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Rapid Report

Effects of Charged Oxime Reactivators on the HK-2 Cell Line in Renal Toxicity Screening

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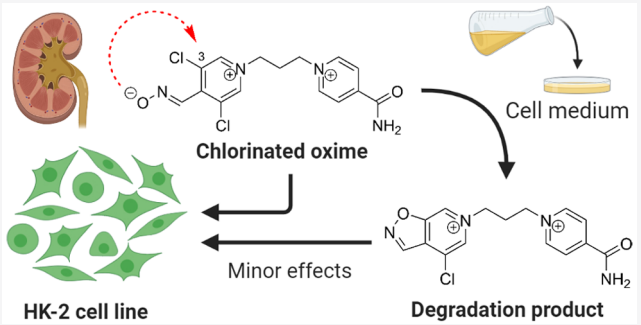
ABSTRACT: Oxime cholinesterase reactivators (oximes) are used to counteract organophosphate intoxication. Charged oximes are administered via intramuscular or intravenous injection when the majority of dose is unmetabolized and is excreted as urine. In this study, the effects of selected double charged oximes were determined in the HK-2 cell line as a model for renal toxicity screening. Some effects on dehydrogenase activity were found for obidoxime, asoxime (syn. HI-6), K027, and K203. The effects of K868 and K869 were found to be unreliable due to rapid degradation of both chlorinated oximes in the assay medium, resulting for K868 in an isoxazole−pyridinium product.



Organophosphates (OPs) have been used worldwide as insecticides (e.g., chlorpyriphos) within agricultural production or misused as nerve agents (e.g., sarin, soman, VX, or tabun). Organophosphates bind to acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and other proteins in an organism. The acute effect is mediated via irreversible binding on AChE, which cannot decompose neuromediator acetylcholine. Acetylcholine is cumulated in synapses and further causes nicotinic, muscarinic, or central symptoms leading to severe intoxication or death of the organism.[1]

Antidotes of OP compounds are used to counteract life-endangering intoxications. The antidote is usually composed of parasympatholytic agents (e.g., atropine), cholinesterase reactivators (e.g., pralidoxime, methoxime, trimedoxime, obidoxime, or asoxime; **1**−**5**, Figure 1), and anticonvulsant agents (e.g., diazepam). The causal treatment is mediated by cholinesterase reactivator (so-called oxime), which cleaves the OP moiety from the AChE active site and renews its function.[2]

The available oximes are highly polar organic molecules that are administered by i.m. injection in high doses (hundreds of milligrams).[3] They are rapidly distributed to the body (with very limited CNS penetrability) and delivered to the phosphylated AChE. Once they cleave the OP moiety, phosphylated oxime is formed and is further decomposed with a half-life of seconds to hours.[4] The phosphylated oxime gives rise to the corresponding organophosphoric or organophosphonic acid and highly polar nitrile, which are rapidly excreted. However, the majority of oxime dose remains unchanged (unmetabolized) and is excreted as urine with a half-life of ~1−2 h.[5] From this point of view, the kidneys might be the primary organ of toxicity because they are responsible for elimination of >50−80% of the oxime dose from

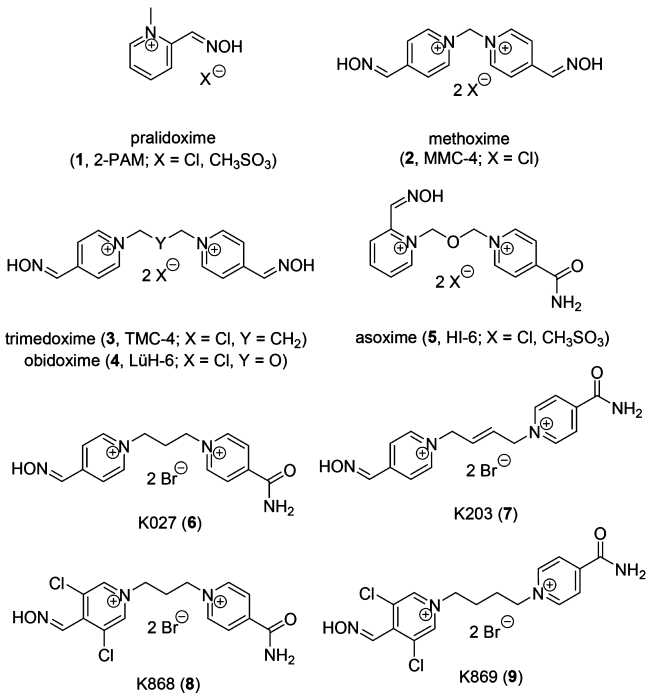


Figure 1. Standard oxime reactivators (**1**−**5**), standard K-oximes (**6** and **7**), and chlorinated K-oximes (**8** and **9**).

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intoxicated person and >60−80% of the oxime dose for healthy volunteers.[6−8]

The K-oximes have been developed in the past two decades. Some of them (e.g., K027 (**6**) and K203 (**7**)) were highlighted for very promising reactivation ability against various OPs both in vitro and in vivo (Figure 1).[9,10] More recently, the chlorinated oximes K868 (**8**) and K869 (**9**) were found to be excellent reactivators of multiple OPs on human AChE and BChE in vitro.[11,12] They are highly polar organic compounds that are supposed to behave in organisms similarly to standard oximes, and thus they might have a toxic effect in kidneys.

For this reason, we have used a screening in vitro method for evaluation of their toxic effects on a model of the human kidney cell line. Although other cell lines were formerly used in the estimation of cellular effects of oximes (e.g., hepatic HepG2 cells,[13,14] renal HEK293T cells,[15] and neuronal SH-SY5Y cells[16,17]), we chose a human proximal tubular HK-2 (human hidney-2) immortalized cell line, which originates from proximal tubular cells by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes.[18] HK-2 cells grow in a monolayer, and they are suitable for studying proximal tubular toxicity.[19] The main advantage of HK-2 cells consists of preservation of the basic morphological and functional properties of proximal tubular epithelial cells, and thus they were used in numerous studies focused on estimation of the proximal tubular injury.[18,20−23]

In this work, the biological effects of obidoxime (**4**), asoxime (**5**), K027 (**6**), K203 (**7**), K868 (**8**), and K869 (**9**) were studied in HK-2 cells. The effects were studied after a relatively short incubation time (1 and 6 h) based on the half-life of oximes in vivo (~1.5−2 h).[24,27] The cells were incubated with the oximes at four different concentrations (0.1−100 μM). Three different biochemical tests were used, including evaluation of dehydrogenase activity (WST-1, water-soluble tetrazolium salt 1), measurement of mitochondrial membrane potential (JC-1 dye), and glutathione assay (monochlorobimane, MCB; Figure 2 and Table S1).[22−24]

The results showed that the dehydrogenase activity in HK-2 cells (Figure 2A) decreased independently with time. After 1 h of treatment, the dehydrogenase activity measured using the WST-1 test was decreased significantly in all tested concentrations of **4**, **5**, **6**, and **7** but not with a similar extent for **8** and **9** compared to that of control, which were found to induce an increase in activity at 100 μM. On the other hand, tested compounds at all concentrations did not induce a decrease or increase of dehydrogenases activity greater than 25%. Interestingly, no correlation was found between increasing concentration of oxime and decreasing dehydrogenase activity for **4**−**7**. This finding cannot be fully compared to former studies that observed no cytotoxicity via determination of cell viability in various cell lines using a MTS assay.[13,25] In addition, this finding cannot be explained by the background absorbance because it was lower than 5% of the control for all tested oximes. Thus, such an effect may be attributed to the noncompetitive inhibition of dehydrogenases by oximes, but further elucidation of this phenomenon is needed. The dehydrogenases activity after 6 h was found to be significantly decreased in comparison with that of nontreated cells in the case of **4**, **5**, **6**, and **7**, but the activity was not significantly decreased compared to that with 1 h treatment. Only mild reduction of the dehydrogenase activity at 6 h was found in **8** and **9** treated cells when compared to that with 1 h treatment.

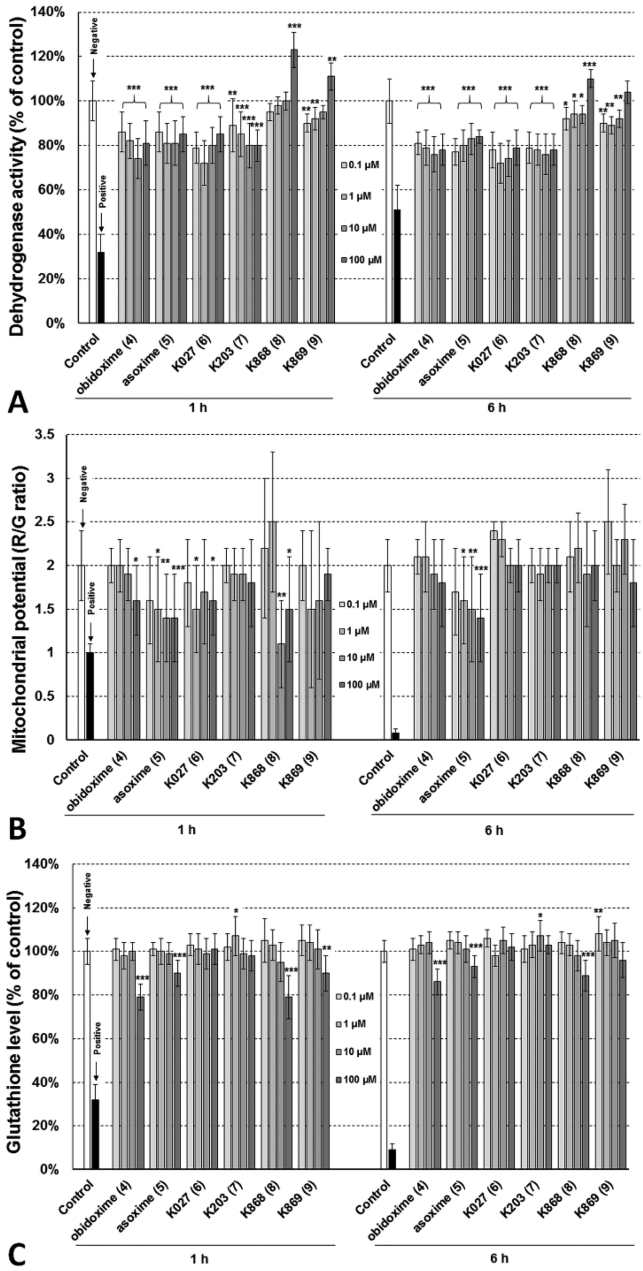

Figure 2. Results of dehydrogenase activity (WST-1 test; A), mitochondrial membrane potential (JC-1 probe; B), and glutathione assay (MCB assay; C) in HK-2 cells. Untreated cells were used as a negative control (white columns), and FCCP (40 μM) was used as a positive control (black columns) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, vs negative control).

As the WST-1 test detects total intracellular and especially mitochondrial dehydrogenase activity, changes in mitochondrial membrane potential (MMP) using the JC-1 probe were further screened (Figure 2B). After 1 h of treatment, MMP was decreased significantly in cells treated with 100 μM doses of **4**, **5**, **6**, and **8** compared to that with the control. In addition, other concentrations of **5** induced the reduction of MMP, as well. After 6 h, the sustained significant reduction of MMP was found in cells treated with **5** in 1, 10, and 100 μM. The other compounds did not induce a significant decrease of MMP in HK-2 cells.

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When glutathione levels in treated and nontreated cells were compared (Figure 2C), a significant glutathione depletion was found in 100 μM **4**, **5**, **8**, and **9** treated cells. The significant glutathione reduction remained after 6 h with exception of **6**, **7**, and **9**, which did not decrease the glutathione levels.

The significantly different results of **8** and **9** in the WST-1 assay and **5** in the JC-1 assay led us to confirm their stability. The asoxime (**5**) and chlorinated oximes (**8** and **9**) have decreased pK_{a1} (~7.4−7.5); they rapidly form a reactive oximate anion, and as such, they might be degraded faster in the assay environment.[11] Thus, the stability of all compounds was tested in cell culture medium at 37 °C for 24 h (Figure 3 and Table S2) and

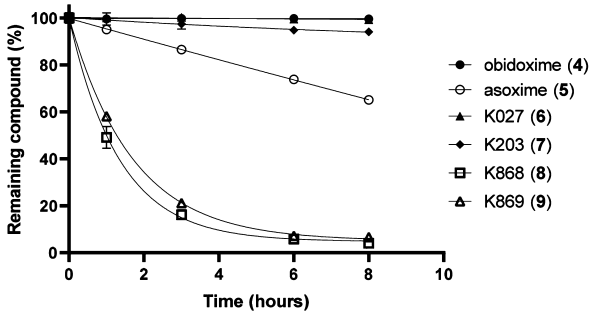

Figure 3. Stability of tested oximes in the cell culture medium at 37 °C.

analyzed via UHPLC. Apparently, **4**, **6**, and **7** were found to be stable in the assay medium. On the other hand, **5**, **8**, and **9** were degraded when asoxime (**5**) content was decreased to ~95% in 1 h and to ~70% in 6 h. The chlorinated oximes (**8** and **9**) were degraded even more rapidly to ~50−60% in 1 h and to ~5−10% of the initial levels after 6 h. Interestingly, **5** and **8** were also evaluated in un-ionized water at 37 °C, where they remained stable for 8 h (Figure S1).

With data from biochemical assays and compound stability, the results determined for **4**, **6**, and **7** were found to be reliable. For the HK-2 cell line, the significant decrease of dehydrogenase activity was proven for all of them but maximally to ~70−80% vs untreated controls independent of time. Mitochondrial potential was significantly decreased for the highest concentrations (i.e., 100 μM) of **4** and **6** but only to ~75−80% vs control in 1 h. The glutathione levels were significantly decreased for the highest concentrations (i.e., 100 μM) of **4** but only to ~80% vs control independent of time. Further, the elimination half-life of **4**, **6**, and **7** was found to be ~1.5−2 h for i.v. administration in humans or i.m. administration in pigs, where the maximal plasma concentration used for human administration was ~20 μM.[26,27]

Further, **5** was found to be unstable under assay conditions, and thus its results can be considered as partially unreliable. At 1 h, its level was almost unchanged and affected all screened parameters. However, the induced and significant decrease of parameters ranged in ~70−90% vs control. At 6 h, its level was reduced to ~70% of the original level, but no significant changes in assay results (e.g., increase of determined parameters) were found when compared to that with the 1 h interval. In addition, the elimination half-life of **5** was found to be ~1.5−2 h for i.m. administration to humans and i.v. or i.m. administration to pigs, where the maximal plasma concentration used for human administration was ~40 μM.[5,28,29] From this point of view, all observed changes mediated by obidoxime (**4**), asoxime (**5**), K027 (**6**), and K203 (**7**) in HK-2 cells as the model for renal toxicity screening have to be taken into account. However, the decrease of dehydrogenase activity does not necessarily mean cell death. The cell viability was preserved as it was documented by the results in JC-1 and MCB assays for **4** (except its highest concentration), **5** (except its highest concentration), **6**, and **7**.

8 and **9** were rapidly degraded in the cell culture medium, and thus their results with HK-2 cells were considered to be unreliable. After 1 h of treatment, the outcomes of toxicity evaluation can be attributed to ~50−60% of the parent compounds, and after 6 h, the results of experiments were not related to the parent compounds (presented at levels <10%), but they were caused by the degradation products. For these reasons, the UHPLC-DAD-MS analysis of degradation products was made for **8** (m/z 353.2) as the exemplar chlorinated oxime after 0, 1, 3, 6, 8, and 24 h. Two degradation products (m/z 326.1 and m/z 317.2) were identified (Figure 4).

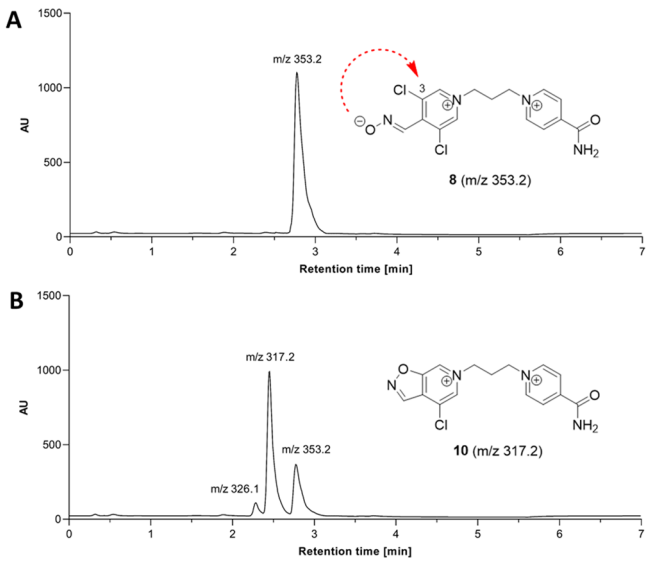

Figure 4. UHPLC analysis of K868 (**8**, m/z 353.2) in 0 h (A) and degradation products in 3 h (B) when the main degradation product **10** (m/z 317.2) was identified.

As the formation of these degradation products took place in a ratio of 9:1 in favor of compound m/z 317.2, further UHPLC-MS/MS (Figure S2), 1D, and 2D NMR analyses (^{1}H, ^{13}C, H,H−COSY, HSQC, and HMBC; Figures S3 and S4) were focused on the identification of the major product **10** (Figure 4B). In the case of **8**, the lower pK_a (~7.5)[11] resulted in an increased conversion of oxime into an oximate anion supported by two chlorine electron-withdrawing moieties. Further, a nucleophilic attack of oximate on the C3 atom of the pyridinium ring took place when the formation of isoxazole−pyridinium degradation product **10** was finished.

Taken together with results of cytotoxicity for **8** and **9**, these results are mostly related to the degradation products of both oximes (e.g., **10**) in the medium, and they cannot be attributed to the parent oximes. However, oximes **8** and **9** or their degradation products had low or minimal effects on dehydrogenase activity, MMP, or glutathione levels in concentrations attainable in vivo after 1 or 6 h (Figure 2). From this point of view, oximes **8** and **9** or their degradation products seem to have limited effects in the studied cell line. However, oximes **8** and **9** were formerly found to be highly potent AChE and BChE reactivators under in vitro/ex vivo conditions,[11,12] which raises the question of whether their

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decreased stability could affect their reactivation ability in vivo. As their reactivation ability in human whole blood was found to be greater than ~80% for OP-inhibited AChE in 10 min[11] when the majority of oxime dose should not be degraded, we can hypothesize that some reactivation ability could be also observed under in vivo conditions. However, such a hypothesis has to be confirmed, and oximes **8** and **9** should be studied in more detail under in vivo conditions, with particular attention to their stability and metabolites.

To conclude, the effects of obidoxime, asoxime, K027, K203, and two chlorinated oximes were studied in the renal HK-2 cell line. Their effects on dehydrogenase activity, MMP, or glutathione levels in cells were determined with interestingly different results of chlorinated oximes **8** and **9**. For this reason, stability of all oximes was obtained and both chlorinated oximes were found to be rapidly degraded in the assay medium and in contrast with the use of un-ionized water. According to LC-MS and NMR analyses, the major degradation product of oxime K868 (**8**) was identified to be an isoxazole−pyridinium compound without an oxime moiety as a result of the increased oximate nucleophilic activity. These findings on cytotoxicity and degradation of oxime reactivators or chlorinated oximes should also be carefully controlled during further in vitro or in vivo studies. In addition, it is important to evaluate such oximes in a more complex environment of living organisms to ensure their properties, stability, and safety.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00489.

Methods and additional figures/tables (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; HK-2, Human Kidney-2 cell line; i.m., intramuscular; i.v., intravenous; JC-1, JC-1 dye; MCB, monochlorobimane; MMP, mitochondrial membrane potential; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NMR, nuclear magnetic resonance; NMR COSY, NMR $^{1}H-^{1}H$ correlation spectroscopy; NMR HSQC, NMR heteronuclear single quantum coherence spectroscopy; NMR HMBC, NMR heteronuclear multiple bond correlation; OP, organophosphate; UHPLC, ultrahigh performance liquid chromatography; WST-1, water-soluble tetrazolium salt 1

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