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Regular paper

Anticancer activity of new molecular hybrids combining 1,4-naphthalenedione motif with phosphonic acid moiety in hepatocellular carcinoma HepG2 cells

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Structural motifs found in naturally occurring compounds are frequently used by researchers to develop novel synthetic drug candidates. Some of these new agents are hybrid molecules which are designed through a concept of combining more than one functional element. In this report, anticancer activity of new synthetic molecular hybrids, substituted 3-diethoxyphosphorylnaphtho[2,3-*b*]furan-4,9-diones and 3-diethoxyphosphorylbenzo[*f*]indole-4,9-diones, which integrate natural 1,4-naphtalenedione scaffold, present in several anticancer agents, with pharmacophoric phosphonate moiety, were tested against hepatocellular cell line HepG2. Cytotoxicity was examined using MTT assay. Two most potent compounds, furandione 8a and benzoindoldione 12a, which reduced the number of viable HepG2 cells with the IC_{50} values of 4.13 μM and 5.9 μM, respectively, were selected for further research. These compounds decreased the mRNA expression levels of several genes: *Bcl-2*, angiogenic vascular endothelial growth factor (*VEGF*), *c-Fos, caspase-8* and increased the expression of *Bax*, *caspase-3* and *-9*, *c-Jun, p21, p53*, as determined by quantitative real-time PCR. The ability of these compounds to induce apoptosis and DNA damage was studied by flow cytometry. The obtained data showed that the new compounds inhibited cell viability by increasing apoptosis and decreasing angiogenesis. Compound 8a was a much stronger apoptosis inducer as compared with 12a and strongly activated the intrinsic pathway of apoptosis, associated with the loss of mitochondrial membrane potential and changes in *Bax/Bcl-2* ratio. These findings show that the synthetic hybrids combining 1,4-naphthalenedione system and phosphonic acid moiety display potential to be further explored in the development of new anticancer agents.

Key words: MTT test, apoptosis, real-time PCR, flow cytometry

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Abbreviations: DMSO, dimethyl sulfoxide; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *MMP-9,* metalloproteinase-9; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PI, propidium iodide; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TNF-α, tumor necrosis factor-alpha; uPA, urokinase plasminogen activator

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related deaths (Siegel *et al.*, 2013; Siegel *et al.,* 2014). This type of cancer is predominantly associated with hepatitis B and C virus infections (Liovet *et al.,* 2003; Parkin *et al.,* 2005). The most commonly offered therapy for patients with HCC is surgery, but only a small portion of patients are candidates for radical surgery of tumor at the time of diagnosis (Simonetti *et al.,* 1997; Liovet *et al.,* 2008; Ramalingman *et al.,* 2008; Takayama *et al.,* 2010). Therefore, chemotherapy is the most important treatment for advanced HCC and there is an urgent need for the development of efficient drugs since sorafenib, an approved drug for HCC, is effective only in approximately 30% of the patients (Bruix *et al.,* 2011). The diagnosis of HCC still remains difficult and there are large gaps in our current understanding of underlying molecular mechanisms involved in the pathogenesis of HCC (Sanyal *et al.,* 2010). The elucidation of these diverse mechanisms for the identification of novel drug targets has therefore been a major focus in medicine, and further research efforts are still needed for an increased understanding and for developing efficient treatment strategies.

The scaffold of 1,4-naphtalenedione (Fig. 1), usually *ortho*-condensed with one or two planar rings is a well known pharmacophoric unit present in many natural and synthetic compounds displaying cytotoxic activity. Such structural motif is present in a large group of antibiotics widely used in anticancer therapy, known as anthracyclines, the best known of which is doxorubicin.

On the other hand, organophosphorus compounds, in particular phosphonic acid derivatives, have found an important and well-recognized place in the search for new drug candidates. Their success as pharmacophores is based on several assumptions, e.g. that phosphonic acid derivatives could inhibit the process in which phosphate is involved or that phosphoryl group can mimic the tetrahedral intermediates formed in enzymatic reactions involving carboxyl group metabolism. These rationales have resulted in the design of some important drugs with anticancer activity, i.e. N-phosphonoacetyl-L-aspartic acid (PALA) (Fig. 1).

Recently, we described the synthesis of a series of new molecular hybrids combining both mentioned above elements, 1,4-naphtalenedione system and phosphonic acid moiety (Gach *et al.,* 2016). Here, these novel compounds were tested in the MTT assay for their possible cytotoxic

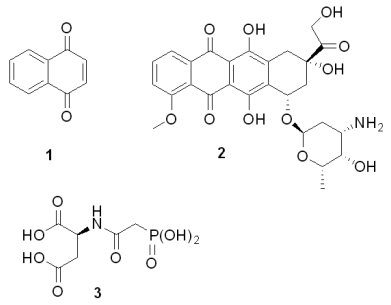


Figure 1. The structure of: (1) 1,4-naphthalenedione, (2) doxorubicin, (3) N-phosphonoacetyl-L-aspartic acid (PALA).

activity in HepG2 cells. Among various hepatocellular carcinoma cell lines HepG2 is the one most extensively employed in the studies involving new drug candidates, since the cells preserve a large part of cellular functions of normal hepatocytes, including the expression of hepatocyte-specific cell surface receptors and synthesis of plasma proteins (Dehn *et al.,* 2004). From the compounds screened in the MTT assay, two analogs with the lowest IC_{50} values were selected for further studies and their ability to regulate apoptosis and the level of the selected factors responsible for angiogenesis and metastasis was assessed.

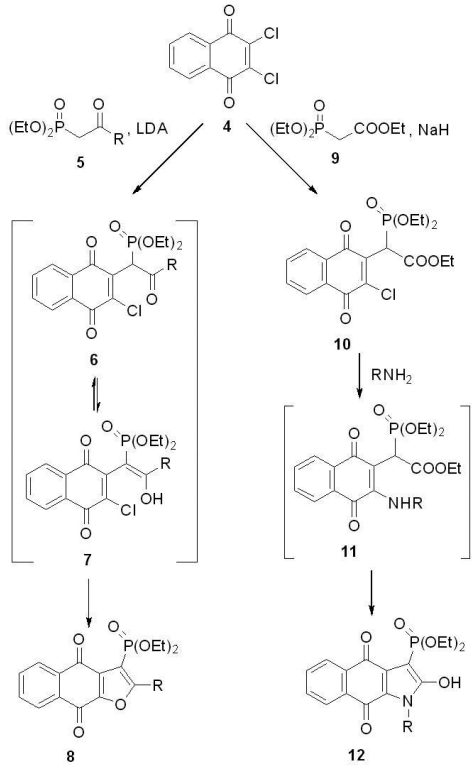
MATERIALS AND METHODS

Materials and general procedures. 3-Diethoxyphosphorylnaphtho[2,3-b]furan-4,9-diones **8a-c** and 3-diethoxyphosphorylbenzo[f]indole-4,9-diones **12a-c** were synthesized as outlined in the Scheme. Reaction of 2,3-dichloro-1,4-naphthoquinone **4** with diethyl acylmethylphosphonates **5a-c** or ethyl diethoxyphosphorylacetate **9**, in the presence of base, gave corresponding substitution products **6** or **10**, respectively. Intramolecular cyclization of **6** *via* enol **7** furnished furanodiones **8a-c**, whereas reaction of **10** with corresponding amines followed by intramolecular cyclization yielded benzoindolediones **12a-c** (Scheme 1). The detailed procedures are described elsewhere (Gach *et al.,* 2016).

Compounds were dissolved in DMSO and further diluted in the culture medium to obtain less than 0.1% DMSO concentration. In each experiment, controls without and with 0.1% DMSO were performed. 0.1% DMSO had no effect on the observed parameters.

Table 1. Primer sequences for real-time PCR

Gene	Primer sequences	
	Forward primer	Reverse primer
GAPDH	GTCGCTGTTGAAGTCAGAGGAG	CGTGTCAGTGGTGGACCTGAC
Bcl-2	CATGCTGGGGCCGTACAG	GAACCGGCACCTGCACAC
BAX	ACCCGGTGCCTCAGGATGCGT	GGCAAAGTAGAAAAGGGCGAC
Cas-3	TGAAGCTACCTCAAACTTCC	CAGCATCACTGTAACTTGCT
Cas-8	AGGAAAGTTGGACATCCTGAAAA	GGAGAGTCCGAGATTGTCATT
Cas-9	GTGACATCTTTGTGTCCTAC	CTGTTTATAAATCCCTTTCA
VEGF	AAGGAGGAGGGCAGAATCAT	ATCTGCATGGTGATGTTGGA
c-Fos	CGGGCTTCAACGCAGACTA	GGTCCGTGCAGAAGTCCTG
c-Jun	TGCCTCCAAGTGCCGAAAAA	TGACTTTCTGTTTAAGCTGCTCC
p53	CTCTCCCCAGCCAAAGAAGAA	CCACGGATCTGAAGGGTGAA
p21	GGCAGACCAGCATGACAGATT	GCGGATTAGGGCTTCCTCT



Scheme 1. Synthesis of 3-diethoxyphosphorylnaphtho[2,3-b]furan-4,9-diones 8 and 3-diethoxyphosphorylbenzo[f]indole-4,9-diones 12.

Cell culture. Hepatocellular carcinoma cell line HepG2 was purchased from the European Collection of Cell Cultures (ECACC). The HepG2 cells were cultured in the Minimum Essential Medium Eagle (MEME, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries, Beit-Haemek, Israel), 2 mM glutamine (Sigma-Aldrich, Louis, MO, USA), Men Non-essential amino acid solution 100x (Sigma-Aldrich, St. Louis, MO, USA), gentamycin (5 μg/mL) (Biological Industries, Beit-Haemek, Israel). Cells were incubated in 37°C, in a humidified atmosphere containing 95% air and 5% CO_2.

Cell viability assay (MTT). Cell growth and viability were measured by the MTT assay. The assay was performed according to the known procedure (Mosmann *et al.,* 1983). Briefly, HepG2 cells (10^4/per well) were seeded into 24-well plates (in 100 μl), left to adhere and grow for 24 h. Various concentrations of the tested compounds were added and after 24 h the MTT solution (5 mg/ml in phosphate buffered saline; PBS, Gibco, Invitrogen, Carlsbad, CA, USA) was added to each well. The plates were incubated for 2 h at 37°C, then the medium was removed and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of the blue formazone product was measured at 540 nm using a iMark Bio-Rad microplate reader (Hercules, CA, USA). The IC_{50} values (50% inhibitory concentration) were calculated.

Quantitative real-time PCR assay. Total RNA was extracted from HepG2 cells, using Total RNA Mini Kit (A&A Biotechnology, Gdynia, Poland) accord-

ing to the manufacturer's protocol (Chomczynski *et al.,* 1987*)*. The RNA concentration was determined by measuring the absorbance at 260, 280 nm on the UV/VIS spectrophotometer (Pharmacia, Cambridge, UK). cDNA was synthesized using the Enhanced Avian HS RT-PCR Kit (St Louis, MO, USA). cDNA was amplified using Syber Green dye (Brilliant II SYBR Green) and gene specific primers (Table 1) in Stratagene MX3005P Real-time system (La-Jolla, California, USA). Real-time PCR cycles were run in triplicate using the following thermal cycling profile: 95°C for 10 min, followed by amplification was achieved by 40 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 45s and extension at 72°C for 30 s. The dissociation curve was inspected for quality control purposes.

The expression levels of the tested genes were determined by the $2^{-\Delta\Delta CT}$ method (Winer *et al.,* 1999). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

Analysis of cell proliferation, apoptosis and DNA damage by flow cytometry. Cell proliferation, DNA damage and apoptosis were determined using the "Apoptosis, DNA Damage, and Cell Proliferation Kit" (BD Bioscience, San Jose, CA, USA), according to the manufacturer guidelines. The test kit includes: BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution; BD Perm/Wash™ Buffer; BD Cytofix/Cytoperm™ Plus Permeabilization Buffer, DAPI, BrdU, DNase and antibodies (PerCP-Cy™5.5 Mouse Anti-BrdU Alexa Fluor® 647 Mouse Anti-H2AX (pS139); PE Mouse Anti-Cleaved PARP (Asp214)).

Briefly, HepG2 cells were seeded in 25 cm^2 cell culture flask at a density 8.0×10^4/mL in 10 mL of standard growth medium and left to grow for 24 h. Then, the growth medium was replaced by a fresh growth medium supplemented with the tested compounds in the desired concentrations. After 24 h of incubation, the culture medium was collected and the floating cells were counted and discarded. Then, the fresh growth medium supplemented with BrdU (final concentration of 10 μM) was added to each flask for additional 8h. After incubation culture medium was collected and washed twice with phosphate buffered saline (PBS, GIBCO, Invitrogen, Carlsbad, CA, USA), harvested by trypsinization and centrifuged ($200 \times g$, 5 min). The total number of cells was determined. Cells were fixed, permeabilized according to the manufacturer's protocol and treated with DNase (300 μg/mL in DPBS) for 1 h at 37°C in order to expose BrdU epitopes. After incubation, cells were simultaneously stained with fluorochrome-labeled anti-BrdU, H2AX (pS139) and cleaved PARP (Asp214) antibodies for 20 min at room temperature. After washing, DNA staining for cell cycle analysis using DAPI solution (1 μg/mL of staining buffer) was performed. Cell were resuspended in staining buffer and analyzed by flow cytomtery using (Becton Dickinson Canto II). The data was visualized and quantified by constructing a dot-plot using the BD FACSDiva software.

Analysis of reactive oxygen species (ROS) induction by flow cytometry. Induction of ROS generation was determined by flow cytometry using the CellROX® Oxidative Stress Reagents (Molecular Probes; Life Technologies, Carlsbad, CA, USA) which are fluorogenic probes designed to reliably measure ROS in the live cells. The cell-permeable reagents are non-fluorescent or very weakly fluorescent in a reduced state but upon oxidation they exhibit a strong fluorogenic signal. Briefly, HepG2 cells were seeded in 6-well cell culture plates at a density of 0.16×10^6/ml in 3 ml of standard growth medium. After 24 h, the growth medium was replaced by a fresh growth medium supplemented with the tested compounds in the desired concentrations. Then the cells were incubated with 5 μM CellROX® Green and 5 μM Reagents at 37°C for 30 min in the dark. After harvesting by trypsinization, centrifuged ($200 \times g$, 5 min) and washed twice with PBS the were re-suspended in PBS and analyzed by flow cytometry (Becton Dickinson Canto II). Menadione was used as a positive control.

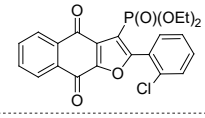
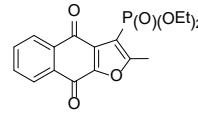
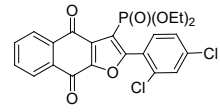
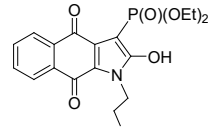
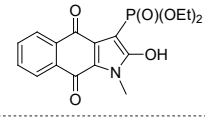
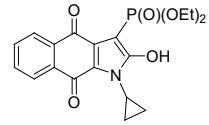
Statistical analysis. Statistical analyses were performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). The data were expressed as means ± SEM. Statistical comparisons were assessed by a one way ANOVA followed by a post-hoc multiple comparison Student-Newman-Keuls test. A probability level of 0.05 or lower was considered statistically significant.

RESULTS

MTT assay

Cytotoxic activity of the compounds was examined using the MTT assay. HepG2 cells were exposed to a board range of compound concentrations for 24 h (Table 2). Compounds **8a** and **12a** showed the highest cytotoxic activity and were selected for further evaluation as potential anticancer agents.

Table 2. Growth inhibitory effects of novel chimeric derivatives in human hepatocellular cell line HepG2

Compound	Structure	$IC_{50} \pm SEM$ (μM)
8a		4.13±0.2
8b		13.4±1
8c		6.0±0.7
12a		5.93±0.5
12b		>50
12c		>50

All values are expressed as mean ± SEM of three independent experiments.

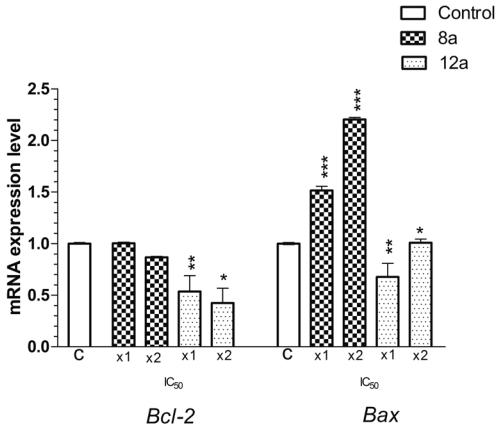


Figure 2. The change of the apoptosis-related gene expression in HepG2 treated with 8a and 12a, detected by real time PCR. *GAPDH* was used as control.
The expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ±SEM.

Effect of 8a and 12a on expression levels of selected genes involved in apoptosis angiogenesis and metastasis

The effect of **8a** and **12a** on the expression of several genes engaged in such cellular processes as apoptosis and angiogenesis was assessed using quantitative real-time PCR. The tested compounds were used in two concentrations (IC_{50} and $2xIC_{50}$). Gene expression was normalized to the house keeping *GAPDH* gene. Analogs **8a** and **12a** caused down-regulation of the anti-apoptotic *Bcl-2* and *caspase-8* and up-regulation of pro-apoptotic *BAX, caspase-3* and *-9,* ($p \leq 0.01$) (Figs. 2 and 3). Both compounds were also shown to increase the mRNA levels of *p21* and *p53,* two genes responsible for regulation of the cell cycle (Fig. 4).

As it is well known, some cancer cells acquire the ability to penetrate the walls of blood vessels and to circulate through the bloodstream to other tissues in the body. In malignant cells there is usually the overexpression of matrix metalloproteinases (MMP) which are enzymes involved in the breakdown of extracellular matrix and enable cancer cells to form metastasis. When a new tumor is formed, it needs a supply of nutrients and oxygen. Vascular endothelial growth factor (VEGF), is a signal protein produced by cells that stimulates angiogenesis

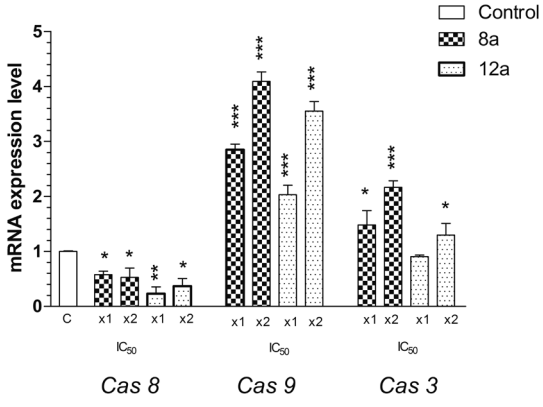


Figure 3. The expression levels of *Caspase 9*, *Caspase 3* and *Caspase 8* genes in HepG2 treated with 8a and 12a.
Expression was detected by real time PCR. *GAPDH* was used as control. The expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ±SEM.

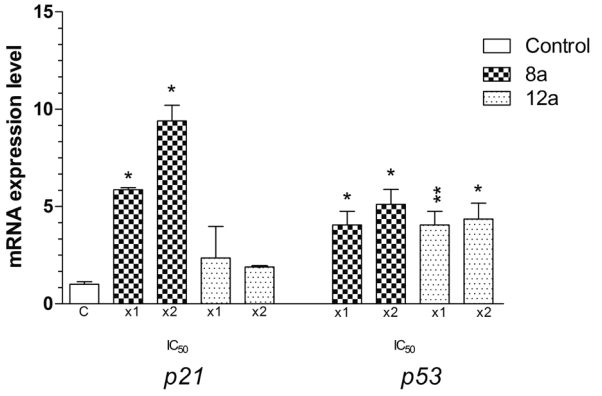


Figure 4. The expression levels of *p21* and *p53* genes in HepG2 cells treated with 8a and 12a.
Expression was detected by real time PCR. *GAPDH* was used as control. The expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are means ±SEM.

and in normal cells is a part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. In cancer cells overexpression of *VEGF* may be an early step in the process of metastasis and is often implicated with poor prognosis. **8a** and **12a** were shown to only slightly decrease the mRNA expression of MMP9 (Fig. 5) but caused a significant down-regulation of *VEGF* as compared with control (Fig. 6).

The influence of both tested compounds on the expression of two transcription factors, *c-Fos* and *c-Jun* was also investigated. As shown in Fig. 7, the tested compounds down-regulated *c-Fos* but increased *c-Jun* expression. These transcriptional factors are regulated by the mitogen-activated protein kinase (MAPK) which promotes cell survival and proliferation. The stimulation of *c-Fos* involves a signal transduction pathway, which includes activation of the small G protein Ras, Raf-1 kinase and the mitogen-activated protein (MAP) kinases, and extracellular-signal-regulated kinases (ERK1 and ERK2) (Kondoh *et al.,* 2007). The *c-Fos* is a proto-oncogene, known as a third intracellular messenger. It encodes a 62 kDa protein, which can form a homo-dimers or hetero-dimers with Jun family of transcription factors, resulting in the formation of Activator Protein-1 (AP-1). It has been reported that the promoter region for the *VEGF* gene contains several AP-1 binding motifs and the expression of *VEGF* is controlled by transcription

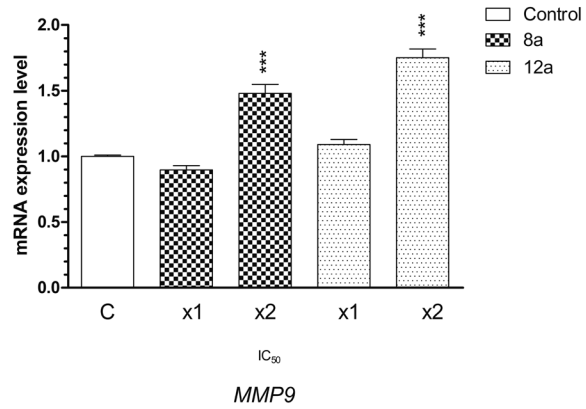


Figure 5. The expression levels of metalloproteinase-9 *(MMP9)* gene HepG2 cells treated with 8a and 12a, detected by real time PCR.
GAPDH was used as control. The expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are expressed as means ±SEM.

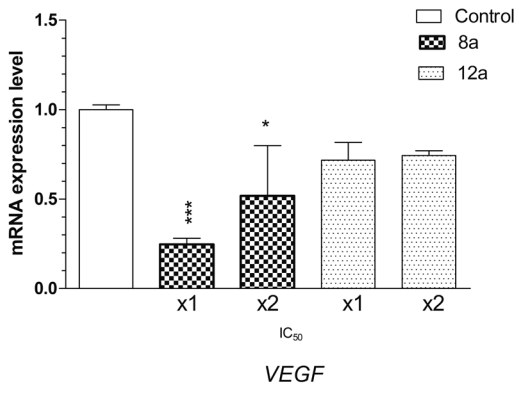


Figure 6. The expression levels of vascular endothelial growth factor (*VEGF)* gene in HepG2 cells treated with 8a and 12a.
Expression was detected by real time PCR. *GAPDH* was used as control. The expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are expressed as means ±SEM.

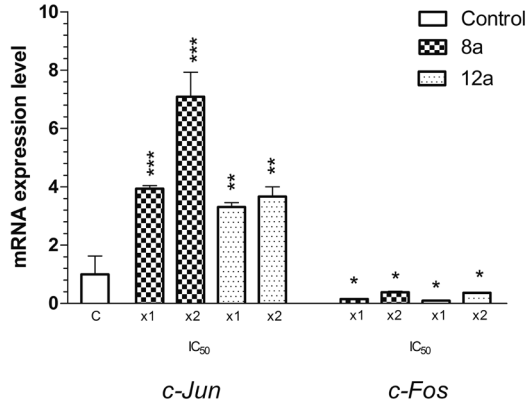


Figure 7. The expression levels of *c-Jun* and *c-Fos* transcription factor genes in HepG2 cells treated with 8a and 12a.
Expression was detected by real time PCR. *GAPDH* was used as control. The expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data are expressed as means ±SEM.

factors AP-1 and AP-2. As demonstrated in Figs. 6 and 7, *VEGF* and *c-Fos* mRNA expression was decreased in cells incubated with the tested compounds as compared with control, confirming the relationship between these two genes (Collado *et al.,* 2005).

The stimulation of *c-Jun* involves the signal transduction pathway, which includes activation of the *c-Jun* N-terminal kinase (JNK) group of MAP-kinases. The expression of *c-Jun* is regulated by diverse extracellular stimuli, such as pro-inflammatory cytokines or oxidative and cellular stress. Especially the JNK pathway has been implicated in apoptotic responses to DNA damage, cell stress and cytotoxic drugs (Gurzov *et al.,* 2008). In our experiments, the up-regulation of *c-Jun* mRNA expression resulted in the increased number of apoptotic (Fig. 7) and DNA damage-positive cells.

Effect of 8a and 12a on apoptosis by flow cytometry using Annexin V-FITC and propidium iodide (PI) double staining

HepG2 cells were tested for 48 h with **8a** and **12a** at IC_{50} concentration. Annexin V/PI analysis was performed to quantify apoptotic phenotype. Analysis by flow cytometry showed that **8a** induced late apoptosis in HepG2 cells but did not change the number

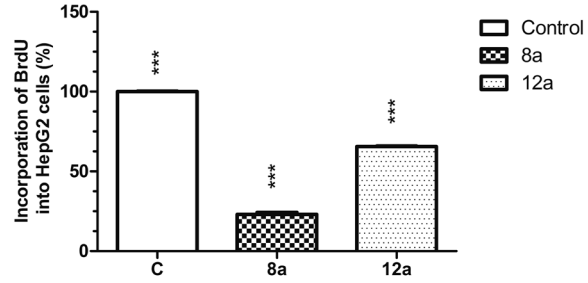


Figure 8. Incorporation of BrdU (BrdU test)
Inhibition of cell proliferation in HepG2 cells incubated for 48 h with **8a** and **12a** at IC_{50} concentration and treated with BrdU for additional 8 h. Fluorochrome-labeled anti-BrdU antibodies were added and allowed to stand for 20 min at room temperature. Results were analyzed by flow cytometry. Data represent mean ± SEM. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001.

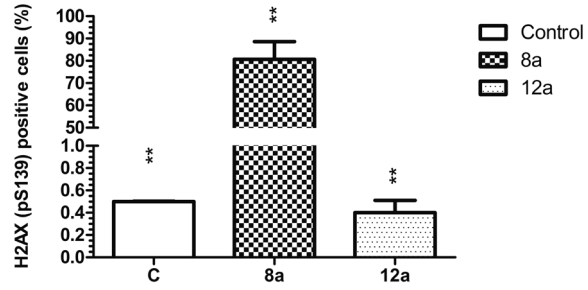


Figure 9. DNA damage (H2AX test)
DNA damage (H2AX test) in HepG2 cells incubated for 48 h with **8a** and **12a** at IC_{50} concentration. Fluorochrome-labeled anti-H2AX (pS139) antibodies were added to HepG2 cells and allowed to stand for 20 min at room temperature. Results were analyzed by flow cytometry. Data represent mean ± SEM. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001.

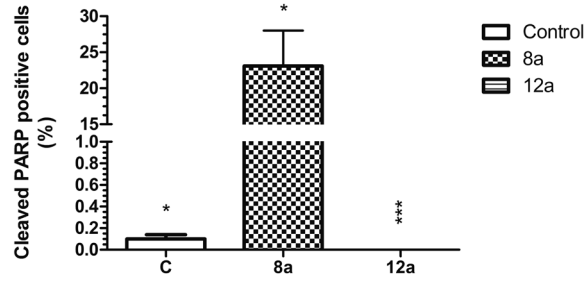


Figure 10. Induction of apoptosis (PARP test)
Induction of apoptosis (PAPR test) in HepG2 cells incubated for 48 h with **8a** and **12a** at IC_{50} concentration. Fluorochrome-labeled anticleaved (Asp214) antibodies were added and 89 kDa-cleaved fragment of PARP served as a marker of cellular apoptosis. Results were analyzed by flow cytometry. Data represent mean ± SEM. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. **p<0.01, ***p<0.001.

of early apoptotic cells (Table 3). By contrast, **12a** did not produce significant changes as compared to control. The number of late apoptotic cells increased up to 77.2±2.3% when incubated with **8a** and to 9.5±3.2***% in **12a** treated cells as compared with control. Incubation with **8a** and **12a** enhanced also the number of necrotic cells.

Table 3. Effect of tested compounds on apoptosis induction in HepG2 cells.

Compound	Necrosis	Late apoptosis	Early apoptosis	Healthy cells
Control	10.1±0.8*	13.2±1.6***	0.8±0.3*	57.5±1.2***
8a	3.6±1.1	77.2±2.3	0.7±0.2*	1.7±0.1***
12a	4.1±0.8*	9.5±3.2***	1.6±1.3	44.1±0.6***

Data represent percentage of cells in each step of apoptosis. Values are mean ± SEM of three independent experiments. Statistical significance was assessed using one-way ANOVA and a post hoc multiple comparison Student–Newman–Keuls test. *p<0.05, **p<0.01, and ***p<0.001.

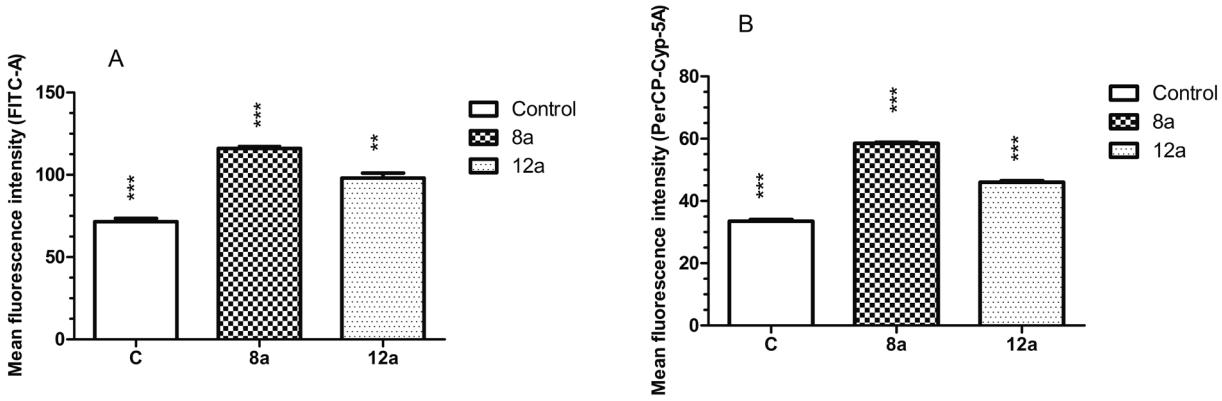


Figure 11. Generation of ROS in nucleus and mitochondria (A) and in cytoplasm (B) in HepG2 cells incubated for 48 h with 8a and 12a at IC_{50} concentration.
The cells were treated with 5 μM CellROX® Green Reagent and 5 μM CellROX® Red Reagent in a 37°C for 30 min. Data represent mean ± SEM. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman–Keuls test. *p<0.05, **p<0.01, and ***p<0.001.

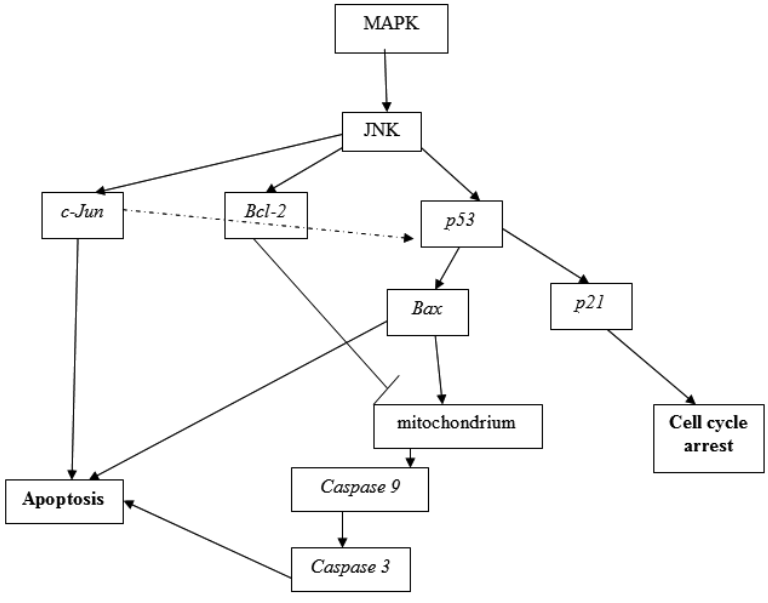


Figure 12. The possible pathways leading to HepG2 cell apoptosis induced by 8a.

Inhibition of cell proliferation, induction of DNA damage and apoptosis

The ability of **8a** and **12a** to inhibit cell proliferation, generate DNA damage and induce apoptotic cell death was also examined by flow cytometry using the 'Apoptosis, DNA Damage, and Cell Proliferation Kit' (BD Bioscience). The obtained results demonstrated that **8a** significantly inhibited proliferation in about 76.8% of cell population, while **12a** only in 34.5% (Fig. 8). **8a** generated DNA damage in 80.7% of cells, while **12a** had no effect in this test (Fig. 9). Only **8a** significantly increased the number of cleaved PARP positive (apoptotic) cells (up to 23.1% compared with control 0.1%) (Fig. 10).

Effect of 8a and 12a on reactive oxygen species (ROS) generation

One of the possible mechanisms of the cytotoxic activity of compounds with 1,4-naphtalenedione scaffold can be associated with generation of reactive oxygen species (ROS) in cancer cells. As shown in Fig. 11, the tested compounds only slightly increased intracellular ROS levels, as compared with control.

DISCUSSION

Simple synthetic compounds based on the motifs present in natural products may become attractive drug candidates. Continuing our studies on hybrid molecules combining 1,4-naphthalenedione motif with phosphonic acid moiety we investigated the anticancer activity of 3-diethoxyphosphorylnaphtho[2,3-*b*]furan-4,9-dione **8a** and 3-diethoxyphosphorylbenzo[*f*]indole-4,9-dione **12a** *in vitro* against human hepatocellular carcinoma HepG2 cell line. Both compounds showed high cytotoxic activity with the IC_{50} values below 10 μM but and **8a** was much more active in inducing apoptosis, the process that can be induced by different pathways. As it is well recognized, activation of MAPK phosphatases leads to the

phosphorylation and induction of MAP kinase signaling pathway. The members of the MAPK, involving a series of protein kinase cascades, play a critical role in regulation of cell proliferation, differentiation, stress response, apoptosis and survival (Plotnikov *et al.,* 2011; Yang *et al.,* 2013).

The first identified MAPK was c-Jun N-terminal kinase (JNK) (Derijard *et al.,* 1994; Kyriakis *et al.,* 1994). The crucial role of JNK is phosphorylation and activation of protein 1 (AP-1) family, which consist of Fos (encoded by the *c-Fos* gene), Jun (encoded by the *c-Jun* gene) and activating transcription factor 2 (ATF-2) subfamilies. Finally, activation of JNK and c-Jun/AP-1 can contribute to apoptosis (Takeda *et al.,* 2004; Matsuzawa *et al.,* 2005).

Despite a function of JNK in the regulation of c-Jun, this kinase may also regulate and control the p53 pathway by ability to phosphorylate p53 protein. Additionally, *c-Jun* may control cell cycle in a p53-dependent manner and N-terminal-JNK can be the functional inducer in *p53* transcriptional activity (Waldman *et al.,* 1995; Wisdom *et al.,* 1999; Buschmann *et al.,* 2000; Cheng *et al.,* 2003; Danasekaran *et al.,* 2008).

The p53 protein plays a significant role in modulation the cell cycle by induction the G1-, S-, or G2-phase arrest or stimulation apoptosis when DNA is damaged. Transactivation of *p21* is required for the p53-dependent G1 checkpoint (Waldman *et al.,* 1995; Abbas *et al*., 2009).

The p53-induced apoptosis can also involve the activation of a proapoptotic gene, *Bax* (Miyashita *et al.,* 1995). By phosphorylation-dependent posttranslational, proapoptotic processes, JNK can contribute to transcription of the members of apoptotic signaling pathway, leading to inactivation of *Bcl-2* promoter.

JNK and p53 pathways may cooperate in induction of apoptotic mitochondrial events which include activation of *Bax* and *Bak*, inactivation of *Bcl-2* and disrupt the mitochondrial outer membrane. Finally, the cytochrome *c* released from the mitochondria promotes induction of the *caspase 9*, leading to *caspase 3* activation and apoptosis (Kharbanda *et al.,* 2000; Verhagen *et al.,* 2000; Cory *et al.,* 2002; Adams *et al.,* 2007; Elmore *et al.,* 2007; Danasekaran *et al.,* 2008; Ahn *et al.,* 2011). The graph in Fig. 12 shows the possible pathways leading to HepG2 cell apoptosis induced by **8a**.

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