Evaluation of anti-cancer activity of stilbene and methoxydibenzo[*b*,*f*]oxepin derivatives

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Running title: Anti-cancer activity of new stilbene derivatives

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Abstract

Background: Stilbenes, 1,2-diphenylethen derivatives, including resveratrol and combretastatins, show anticancer features especially against tumor angiogenesis. Fosbretabulin, CA-4, in combination with carboplatin, is in the last stages of clinical tests as an inhibitor of thyroid cancer. The mode of action of these compounds involves suppression of angiogenesis through interfering with tubulin (de)polymerization.

Objective: We have previously synthesized five *E*-2-hydroxystilbenes and seven dibenzo[*b*,*f*]oxepins in *Z* configuration, with methyl or nitro groups at varied positions. The aim of the present work was to evaluate the anticancer activity and molecular mechanism(s) of action of these compounds.

Results: Two healthy, EUFA30 and HEK293, and two cancerous, HeLa and U87, cell lines were treated with four newly synthetized stilbenes and seven oxepins. Two of these compounds, JJR5 and JJR6, showed the strongest cytotoxic effect against cancerous cells tested and these two were selected for further investigations. They induced apoptosis with sub-G1 or S cell cycle arrest and PARP cleavage, with no visible activation of caspases 3 and 7. Proteomic differential analysis of stilbene-treated cells led to the identification of proteins involved almost exclusively in cell cycle management, apoptosis, DNA repair, and stress response, e. g. oxidative stress.

Conclusions: Among newly synthesized stilbene derivatives we selected two as potent anticancer compounds triggering late apoptosis/necrosis in cancerous cells through sub-G1 phase cell cycle arrest. They changed cyclin expression, induced DNA repair mechanisms, enzymes involved in apoptosis, and oxidative stress response. Compounds JJR5 and JJR6 can be a base for structure modification(s) to obtain even more active derivatives.

Keywords: anticancer agent; stilbene; oxepin; apoptosis; cell cycle arrest; EUFA30; HEK293, HeLa; U87

Introduction

Cancer has become one of the most important health problems characterized by the high cost of treatment and uncertain prognosis. This situation forces searching for new anticancer agents, of natural origin or chemically synthetized, showing multi-targeting activity and no toxicity to normal cells. Most of the solid tumors develop their own vascular supply in the process of angiogenesis. Suppression of angiogenesis by vascular targeting agents (VTAs) becomes a promising approach. VTAs can be divided into two groups: (i) angiogenesis inhibitors (AIs) and (ii) vascular disrupting agents (VDAs). Stilbenes are natural 1,2-diphenylethen derivatives and include resveratrol (3,5,4'-trihydroxy-trans-stilbene), commonly found in grapes and red wine [1]. It forms *Z*- or *E*-isomers, and/or glucosides (*Z*-/*E*-piceid). Unfortunately, after intake it is metabolized to glucuronide and sulfate conjugates lowering substantially its bioavailability [2]. Combretastatins are the other class of stilbenes first isolated from the bark of the African willow tree (*Combretum caffrum*) that potentially fulfill conditions for being VTAs [3-5]. The most extensively tested agent is combretastatin A-4 (CA-4). Binding to β-tubulin at the colchicine binding site, it inhibits tubulin polymerization disclosing highly anti-mitotic and anti-angiogenetic activities [4], but unfortunately showing low stability [6]. In the more stable phosphorylated form, CA-4P, it inhibits gastric tumor metastasis and increases antitumor immune response [5]. Upon administration, combretastatin A-1 diphosphate, CA-1P (OXi 4503), is dephosphorylated to CA-1 promoting microtubule depolarization, disruption of the tumor blood flow, and cell cycle arrest leading to apoptosis/necrosis. Additionally, it produces free quinone radicals creating reactive oxygen species [7]. Fosbretabulin, disodium phosphate derivative of CA-4, in combination with carboplatin, is in the last stages of clinical tests in patients suffering from thyroid cancer [8] (https://clinicaltrials.gov/ct2/home).

Greene and co-workers (2012) tested CA-4 and its azetidinone *Z*-restricted analog, CA-432, against adenocarcinoma-derived CT-26, Caco-2, and HT-29 and fibrosarcoma-derived HT-1080 cells [9].

Combretastatin analogues, almost without exception, share a 3,4,5-trimethoxyaryl ring (A-ring) structure widely accepted as essential for biological activity. During our previous study, we developed the synthetic routes for 12 new derivatives of dibenzo[*b*,*f*]oxepin, including five *Z*-izomers of (*E*)-2-hydroxystilbenes and seven variously substituted dibenzo[*b*,*f*]oxepins with a *Z*-stilbene motif in their skeleton [10].

The aim of the present work was to evaluate the anticancer activity and molecular mechanism of action of these compounds. Four cell lines: fibroblasts EUFA30 and human embryonic kidney HEK293, representing healthy tissues, and cervical cancer HeLa and glioblastoma U87, representing cancer cells, were subjected to four stilbenes and seven oxepin derivatives. Two of the stilbene derivatives, JJR5 and JJR6, showed relatively high activity against cancerous cells tested and were selected for further investigations. They induced apoptosis with sub-G1 or S cell cycle arrest, with no visible activation of caspases 3 and 7, but inducing PARP cleavage. Proteomic differential analysis led to the identification of several proteins involved in cell cycle management, cytoskeleton, and stress response, e.g. oxidative stress, like heme oxidase HO-1. We conclude that JJR5 and JJR6 compounds are worth further investigation and can be a base for structure modification(s) creating more active compounds.

Material and Methods

Stilbenes and oxepins

We have synthesized 12 derivatives of dibenzo[*b*,*f*]oxepines from substituted 2-hydroxy-2',4'-dinitrostilbenes as described in detail in Krawczyk et al. (2016) [10].

Cell cultures and treatments

Fibroblasts EUFA30 originated from the Department of Toxicogenetics, Leiden University Medical Centre, The Netherlands. Human embryonic kidney HEK293 cells (CRL-15730) were purchased from the American Type Culture Collection (ATCC). Cervical cancer HeLa (93021013) and glioblastoma U87 (89081402) cells were provided by the European Collection of Authenticated Cell Cultures (ECACC). Cells were cultured in DMEM medium (Thermo Fisher Scientific) supplement with 10% fetal bovine serum (Thermo Fisher Scientific), 0,1% antibiotics (penicillin, streptomycin, Thermo Fisher Scientific). Cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C.

Cytotoxicity assay

Exponentially growing cells were seeded onto a 96-well plate at the density of 2 x 10^3 cells/well, cultured for 18 h, and the respective treatment (DMSO or tested stilbenoid in indicated concentration) was applied for 24 h or 48 h. Alamar Blue (Thermo Fisher Scientific) was added accordingly to manufacturer protocol. After 4 h, light emission at 590 nm was measured with excitation at 560 nm using a scanning multiwell spectrophotometer Synergy HTI (Biotek). The experiment was carried out at least three times with three replicates for each inhibitor concentration. After background subtraction, inhibition rates IC_{50} were calculated.

Flow cytometry

The Annexin V-FITC apoptosis detection kit (BD Biosciences) was used to detect apoptosis by flow cytometry. Cells were seeded at 6-well plates at a concentration of 5 x 10^5 cells/well, cultured for 18 h, and tested agent was applied for indicated periods. Afterward, cells were washed with PBS, resuspended in binding buffer at a concentration of 2 x 10^6 cells/ml, and anti-Annexin V FITC-conjugated antibody and propidium iodide were added to 100 µl aliquots. The mixtures were incubated for 15 min at room temperature, supplemented with binding buffer to 500 µl and processed by BD FACSCalibur (BD Biosciences). Data were analyzed in Flowing Software version 2.5.1 (Flowing Software, http://www.uskonaskel.fi/flowingsoftware).

Western-blot analysis

Total proteins were extracted from HEK293, Hela, and U87 cell with RIPA buffer (Sigma-Aldrich) in the presence of protease inhibitor cocktail (Sigma-Aldrich). Cellular debris were spun down and supernatant analyzed for protein content by Bradford assay (Bio-Rad). Samples were diluted with SDS-PAGE loading buffer to a final protein concentration of 2 µg/µl and 10 µl loaded on the Mini-PROTEAN TGX 4-15% gradient gel (Bio-Rad). The Western-blot analysis was performed with the following antibodies used at 1:200 dilution: for monoclonal anti-ALKB (Santa

Cruz Biotechnology INC, sc-374301), monoclonal anti-ALKBH3 (Santa Cruz Biotechnology INC, sc-376520), monoclonal anti-FTO (Santa Cruz Biotechnology INC, sc-374301), monoclonal anti-ALKBH5 (Sigma–Aldrich, HPA007196), polyclonal anti-Caspase-3 (Cell Signaling, 9662), polyclonal anti-Caspase-7 (Cell Signaling, 9492), polyclonal anti-PARP (Cell Signaling, 9542), anti-tubulin (Santa Cruz Biotechnology INC, sc-58885), and anti-p53 (Santa Cruz Biotechnology INC, sc-6243) antibodies. Appropriate secondary antibodies conjugated with horse-radish peroxidase were used. Chemo-luminescence was measured using the ChemiDoc MP Imaging System (Bio-Rad). Total protein was standardized in four steps: (i) equal amounts of cells were taken for extractions in RIPA buffer; (ii) the extract was then assayed by Bradford for protein content; (iii) equal amounts were loaded onto the gel and verified by Coomassie staining; (iv) proteins transferred to the nitrocellulose membrane were visualized by Ponceau-S reversible staining prior to the final Western-blot analysis.

Proteomic analysis

Samples were prepared as for the Western-blot analysis, loaded on the gradient gel, and after short run bands were cut out. Peptide mixtures were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS/MS) using the Nano-Acquity (Waters) LC system and the Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Prior to the analysis, proteins were subjected to the standard "in-gel digestion" with proteins reduced with 50 mM TCEP (60 min, 60°C), alkylated with 200 mM MMTS (45 min, room temperature) and digested overnight with trypsin (sequencing Grade Modified Trypsin, Promega). Peptide mixtures were applied to RP-18 precolumn (nanoACQUITY Symmetry C18, Waters) using 0.1% TFA as a mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY BEH C18, Waters) using the acetonitrile gradient (5-35% acetonitrile, 180 min) in the presence of 0.05% formic acid with the flow rate of 250 nl/min. Column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples was included. Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science) against UniProt (v. 201604) database restricted to *Homo sapiens* sequences. Search parameters for precursor and product ions mass tolerance were 20 ppm and 0.1 Da, respectively, enzyme specificity: trypsin, missed cleavage sites allowed: 1, fixed modification of cysteine by methylthiol and variable modification of methionine oxidation. Peptides with Mascot Score exceeding the threshold value corresponding to < 5% expectation value and FDR < 1%, calculated by Mascot procedure were considered as positively identified.

The mass calibration and data filtering described above were carried out with MScan software (http://proteom.ibb.waw.pl). Only proteins represented by at least two peptides were considered and proteins identified by a subset of peptides from another protein were excluded. Peptides lists that matched the acceptance criteria from the LC-MS/MS runs were merged and overlaid onto 2-D heat maps generated from the LC-MS/MS datasets, using m/z and retention times coordinates, validated by predicted and observed isotopic pattern correlation (acceptance criteria: RMS > 90%) using MSparky (http://proteom.ibb.waw.pl). Peptide intensities were determined as the surface of the isotopic envelope of the tagged isotopic envelopes. Finally, lists of identified peptides with corresponding quantitative values were exported for statistical analysis carried out with Diffprot software [11]. Prior to

the analysis, quantitative values were normalized with LOWESS. Proteins with more than 90% common peptides were clustered and only peptides unique for the cluster were used for statistical analysis. Only proteins with q-values < 0.05 were taken into consideration during further analysis.

Molecular modeling of α/β-tubulin interactions with selected stilbenes and *Z*-restricted analog

The optimum ground-state geometry for compounds JJR5, JJR6, JJR9, and HKKJ8 were calculated using density functional theory (DFT) [12-13]. The B3LYP functional and 6-311G 6-311++g (2d,p) basis set and the continuum model (PCM; Gaussian 03W) [13] were used in order to simulate the effects of the DMSO solvent. Molecular docking of compounds into the 3D X-ray structure of tubulin (PDB ID: 1SA0) was carried out using the Auto-Dock Vina software (the BFGS method) [14]. Configurations of protein/dimethoxydibenzo[b,f]oxepin complex were created using UCSF Chimera software [15]. The graphical user interface ADT was employed to set the enzyme, all hydrogens were added. For macromolecules, pdbqt files generated were saved. The 3D structures of ligand molecules were built, optimized (B3LYP functional and 6-311G 6-311++g (2d,p) basis set level), and saved in Mol2 format. The graphical user interface ADT was also employed to set up the ligand and the pdbqt file was saved. Auto-Dock Vina software [14] was employed for all docking calculations. The AutoDockTools program was used to generate the docking input files. In docking, a grid box size of 44 x 46 x 44 points in x, y, and z directions was built, and the maps were center located (115.574, 89.495, 7.664) in the catalytic site of the protein. A grid spacing of 0.375 Å (approximately 1/4 of the length of carbon-carbon covalent bond) was used for the calculation of the energetic map.

Statistical analysis

Statistical analyses were done using Origin 2015 software (OriginLab Corporation). For cytotoxicity curves, non-linear fit was used with logistic dose response model implemented in OriginPro.

Results and Discussion

Cytotoxic effect of stilbenes and oxepins tested towards cancerous cells

In the present study we evaluated the cytotoxic efficacy of previously synthetized four stilbene and seven oxepin derivatives on two cancerous (HeLa and U87), and two healthy (EUFA30 and HEK293) cell lines (Fig. 2). The compounds JJR1, JJR5, JJR6, and JJR9 and HKKJ8, JJR1-2, JJR2-2, JJR5-2, JJR6-2, JJR7-2, and JJR9-2 have *E* and *Z* configuration, respectively. Stilbene/oxepin-mediated growth inhibition proceeded in a dose-dependent manner and the effect was stronger for HeLa than U87 cells. Generally, stilbenes were more cytotoxic than oxepins, and all of them were more toxic to cancerous than healthy cells. For active compounds, IC_{50} values were the lowest after 48 h of HeLa treatment for four stilbenes, JJR1, JJR5, JJR6, and JJR9, and one oxepin, HKKJ8, and amounted 3.50, 17.18, 6.97, 17.05, and 18.54 μM, respectively. The JJR5 stilbene was the most effective against U87 cells with IC_{50} of 48.93 μM after 48 h of treatment (Table 1). Similar results were obtained with proliferation assay based on BrdU incorporation (data not shown).

Table 1. IC_{50} values (μM) of tested stilbene and oxepin derivatives based on the survival of EUFA30, HeLa, and U87 cells after 48 h of treatment.

Stilbene/oxepin:	Cells type:		
	EUFA30	HeLa	U87
JJR1	-	3.50	-
JJR5	52.08	17.18	48.93
JJR6	42.72	6.97	55.76
JJR9	-	17.05	-
HKKJ8	-	18.54	54.07
JJR6-2	-	54.82	-
JJR7-2	-	43.39	-

To summarize, the anticancer activity is developed by substitution of methoxy group in various positions of 2-hydroxy-2',4'-dinitrostilbene skeleton. The most active against HeLa and U87 cells were the stilbenes with a methoxy (or nitro) group in positions 3, 4, and 5; however, the presence of a substituent in positon 6 renders it inactive. The substituted ring due to electron donating (EDG) hydroxyl group has enriched electron density.

It should be also considered that for some compounds, e.g. JJR1 tested against HeLa cells, the cytotoxic effect depended upon the time of treatment. After 24 h, this stilbene was the least effective, but after 48 h it reached the lowest among stilbenes tested IC_{50} value of 3.50 μM.

CA-4/benzoxazolone hybrids were checked against HepG2, EA.hy 962, and K562 cells. One of them reached IC_{50} value in the range of 50-750 nM. When tested against relatively resistant cancer cells, this derivative was more toxic than CA-4, e.g. the IC_{50} reached 0.25 μM for colorectal adenocarcinoma HT-29 cells compared to 2.62 μM for CA-4 after 72 h of treatment. A similar phenomenon was observed for mouse colon carcinoma Colon-26 and human adenocarcinomic alveolar basal epithelial A-549 cells. Also, the *E*-isomers were studied but did not show the activity below the concentration of 50 μM [16].

CA-4 and CA-432 were very active against HT1080 and CT-26 cells showing IC_{50} values below 10 nM. Caco-2 cells survived at about 10-fold better rate. In the case of these three cell lines, stabilization of *Z* conformation slightly lowered the activity of the latter. Although both compounds were less active against HT-29 cells, with IC_{50} value for CA-4 equal to about 10 μM, its CA-432 derivative acted 10-fold stronger [9] indicating that the ethylene bridge influences anti-cancer activity of stilbene depending on the cell type tested.

Taking under consideration high cytotoxicity against cancerous and low effect on healthy cells, for further tests stilbenes JJR5, JJR6, and JJR9 and oxepins HKKJ8, JJR5-2, JJR6-2, and JJR9-2 were selected.

Effect of stilbenes and oxepins derivatives on the induction of apoptosis

To measure apoptosis induction, cells were treated for 24 and 48 h with 50 or 100 μM JJR5 or JJR6 (Fig. 3, Supplementary Fig. S1). The HEK293 cells were the least sensitive showing only a slight increase in necrotic (PI^+)

and late apoptotic (Annexin-FITC$^+$PI$^+$) cells, namely, 18.4% and 17.5% comparing to 1.3% and 8.2% in control cells. The HeLa cells were the most sensitive to both JJR5 and JJR6. After 24 h of JJR5 treatment, 40.1% of cells were in early apoptosis (Annexin-FITC$^+$), transforming almost entirely, 85.2%, into late apoptosis after 48 h. JJR6 was less active than JJR5 against HeLa cells producing 42.8% in early, and 41.3% in late apoptotic cells after 48 h. Both compounds were more effective than camptothecin used as a control. U87 cells were the most resistant to stilbenes tested. JJR5 was more active than JJR6, increasing the number of early and late apoptotic cells to 28.4% and 47.2% comparing to 3.8% and 9.6% in control cells, and necrotic cells from 2.2% to 8.5%. On the contrary, JJR6 caused slight increase in early and late apoptosis, to 8,2% and 18.6%, respectively (Fig. 3).

A typical apoptotic pathway employs activation of p53 protein and caspases. The activation of caspases 3 and 7 depends on the proteolytic cut of procaspases into p17 and p20 units, respectively. During apoptosis also PARP (p116), a protein involved in DNA repair, is inactivated by the cleavage producing p89 unit.

Consistently with previous results (the lack of significant changes in apoptosis induction), the Western blot analysis did not show the cleavage of PARP protein by JJR5, JJR6, and HKKJ8 compounds in HEK293 and U87 cells, with the exception of HKKJ8-treated HEK293 cells. However, the PARP cleavage product appeared in HeLa cells treated with the same derivatives. HKKJ8 induced proteolysis of PARP also in HEK293 cells (Fig. 4). Additionally, the slight induction of p53 in all cell lines can be observed after the addition of JJR5 and JJR6 (Fig. 4). We did not observe the activation of caspases 3 and 7. Results obtained suggest that the possible way of action of stilbenes could be the induction of necrosis rather than apoptosis. Additionally, these effects resemble the action of doxorubicin on human kidney proximal tubular HK-2 cells causing cell death through the p53 independent pathway inducing PARP cleavage to p55 subunit and only slightly increasing the caspase 3 activity [17].

In MCF-7 cells, the 250 μM resveratrol induced apoptosis at 30% after 48 h, with only slight activation of caspase 9 and no visible detection of caspase 3 [18]. In these cells the possible apoptosis pathway is lead by calpain activation [19]. Also Sawai and Domae (2011) have indicated that primary necrotic and post-apoptotic secondary necrotic cells cannot be distinguished by Annexin/PI staining and that primary necrotic cells are Annexin-FITC$^+$PI$^-$ before they become PI$^+$ [20]. The AIF-dependent and caspase-independent pathway of apoptosis could not be excluded [21].

The routes leading to apoptosis, among others, depend on cell type used for testing. Apoptosis has been induced in p53-positive human liver cancer Hep G2 cells through p53 pathway with elevated expression of p21 and Bax but not Fas/APO-1 [22]. On the contrary, when used with As_2O_3, resveratrol induced the expression of Fas and FasL, cytochrome C leakage, and oxidative stress leading to caspase-independent apoptosis [23]. Additionally, in human epidermoid carcinoma A432 cells, it activated apoptosis through pRb-E2F/DP pathway [24].

Significantly, CA-4 and CA-432 have induced different apoptotic/death pathways depending on the cells treated. In fibrosarcoma HT-1080 cells, caspase-dependent apoptosis has been triggered. In contrast, adenocarcinoma CT-26 cells have increased in size and contained multiple nuclei and vacuoles, and translucent cytoplasm resembling necrosis. On the other hand, the survival of HT-29 (p53-positive) cells can be, at least partially, explained by autophagy. Moreover, the prolonged exposure to both combretastatins led to the formation of the acidic vesicular organelles (AVOs) in 45-50% of polyploid, non-apoptotic/non-necrotic cells. A similar effect has been observed in HT-29 and Caco-2 cells. On the contrary, HT-1080 cells have not been changed [9].

Cell cycle distribution

Cell cycle distribution was tested using JJR5 and JJR6 stilbene derivatives. After 24 h of JJR5 treatment, HEK293 cells did not show any significant changes, with slight increase in sub-G1 population: 11.9% *versus* 7.7% in control cells (Fig. 5 and Supplementary Fig. S2). The sub-G1 population in HeLa cells was much more significant reaching 18.8% and 11.4% in cells treated with JJR5 and JJR6, respectively, in comparison to 5.6% in control. The U87 cells behaved differently showing greater sub-G1 and S phases cell populations, 8.9% and 21.4% compared to 3.3 and 16.1% in control cells treated with JJR5. JJR6 treatment increased cells in S phase to 29.3% not changing the sub-G1 population. Surprisingly, in U87 cells treated with either JJR5 or JJR6, the number of cells showing polyploidy raised significantly, from 2.8% in control to 12.7% and 11.9%, respectively. On the contrary, HeLa cells showed only slight increase in polyploidy, 6.5% and 7.8% in JJR5 and JJR6 treated cells, compared to 4.4% in the control.
To sum up, it should be noted that tested stilbenes direct the cells to sub-G1 phase indicating cell death, and in the case of U87, stuck the cells in S phase with a more significant effect of JJR6 than JJR5. Additionally, U87, the most resistant among cells tested, could escape apoptosis/necrosis through polyploidy.

The CA-4 retarded HepG2 cells at mitosis [25] by 6% lower than its benzoxazolone derivative, increasing the cell population in G2/M phase from 28 to 39% after 8 h treatment. Both compounds reached the retardation maximum at 24 h of treatment (about 80%) followed by sub-G1 enrichment "directly from mitotic arrest". Some cells escaped the cell cycle arrest and underwent cell divisions increasing their DNA content to more than 4N [16] which is consistent with our results.
Resveratrol has exerted G2/M cell cycle retardation in oral squamous carcinoma cells by cyclins A2 and B1 [26]. On the other hand, resveratrol is also capable to arrest cells at G1 phase as it has been observed in human epidermoid carcinoma A431 cells. The mechanism of action involves induction of Cdk1, and the reduction of cyclins D1, D2, and E and Cdk2, Cdk4, and Cdk6 [27]. Similar phenomenon has been shown for liver carcinoma cells [22]. Treated with resveratrol metabolites, colon cancer cells have been inhibited at G1/S phase with the depletion of cyclin D1 through A3 adenosine receptor [28]. Also, the retardation in S phase and apoptosis has been observed for treated with resveratrol anaplastic thyroid carcinoma cells [29]. In CT-26 and HT-1080 cells, 8 h treatment with 50 nM CA-4 caused cell cycle arrest at G2/M phase of more than 50% of cells. Treatment of CT-26 cells prolonged to 48 h, has increased sub-G1 population by 20% and polyploidy by 35%. In HT-1080 cells, only sub-G1 has soared to about 50%. Caco-2 cells were induced to cell death (about 15%) as well as to polyploidy (about 30%). In HT-29 only cell death has been induced [9]. After 48 h of 25 μM resveratrol treatment, the A549 cells have been arrested in G1 phase at 70% (compared to 50% in control). This was connected with the altered expression of cyclins A, B, and D3 and Chk1, Cdk1, CDC27, Eg5, integrins, and ASS [30].

Proteomic analysis

The proteomic differential analysis allowed to identify four to 18 proteins that either appeared only in control/treated cells or of which q-values were at the level of < 0.11 in cells treated with JJR5 and JJR6 derivatives (Supplementary Tables S1-S6). The significance of several of these proteins is described below.

Tested compounds influenced the oxidative stress response by upregulating e.g. the glutathione S-transferase Mu 3 (Supplementary Table S1), heme oxidase-1 HO-1 or thioredoxin reductase 1 (Tables S5 and S6) in U87 cells. These findings are consistent with the results of other groups. Resveratrol promoted antioxidant microenvironment by the regulation of SOD, CAT, glutathione reductase, peroxidase, and transferase, as well as GSH and other antioxidants, thus decreasing damage to DNA in the rat model of colon carcinogenesis [31]. On the contrary, resveratrol derivative, the 3,3',4,4'-tetrahydroxy-*E*-stilbene, has induced the production of ROS increasing activity of antioxidant cell machinery consequently decreasing DNA lesion 8-oxo-guanine and senescence-associated β-galactosidase in human peritoneal mesothelial cells [32]. It was established that resveratrol has induced the expression of heme oxidase HO-1 and glutamate-cysteine ligase GCLC via Akt/protein kinase B and extracellular signal-regulated protein kinase ERK1/2 and Nrf2 transcription factor in PC12 cells [33]. High throughput Western blot screening of A549 cells treated with 25 μM resveratrol for 48 h showed changes of more than 1.25-fold for 127 proteins. Surprisingly, HO-1 protein and caspase 2 were found to be down-regulated. Based on microarray experiments, the authors also found 157 genes of expression altered more than 2-fold. The pathways changed by resveratrol included: G1-S and G2-M checkpoints, apoptosis cascade as well as TGF-b, NF-kB, and MAPK/JNK pathways [30].

Parafibromin is a tumor suppressor found only in untreated HEK293 cells (Supplementary Tables S1 and S2). It influences the cell cycle through the inhibition of cyclin D1/PRAD1 expression [34]. Additionally, in HEK293 cells, JJR5 induced cullin-5 CUL5 protein, a substantial part of SCF-like ECS (Elongin-Cullin 2/5-SOCS-box protein) E3 ubiquitin-protein ligase complex probably ubiquitinating JAK2 kinase, arresting cell cycle progression (*via* homology to CUL1), and inducing apoptosis by constituting vasopresin cell surface receptor [35]. In cells treated with JJR5 or JJR6, we also identified the change in the expression of G2/M-specific cyclin B1 (Supplementary Table S2) and other cyclins (although with no statistical significance), being in agreement with the modulation of cell cycle phases. Another protein connected with cell cycle is Ran-specific GTPase-activating protein RANBP1 (Supplementary Table S6).

The substantial part of differentially identified proteins constitute these connected to DNA/RNA metabolism and repair. This proteins include down-regulated ATP-dependent RNA helicase DDX54 (Supplementary Table S2), UV excision repair protein RAD23 homolog B appearing only in treated HeLa cells (Supplementary Tables S3 and S4), or down-regulated DNA replication licensing factor MCM3 (Supplementary Table S5). Resveratrol and its metabolites have inhibited TOPO2 through histone H2AX phosphorylation causing TOPO-DNA permanent complex in glioblastoma cells inducing DNA damage [36-37], and in breast and colon cancer cells accompanied by decreased nuclear TERT [38]. The study conducted on MCF-7 cells treated with resveratrol showed the substantial inhibition of mismatch repair (MR), DNA replication, homologous recombination (HR), e.g. MRN complex, and cell cycle pathways [39]. In cells treated for 48 h with 250 μM resveratrol, the proteomic analysis revealed seven differentially appearing proteins: down-regulated HSP27, translationally controlled tumor protein TPT1, peroxiredoxin 6, stress-induced-phosphoprotein 1 STIP1, pyridoxine-5'-phosphate oxidase 1 PNPO, and hypoxantine-guanine phosphoribosyl transferase HGPRT1, and one up-regulated triosephosphate isomerase TPI1 [18].

Among proteins connected with RNA metabolism they were: (i) found only in JJR5-treated HEK293 cells nuclear RNA export factor 1 (Supplementary Table S1); (ii) present only in control HEK293 cells ribosomal RNA small

subunit methyltransferase NEP1 (Supplementary Table S1); (iii) increased by JJR6-treatment in HeLa tyrosine-tRNA ligase (Supplementary Table S4); (iv) increased by JJR5-treatment in U87 glycine-tRNA ligase (Supplementary Table S5); (v) RNA-binding protein involved in modulation of mRNA transcription by Polymerase II (Q9Y224) (Supplementary Table S5). These findings clearly indicate that RNA/protein metabolism is modulated by stilbene derivatives tested.

Separate group of proteins takes part in ubiquitin metabolism, namely, ubiquitin-like protein ISG15 (Supplementary Table S4) and ubiquitin-conjugating enzyme E2 N UBE2N (Supplementary Table S6). This is in agreement with the proteomic analysis of cerebral cortex of rats treated with resveratrol in middle cerebral artery stroke model where 29 proteins were identified with more than 2.5-fold change. These group of proteins included peroxiredoxin-5, ubiquitin carboxy terminal hydrolase L1 UCH-L1, isocitrate dehydrogenase NAD^+ subunit α, apolipoprotein A-I, collapsing response mediator protein CRMP-2, mitogen activated protein MAP kinase 1, and nucleoside diphosphate kinase B indicating that this stilbene influences pathways connected with oxidative stress, energy metabolism, and signaling [40].

An important finding is the change of the presence of γ-glutamylcyclotransferase (Supplementary Tables S1 and S2) present only in treated HEK293 cells. The protein is responsible for the induction of cytochrome C release from mitochondria, thus triggering apoptosis [41]. Treated with stilbene HeLa cells (Supplementary Tables S3 and S4) exhibited induction of (i) Annexin A2, the protein involved in apoptosis [42] but normally promoting actin polymerization [43], and (ii) anamorsin CIAPIN1 acting as a factor inhibiting cell death by cytokine regulation [44].

Interestingly, in HEK293 cells, JJR6 suppressed the G-rich sequence factor 1 GRSF1 responsible for the assembly of mitochondrial ribosomes and recruitment of *ND6* mRNA and *cytb* and *ND5* lncRNAs [45].

Stilbenes/oxepins activity against ALKBH dioxygenases

ALKBH proteins are members of the dioxygenase family present in almost all organisms first discovered in bacteria as a protection system against methylating damage to DNA. There are nine AlkB homologues in humans (ALKBH1-8 and FTO) showing activity against variety of substrates including reversible methyl modifications in RNA. We have found the expression of selected ALKBHs in cancer cells that implies involvement of these dioxygenases in cancer DNA/RNA metabolism. Thus, we evaluated the influence of stilbenes/oxepins on the expression of these proteins. With the use of Western blot analysis we found no such activity of compounds tested (data not shown).

Molecular modeling studies to determine the probability of α/β-tubulin interactions with selected stilbenes and *Z*-restricted analog

Microtubules are dynamic polymers with an α/β-tubulin heterodimer as the essential structural element. The tubulin heterodimer contains at least three distinct drug binding sites: the paclitaxel, vinblastine, and colchicine sites [46]. Up to now, no colchicine site inhibitor useful in anticancer therapy has been found. The derivatives of stilbene interact with the colchicine binding site of α/β-tubulin heterodimer. We conducted a computational docking experiment to identify possible α/β-tubulin interactions with JJR5, JJR6, and JJR9 stilbenes and dibenzo[*b*,*f*]oxepin HKKJ8 – the

analog incorporating *Z*-stilbene scaffold. Compounds were generated using molecular modeling software [14-15] and docked into the α/β-tubulin (PDB ID: 1SA0) .

The interaction of JJR5, JJR6, or HKKJ8 at colchicine binding site can be observed. On the other hand, JJR9 appears at another site (Fig. 6). In the binding mode, JJR5, JJR6, or HKKJ8 binds to the colchicine binding site of tubulin *via* hydrophobic interactions stabilized by a hydrogen bond. The calculated binding energies were used as the parameters for the selection of the cluster of docking posed to be evaluated (Fig. 6a-c). The selected structures of JJR5, JJR6, JJR9, and HKKJ8 have the estimated binding free energy of -7.0, -7.3, -7.7, and 7.4 kcal/mol, respectively, whereas the binding free energy of colchicine and CA-4 are -8.6 and -7.6 kcal/mol, respectively [47]. The models are similar to that for colchicine/CA-4 at the colchicine binding site. The compounds JJR5, JJR6, and HKKJ8 were embedded in the hydrophobic pocket occupied by the A ring of colchicine (Fig. 6a-d). Overall, these results suggest that the new derivatives interact strongly with tubulin, similarly to colchicine and CA-4.

Conclusions

We tested 11 new stilbene derivatives against healthy EUFA30 and HEK293 and cancerous HeLa and U87 cells and elucidated the mode of action of two of them. The JJR5 and JJR6 were selected as the most potent compounds minimally affecting healthy EUFA30 cells. They triggered late apoptosis/necrosis in cancerous cells through sub-G1 and/or S phase cycle arrest. The cell death seems to be caspase-independent indicating the caspase-independent apoptosis or regulated necrosis pathway. Besides changing the expression of cell cycle connected proteins like cyclins, the JJR5 and JJR6 compounds induced DNA repair mechanisms (UV excision repair protein RAD23 homolog B), especially homologous recombination (HR) and enzymes involved in apoptosis through mitochondrial cytochrome C leakage (γ-glutamylcyclotransferase) and oxidative stress response (heme oxidase HO-1).

The stilbenes tested are a good basis for further investigation, e.g. in the area of combinatorial therapy. Combretastatins together with paclitaxel are also toxic to the outer layers of solid tumors [48]. The synergistic action could be potentiated directly in organisms by the fact that e.g. resveratrol acts as an inhibitor of cytochromes P450 CYP1A1 or CYP3A4 thus potentially blocking the conversion of xenobiotics to carcinogens [49].

Also there is a possibility of the modulation of other regulatory molecules like lncRNAs. A recent report has shown that resveratrol at the concentration of 25 μM is able to change the expression of 40 lncRNAs, particularly AK001796, in lung cancer A549 cells [50]. Similarly, our results imply the involvement of JJR5 and JJR6 compounds in the regulation of lncRNAs.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figures description

Figure 1. The structure of CA1P, CA4P and their sodium salts, OXi4503 and fosbretabulin, alongside of resveratrol and colchicine.

Figure 2. The cytotoxic effect of A) stilbene (JJR1, JJR5, JJR6, and JJR9) and B) oxepin (HKKJ8, JJR1-2, JJR2-2, JJR5-2, JJR6-2, JJRJR7-2, and JJR9-2) derivatives on healthy EUFA30 and cancerous HeLa and U87 cells based on AlamarBlue assay, after 24 and 48 h of treatment. Non-linear fitting with logistic dose response model was employed and IC_{50} vales calculated if possible (see Table 1).

Figure 3. The distribution of HEK293, HeLa, or U87 cells stained with Annexin-FITC and propidium iodide (PI) after flow cytometry analysis. Cells were treated with 50 or 100 μM JJR5 or JJR6 for 24 or 48 h. Camptothecin (10 μM) and H_2O_2 (1 mM) were used as experimental controls. Tested samples were prepared and analysed in duplicates.

Figure 4. The Western-blot analysis of PARP, p53, and cytochrome C in HEK293, HeLa, or U87 cells treated with JJR5, JJR6, JJR9, HKKJ8, JJR5-2, JJR6-2, or JJR9-2 for 24 h.

Figure 5. Cell cycle distribution in HEK293, HeLa, or U87 cells treated with 50 μM JJR5 or JJR6 for 24 h. Tested samples were prepared and analysed in duplicates.

Figure 6. 3D model of the interactions between compounds a) HKKJ8, b) JJR5, c) JJR6 and the colchicine binding site of a α/β tubulin d) JJR9 the other site of a α/β tubulin (crystal structure from PDB code: 1SA0).

Supplementary Figures and Tables

Figure S1. Dot plots of distribution of healthy, apoptotic, and necrotic cells based on Annexin-FITC and propidium iodide (PI) staining of HEK293, HeLa, or U87 cells treated with 50 μM JJR5 or JJR6 for 24 h, an example.

Figure S2. Histograms of cell cycle distribution in HEK293, HeLa, or U87 cells treated with 50 μM JJR5 or JJR6 for 24 h, an example.

Table S1. Proteins identified in Differential Proteomic analysis of HEK293 cells treated with JJR5.

Table S2. Proteins identified in Differential Proteomic analysis of HEK293 cells treated with JJR6.

Table S3. Proteins identified in Differential Proteomic analysis of HeLa cells treated with JJR5.

Table S4. Proteins identified in Differential Proteomic analysis of HeLa cells treated with JJR6.

Table S5. Proteins identified in Differential Proteomic analysis of U87 cells treated with JJR5.

Table S6. Proteins identified in Differential Proteomic analysis of U87 cells treated with JJR6.