Chapter 7

Cellular Caspases: New Targets for the Action of Pharmacological Agents

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1. Introduction

The number of scientific papers devoted to the study of caspases has lately being constantly growing. Caspases are a family of cysteine-dependent aspartate specific proteases. Caspases play an essential role in the apoptosis, necrosis and inflammation processes. Apoptosis is asynchronous programmed cell death, which helps maintain the physiological balance and genetic stability of the organism through self-destruction of genetically modified defect cells. Apoptosis may happen also in normal cells, e.g., during embryogenesis. During apoptosis, activated endogenous nucleases cleave DNA into fragments, but cell membranes and the intracellular matter remain intact, nor is there tissue damage or leukocytic infiltration. In contrast to apoptosis, necrosis is a pathological form of cell death caused by acute damage, rupture of the membrane, release of the cytoplasm content, and the inflammatory process induced thereby [1, 2].

As of now, at lease 14 caspases have been described from mammals: 8 caspases are involved in apoptosis, 5 activate anti-inflammatory cytokines, and one acts in keratinocyte differentiation. This division is, however, rather arbitrary – we know from a number of papers that some apoptotic caspases may, depending on the conditions, participate in other cell life processes, such as proliferation [3], differentiation [4, 5], modification of susceptibility to leukocyte lysis [2, 6].

During apoptosis, different caspases perform different functions. Depending on the phase at which those proteins enter the apoptotic cascade one distinguishes initiator (apical) and effector (executioner) caspases. E.g., caspase-2, -8, -9, -10, and -12 are initiator ones; caspase-3, -6, -7 – effector ones. All caspases are originally inactive, but activated when needed by cleavage of a small fragment by initiator caspases. Initiator caspase activation is more sophisticated – by special protein complexes: apoptosomes, PIDDosomes, DISC (*death-*

inducing signalling complexes) [7, 8]. Under certain conditions effector caspases may act as initiator ones to accelerate apoptotic reactions.

Research into the phenomenon of programmed cell death started in the late 1960s. One of the pioneers in the sphere was John Kerr, who studied the death of hepatocytes in rats with acute liver failure [9]. In 1972, a team of British scientists headed by Kerr first coined the term apoptosis to denote programmed cell death. The authors described two phases of the process (formation of apoptotic bodies, and their phagocytosis by other cells), and stressed that apoptosis is an active and controlled process. Yet, nothing was known at the time about the factors and mechanisms of this type of cell death. Studies of the structure of caspases began only in 1994 [10, 11]. In 1996, it was found that cytochrome c together with ATP promote activation of caspase-3 [12]. Caspases are now investigated in various model systems both *in vitro* and *in vivo*. The most popular systems in use are tumor cell lines of various histogenesis. Most studies employ hemopoietic tumor cells (lines *K562*, *HL-60*, *Raji*, *HEL*, etc.), cells derived from solid tumors (lines *HeLa*, *RPTC*, *HCT 116*, etc.), as well as stem cells. Through this approach one can not just study the structure, activation mechanisms and basic functions of caspases in normal cells, but also identify the biological role of these enzymes in emerging and progressing pathological processes, first of all, oncologic and immuno-inflammatory ones. Besides, the chemical reagents used in those model systems usually activate or inhibit cell processes, including apoptosis. These facts open up immense opportunities in the analysis of the activity of the substances considered for applicability as components of new targeted drugs. The clue to the effectiveness of the chemical reagents for the treated cells will be the changes in the susceptibility of those cells to cytotoxic lysis of blood leukocytes, namely natural killer cells and cytotoxic T-lymphocytes. Since proteases play an important part in cytotoxic lysis, caspases in this case can also be viewed as candidate molecules whose activity can be modulated to accelerate or damp apoptosis, as well as modify the susceptibility of pathological cells to leukocytolysis.

Hence, the primary objective of the chapter is to demonstrate by means of the experimental data the crucial role of caspases in the induction and progress of tumor cell apoptosis, as well as in the modification of tumor cell susceptibility to the cytotoxic lysis of natural killer cells upon treatment with chemical compounds, part of which have been newly synthesized. The model system used in our study was the human erythromyeloid leukemic cell line *K562* (Russian Cell Culture Collection, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia), as well as *K562/2-DQO* and *K562/4-NQO* subline cells. To induce apoptosis and modulate cell susceptibility to the lytic action of blood leukocytes we used a group of N-bearing heterocyclic reagents – derivatives of quinoline and pyridine: quinoline – *Q*, 2-methylquinoline – *2-MeQ*, quinoline-1-oxide – *QO*, 2-methylquinoline-1-oxide – *2-MeQO*, 4-nitroquinoline-1-oxide – *4-NQO,* 2-methyl-4-nitroquinoline-1-oxide – *2-Me-4-NQO*, 2-(4′-dimethylaminostyryl)quinoline-1-oxide – *2-DQO,* 4-(4′-dimethylaminostyryl)quinoline-1-oxide – *4-DQO,* 2-(4′-nitrostyryl)quinoline-1-oxide – *2-NSQO,* 4-(4′-nitrostyryl)quinoline-1-oxide – *4-NSQO*, and 4-(4′-dimethylaminostyryl)pyridine-1-oxide – *4-DPyO* (Figure 1).

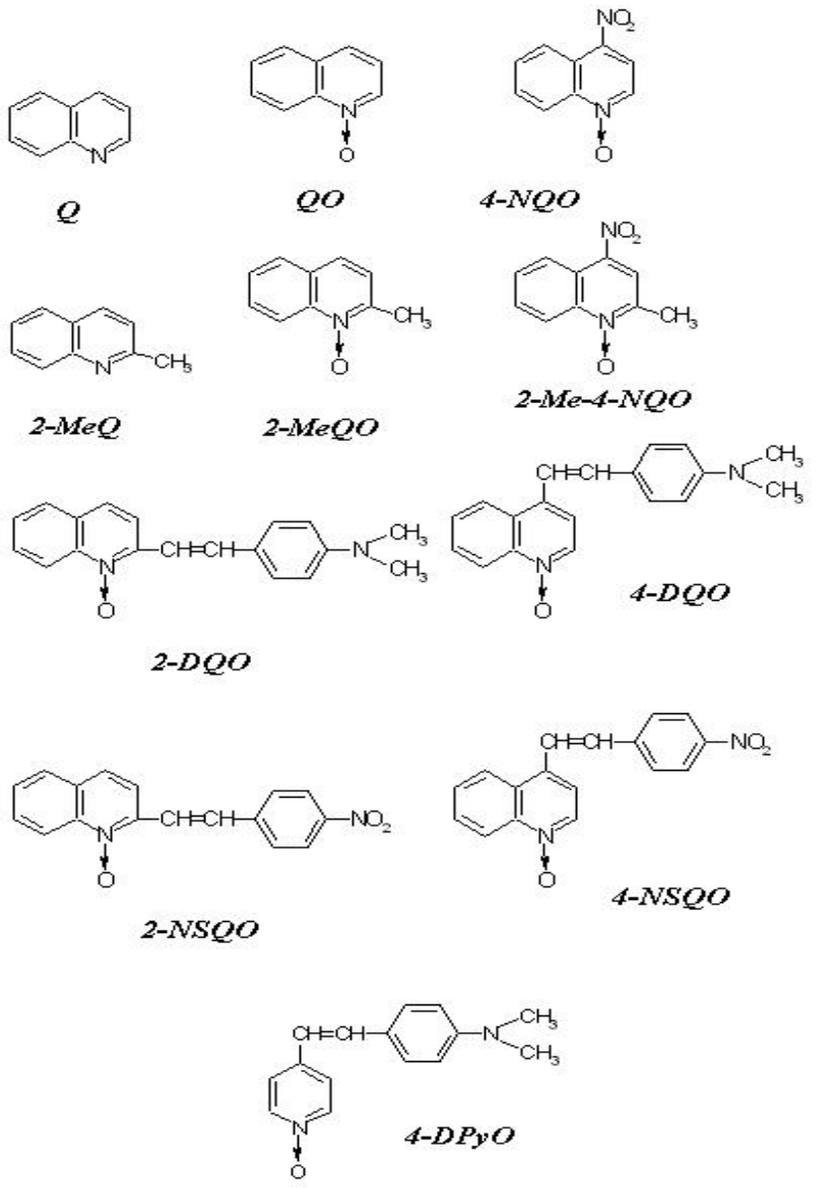


Figure 1. Derivatives of quinoline and pyridine

In current clinical practices, quinoline derivatives are used as drugs with high pharmacological potential – photoprotective, anti-inflammatory, immunomodulating, antioxidant, antiproliferative, antiaggregatory, hypolipidemic, and hypoglycemic. This group of chemical compounds and their intracellular metabolites can be highly competitive as structural analogs of key molecules of the cell (nitrogen bases, nucleosides, nucleotides, various co-enzymes, etc.), and take effect by activating or inhibiting a certain cell process. The compounds with such biological effects most frequently used in the oncology practice are 5-fluorouracil, cytarabine, methotrexate, vinblastine, dactinomycin, and others.

Derivatives of N-bearing heterocycles are very effective in treatment of tumors of various histogenesis and location, including leukemia, but many of them have limited applicability because of high toxicity. Finding the compounds that feature both high anti-tumor activity and low side effects is therefore an important task.

2. Body

A major problem in modern cell biology is the search for intracellular targets for pharmacologically active substances, and subsequent development of new generation medicines based on the resultant data. As a rule, molecules involved in basic processes of cell life are viewed as such intracellular targets. The molecules participating in two or more vital processes are of greatest interest. Such are caspases. They are synthesized in the cell as precursors (procaspases). Caspase precursors consist of a prodomain and two subunits: a small one and a large one. Some caspases contain a short linker sequence (around 10 amino acids) between the subunits. Caspases can activate one another: the prodomain is cleaved from the procaspase, and the small and large subunits of the two caspases form an active caspase – heterotetramer (Figure 2), which contains two active centres. Caspases of the initiator (pro-apoptotic and pro-inflammatory) type contain a long prodomain made up of approximately 100 amino acids, whereas the short prodomain of effector caspases contains only 30 amino acid residues. Long prodomains contain various motifs, mainly DED (*death effector domain*) and CARD (*caspase recruitment domain*), but also DID (*death inducing domain*). Each of the procaspases-8 and -10 contains two tandem copies of the DED, whereas CARD was found in caspases-1, -2, -4, -5, -9, -11, -12. Homotypic interactions between DED and CARD recruit procaspases into initiator complexes, activate them and trigger the caspase cascade [13].

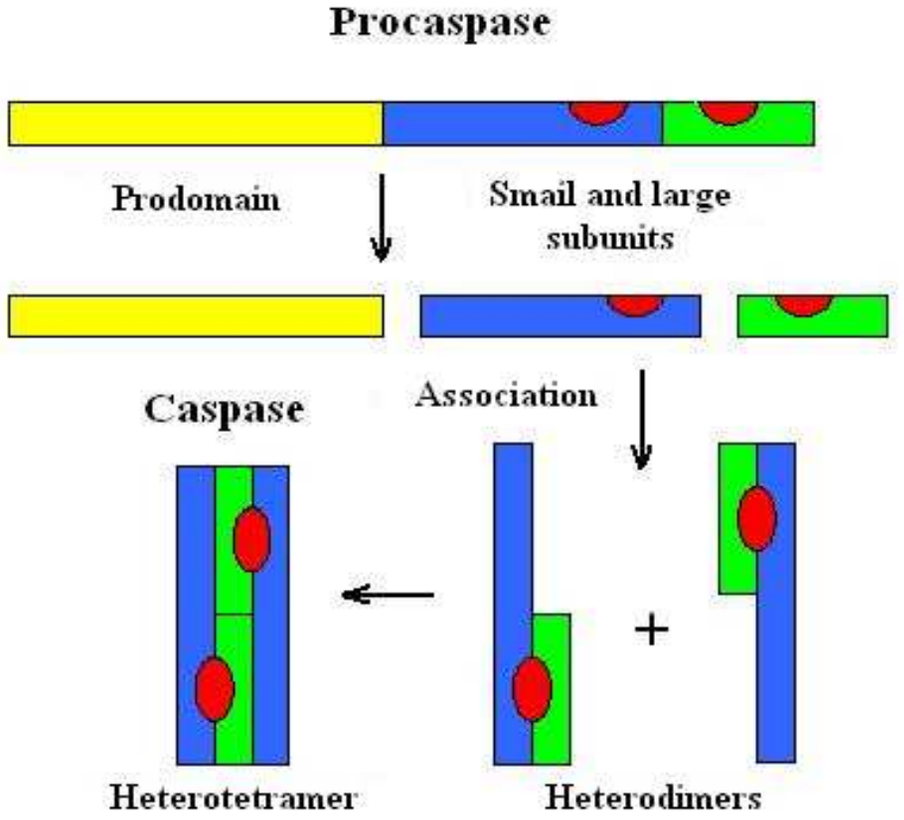


Figure 2. Proteolytic caspase activation (adopted from http://www.muldyr.ru/a/a/apoptoz_-_fazyi_apoptoza)

Apart from distinctions in the prodomain structure, caspases can be grouped by substrate specificity. E.g., caspases-6, -8 and -9 show preference for cleaving substrates with the valine/leucine−glutamate−threonine/histidine−aspartate (V/L−E−T/H−D) sequence, whereas caspases-3 and -7 selectively cleave the motif made up of the aspartate−glutamate−valine−aspartate (D−E−V−D) sequence. An optimal target for caspase-1 and its related caspases-4 and -5 are the sequences tyrosine−valine−alanine−aspartate (Y−V−A−D) or tryptophan/leucine−glutamate−histidine−aspartate (W/L−E−H−D) [14].

Various forms of post-translational modifications can influence the activity of caspases. Thus, phosphorylation of caspase-9 by the serine/threonine protein kinase Akt inhibits its activity. Such phosphorylation occurs away from the caspase active centre, and presumably hinders the clustering of the caspase subunits into the tetramer [13, 15]. Another variant of post-translational modification of caspases is S-nitrosylation. The NO radical group is tranfered to the cysteine of the caspase active centre and the R−S−NO group is formed [16, 17]. Furthermore, caspases are susceptible to oxidative modifications induced by active forms of oxygen, with disulphides formed in the process.

Apoptosis can be triggered by various mechanisms that activate caspases. Two major pathways are distinguished: activation involving cell receptors, and mitochondria-mediated activation.

Caspase activation involving cell receptors. The process of apoptosis often begins with ligation of specific extracellular ligands with cell death receptors on the membrane surface. Receptors of the apoptotic signal belong to the TNF (*tumor necrosis factor*) receptors superfamily [18]. The best studied death receptors, whose role in apoptosis has been identified and described, are CD95 (Fas or APO-1), and TNFR1 (p55 or CD120a) (Figure 3). Additional receptors are CARI, DR3 (*death receptor* 3), DR4, DR5, and DR6. All death receptors are transmembrane proteins which share a sequence of 80 amino acids at the cytoplasmic face termed the death domain (DD). It is required for apoptotic signalling [7, 18]. Extracellular regions of death receptors interact with ligand trimers (CD95L, TNF, Apo3L, Apo2L, etc.), and the latter trimerize the death receptors (crosslink 3 molecules of the receptor) [19, 20]. The thus activated receptor interacts with *a* corresponding intracellular adaptor(s). The adaptor for the CD95 (Fas/APO-1) receptor is FADD (*Fas-associated DD-protein*). The adaptor for the TNFR1 and DR3 receptors is TRADD (*TNFR1-associated DD-protein*) (Figure 3).

The adaptor associated with the death receptor interacts with caspases. The "ligand−receptor−adaptor−effector" interaction chain results in the formation of aggregates in which caspases are activated. These aggregates are called apoptosomes, apoptotic chaperones, or death-inducing signalling complexes (DISC). An example of apoptosome is the FasL−Fas−FADD−procaspase-8 complex, in which caspase-8 is activated (Figure 3) [7, 21]. Death receptors can mediate activation of caspases-2; -8, and -10 [22]. The activated initiator caspases then activate effector caspases.

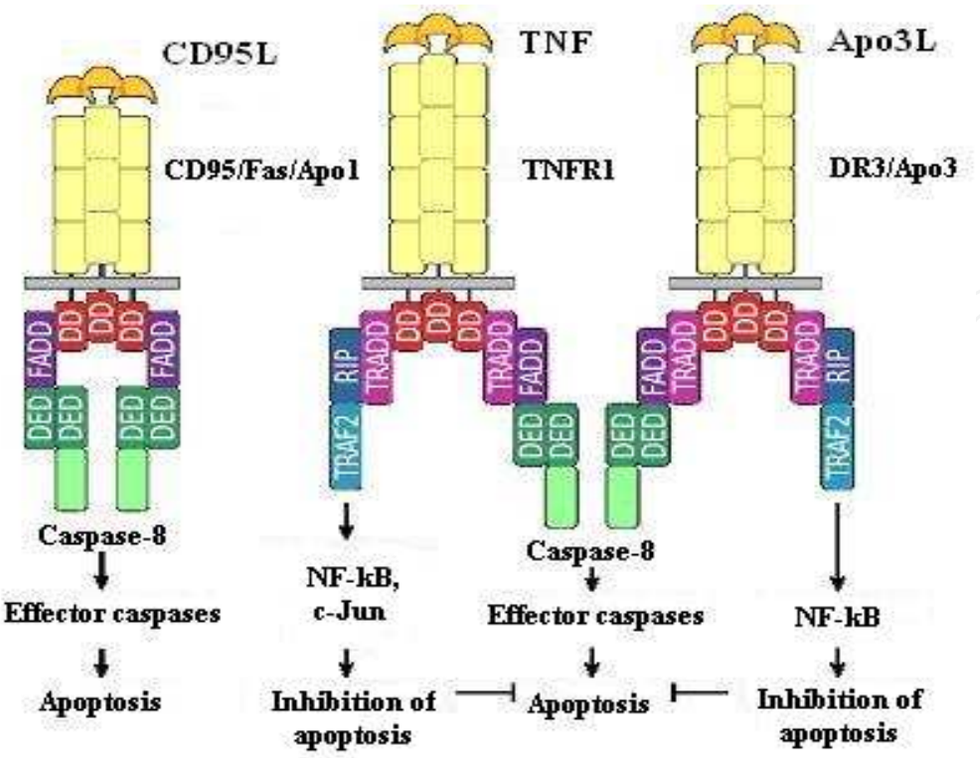


Figure 3. Receptors of the apoptotic signal (adopted from http://www.muldyr.ru/a/a/apoptoz_-_fazyi_apoptoza)

Mitochondria-mediated caspase activation. The mitochondrial apoptotic signalling pathway is realized through release of apoptogenic proteins from the intermembrane region into the cell cytoplasm. Presumably, there are two major pathways for the release of apoptogenic proteins: through formation of a giant pore followed by rupture of the mitochondrial membrane, or through opening of highly permeable channels on the mitochondrial outer membrane [23]. The apoptosome formation model can be represented as "Cytochrome c−Apaf-1−CARD−procaspase-9". Apaf-1 (*apoptosis protease activating factor-1*) undergoes conformational modifications induced by the reaction involving the loss of ATP energy. Procaspase-9 gets access to the CARD of Apaf-1. The thus activated caspase-9 recruits procaspase-3, which is, in turn, activated to form caspase-3 [24, 25].

In some apoptotic models the cytochrome is released by activation of the PTP (*permeability transition pore*) [26]. This pore is a compound complex made up of adenine nucleotide transporter (in the inner mitochondrial membrane), voltage-dependent anion channel, otherwise termed porin (in the outer mitochondrial membrane), and cyclophilin D (in the matrix of mitochondria). The 2.6−2.9 nm giant pore is non-specific, and molecules up to 1.5 Da can pass. Pore opening leads to mitochondrial swelling and rupture of the outer membrane. In addition to cytochrome c mitochondria emit other pro-apoptotic factors [23, 25]. Pore opening can be stimulated by inorganic phosphate, caspases, SH-reagents, cell exhaustion by reduced glutathione, formation of active forms of oxygen, uncoupling of oxidative phosphorylation, rise in Ca^{2+} content in the cytoplasm, effect of ceramide, depletion of the mitochondrial ATP pool, etc. [24, 27, 28].

A presumable alternative pathway for the release of apoptotic proteins from the mitochondrial intermembrane region is formation of a protein channel in the outer mitochondrial membrane. Whichever the pathway, the substances released into the cytoplasm are cytochrome c, procaspases-2, -3, -9, and AIF (*apoptosis inducing factor*) [14]. The release is promoted by Bcl-2 family proteins (Bax-protein). The flavoprotein AIF released from the mitochondrial intermembrane region is an apoptosis effector that would then act independently of caspases [24, 29].

The caspases activated through these pathways directly or indirectly promote the destruction of cell structures. Nuclear lamina proteins are hydrolysed, the cytoskeleton is disrupted, proteins regulating cytoadherence are degraded. Another essential function of effector caspases is inactivation of apoptosis-blocking proteins. To wit, they cleave the inhibitor DFF (*DNA fragmentation factor*), which prevents activation of the apoptotic CAD (*caspase-activated DNase*). Anti-apoptotic proteins of the Bcl-2 family are cleaved. Finally, the action of effector caspases results in dissociation of the regulatory and effector domains of the proteins involved in DNA replication and repair, mRNA splicing [30, 31].

No matter which apoptotic mechanism the cell chooses, the process can be modulated. At present, the structure and action mechanisms of a great number of apoptosis modulating chemical compounds are known. Most of them influence DNA either directly or indirectly. Nonetheless, if apoptosis can be modulated, then modification of caspase expression is also possible (although there exists caspase-independent apoptosis). The tumor cell apoptosis modulators used in this study are derivatives of two heterocyclic compounds – quinoline and pyridine (Figure 1). This group of reagents, most of which are N-oxide derivatives, holds good promise for *in vitro* study of cell systems, because these compounds comprise several functional activity centres (at the heterocyclic nucleus, functional groups, and radicals). Most published data on the biological activity of quinoline N-oxide derivatives are focused on 4-nitroquinoline-1-oxide (*4-NQO*), whereas information on the activity of other structural analogs of the reagents is, unfortunately, almost totally missing.

4-NQO is a chemical carcinogen whose biological effects on cells are in many ways similar to the effect of UV light [32]. The action of *4-NQO* is studied using various model systems: prokaryotic cells *in vitro* [33], eukaryotic cells *in vitro* [34], multicellular organisms [35]. The range of the investigated cell life processes and intracellular biomolecules is also diverse. For the neoplastic effect to take place *4-NQO* has to be metabolically activated in the cells to the proximate carcinogen 4-hydroxyaminoquinoline-1-oxide, which, after being acetylated, can covalently bind to DNA, namely adenine and guanine, to form stable monoadducts [36, 37]. Xanthine oxidase can metabolise *4-NQO* to a more reactive superoxide anion (5.5-dimethyl-1-pyrroline-*N*-oxide superoxide radical). Formation of substantial amounts of such structures first of all disturbs the processes of DNA replication and repair, thus inducing apoptosis (or necrosis). If, however, this does not happen, accumulation of multiple mutations in the genome would cause normal cells to transform into tumor cells. Some questions logically arise in this connection: how much similarity is there between the biological effects of the structural analogs of *4-NQO* and those of *4-NQO* itself? Would the

application of these compounds enable activation of apoptosis in tumor cells by modulating the activity of caspases? Will the susceptibility of the reagent-treated cells lead to leukolysis change? It is these and other questions that we shall try to answer below.

3. Materials and methods

Cells of the human erythromyeloid leukemic cell line *K562* were incubated in the following medium: 89% RPMI 1640 (Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow) supplemented with 11% fetal bovine serum (Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow), 2 mM L-glutamine, 40 μg/ml gentamicin sulphate, 50 μM 2-mercaptoethanol (Ferak, Germany) in 95% O_2 and 5% $C0_2$ at 37^0C. The cultures were seeded to 1 ml (3 ml) of the medium with the seeding density of 10^5 (10^6) cells/well, respectively. Quinoline derivatives in concentrations detailed in the text and figure captions were added to the medium. Cells were counted in Goryaev's chamber. EC_{50} cells incubated with the reagents were ascertained using the technique [38]. The viability was assessed using the test with 3-(4.5-dimethylthiasol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (Sigma, USA).

Assessment of viability (MTT-test). The technique was described in [39]. 100 μL aliquots of cell suspension (10^5 cells/well) were seeded to a 96-well flat bottom culture plate and treated with 100 μL of the chemical compound solution of the corresponding concentration. All assays were performed in three replicates. The plates were left to incubate for 24, 48, 72, and 96 hours. Three hours before the end of the incubation period the wells were stained with MTT solution (3-(4.5-dimethylthiasol-2-yl)-2.5-diphenyltetrazolium bromide (Sigma, USA)) to a final concentration of 0.25 mg/ml, and re-incubated in the dark in a humidified atmosphere at 37°C. Then, the supernatants were carefully removed and 200 μL aliquots of DMSO were added to each well. The residue was re-suspended and incubated for 15 min in the dark at room temperature. The optical density was measured at 540 nm with a Labsystems Multiscan Plus reader (LKB, Finland). In this case, staining of the cells incubated in the absence of chemical compounds was regarded as the control. Specific cell death was calculated by formula (1):

$$\text{Induced death, \%} = (1 - (D_{exp} - D_{env} / D_c - D_{env})) \times 100\ \% \quad (1)$$

where D_c is the optical density in control wells (cells without chemical reagent); D_{exp} – optical density in treated assays (cells with chemical reagent); D_{env} – optical density of the control environment.

Tumor cell clones resistant to 2-DQO and 4-NQO were obtained as described in [38]. Cell resistance to xenobiotics was induced by long-term (over a month) exposure in a culture medium containing *2-DQO* (10^{-9} M) or *4-NQO* (10^{-12} M). The concentration of the substances in the culture medium was increased every 14–21 days. The final doses for which resistant cell lines were obtained were 10^{-5} and 10^{-8} M, respectively.

Real-time PCR. Total RNA from peripheral blood leukocytes and tumor cells was extracted with the "YellowSolve" kit (Clonogen, Russia) following the manufacturer's guidelines. The extracted RNA template was treated with DNase (Sigma, USA). The concentration and purity of the RNA template was determined by spectrophotometry ("SmartSpec Plus", BioRad, USA). RNA nativity was determined by agarose gel electrophoresis. Complementary DNA was synthesized from 1 μg of total RNA using random hexaprimers and MMLV reverse transcriptase following the protocol proposed by the manufacturer (Sileks, Russia). RNA and cDNA samples were stored at -80°C. Gene expression was estimated by real-time PCR. The fluorophore for product detection was the intercalating SYBR Green I dye. Amplification was performed in an "iCycler Thermal Cycler" (BioRad, USA) with "iQ5 Optical System" V2.0 software (BioRad, USA) using real-time PCR assay kits in the presence of SYBR Green I. The PCR reaction mixture was prepared with the component volumes recommended by the manufacturer: we mixed 2.5 μl deoxynucleosidetriphosphates (2.5 mM), 2.5 μl 10-fold PCR buffer with SYBR Green I, 2.5 μl $MgCl_2$ (25 mM), 1 μl aliquots of forward and reverse primers (20 pmol/μl), 0.25 μl Taq-DNA-polymerase (5 U/μl), 2 μl template cDNA, deionized water – up to 25 μl per test tube. The PCR protocol was 15 sec at 95°C, 50 sec at 60°C (45 cycles). To determine the specificity of primer annealing PCR fragments were melted: for 1 min at 95°C, 1 min at 60°C, 10 sec at 60°C (80 cycles, the temperature raised by 0.5°C in each cycle). To exclude the possibility of the template cDNA being contaminated by the genomic DNA PCR was performed for each template under the same conditions with the RNA matrix. Primers for the nucleotide sequences of the investigated genes and the reference gene GAPDH were selected using the Primer Premier software ("Premier Biosoft", USA) or published sources (Table 1). Oligonucleotides were synthesized by the Syntol company (Russia). Gene expression was measured against the amount of GAPDH mRNA using the $2^{-\Delta\Delta Ct}$ method [40]. The resultant reaction products were separated in 8% polyacrilamide gel using the tris-borate buffer. PCR products were stained with 1% ethidium bromide solution and visualized in transmitted UV light using the low-molecular (501–567 bp) pUC19/Msp I fragment length marker (Syntol, Russia).

Gene	Gene Bank №	Sequence	Source
GAPDH F:	NM_	5'-**GAAGGTGAAGGTCGGAGTC**-3'	[41]
GAPDH R:	002046.3	5'-**GAAGATGGTGATGGGATTTC**-3'	
CASPASE 6 F:	NM_	5'-**ACTGGCTTGTTCAAAGG**-3'	[42]
CASPASE 6 R:	001226.3	5'-**CAGCGTGTAAACGGAG**-3'	
CASPASE 3 F:	NM_	5'-**ATGGAAGCGAATCAATGGAC**-3'	[43]
CASPASE 3 R:	004346.3	5'-**ATCACGCATCAATTCCACAA**-3'	
CASPASE 9 F:	NM_	5'-**AACAGGCAAGCAGCAAAGTT**-3'	[43]
CASPASE 9 R:	001229.2	5'-**CACGGCAGAAGTTCACATTG**-3'	

Table 1. Primers for the nucleotide sequences of the caspase genes under study and the reference gene GAPDH

The enzyme activity of caspases was determined by standard technique using specific substrates labeled with fluorescent marker (7-amino-4-trifluoromethylcumarin – AFC) (BioRad, USA), detected by variations in fluorescence or optical density [2]. 50 μl of lytic buffer prepared by mixing 920 μl of bidistilled H_2O, 40 μl of 25-fold reaction buffer and 10 μl of each of the four inhibitors: PMSF (phenylmethylsulfonyl fluoride) (35 mg/ml), pepstatin *A* (1 mg/ml), aprotinin (1 mg/ml), and leupeptin (1 mg/ml), was added to the tumor cells (10^6 cells). The 25-fold reaction buffer included the following components: 250 мM HEPES, pH 7.4, 50 mM EDTA, 2.5% CHAPS (3-((3-chloramidopropyl)dimethylammonio)-1-propanesulfonate), 125 mM dithiothreitol. After that, the cells were frozen three times in liquid nitrogen, the cell lysate then centrifuged in a microcentrifuge at 17 000 *G* (4^0 C) for 30 min, and the supernatant (template) collected. The activity of caspases-3, -6 and -9 was determined in the reaction buffer by mixing the template with the corresponding specific substrate. The substrate for caspase-3 was DEVD (Asp–Glu–Val–Asp), for caspase-6 – VEID (Val–Glu–Ile–Asp), for caspase-9 – LEHD (Leu–Glu–His–Asp). The amount of cleaved AFC was measured by spectrophotometry in FluoroMax ("Horiba-Scientific", Japan) at 395 nm 30, 60, 90, 120, 150, 180 min after the onset of the reaction. Then, the curve of caspase activity depending on the template and substrate incubation time was plotted. Plot ΔS versus Δt and calculate the slope (ΔS/Δt).

$$\Delta S = [S(t_i) - B(t_i)] - [S(t_0) - B(t_0)], \qquad \Delta t = (t_i - t_0), \tag{2}$$

S – sample signal at time t, and B – blank signal at time t; t_i – time of measurement, t_0 – time of initial measurement.

Cytochrome c reductase activity of microsomes was measured by spectrophotometry in FluoroMax ("Horiba-Scientific", Japan) at 25^0 C. The reaction medium contained microsomes (30 μg protein/ml), NADPH or NADH (50 μM), *2-NSQO* or *4-NSQO* (1−100 μM), and cytochrome c (20 μM).

Determination of the susceptibility of tumor cells to the cytotoxic lysis of human leukocytes. Lysis was studied in a homologous system. Human peripheral blood leukocytes were isolated from the blood of healthy males by a two-step procedure involving fractionation on a Ficoll-Hypaque gradient, followed by erythrocyte lysis by distilled water. The viability of leukocytes, estimated by the Trypan blue test, was at least 93–95 %.

The test for cytotoxicity of human leukocytes (effector cells) for *K562* cells (target cells) incubated with quinoline derivatives and labeled with ^{3}H-uridine followed the protocol [44]. Pre-assay incubation of the cultures with the reagents lasted 48 h and 96 h in all variants. The effector cell/target cell ratio was 50:1. The cytotoxic index (CI, %) was calculated by formula (3):

$$\text{Cytotoxic index} = 1 - (\text{cpm}) \text{ in experimental tests} / (\text{cpm}) \text{ in control tests} \times 100\ \%, \tag{3}$$

where the control was *K562* cell cultures labeled with ^{3}H-uridine and free of effector-cells.

Quinoline derivatives were kindly provided by Prof. V.P. Andreev (St. Petersburg State University, Russia). The composition and structure of the resultant compounds were ascertained by elemental analysis, mass, IR-, NMR spectroscopy (^{1}H, ^{13}C, ^{15}N).

Reliability of the results was estimated using Student's t-test, and the non-parametric Mann–Whitney test.

4. Results

The first stage of the investigation of the biological activity of quinoline and pyridine derivatives was experiments to determine the effect of varying concentrations of *Q, 2-MeQ, QO, 2-MeQO, 4-NQO, 2-Me-4-NQO, 2-DQO, 4-DQO, 2-NSQO, 4-NSQO*, and *4-DPyO* on the viability of *K562* cells. The resultant data were processed to calculate EC_{50} values of each compound. The results are detailed in Table 2 (the reagents are arranged in the order of decreasing toxicity). It follows from the results that the greatest toxic effect on tumor cells under the stated conditions was demonstrated by *2-Me-4-NQO* and *4-NQO*, and the lowest – by *2-NSQO* and *4-NSQO*.

Reagent	**EC_{50}, μM**
2-Me-4-NQO	1.04
4-NQO	1.05
2-MeQ	1.26
Q	4.47
2-MeQO	23.44
4-DPyO	33.11
1. 2-DQO	2. 208.93
3. 4-DQO	4. 221.28
5. QO	6. 316.23
7. 2-NSQO	8. 570.10
9. 4-NSQO	10. 600.50

Table 2. Reagent concentrations resulting in 50% death of *K562* cells (EC_{50})

The data presented in Table 2 indicate also that when the methyl radical (*Q* → *2-MeQ, QO* → *2-MeQO, 4-NQO* → *2-Me-4-NQO*) and the nitro group (*QO* → *4-NQO, 2-MeQO* → *2-Me-4-NQO*) were attached to the quinoline heterocycle, the toxicity of the compounds for *K562* cells increased. For example, the EC_{50} of *Q* was 4.47 μM, *2-MeQ* – 1.26 μM; *QO* – 316.23 μM, *2-MeQO* – 23.44 μM; *4-NQO* – 1.05 μM, *2-Me-4-NQO* – 1.04 μM, EC_{50} in the *QO*/*4-NQO* and *2-MeQO*/*2-Me-4-NQO* pairs was 316.23 μM, 1.05 μM and 23.44 μM, 1.04 μM, respectively. When *K562* cells were incubated with reagents comprising the N-oxide group (*QO*, *2-MeQO*), the cell survival rate was, on the contrary, higher than for the cells incubated with *Q*

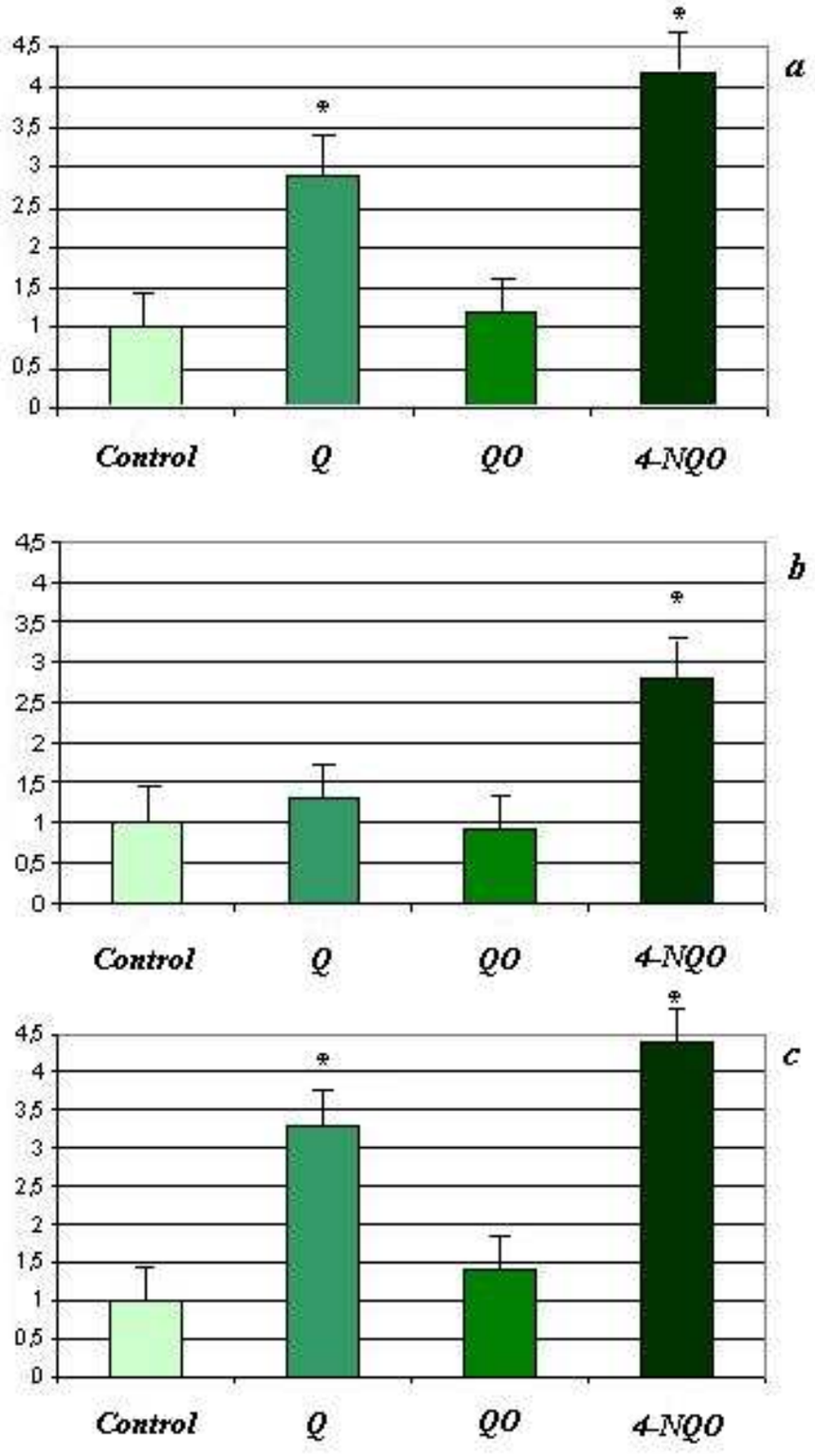
or *2-MeQ*, respectively (see EC_{50}). The toxic effect of the compounds was reduced also by addition of the "*styryl tail*" with the nitro group (*2-NSQO*, *4-NSQO)* to the reagent, whereas substitution of the nitro group with the dimethylamino group (*2-DQO, 4-DQO*) resulted in a 2.72-fold rise in the toxicity (see EC_{50}). Translocation of the styryl group within the quinoline ring was also significant: if the group was located closer to the heteroatom (Fig. 1), the compound also became more toxic (EC_{50} of *2-DQO* was 208.93 μM, that of *4-DQO* – 221.28 μM; EC_{50} of *2-NSQO* – 570.10 μM, that of *4-NSQO* – 600.50 μM). When the quinoline cycle in the chemical compound was substituted with the pyridine cycle the reagent's toxicity for the cells increased 6.68-fold, e.g., *4-DQO* EC_{50} was 221.28 μM, whereas *4-DPyO* EC_{50} was 33.11 μM. This pattern was observed both on the 48 h and on the 96 h of incubation.

The results above suggest that the toxic effect of the investigated reagents on tumor cells depends on the presence/absence of certain type substituents (electron donors or electron acceptors) in the quinoline cycle, as well as on the direct bond of the named substituents to the quinoline cycle. Thus, attachment of the nitro group (electron acceptor) to the cycle – *QO* → *4-NQO, 2-MeQO* → *2-Me-4-NQO* – rendered the substance more toxic, whereas inclusion of the substituent in the "*styryl tail*" (*4-NQO* → *4-NSQO*) reduced the toxic effect of the reagent 571.90 fold (see EC_{50}).

Intracellular activation of aromatic nitrogen-bearing compounds, including *4-NQO* and other quinoline derivatives, is known to involve cytochrome P-450 and NADPH-dependent cytochrome P-450 reductase [45], glutathione-S-transferase, and quinone reductase [46]. As the result, there appears a set of metabolites most of which have a higher biological activity, and are potentially capable of interacting with high-molecular cell compounds (proteins, nucleic acids). Since an essential component part of research into the biological activity of chemical reagents *in vitro* is the study of their apoptosis inhibiting action, we shall now report the results on the effect of the investigated group of heterocycles on the expression (at the mRNA level) and enzymatic activity of cellular caspases.

Data on modifications of caspase-3, -6 and -9 mRNA expression in *K562* cells treated with *Q*, *QO* and *4-NQO* for 48 h are presented in Figure 4 (**a–c**). The reagent concentrations applied are detailed in the figure. The results suggest that cell incubation with *4-NQO* caused a rise in mRNA expression in all caspases; treatment with *Q* induced a rise in caspase-3 and -9 mRNA expression; whereas *QO* did not induce caspase expression under the given treatment conditions. A similar pattern was observed in the enzymatic activity (Figure 5 **a–c**). Cell incubation with *4-NQO* promoted the activity of all the caspases, whereas treatment with *Q* activated only caspases-3 and -9 (caspase-6 in this case was induced only on the 96 h). *QO* exhibited an activating effect on caspase-3 also on the 96 h of tumor cell treatment. Note that the activity of caspase-6 in the cells ($p<0.05$) (Figure 5 **b**) treated with *4-NQO* rose much less than that of caspases-3 and -9 ($p<0.01$) (Figure 5 **a**, **c**).

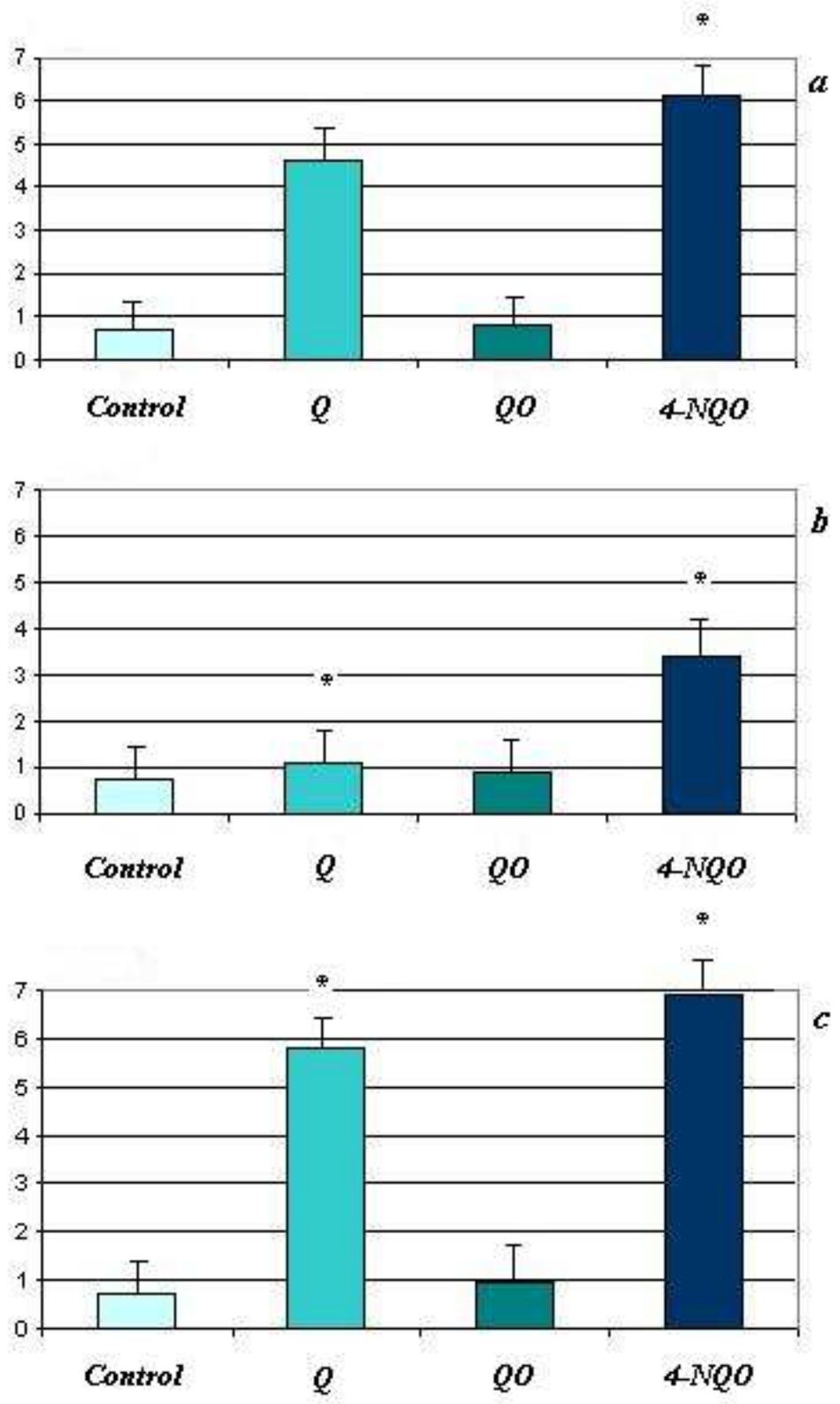
Caspase expression in *K562* cells incubated with *2-MeQ, 2-MeQO*, and *2-Me-4-NQO* did not differ from their expression in *Q*, *QO*, and *4-NQO* treatments, respectively.



Results are presented as the ratio of caspase to GAPDH expression relative to a standard curve for each assay. Drug concentrations: *Q* – 0.3 μM, *QO* – 10 μM, *4-NQO* – 0.001 μM. Incubation time – 48 h. Viability of cultured cells – 93–95%.

Y axis – relative expression, conventional unit; * – $p<0.05$

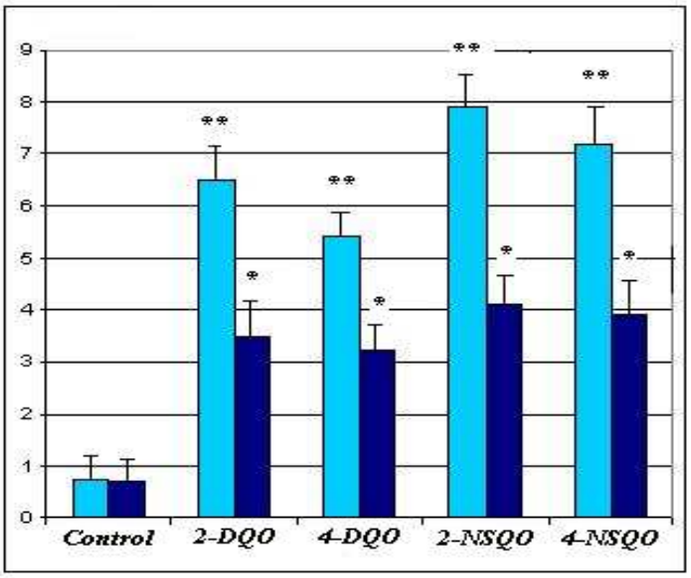
Figure 4. Caspase-3 (*a*), -6 (*b*) and -9 (*c*) mRNA levels were determined by quantitative real-time RT-PCR in *K562* cells treated with *Q*, *QO* and *4-NQO* respectively.



Drug concentrations: *Q* – 0.3 μM, *QO* – 10 μM, *4-NQO* – 0.001 μM. Incubation time – 48 h.
Viability of cultured cells – 93–95%. Y axis – $\Delta S/\Delta t \times 10^{-4}$; * – $p<0.05$

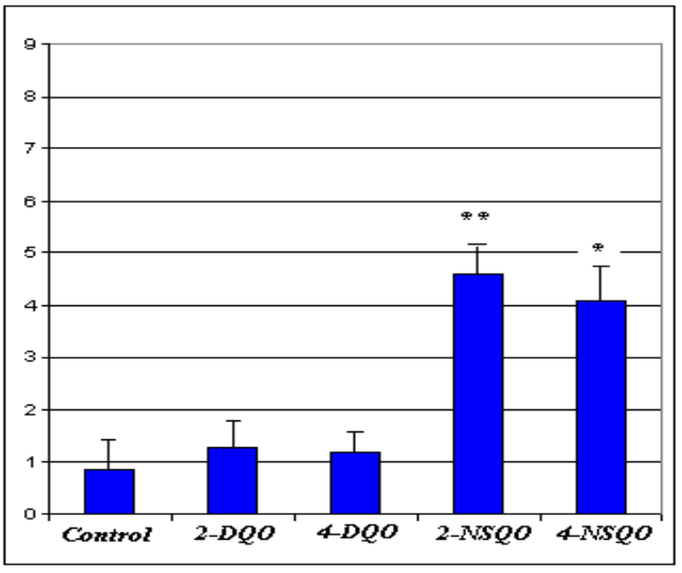
Figure 5. Caspase -3 (*a*), -6 (*b*) and -9 (*c*) activity in *K562* cells treated with *Q*, *QO* and *4-NQO* respectively.

Styryl derivatives also produced caspase inhibiting effects on *K562* cells. The results portrayed in Figures 6 and 7 evidence that treatment of tumor cells with *2-DQO*, *4-DQO*, *2-NSQO*, and *4-NSQO* promoted the activity of caspases-3 and -6, but with the latter two reagents the activity of the stated caspases was higher, and the rise in caspase-9 activity was recorded on the 24 h of application of the compounds to the incubation medium. Expression at the mRNA level correlated with data on the activity at the enzyme level.



Drug concentrations – 1 μM. Incubation time – 48 h., Viability of cultured cells – 93–95%. Y axis – $\Delta S/\Delta t \times 10^{-4}$;
* – p<0.05, ** p<0.01;
caspase-3; caspase-6

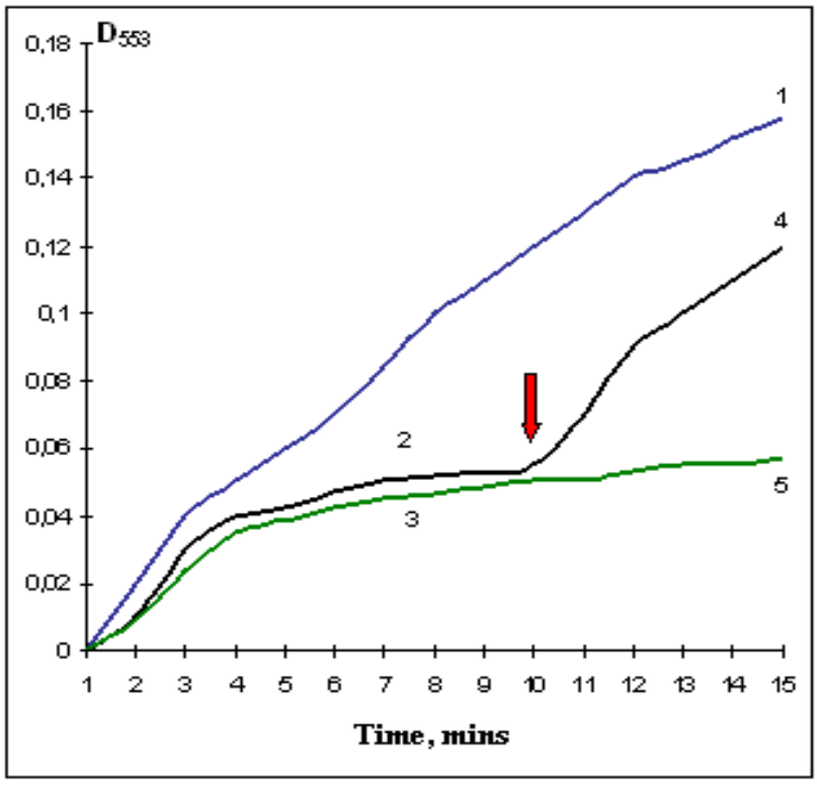
Figure 6. Caspase-3 and -6 activity in *K562* cells treated with **2***-DQO, 4-DQO, 2-NSQO, 4-NSQO* respectively.



Drug concentrations – 1 μM. Incubation time – 24 h, Viability of cultured cells – 93–95%. Y axis – $\Delta S/\Delta t \times 10^{-4}$;
* – p<0.05, ** p<0.01

Figure 7. Caspase-9 activity in *K562* cells treated with *2-DQO, 4-DQO, 2-NSQO, 4-NSQO* respectively

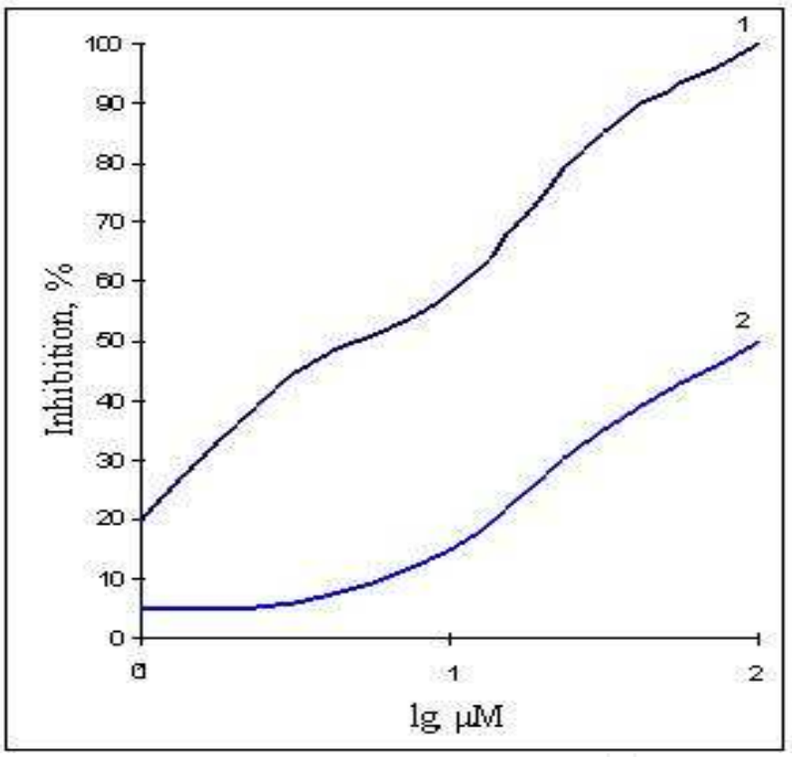
We have already mentioned that the release of cytochrome c from mitochondrial interior to the cell cytoplasm is a crucial stage in the activation of caspase cascades and triggering of apoptosis. In addition to involvement in the reactions of cellular caspase activation, "cytoplasmic" forms of cytochrome c also act as a substrate for microsomal NADPH-dependent cytochrome P-450 reductase [47]. This reductase facilitates electron transport to cytochrome P-450, which, in turn, participates in the metabolism of many heterocycles, including those investigated in the present paper. Figure 8 shows the kinetic curve of cytochrome c reduction by microsomes with no and with 10 μM *2-NSQO*. Addition of *2-NSQO* to the medium containing microsome suspension, NADPH, and cytochrome c causes a deceleration of cytochrome c reduction, which progresses with time. Depending on the reagent concentration, the reduction rate decreased at the greatest pace in the first 1–5 min of the reaction. Pre-incubation of microsomes with *2-NSQO* for 5 min, followed by the addition of NADPH and cytochrome c to the reaction medium did not change the protein reduction rate as compared with the variant described above. After the NADPH-cytochrome c reductase activity had been totally inhibited by *2-NSQO*, addition of NADH to the incubation medium partially restored cytochrome c reduction, whereas addition of NADPH and/or cytochrome c did not promote the reaction rate ($p>0.05$) (Figure 8).



Cytochrome c and NADPH were added immediately after application of *2-NSQO* to the microsome suspension (2) or after 5 min of pre-treatment of the microsomes with the reagent (3). NADH (50 μM) (4) or NADPH (50 μM) (5) were added to system 2 on the minute of the reading event. Light absorption by the system was measured in phosphate buffer on Labsystems Multiscan Plus reader (LKB, Finland) at 553 nm

Figure 8. Cytochrome c reduction by microsomes of *K562* cells with no (1), and with 10 μM *2-NSQO* (2–5).

Substitution of *2-NSQO* with its structural analog (*4-NSQO*) yielded a similar pattern, but inhibition of the NADPH-cytochrome c reductase activity proceeded much slower, and addition of NADH to the incubation medium fully restored cytochrome c reduction. Dependence of the NADPH-cytochrome c reductase activity on the concentration of *2-NSQO* and *4-NSQO* is shown in Figure 9. The microsomal protein content being 30 μg/ml, *2-NSQO* in a concentration of 10 μM inhibited the system's enzymatic activity by 50%, and at a concentration of 50 μM and higher the activity dropped by more than 80%. The 10 μM concentration of *4-NSQO* inhibited the enzymatic activity of microsomes by 15%, and the 100 μM concentration – by 50%. The above results suggest that the apoptosis inducing effect of *2-NSQO* and its structural analogs on the cells may be due to the irreversible inhibition of microsomal enzymes with NADPH-cytochrome c reductase activity. Where this effect happens, functioning of the whole electron transport chain in the cells is usually disrupted, there form large amounts of genotoxic products, and the cell eventually dies through apoptosis (or necroptosis).



X axis– *2-NSQO* and *4-NSQO* concentrations, μM (logarithmic scale); Y axis – inhibition of the reaction rate (%) relative to the control. Modifications to the system were made in the first 5 min of the reaction

Figure 9. Effect of *2-NSQO* (1) and *4-NSQO* (2) on NADPH-cytochrome c reductase activity of the microsomes of *K562* cells.

Another important fact is that when some quinoline derivatives interacted with DNA the greatest hypochromic shift in DNA UV spectra (which evidences the formation of complexes) was observed in treatments with *4-NQO, 4-NSQO, 4-DQO, 4-DPyO*, whereas mixing of *Q* or *2-MeQ* with DNA did not cause changes in the absorption spectra (Table 3). This fact supports the assumption that for such biological effect to appear the latter two

reagents must first undergo metabolic activation in the cell, and form electrophilic centres within the molecule. Other N-bearing heterocyclic compounds are potentially able to bind to DNA without prior intracellular activation (e.g., through intercalation), so that their biological effects would appear sooner.

Reagent	Percent of the reagents-related hypochromic effect of DNA
4-NQO	10.7±1.2
4-NSQO	9.7±1.3
4-DQO	9.4±1.8
4-DPyO	7.0±1.4
QO	2.4±0.6
Q	0
2-MeQ	0

Table 3. Hypochromic effect of DNA upon binding with quinoline and pyridine derivatives. DNA from chicken erythrocytes was used in the experiment. The spectra were obtained in phosphate buffer on a Labsystems Multiscan Plus reader (LKB, Finland) at λ 260 nm.

Many chemical compounds, including anticancer drugs, can induce tumor cell apoptosis both *in vivo* and *in vitro* [48–50]. However, a major cause of failure in the application of cytostatic agents in clinical practice is the multiple drug resistance (MDR) phenotype induced in the tumor cells. Multidrug resistance is a condition when tumor cells are unsusceptible to a variety of chemotherapeutic agents differing in chemical structure and the mechanisms through which they affect the cell. MDR is a serious hindrance to success in the treatment of malignant tumors, including leukosis. Latest studies have demonstrated that the molecular mechanisms of MDR are multifarious, and the drug resistance of a cell can depend on various mechanisms triggered at different stages of the toxic impact of the agent on the cell – from restricted accumulation of the drug in the cell to cancellation of the substance-induced cell death programme. Interplay of several protective mechanisms is not uncommon, but one mechanism usually prevails. The best studied mechanisms, whose clinical significance for certain neoplastic forms (such as chronic myeloid leukemia or chronic lymphatic leukemia) has been ascertained, are: activation of transmembrane transport proteins that remove various substances from the cell (namely, P-glycoprotein – Pgp); activation of the glutathione system enzymes that detoxify the drugs; modifications in the genes and proteins that control apoptosis and cell survival [51].

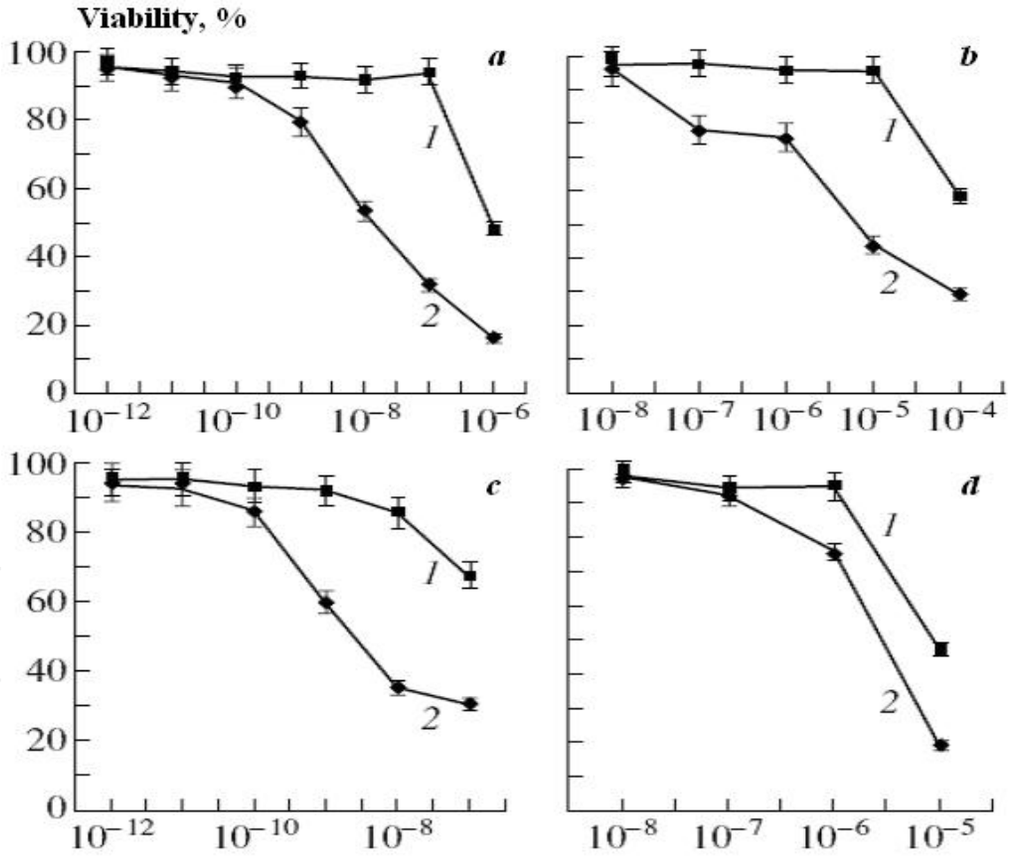
We have therefore obtained *K562* cell sublines resistant to *2-DQO* and *4-NQO* (*K562/2-DQO, K562/4-NQO*), and characterized their capacity to undergo induced apoptosis under the effect of structurally different chemical reagents as compared with the parental line cells.

The MDR development mechanisms related to inhibition of cytostatic-induced apoptosis have lately been actively investigated. Of greatest interest among the cytostatic agents are DNA-tropic compounds (adriamycin, actinomycin D), antimetabolites (5-fluorouracil, methotrexate), reagents interacting with the mitotic spindle microtubules (vincristine,

vinblastine, taxol), and others. *K562/4-NQO* and *K562/2-DQO* cells were tested for susceptibility to the following chemical compounds: DNA intercalating agent ethidium bromide, microtubule polymerization inhibitor colchicine; and quinoline derivatives – *2-DQO* and *4-NQO*, respectively. The results are shown in Table 4 and Figure 10.

Cell line	EC_{50}, μM			
	Ethidium bromide	Colchicine	*4-NQO*	*2-DQO*
K562	2.5	0.002	1.06	79.7
K562/4-NQO	39.8	0.0004	—	398.2
K562/2-DQO	7.9	0.120	79.0	—

Table 4. Susceptibility of *K562/4-NQO* and *K562/2-DQO* cells to cytostatic agents. Clones resistant to *4-NQO* and *2-DQO* were obtained after [38]. Cell resistance to the xenobiotics was induced by long-term (over 1 month) exposure to 10^{-12} M *4-NQO* or 10^{-9} M *2-DQO* in the culture medium. The concentration of the substances was then increased every 14th–21st days. The final concentrations of the reagents for which resistant cell lines were obtained were 10^{-8} M for *4-NQO*, and 10^{-5} M for *2-DQO*.



a – cells were treated with *4-NQO*: 1 – Viability of *K562/2-DQO* cells, 2 – Viability of *K562* cells;
b – cells were treated with *2-DQO*: 1 – Viability of *K562/4-NQO* cells, 2 – Viability of *K562* cells;
c – cells were treated with colchicines: 1 – Viability of *K562/2-DQO* cells, 2 – Viability of *K562* cells;
d – cells were treated with ethidium bromide: 1 – Viability of *K562/2-DQO* cells, 2 – Viability of *K562* cells.
X axis – drug concentration (M). Incubation time – 48 h.

Figure 10. Viability of *K562*, *K562/2-DQO* and *K562/4-NQO* cell lines treated with different xenobiotics.

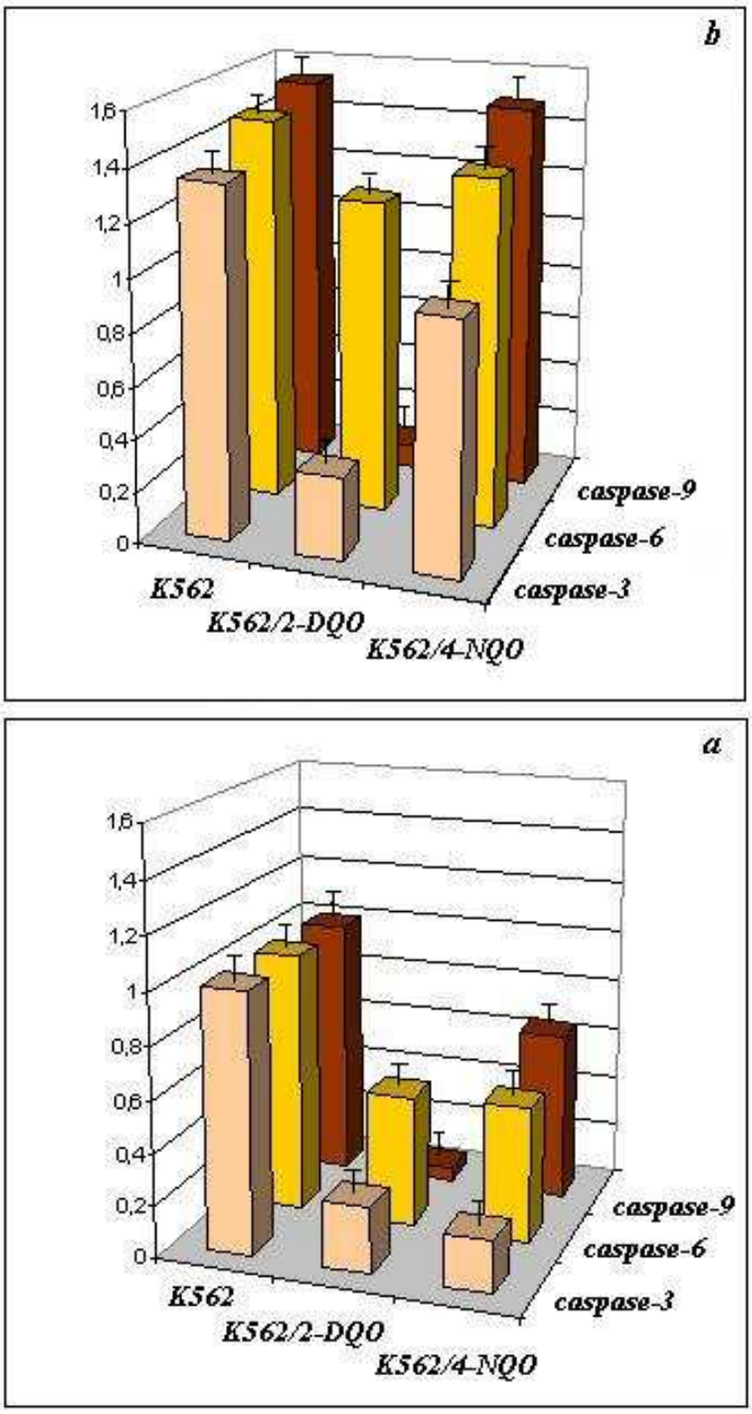
Measurements of the viability of the cells of the parental line and the derived sublines, incubated for 48 h in the presence of various concentrations of the reagents, revealed substantial differences in the toxic effect of the xenobiotics on the tumor cells. *K562/4-NQO* cells were highly resistant to ethidium bromide and *2-DQO*, but susceptible to colchicine (Table 4, Figure 10). Contrastingly, *K562/2-DQO* cells demonstrated quite high resistance to colchicine and *4-NQO*, but lower resistance to ethidium bromide (Table 4, Figure 10). One should mention that the resistance of *K562/2-DQO* cells to ethidium bromide was 5.03 times lower than that of *K562/4-NQO* cells.

It is now solid knowledge that when subjected to stepped selection for resistance to some cytotoxic agents (plant alkaloids, antibiotics), cells of tumor lines in vitro become cross-resistant to quite a number of cytostatics, which differ both in the structure and genesis, and in the effect on different cellular targets [52–54]. The spectra of the drugs to which the cells develop cross-resistance, as well as the mechanisms behind it may vary depending on the selection agent. Grinchuk et al. [55] isolated three adriamycin-resistant clones *C9*, *B2*, and *B3*, through multi-step selection from the *K562* cell population. The cells of the adriamycin-resistant clones displayed cross-resistance to colchicine, actinomycin D, and ethidium bromide – agents of the multidrug resistance group. Amplification of the gene *mdr1* was detected in the cells of the resistant clones at DNA hybridization with Southern blot. Karyological analysis of the resistant cells performed at early stages of their incubation with adriamycin showed the genome contained extra genetic material (morphological markers of amplification – double minichromosomes in clones *B2* and *B3*, and uniformly stained regions in the chromosomes of clone *C9* cells). The karyotype modifications are attributed to destabilization of the *K562*/adriamycin cell genome as the cells acquired the MDR phenotype. Thus, distinctions in the resistance to the toxic effect of xenobiotics among cells of different sublines may be related to specific characteristics of the expression of *mdr* genes and then P glycoprotein, expression of other MDR proteins, genome destabilization, and, not least, changes in the activity of cellular enzymes involved in the first (e.g., cytochrome P-450) and second (e.g., glutathione-S-transferase) phases of the reagent metabolism in the cells. These and other factors can, under certain conditions, play a decisive role in the modulation of the functional activity of apoptosis inducing agents, including caspases.

Figure 11***a*** shows the results on relative expression of caspase-3, -6, and -9 genes in the cells of the *K562* parental line and the derivative sublines (data from RT-PCR analysis). One can see that the mRNA expression of the named caspases in *K562/4-NQO* and *K562/2-DQO* cells is significantly lower ($p<0.05$) than in the parental *K562*, and that is a potential explanation of the resistance of the tumor cells to *4-NQO* and *2-DQO*.

In the treatment with colchicine mRNA expression of caspases-3, -6, and -9 was significantly promoted in the parental line *K562* and *K562/4-NQO* – $p<0.05$ (Figure 11 ***b***). In *K562/2-DQO* cells only caspase-6 expression was activated ($p<0.05$). Incubation of tumor cells with ethidium bromide promoted the mRNA expression of the three investigated caspases in the parent cells; caspase-3 expression was promoted in *K562/2-DQO* cells; no significant changes in caspase mRNA expression was observed in the *K562/4-NQO* line. Changes in the enzymatic activity correlated with mRNA expression.

The results above prove that the cell sublines we have obtained have become cross-resistant to chemical reagents of the MDR group, and that such resistance may be due, i.a., to modifications in caspase expression.



Results are presented as the ratio of caspase to GAPDH expression relative to a standard curve for each assay. Drug concentrations: 0.1 μM for *K562/2-DQO* cells, 0.001 μM for *K562* cells, 0.001 μM for *K562/4-NQO* cells. Incubation time – 48 h. Viability of cultured cells – 93–95%. Z axis – relative expression, conventional unit.

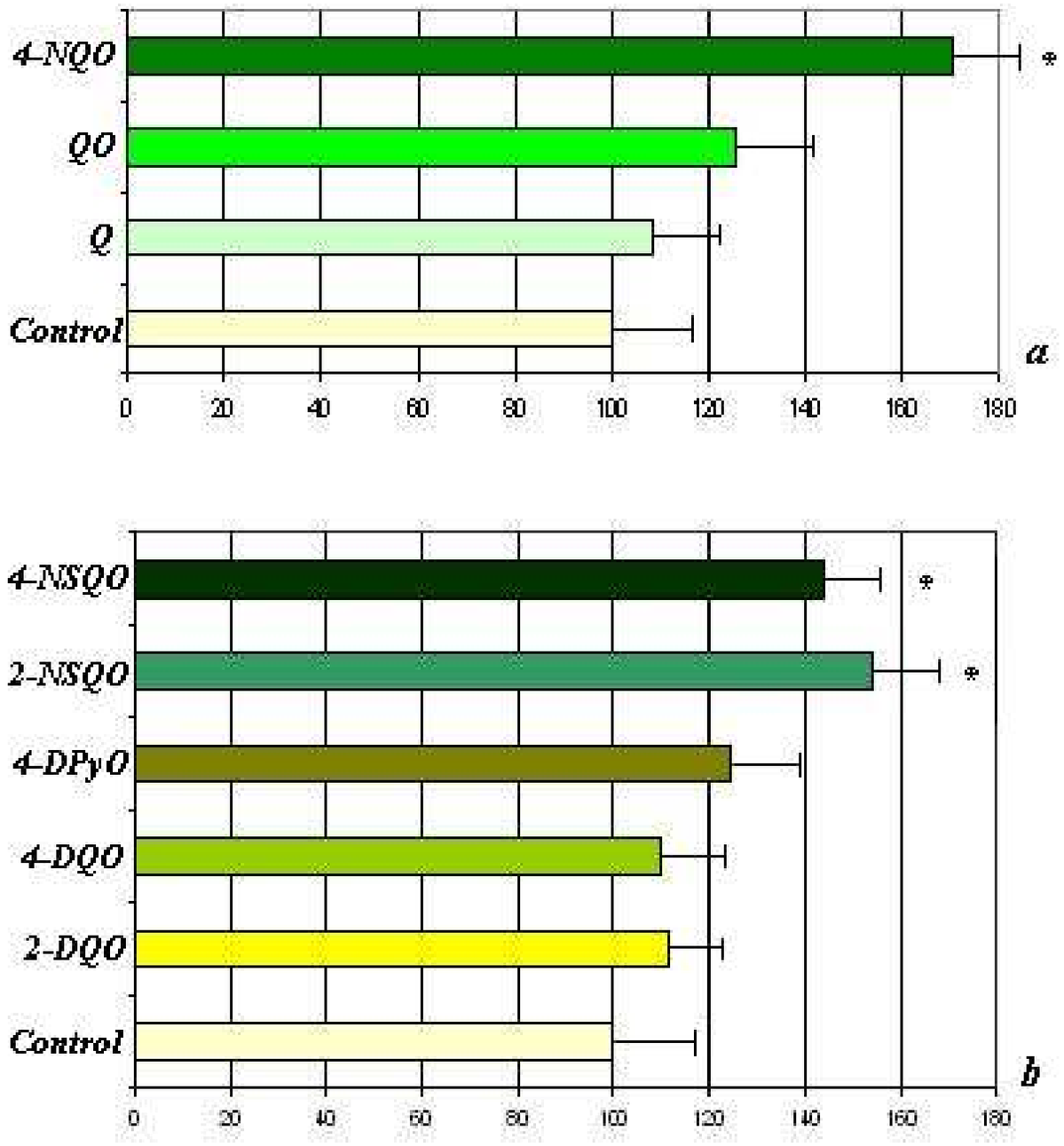
Figure 11. Caspase-3, -6 and -9 mRNA levels were determined by quantitative real-time RT-PCR in *K562*, *K562/2-DQO* and *K562/4-NQO* cell lines (*a*) treated with colchicine (*b*) respectively.

One of the preconditions for *in vivo* development and progress of tumors is that the tumor cells gain another type of resistance – resistance to factors of anti-tumor immunity. Such factors are, first of all, natural killer (NK) cells and cytotoxic T lymphocytes. The mechanisms of identification of *de novo* tumor cells in the organism by natural killers have not been fully elucidated, but the mechanisms of the lysis of such cells are quite well studied. NK cells and antigen-specific cytotoxic T lymphocytes, as well as these IL-2 activated cells are known to lyse target cells both by perforin-dependent cytolysis, and by inducing Fas-dependent apoptosis [56, 57]. In this case, the target cells can modulate the rate of release of the cytolytic granule content, including perforin and granzymes, or suppress killer activation by reducing the frequency and affinity of the formation of target cell-effector cell conjugates, the latter being a quite frequent phenomenon in the system [58]. Perforin is structurally homologous to the *C9* complement component, and has a similar action mechanism (formation of polyperforin pores made up of 12–18 monomers, with the inner diameter of 10–20 nm) [56]. On the other hand, no DNA fragmentation occurs when the complement acts on the cells, wherefore we presume that the action of perforin involves also other factors (namely serine proteases of the granzyme family [58], which can permeate the target cell cytosol via perforin-generated pores in the plasma membrane and trigger apoptotic events, for instance by means of caspase activation.

Some authors have demonstrated in their papers that treatment of tumor cells with chemical reagents modifies their susceptibility to the cytotoxic lysis of homologous effector cells, and the modification may be either for amplification or for reduction of the effect depending on the cell line and the treatment settings. E.g., treatment of *K562* cells with sodium butyrate [59], fagaronine, adriamycin, aclacinomycin A [60] makes the named cells less susceptible to natural killer lysis. Quite a number of lymphoma cell lines (*Raji* and *Daudi* lymphoma cell lines, human lymphoblastoid cell line *NAD-7*, human T-lymphoblastoid line *Molt-4*) treated with PMA, sodium butyrate, retinoic acid demonstrate higher susceptibility to the cytotoxic activity of natural killers [61−63]. An interesting effect was observed in the case of *CB-1 B* cell lines. Clone *26CB-1* is more resistant to NK cells than clone *13CB-1*. After they had been treated with sodium butyrate, iodinated deoxyuridine, 5-azacytidine, tunicamycin, the susceptibility of clone *26CB-1* cells to cytotoxic lysis increased, whereas the susceptibility of clone *13CB-1* cells remained nearly unmodified [64].

Distinctions in the chemical inducers' structure and mechanisms of action on cells may have different effects on modulation of the expression of positive and negative apoptosis regulators, which quantitative ratios may contribute to establishment of both the initial susceptibility of the cells to natural killer lysis and the post-treatment susceptibility. This may as well be valid for the cell sublines with a more or less differentiated phenotype.

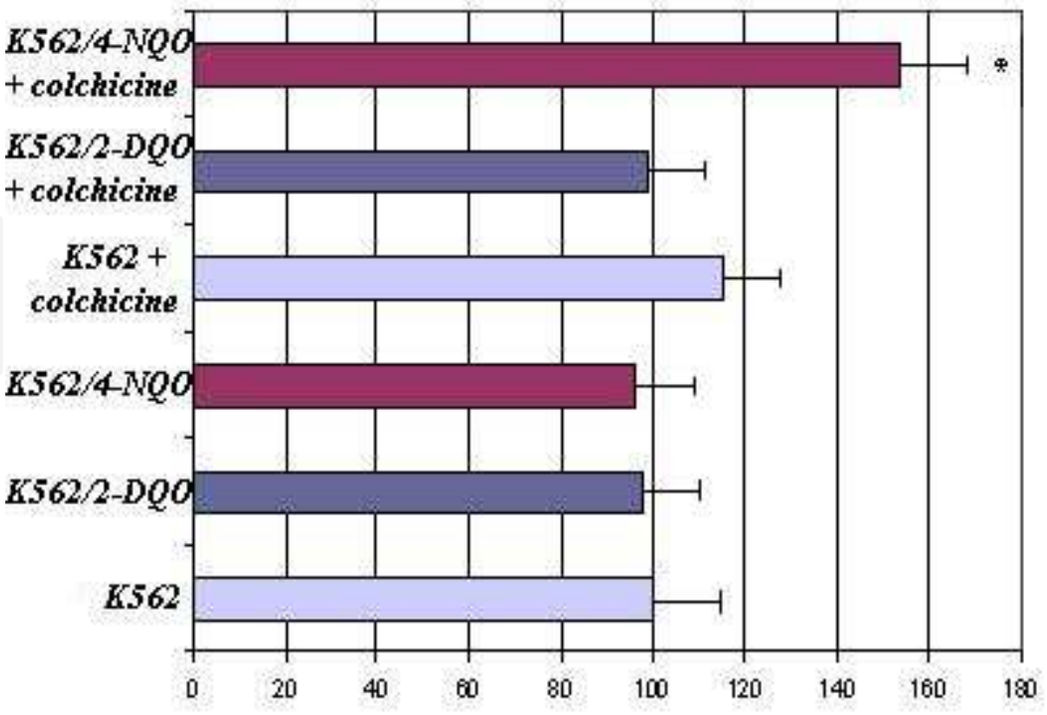
Data in Figure 12 (**a**, **b**) show that the treatment of tumor cells with *4-NQO, 2-NSQO,* and *4-NSQO* for 48 h caused a significant rise ($p<0.05$) in susceptibility to the lysis of human leukocytes containing NK cells. Caspase expression and cell apoptosis induction were also promoted in this case. In treatments with other quinoline derivatives the cytotoxic index in the cultures did not change. A similar pattern was observed also when incubation lasted 96 h.



Drug concentrations: *Q* – 0.3 μM, *QO* – 10 μM, *4-NQO* – 0.001 μM, styryl derivatives – 1 μM. X-axis: cytotoxic index ratio of effector cells with treated cell lines to control cell lines without treatment, % (cytotoxic index value of control cells is 100 %). Effector cells to target cells ratio is 50:1. Incubation time – 48 h; * $p<0.05$.

Figure 12. The *K562* cell line sensitivity to cytotoxic lysis by human peripheral blood white cells after treatment with quinoline and pyridine derivatives

When cells of sublines *K562/4-NQO* and *K562/2-DQO* were used as the targets, changes in susceptibility to the leukocyte lysis effect were recorded only in the *K562/4-NQO*–colchicine system (Figure 13).



Drug concentrations – 0.1 μM for *K562/2-DQO* cells, 0.001 μM for *K562* cells, 0.001 μM for *K562/4-NQO* cells. X-axis: cytotoxic index ratio of effector cells with treated cell lines to control cell lines without treatment, % (cytotoxic index value of control cells is 100 %). Effector cells to target cells ratio is 50:1. Incubation time – 48 h; * $p<0.05$.

Figure 13. The *K562* cell line sensitivity to cytotoxic lysis by human peripheral blood white cells after treatment with colchicines.

Thus, treatment of tumor cells with chemical compounds may simultaneously induce cell apoptosis and modulate their susceptibility to the cytotoxic lysis of blood leukocytes. In these treatment conditions the expression and/or activity of intracellular apoptosis regulators, namely caspases, changes, and this change may tell on the effectiveness of the leukocyte lysis effect, which is based on induction of target cell apoptosis. It is essential that the susceptibility of tumor cells to the lysis of leukocytes containing NK cells changed in those variants where the expression and activity of caspases-3, -6, -9 were promoted (cultures treated with *4-NQO, 2-NSQO*, and *4-NSQO*). On the other hand, the equivocal nature of the results proves that the process involves extra intracellular factors. One can therefore assume that similar patterns of the response of tumor cells to treatment with chemical compounds can occur *in vivo* in humans and animals treated with the anticancer drugs stimulating cell apoptosis. This effect can persist when nontoxic or low-toxicity doses are applied, and caspases, being a cell's major apoptosis inducing factor, can be viewed as potential targets for pharmacologically active agents.

5. Conclusions

Over 40 years have passed since the first publication devoted to apoptosis. Since then, the problem of apoptosis regulation mechanisms has become central to pharmacology, genetics,

virology, cytology, immunology, biochemistry, embryology, and other areas of modern science at the cellular and molecular levels. It is obvious that the problem addressed in this chapter is of high applied value, for the key aim of the effort is to enhance the effectiveness of anticancer therapy. The industry of rational drug design is becoming more and more widespread in practical medicine. The principal concepts in drug design are the target and the drug. The target is a low-molecular biological structure presumably linked to a certain function which disruption would lead to disease, and to which a certain impact should be applied. The most common targets are receptors, enzymes, hormones. The drug is most often a chemical compound (usually a low-molecular one) that specifically interacts with the target, modifying the cell response in one way or another. One of the earliest and most seminal stages of drug design is accurate identification of the target by influencing which one can specifically regulate certain biochemical processes, leaving others unaffected as much as possible. This is not however always feasible: by no means are all diseases caused by dysfunction of just one protein or gene. Preference should therefore be given to the processes' principal biomolecules. In terms of apoptosis, caspases clearly are such biomolecules. Hence, the choice of methods for experimental validation of caspases as potential specific targets for drugs is a topical challenge for the nearest future.

6. Abbreviations:

Quinoline – *Q*, 2-methylquinoline – *2-MeQ*, quinoline-1-oxide – *QO*, 2-methylquinoline-1-oxide – *2-MeQO*, 4-nitroquinoline-1-oxide – *4-NQO*, 2-methyl-4-nitroquinoline-1-oxide – *2-Me-4-NQO*, 2-(4'-dimethylaminostyryl)quinoline-1-oxide – *2-DQO*, 4-(4'-dimethylaminostyryl)-quinoline-1-oxide – *4-DQO*, 2-(4'-nitrostyryl)quinoline-1-oxide – *2-NSQO*, 4-(4'-nitrostyryl)-quinoline-1-oxide – *4-NSQO*, and 4-(4′-dimethylaminostyryl)pyridine-1-oxide – *4-DPyO*; death effector domain – DED, caspase recruitment domain – CARD, death inducing domain – DID, p53-induced protein with a death domain – PIDD, death-inducing signalling complex – DISC, DNA fragmentation factor – DFF, caspase-activated DNase – CAD, permeability transition pore – PTP, apoptosis inducing factor – AIF, tumor necrosis factor receptor – TNFR, death domain – DD, death receptor – DR, Fas-associated DD-protein – FADD, TNFR1-associated DD-protein – TRADD.

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