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 natural 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-dithiooxophosphorylnaphtho[2,3-b]furan-4,9-diones and 3-dithiooxophosphorylnaphtho[2,3-b]indole, which integrate natural 1,4-naphthalenedione scaffold, present in several anticancer agents, with pharmacophoric phosphonate moiety, were tested against hepatocellular line HepG2. Cytotoxicity was examined using MTT assay. Two most potent compounds, furandione 8a and benzoinindoldione 12a, which reduced the number of viable HepG2 cells with the IC₅₀ 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 angiogenicity. 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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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-naphthalenedione (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 carboxylic acid metabolism. These rationales have resulted in the design of some important drugs with anticancer activity, i.e. N-phosphonomoacetyl-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-naphthalenedione system and phosphonic acid moiety (Gach et al., 2016). Here, these novel compounds were tested in the MTT assay for their possible cytotoxic

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

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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 benzindolodiones 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.

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 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^ΔΔCT 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 cm² cell culture flask at a density 8.0 × 10⁴/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, centrifuged (200 × g, 5 min), and washed twice with PBS before re-suspension in PBS and analysis by flow cytometry (Becton Dickinson Canto II). The data were expressed as means ± SEM. Statistical comparisons were assessed by 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 broad 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC₅₀ ± SEM (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td><img src="image" alt="Structure of 8a" /></td>
<td>4.13 ± 0.2</td>
</tr>
<tr>
<td>8b</td>
<td><img src="image" alt="Structure of 8b" /></td>
<td>13.4 ± 1</td>
</tr>
<tr>
<td>8c</td>
<td><img src="image" alt="Structure of 8c" /></td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>12a</td>
<td><img src="image" alt="Structure of 12a" /></td>
<td>5.93 ± 0.5</td>
</tr>
<tr>
<td>12b</td>
<td><img src="image" alt="Structure of 12b" /></td>
<td>&gt;50</td>
</tr>
<tr>
<td>12c</td>
<td><img src="image" alt="Structure of 12c" /></td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SEM of three independent experiments.
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 housekeeping 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 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...
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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 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.
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 ‘Apop-Tosis, 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).

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-naphtalenedione motif with phosphonic acid moiety we investigated the anticancer activity of 3-diethoxycarbonylnaphtho[2,3-b]furan-4,9-dione 8a and 3-diethoxycarbonylbenzof[2]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 sensing 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; Elnore 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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