Application of BY-2 cell model in evaluating an effect of newly prepared potential calcium channel blockers

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Abstract: Calcium channel blockers represent a group of therapeutically important compounds that have found an application in treatment of systemic vascular resistance and arterial pressure, eventually angina pectoris. We studied possibility of application of a BY-2 cell model to evaluate the potential of newly prepared potential calcium channel blockers. In the preliminary experiment, toxicity of studied compounds was determined. In the next experiment, we evaluated possible protective effect of studied compounds on programmed cell death induced by hydrogen peroxide on the BY-2 cells. Calcium channel blocker lanthanum ions and imidazole, inhibitor of NAD(P)H oxidase (EC 1.6.3.1) that prevents reactive oxygen species formation and programmed cell death, were used as reference compounds to compare the effect of studied compounds. We studied changes in the cell viability and growth as well as markers of cell proliferation, levels of intracellular free calcium ions, reactive oxygen species, lipid peroxidation, and markers of programmed cell death, mitochondrial membrane potential and caspase-like activity. Late signs of programmed cell death (changes in nuclear architecture) were also evaluated. Our experiments revealed protective potential of studied compounds against programmed cell death induced by hydrogen peroxide and possibility of application of the BY-2 cell culture to evaluate pharmacological effects of studied compounds in preliminary tests.

Keywords: Beta blockers, calcium channel blockers, reactive oxygen species, programmed cell death, BY-2 cells.

INTRODUCTION

Plant development and growth is controlled by many signals, both hormonal and environmental. Calcium ions appear to be ubiquitous signals in all plant cells and tissues, so they play crucial role in cellular and physiological processes (cytoplasmic streaming, cell division, cell elongation, cell differentiation and polarity, gravitropism, thigmotropism) as well as in responses to the environmental stimuli (plant defence and stress responses, disease resistance, salt tolerance). Ca^{2+} levels are relatively high in the apoplast, mitochondria, vacuoles, and endoplasmic reticulum; on the other hand, low levels are maintained in the cytoplasm. Calcium ions homeostasis is regulated by many mechanisms including Ca^{2+} channels that have been characterized in plasma membrane, nuclear and plastid membranes, endoplasmic reticulum, and tonoplast and are responsible for influx of extracellular Ca^{2+}, Ca^{2+}/H^{+} antiporters, and channel proteins, such as Ca^{2+} ATPases (Anil and Rao, 2001, White, 1998, White, 2000, White, 2004). Intracellular levels of Ca^{2+} are modulated in the response to the various signals including plant growth regulators and biotic and abiotic stress (Reddy, 2001). Changes in calcium levels were observed during apoptosis, tightly controlled form of PCD. Increase of levels of intracellular calcium ions can result from calcium influx from the extracellular environment and release of calcium ions from the endoplasmic reticulum. The resulting increase of the intracellular cytoplasmic calcium ions activates several proteins, such as calpain, protein kinase, and calmodulin (Orrenius *et al*., 2003). Connection between calcium/ calmodulin signalling and release of cytochrome c under treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has been shown in the work of Kobayashi *et al*. (Kobayashi *et al*., 2009). In the physiological conditions, propagation of calcium signal to the mitochondria leads to stimulation of ATP via activation of the Ca^{2+}-sensitive dehydrogenases. On the other hand, overloading of mitochondria with Ca^{2+} activates permeability of transition pore opening and finally causes permeabilization of mitochondrial outer membrane. Cell death (apoptosis) is stimulated by the release of factors that promote apoptosis (e.g. cytochrome c) to the cytoplasm (Bernardi, 1999; Green and Kroemer, 2004; Kim *et al*., 2003). Enhancing of the calcium ions mobilization from the endoplasmic reticulum (or from extracellular environment) may augment the mitochondrial Ca^{2+} under activation of the apoptosis (Pinton, Ferrari *et al*., 2001). In the presence of stress factors, such as ROS, permeability transition pores are sensitized with subsequent mitochondrial membrane permeabilisation and apoptosis (Gerasimenko *et al*., 2002; Hajnoczky *et al*., 2003; Jacobson and Duchen, 2002). In plants, programmed cell death is connected not only with

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processes involved in development (ontogeny), but also with defence (Danon *et al*., 2000; Lord and Gunawardena, 2012; Williams and Dickman, 2008). Sublethal oxidative stress has been found in a variety of systems to participate either directly or indirectly in the programmed cell death. Hydrogen peroxide is able to induce PCD in some plant experimental models, such as soybean, barley, tomato, *Arabidopsis thaliana*, and BY-2 cells (Desikan *et al*., 1998; Gadjev *et al*., 2008; Gechev and Hille, 2005; Houot *et al*., 2001; Levine *et al*., 1994; Palma and Kermode, 2003). Relations between hydrogen peroxide, formation of mitochondrial permeability transition pores and intracellular free Ca^{2+} levels have been studied by Wang *et al*. (Wang *et al*., 2006). Authors revealed increased intracellular levels of Ca^{2+} ions at the very early stage of apoptosis in tobacco protoplasts. Ruthenium red (RR) is used in experiments as a mitochondrial Ca^{2+} uniporter inhibitor. Depletion of Ca^{2+} ions caused by inhibition of above-mentioned uniporter plays probably important role in processes of apoptosis. It has been suggested that curcumin-induced apoptosis in Caki cells is based on the depletion of intracellular calcium ions (Bae *et al*., 2003). However, studies on plants or plant cells models are almost missing (Pottosin *et al*., 1999).

Calcium channel blockers (CCBs) represent a group of chemicals that work by blocking voltage-gated calcium channels in myocardium and vascular smooth muscles. Generally, two classes of CCBs based on chemical structure have been recognized, dihydropyridine CCBs and non-dihydropyridine CCBs. Non-dihydropyridine CCBs group includes phenylalkylamine CCBs, and benzothiazepine CCBs. CCBs are often used to reduce systemic vascular resistance and arterial pressure (dihydropyridine CCBs), or to treat angina pectoris (non-dihydropyridine CCBs) (Elliott and Ram, 2011). New therapeutic potentials of CCBs are studied. Anti-atherosclerotic potential of dihydropyridine has been reviewed by Ishii *et al.* (Ishii *et al*., 2012), modulation of innate immunity has been reviewed Liu and Matsumori (Liu and Matsumori, 2011). However, there are CCBs that have found an application in experimental botany. Verapamil has been identified to induce systemic antiviral resistance in susceptible plants (Singh *et al*., 2011), diltiazem has been found to modify nutrient uptake by sorghum primary root tips (Wilkinson *et al*., 1994). In our work, we decided to use tobacco BY-2 cells to test newly prepared calcium channel blockers with respect to calcium ions efflux/release and processes of PCD.

MATERIAL AND METHODS

Chemicals

All of the chemicals used in experiments were purchased from Sigma-Aldrich, USA, unless otherwise stated and stored in accordance with the manufacturer's recommendations. Working solutions were prepared immediately prior to use.

BY-2 culture and experimental design

Nicotina tabacum L. cv. Bright Yellow-2 suspension-cultured cells (BY-2) were cultured in liquid MS medium (Murashige and Skoog, 1962) as modified by Nagata (Nagata *et al*., 1992a) under continuous shaking (130 rpm) at 27°C in the dark in 250ml Erlenmeyer flasks. BY-2 cells in the exponential growth phase were transferred into fresh cultivation media supplemented with tested compounds (JP01-JP08) to create concentrations of 10, 50, 100, 100, 250 and 500μM. Stock solutions of studied compounds were prepared by their dissolving in DMSO (1 $mg \cdot mL^{-1}$). For structural formulas, see fig. 1A. The general synthetic procedure used in this study is illustrated in fig. 1B. Among the starting materials, alkyl [4-(oxiran-2-ylmethoxy)phenyl] carbamates (2a-d) were synthesized by our method reported previously (Csollei *et al*., 1982). Alkyl (4-hydroxyphenyl)carbamates (1a-d) were prepared from 4-aminophenole and alkyl chloroformates (Csollei *et al*., 1982). As for the other group of starting materials, isonipecotamide was alkylated with butyl- and propylbromide in the presence of base in ethanol (Itoh *et al*. 1999) to afford corresponding 1-substituted-piperidine-4-carboxamide derivatives (3a-b), which gave 1-substituted-4-piperidinylmethylamines (4a-b) through a reduction with $LiAlH_4$ in THF (Chodkowski and Gutkowska, 1977; Itoh *et al*., 1999). By the addition method alkyl [4-(oxiran-2-ylmethoxy)phenyl]carbamates (2a-d) were coupled with 1-substituted-4-piperidinylmethylamines (4a-b) in methanol to afford targ*et al*kyl {4-[3-(1-butyl- and 1-propylpiperidin-4-ylmethylamino)-2-hydroxypropoxy]phenyl} carbamates that were further transformed to salts with methanesulfonic acid. The structures of prepared final products were determined by IR, NMR, and mass spectrometry techniques. Details will be published elsewhere. The BY-2 cells were then cultivated for 48 h with samples being collected at the end of this time interval. Following parameters were evaluated: cell viability, cell growth, activities of oxidoreductases and dehydrogenases, and cell plasma membrane damage. The concentration that had showed only minimal toxic effect on the BY-2 cells (50μM) was used in subsequent experiment. In the next experiment, BY-2 cells were exposed to hydrogen peroxide (12.5mM); combinations of hydrogen peroxide (12.5mM) and tested compounds (50μM), calcium channel blocker La^{3+} ions (10μM), and imidazole (100μM), an inhibitor of NAD(P)H oxidase (EC 1.6.3.1) that prevents ROS formation and PCD, in combination with hydrogen peroxide (12.5mM) were used. Samples were collected at the strictly defined 24-h time interval. Following parameters were evaluated: cell growth of the BY-2 cells and viability, changes in intracellular Ca^{2+} level, changes in the activities of oxidoreductases and dehydrogenases, production of ROS, namely hydrogen peroxide and superoxide anion radical, production of malondialdehyde, changes in nuclear architecture, mitochondrial potential, and caspase-like

activity as signs of programmed cell death. All experiments were carried out in a triplicate.

Cell viability and growth

Fluorescein diacetate (FDA, Sigma-Aldrich, USA) and the propidium iodide (PI, Sigma-Aldrich, USA) were used to evaluate the viability of BY-2 cells as described by Babula *et al*. (Babula *et al*., 2012). The BY-2 cells growth was determined by a weighing. Strictly defined volume of well-mixed cell suspension culture (3mL) was filtered through a filter paper with aspiration and fresh weight of the BY-2 cells recorded (ABT 120, Labicom, Czech Republic). The parameters were determined in triplicate.

MTT and TTC assays

Measuring the mitochondrial-dependent reduction of 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chem. Corp., USA) and 1,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich Chem. Corp., USA) to formazan in separate assays was used to evaluate the dehydrogenase and oxidoreductase activities (Babula *et al*., 2009). The activities were each expressed as a percentage of the activity of a control sample of untreated tobacco BY-2 cells at the time 0 (=100%). For all spectrophotometric measurements, a spectrophotometer Helios Epsilon Unicam (Thermo Fisher Scientific, USA) was used.

Determination of intracellular free Ca^{2+}

Quin-2 AM (Life Technologies, USA) was used to visualize free intracellular Ca^{2+} (Tanimoto, 1994). Thrice-washed BY-2 cells were incubated for 1 hour in 50 μM solution of Quin-2 AM (2-[(2-amino-5-methylphenoxy) methyl]-6-methoxy-8-aminoquinoline-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)). After incubation, the BY-2 cells were washed three times with fresh MS cultivation medium and observed (Axioscop 40, Zeiss, Germany). NIS elements software (Nikon, Japan) was used to process of images and to evaluate the resultant pictures.

Reactive oxygen species (ROS) and lipid peroxidation

$TiCl_4$ method based on the reaction between hydrogen peroxide and $TiCl_4$ under creation of coloured Ti complexes was used to determine amount of hydrogen peroxide. The absorbance was measured at 410nm (Kovacik *et al*., 2009). Method of Choi *et al*. was used to determine superoxide anion radical ($O_2^{\cdot-}$); this method measures the reduction of yellow nitro blue tetrazolium (NBT) to blue NBT formazan at 620nm (Choi *et al*., 2006). 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, USA) was used to visualize ROS (Garnczarska, 2005). Thrice-washed BY-2 cells were incubated for 1 hour in 10μM H_2DCFDA in fresh MS cultivation medium in darkness to avoid possible light-accelerated oxidation of H_2DCFDA. After it, the cells were washed three times with MS medium and observed (Axioscop 40, Zeiss, Germany).

Changes in mitochondrial potential and caspase-like activity

JC-1 (Sigma-Aldrich, USA) was used to determine changes in mitochondrial potential according to the method published by Simeonova *et al*. (Simeonova *et al*., 2004). Working solution of JC-1 was prepared immediately prior to use from the stock solution in DMSO ($1mg \cdot mL^{-1}$). At strictly defined time intervals, BY-2 cells were collected, washed three times with fresh cultivation medium, and used to isolate the protoplasts according to the method described by Bandmann and Homann (Bandmann and Homann, 2012). TwomL of the BY-2 cells resuspended in fresh MS medium was mixed with 2mL of digestion medium (3% cellulase Onzuka R10 obtained from Duchefa, The Netherlands, 0.2% macerozyme, 0.1% pectolyase, w/v, both Sigma-Aldrich, USA, 8mM $CaCl_2$, 25mM MES-KOH, final pH 5.6, adjusted with sucrose) and incubated for 3 hours. After the digestion, the BY-2 cells were collected, washed three times (30mM $CaCl_2$, 30mM KCl, 20mM MES, pH 6.5, adjusted with sorbitol) and immediately stained with JC-1 (final concentration of $2.5\mu g \cdot mL^{-1}$, 25°C, 30min, darkness. JC-1 monomers emit at 535nm and the aggregates emit at 595nm; spectrofluorimetric detector (RF-551, Shimadzu, Duisburg, Germany) was used to determine intensity of the emissions. Fluorescence measurements were repeated three times for each concentration and time period. Caspase 3 Colorimetric Kit with acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) as a substrate was purchased from Sigma Aldrich, USA and was used to determine caspase-like activity according with the manufacturer's instructions. The absorbance was measured at 405nm (Helios Epsilon Unicam spectrophotometer, Thermo Fisher Scientific, USA). All values of the caspase-like activity were recalculated to those at time 0 (100%).

Nuclear architecture and programmed cell death

In order to determine nuclear architecture and detect signs of programmed death, a 20μL sample of BY-2 cells was treated with 20μL of PEM-buffer (100mM PIPES, 10mM EGTA, 10mM $MgCl_2$, pH 6.9, all chemicals obtained from Sigma-Aldrich, USA) that contained formaldehyde (4%, w/w; Sigma-Aldrich, USA). A Hoechst 33258 fluorescent probe (Sigma-Aldrich, USA) was used as a nuclear (DNA) stain. One thousand nuclei in each preparation were observed (fluorescence microscope Olympus AX 70, Germany). Ten random fields (minimally 1,000 cells) from each series were evaluated in triplicate; each structural change was expressed as a percentage of the total cells.

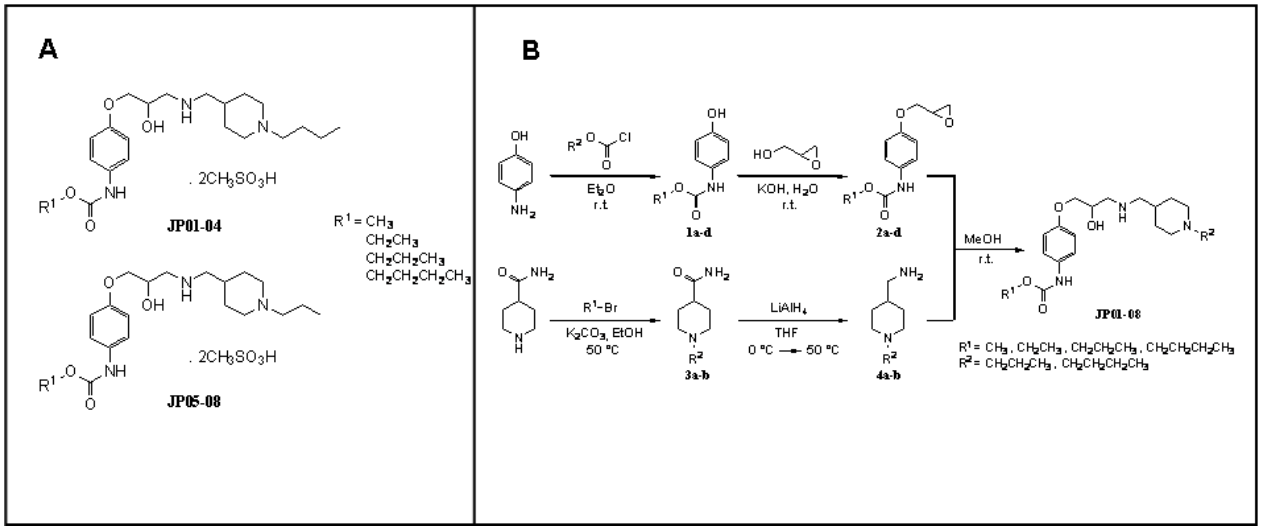

Fig. 1: Structure of prepared compounds (A). The small series of compounds with substituents R^2=propyl and butyl on piperidine ring was synthesised. The different lipophilicity was achieved by variation of carbamate alkyl chain. From C1 to C4 chain was investigated. The general synthesis of alkyl {4-[3-(1-butyl- and 1-propylpiperidin-4-ylmethylamino)-2-hydroxypropoxy]phenyl}carbamates (JP01-08) (B).

STATISTICAL ANALYSIS

Statistical analyses were performed using Microsoft® Office Excel (Microsoft®, USA) that was used to evaluate standard deviation; Error bars in graphs represent the variability of data. All experiments were carried out in triplicates (n=3).

RESULTS

We tested newly prepared calcium channel blockers on the BY-2 plant cell model. Firstly, we tested toxicity of prepared compounds. We chose concentration range from 0 to 500µM (0, 10, 50, 100, 250, and 500µM). Cell viability, growth parameter, markers of cell proliferation (activities of oxidoreductases and dehydrogenases), and damage of cell plasma membrane were evaluated after 48 h of treatment. In the next step, we used non-toxic concentration of compounds tested (50µM) to evaluate the calcium ions flux under hydrogen peroxide treatment (12.5mM). Hydrogen peroxide was used in order to induce PCD. Its concentration was chosen according to work of Houot *et al*. (Houot *et al*., 2001). We evaluated not only changes in intracellular free calcium ions, but also individual steps of machinery of PCD - mitochondrial dysfunction including changes in mitochondrial potential, activity of caspase-like enzymes, and finally signs of programmed cell death. In addition, we monitored also levels of ROS, both non-radical (hydrogen peroxide) and radical (super oxide anion radical), and product of lipid peroxidation, malondialdehyde, a marker of plasma membrane damage. Two additional controls were used. La^{3+} ions, the first control, are known to be able to affect processes in which Ca^{2+} ions play crucial role (Li *et al*., 2007), so they act as calcium channel blocker (Friedman *et al*., 1998). It has been shown that intracellular free calcium ions have important role in regulating PCD in plant cells, probably by mediating transition of mitochondrial permeability (Lin *et al*., 2006; Wang *et al*., 2006). Imidazole was the second control used in our experiments. Reactive oxygen species originate from NAD(P)H oxidase localised in the plasma membrane; this enzyme transfers electrons from cytoplasmic NADPH to O_2 to create $O_2^{\cdot-}$; superoxide anion radical is immediately converted by the dismutation to hydrogen peroxide (Marino *et al*., 2012). NAD(P)H oxidase (EC 1.6.3.1) represents important regulator of ROS-mediated signalling in plants. In order to these facts, imidazole was chosen as a potent inhibitor of NAD(P)H oxidase, concentration of 100µM was chosen in accordance with the work of Liu *et al*. (Liu *et al*., 2012).

Preliminary experiments toxicity of studied compounds

Firstly, we tested toxicity of studied compounds in preliminary experiments. We chose concentration range from 0 (control) to 500µM, samples were collected after 48h of treatment. Cell viability, growth, changes in activities of oxidoreductases and dehydrogenases, and plasma membrane damage were studied. The application of studied compounds led to the significant changes in cell viability only at the highest concentration (500µM) used in the experiment. By the end of the experiment (48h), the viability of the BY-2 cells treated with this particular concentration was as follows: control (no treatment) 99.2%; JP01 95.5%; JP02 90.3%; JP03 87.3%; JP04 84.5%, JP05 95.3%, JP06 94.8%; JP07 88.7% and JP08 86.7%. As it is well evident, compound JP04 showed the highest toxicity and compound JP01 the

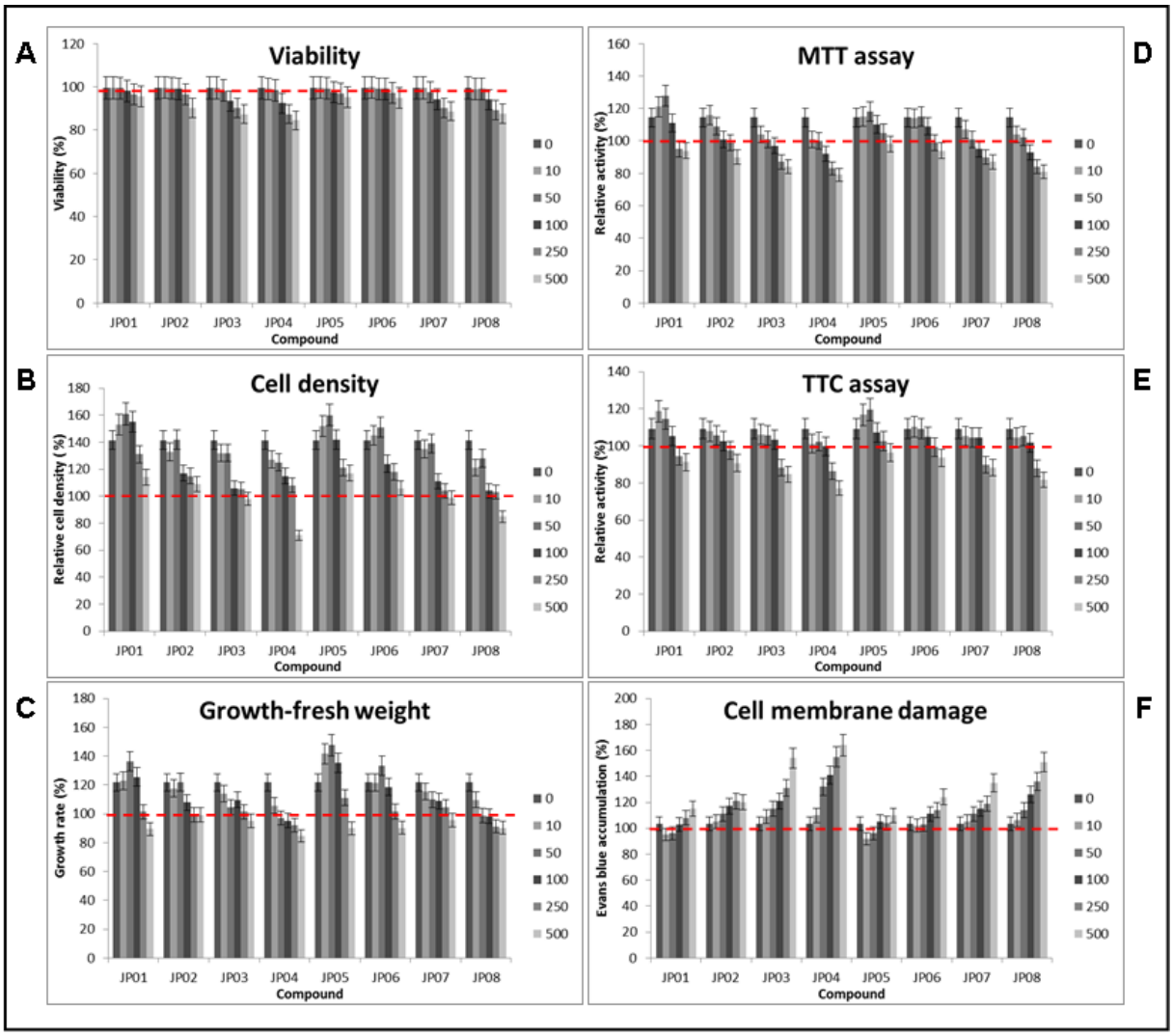

Fig. 2: Evaluation of toxicity of studied compounds JP01 - JP08 in a concentration-dependent manner after 48h of treatment. Concentrations 0, 10, 50, 100, 250, and 500µM were used, following parameters were evaluated: Cell viability (A, expressed as a percentage of living cells), relative cell density (B, cell density at the beginning of the experiment =100%), increase in fresh weight (C, value at the beginning of the experiment =100%), activity of dehydrogenases and oxidoreductases (D, E, values at the beginning of the experiment =100%) and cell membrane damage (F, accumulation of Evans blue stain, value at the beginning of the experiment =100%). Red dashed line indicates the value determined for control (untreated) BY-2 cells at the beginning of the experiment (time 0).

lowest one. On the other hand, viability of BY-2 cells treated with the lower concentrations of studied compounds (10, 50, and 100µM) showed almost no changes in this parameter (see fig. 2). Also next parameters were comparable with control. Obtained results were related to control at the beginning of the experiment (time 0) that was considered 100%. Cell density as well as increase in fresh weight was similar to control in low concentrations (10, 50, and 100µM). Application of the highest concentration (500µM) significantly diminished both cell density and increase in fresh weight. The most significant deceleration in growth was determined for compound JP04. BY-2 cells treated with JP04 at the concentration of 500µM showed only 71% of the cell density of control (untreated BY-2 cell) at the time 0 and only 84.6% of fresh weight of control at the time 0. These findings are in agreement with the highest toxicity found for this compound. Also changes in activity of oxidoreductases and oxidoreductases were clearly evident only for the highest applied concentration of studied compounds (500µM). Low concentrations of studied compounds (10 and 50µM) caused no significant changes in activities of both enzymes in all studied compounds excepting JP04 and JP08. In agreement with previous findings, the most significant reduction of oxidoreductase and dehydrogenase activities was found

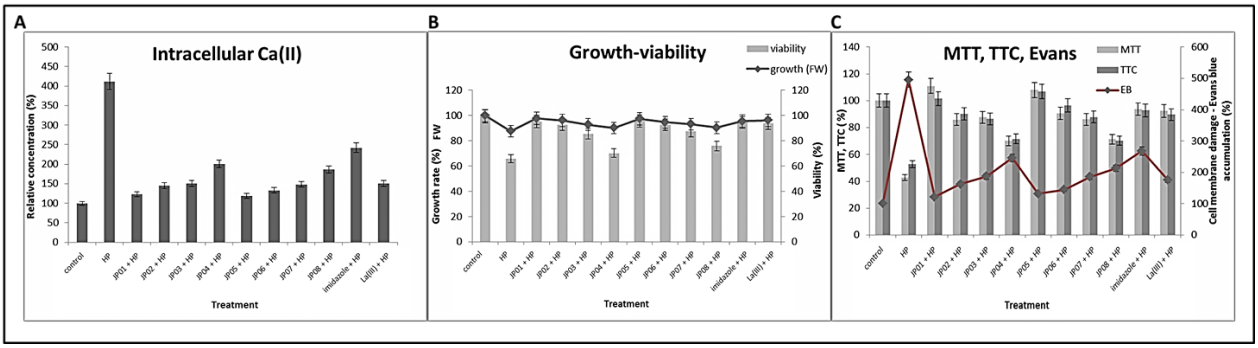

Fig. 3: Changes in the intracellular free Ca^{2+} (A, relative concentration as evaluated using image analysis, control at the beginning of the experiment (time 0) =100%), viability and growth, respectively increase in fresh weight (B), and activity of dehydrogenase and oxidoreductase and Evans blue accumulation marker of cell membrane damage (C, values related to control at the beginning of the experiment (time 0), control =100%) after 24h of treatment. Following experimental variants were used in experiment: control (untreated) BY-2 cells, combination of hydrogen peroxide (12.5mM) and studied compound (JP01-JP08, 50µM), and combination of hydrogen peroxide with imidazole (100µM) or with La^{3+} (10µM).

for compound JP04. In order to these results, we decided to use the concentration of 50µM for next experiments. This concentration was almost nontoxic for all studied compounds.

Experiment combination of studied compounds with hydrogen peroxide

In the second experiment, we focused on the effect of combination of studied compounds (50µM) with hydrogen peroxide (12.5mM) on BY-2 cells. We aimed at the protective effect of studied compounds, respectively at the ability of studied compounds to regulate process of programmed cell death, in which reactive oxygen species and changes (flux) in intracellular free calcium ions are involved. We monitored cell viability as a marker of toxicity and increase in fresh weight as a marker of growth, changes in intracellular free calcium ions, changes in activities of oxidoreductases and dehydrogenases, reactive oxygen species (superoxide anion radical and hydrogen peroxide), and lipid peroxidation. Changes in nuclear architecture, mitochondrial potential, and caspase-like activity were evaluated as in order to monitor process of programmed cell death. Due to the fact that changes in nuclear architecture are well evident after 24 h of treatment of the BY-2 cells by hydrogen peroxide (Houot *et al*., 2001), this time interval was chosen as the most suitable to evaluate the combined effect of hydrogen peroxide and studied compounds.

Effect of treatment on viability of BY-2 cells and their growth

Viability of control (untreated) BY-2 cells after 24 h of cultivation was 99.3%. On the other hand, BY-2 cells treated by hydrogen peroxide in concentration of 12.5mM showed significant reduction of viability to only 65.7%. Application of studied compounds in combination with hydrogen peroxide caused decrease in viability of BY-2 cells. Combined application of JP04 with hydrogen peroxide led to the decrease in viability to 70.2%. On the other hand, BY-2 cells treated by JP05 in combination with hydrogen peroxide caused only slight reduction in viability to 95.6%. Combination of hydrogen peroxide and imidazole caused decrease in viability to 94.7%, La^{3+} ions in combination with hydrogen peroxide diminished cell viability to 93.8%. These results corresponded with the changes in growth parameter of BY-2 cell suspension culture. Compared to control (untreated) BY-2 cells that represented 100%, the most evident decrease in fresh weight was determined for combination HP and JP04, on the other hand, combination of JP02 and hydrogen peroxide led to the growth characteristics comparable with control (97.6% of control). For details, see fig. 3.

Changes in dehydrogenase and oxidoreductase activities

MTT and TTC assays are generally accepted and used to determine changes in activities of dehydrogenases and oxidoreductases. Our experiments showed a decrease in activity of both enzymes in all treated variants with the exception of JP01 and JP05 combined with hydrogen peroxide compared to the control BY-2 cells. The activity of dehydrogenase had 111.0% of the level of activity found in the control for the variant with hydrogen peroxide in combination with JP01 and 107.9% for that with hydrogen peroxide in combination with JP05. For oxidoreductase, the corresponding values were 101.6% for hydrogen peroxide in combination with JP01 and 106.8% for hydrogen peroxide in combination with JP05. On the other hand, hydrogen peroxide significantly reduced the activities of both dehydrogenase and oxidoreductase. The activity of dehydrogenase was reduced to 42.8% compared to the control and that of oxidoreductase to 52.8% compared to the control for the hydrogen peroxide. The results are shown in fig. 3C.

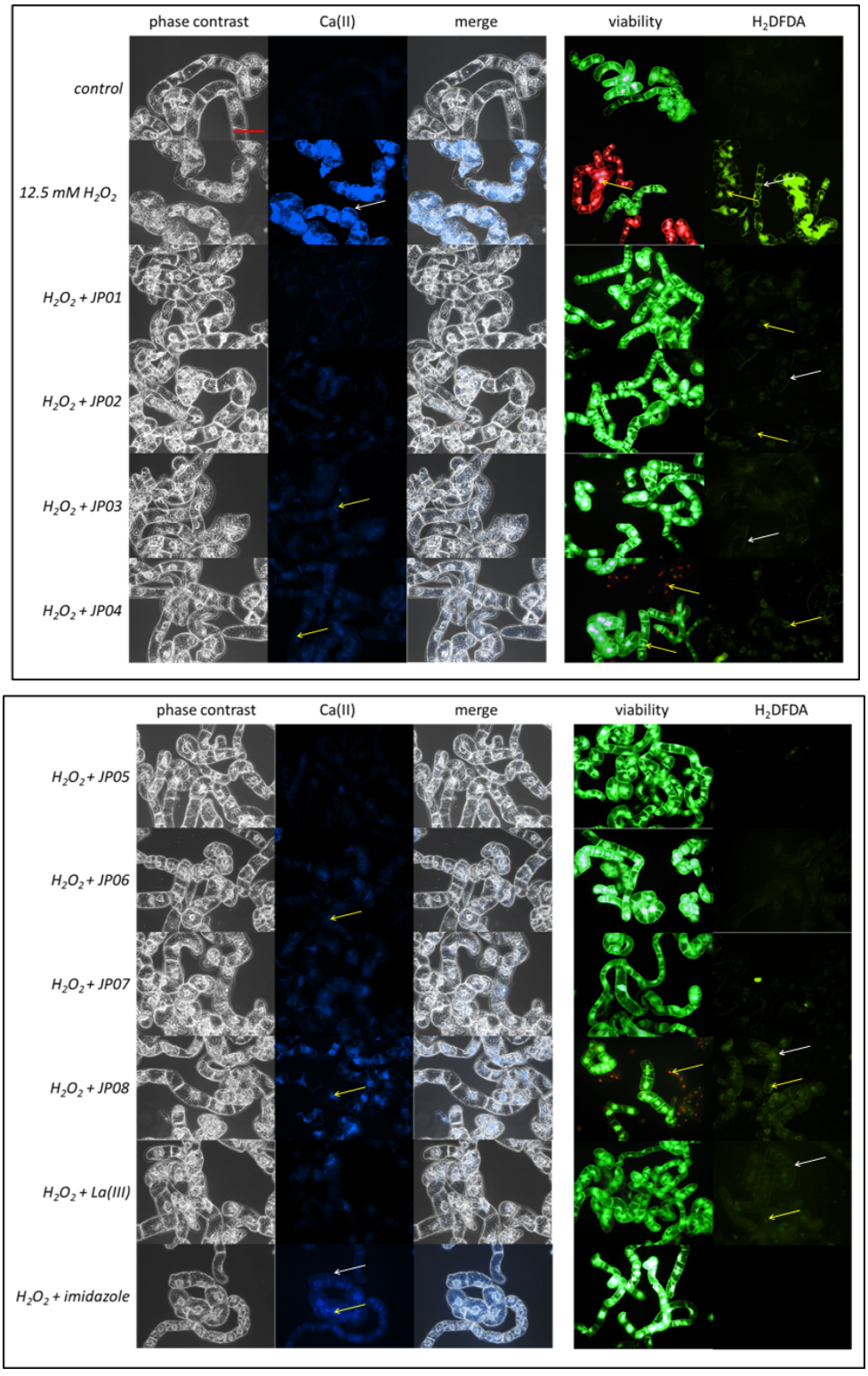

Fig. 4: Morphology of BY-2 cells after 24 h of treatment and visualization of intracellular free Ca^{2+} (first three columns – phase contrast, Ca^{2+} ions, and merge), cell viability (fourth column, double staining with FDA and PI, living cells are stained in green, death cells are stained in red) and visualization of ROS (last column, staining with H_2DCFDA, intensity of emission indicates presence of ROS). Note cytoplasm shrinkage, typical sign of programmed cell death in plant cells, in BY-2 cells treated by hydrogen peroxide (12.5mM). White arrows indicate cell wall and adjacent plasma membrane; yellow arrows indicate localization of nuclei in BY-2 cells. Red bar indicates 50µm.

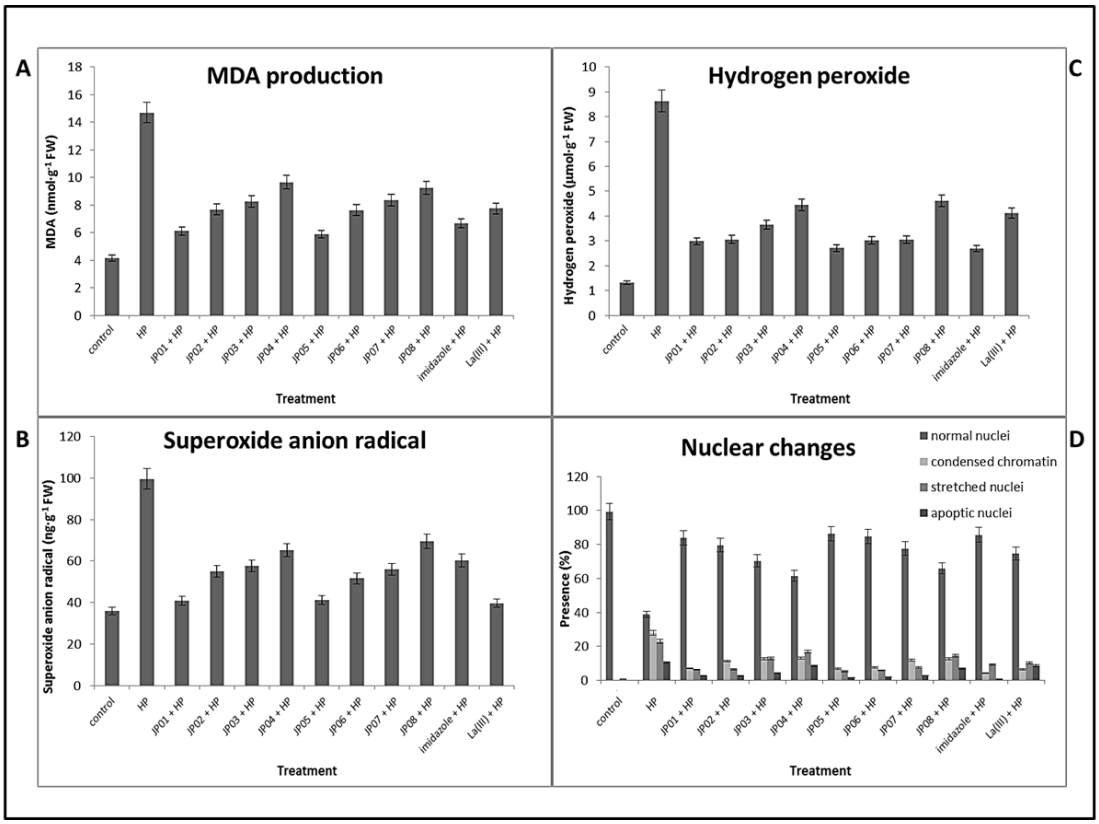

Fig. 5: Changes in the lipid peroxidation (A) and ROS (superoxide anion radical -B, hydrogen peroxide -C) after 24 h of treatment. Changes in nuclear architecture in particular experimental variants (D). Following experimental variants were used in experiment: control (untreated) BY-2 cells, combination of hydrogen peroxide (12.5mM) and studied compound (JP01-JP08, 50µM) and combination of hydrogen peroxide with imidazole (100µM) or with La^{3+} (10µM).

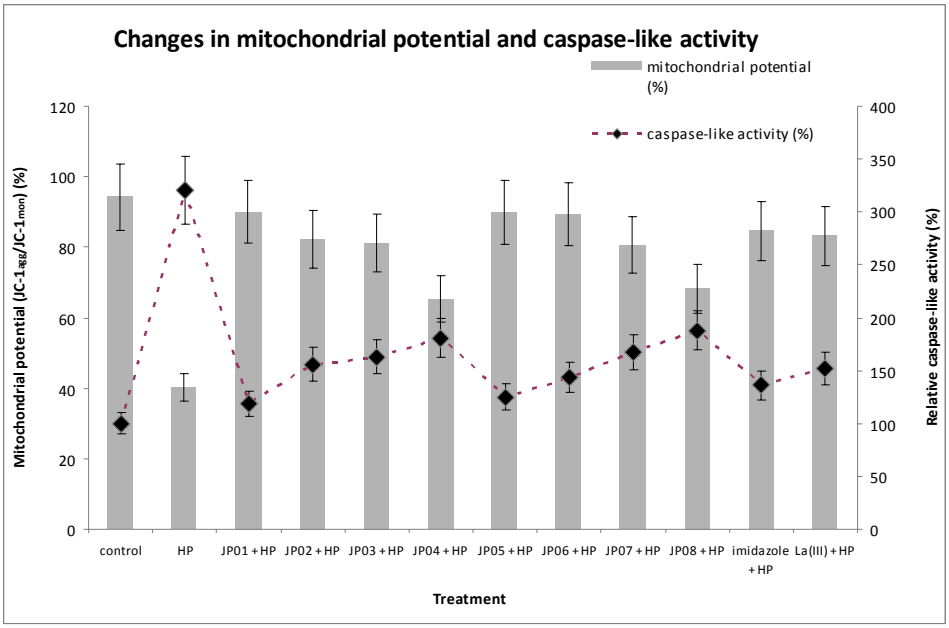

Fig. 6: Changes in the mitochondrial membrane potential (expressed as a rate between JC-1 aggregates and JC-1 monomers) and caspase-like activity in BY-2 cells after 24h of treatment. Following experimental variants were used in experiment: control (untreated) BY-2 cells, combination of hydrogen peroxide (12.5mM) and studied compound (JP01-JP08, 50µM) and combination of hydrogen peroxide with imidazole (100µM) or with La^{3+} (10µM).

Changes in intracellular free calcium

Changes in intracellular free calcium (calcium flux) were observed by using Quin-2 AM probe and fluorescence microscopy. The pictures obtained were evaluated using the NIS element software; this software enables processing of image and the calculation of selected parameters, in our case relative intensity of emission. All results were referenced to intracellular free calcium ions level of the control (untreated) BY-2 cells (=100%). The highest increase in intracellular free Ca^{2+} was found for the hydrogen peroxide (12.5mM). It was almost 412% relative to control. In comparison with this result, application of hydrogen peroxide in combination with studied compounds led to inhibition of Ca^{2+} flux. It was 119% relative to the control for JP05 and hydrogen peroxide and almost 201% for JP04 and hydrogen peroxide. In the case of two controls, La^{3+} and imidazole, it was 151% and 242%, respectively. For details, see fig. 3A. Quin-2 AM probe showed that the greatest amount of free Ca^{2+} ions was localized in cytoplasm, especially around nuclei and adjacent cytoplasm. On the other hand, cell walls of the BY-2 cells were also stained. For details, see fig. 4A and 4B.

Reactive oxygen species (ROS) and lipid peroxidation

ROS, in our case hydrogen peroxide and superoxide anion radicals, are important markers of oxidative stress. The greatest increase in the amount of hydrogen peroxide was observed for the hydrogen peroxide itself (8.63µmol·g^{-1} FW) as compared to 1.32µmol·g^{-1} FW for the control BY-2 cells. On the contrary, application of hydrogen peroxide in combination with studied compounds led to the decline in the hydrogen peroxide levels in all studied combinations. Compound JP05 was the most effective (2.71µmol·g^{-1} FW), on the other hand, only moderate decline in the hydrogen peroxide level was observed for compound JP04 (4.45µmol·g^{-1} FW). Surprisingly, hydrogen peroxide in combination with imidazole reduced amount of detected hydrogen peroxide in the BY-2 cells (2.69µmol·g^{-1} FW). A similar course was observed in the case of superoxide anion radical. Its greatest increase in the amount (99.6ng·g^{-1} FW) was observed for the hydrogen peroxide as compared to 35.8ng·g^{-1} FW for the control BY-2 cells. On the other hand, the lowest amount of superoxide anion radical (40.9ng·g^{1} FW) was observed for the compound JP04 combined with hydrogen peroxide. Whereas imidazole combined with hydrogen peroxide declined level of superoxide anion radical detected in the BY-2 cells only moderately (60.3ng·g^{-1} FW), La^{3+} ions in combination with hydrogen peroxide declined greatly level of superoxide anion radical (39.6ng·g^{-1} FW). ROS (especially hydrogen peroxide) were observed visually using 2′,7′-dichlorodihydrofluorescein diacetate. This probe showed that the greatest amount of ROS in BY-2 cells was localized in cytoplasm adjacent to nuclei and in the plasma membrane (See fig. 3A and 3B). The fact that plasma membranes were stained led us to determine level of lipid peroxidation. Lipid peroxidation is the process that damages membranes by forming lipid radicals. They destroy characteristics of the plasma membrane and cause it to lose integrity. Malondialdehyde, a marker of lipid peroxidation, was determined spectrophotometrically in our work. Whereas control BY-2 cells had produced only a small amount of malondialdehyde (4.15nmol·g^{-1} FW) after 24 h of cultivation, the content of MDA in hydrogen peroxide-treated BY-2 cells, and hydrogen peroxide-treated BY-2 cells in combination with studied compounds increased. For hydrogen peroxide itself, the MDA content was almost 3.54-times as much as that of the control (14.69nmol·g^{-1} FW). On the other hand, for hydrogen peroxide in combination with compound JP05 the MDA content was 5.88 nmol·g^{-1} FW and for hydrogen peroxide in combination with compound JP04 the MDA content was 9.66 nmol·g^{-1} FW. For details, see fig. 5.

Changes in mitochondrial potential, caspase-like activity, plasma membrane integrity, and the changes in nuclear architecture

Changes in the mitochondrial potential lead to the mitochondrial dysfunction and PCD. In our case, a JC-1 fluorescent probe was used to determine the mitochondrial potential; the rate between JC-1 aggregates ($JC\text{-}1_A$) and JC-1 monomers ($JC\text{-}1_M$) was determined. For control BY-2 cells, this rate was 94.36%. The application of hydrogen peroxide significantly reduced the presence of JC-1 aggregates (40.22%). On the other hand, for the hydrogen peroxide in combination with JP01, the rate was 90.11% and for the hydrogen peroxide in combination with JP04, it was only 65.33%. Loss of potential of the mitochondrial membrane is connected with the release of cytochrome c and following processes of programmed cell death. PCD is executed by enzymes with caspase-like activity. The highest caspase-like activity was found for the hydrogen peroxide (12.5mM). It was 305% relative to the control. BY-2 cells treated by hydrogen peroxide and JP01 showed 119% of activity of the control (untreated) BY-2 cells, on the other hand, combination of hydrogen peroxide and JP04 led to the increase in caspase-like activity to 181%. Results are shown in fig. 6. The Evans blue method was used to evaluate the integrity of the plasma membrane; BY-2 cells with disrupted or damaged plasma membranes accumulated Evans blue stain. It can subsequently be extracted and determined spectro-photometrically. The accumulation of Evans blue stain was greatest in the hydrogen peroxide-exposed BY-2 cells (495% relative to the control). The combination of hydrogen peroxide and compound JP01 led to only moderate increase in Evans blue accumulation (122%), on the other hand, hydrogen peroxide combined with JP04 increased the amount of accumulated Evans blue stain in BY-2 cells. Surprisingly, the high rate of Evans blue accumulation was determined for combination hydrogen peroxide-imidazole. It was 268% relative to the control

(see fig. 3C). Signs of PCD were determined microscopically using Hoechst 33258 probe. The following parameters were evaluated: the presence of normal nuclei, stretched nuclei, nuclei with condensed chromatin (pre-apoptic nuclei), and apoptic-like nuclei. The most significant changes occurred in the hydrogen peroxide-treated variant. Apoptic-like nuclei were found to be present in 10.5% of the BY-2 cells (compared with the control (untreated) at 0%), stretched nuclei were found in 22.9% of the BY-2 cells (compared to 0.7% in the control BY-2 cells), pre-apoptic nuclei with condensed chromatin were observed in 27.8% of the BY-2 cells (compared to 0.65% in the control BY-2 cells). Normal nuclei were detected in 38.8% of the BY-2 cells (98.65% in the control) in this particular treatment. Combination of hydrogen peroxide with studied compounds led to the lowering of incidence of changes in nuclear architecture. The lowest incidence of changes in the nuclear architecture was detected in hydrogen peroxide-JP06 variant followed by hydrogen peroxide-JP01 variant and hydrogen peroxide-imidazole variant. For details, see fig. 5D.

DISCUSSION

Calcium ions represent a crucial messenger in various stimuli, both biotic and abiotic (Hashimoto and Kudla, 2011). In addition, calcium ions play crucial role in many physiological processes. Two distinct targets have been identified in plant cells. The first one is the cell wall, where calcium ions mediate the cross-linking acidic pectin residues. The second one is the cell membrane system, where calcium ions control the membrane structure and function and affect the membrane permeability (Hepler, 2005). In order to these facts, level of intracellular free calcium ions must be strictly regulated. The regulation of Ca^{2+} flux is a keystone of the integration of environmental signals and their translation into adaptive physiological responses (Jammes *et al*., 2011). Calcium channels in the plasma membrane, together with other transporters, play a role in shaping the Ca^{2+} signature by transporting Ca^{2+} into the cells. Calcium channels function in various responses. In our work, we studied newly prepared potential calcium channel blockers. These compounds are studied as potential therapeutic agents. In the first step, we determined toxicity of these compounds on BY-2 cell model. These compounds decreased cell viability only at the highest applied concentration (500µM), lower concentrations were almost non-toxic. We decided to use the concentration of 50µM for next experiments. Hydrogen peroxide (12.5mM) was used as a compound that is able to induce process of PCD in tobacco BY-2 cells (Houot *et al*., 2001). Hydrogen peroxide represents both an important signaling molecule and a toxic byproduct of cell metabolism; cellular levels of hydrogen peroxide are strictly controlled and their maintenance has hallmarks of homeostatic regulation. The cell can sense sublethal doses of hydrogen peroxide and activate mechanisms that detoxify hydrogen peroxide. Alternatively, upon different cell death stimuli various hydrogen peroxide-producing mechanisms can be activated, and as a result of this deliberate hydrogen peroxide production a self-destructive PCD is triggered (Gadjev *et al*., 2008; Gechev *et al*., 2002; Gechev and Hille, 2005). Wang *et al*. investigated the ability of intracellular free Ca^{2+} to regulate mitochondrial permeability transition pores in tobacco protoplasts where apoptosis was induced by hydrogen peroxide (Wang *et al*., 2006). Lanthanum chloride used in their experimental work partially inhibited the increase in intracellular Ca^{2+}, so application of lanthanum chloride resulted in the reversal of the effect of hydrogen peroxide. Authors concluded that calcium channel blocker lanthanum chloride partially inhibited the cytotoxic effect of hydrogen peroxide and the signaling pathway of intracellular free Ca^{2+}-mediated mitochondrial permeability transition associated with hydrogen peroxide-induced apoptosis in tobacco protoplasts (Wang *et al*., 2006). Imidazole was chosen as the second control. Imidazole is potent inhibitor of NAD(P)H oxidase, enzyme that is involved in the transfer of electrons from cytoplasmic NADPH toO_2 to create $O_2^{\cdot-}$; this process is followed by the dismutation of $O_2^{\cdot-}$ to hydrogen peroxide (Marino, Dunand et a Citation not accoring to Journal's style.l., 2012). In the light of above-mentioned facts, our experimental design was set to determine the ability of these compounds to affect Ca^{2+} flux. Hydrogen peroxide was chosen as a compound triggering programmed cell death in BY-2 cells, combined treatment (hydrogen peroxide + studied compound) was chosen to reveal the activity of the studied CCB and thus protection against programmed cell death induced by changes in Ca^{2+} flux. We monitored changes that are closely connected with the changes in intracellular free Ca^{2+} ions, especially changes connected with PCD including changes in mitochondrial potential, changes in permeability of the plasma membrane, and caspase-like activity. In addition, we determined levels of ROS hydrogen peroxide and superoxide anion radicals. The execution of PCD was monitored visually; we determine typical changes in the nuclear architecture that are connected with PCD execution. The most significant changes in the monitored parameters were caused by application of hydrogen peroxide. This compound demonstrated the possibility to induce programmed cell death with all typical signs. On the other hand, both La^{3+} ions and imidazole had protective effect on BY-2 cells treated by hydrogen peroxide. These compounds also inhibited flux of Ca^{2+} and partially inhibited cytotoxic effect of hydrogen peroxide. Inhibition of Ca^{2+} flux was more obvious in the La^{3+} variant compared to imidazole, whereas inhibition of formation of ROS was more evident in the imidazole variant. However, the ability of La^{3+} ions to prevent stress has been shown in many publications. Ott *et al*. observed

inhibition of accumulation of hydrogen peroxide and PCD in tobacco leaf tissue (Ott *et al*., 2003), Li *et al*. observed inhibition of salt stress-induced programmed cell death in rice root tips after application of La^{3+} (Li *et al*., 2007). We observed protective effect of La^{3+} on BY-2 cells treated by hydrogen peroxide. Application of La^{3+} led to the prevention of the loss of mitochondrial potential; this fact was closely connected with only slight increase in intracellular free Ca^{2+} ions level mediated probably by calcium flux. On the other hand, knowledge and application of imidazole in plant experimental botany is almost missing. Liu *et al*. showed that calcium-dependent NAD(P)H oxidase is involved in the salt stress-induced PCD and its inhibition by imidazole can reduce superoxide anion radical production and prevent salt stress-induced PCD in part (Li *et al*., 2007). We observed partial protection of BY-2 cells exposed to hydrogen peroxide by imidazole. However, the mechanism of this protective action must be further studied. Imidazole and its derivatives are known to be able to form metal complexes (Rowan *et al*., 1981; Wang *et al*., 2011; Yue *et al*., 2012). Complexes of imidazole and its derivatives with copper and iron ions have been described and studied (Atria *et al*., 2011; Dhanalakshmi *et al*., 2009; Martinez *et al*., 2012). Just copper and iron ions participate in Fenton's reaction that is responsible for formation of highly toxic hydroxyl radicals (Urbanski and Beresewicz, 2000). Just ability of imidazole to form complexes with these metal ions may be responsible for its protective effect against toxicity of hydrogen peroxide. However, further studies are quite necessary. The compounds tested in our experiments were alkyl {4-[3-(1-butyl- and 1-propylpiperidin-4-ylmethylamino)-2-hydroxypropoxy]phenyl} carbamates. Parallel application of studied compounds (50μM) with hydrogen peroxide (12.5mM) revealed the protective effect of studied compounds hydrogen-peroxide induced cytotoxicity. Compound JP05 and JP01 with the shortest alkyl group - methyl in carbamate functional group showed the highest protective potential, compounds JP08 and JP04 containing more lipophilic butyl group the lowest protective potential. Our experimental work revealed the possibility of testing of potential calcium channel blockers on the BY-2 cell model that is commonly compared to HeLa cells used in animal biology (Nagata *et al*., 1992b).

CONCLUSION

Tobacco BY-2 cell suspension culture was used to test the effect of potential calcium channel blockers. Experimental design was based on the application of hydrogen peroxide that is connected with calcium flux and signal cascade leading to programmed cell death. La^{3+} ions, known and used calcium channel blocker, and imidazole, inhibitor of NAD(P)H oxidase, were used as next controls. Our results revealed protective effect of studied compounds against hydrogen peroxide-induced programmed cell death. Studied compounds showed ability to inhibit calcium ions flux. In conclusion, tobacco BY-2 cell suspension culture may be used as a model in preliminary pharmacological studies.

ACKNOWLEDGMENTS

Grants IGA VFU Brno 63/2011/FaF, 51/2011/FaF, 88/2013/FaF are highly acknowledged for financial support.

REFERENCES

Anil VS and Rao KS (2001). Calcium-mediated signal transduction in plants: A perspective on the role of Ca2+ and CDPKs during early plant development. *J. Plant Physiol.*, **158**: 1237-1256.

Atria AM, Cortes-Cortes P, Garland MT, Baggio R, Morales K, Soto M and Corsini G (2011). X-Ray studies and antibacterial activity in copper and cobalt complexes with imidazole derivative ligands. *J. Chil. Chem. Soc.,* **56**: 786-792.

Babula P, Adam V, Kizek R, Sladky Z and Havel L (2009). Naphthoquinones as allelochemical triggers of programmed cell death. *Environ. Exp. Bot.,* **65**: 330-337.

Babula P, Vodicka O, Adam V, Kummerova M, Havel L, Hosek J, Provaznik I, Skutkova H, Beklova M and Kizek R (2012). Effect of fluoranthene on plant cell model: Tobacco BY-2 suspension culture. *Environ. Exp. Bot.,* **78**: 117-126.

Bae JH, Park JW and Kwon TK (2003). Ruthenium red, inhibitor of mitochondrial Ca2+ uniporter, inhibits curcumin-induced apoptosis via the prevention of intracellular Ca2+ depletion and cytochrome c release. *Biochem. Biophys. Res. Commun.,* **303**: 1073-1079.

Bandmann V and Homann U (2012). Clathrin-independent endocytosis contributes to uptake of glucose into BY-2 protoplasts. *Plant J.,* **70**: 578-584.

Bernardi P (1999). Mitochondrial transport of cations: Channels, exchangers and permeability transition. *Physiol. Rev.,* **79**: 1127-1155.

Csollei J, Borovansky A, Benes L, Bederova E and Svec P (1982). Synthesis of new β-adrenolytic agents of the aroxypropanolamine type. *Cesk. Farm.,* **31**: 229-235.

Danon A, Delorme V, Mailhac N and Gallois P (2000). Plant programmed cell death: A common way to die. *Plant Physiol. Biochem.,* **38**: 647-655.

Desikan R, Reynolds A, Hancock JT and Neill SJ (1998). Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on defence gene expression in Arabidopsis suspension cultures. *Biochem. J.,* **330**: 115-120.

Dhanalakshmi T, Suresh E and Palaniandavar M (2009). Synthesis, structure, spectra and reactivity of iron (III) complexes of imidazole and pyrazole containing

ligands as functional models for catechol dioxygenases. *J. Chem. Soc., Dalton Trans.,* **39**: 8317-8328.

Elliott WJ and Ram CVS (2011). Calcium channel blockers. *J. Clin. Hypertens.*, **13**: 687-689.

Friedman H, Meir S, Rosenberger I, Halevy AH, Kaufman PB and Philosoph-Hadas S (1998). Inhibition of the gravitropic response of snapdragon spikes by the calcium-channel blocker lanthanum chloride. *Plant Physiol.,* **118**: 483-492.

Gadjev I, Stone JM and Gechev TS (2008). Programmed cell death in plants: New insights into redox regulation and the role of hydrogen peroxide. *Int. Rev. Cell Mol. Biol.,* **270**: 87-144.

Garnczarska M (2005). Response of the ascorbate-glutathione cycle to re-aeration following hypoxia in lupine roots. *Plant Physiol. Biochem.,* **43**: 583-590.

Gechev T, Gadjev I, Van Breusegem F, Inze D, Dukiandjiev S, Toneva V and Minkov I (2002). Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. *Cell. Mol. Life Sci.,* **59**: 708-714.

Gechev TS and Hille J (2005). Hydrogen peroxide as a signal controlling plant programmed cell death. *J. Cell Biol.,* **168**: 17-20.

Gerasimenko JV, Gerasimenko OV, Palejwala A, Tepikin AV, Petersen OH and Watson AJM (2002). Menadione-induced apoptosis: Roles of cytosolic Ca2+ elevations and the mitochondrial permeability transition pore. *J. Cell Sci.,* **115**: 485-497.

Green DR and Kroemer G (2004). The pathophysiology of mitochondrial cell death. *Science*, **305**: 626-629.

Hajnoczky G, Davies E and Madesh M (2003). Calcium signaling and apoptosis. *Biochem. Biophys. Res. Commun.,* **304**: 445-454.

Hashimoto K and Kudla J (2011). Calcium decoding mechanisms in plants. *Biochimie,* **93**: 2054-2059.

Hepler PK (2005). Calcium: A central regulator of plant growth and development. *Plant Cell*, **17**: 2142-2155.

Houot V, Etienne P, Petitot AS, Barbier S, Blein JP and Suty L (2001). Hydrogen peroxide induces programmed cell death features in cultured tobacco BY-2 cells, in a dose-dependent manner. *J. Exp. Bot.,* **52**: 1721-1730.

Chodkowski A and Gutkowska B (1977). Syntheses of some amides of 1-butyl-4-phenylpiperidine-4-carboxylic and 3-indolylacetic acids. *Rocz. Chem.,* **51**: 1231-1234.

Choi HS, Kim JW, Cha YN and Kim C (2006). A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *J. Immunoassay Immunochem.,* **27**: 31-44.

Ishii N, Matsumura T, Shimoda S and Araki E (2012). Anti-atherosclerotic potential of dihydropyridine calcium channel blockers. *J. Atheroscler. Thromb.*, **19**: 693-704.

Itoh K, Kanzaki K, Ikebe T, Kuroita T, Tomozane H, Sonda S, Sato N, Haga K and Kawakita T (1999). Synthesis and pharmacological evaluation of carboxamide derivatives as selective serotoninergic 5-HT4 receptor agonists. *Eur. J. Med. Chem.,* **34**: 977-989.

Jacobson J and Duchen MR (2002). Mitochondrial oxidative stress and cell death in astrocytes requirement for stored Ca^{2+} and sustained opening of the permeability transition pore. *J. Cell. Sci.,* **115**: 1175-1188.

Jammes F, Hu HC, Villiers F, Bouten R and Kwak JM (2011). Calcium-permeable channels in plant cells. *Febs. J.,* **278**: 4262-4276.

Kim JS, He LH and Lemasters JJ (2003). Mitochondrial permeability transition: A common pathway to necrosis and apoptosis. *Biochem. Biophys. Res. Commun.*, **304**: 463-470.

Kobayashi D, Ahmed S, Ishida M, Kasai S and Kikuchi H (2009). Calcium/calmodulin signaling elicits release of cytochrome c during 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced apoptosis in the human lymphoblastic T-cell line, L-MAT. *Toxicology,* **258**: 25-32.

Kovacik J, Klejdus B, Hedbavny J, Stork F and Backor M (2009). Comparison of cadmium and copper effect on phenolic metabolism, mineral nutrients and stress-related parameters in matricaria chamomilla plants. *Plant Soil,* **320**: 231-242.

Levine A, Tenhaken R, Dixon R and Lamb C (1994). H2o2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell,* **79**: 583-593.

Li JY, Jiang AL, Chen HY, Wang Y and Zhang W (2007). Lanthanum prevents salt stress-induced programmed cell death in rice root tip cells by controlling early induction events. *Journal Integr. Plant Biol.,* **49**: 1024-1031.

Lin JS, Wang Y and Wang GX (2006). Salt stress-induced programmed cell death in tobacco protoplasts is mediated by reactive oxygen species and mitochondrial permeability transition pore status. *J. Plant Physiol.,* **163**: 731-739.

Liu CH, Chao YY and Kao CH (2012). Abscisic acid is an inducer of hydrogen peroxide production in leaves of rice seedlings grown under potassium deficiency. *Bot. Stud.*, **53**: 229-237.

Liu WL and Matsumori A (2011). Calcium channel blockers and modulation of innate immunity. *Curr. Opin. Infect. Dis.,* **24**: 254-258.

Lord CEN and Gunawardena A (2012). Programmed cell death in C. elegans, mammals and plants. *Eur. J. Cell Biol.,* **91**: 603-613.

Marino D, Dunand C, Puppo A and Pauly N (2012). A burst of plant NADPH oxidases. *Trends Plant Sci.,* **17**: 9-15.

Martinez A, Membrillo I, Ugalde-Saldivar VM and Gasque L (2012). Dinuclear copper complexes with imidazole derivative ligands: A theoretical study

related to catechol oxidase activity. *J. Phys. Chem. B.,* **116**: 8038-8044.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant,* **15**: 473-497.

Nagata T, Nemoto Y and Hasetawa S (1992a). Tobacco BY-2 cell-line as the HeLa-cell in the cell biology of higher plants. *A Surv. of Cell Biol.,* **132**: 1-30.

Nagata T, Nemoto Y and Hasezawa S (1992b). Tobacco by-2 cell-line as the hela-cell in the cell biology of higher plants. *Int. Rev. Cytol -A Survey of Cell Biology*, **132**: 1-30.

Orrenius S, Zhivotovsky B and Nicotera P (2003). Regulation of cell death: The calcium-apoptosis link. *Nat. Rev. Mol. Cell Biol.,* **4**: 552-565.

Ott PG, Klement Z, Nage I and Adam AL (2003). Lanthanum inhibits programmed cell death but not resistance in the tobacco - Pseudomonas savastanoi pv. phaseolicold incompatible interaction. *In*: Iacobellis NS editor. *Pseudomonas Syringae and Related Pathogens: Biology and Genetics*. 1st ed., Springer, Dordrecht. pp.335-344.

Palma K and Kermode AR (2003). Metabolism of hydrogen peroxide during reserve mobilization and programmed cell death of barley (Hordeum vulgare L.) aleurone layer cells. *Free Radical Biol. Med.,* **35**: 1261-1270.

Pinton P, Ferrari D, Rapizzi E, Di Virgilio F, Pozzan T and Rizzuto R (2001). The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: Significance for the molecular mechanism of Bcl-2 action. *Embo J.,* **20**: 2690-2701.

Pottosin, II, Dobrovinskaya OR and Muniz J (1999). Cooperative block of the plant endomembrane ion channel by ruthenium red. *Biophys. J.,* **77**: 1973-1979.

Reddy ASN (2001). Calcium: Silver bullet in signaling. *Plant Sci.,* **160**: 381-404.

Rowan NS, Storm CB and Rowan R (1981). Properties of metal-ion coordinated imidazoles nmr and C-2h exchange in Co (III) complexes. *J. Inorg. Biochem.,* **14**: 59-65.

Simeonova E, Garstka M, Koziol-Lipinska J and Mostowska A (2004). Monitoring the mitochondrial trans membrane potential with the JC-1 fluorochrome in programmed cell death during mesophyll leaf senescence. *Protoplasma,* **223**: 143-153.

Singh P, Sharma S and Prasad V (2011). Verapamil, a calcium channel blocker, induces systemic antiviral resistance in susceptible plants. *J. Phytopatol.,* **159**: 127-129.

Tanimoto S (1994). Localization of intracellular free calcium ions during adventitious bud initiation in Torenia stem segments. *Plant Tiss. Cult. Lett.,* **11**: 55-60.

Urbanski NK and Beresewicz A (2000). Generation of (OH)-O-. initiated by interaction of Fe2+ and Cu+ with dioxygen; Comparison with the fenton chemistry. *Acta. Biochim. Pol.*, **47**: 951-962.

Wang J, Liu XR, Su YJ, Li KS and Zhao SS (2011). Synthesis, Characterization and DNA-binding studies of transition metal complexes with imidazole ligands. *Adv. Mater. Res.,* pp.233-235: 175-179.

Wang Y, Lin JS and Wang GX (2006). Calcium-mediated mitochondrial permeability transition involved in hydrogen peroxide-induced apoptosis in tobacco protoplasts. *J. Integr. Plant Biol.,* **48**: 433-439.

White PJ (1998). Calcium channels in the plasma membrane of root cells. *Ann. Bot.,* **81**: 173-183.

White PJ (2000). Calcium channels in higher plants. *BBA-Biomembranes,* **1465**: 171-189.

White PJ (2004). Calcium signals in root cells: The roles of plasma membrane calcium channels. *Biologia,* **59**: 77-83.

Wilkinson RE, Duncan RR and Berry C (1994). Diltiazem influence on mineral nutrient-uptake by sorghum primary root tips. *J. Plant Nutrition,* **17**: 2177-2183.

Williams B and Dickman M (2008). Plant programmed cell death: Can't live with it; can't live without it. *Mol. Plant Pathol.,* **9**: 531-544.

Yue SM, Li N, Bian JY, Hou TT and Ma JF (2012). Synthesis, crystal structure and luminescent properties of transition metals complexes based on imidazole derivatives. *Synthetic Met.*, **162**: 247-256.