Abstract

Various *in vitro* approaches are available to evaluate the toxicity of substances as well as their mechanism of toxicity in the early stages of the process of developing new chemical entities. These systems are mainly used for screening and allow the creation of more complex toxicological profiles. The present work focuses on *in vitro* evaluation of substances modulating acetylcholinesterase activity. The enzyme plays a crucial role in the nervous system and occurs mainly in cholinergic synapses and neuromuscular junctions. The primary biological function of this enzyme is the termination of the nerve impulse at these synapses by the rapid hydrolysis of the neurotransmitter acetylcholine to choline and acetate. Acetylcholinesterase is a target enzyme for many drugs, e.g., for Alzheimer's disease or other neurodegenerative diseases. The most often used are reversible and pseudoreversible inhibitors. It also plays a role in poisoning by chemical warfare agents, namely nerve agents and organophosphorus pesticides.

Acetylcholinesterase reactivators are used as antidotes for these poisons. Reactivators (asoxime, pralidoxime, obidoxime, trimedoxime, methoxime and oximes K027, K048, K074, K075, and K203) and inhibitors (pyridostigmine, galantamine, rivastigmine, donepezil, tacrine, 7-methoxytacrine, and 6-chlorotacrine) of acetylcholinesterase were evaluated using several cell-based *in vitro* assays. Colorimetric and electroimpedance methods determined their cytotoxicity. Furthermore, the induction of apoptosis was monitored by microcapillary flow cytometry. The change in the level of free radicals in the cells as well as the antioxidant potential of the test substances was observed using various fluorescent probes.

The cytotoxicity and the induction of apoptosis of tested compounds were determined in 4 different cell models (HepG2, ACHN, SH-SY5Y, and NHLF) after 24 hours incubation. The least toxic reactivators were oximes K027 (least toxic to the HepG2 and SH-SY5Y cell lines) and K048 (least toxic to ACHN and NHLF cells). The cytotoxicity of other substances further increased. According to our results, certain structural aspects affect the cytotoxicity of substances, including the length and nature of the linker chain between pyridinium rings, the number of oxime groups, and also their position on the pyridinium ring. The least toxic inhibitor for all cell lines was pyridostigmine. Because this group was structurally diverse, it was not possible to evaluate the relationship between cytotoxicity and the structure of the compounds. The induction of apoptosis or necrosis after treatment of cells with reactivators or inhibitors varied between cell lines, which could be attributed to the different metabolic capacities of used cell models.

Disruption of the redox equilibrium was monitored using a non-specific fluorescent probe of 2,7-dichlorodihydrofluorescein diacetate at time intervals of 4 and 24 hours together with cell viability. The results showed that reactivators could be divided into three different groups. These include a group with strong oxidative stress response (K027, K048, K074, trimedoxime, obidoxime), a group in which oxidative stress could play a partial role (K203 and K075), and finally, a group of substances with weak oxidative stress induction (pralidoxime, methoxime, asoxime). For a better comparison of the ability of reactivators to disrupt the redox equilibrium of cells, we further tested oxidative stress status at concentrations that corresponded to IC50 values. The production of free radicals was monitored by three different fluorescent probes at time intervals of 1, 4, and 24 hours. Obidoxime appeared to be the strongest inductor of oxidative and nitrosative stress. We did

not observe any dysregulation of the redox equilibrium after acetylcholinesterase inhibitors treatment.

Antioxidant capacity was determined using a 2,7-dichlorodihydrofluorescein diacetate fluorescence probe. After induction of oxidative stress by *tert*-butyl hydroperoxide, we observed an antioxidant effect in cells treated with oximes K075, K203 and pralidoxime. Both K075 and K203 contain double bond in their molecule, while pralidoxime represents monopyridinium oximes. Inhibitors with antioxidant properties include donepezil, rivastigmine, and galantamine.