis of the neurotransmitter acetylcholine (ACh) to acetate and choline. Excessive stimulation of cholinergic receptors as a result of the accumulation of ACh in the synaptic cleft leads, depending on the route, dose and time of exposure, to the manifestation of several toxic symptoms through three types of reactions: muscarinic, nicotinic and central nervous system. So far, we have witnessed the "show" of the Novichoks' enormous toxic potential three times. The first two use cases of these nerve agents occurred in 2018 in Salisbury and Amesbury (UK). The third example of using the Novichok compound was the poisoning of Alexei Navalny on a domestic flight in Russia. This case is the only published clinical study on the treatment of Novichok poisoning, which has proven the effectiveness of butyrylcholinesterase therapy and demonstrated the lack of reactivation effectiveness after the administration of obidoxime.

Due to its toxicity, working with Novichoks requires extreme precautions. Moreover, experimental studies should be preceded by the estimation of toxicological parameters. Moreover, after updating the CWA (Chemical Warfare Agent) list, more than 10,000 structures can be candidates for the Novichok agent. Therefore, synthesising and estimating target parameters for each structure is impossible. Given the global threat A-series compounds poses, many unknowns and data gaps must be addressed.

Hence the inspiration for research using *in silico* toxicological methods in the form of prediction of selected Novichok parameters.

Novichok properties such as toxicity and environmental fate were determined using an *in silico*, quantitative structure-activity relationship (QSAR) approach. The QSAR Toolbox application package, Estimation Program Interface Suite and Toxicity Estimation Software Tool were used to estimate the median lethal dose (LD_{50}) parameters, biodegradation, hydrolysis half-life and hydrolysis rate constant.

So far, the literature has claimed that organophosphorus compounds from the Novichok group exceed their toxicity in the known conventional compounds of the -V or -G series. Compound A-230 was purported to be 5-8 times more toxic than VX, while A-232 was supposed to be 10 times more toxic than Soman (GD). The predictions contradicted these reports, the most dangerous of the tested Novichoks A-232 (LD_{50} = 0.41 mg/kg bw) was estimated as four times less toxic than the compound VX (LD_{50} = 0.1 mg/kg bw) and seven times weaker than GD (LD_{50} = 0.06 mg/kg bw). The estimated half-life and the constant rate of hydrolysis indicate that the Novichoks used can persist in the external environment, depending on the organophosphorus compound used, from several hours (A-230, A-242 and Iranian "Novichok"), for more than a month (A- 232, A-234, A-262 and C01A043-A045), even up to several years (C01A035-A042). In addition, using in silico models to predict rapid biodegradation showed that Novichoks are difficult to degrade aerobically and anaerobically. No resistant compounds were obtained, and the time frames for this process ranged from weeks (A-230, A-242, C01-A037 and C01-A041) to months.

To summarise, the prediction of parameters of organophosphorus compounds is a complex process and requires the use of various *in silico* approaches. With gratitude to *in silico* toxicological studies, a comprehensive insight into the nature of the Novichoks has been obtained in a health-safe manner. Although much information about the A-series compounds is still shrouded in mystery, the successful prediction of the toxic and environmental properties of Novichoks (n = 17) supplemented some of the essential data missing in the scientific literature.

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Promotor pomocniczy:	Dr n. med. Agata Michalska

OŚWIADCZENIE:

W związku z przygotowaniem przeze mnie rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów, na co zezwala **Ustawa z dnia 20 lipca 2018r. – Prawo o szkolnictwie wyższym** (Dz. U. 2022 poz. 574 z późn. zm.) oraz **Regulamin przeprowadzania czynności w postępowaniach w sprawie nadania stopnia doktora lub stopnia doktora habilitowanego prowadzonych na Uniwersytecie Rzeszowskim** przyjęty przez Senat Uniwersytetu Rzeszowskiego Uchwałą Nr 145/03/2022 z dnia 31 marca 2022r. przedstawiam pisemne oświadczenie wszystkich współautorów pracy stanowiące zgodę na wykorzystanie danych publikacji do potrzeb rozprawy doktorskiej.

M. Noga, A. Michalska, K. Jurowski, The prediction of hydrolysis and biodegradation of Novichoks using in silico toxicology methods, Science of the Total Environment (2023). https://doi.org/10.1016/j.scitotenv.2023.164241; (200 pkt MEiN, IF 10.754).

Jako współautor akceptuje wykorzystanie danych publikacji do potrzeb rozprawy doktorskiej Pana mgr. inż. Macieja Nogi.

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