Original article

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Cytotoxicity-related effects of imidazolium and chlorinated bispyridinium oximes in SH-SY5Y cells

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Current research has shown that several imidazolium and chlorinated bispyridinium oximes are cytotoxic and activate different mechanisms or types of cell death. To investigate this further, we analysed interactions between these oximes and acetylcholine receptors (AChRs) and how they affect several signalling pathways to find a relation between the observed toxicities and their effects on these specific targets. Chlorinated bispyridinium oximes caused time-dependent cytotoxicity by inhibiting the phosphorylation of STAT3 and AMPK without decreasing ATP and activated ERK1/2 and p38 MAPK signal cascades. Imidazolium oximes induced a time-independent and significant decrease in ATP and inhibition of the ERK1/2 signalling pathway along with phosphorylation of p38 MAPK, AMPK, and ACC. These pathways are usually triggered by a change in cellular energy status or by external signals, which suggests that oximes interact with some membrane receptors. Interestingly, *in silico* analysis also indicated that the highest probability of interaction for all of our oximes is with the family of G-coupled membrane receptors (GPCR). Furthermore, our experimental results showed that the tested oximes acted as acetylcholine antagonists for membrane AChRs. Even though oxime interactions with membrane receptors need further research and clarification, our findings suggest that these oximes make promising candidates for the development of specific therapies not only in the field of cholinesterase research but in other fields too, such as anticancer therapy via altering the Ca^{2+} flux involved in cancer progression.

KEY WORDS: antidotes; calcium signalling; cell viability; Fura-2 AM; receptor; kinase

Oxime antidotes are well-known molecules, whose main task is to reactivate synaptic acetylcholinesterase (AChE, EC 3.1.1.7) activity after covalent inhibition by lethal organophosphorus (OP) nerve agents (1). Namely, their basic property is the strong nucleophilicity of the oxime group (–N=OH), which displaces the OP moiety from the active AChE site and recovers its catalytic activity (2). By combining specific structural features required for binding to the active site of AChE and different structural motifs, researchers have tested a great number of new oxime structures for their reactivation potency to find more efficient reactivators than currently used in medical practice (3–10). When such carefully designed oximes fail to achieve the expected efficiency in *in vitro* AChE reactivation experiments, they are usually discarded from further evaluation as "research waste" instead of being repurposed for other plausible biological niches. In these niches they can act as antimicrobial (11), surfactant (12), antioxidant (13), anticancer, and anti-inflammatory agents or inhibitors for many different regulatory kinases (14). In our previous research (15), we identified possible interactions between oximes and several targets such as membrane components, receptors, and/or enzymes other than AChE. Therefore, the main aim of this study was to focus our investigation on imidazolium and chlorinated bispyridinium oximes to see how they modulate intracellular signalling in order to predict new specific targets of their action and potential health benefits.

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MATERIALS AND METHODS

Oximes and human cells

Figure 1 shows the structures of the tested chlorinated bispyridinium oximes K867 and K870 and imidazolium oximes VII and X, prepared as described elsewhere (15–17) and donated to us by Dr Kamil Musilek (University of Hradec Králové Faculty of Science, Department of Chemistry, Hradec Králové, Czech

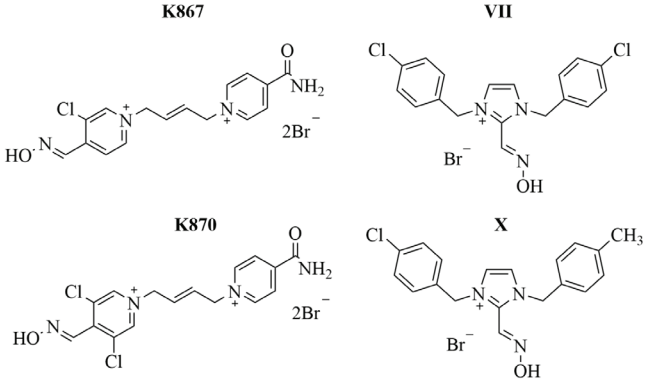

Figure 1 Structures of oximes tested in this study

Republic) and Dr Ines Primožič (University of Zagreb Faculty of Science, Department of Chemistry, Zagreb, Croatia).

The human neuroblastoma SH-SY5Y (ECACC 94030304) cell line was purchased from Sigma-Aldrich (Steinheim, Germany), the certified distributor for European Collection of Authenticated Cell Cultures (ECACC), and the cells were grown in Dulbecco's Modified Eagle Medium (DMEM/F-12, Sigma-Aldrich) supplemented with 15 % (v/v) foetal bovine serum (Sigma-Aldrich), 2 mmol/L glutamine, and 1 % (v/v) non-essential amino acids (NEAA, Sigma-Aldrich) at 37 °C in a 5 % CO_2 atmosphere.

Energy status determination

Cell viability was determined based on quantification of adenosine triphosphate (ATP) using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA), as the quantity of ATP is directly proportional to the number of metabolically active cells. 20,000 cells per well were seeded in 96-well opaque plates and exposed for 4 h to oximes in concentrations causing 20–25 % inhibition (~$IC_{20–25}$) as determined in our previous study (15). These were the following: 400 μmol/L for K867, 15 μmol/L for K870, 250 μmol/L for VII, and 200 μmol/L for X. After the incubation with the oximes, we measured cell luminescence on a VICTOR^3 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). Data were taken from at least two independent experiments (each treatment performed in duplicate) and plotted as a percentage of ATP determined in control, untreated cells.

Western blot analysis

SH-SY5Y cell preparation and the procedure followed a protocol described elsewhere in detail (18, 19). Briefly, the cells were seeded at a density of 100,000 cells per well in a complete medium with all supplements added. The following day, the medium was replaced with DMEM/F-12 without supplements. The cells were serum-starved for 16 h. During the last 5, 15, 30, 45, and 60 min, the cells were treated with the oximes in concentrations mentioned above. At the end of the experiment, cells were washed with ice-cold phosphate-buffered saline (PBS) to remove oximes and lysed in the Laemmli buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2 % (w/v) sodium dodecyl sulphate (SDS), 10 % (w/v) glycerol, 5 % (v/v) 2-mercaptoethanol, and 0.002 % (w/v) bromophenol blue. Proteins were separated with sodium dodecyl sulphate and polyacrylamide gel (4–12 %) electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to the polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany) by wet electrotransfer. After blocking, membranes were incubated overnight with a primary antibody (Table 1) diluted in a buffer containing 20 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, 0.1 % (w/v) bovine serum albumin, and 0.1% (w/v) sodium azide at 4 °C and then with the secondary antibody-horseradish peroxidase conjugate in Tris-buffered saline with 0.1 % Tween 20 (TBST) and 5 % (w/v) dry milk at room temperature for 1 h. Membranes were incubated with an enhanced chemiluminescence (ECL) reagent (Bio-Rad Laboratories) and immunolabelled proteins (chemiluminiscent signal) visualised with a Fusion FX System (Vilber, Marne-la-Vallée, France). Densitometry was done with the Quantity One 1-D Analysis Software (Bio-Rad Laboratories). The intensities of individual bands are expressed in arbitrary units (AU) relative to the total intensity of all the bands. The Ponceau staining was used as protein loading/concentration control as described elsewhere (20).

iCa^{2+} signalling

Intracellular calcium (iCa^{2+}) mobilised by the activation of G-protein coupled receptors (GPCR) or calcium channels was measured using the acetoxy-methyl-ester Fura-2 (Fura-2 AM) dye (Sigma-Aldrich). 30,000 cells per well were seeded in 96-well opaque plates, added the Fura-2 AM dye loading solution (100 μL/well), and incubated at room temperature for 1 h for the dye to penetrate the cells and get activated by cellular esterases. After washing cells twice with HEPES-buffered saline (HBS) containing 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose (pH 7.4), cells were exposed to ~$IC_{20–25}$ concentrations of oximes reported in our previous study (15). Each well was monitored for baseline fluorescence, and, 25 s later, added 5 μmol/L of acetylcholine (ACh) (Sigma-Aldrich) using the automated pipetting system of an InfiniteM200PRO plate reader (Tecan Austria GmbH, Salzburg, Austria). Fluorescence was read at Ex/Em 340/510 nm for calcium-bound and at Ex/Em 380/510 nm for unbound Fura-2 dye, and the data are presented as ratiometric levels of the dye (340/380 nm) (21) indicating cytosolic calcium levels.

Table 1 Primary antibodies (Cell Signaling Technology Inc, USA) used for Western blotting

Antibody	Cat. No.	Host	Type	Dilution
Anti p-ERK (Thr202/Tyr204)	#4370	Rabbit	Monoclonal	1:2000
Anti pNFκB p65 (Ser536)	#3033	Rabbit	Monoclonal	1:2000
Anti p-p38 MAPK (Thr180/Tyr182)	#4511	Rabbit	Monoclonal	1:1000
Anti p-STAT3 (Tyr705)	#9145	Rabbit	Monoclonal	1:1000
Anti p-ACC (Ser79)	#3661	Rabbit	Polyclonal	1:1000
Anti p-AMPKα (Thr172)	#2535	Rabbit	Monoclonal	1:1000

In silico target prediction

For target prediction *in silico* we used the online SwissTargetPredicition web tool (Swiss Institute of Bioinformatics, Lausanne, Switzerland), which can predict the most probable target proteins of small bioactive molecules from a library of 370,000 known active molecules and more than 3,000 proteins from three species (22, 23).

Statistics

If not stated otherwise, results are presented as means ± standard error. Differences between the groups were analysed using one-way ANOVA followed by Dunnett's test. Statistical analyses were run on the GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance is set as follows: &p≤0.05; #p≤0.01; $p≤0.001; and *p≤0.0001.

RESULTS

Cell energy status and effect of oximes on intracellular signalling cascades

Unlike K867 and K870, oximes VII and X significantly decreased ATP (Figure 2).

Figure 3 shows time-dependent effects of the four oximes on important signalling kinases, transcription factors, and nuclear factors or enzymes regulating cell metabolism, growth, proliferation, and survival. The phosphorylation of extracellular signal-regulated kinase (ERK1/2), an important mitogen-activated protein kinase (p44/42 MAPK), increased with the chlorinated bispyridinium oximes K867 and K870 and decreased with the imidazolium oximes VII and X. The nuclear factor-ϰB (NF-ϰB), a transcription factor involved in the immune and inflammatory responses, showed no change in phosphorylation. K870 and X increased phosphorylation of p38 MAPK. Chlorinated bispyridinium oximes suppressed phosphorylation of the signal transducer and activator of transcription (STAT3), while imidazolium oximes had no effect. The oxime VII also did not change the phosphorylation of cellular energy sensor AMP-activated protein kinase (AMPK) or its downstream substrate acetyl-CoA carboxylase (ACC). In contrast, K867 markedly decreased the phosphorylation of AMPK and increased the phosphorylation of ACC. The oxime X increased the phosphorylation of both AMPK and ACC, which points to the activation of the AMPK-signalling pathway.

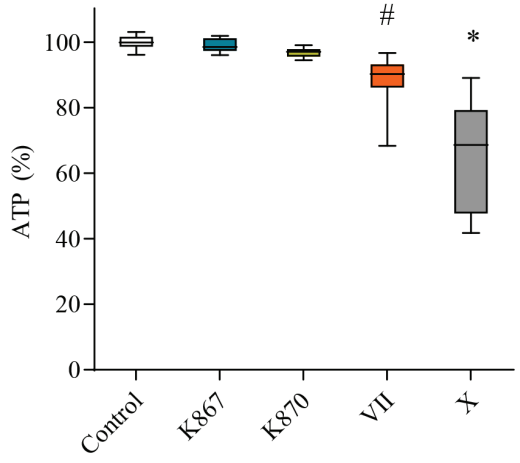

Figure 2 ATP in SH-SY5Y cells after 4 h exposure to selected oximes expressed as the percentage of control (untreated cells). Oxime concentrations: K867 (400 μM), K870 (15 μM), VII (250 μM) and X (200 μM). #p≤0.01; *p≤0.0001

New predicted targets

In order to find whether there is a predicted target class for our tested oximes in the existing database, we ran them through the SwissTargetPrediction web tool (23) based on structural similarities with molecules of known activities. Results presented in Figure 4 indicate high probability of interaction between our oximes and the members of the G-coupled membrane receptors (GPCR) family. Some oximes are also likely to interact with several other targets, such as specific enzymes, nuclear receptors, or proteases.

iCa^{2+} signalling

Figure 5 shows the potential of our oximes to interact with acetylcholine receptors (AChRs). Compared to control, all but K870 nearly completely suppressed the activity of ACh and the increase in iCa^{2+}caused by the activation of muscarinic G-protein-coupled receptors and nicotinic ionotropic channels.

DISCUSSION

In this study, we wanted to look deeper into the phenomenon of previously detected cellular toxicity of chlorinated bispyridinium and imidazolium oximes (15) by following the ATP status of cells, activation of specific signalling cascades, and potential interactions of oximes with AChR. As expected, ATP levels decreased with imidazolium oxime treatment, which confirms that VII and X lower the number of metabolically active cells and mitochondrial dehydrogenase activity and impair cellular energy and production of NADH or NADPH. Namely, dehydrogenase activity and the level of NADH are essential for oxidative phosphorylation and the synthesis of ATP in the cells (24). In that sense, the level of ATP can inform us whether a cell will go into regulated and energy consuming (apoptosis) or unregulated (necrosis) death (15, 25). Since the ATP status of cells treated with chlorinated bispyridinium oximes K867 and K870 did not change in the tested time frame, this could mean that the exposed cells are programmed to undergo apoptosis in such circumstances. Moreover, as STAT3 phosphorylation decreased after exposure to these bispyridinium

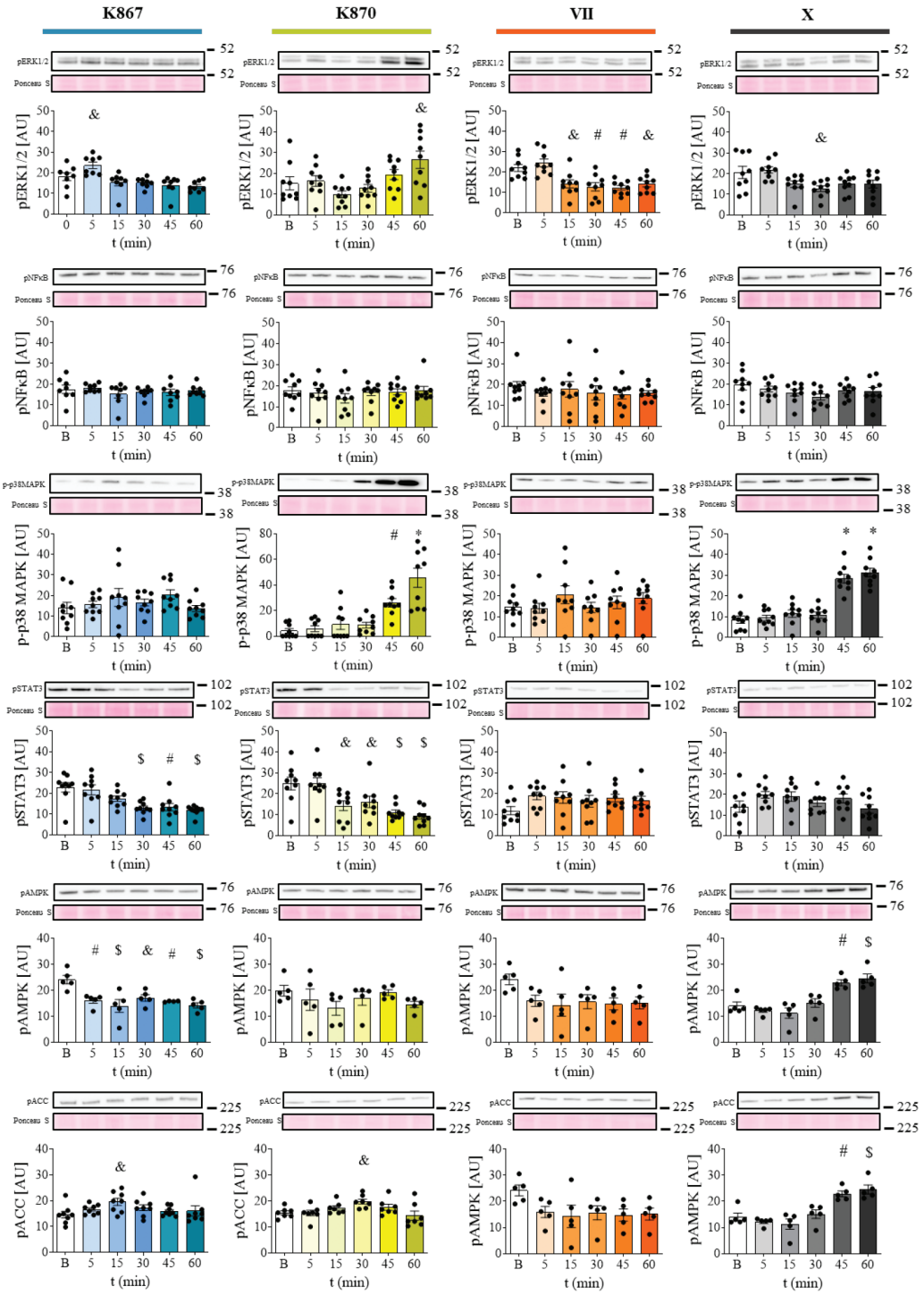

Figure 3 Time-dependent effects of the selected oximes K867 (400 μmol/L), K870 (15 μmol/L), VII (250 μmol/L), and X (200 μmol/L) on intracellular signalling in serum-starved SH-SY5Y cells over 5–60 min. Position towards bends and molecular weight markers in kDa are indicated on the right side of the blots and Ponceau S. Results are presented as means ± SD (n=9) &p≤0.05; #p≤0.01; $p≤0.001; *p≤0.0001. B – baseline; pERK1/2 – phospho-ERK1/2 (Thr202/Tyr204); pNFkB – phospho-NFκbeta p65 (Ser536); p-p38MAPK – phospho-p38MAPK (Thr180/Tyr182); p-STAT3 – phospho-STAT3 (Tyr705); p-ACC – phospho-ACC (Ser79); p-AMPKα – phospho-AMPKalpha (Thr172); t – time

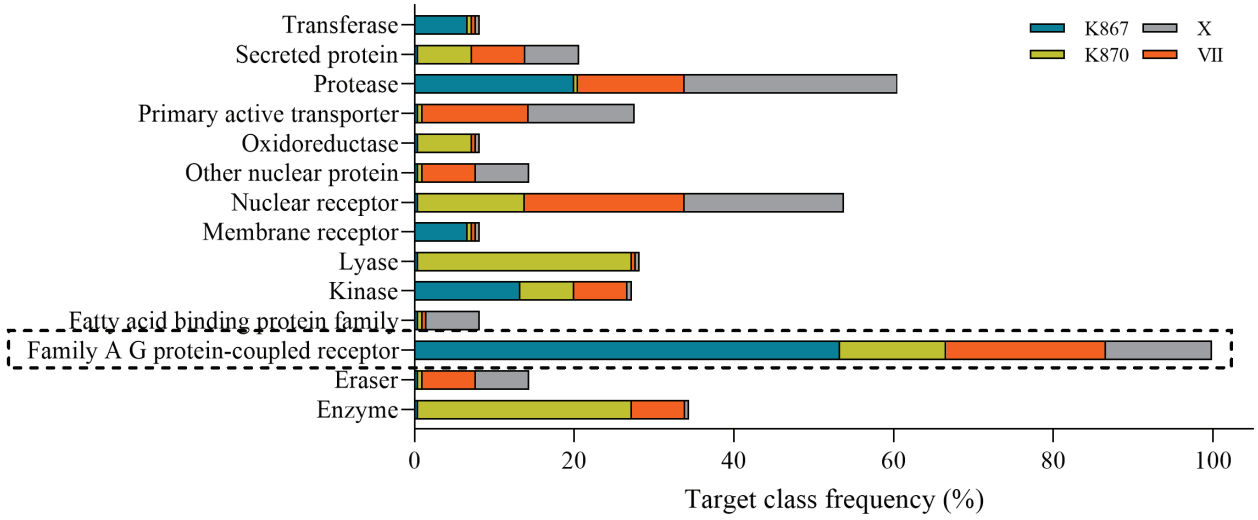

Figure 4 Target class frequencies of oximes K867, K870, VII, and X predicted by the SwissTargetPrediction engine. The dashed frame highlights the family of G-protein-coupled receptors as the most probable target of interaction

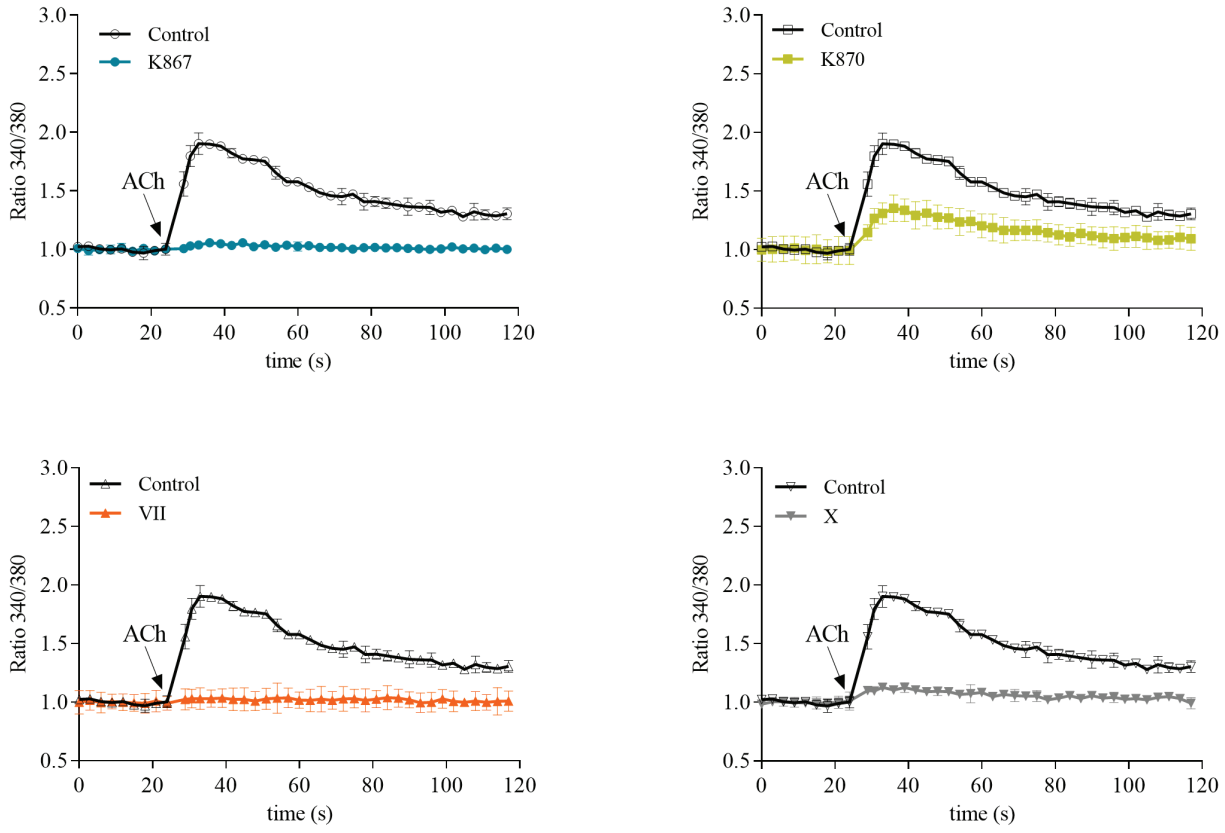

Figure 5 iCa^{2+} levels in SH-SY5Y cells before and after the addition of ACh (5 μmol/L) in the presence of oximes K867 (400 μmol/L), K870 (25 μmol/L), VII (250 μmol/L), and X (200 μmol/L) compared to control (not treated with the oximes)

oximes, it is reasonable to assume that genes inhibiting apoptosis were not expressed (26). All this confirms our earlier study findings (15), in which chlorinated bispyridinium oximes activated caspase 9 mitochondria-mediated intrinsic apoptosis (15).

Furthermore, for oximes VII and X the reduced ATP status and disrupted mitochondrial membrane potential reported in our previous study (15) may directly be owed to suppressed phosphorylation of ERK1/2. These imidazolium oximes may also have triggered mechanisms activating the AMPK-ACC cascade enzymes, whose phosphorylation may lead to increased oxidation of fatty acids important for cardiac metabolism (29). Increased phosphorylation of AMPK and ACC was the most prominent with oxime X, which resulted in the lowest ATP levels. It therefore seems likely that AMPK activation by oxime X is primarily the result of energy stress.

As reported earlier (30), chlorinated bispyridinium and imidazolium oximes – being permanently charged molecules – are unlikely to cross the cell membrane, and their toxicity is more likely related to an interaction with some membrane receptors (30). This makes them promising agents for other interaction research beyond the field of cholinesterases. Our query with the SwissTargetPrediction tool indicated a high probability of interaction with the G-coupled membrane receptors (GPCR). Furthermore, this study shows that our oximes interact with membrane acetylcholine receptors (AChRs), which include muscarinic (GPCR) and nicotinic (ionotorpic) receptors to block the release of iCa^{2+}. This antagonism with AChRs is an interesting finding, as it suggests that they can act as antidotes in cholinergic synapses by minimising the effects of acetylcholine accumulated when AChE is irreversibly inhibited by OP compounds. Furthermore, calcium involvement in cell survival, proliferation, and regulation of intracellular enzymes opens a new research direction for these compounds, since Ca^{2+} flux is important in cancer progression and some anticancer therapies (31).

CONCLUSIONS

We have shown that the selected imidazolium and chlorinated bispyridinium oximes affect the energy status of the cell and trigger several important signalling pathways. Considering that these pathways are triggered by an external signal, our oximes probably bind to receptors. By binding to AChRs, oximes disrupt intracellular calcium accumulation and inhibit or activate signalling pathways, i.e. ERK1/2, p38 MAPK, or AMPK cascades. This finding gives a new research direction for these compounds, since their effect on calcium signalling can be used in cancer therapy research. The next step is to test these oximes on progressive cancer cells and determine their effect on defined cell targets. In addition, our findings are equally important for the understanding of potential antidotal effects of these oximes against OP poisoning.

Acknowledgements

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Učinci imidazolijevih i kloriranih bispiridinijevih oksima povezani s njihovom toksičnosti na stanicama SH-SY5Y

Praćenjem učinka odabranih imidazolijevih i kloriranih bispiridinijevih oksima utvrđeno je da uzrokuju citotoksičnost i aktiviraju različite mehanizme ili tipove stanične smrti. Kako bismo to detaljnije istražili, analizirali smo aktivaciju nekoliko signalnih putova, kao i interakcije acetilkolinskih receptora (AChR) s navedenim oksimima te procijenili može li se opaženi toksični učinak objasniti njihovim utjecajem na ove specifične mete. Rezultati su pokazali da su klorirani bispiridinijevi oksimi prouzročili vremenski-ovisnu citotoksičnost, bez smanjenja razine ATP-a uz aktivaciju ERK1/2 i p38 MAPK-vezanih signalnih kaskada i inhibiciju fosforilacije STAT3 i AMPK proteina. Imidazolijevi oksimi djelovali su vremenski neovisno, uz značajno smanjenje razine ATP-a i inhibiciju ERK1/2 signalnog puta te fosforilaciju p38 MAPK, AMPK i ACC proteina. Navedeni signalni putovi obično se aktiviraju ili promjenom unutarnjega staničnog statusa, osobito energetskoga, ili vanjskim signalima, što upućuje na moguće interakcije oksima s nekim membranskim receptorima. Zanimljivo, *in silico* analizom procijenjeno je da je najvjerojatnija interakcija testiranih oksima s porodicom G-protein-spregnutih membranskih receptora (GPCR). K tomu, eksperimentalno je potvrđeno da testirani oksimi djeluju kao mogući antagonisti acetilkolina za vezanje na membranske AChR, potvrđujući tako i računalnu *in silico* procjenu. Iako interakcije ispitanih oksima s membranskim receptorima treba dodatno potvrditi, takve bi ih interakcije učinile kandidatima za razvoj specifičnih terapija u drugim područjima istraživanja, osim u istraživanjima povezanima s kolinesterazama, npr. kao moguće protutumorske lijekove, putem utjecaja na fluks iona Ca^{2+} uključenoga u progresiju tumora.

KLJUČNE RIJEČI: Fura-2 AM; kinaza; protuotrovi; receptor; signalizacija kalcijem; vijabilnost stanica