VILNIUS UNIVERSITY

RASA JARAŠIENĖ – BURINSKAJA

MOLECULAR MECHANISMS OF QUINONE AND POLYPHENOL INDUCED MOUSE HEPATOMA CELL DEATH

Summary of doctoral dissertation

Physical sciences, biochemistry (04P)

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VILNIAUS UNIVERSITETAS

RASA JARAŠIENĖ – BURINSKAJA

MOLEKULINIAI CHINONŲ IR POLIFENOLIŲ SUKELTOS PELĖS HEPATOMOS LĄSTELIŲ ŽŪTIES MECHANIZMAI

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Introduction

Cancer is one of the leading causes of mortality worldwide. Many researchers globally are seeking effective treatments that would specifically target and kill or at least suppress the proliferation of malignant cells. The goal of such research is to discover selective chemotherapeutic substances – those with high specific toxicity towards tumor cells and low toxicity towards healthy cells.

This work is aimed at investigating the mechanisms of quinone and polyphenol toxicity towards malignant cells. Many quinones (ubi- and plastoquinones) work as electron transfer molecules in various organisms, while various microorganisms and plant-synthesized naphto- and anthraquinones often perform protective functions, associated with oxidative stress, i. e. they speed up the production of reactive oxygen species (ROS) that are produced when reoxidating quinone free radicals.

The anti-tumor activity of quinones was known since the 1960s. The effectiveness of anthracyclines and mitomycins, which are used in chemotherapy, is based on their ability to induce oxidative stress. Anthracyclines also intercalate into the DNA, thus damaging its structure, while products of mitomycin reduction covalently modify DNA. Another important class of compounds, investigated since the 1970s are aziridinyl-substituted benzoquinones. In addition to free radical-induced oxidative stress, these compounds tend to have other mechanisms of activity: DNA alkylation by their hydroquinones, and direct DNA alkylation by low redox potential aziridinyl-benzoquinones. Even though clinical trial results of these compounds have been controversial due to side effects, the results of pre-clinical and stage I clinical trials (2005 – 2011) of aziridinyl-benzoquinone RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone), synthesized in 1996, were encouraging.

In the context of chemotherapy, an important process is slow (auto)oxidation of reduced quinones (hydroquinones), which is associated with the cytotoxicity of polyphenol compounds (polyhydroxybenzene and flavonoid), which have antioxidant properties. These compounds are often considered as useful food components.

In this work, the cytotoxicity mechanisms of three interrelated groups of compounds, aziridinyl-substituted quinones, aziridinyl-unsubstituted quinones and polyphenols, were investigated, with specific emphasis on the expression and role of signaling proteins. The research was carried out using a malignant growth model of mouse hepatoma MH-22A cell line, which is widely used in cancer research and anti-cancer medication screening.

The aim of this work is to determine possible mechanisms of cytotoxicity of aziridinyl-substituted benzoquinone RH1 and polyphenol quercetin (QUE, 3,3,4,5,7-pentahydroxyflavine) in MH-22A cells and to assess the role of signaling molecules in the process of cell death.

The goals of the study:

1. Assess the cytotoxicity of the chosen quinone and polyphenol xenobiotics and determine the most efficient ones.
2. Determine the cytotoxic efficiencies of aziridinyl-substituted benzoquinone RH1, aziridinyl-unsubstituted quinone duroquinone (DQ) and polyphenol QUE, and the type of cell death in mouse hepatoma MH-22A cells.
3. Determine the expression and activity of NQO1 in MH-22A cells.

4. Evaluate the roles of NQO1 and oxidative stress on RH1, DQ and QUE- induced cell death.
5. Determine the effects of DNA damage in RH1, DQ and QUE- induced cell death.
6. Investigate the roles of mitogen-activated protein kinases (MAPKs) in MH-22A cell response to the test compounds.
7. Investigate the role of transcription factor p53 in RH1, DQ and QUE-induced cell death.

Relevance and novelty

The relevance of this work is associated with possible use of aziridinyl-benzoquinone in tumor chemotherapy. Aziridinyl-substituted benzoquinones have been investigated since 1970 and their initial stages of anti-tumor activity (NQO1 bioreductive oxidation and oxidative stress) are well understood (Šarlauskas *et al*, 2015; Di Francesco *et al*, 2004; Nemeikaitė-Čėnienė *et al*, 2003; 2015). However, information on later stage mechanisms of cytotoxicity is still lacking – regarding signaling protein expression and their roles in cell death. Since quinone cytotoxicity is in part associated with their reduced form (polyphenol) oxidation that generates ROS, polyphenol cytotoxicity was assessed as well.

The novelty of this work is in the elucidation of the expression and roles of signaling proteins during MH-22A cell death upon treatment with aziridinyl-substituted and aziridinyl-unsubstituted benzoquinones and polyphenol quercetin.

The roles of mitogen-activated protein kinase (MAPK) and transcription factor p53 signaling pathways were investigated during MH-22A cell death after treatment with RH1 and QUE for the first time. We determined that possible antiapoptotic ERK activity was response to oxidative stress. Stress-activated protein kinase JNK was responsible for MH-22A cell survival after treatment with RH1, while in the case of QUE, it took part in induced cell death. p38 kinase, contrary to JNK, participated in transducing the cell death signal after treatment with RH1 – proapoptotic activity of p38 was registered, while after QUE treatment, a neutral effect of this kinase was found. We also found that p53 expression is uncommon in MH-22A cells, but it was upregulated by RH1. We determined that after treatment with RH1, the p53 protein can lead to cell survival, while after QUE treatment – promote cell death.

Dissertation Contents. The dissertation is written in Lithuanian and contains the following parts: Introduction, List of Publications, Literature Review, Materials and Methods, Results and Discussion, Conclusions, List of References (218 entries), Figures (47), 112 pages total.

Materials and Methods

Reagents. BSA, Triton X-100, acrylamide, bis-acrylamide, Tris base, glycine, EDTA, Tris-HCl, tetramethyl ethylenediamine (TEMED), methanol, sodium dodecyl sulfate (SDS), nonfat-dried bovine milk, DMSO, aprotinin, phenylmethylsulfonyl fluoride (PMSF), glycerol, mercaptoethanol, ammonium persulphate (AP), Tween 20, inorganic reagents, flavonoids, polyphenols, aziridinyl-unsubstituted quinones, folin reagent, NADH, cytochrome c, DIC (dicoumarol), DPPD (N, N'-diphenyl-p-phenylenediamine), DESF (desferroxamine) phosphate buffered saline (PBS) were purchased from Sigma-Aldrich, USA. Bradford Reagent was obtained from Thermo Fisher Scientific, USA. Chemiluminescent Crystalline Solutions were from Invitrogen, USA. Aziridinyl-substituted 1,4-benzoquinones were synthesized by Dr J. Šarlauskas

(Institute of Biochemistry). **Dyes and signal molecule inhibitors:** MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), acridine orange/ethidium bromide mixture, trypan blue, PD98059, SP600125, SB203580, PIF-α were obtained from Sigma-Aldrich, USA. **Antibodies:** Anti-ERK, P-p38, P-p53 and NQO1 were from Cell Signaling Technologies, Beverly, USA. P-ERK, p38, p53 - from Santa Cruz Biotechnology, Santa Cruz, CA, USA. JNK1/2 was from BD BioScience, San Jose, CA, USA. Secondary goat anti-mouse and goat anti-rabbit antibodies (both conjugated with horseradish peroxidase) were obtained from Abcam, Cambrige, UK. All antibodies were diluted according to manufacturer's recommendations.

Cell culture. MH-22A cells were obtained from VU Biochemistry Institute Cell Collection, and human melanoma SK-MEL-28 cells and human promyelocytic leukemia HL-60 cells were obtained from Cell Line Service, Germany.

MH-22A and SK-MEL-28 cells were grown in DMEM, while HL-60 cells were grown in IMDM, supplemented with 10 % FBS and antibiotics. Cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2, and passaged twice a week by detaching the cells with trypsinisation. For cytotoxicity assessment, MH-22A cells were seeded at a density of 3.0×10^5 cells/ml in 96-well flat bottom plates. After reaching 60–80 % confluency, the cells were treated with test substances. The inhibitors were added to the culture 30 min prior to test compound treatment.

Cell viability analysis. After 24 h incubation with test compounds, the cells were washed with PBS and their viability was assessed by MTT method. Cells were treated with MTT (0.2 mg/ml in PBS) for 1 h at 37 °C. The formazan crystals formed in the intact cells were dissolved in ethanol, and the absorbance was measured at 570 nm using an Varioskan Flash (Thermo Scientific, USA) plate reader. The results were expressed as average of relative cell viability (treatment/control). Alterrnatively, the cell viability was examined by trypan blue exclusion test, using a light microscope (Olympus CX 41, Olympus corp., Japan).

Cell death assay. The apoptotic index was determined using acridine orange (AO) and ethidium bromide (EB) staining (Mercille and Massie, 1994). In this case, the cells were grown for 24 h at concentration of RH1, MeDZQ, QUE and DQ causing 50 % cell survival. Types of cell death were also analyzed with fluorescence microscopy (Olympus IX51, Olympus corp., Japan). 3 x 100 cells were counted in each sample. Cell death type was also identified by flow cytometry (Guava easyCyte 8HT, Millipore, Life-Science-Research, USA). After treatment with quinones, 180 µl cell suspension (100-500 cells/µl) was mixed with 20 µl ViaCount (Millipore) dye, incubated for 15 min and then analyzed using flow cytometry.

Comet assay.

Single-cell gel electrophoresis (Comet assay) was used for DNA damage measurement. After 24 h treatment with DQ, RH1 and QUE (cell viability >80 %), the cells were detached by trypsinization, and diluted to 50-60 x 10^3 /ml. Cell suspension (40 µl) was mixed with 40 µl of 1 % low melting-point agarose and transferred onto a microscope slide precoated with 1 % standard agarose, and covered with a cover glass slide. The slides were kept at 4 °C for 10 min. Afterwards, the cover glass slides were immersed into a lysis buffer (10 mM Tris-Cl, pH 10.0, 2.5 M NaCl, 100 mM EDTA, 1 % Triton X-100, and 10 % DMSO) for 90 min at 4 °C, and finally washed by distilled water for 5 min. After lysis, the slides were subjected to electrophoresis in the COMET-20 SYSTEM (Scie-Plas, UK), at 300 mA, 20-19 V, in 300 mM NaOH, 1 mM EDTA, at 4 °C. Then the slides were neutralized by 0.4 M Tris-HCl, pH 7.5 for 30 min, stained with 80 µl ethidium bromide for 5 min, and washed off by distilled water. The samples were analyzed using a fluorescent microscope (Nikon Eclipse 80i, Nikon corp., Japan) with a magnification of 400x. The computerized image analysis system Lucia was used to determine the percentage of DNA in the comet tail as the most validated index of DNA damage. We analyzed 100 nuclei in each sample.

Determination of protein content. The protein content was determined by Lowry (Lowry *et al*., 1951) and Bradford (Bradford, 1976) methods.

Ultrasound destruction of cells. After a 24 h treatment with test compounds, the cells were detached with a mixture of trypsin and EDTA. The resulting suspension was centrifuged for 15 min at 1500 rpm. The cells were transferred into a buffer (0.1 M KH_2PO_4, 1 mM EDTA, 20 mM PMSF, pH 7.0) and lysed using a Bandelin Sonopuls HD 2070 (BANDELIN electronic, Germany) apparatus (4 x 20 s, 20 s pauses). Finally, the samples were centrifuged at 4 °C for 45 min at 10 000 rpm, and kept at -20 °C.

NQO1 activity assessment in MH-22A cells. The activity of NQO1 in MH-22A cell lysates was measured spectrophotometrically at 550 nm using Hitachi-557 (Japan) UV-VIS spectrophotometer. The reaction mixture (2 ml, 0.1 M K-phosphate, pH 7.0, 25 ^oC) contained 10 μM menadione, 50 μM NADH, 50 μM cytochrome C and 50 μl cell lysate. Each measurement was repeated by adding a NQO1 inhibitor, dicoumarol (20 μM), into the reaction mixture. The activity of NQO1 is expressed as nm reduced cytochrome c / min*mg protein.

Western blot analysis. Using western blot analysis, we evaluated the effect of RH1, QUE and DQ on MAPK kinases and protein p53 expression and phosphorylation. Cells were lysed with ice-cold lysis buffer (10 mM Tris HCl pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 % BSA, 1 % Triton X-100, 1 mM PMSF, 2 mM Na_3VO_4, 20 μg/ml aprotinin, pH 7.2). Insoluble materials were removed by centrifugation at 14 000 rpm for 15 min at 0-4 °C. The resulting supernatant was mixed with equal volume of 4 × SDS sample buffer. The amount of protein was estimated by the Bradford method. Then, SDS-PAGE (10 % acrylamide gels) was performed and the separated proteins were transferred to a PVDF membrane. The blots were blocked with the following buffer: 3 % low fat milk in TBST (4 mM Tris HCl, 1 mM Tris base saline, 0.1 % Tween 20, 154 mM NaCl) for 30 min at room temperature. The membranes were washed in TBST 3 x 5 min, probed first with the primary antibody in 5 % low fat milk in TBST for 24 h at 4 °C, and then with the secondary antibody in the same 5 % low fat milk in TBST for 1 h at room temperature. After incubation, membranes were washed 3 x 5 min in TBST. Proteins were visualized using an Amersham ECL system.

Statistical analysis. The data was statistically analyzed using Microsoft Office Excel 2010. The data are expressed as either representative results or as a mean of at least three independent experiments ± SD. Statistical analyses were performed using the Student's t-test. Differences were considered statistically significantwhen $p < 0.05$. Multi-parametric regression analysis was performed using Statistica (StatSoft, version 4.3).

RESULTS

Assessment of quinone and polyphenol cytotoxicity in MH-22A cells. The cL_{50} values of quinones, flavonoids and hydroxybenzenes in MH-22A cells are summarized in *Fig. 1, 2* and *Tables 1,2*. For further research, aziridinyl-benzoquinone RH1 and polyphenol QUE were chosen. For a model aziridinyl-unsubstituted quinone, 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone, DQ) was chosen. In some cases, another aziridinyl-substituted benzoquinone MeDZQ (*Table 1*) was used.

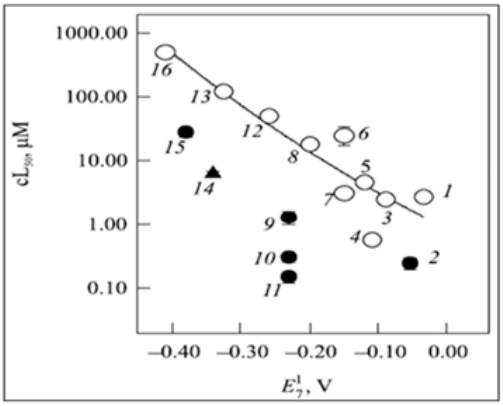

Fig. 1. **Dependence of cL_{50} of aziridinyl-unsybstituted quinones (blank circles), aziridinyl-substituted quinones (solid circles), and daunorubicin (solid triangle) in MH-22A cells on the redox potentials of quinone/semiquinone couples of quinones at pH 7.0 (E^1_7).** The numbers of compounds correspond to those given in *Table 1*.

Compound cytotoxicity was assessed using MTT after a 24 hour treatment.

Table 1. Redox potentials of quinone/semiquinone couples of quinones at pH 7.0 (E^1_7) (Wardman *et al*, 1989 and Di Francesco *et al*, 2004) and their concentrations for 50% survival of MH-22A cells during a 24 h incubation (cL_{50}).

No.	Compound	E^1_7V	cL_{50}μM
1.	2,3-dichlor-1,4-naphthoquinone	-0.035	2.7 ± 0.1
2.	DZQ	-0.054	0.25 ± 0.05
3.	5-hydroxy-1,4-naphthoquinone	-0.09	2.5 ± 0.04
4.	5,8-dihydroxy-1,4-naphthoquinone	-0.11	0.58 ± 0.05
5.	9,10-phenanthrene quinone	-0.12	4.6 ± 0.3
6.	Trimethyl-1,4-benzoquinone	-0.15	25.0 ± 8.0
7.	1,4-naphthoquinone	-0.15	3.1 ± 0.05
8.	2-methyl-1,4-naphthoquinone	-0.20	18.0 ± 1.3
9.	Trimethyl-aziridinyl-1,4-benzoquinone	-0.23	1.3 ± 0.3
10.	MeDZQ	-0.23	0.31 ± 0.05
11.	RH1	-0.23	0.12 ± 0.02
12.	Tetramethyl-1,4-benzoquinone	-0.26	59.0 ± 5.0
13.	1,8-dihydroxy-1,4-anthraquinone	-0.325	120 ± 15.0
14.	Daunorubicin	-0.34	5.9 ± 0.5
15.	BZQ	-0.38	28.0 ± 4.0
16.	2-hydroxy-1,4-naphthoquinone	-0.41	500 ± 80

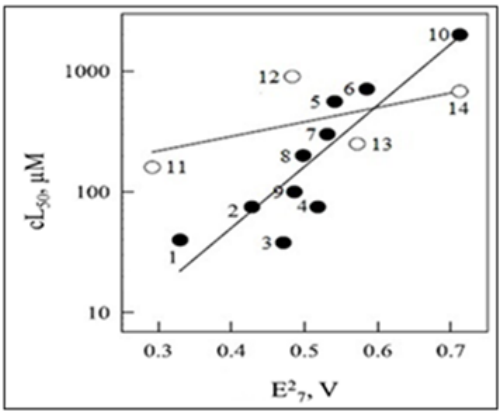

Fig. 2. **Dependence of hydroxybenzene (solid circles) and flavonoid (blank circles) cytotoxicity in MH-22A cells on their single-electron oxidation potential, E^2_7.** The numbers of compounds are taken from *Table 2*.

Compound cytotoxicity was assessed using MTT after a 24 h treatment.

Table 2. Polyphenol redox potentials of phenoxyl radical/phenol couples at pH 7.0 (E^2_7) (Wardman *et al.,* 1989 and Jovanovic *et al*, 1998) and their concentrations for 50% survival of MH-22A cells during 24 h incubation (cL_{50}).

No.	Compound	E^2_7(V)	cL_{50}μM
	Hydroxybenzenes		
1.	Di-3,5-t-butylcatechol	0.39	40 ± 6μM
2.	t-butilhydroquinone	0.46	75 ± 12μM
3.	4-methylcatechol	0.46	45 ± 5μM
4.	Catechol	0.53	75 ± 15μM
5.	Caffeic acid	0.54	560 ± 60μM
6.	Methylgallate	0.56	450±30μM
7.	Ethylgallate	0.56	300 ± 50μM
8.	Bis(1,6- hexanediol)- gallate	0.56	200 ± 40μM
9.	Octylgallate	0.56	100 ± 20μM
10.	4-methoxyphenol	0.73	≥2000μM
	Flavonoids		
11.	Quercetin	0.33	140 ± 25 μM
12.	Taxifolin	0.50	900 ± 100μM
13.	Morin	0.60	250 ± 35μM
14.	Kaempferol	0.75	680 ± 80μM

Quinone and polyphenol induced cell death. Before investigating the mechanisms of activity, we first determined the types of cell death induced by RH1, MeDZQ, QUE and DQ in MH-22A cells.

At concentrations of all compounds equal to cL_{50}, AO/EB staining results show that RH1, MeDZQ and QUE induce larger percentage of apoptotic cells than DQ (*Fig. 3*). A similar tendency was observed in analyzing cell death using flow cytometry.

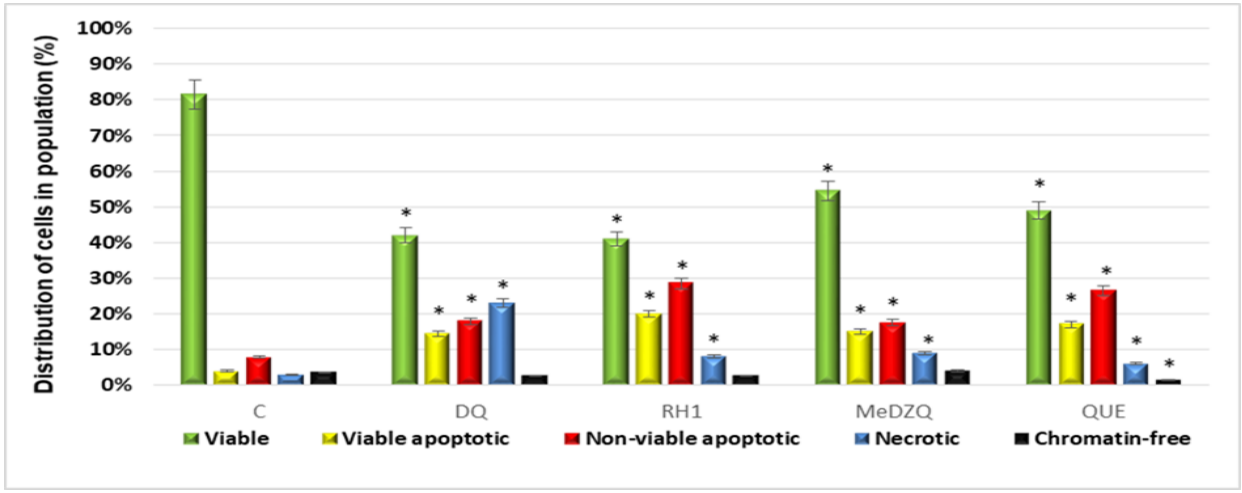

Fig. 3. **Cell death analysis after 24 hours of DQ (59 µM), RH1 (0.12 µM), MeDZQ (0.31 µM) and QUE (140 µM) treatment.** AO/EB staining. Exposure duration - 24 hours. *Statistically significant differences between control cells (no treatment) and DQ, RH1, MeDZQ and QUE-treated cells ($p < 0.05$).

The role of NQO1 in MH-22A cell death. The activity of NQO1 in MH-22A cells is 79.5 ± 7.5 nmol × mg protein^{-1} × min^{-1}. Its inhibitor dicoumarol (DIC) increased cell survival after treatment with all investigated compounds except DQ, where the effect was statistically insignificant. We also found that in the presence of DIC the number of viable and dead apoptotic cells was decreased, but the number of necrotic cells remained similar (*Fig. 4*). Analogous results were obtained by flow cytometry.

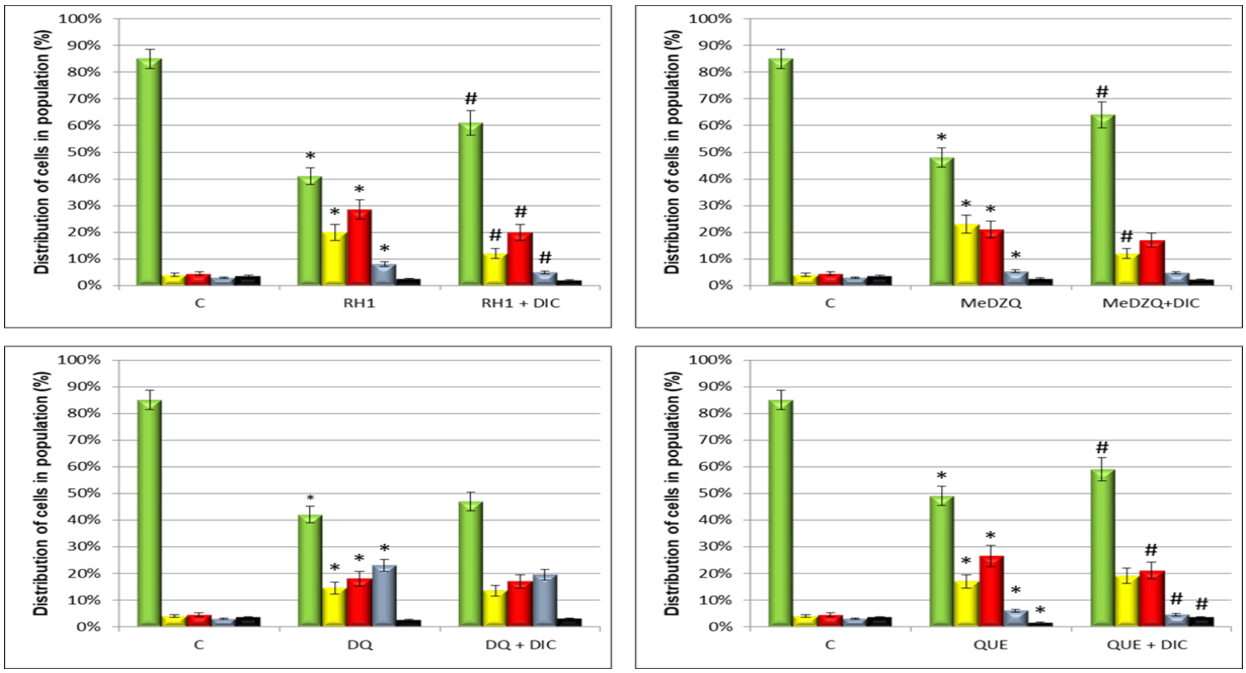

Fig. 4. **Assessment of DIC influence on MH-22A cell death.** AO/EB staining after treating the cells with DQ (59 µM) and DQ (59 µM) + DIC (20 µM); RH1 (0.12 µM), RH1 (0.12 µM) + DIC (20 µM); MeDZQ (0.31 µM); MeDZQ (0.31 µM) + DIC (20 µM) and QUE (140 µM), QUE (140 µM) + DIC (20 µM). *Statistically significant differences between control (untreated cells) and DQ, RH1, MeDZQ and QUE treated cells ($p < 0.05$). # Statistically significant differences between cells treated with DIC and one of the compounds and cells treated solely with DQ, RH1, MeDZQ and QUE ($p < 0.05$).

The role of oxidative stress during RH1 and QUE action. To investigate the role of oxidative stress in cell death, we used two antioxidants *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) and desferrioxamine (DESF) (Shimoni *et al.*, 1994; Nemeikaitė-Čėnienė *et al.*, 2005). Results were compared to cell viability after treatment with DQ and a known oxidative stress inducer - hydrogen peroxide (H_2O_2). We found that the above antioxidants protected against the action of all compounds investigated (*Fig. 5*).

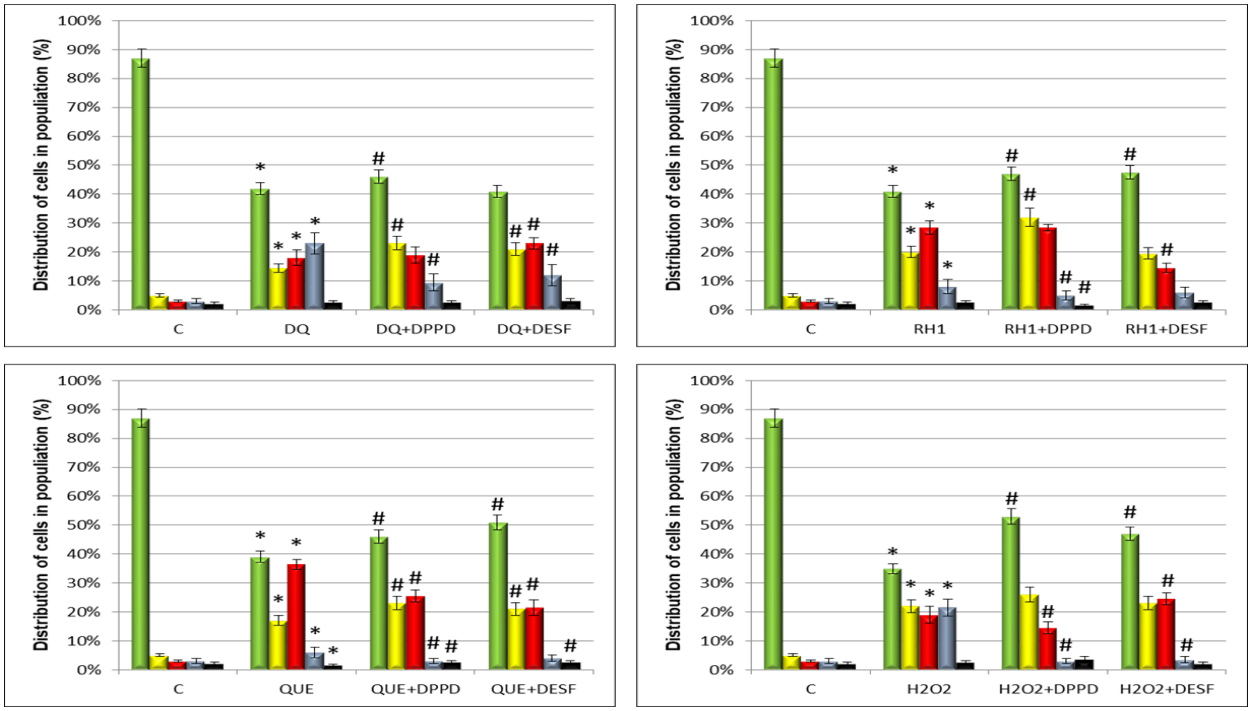

***Fig. 5*. MH-22A cell death analysis using AO/EB staining.** MH-22A cells were exposed for 30 minutes with DPPD (2 μM) and DESF (300 μM), followed by addition of the test substance DQ (59 μM), RH1 (0.12 μM), QUE (140 μM) and H_2O_2 (200 μM). *Statistically significant differences between control (cells without exposure) and DQ, RH1, QUE and H_2O_2 - treated cells ($p < 0.05$). # Statistically significant differences between cells treated with DPPD or DESF and one of the compounds and cells treated solely with DQ, RH1, QUE and H_2O_2 ($p < 0.05$)

Analogous results were obtained when assessing cell viability using flow cytometry and trypan blue staining.

DNA damage during the action of RH1 and QUE. In order to evaluate the effects of the test compounds on the amount of DNA damage, we used the comet method. In this case the cell viability should be >80 %. Thus, lower compound were used, 25 μM DQ, 0.03 μM RH1, and 75 μM QUE.

It is clear that QUE and RH1 damaged DNA stronger than DQ (*Fig. 6*). We found that DIC significantly decreased the DNA damage by RH1 and QUE, but almost did not affect its damage by DQ (data not shown). Next, the data of *Fig. 7* show that while DPPD and DESF protect against the DNA damage by RH1, their role in protection against QUE is equivocal.

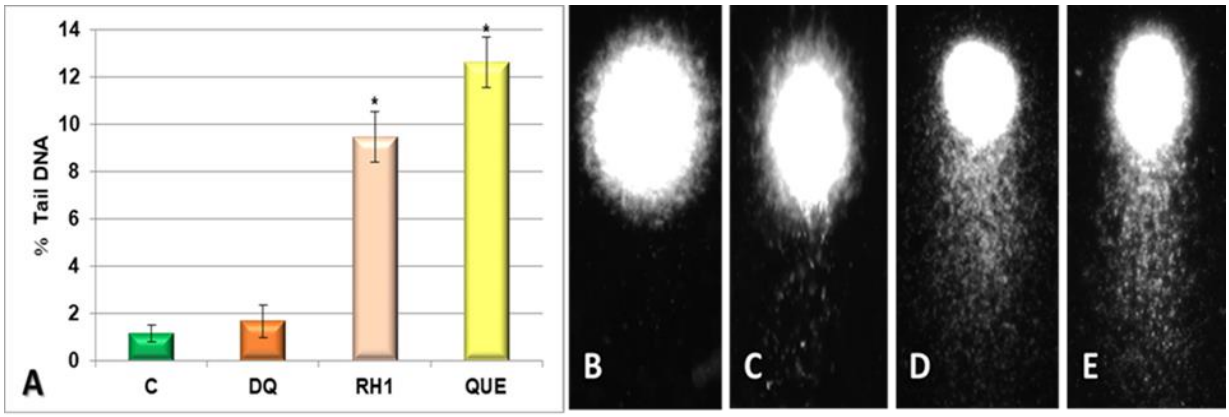

Fig. 6. **Percentage points of DNA in the comet tail after treatment with DQ, RH1 and QUE:** A – quantitative evaluation of DNA in the comet tail after 24 hours effects on the test compounds: DQ (25 μM), RH1 (0.03 μM) ir QUE (75 μM); B-E comet images (B - control (cells without exposure), hereinafter referred to as the effects of the compounds under study: C - DQ, D – RH1, E – QUE. *Statistically significant differences between control (cells without exposure) and DQ, RH1 and QUE treated cells ($p < 0.05$).

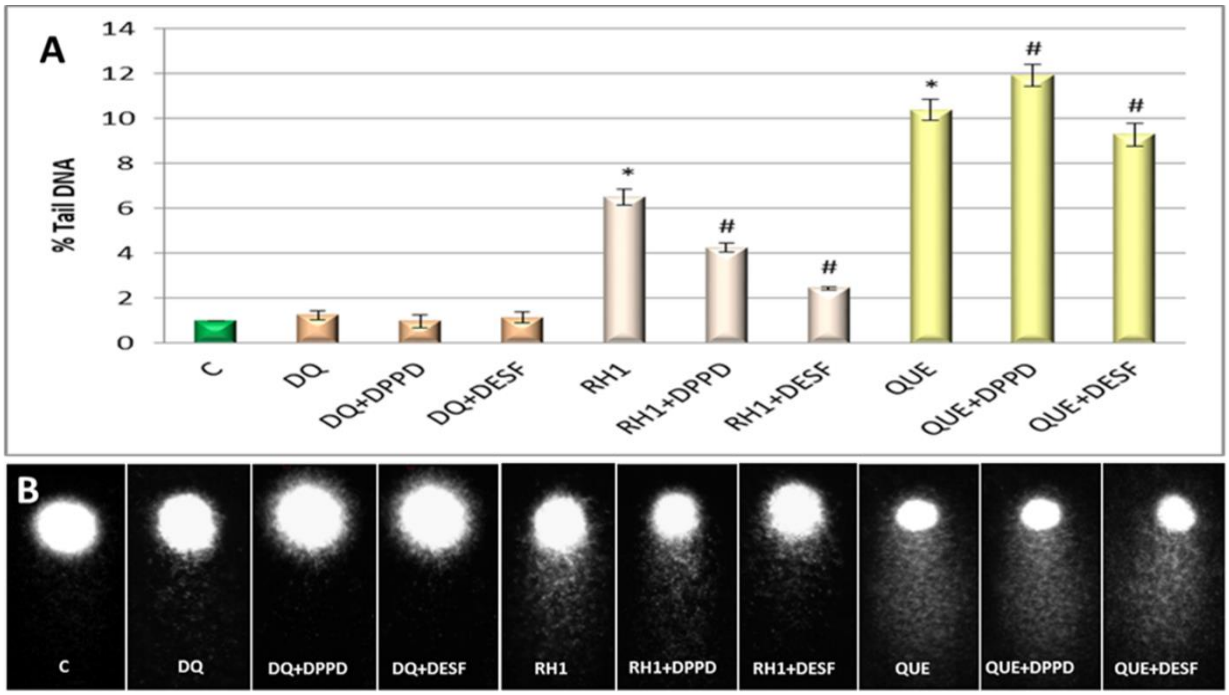

Fig. 7. **The role of oxidative stress in the formation of DNA damage.** Anti-oxidant DPPD (2 μM) and DESF (300 μM) were exposed to MH-22A cells for 30 minutes before adding the test substances - DQ (25 μM), RH1 (0.03 μM) and QUE (75 μM). A - quantitative evaluation of DNA in the comet tail after 24 hours effects on the test compounds: DQ, RH1 and QUE; B - comet images. *Statistically significant differences between control (cells without exposure) and DQ, RH1 and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with DIC and one of the compounds and cells treated solely with DQ, RH1 and QUE ($p < 0.05$).

The role of MAP kinases in RH1 and QUE-induced MH-22A cell death. MAPK signaling pathway is one of the best known signaling pathways in response to extracellular stimuli including the oxidative stress (Huang *et al.*, 2010). Therefore, we evaluated the expression of ERK, JNK, p38 and MAP kinases and its roles in MH-22A cell viability in the presence of investigated compounds.

The studies of ERK. First we evaluated the effect of RH1, QUE, and DQ on ERK expression and phosphorylation. ERK1/2 expression and phosphorylation remains unaltered, except that after 24 h RH1 treatment phospho-ERK1/2 level increases**,** while it decreases during 24 h QUE treatment (*Fig. 8*). MeDZQ did not affect ERK1/2 expression and phosphorylation (data not shown).

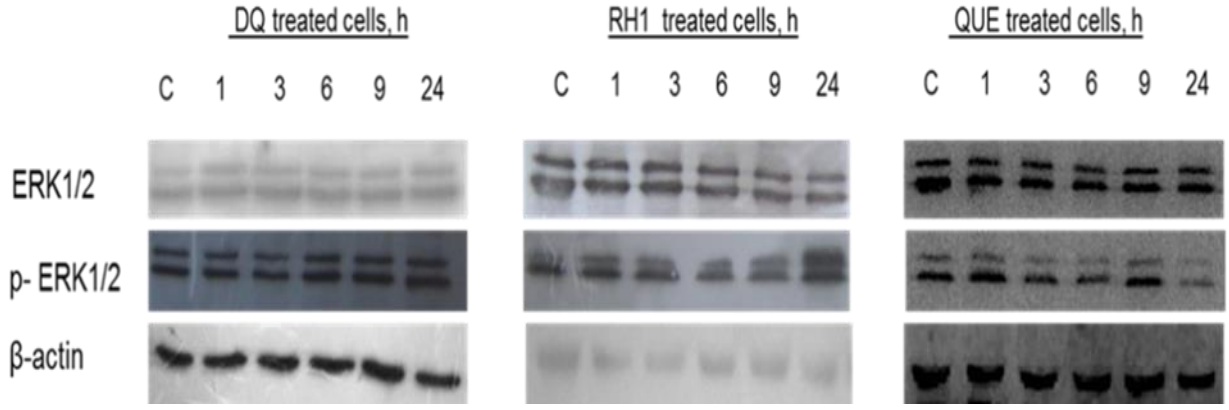

Fig. 8. **ERK1/2 and phospho-ERK1/2 protein expression in MH-22A cells after 24 hours of treatment with DQ (59 M), RH1 (0.12 μM) and QUE (140 μM).**

Next, we evaluated the role of ERK kinase by using its indirect inhibitor PD98059 which acts on ERK by inhibiting MEK1/2 activity. However, PD98059 almost did not influence MH-22A cell viability in the presence of RH1, MeDZQ, QUE and DQ. The inhibitor of NQO1 dicoumarol reduced the level of ERK1/2 expression only in the case of aziridinyl-unsubstituted DQ treatment, and decreased the level of its phosphorylation durin the action of DQ and QUE (*Fig. 9*).

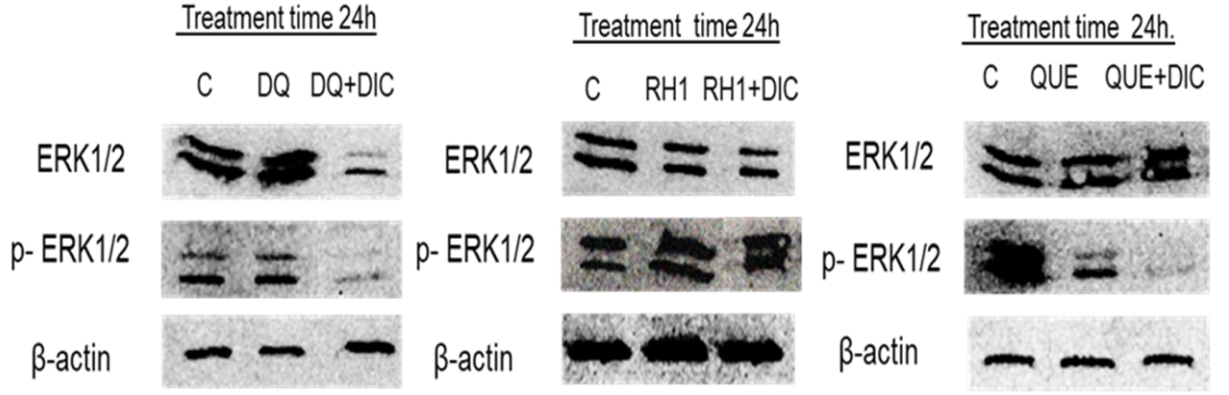

Fig. 9. **ERK1/2 and phospho-ERK1/2 protein levels in MH-22A cells after 24 hours of treatment with DQ (59 μM), RH1 (0.12 μM), QUE (140 μM) and DIC (20 μM) inhibitor.** MH-22A cells were exposed for 30 minutes with DIC (20 μM), followed by addition of the test substance.

The antioxidants DPPD and DESF did not affect ERK1/2 level after RH1, QUE and DQ treatment (*Fig. 10*). On the other hand, DPPD decreased its level in the case of H_2O_2. and DPPD treatment had a significant effect on the reduction of ERK1/2 protein. On the other hand, after QUE and DQ treatment, the antioxidants decreased the expression of phospho-ERK, but did not change the level of ERK 1/2 phosphorylation under the action of RH1.

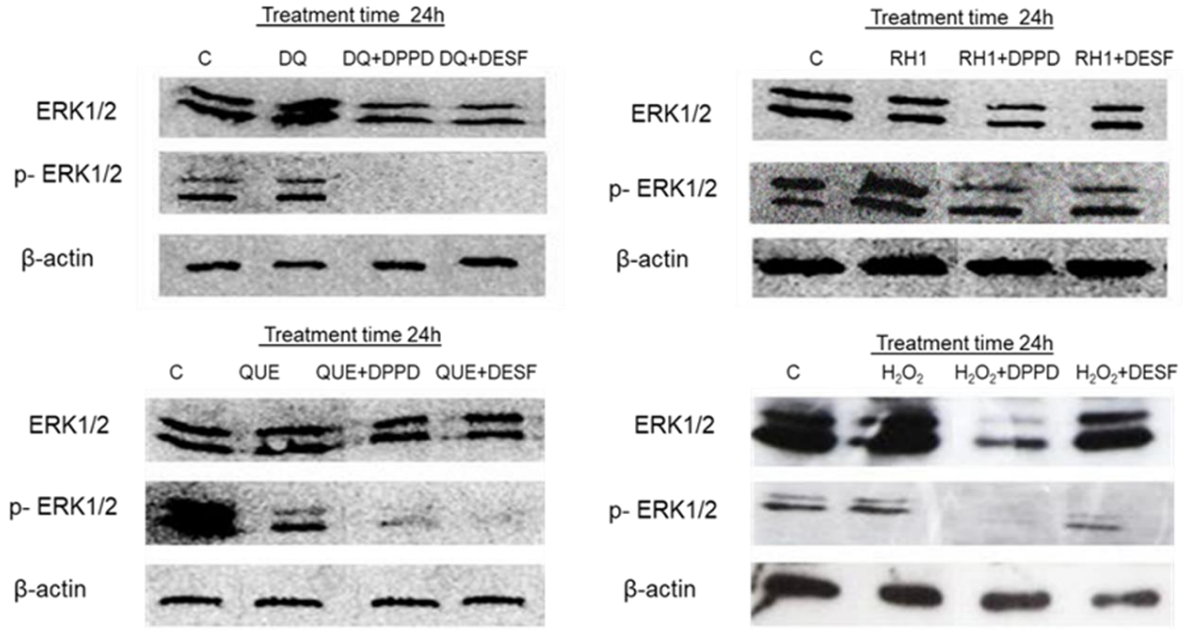

Fig. 10. **The effect of oxidative stress on ERK1/2 and phospho-ERK1/2 protein levels in MH-22A cells.** Cells were exposed for 30 minutes with DPPD (2 μM) and DESF (300 μM), followed by addition of the test substance: DQ (59 μM), RH1 (0.12 μM), QUE (140 μM), H_2O_2 (200 μM).

Finally, an indirect ERK1/2 inhibitor PD98059 did not affect the DNA damage by RH1 and DQ, but slightly protected against the action of QUE (data not shown).

The studies of JNK. The data of *figure 11* show that DQ, RH1 and QUE did not change the level of JNK1/2 during 24 h. On the other hand, the level of its phosphorylation starts to decrease after 6 h in all the cases. The analogous results were observed with MeDZQ (data not shown). In order to determine the role of JNK signaling pathway in the death of MH-22A cells, we used a competitive JNK inhibitor SP600125, which directly inhibits further signal transduction, thus disabling the activation of one of the main downstream targets in the nucleus c-Jun (Cargnello and Roux, 2011; Bennett *et al.*, 2001). SP600125 itself had no effect on the viability of the cells (data not shown).

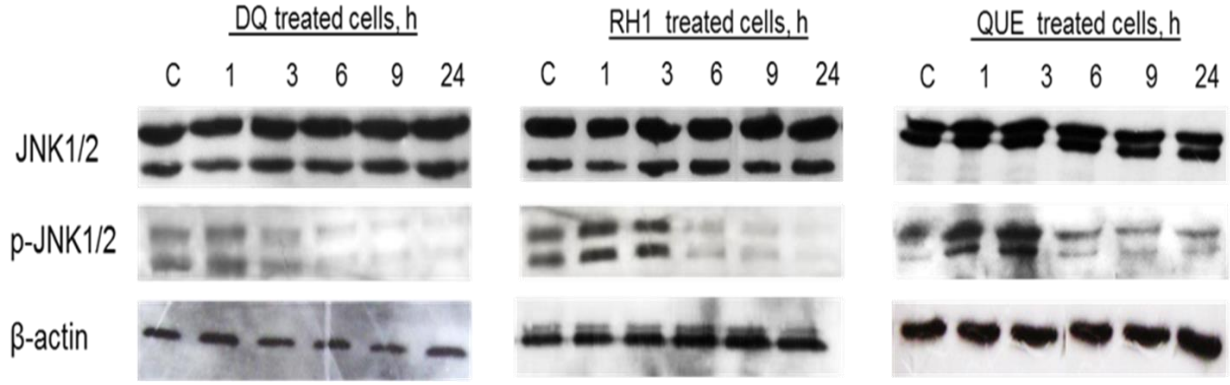

Fig. 11. **JNK1/2 and phospho-JNK1/2 protein expression levels in MH-22A cells after 24 hour treatment with DQ (59 μM), RH1 (0.12 μM) and QUE (140 μM).**

Figure 12 shows that SP600125 reliably decreased cell viability after DQ, RH1 and MeDZQ treatment. However, it protected the cells agaist the action of QUE.

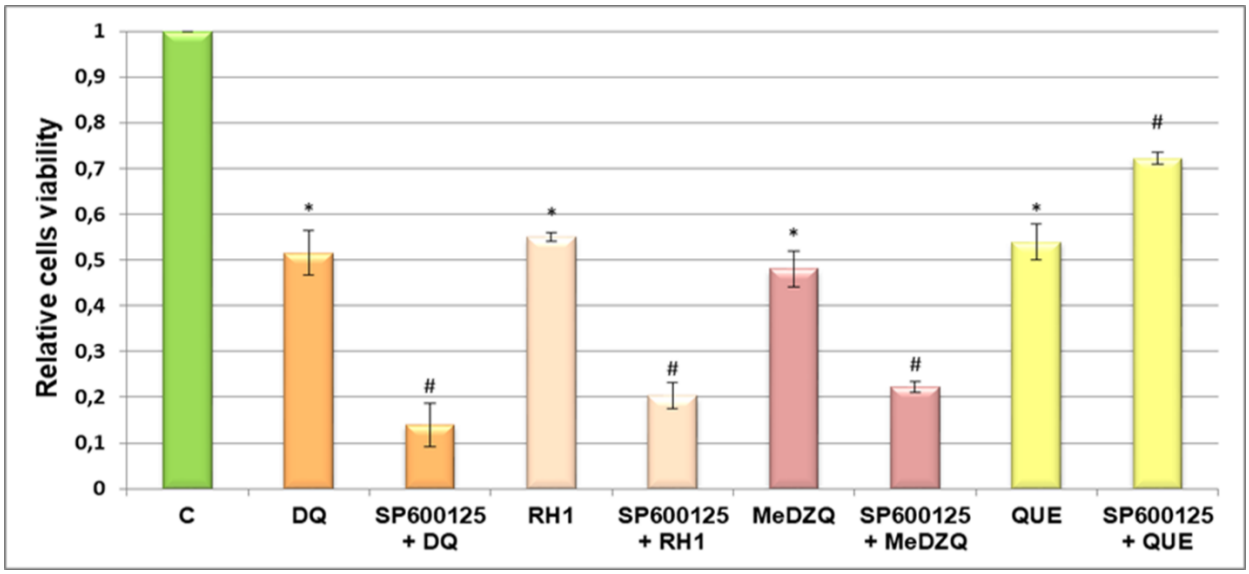

Fig. 12. **Influence of JNK kinase inhibitor SP600125 on the activity of MH-22A cells with the investigated compounds.** MH-22A cells were exposed for 30 minutes with an inhibitor SP600125 (20 μM), followed by addition of the test substance DQ (59 μM), RH1 (0.12 μM), MeDZQ (0,31 μM) and QUE (140 μM). The effect was assessed using MTT method. *Statistically significant differences between control (cells without exposure) and DQ, RH1, MeDZQ and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with SP600125 and one of the compounds and cells treated solely with DQ, RH1, MeDZQ and QUE ($p < 0.05$).

DIC reliably decreased the expression and phosphorylation of JNK after DQ treatment, but it did not affect the effects of RH1 and QUE (*Fig. 13*).

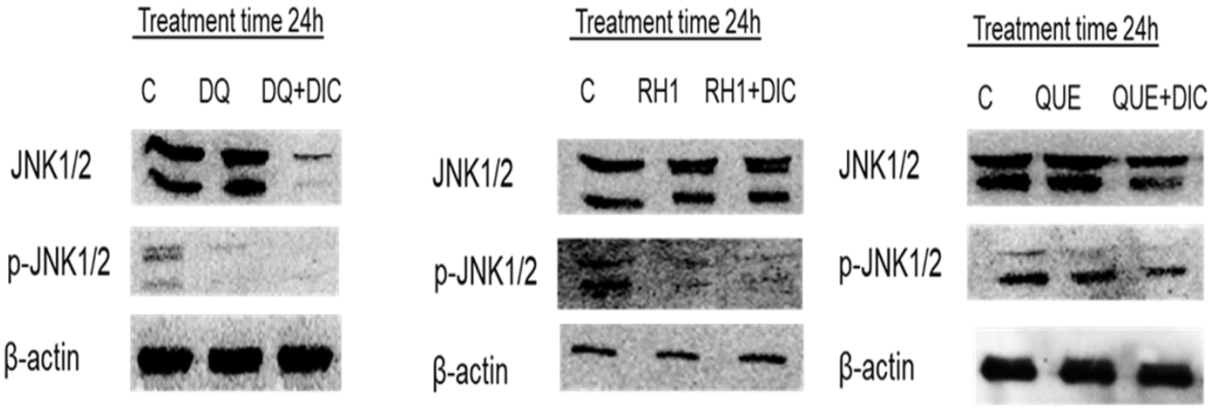

***Fig. 13*. The effect of DIK on JNK1/2 and phospho-JNK1/2 protein levels in MH-22A cells after 24 hour treatment.** The NQO1 inhibitor DIC (20 μM) was exposed to MH-22A cells for 30 minutes before adding the test substances DQ (59 μM), RH1 (0.12 μM) and QUE (140 μM).

The antioxidants DPPD and DESF did not influence JNK1/2 expression after DQ and RH1 treatment, but reduced it after the QUE and H_2O_2 treatment. They did not affect the level of JNK phosphorylation in the case of DQ, decreased it in the case of QUE and H_2O_2, and increased it in the case of RH1 (*Fig. 14*). We found that SP600125 efficiently protected against the DNA damage after DQ, RH1 and QUE treatment (*Fig. 15*).

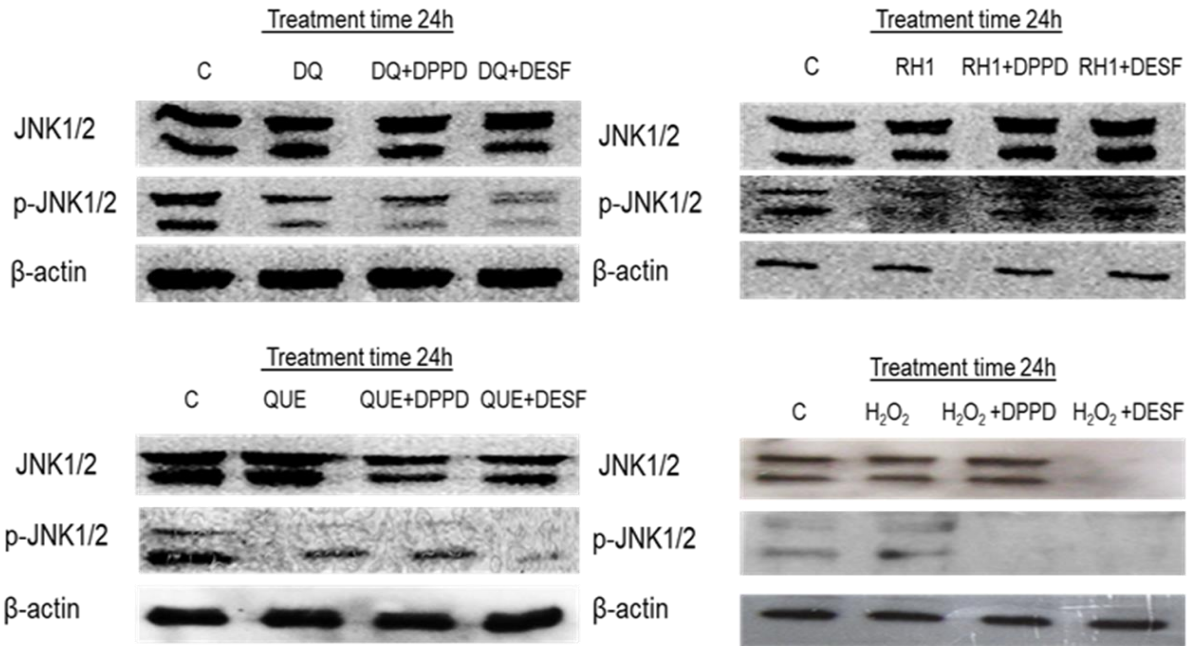

Fig. 14. **Influence of oxidative stress on JNK1/2 and phospho-JNK1/2 protein levels in MH-22A cells after 24 hour treatment.** Cells were exposed for 30 minutes with DPPD (2 μM) and DESF (300 μM), followed by addition of the test substance: DQ (59 μM), RH1 (0.12 μM), QUE (140 μM), H_2O_2 (200 μM).

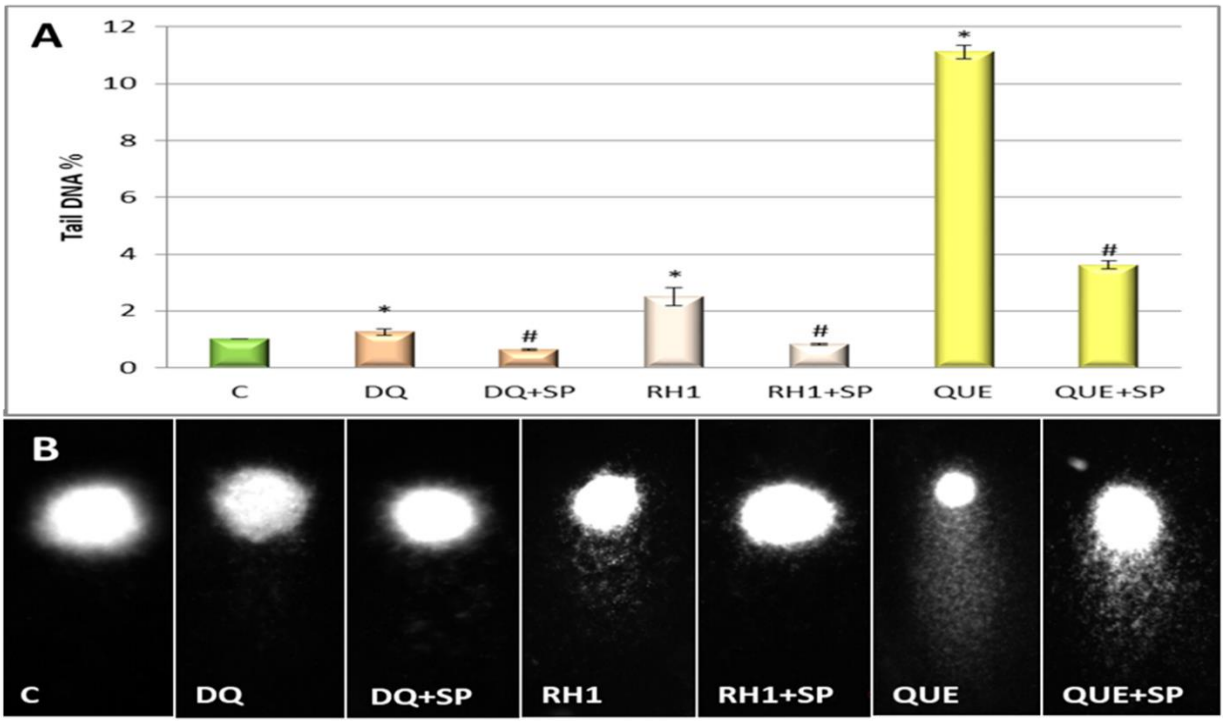

Fig. 15. **The role of JNK in the development of DNA damage.** Inhibitor SP600125 (SP, 20 μM) was exposed to MH-22A cells for 30 minutes before adding the test substances - DQ (25 μM), RH1 (0.03 μM) and QUE (75 μM). A - quantitative evaluation of DNA in the comet tail after 24 hours effects on the test compounds; B - comet images. *Statistically significant differences between control (cells without exposure) and DQ, RH1 and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with SP600125 and one of the compounds and cells treated solely with DQ, RH1 and QUE ($p < 0.05$).

The studies of p38. We found that DQ in principle did not affect the expression of p38 and its phosphorylation (*Fig. 16*).

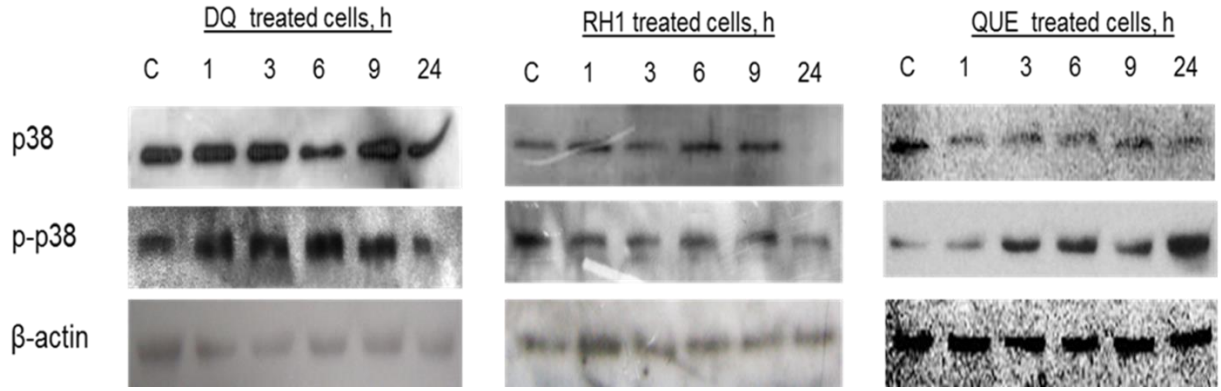

Fig. 16. **p38 and phospho-p38 protein expression level in MH-22A cells after 24 hours treatment with DQ (59 µM), RH1(0.12 µM) and QUE (140 µM).**

However, RH1 terminated its expression at 24 h, and kept constant but slightly lower than control the level of its phosphorylation. QUE kept the level of p38 constant but slightly lower than control during 1-24 h, but permanently increased the level of its phosphorylation (*Fig. 16*). The inhibitor of p38, SB203580, protected against the cytotoxicity of RH1 and MeDZQ, almost did not affect the cytotoxicity of QUE, and increased the toxicity of DQ (*Fig. 17*).

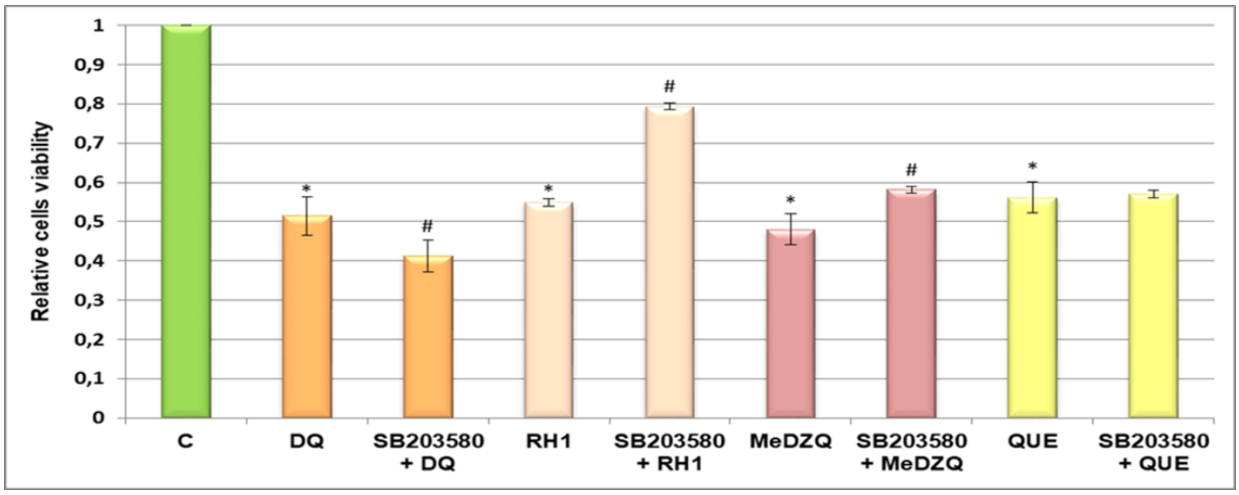

Fig. 17. **Influence of p38 kinase inhibitor SB203580 on the viability of MH-22A cells after exposure to the investigated compounds.** MH-22A cells were exposed for 30 minutes with an inhibitor SB203580 (20 µM), followed by addition of the test substance DQ (59 µM), RH1 (0.12 µM), MeDZQ (0,31 µM) and QUE (140 µM). The effect was assessed using MTT method. *Statistically significant differences between control (cells without exposure) and DQ, RH1, MeDZQ and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with SB203580 and one of the compounds and cells treated solely with DQ, RH1, MeDZQ and QUE ($p < 0.05$).

NQO1 inhibitor DIC restored the p38 expression after RH1 treatment, but had no effect on the level of phosphorylation. Contrary, after DQ and DIC co-treatment, both the expression and phosphorylation of p38 decreased in comparison to the effects of sole DQ. After QUE and DIC treatment, no significant changes were observed (*Fig. 18*).

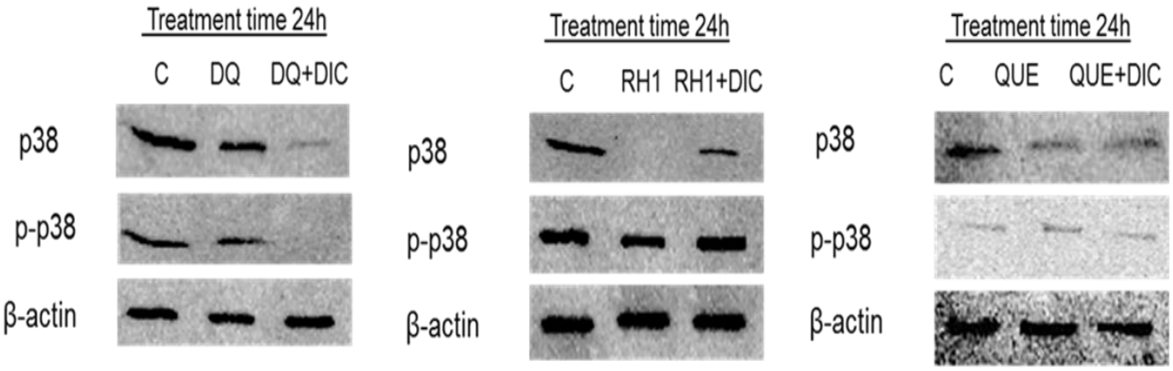

Fig. 18. **The effect of DIK on p38 and phospho-p38 protein levels in MH-22A cells after 24 hour treatment.** The NQO1 inhibitor DIC (20 μM) was exposed to MH-22A cells for 30 minutes before adding the test substances DQ (59 μM), RH1 (0.12 μM) and QUE (140 μM).

The antioxidants DPPD and DESF did not influence p38 expression and phosphorylation under the action of DQ and QUE, however, they restored its expression and activated phosphorylation under the action of RH1 (*Fig. 19*). The under the action of antioxidants did not have a significant influence. In comparison, after treatment with H_2O_2, DESF inhibited the expression and activation of p38. The other antioxidant, DPPD, did not have a significant effect (*Fig. 19*). A p38 inhibitor SB203580 protected against the DNA damage by all investigated compounds (*Fig. 20*).

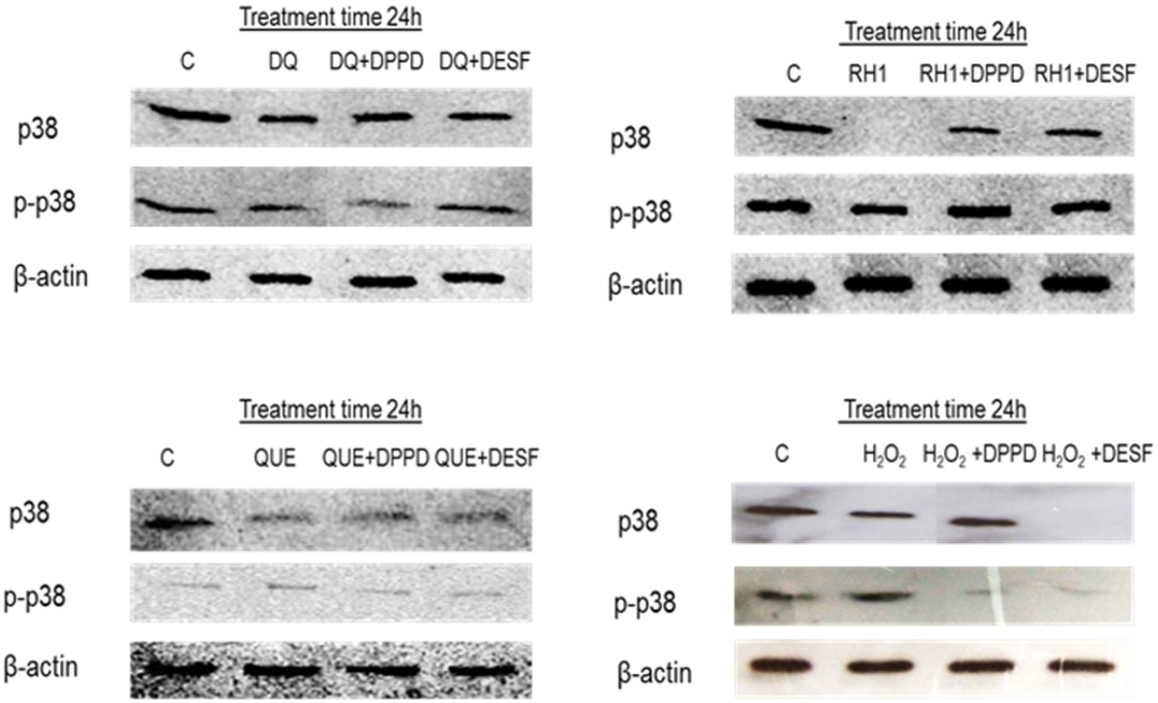

Fig. 19. **Influence of oxidative stress on p38 and phospho-p38 protein levels in MH-22A cells after 24 hour treatment.** Cells were exposed for 30 minutes with DPPD (2 μM) and DESF (300 μM), followed by addition of the test substance: DQ (59μM), RH1 (0.12μM), QUE (140 μM), H_2O_2 (200 μM).

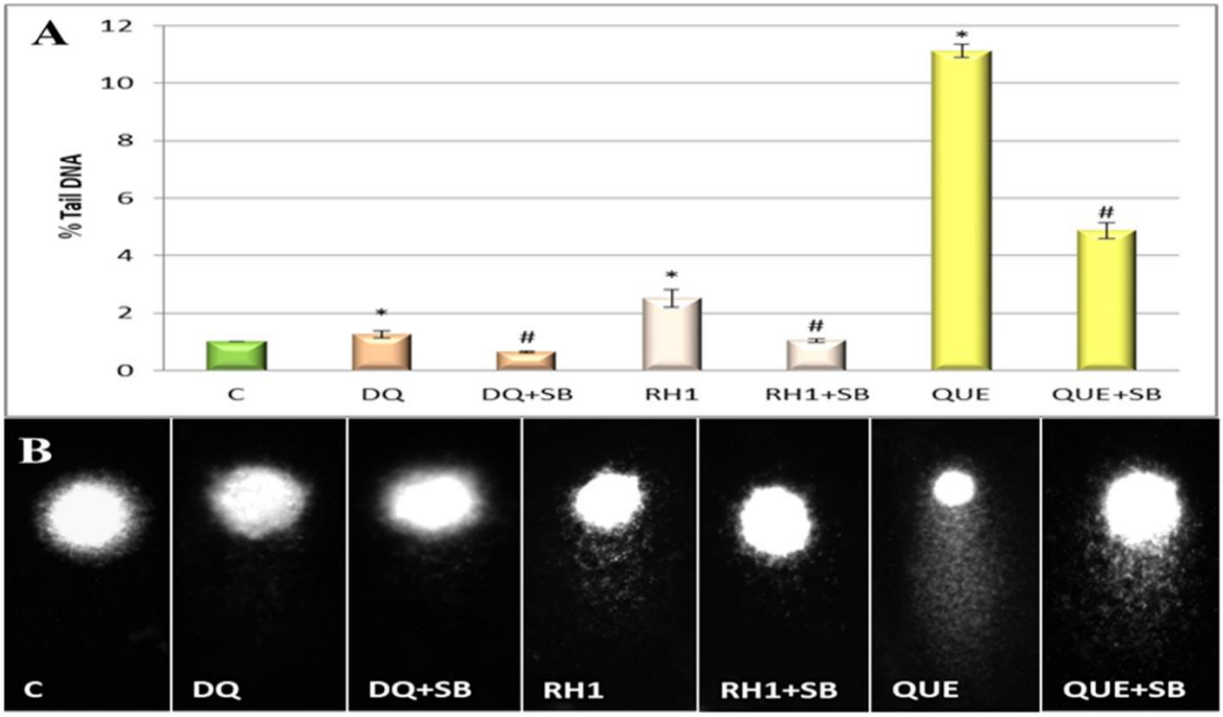

***Fig. 20*. The role of p38 in the development of DNA damage.** Inhibitor SB203580 (SB, 20 μM) was exposed to MH-22A cells for 30 minutes before adding the test substances - DQ (25 μM), RH1 (0.03 μM) and QUE (75 μM). A - quantitative evaluation of DNA in the comet tail after 24 hours effects on the test compounds; B - comet images. *Statistically significant differences between control (cells without exposure) and DQ, RH1 and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with SB203580 and one of the compounds and cells treated solely with DQ, RH1 and QUE ($p < 0.05$).

The studies of transcription factor p53. In control experiments, MH-22A cells do not express p53. Its expression and phosphorylation was induced by RH1, and not by DQ or QUE at >6 h (*Fig. 21*).

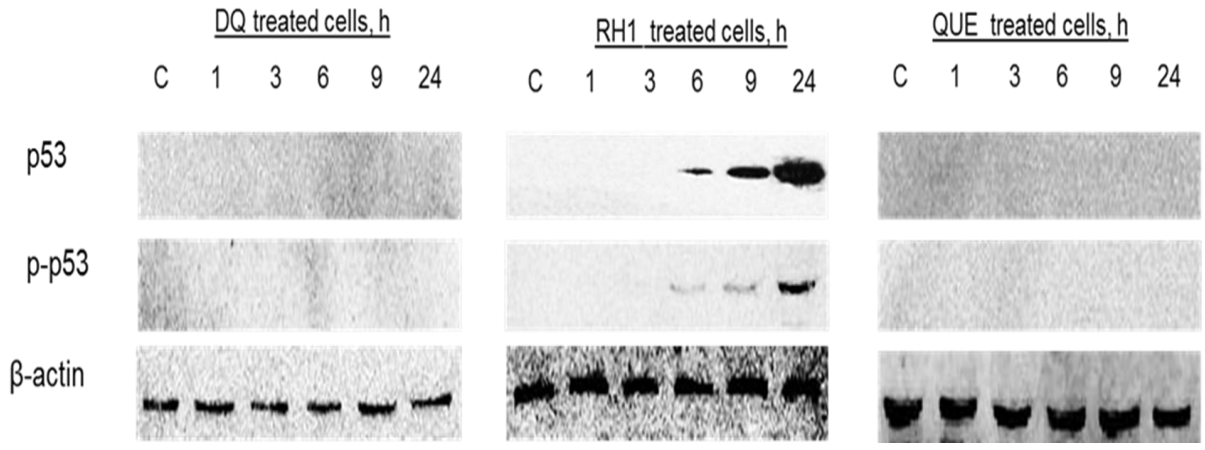

***Fig. 21*. P53 and phospho-p53 protein expression level in MH-22A cells after 24 hours treatment with DQ (59 μM), RH1 (0.12 μM) and QUE (140 μM).**

The p53 inhibitor pifithrin-α (Pif-α) significantly reduced cell viability after DQ and RH1 treatment, but increased it after QUE treatment (*Fig. 22*). Pif-α alone did not influence cell viability (data not shown).

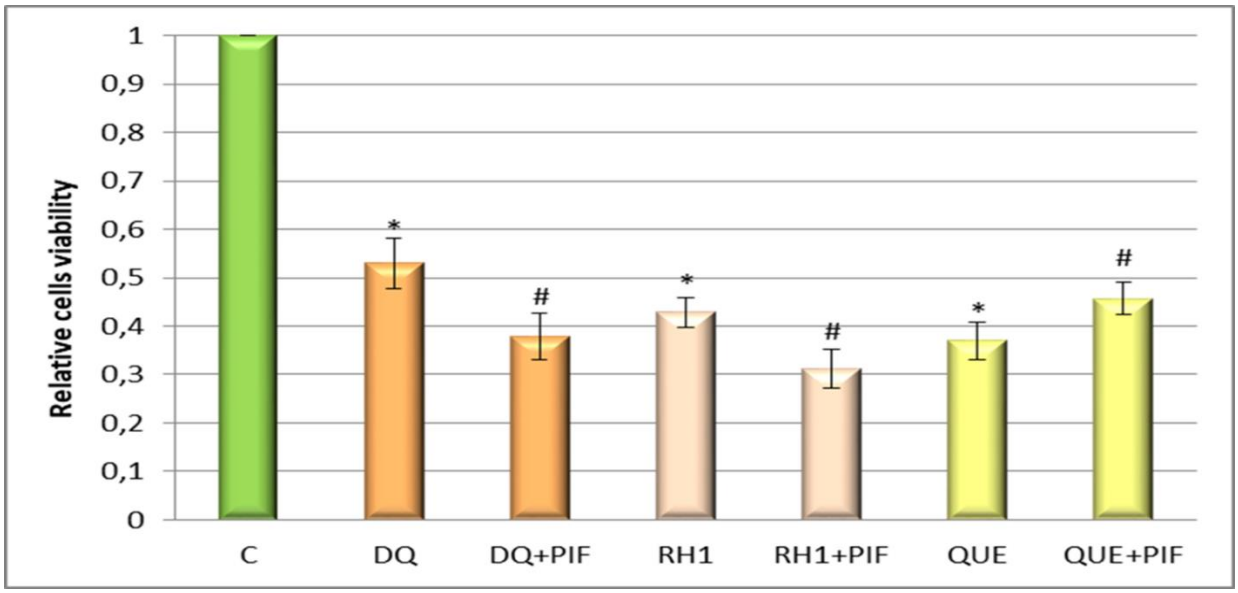

Fig. 22. **Influence of p53 kinase inhibitor PIF-α on the viability of MH-22A cells after exposure to the investigated compounds.** MH-22A cells were exposed for 30 minutes with an inhibitor PIF-α (PIF, 20 μM), followed by addition of the test substance DQ (59 μM), RH1 (0.12 μM) and QUE (140 μM). The effects were assessed using MTT method. *Statistically significant differences between control (cells without exposure) and DQ, RH1 and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with PIF-α and one of the compounds and cells treated solely with DQ, RH1 and QUE ($p < 0.05$).

DIC did not affect the expression and phosphorylation of p53 induced by RH1 (*Fig. 23*).

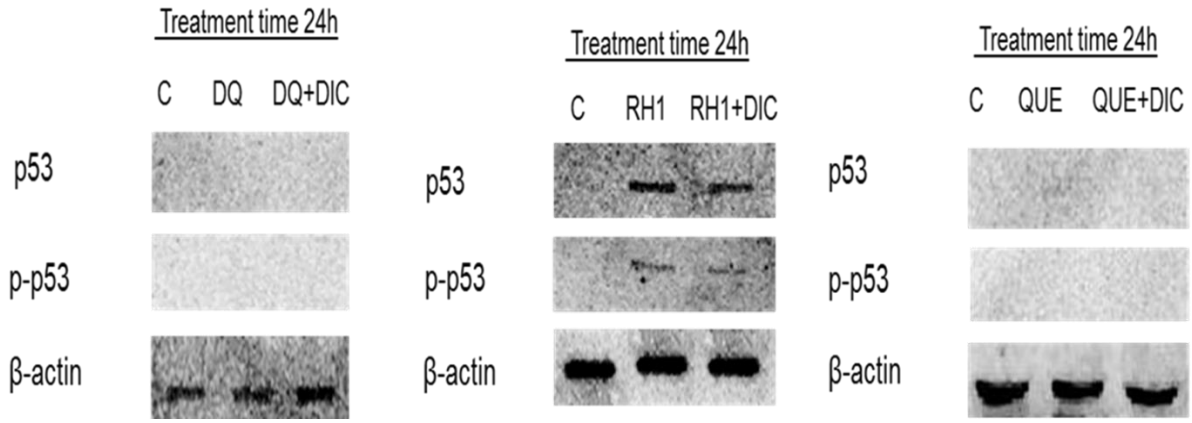

***Fig. 23*. The effect of DIK on p53 and phospho-p53 protein levels in MH-22A cells after 24 hour treatment.** The NQO1 inhibitor DIC (20 μM) was exposed to MH-22A cells for 30 minutes before adding the test substances DQ (59 μM), RH1 (0.12 μM) and QUE (140 μM).

The antioxidant DPPD did not change the expression or phosphorylation of p53, while DESF increased RH1-induced p53 expression. At the same time, the level of p53 phosphorylation had increased which could be attributed to a higher expression of this protein and not to increased activation *(Fig. 24)*. PIF-α protected DNA from damage by RH1, QUE and DQ (*Fig. 25*).

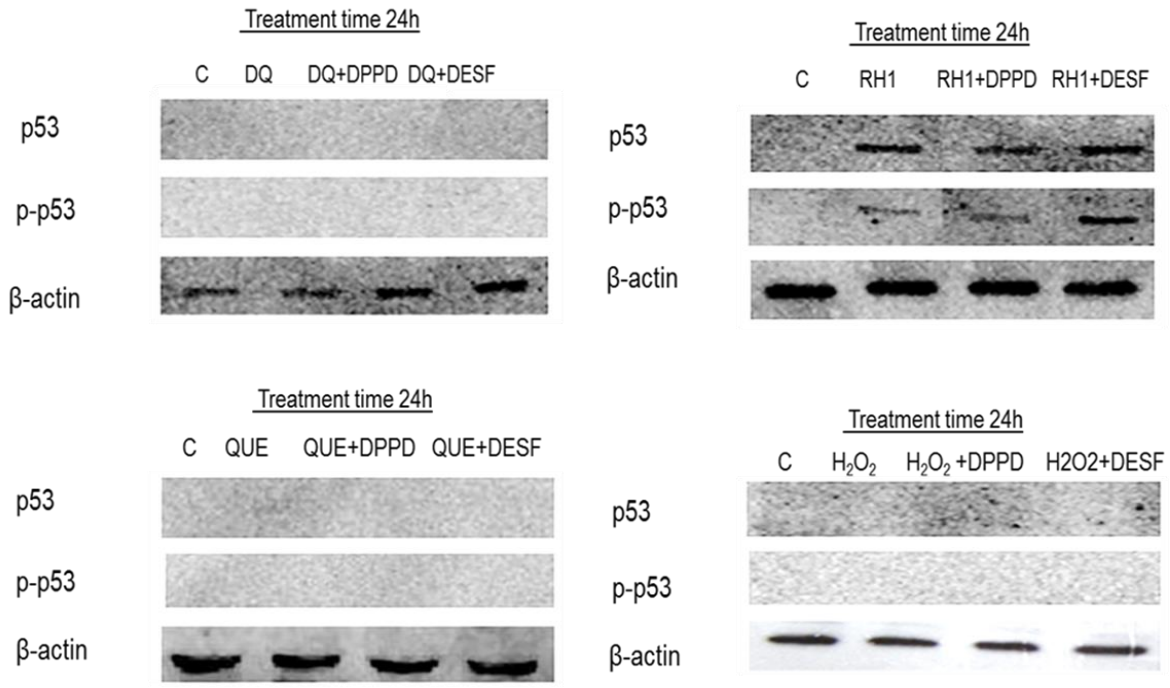

Fig. 24. **Influence of oxidative stress on p53 and phospho-p53 protein levels in MH-22A cells after 24 hour treatment.** Cells were exposed for 30 minutes with DPPD (2 μM) and DESF (300 μM), followed by addition of the test substance: DQ (59 μM), RH1 (0.12 μM), QUE (140 μM), H_2O_2 (200 μM).

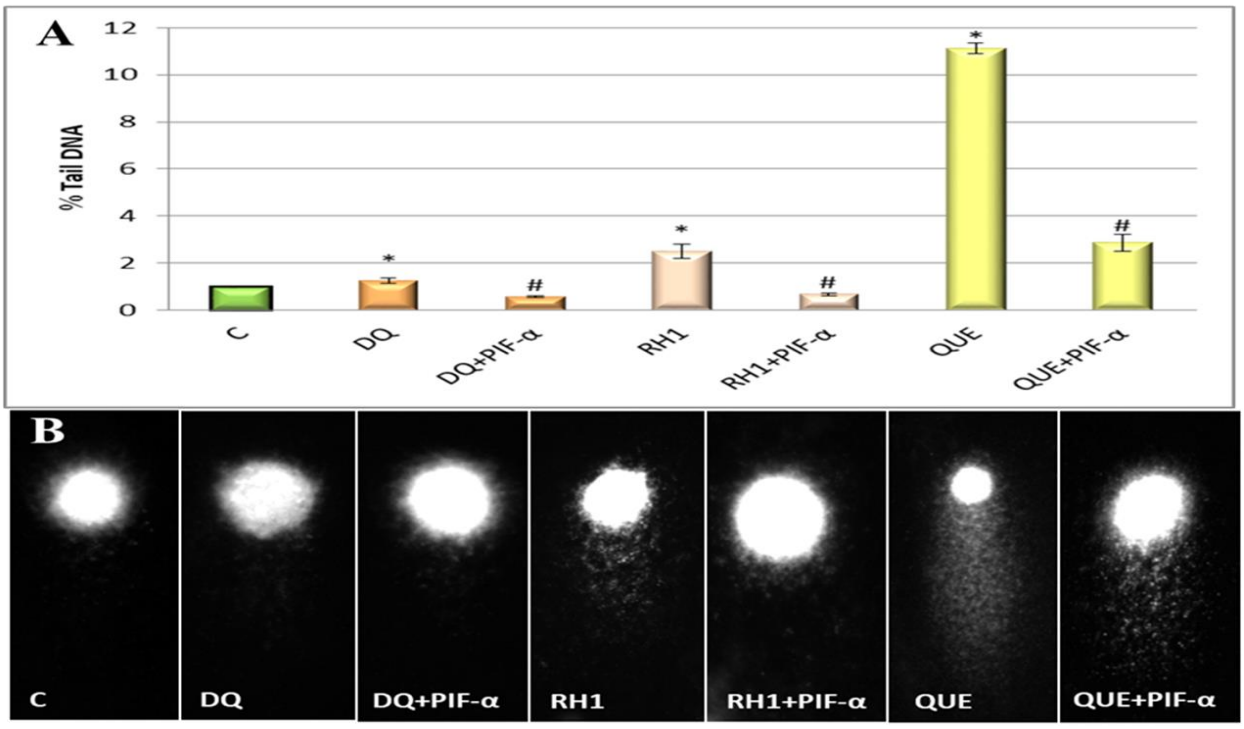

Fig. 25. **The role of p53 in the development of DNA damage.** Inhibitor PIF-α (20 μM) was exposed to MH-22A cells for 30 minutes before adding the test substances - DQ (25 μM), RH1 (0.03 μM) and QUE (75 μM). A - quantitative evaluation of DNA in the comet tail after 24 hours effects on the test compounds; B - comet images. *Statistically significant differences between control (cells without exposure) and DQ, RH1 and QUE- treated cells ($p < 0.05$). # Statistically significant differences between cells treated with PIF-α and one of the compounds and cells treated solely with DQ, RH1 and QUE ($p < 0.05$).

A final step in this work was to evaluate ERK, JNK and p38 kinase links to transcription factor p53 during the action of the test compounds. To do this, p53 expression and level of phosphorylation were assessed using corresponding MAP kinase inhibitors. In this case, QUE and DQ effects were not investigated, since they did not influence p53 expression. Results show that p38 kinase inhibitor SB203580 and JNK kinase inhibitor SP600125 did not have a significant effect on p53 expression and activation. A contrary situation was observed in the case of ERK kinase inhibitor PD98059 - here, p53 expression and phosphorylation were slightly increased.

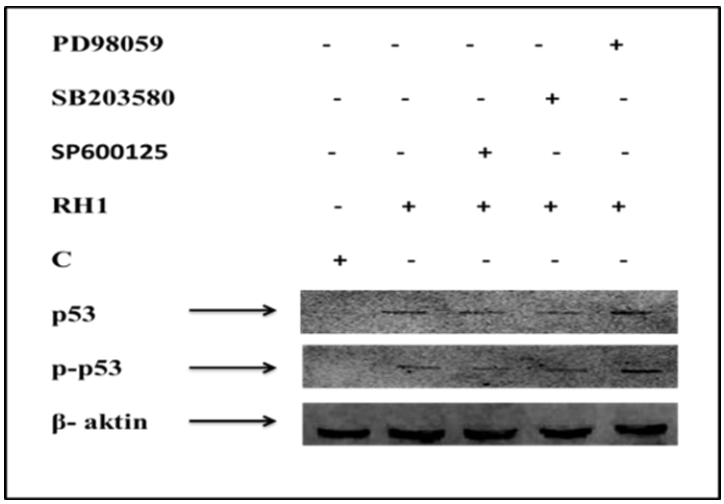

Fig. 26. **Levels of p53 and phospho-p53 proteins in MH-22A cells after 24 hours of treatment with RH1 and inhibitors – PD9805920 (20 μM), SB203580 (20 μM) and SP600125 (20 μM).**

DISCUSSION

In this study, the cytotoxicity of a series of quinones and polyphenols was investigated in MH-22A cells. Mechanisms of DQ, RH1, MeDZQ and QUE toxicity were investigated with an emphasis on the role of signalling molecule expression. The results obtained in this study are valuable in assessing the cytotoxicity and therapeutic efficiency of other aziridinyl-substituted quinones and polyphenols. We will first discuss the structure-cytotoxicity relationships, oxidative stress and bioreductive activation roles of quinones and polyphenols in cytotoxicity, apoptosis and induction of DNA damage formation:

a) Mechanisms of quinone cytotoxicity. Aziridinyl-unsibstituted quinone cytotoxicity increases with increasing single-electron reduction potential (E^1_7) (*Fig. 1, table 1*). These results coincide with data obtained in other cell lines (Nemeikaitė-Čėnienė *et al.,* 2003; Šarlauskas *et al.,* 2015; O'Brien, 1991; Wardman, 1990). This relationship shows that the most important factor in determining the cytotoxicity of these compounds is the generation of free radicals (O'Brien, 1991; Nemeikaitė-Čėnienė *et al.,* 2003). They form during single-electron reduction, catalysed by NADPH:cytochrome P-450 reductase and other single electron transferring enzymes (Čėnas *et al*., 2004). This notion is supported by protective effects of antioxidants DPPD and DESF (*Fig. 5*). However, aziridinyl-substituted quinones DZQ, RH1, MeDZQ, BZQ and trimethyl-aziridinyl-1,4-benzoquinone are much more toxic than could be predicted from their single-electron reduction potential. This could be explained by their additional cytotoxicity mechanism – bioreductive activation, that is two-electron reduction to aziridinyl-substituted hydroquinones which alkylate DNA (Butler *et al.,* 1989; Hargreaves *et al.,* 2000; Di Francesco *et al.,* 2004, Pierce *et al.,* 2010).

Since these compounds induce oxidative stress, DPPD and DESF reduce their cytotoxicity as in the case of DQ and H_2O_2. However, contrary to the case of DQ, RH1and MeDZQ cytotoxicity is efficiently inhibited by NQO1 inhibitor dicoumarol (*Fig. 4, 5*). This proves the role of NQO1 in aziridinyl-substituted quinone cytotoxicity. NQO1 activity in MH-22A cells is 79.5 nmol × mg^{-1} × min^{-1}, which is modest when compared to other cancer cell lines in a panel of NCI (Ross and Siegel, 2004), 100-1000 nmol × mg^{-1} × min^{-1}. On the other hand, aziridinyl-substituted quinone BZQ, which has a low reduction potential, can directly alkylate DNA even without bioreductive activation taking place (Butler *et al.*, 1989; Di Francesco *et al.*, 2004). This explains the enhanced cytotoxicity of BZQ (*Fig. 1*). This compound is an inactive substrate for NQO1, its maximal reduction rate is <1 % of that of MeDZQ (Anusevičius *et al.*, 2002). An enhanced cytotoxicity of anticancer anthracycline daunorubicin (*Fig. 1*) could be caused by its positive charge on the amino sugar residue, which increases its intracellular accumulation and intercalation in DNA (Hindenburg *et al.*, 1989; Mohan and Rapoport, 2010).

b) Prooxidant cytotoxicity of polyphenols. The cytotoxicity of polyphenols increases with decreasing single-electron oxidation potential (E^2_7) and with increasing lipophilicity of the compounds. This is evident from the comparison of cytotoxicity of methyl-, ethyl- and octyl- gallate (*Fig. 2, table 2*). These data are analogous to those obtained in FLK and HL-60 cells, and primary cell splenocytes (Miliukienė *et al.*, 2014, Nemeikaitė-Čėnienė *et al.*,2005). In our case, the toxicity is mostly caused by the efficiency of polyphenol oxidation into their quinone/quinomethide products. Prooxidative cytotoxicity of polyphenols in MH-22A cells is supported by protective activity of DESF and DPPD (*Fig. 5*). Cellular protection using dicoumarol (*Fig. 4*) shows that NQO1 promotes QUE cytotoxicity. It could be hypothesised that during QUE oxidation, one molecule of H_2O_2 is formed, while NQO1 can reduce QUE quinone and thus regenerate it (Boots *et al.*, 2005). In such case, QUE could be oxidized repeatedly, generating more H_2O_2, thus increasing its cytotoxicity. However, this interpretation may not be suitable in all cases. For example, CHO cells with increased amount of NQO1 are more resistant to QUE, while dicoumarol potentaites the cytotoxicity of QUE (Gliszczynska-Swiglo *et al.*, 2003).

c) The role of oxidative stress and NQO1 in cell death and their link to DNA damage. Quinones induce both apoptotic and necrotic cell death, the latter is usually linked to the effects of free radicals, i. e. oxidative stress (Molorni *et al.*, 1993; Nemeikaitė-Čėnienė *et al.*, 2005; Park *et al.*, 2011; Sun and Ross, 1996). When treating with polyphenols, active forms of oxygen are produced that induce apoptotic cell death (Bhattacharya *et al.*, 2009; Lee *et al.*, 2014). Our results support the data that show quinones MeDZQ, RH1, DQ and polyphenol QUE as the inducers of both apoptotic and necrotic cell death (*Fig. 3*). However, it is noted that DQ, the cytotoxicity of which is manifested only through the free radical formation, relatively more efficiently induces necrotic but not apoptotic cell death. For example, in FLK cells upon the treatment with MeDZQ or RH1, a higher number of living apoptotic cells is formed than upon treatment with DQ (Nemeikaitė-Čėnienė *et al.*, 2005). MeDZQ, RH1 and QUE may possess additional cytotoxicity mechanisms, which favour apoptosis. Because of the reduction of dead and living apoptotic cell amount by dicoumarol in the presence of the above compounds (*Fig. 4*), it can be stated that NQO1-dependent processes take part in RH1-

and QUE-induced apoptosis. A link between more intensive apoptotic processes and more significant DNA damage can be observed after treatment with RH1, QUE and the effect of DIC. In line with our findings, it has been shown that RH1 is involved in the DNA damage that is independent of oxidative stress (Dehn *et al*, 2005). Because the RH1-induced DNA damage is also supressed by antioxidants (*Fig. 7*), it might be caused by reactive oxygen species as well. On the other hand, the effects of DESF and DPPD on QUE-induced DNA damage are insignificant and contrary to each other (*Fig. 7*). It could be supposed that in this case that topoisomerase-II is inhibited and/or DNA is directly alkylated by quinone-type oxidation product(s) of QUE. It has been shown that during the incubation of ^{14}C-labeled quercetin with Caco-2 and HepG2 cells lines, covalent adducts with proteins and DNA were formed (Walle *et al.*, 2003). In contrast, the necrotic cell death is most associated with the damage to outer cell membrane, and, as it is characteristic for the action of DQ, is not accompanied followed by significant DNA damage (*Fig. 3, 6*). It could be hypothesised as well that oxidative stress also increases the mitochondrial membrane permeability, which causes both apoptosis and necrosis (Kovaltowski *et al*., 2001).

Next, we will focus on signalling protein expression upon treatment with RH1, DQ or QUE, and their links with the cell death.

The role of MAP kinases. It is known that upon treatment of tumour cells with quinones, activated SAPKs take part in the induction of apoptosis (Park *et al*., 2011). It was also shown that these kinases can be activated by both DNA damage and oxidative stress (Wynand and Bernd, 2006; Kovtun *et al.,* 2000). Data on the effects of SH-unreactive and fully substituted quinones on MAP kinases are scarce and controversial: in A549 cells, RH1 stimulated the phosphorylation of ERK, JNK and p38 (Stulpinas *et al.*, 2016), while the quinone embelin stimulated JNK, p38 and ERK1/2 phosphorylation (Avisetti *et al.*, 2014) in MDA-MB-231 cells; RH1 induced apoptosis, possibly mitochondrial, with JNK taking part in it (Park *et al.*, 2011); doxorubicin activated ERK in rat liver epithelial cells (Abdelmoksen *et al.*, 2005); rhein activated the phosphorylation of JNK and p38 without the involvement of ROS (Lin *et al*., 2003), and pyroloquinolinquinone suppressed the phosphorylation of p38 and JNK in primary microglia cells (Yang *et al.*, 2014), but activated ERK in NIH/3T3 mouse fibroblasts (Kumazawa *et al.*, 2007).

Data on the effects of QUE on MAP kinases are abundant, but controversial as well. For example, the ROS formed after QUE treatment induce apoptotic cell death involving p38 (Lee *et al.,* 2010). However, QUE supressed the phosphorylation of ERK in human glioma (Pan *et al.,* 2015) and HepG2 cells (Granado-Serrano *et al.,* 2008), but stimulated it in HL-60 cells with involvement of ROS were (Lee *et al.,* 2015). QUE-induced apoptosis in choryocarcinoma cells was followed by the phosphorylation of ERK, JNK and p38, involving ROS (Lim *et al.,* 2017). More examples like these are present in the literature.

Our data show that JNK inhibitor SP600125 increased MH-22A cell viability and decreased the amount of DNA damage after treatment with QUE (*Fig. 12, 15*). Other data, however, appear to be more contradictory, since SP600125 increased DQ and RH1-induced cytotoxicity (*Fig. 12*) and decreased the amount of DNA damage (*Fig. 15*). SB203580, the inhibitor of p38, decreased the amount of DQ, RH1 and QUE-induced DNA damage (*Fig. 20*) as well as reduced the RH1-induced cytotoxicity, but increased

the cytotoxic effect of DQ (*Fig. 17*). It did not have any effect in the case of QUE (*Fig. 17*). These contradictions may be at least partly explained by a relatively poorly studied action of these classical inhibitors of kinases, their parallel effects on the expression of NQO1: SB203580 stimulated NQO1 expression in control cells (Qureshi *et al.*, 2008), but suppressed it upon treatment with various polyphenols (Tsai *et al.*, 2011; Roubalova *et al.*, 2106; Ahn *et al.*, 2017); SP600125 supressed the expression of NQO1 in control cells (Qureshi *et al.*, 2008), while data on the effects of PD98059 is not available. This leads to the conclusion, that the use of these compounds in investigating NQO1-associated MAP kinase signalling is problematic.

Therefore, in order to assess the link between apoptosis and kinase expression, we used the multiparametric regression analysis, which was previously used for the quantitative analysis of apoptosis induction in FLK cells (Nemeikaitė-Čėnienė *et al.*, 2005). The variables, presented in *table 3*, were the percentage of apoptotic cells (A) or apoptotic index A/(A+G), where G – the percentage of viable cells, according to the data of *Fig. 3,4,5*. It was arbitrarily assumed that the kinase expression index is 1 when its level is close to that of control cells, 1.5 when it increases, 0.5 when it decreases, and 0 when it becomes undetectable. After assessing the dependence of A or A/(A+G) on individual kinase expression, we found that the expression of p-ERK, p38 and p-p38 kinases decreases during apoptotic cell death, while that of JNK increases:

$$A/(A+G) = (27.550\pm3.487) + (1.024\pm5.276)\ ERK - (7.600\pm3.905)\ p\text{-}ERK + (13.248\pm5.049)\ JNK - (3.214\pm5.972)\ p\text{-}JNK - (13.972\pm6.122)\ p38 - (2.973\pm4.915)\ p\text{-}p38 \quad (r^2 = 0.5838,\ F\ (6.10) = 2.338) \qquad (1)$$

The increase in the expression of JNK during apoptosis ($p = 0.025$) and increase in the expression of p38 ($p = 0.046$) are statistically significant. The expression of p-ERK decreases, but is not highly statistically significant ($p = 0.080$). The use of the percentage of apoptotic cells as variable (*Table 3*) yields even less statistically significant results, but the effects of apoptosis on the expression of JNK, p-ERK and p38 is the same (data not presented). It was not possible to characterize the changes in other MAP kinase expressions.

During the assessment of the roles of ROS and alkylation on the expression and phosphorylation of kinases, it was assumed that the generation of ROS in control cells and in any experiment in the presence of DPPD and DESF was equal to 0. The occurrence of alkylation in control cells, those treated with DQ and H_2O_2 as well as in the case of RH1 + dicoumarol, was equal to 0. In all other cases, the incremient of alkylation was equal to 1 (Table 3). The results suggest that the of expression of p38 is statistically insignificantly suppressed by ROS or alkylation: $(0.789\pm0.109) - (0.276\pm0.133)$ ROS $- (0.276\pm0.133)$ alkylation ($r^2 = 0.3934$, F (2.14) = 4.540). The effects of ROS and alkylation are characterized by $p = 0.057$. However, the expression of p-ERK is statistically significantly induced by ROS ($p = 0.029$): $(0.237\pm0.199) + (0.592\pm0.244)$ ROS $- (0.033\pm0.244)$ alkylation ($r^2 = 0.2960$, F (2.14) = 2.944). It was not possible to characterize the induction pathways of other kinases. In this case, p-ERK is possibly a antiapoptotic factor, induced by oxidative stress, JNK is a antipoptotic factor with an undefined mechanism of induction, and p38 is possibly suppressed by ROS or alkylation.

Table 3. Relation between apoptotic cells, apoptotic index, ROS or alkylation effect to cell and MAP expression.

No.	**Conditions**	**A (%)**	**A/(A+G) (%)**	**Condition**		**ERK1/2**	**p-ERK1/2**	**JNK**	**p-JNK**	**p38**	**p-p38**
				ROS	**Alkylation**						
1	Control	3±1	4±1	0	0	1	1	1	1	1	1
2	DQ	14±1	25±5	1	0	1	1	1	0	0,5	0,5
3	DQ + DPPD	23±3	31±6	0	0	0,5	0	1	0	0,5	0,5
4	DQ + DESF	21±1	33±6	0	0	0,5	0	1	0	0,5	0,5
5	DQ + DIC	13±2	22±5	1	0	0	0	0	0	0	0
6	RH1	20±1	32±5	1	1	1	1,5	1	0,5	0	0,5
7	RH1 + DPPD	32±3	38±6	0	1	0,5	0,5	1,5	0,5	0,5	1
8	RH1 + DESF	20±1	28±3	0	1	0,5	0,5	1,5	0,5	0,5	1
9	RH1 + DIC	11±3	15±5	1	0	1	1,5	1	0,5	0,5	1
10	QUE	17±1	27±6	1	1	1	0,5	1	0,5	0,5	1
11	QUE + DPPD	23±3	31±6	0	1	1	0	0,5	0,5	0,5	1
12	QUE + DESF	21±2	25±3	0	1	1	0	0,5	0	0,5	1
13	QUE + DIC	19±2	25±5	1	1	1,5	0	1	0,5	0,5	0,5
14	MeDZQ	19±3	32±8	1	1	1	1	1	1	0	0
15	MeDZQ + DIC	12±2	15±5								
16	H_2O_2	23±2	22±5	1	0	1	1	1	1	1	1
17	H_2O_2 + DPPD	26±3	32±5	0	0	0,5	0	1	0	1	0
18	H_2O_2 + DESF	23±2	31±5	0	0	1	0	0	0	0	0

The expression of p53. The expression of p53 was induced only by RH1 in our cells (*Fig. 21*). Similar results were obtained by Ngo *et al.* (1998), who investigated the induction of p53 using aziridinyl-substituted benzoquinones in MCF-7 cells. In the latter case, the induction was attenuated by dicoumarol. It is believed that p53 takes part in RH1-induced, NQO1-dependent death of RKO cells (Park *et al.,* 2011). Similar results were obtained in our experiments (*Fig. 23*). This shows that the expression of p53 is at least in part caused by DNA damages that appear in response to RH1 and NQO1 reaction products. It is known that MAPKs phosphorylate and activate p53 (Wu, 2004), but our results show that the activation of JNK does not change the expression of p53 and p-p53. After suppressing ERK, the expression and phosphorylation of p53 slightly increases (*Fig. 26*). However, additional research is needed in order to identify the intermediate intracellular targets. One may note that the fact that QUE, which induces significant DNA damage (*Fig. 6*) does not induce p53 (*Fig. 21*), is also demonstrated in MCF-7 cells (Choi *et al.,* 2001), human epithelial cells (O'Prey *et al.,* 2003), and OE33 adenocarcinoma cells (Zhang *et al.,* 2008), even though in many other cases, an induction of p53 is observed. This effect is evidently dependent on the cell type, QUE concentration and treatment duration. Since p53 is not induced by all types of DNA damage, it is possible to suppose that RH1 and QUE induce different types of DNA damage.

The effect of pifitrin-α on the cytotoxicity (*Fig. 22*) and DNA damage (*Fig. 25*) of QUE should be discussed separately. It appears to contradict the inability of QUE to induce p53. However, it is known that PIF-α binds to the aromatic carbohydrate receptor (AhR), which takes part in the synthesis of NQO1 (Hoagland *et al.,* 2005) and can suppress NQO1 expression (Wang *et al.,* 2012). The expression of p53 is not AhR-dependent (Hoagland *et al.,* 2005). For this reason, one may suppose that PIF-α could reduce the expression of NQO1, thus reducing the cytotoxicity (*Fig. 23*) and DNA damage (*Fig. 25*) of QUE. This is analogous to the suppression of QUE-induced effects using NQO1 inhibitor dicoumarol. In addition, a reduction in the expression of NQO1 can be used to explain the RH1-induced DNA damage suppression using PIF-α *(Fig. 25*). However, this issue is not yet solved and requires additional research, since PIF-α stimulates RH1 cytotoxicity (*Fig. 28*).

The assessment of the effects of RH1 and QUE shows that in spite of their structure and cytotoxicity mechanisms differences, some their cytotoxicity mechanisms are similar and differ from those of non-alkylating DQ. This can be explained by the ability of RH1 hydroquinone and QUE oxidation products to alkylate DNA (Walle *et al.,* 2003; Gliszczynska-Swiglo *et al.,* 2003). Cell death is also caused by oxidative stress that is present in all cases.

Conclusions

1. Aziridinyl-unsubstituted quinone cytotoxicity in mouse hepatoma MH-22A cell line is caused by the oxidative stress and increases with the single-electron reduction potential of the compounds. Higher cytotoxicity of aziridinyl-substituted quinones is caused by NQO1-catalysed formation of alkylating hydroquinones.
2. Polyphenol cytotoxicity in MH-22A cells is caused by oxidative stress and alkylation by their oxidation products. Cytotoxicity increases with decreasing single-electron oxidation potential of polyphenols.
3. Aziridinyl-substituted quinone RH1 and polyphenol quercetin induce a more pronounced apoptotic response than aziridinyl-unsubstituted duroquinone. This is linked to the alkylation process.
4. During the investigation of RH1, DQ and QUE induced cell death, the DNA damage, MAP kinase expression and phosphorylation, a possible antiapoptotic activity of ERK in response to oxidative stress was discovered. The mechanism of JNK-associated cell death remains vaguely understood**.** p38 MAPK has characteristic MH-22A protective activity, but the expression and phosphorylation of this kinase is possibly suppressed by oxidative stress and alkylation.
5. p53 expression is not characteristic for MH-22A cells, but it is specifically induced by RH1. In this case, p53 acts as an antiapoptotic factor.

List of publications on the subject of the present research

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