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Study of cytotoxic activity of new 1,4-naphthoquinone derivatives in murine hepatoma cell line

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A family of vitamins K (VK), 2-methyl-1,4-naphthoquinone derivatives, especially VK_3, have been reported to inhibit the growth of various tumor cell lines. Analysis of cell growth inhibition by novel 1,4-naphthoquinone (NQ) derivatives in the present study was undertaken with two purposes. The first one was to sample new biologically active antitumor compounds, the second being to study the mechanism of cell growth inhibition and death induction by NQ derivatives. Retardation of cell proliferation and cytotoxic activity of synthesized by us five 1,4-naphthoquinones **1–5** containing 2-aminoalkyl moiety with terminal bromo (**1**, **2**), chloro (**4**), hydroxyl (**3**) and mercapto (**5**) groups were examined in the model murine hepatoma cell line MH-22A. Our study showed that four NQ compounds, **1–4,** induced both apoptotic and necrotic cell death. The frequency of apoptosis was largely parallel to the potency of growth inhibition. According to our data, the most effective were compounds 2-(2-hydroxyethylamino)-1,4-naphthoquinone (**3**) and 2-(2-bromoethylamino)-1,4-naphthoquinone (**1**). Derivative **3** appeared to be particularly active; its antiproliferative activity was about 40% higher than of VK_3. In addition, the findings suggest that compound **3,** as well as **1,** in our study induced overexpression of the *c-jun* gene, which has been reported to be associated with apoptosis. Meanwhile, transfected MH-22A cells where the c-Jun level was blocked were much more resistant to the treatment with the NQ derivatives **1** and **3**. In summary, the novel 1,4-naphthoquinones **1** and **3** containing 2-aminoethyl functions with terminal bromo (**1)** and hydroxyl (**3**) groups exhibited structural requirements for murine hepatoma cell growth inhibitory and cell killing activities. The effects may be related with the induction of *c-jun* expression.

Key words: 1,4-naphthoquinone derivatives, tumor cells, antiproliferative, cytotoxic activity, apoptosis

INTRODUCTION

Among the varieties of naturally occurring 1,4-naphthoquinones (NQ), the vitamin K (VK) family is the most important. Physiologically, VK (2-methyl-1,4-naphthoquinone derivatives) is usually identified as a critical factor in blood coagulation [1]. In addition, its action in bone formation and repair, influence on pancreatic function, assistance in converting glucose to glycogen as well as inhibition of cell growth has been observed [2–4].

A number of studies have shown various VK to possess antitumor activity in rodent- and human-derived neoplastic cells both *in vitro* and *in vivo* [5, 6]. However, VK_3 and its analogs have previously been shown to be the most promising in this aspect [7]. Though the growth inhibitory and cytotoxic effects of VK_3 have been demonstrated, the mechanisms of their action is not well understood.

The effectiveness of VK_3 against cancer is believed to be due to (i) its ability to associate and intercalate with DNA duplexes, (ii) its participation in key cellular redox mechanisms and oxidative stress in the cell [8, 9]. Studies of the cell death after treatment with VK_3 show that it seems to act by two ways depending on the concentration, at higher levels initiating an oxidative action and necrosis or autoschizis, while at lower levels inducing apoptosis [10]. On the other hand, the oxidative stress produced by VK_3 can trigger a cascade of events leading to the activation of caspase-3, DNA fragmentation and characteristic signs of apoptosis [11]. Several investigators have demonstrated that VK_3-induced cell death is asso-

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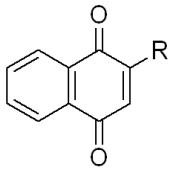
ciated with overexpression of the *c-myc* gene which is considered to be closely related to apoptosis [12, 13]. Similarly, the data exist that VK_3 toxicity is related with activation of the JNK pathway [14]. Following activation, JNKs phosphorylate several nuclear substrates, including the transcription factor c-Jun. The proto-oncogene c-Jun has been implicated in the control of cell proliferation, differentiation and in the regulation of apoptosis [15]. It has been shown that c-Jun has diverse roles in the apoptotic process, depending on cell type and the micro-environment [16].

The objective of the present study was to investigate antitumor activity of our synthesized NQ derivatives **1–5** containing 2-aminoalkyl moieties with terminal bromo, chloro, hydroxyl and mercapto functions instead of 2-methyl group characteristic of the vitamin K family:



1- 5

R = $NH(CH_2)_2Br$ (**1**)
R = $NH(CH_2)_3Br$ (**2**)
R = $NH(CH_2)_2OH$ (**3**)
R = $NH(CH_2)_2Cl$ (**4**)
R = $NH(CH_2)_2SH$ (**5**)
R = CH_3 (**VK_3**)

We tried to relate the structural features of the derivatives to their antiproliferative and cytotoxic activity. Furthermore, we examined the relationship between the way of cell death and *c-jun* expression in MH-22A cells after treatment with the most effective derivatives **3** and **1**.

MATERIALS AND METHODS

Cell culture. Cells of murine hepatoma cell line MH-22A (parental) were propagated in minimal essential medium (MEM) with 2 mM glutamine, supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (50 μg/ml) at a density of $3x10^5$ cells/ml.

The stable transfected MH-22A cell ("antisense") clone containing *c-jun* construct in antisense orientation was kindly presented by Dr. A. Kalvelytė (Department of Developmental Biology, Institute of Biochemistry). The transfected cells were grown in the same conditions.

Reagents. The NQ derivatives **1–5** were synthesized at the Institute of Biochemistry [17]. As a control, VK_3 (Sigma) was used. In this study, VK_3 and the NQ derivatives **1–5** were dissolved in dimethylsulphoxide (DMSO). The final concentration of the substances was 20 μM in the complete medium.

Cell proliferation assay. After 24 and 72 h of treatment, cell number was quantified microscopically, counting the cells with a hematocytometer.

Cell viability test. Viability of cells was determined by the acridine orange (100 μg/ml)/ethidium bromide (100 μg/ml) cell staining technique using fluorescent microscopy. The cells were identified as viable (V), apoptotic (A), necrotic (NEC) and chromatin-free (CF).

Western blot analysis. For Western blotting, cells at a density of $4x10^5$ were washed with ice-cold PBS and lysed in a cold lysis buffer. Lysed cells were centrifuged at 20000 g for 15 min. Proteins were separated by 12% SDS–PAGE and transferred to nitrocellulose membranes. Blots were incubated with primary rabbit anti-c-Jun antibodies. As secondary antibodies, horseradish peroxidase was used. Specific bands were visualized using the ECL Western Blot Detection System (Amersham International).

Statistical analysis. The results represent the means of three independent experiments. They are expressed as the mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$. Analysis was carried out using the statistical package of MS Excel 2002 program.

RESULTS AND DISCUSSION

The synthesis of the novel NQ derivatives **1–5** and analysis of tumor cell growth inhibition by these derivatives in the present study was undertaken with two purposes. The first one was to evaluate antitumor activity of the novel NQ compounds **1–5** in comparison to VK_3, the second purpose being to study the mechanism of cell growth inhibition and death induction by the NQ derivatives **1–5**.

Cytotoxic activity of synthesized by us five 1,4-naphthoquinones was examined in the model murine hepatoma cell line MH-22A. After a pilot study, we found that 20 μM was the optimal concentration of the novel NQ **1–5** derivatives for a comparison of cytotoxic activity in MH-22A cell culture (data not presented). The evidence for choosing this concentration for further studies was based on the literature data about activity of VK_3 in cell culture. It has been found that 5 μM VK_3 stimulates DNA synthesis, while 10 μM exerts antiproliferative effects, 20 μM can induce apoptosis, and 100 μM induces necrosis [18, 10].

The experiments shown in Fig. 1 were carried out to determine the antiproliferative and cytotoxic activity of the novel NQ derivatives **1–5**. After 72 h of exposure, most of the compounds (except compound **5**) exhibited retardation of cell proliferation in comparison with control. The antiproliferative activity of compounds **1, 3** and **4** exceeded VK_3 activity in MH-22A cell culture. It must be noted that after 72 h of cell treatment most effective were compounds 2-(2-bromoethylamino)-1,4-naphthoquinone (**1**) and 2-(2-hydroxyethylamino)-1,4-naphthoquinone (**3**), which exceeded the antiproliferative VK_3 activity. Compound **3** appeared to be particularly active. The cell number after exposure to this derivative (**3**) was almost by 40% lower than after treatment with VK_3. Analysis of cell viability illustrated a cytotoxic effect in the MH-22A culture after the treatments (Fig. 2). Exposure for 24 h, but especially for 72 h highlighted the cytotoxic activity of compound **3**. The data presented show that after a 72 h exposure to compound **3** there were about 45% of viable cells, 20% apoptotic and about

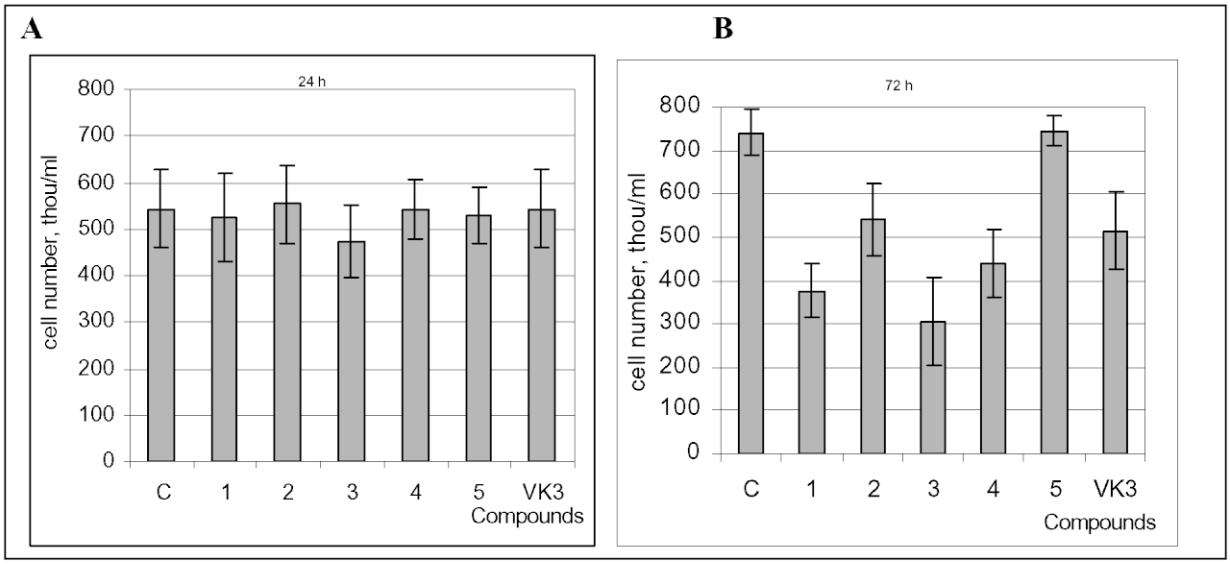


Fig. 1. Antiproliferative effect of new NQ derivatives: number of MH-22A cells after 24 h (A) and 72 h (B) treatment with NQ – compounds **1, 2, 3, 4** and **5; C –** control (without treatment), **VK_3** – exposure to vitamin K_3

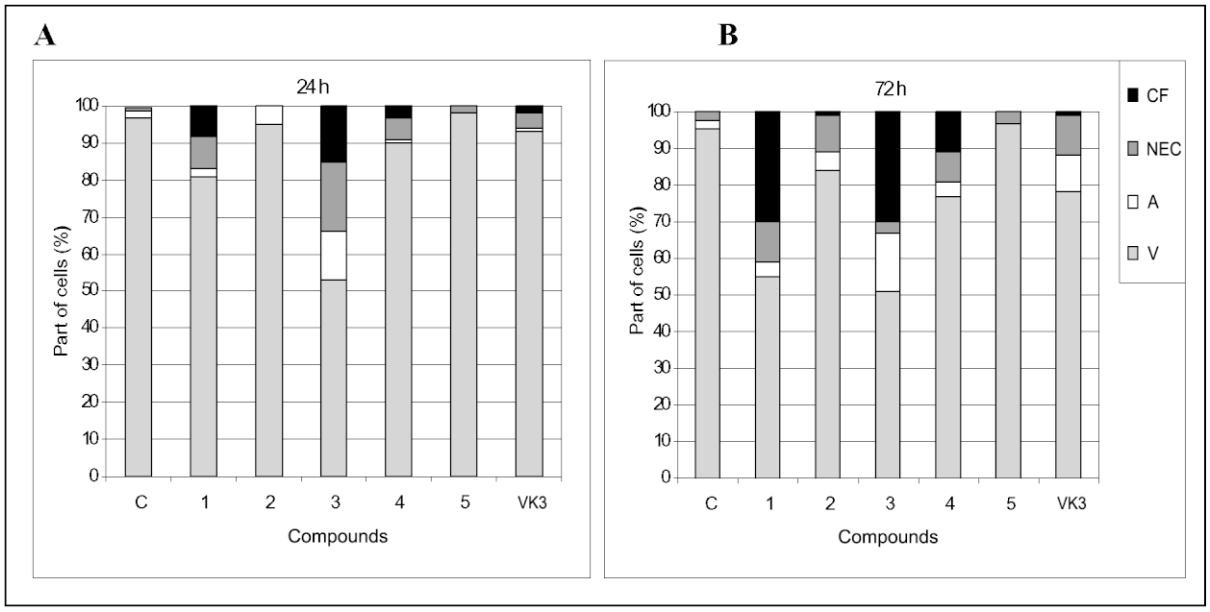


Fig. 2. Cytotoxic effect of NQ derivatives **1, 2, 3, 4** and **5** on MH-22A cells: percentage of viabile (V), apoptotic (A), necrotic (NEC) and chromatin-free (CF) cells after 24 h (A) and 72 h (B) treatment; **C –** control (without treatment), **VK_3** – exposure to vitamin K_3

35% of chromatin-free cells in the culture. The total portion of dead cells in the test culture was about 55%. In comparison, the VK_3-induced cytotoxicity was less pronounced: there were more than 70% of viable, about 10% of apoptotic and the rest were chromatin-free cells in the culture. The total portion of dead cells after VK_3 exposure was about 30%.

In order to study the mechanism of cell growth inhibition and death induction of the most active new derivatives **1** and **3,** we studied the expression of *c-jun* after treatments in MH-22A cell culture. c-Jun is a transcriptional regulator of gene expression. Hypothetically, c-Jun may trigger cell apoptosis by activating a variety

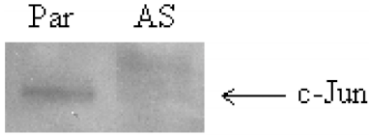


Fig. 3. C-Jun protein level in parental (Par) and „antisense“ (AS) MH-22A cells

of “death genes” and alternatively, it may supress some “protective genes” [16]. Therefore, *c-jun* expression and c-Jun activation may be a decisive factor in the cell life. To study the functional role of c-Jun in the MH-22A death process after the treatment, we used a stable

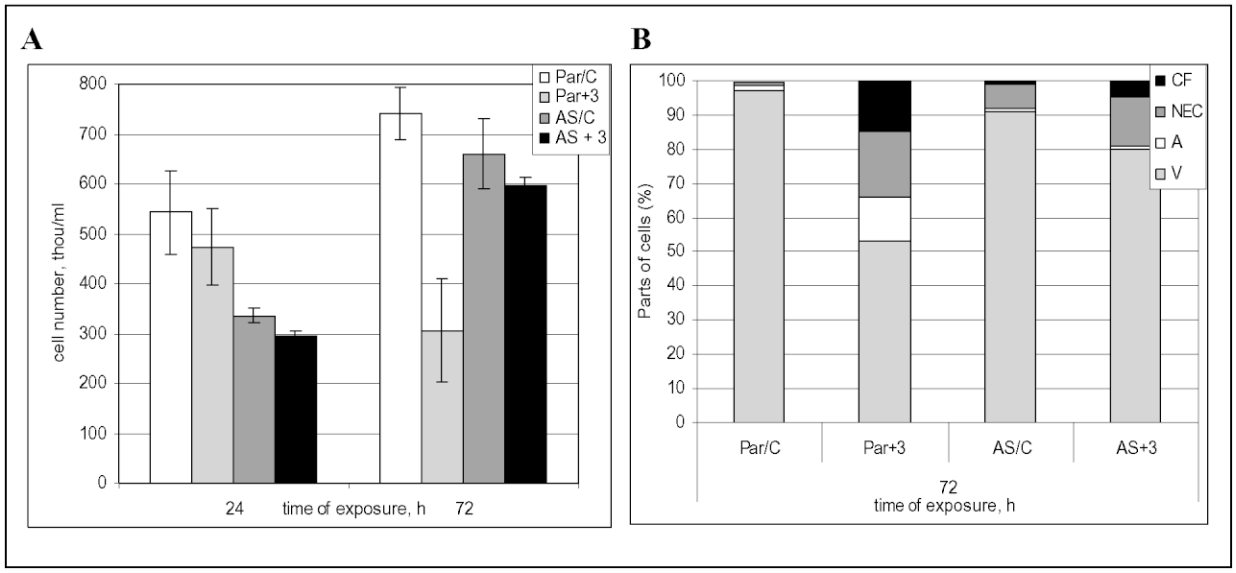


Fig. 4. Antiproliferative and cytotoxic effects of compound **3** on parental (Par) and „antisense" (AS) MH-22A cells. **A** – number of parental and „antisense" cells after 24 and 72 h of treatment with compound **3**; **B** – viability of parental and „antisense" MH-22A cells after 72 h of treatment with compound **3**

transfectant MH-22A cell ("antisense") clone containing a *c-jun* construct in antisense orientation. In the "antisense" cells, study of the c-Jun level confirmed a partially blocked expression of *c-jun.* The data indicated a constitutive level of c-Jun in parental MH-22A cells and a negligible level in "antisense" cells (Fig. 3).

The study of the antiproliferative and cytotoxic activity of compound **3** demonstrated inappreciable differences in cell number and viability between treated and control cells in the "antisense" MH-22A cell culture (Fig. 4). In comparison, the sensitivity of parental MH-22A cells to compound **3** was unarguable. The difference was particularly evident after 72 h cell exposure with compound **3.** Taken together, the data revealed the c-Jun role in MH-22A cell response to NQ derivative **3**. To check this presumption, we tested the expression of c-Jun after treatment in parental cells. The data presented in Fig. 5 indicate that compound **3** induced c-Jun expression. The expression was continuous after the treatment, and the highest protein level was registered after 48 h of exposure.

For the "antisense" cell culture, similar antiproliferative and cytotoxic studies were also performed with compound **1** (data not presented). It should be noted that the results of retardation of cell proliferation and viability as well as induction of c-Jun synthesis demonstrated the same tendency.

Our data showed that derivatives **1**, **3** and **4** of the NQ compounds **1–5** induced both apoptotic and necrotic cell death. The frequency of apoptosis was largely parallel to the potency of growth inhibition. In addition, the findings suggest that the novel compounds **1** and **3** under study induced overexpression of the *c-jun* gene, which had been reported to be associated with apoptosis

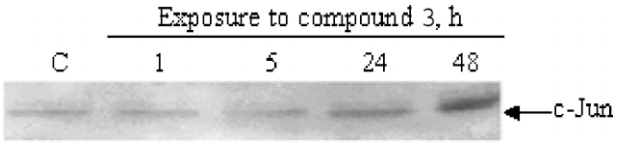


Fig. 5. c-Jun protein level in parental MH-22A cells after 1, 5, 24 and 48 h of treatment with compound **3**; C – control level (without treatment)

[16]. The "antisense" cells where the c-Jun level was partially blocked were much more resistant to treatment with the NQ derivatives **1** and **3**. However, no direct correlation was demonstrated between the potency of murine hepatoma cell growth inhibition by the study compounds and the degree of *c-jun* induction.

In summary, the novel 1,4-naphthoquinones **1** and **3** containing 2-aminoethyl functions with terminal bromo (**1**) and hydroxyl (**3**) groups exhibited structural requirements for tumor cell growth inhibitory and cell killing activities. Compounds **1** and **3** have an advantage in antiproliferative and cytotoxic activities over VK_3. The effects may be related with the induction of *c-jun* expression.

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The stable transfected MH-22A cell clone containing *c-jun* construct in antisense orientation was kindly presented by Dr. Audronė Kalvelytė (Department of Developmental Biology, Institute of Biochemistry).

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NAUJŲ 1,4-NAFTOCHINONO JUNGINIŲ CITOTOKSINIO AKTYVUMO TYRIMAS PANAUDOJANT PELĖS HEPATOMOS LĄSTELIŲ LINIJĄ

Santrauka

Žinoma, kad K grupės vitaminai, 2-methyl-1,4-naftochinonų junginiai, stabdo įvairių navikinių ląstelių augimą. Šiame darbe tirtas naujai susintetintų 1,4-naftochinono (NQ) **(1–5)** junginių poveikis navikinių ląstelių augimui ir žūčiai. Darbo tikslas – ištirti penkių 1,4-naftochinono darinių (**1–5)**, turinčių 2-naftochinono žiede alkilamino grupuotę su terminaliniais bromo (**1, 2**), chloro (**4**) hidroksilo (**3**) bei merkapto (**5**) pakaitais, priežvėžinį aktyvumą ir nustatyti, kaip jie stabdo ląstelių dauginimąsi ir indukuoja jų žūtį. Mūsų rezultatai rodo, kad tirti keturi NQ dariniai (**1–4**) stabdė navikinių ląstelių dauginimąsi bei indukavo jų žūtį tiek apoptozės, tiek ir nekrozės būdu pasirinktoje modelinėje pelės hepatomos MH-22A ląstelių linijoje. Pastebėta, kad minėtų junginių (**1–4)** priešnavikinis aktyvumas buvo susijęs su apoptozės indukcija tirtoje ląstelių populiacijoje. Didžiausiu aktyvumu pasižymėjo 2-(2-brometilamino)-1,4-naftochinonas (**1**) ir 2-(2-hidroksietilamino)-1,4-naftochinonas (**3**). Ypač aktyvus buvo **3** junginys, kuris navikinių ląstelių dauginimąsi stabdė 40% efektyviau negu VK_3. Be to, NQ dariniai (**1** ir **3)** mūsų modelinėje MH-22A ląstelių sistemoje pastebimai indukavo *c-jun* geno ekspresiją. Transfekuotos MH-22A ląstelės, kuriose iš dalies buvo blokuota *c-jun* raiška, buvo daug atsparesnės tiriamiems poveikiams negu intaktinės MH-22A ląstelės. Šie rezultatai rodo, kad nauji 1,4-naftochinono dariniai (**1** ir **3)**, turintys alkilamino liekaną su galine bromo (**1**) ir hidroksilo (**3**) grupe vietoje būdingos vitaminų K šeimai metilo grupės, pasižymėjo didesniu navikinių MH-22A ląstelių proliferaciją stabdančiu ir citotoksiniu aktyvumu negu VK_3. NQ junginiai (**1** ir **3)** modelinėje MH-22A ląstelių sistemoje indukavo *c-jun* geno raišką, ir tai gali būti siejama su citotoksiniu tiriamų junginių poveikiu.