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Novel 1,3,4-thiadiazole-chalcone hybrids containing catechol moiety: Synthesis, antioxidant activity, cytotoxicity and DNA interaction studies

Katarina Jakovljević,[a] Milan D. Joksović,[a] Ivana Z. Matić,[b] Nina Petrović,[b,c] Tatjana Stanojković,[b] Dušan Sladić,[d] Miroslava Vujčić,[e] Barbara Janović,[e] Ljubinka Joksović,[a] Snežana Trifunović[d] and Violeta Marković*[a]

[a] Faculty of Science, Department of Chemistry, University of Kragujevac, R. Domanovica 12, 34000 Kragujevac, Serbia

[b] Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade, Serbia

[c] Laboratory for Radiobiology and Molecular Genetics, "Vinča" Institute of Nuclear Sciences, University of Belgrade, 11000 Belgrade, Serbia

[d] Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

[e] Institute for Chemistry, Technology and Metallurgy, Njegoševa 12, 11000 Belgrade, Serbia

Abstract Hybrid compounds that combine 1,3,4-thiadiazole containing catechol moiety with chalcone motif have been synthesized and examined for their antioxidant activity, cytotoxicity and DNA-binding activity. The series of thirteen compounds showed strong antioxidant and cytotoxic effects on human acute promyelocytic leukemia HL-60 cells. Several compounds exerted good cytotoxic activities on cervical adenocarcinoma HeLa cells. The treatment of HeLa cells with IC_{50} and double IC_{50} concentrations of the compounds **5a**, **5c**, **5f** and **5m** induced statistically significant increase in the percentage of cells within subG1 cell cycle phase. The examined compounds caused G2/M cell cycle arrest in HeLa cells. Each of these compounds triggered apoptosis in HeLa cells through activation of caspase-3, the main effector caspase, caspase-8, which is involved in the extrinsic apoptotic pathway, and caspase-9, which is involved in the intrinsic apoptotic pathway. All of the examined compounds decreased the expression levels of MMP2 in HeLa cells and levels of protumorigenic miR-133b. Compounds **5a** and **5m** lowered the expression level of oncogenic miR-21 in HeLa cells. In addition, compounds **5a**, **5f** and **5m** decreased the expression levels of oncogenic miR-155 while the treatment of HeLa cells with compounds **5a**, **5c** and **5f** increased expression of tumor-suppressive miR-206. The observed effects of the compounds on expression levels of four examined miRNAs suggest their prominent cancer-suppressive activity. The investigation by absorption and fluorescence spectroscopy showed more efficient calf thymus DNA binding activity of the compound **5m** in comparison to other tested compounds. Results of pUC19 plasmid cleavage study and comet assay showed DNA damaging activities of compounds **5a** and **5c**.

***Keywords*:** 1,3,4-thiadiazole-chalcone, antioxidant, apoptosis, gene expression, CT-DNA binding activity

* Corresponding author. *E-mail address*: markovicvioleta@kg.ac.rs (V. Marković)

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1. Introduction

Anticancer hybrid molecules incorporate two or more different, covalently linked pharmacophores with ability to modulate multiple biological targets and improve therapeutic potential of the designed compounds in comparison to single bioactive precursors. Using molecular hybridization techniques, it is possible to synthesize numerous hybrids based on known anticancer scaffolds leading to a more favorable pharmacological profile than the sum of each individual compounds.[1] Chalcones are frequently selected as one of privilege structures because of their significant anticancer properties and facile preparation, offering major advancements in the field of hybrid molecules.[2,3] Such chalcone hybrid analogues with promising anticancer activity have already been synthesized by the coupling of chalcones with various bioactive compounds including coumarin,[4,5] 1,2,3-triazole,[6] retinoid,[7] naphthoquinone,[8] β-carboline,[9] artemisinin,[10] *N*-4-piperazinyl-ciprofloxacin,[11] anthraquinone, [12] pyrazole-5-carboxamide,[13] thiazole[14] and isoxazole.[15]

We selected 1,3,4-thiadiazole as a second bioactive compound for combining with chalcone unit to generate a new hybrid molecule suitable to simultaneously target several pathogenic mechanisms. The covalent bond was established by formation of an amide in the reaction of amine group of heterocycle and previously transformed carboxylic function into acid chloride of chalcone analogue. 1,3,4-Thiadiazoles exhibited a wide spectrum of anticancer activities owing to high electron-donating ability of nitrogen atoms to form hydrogen bonds or to coordinate metal ions.[16,17] Although 1,3,4-thiadiazoles have a promising anticancer potential, their toxicity still remains a major concern.[18] Molecular hybridization may be a way to enhance activity or selectivity and also, overcome the side effects associated with the single compound.

It is well known that antioxidants (including phenolic ones) block free radicals to induce damage of biological macromolecules under oxidative stress, preventing their oxidation, DNA mutations and malignant changes.[19] Very recently, we combined bioactive functions of 1,3,4-thiadiazole and phenolic acid moiety to obtain novel conjugates with antioxidant and antiproliferative activity.[20] The reported antiproliferative activities of 1,3,4-thiadiazoles and chalcone analogues, as well as radical scavenging properties of phenolic compounds, led us to rational design and synthesis of new molecular hybrids with evaluation of their antioxidant, cytotoxic and DNA-binding potential.

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2. Results and Discussion

2.1. Chemistry

The novel 1,3,4-thiadiazole-chalcone hybrids were synthesized in three steps (Scheme 1). Initially, chalcone analogues **4a-m** were prepared using the Claisen-Schmidt condensation of 4-formylbenzoic acid **3** with various substituted acetophenones in the presence of NaOH, according to slightly modified literature procedure.[21] Subsequently, chalcones **4a-m** were converted into acyl chloride by action of $SOCl_2$ and, without isolation, reacted with 4-(5-amino-1,3,4-thiadiazol-2-yl)benzene-1,2-diol **2** in dioxane giving the final hybrid compounds **5a-m** in moderate to good yields (53-87%). Previously, thiadiazole derivative was synthesized by the reaction of 3,4-dihydroxybenzoic acid and thiosemicarbazide in phosphoryl chloride.[20] Although acid chlorides are very reactive compounds, the formation of an amide requires long time and high temperature due to poor nucleophilicity of amino group of 1,3,4-thiadiazole. Almost all compounds still contained a significant amount of dioxane. The solvent was eliminated by dissolving crude product in DMF or DMSO and subsequent precipitation with water or by complete evaporation of THF solutions. The structure of all compounds was confirmed by means of ^{1}H and ^{13}C NMR spectroscopy (see Supplementary content), IR and elemental analysis. The olefinic protons of the chalcone double bond in hybrid compounds **5a-m**, as well as their corresponding precursors **4a-m**, appeared as an AB system. On the basis of coupling constant values (J = 15.6–16.0 Hz), the compounds **5a, 5c, 5d, 5f, 5g, and 5i-m** were isolated and characterized in *E*-isomeric form. However, the compounds **5b**, **5e** and **5h**, and their chalcone precursors (**4b**, **4e** and **4h)** having the coupling constant values in the range from 7.0 to 8.2 Hz correspond to the less stable *Z*-configuration.[22] This kind of geometry of the chalcone double bond is not very frequent and since these derivatives contain an *ortho*-substituted acetophenone moiety in their structure, it is probable that *Z*-isomeric form is favored due to the steric factors. Aromatic protons corresponding to the phenolic group appeared in the range of 6.86-7.44 ppm, giving a doublet for H-5, doublet of doublets for H-6 and another doublet for H-2. Protons belonging to the benzoic acid moiety were well resolved in the form of the AB system for most of the thiadiazole-chalcone derivatives.

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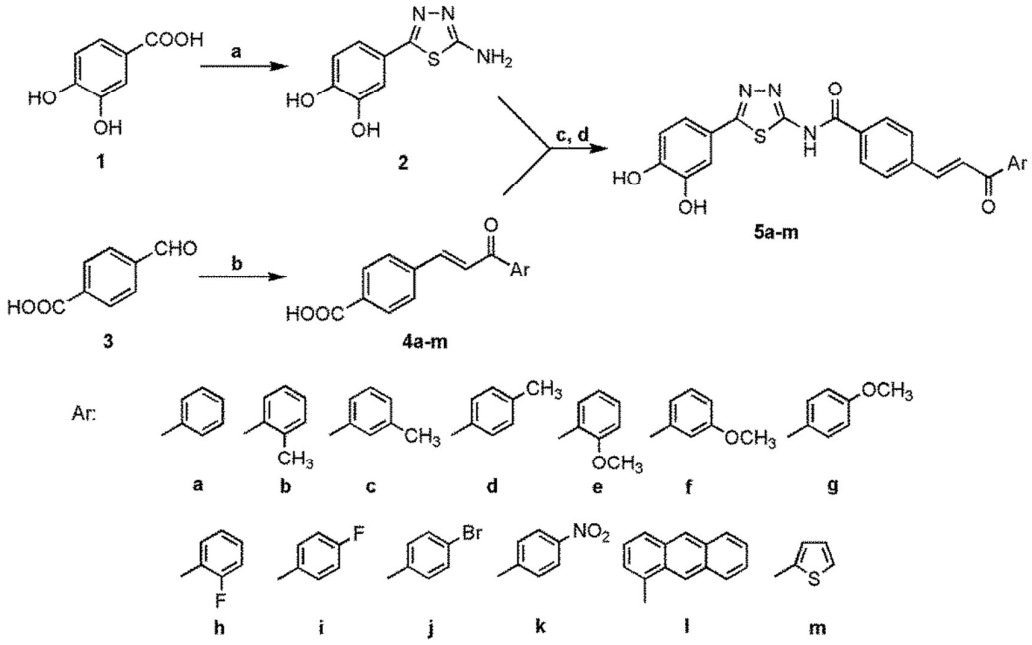

Scheme 1. Reagents and conditions: **a)** $POCl_3$, rt, $H_2NHNC(=S)NH_2$, 1h, reflux; **b)** substituted acetophenones, NaOH, MeOH, 2h, reflux, HCl; **c)** $SOCl_2$, DMF, CH_2Cl_2, 2h, rt; **d) 2**, dioxane, 12h, reflux.

2.2. Biology

2.2.1. Radical scavenging activity and effects of pretreatment of HeLa cells with 1,3,4-thiadiazole-chalcone hybrids on ROS levels

Antioxidant activity of phenolic acids has been well known because of their preventive effect on malignant changes that are associated with radical species.[23] Reactive oxygen species (ROS) in the form of free radicals (superoxide and hydroxyl radical) and neutral molecules (H_2O_2) have been neutralized by stable phenolic antioxidants, diminishing DNA damaging and cancer formation.[19] Thus, the compounds showing antioxidant and cytotoxic activity have a great importance and a combination of phenolic antioxidant moiety with other bioactive pharmacophores could be a good way to obtain more potent radical scavengers as a result of their synergistic effects.

Generally, all synthesized hybrid molecules exhibited better DPPH radical scavenging activity than referent ascorbic acid (Table 1).

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Table 1. DPPH scavenging activity of the 1,3,4-thiadiazole-chalcone hybrids **5a-m**.[a]

Compound	IC_{50} ± SD (μM)
5a	12.03 ± 0.50
5b	11.66 ± 0.41
5c	17.17 ± 0.38
5d	11.81 ± 0.31
5e	9.76 ± 0.75
5f	10.33 ± 0.30
5g	17.86 ± 0.03
5h	13.91 ± 0.32
5i	17.82 ± 0.65
5j	18.04 ± 0.23
5k	14.01 ± 0.34
5l	13.45 ± 0.09
5m	11.06 ± 0.24
Ascorbic acid	20.23 ± 0.14

[a] Results are mean values ± SD from three measurements.

Unfortunately, all our compounds were practically insoluble in methanol, a common solvent for DPPH test, and determination of radical scavenging activity was performed in diluted dimethyl sulfoxide. However, DMSO was found to considerably decrease antiradical capacity of ascorbic acid due to the formation of a molecular complex with DMSO through intermolecular hydrogen bonds.[24] Also, dramatic solvent effects were established on the rates of H-abstraction from phenolic compounds.[25] In accordance with these facts, in separate experiments we observed significantly lower scavenging activity at higher concentrations of DMSO, suggesting the formation of a molecular complex between DMSO and our compounds. From this reason we were not able to consider the influence of chalcone moiety with various electron donating or withdrawing substituents and make a comparison with other 1,3,4-thiazole compounds containing phenolic hydroxyl groups. Anyway, the potent radical scavenging potential of our compounds is evident, and for that reason we decided to explore their effects on ROS levels in human cervical adenocarcinoma HeLa cells. We examined the effects of four compounds – **5a**, **5c**, **5f** and **5m,** which were selected for all further analyses due to their prominent cytotoxic activity and good selectivity in the antiproliferative action (Table 2). As it could be seen in Fig. 1 A, significant accumulation of ROS in the H_2O_2-treated cells indicates activation of endogenous ROS production by added H_2O_2. The pretreatment of HeLa cells with subtoxic IC_{20} concentrations of **5a** and **5f** for 24 h (10 μM for each compound) slightly increased the ROS levels induced by H_2O_2, while pretreatment with compounds **5c** and **5m** did not affect the ROS levels triggered by H_2O_2. The radical species (phenoxyl radical etc.) produced by the oxidation of **5a** and **5f** with H_2O_2 are highly reactive and subject to further oxidation giving quinone compounds. Quinones are still reactive and can be stabilized by interaction with nucleic acid.[26] This reaction might be responsible for the toxic pro-oxidant effect of **5a** and **5f** which may damage cellular constituents

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like DNA by induction of oxidative cytotoxic stress in cancer cells, activating programmed cell death – apoptosis.

In the absence of exogenously added H_2O_2, the intracellular ROS levels in HeLa cells treated for 24 h with each of the four compounds were decreased when compared with basal ROS levels in control. This behavior of all tested compounds, especially **5c**, is a result of their radical scavenging potential to neutralize basal ROS in HeLa cells (Fig. 1, B). Some cancer cells, in advanced stage of disease, have adapted to oxidative stress due to their antioxidative defense capacity. Therefore, the cancer cells show resistance to the drugs that induce intracellular ROS production, such as paclitaxel or doxorubicin.[27] Thus, possibility of drugs to reduce antioxidant system in combination with oxidant agents might be useful in antitumor therapies. A redox modulation of ROS level by production of sufficient amount of ROS provoking apoptosis or by decrease in antioxidant level could be a good way to kill cancer cells which are more sensitive to exogenous oxidative stress than normal cells.

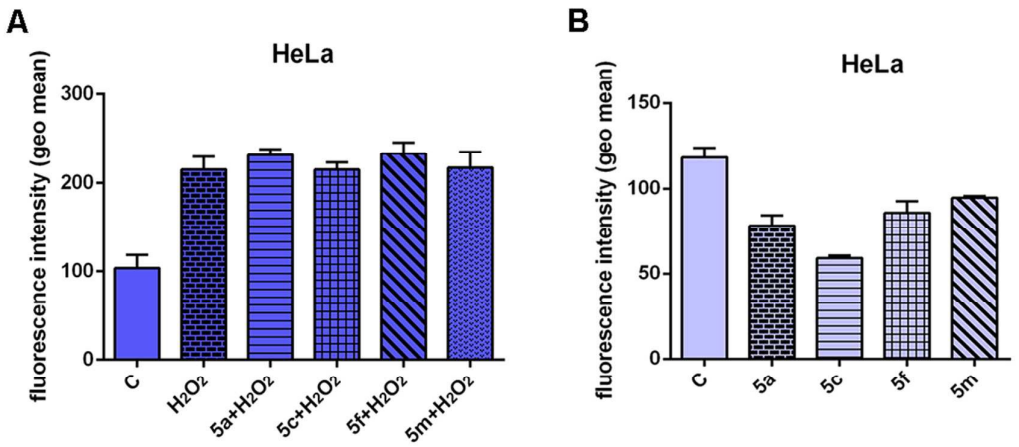

Fig. 1. Effects of 24 h pretreatment of HeLa cells with IC_{20} concentrations of the 1,3,4-thiadiazole-chalcone hybrids **5a**, **5c**, **5f** and **5m** (10 μM for each compound) on ROS generation induced by hydrogen peroxide (10 mM) (A) and endogenous ROS levels (B). The results are presented as the mean ± SD of two independent experiments.

2.2.2. Cytotoxic activity

The cytotoxicity of 1,3,4-thiadiazole-chalcone hybrids containing antioxidant catechol moiety was evaluated against three human malignant cell lines (cervical carcinoma HeLa, acute promyelocytic leukemia HL-60, and lung carcinoma A549), and normal human lung fibroblasts MRC-5 using MTT cell survival test. The obtained IC_{50} values are shown in Table 2. Generally, all tested compounds exerted the strongest cytotoxic activity against leukemia HL-60 cells with IC_{50} values in the range from 6.92 μM to 16.35 μM. The compounds **5a**, **5f**, **5h**, **5l** and **5m** also showed strong cytotoxic effects on HeLa cells, with IC_{50} values from 9.12 μM to 12.72 μM. Lung carcinoma A549 cells were the least sensitive to the cytotoxic activity of the examined hybrids, while other

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tested derivatives exerted moderate to low cytotoxic activity. As it can be seen in Table 2, there is no significant difference between the influence of electron-donating and electron-withdrawing groups of the acetophenone moiety on the cytotoxic action against cancer cells. This indicates that the thiadiazole-chalcone pharmacophore present in the structure of the tested compounds has the crucial role in their antiproliferative action. All of the compounds from this series exerted two to four times higher cytotoxic activity against HeLa and HL-60 malignant cell lines in comparison to their activity against normal MRC-5 cells, with the exception of **5g**, **5j** and **5k** against HeLa cells. In addition, all tested derivatives showed lower toxicity against normal MRC-5 cells comparing to cisplatin as a referent chemotherapeutic.

Table 2. The cytotoxic activity of the investigated 1,3,4-thiadiazole-chalcone hybrids containing antioxidant phenolic moiety.[a]

	IC_{50}±SD (μM)			
Compd.	**HeLa**	**HL-60**	**A549**	**MRC-5**
5a	9.37±0.86	8.40±1.44	42.75±2.77	36.00±1.10
5b	9.12±1.19	7.62±1.40	21.80±2.55	18.56±2.25
5c	9.63±1.26	8.39±1.39	27.87±4.05	34.25±4.88
5d	11.20±2.32	8.44±1.52	26.35±1.90	33.72±3.35
5e	10.22±0.73	11.97±1.27	50.23±5.35	48.01±6.43
5f	9.98±0.99	9.92±0.50	92.14±6.64	42.54±3.88
5g	57.55±7.58	16.35±0.87	89.10±9.35	81.33±9.25
5h	11.08±2.10	8.88±1.32	46.89±1.71	39.58±4.82
5i	17.75±0.17	10.63±0.96	89.33±2.99	45.81±3.38
5j	40.22±3.64	9.74±1.54	87.74±6.82	38.24±4.95
5k	54.76±2.77	11.17±0.52	127.75±8.37	39.44±3.49
5l	11.04±2.41	6.92±0.53	23.23±1.37	21.17±1.23
5m	12.72±3.24	15.72±1.66	47.85±1.92	68.52±7.69
cisplatin	4.91±0.74	2.88±0.34	13.21±0.89	9.35±1.29

[a] Results are mean values ± SD of three independent experiments.

2.2.3. Effects of the 1,3,4-thiadiazole-chalcone hybrids on cell cycle phase distribution

With the aim to investigate the mechanisms of the cytotoxic activity of four selected 1,3,4-thiadiazole-chalcone hybrids **5a**, **5c**, **5f** and **5m**, the changes in cell cycle phase distribution of cervical adenocarcinoma HeLa cells treated with IC_{50} and $2IC_{50}$ concentrations of these compounds for 24 h were assessed. The incubation with both tested concentrations of the compounds induced statistically significant increase in the percentage of HeLa cells within subG1 phase when compared with untreated, control cell samples (Fig. 2). These results point to ability of the examined compounds to induce cell death in HeLa cells. Furthermore, compound **5c** applied at IC_{50} concentration and compound **5m** applied at double IC_{50} concentration caused significant increase in the percentage of cells within G2/M cell cycle phase in comparison to those percentages in the control cells. The

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incubation of HeLa cells with double IC_{50} concentrations of the compounds **5a**, **5c** and **5f** also led to accumulation of cells in G2/M phase, although those differences were not statistically significant. The obtained data demonstrate that tested compounds cause G2/M cell cycle arrest in HeLa cells, thus preventing cell entry into mitosis and eventually leading to cell apoptosis. The double IC_{50} concentrations of compounds **5a** and **5m**, as well as IC_{50} and double IC_{50} concentrations of compound **5c**, induced significant decrease in the percentage of treated cells within G1 phase when compared with control cells, suggesting enhanced sensitivity of cells within G1 phase to the cytotoxic action of these compounds. Our findings are in accordance with literature data showing that compounds bearing chalcone bioactive scaffold induce G2/M cell cycle arrest and apoptotic cell death in cancer cells.[28]

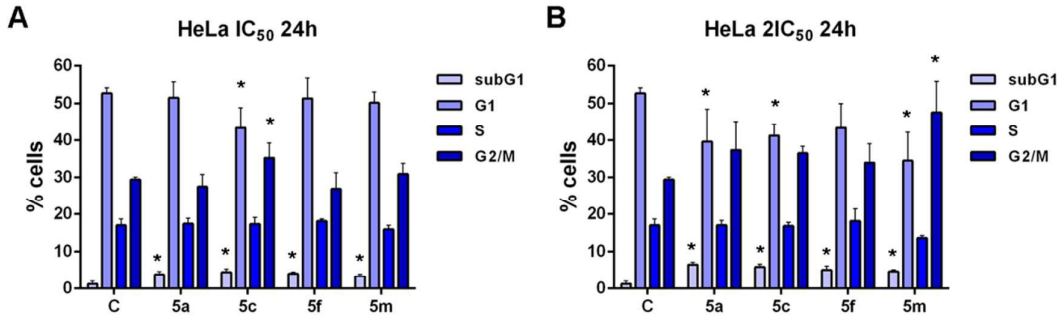

Fig. 2. Changes in the cell cycle phase distribution of human cervical carcinoma HeLa cells treated with IC_{50} (A) and $2IC_{50}$ concentrations (B) of the 1,3,4-thiadiazole-chalcone hybrids **5a**, **5c**, **5f** and **5 m** after 24 h treatment. The results are presented as the mean ± SD of three independent experiments. Statistically significant differences between control and treated cell samples are marked with * ($p<0.05$).

2.2.4. Effects of the specific caspase inhibitors

To further examine whether the tested compounds could induce apoptotic cell death in treated HeLa cells, the cells were pretreated with specific peptide inhibitors of caspase-3, caspase-8 or caspase-9 two hours before addition of compounds and their effects were determined by cell cycle analysis. As it could be seen in Fig. 3, the notable decrease in the percentage of HeLa cells within subG1 cell cycle phase was observed in cell samples which were pretreated with caspase-3 inhibitor, caspase-8 inhibitor or caspase-9 inhibitor, and afterwards exposed to compounds **5a**, **5c**, **5f** or **5m** when compared with HeLa cell samples which were not pretreated with inhibitors before addition of the compound. The obtained results indicate that examined 1,3,4-thiadiazole-chalcone hybrids (**5a**, **5c**, **5f** and **5m**) trigger apoptosis in HeLa cells through the activation of main effector caspase-3, activation of caspase-8, implicated in extrinsic pathway of apoptosis and caspase-9, implicated in intrinsic pathway of apoptosis.

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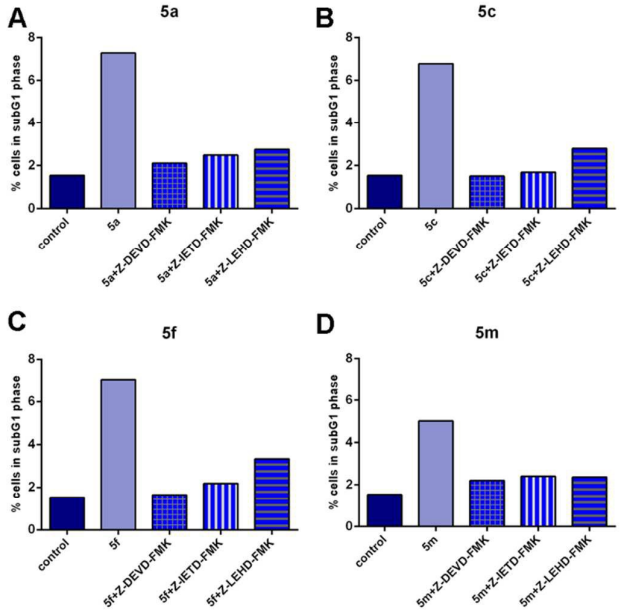

Fig. 3. Effects of the specific caspase inhibitors (Z-DEVD-FMK - caspase-3 inhibitor, Z-IETD-FMK -caspase-8 inhibitor, Z-LEHD-FMK - caspase-9 inhibitor) on the percentages of HeLa cells within subG1 phase treated with $2IC_{50}$ concentrations of the compounds **5a** (A), **5c** (B), **5f** (C), and **5m** (D). Representative graphs are shown.

2.2.5. Effects of 1,3,4-thiadiazole-chalcone hybrids on gene and microRNA expression levels

Increased levels of matrix-metalloproteinase-2 (*MMP2*), matrix-metalloproteinase-9 (*MMP9*), and vascular endothelial growth factor A (*VEGFA*), and increased levels of typical oncogenic miRNAs (miR-21 and miR-155) overexpressed in various cancer types,[29] and decreased levels of matrix-metalloproteinase inhibitor 3 (*TIMP3*)[30] are associated with the cell growth, migration, epithelial mesenchymal transition, invasion, metastasis, and angiogenesis.[31] MiR-133b was known to be downregulated in prostate cancer, but this microRNA exerted protumorigenic activity in cervical cancer.[32] MiR-206 is described to be downregulated in cervical cancer,[33] and that overexpression induces apoptosis.[34]

To further elucidate the molecular mechanisms of cytotoxic activity of our compounds, we examined changes in the expression levels of extracellular matrix-degrading proteases *MMP2* and *MMP9*, *TIMP3* which inhibits matrix metalloproteinases and regulates proteolysis of extracellular matrix, as well as in the expression of inducer of angiogenesis *VEGFA*, and several microRNAs-miR-21/133b/155/206 in human cervical adenocarcinoma HeLa cells treated with subtoxic IC_{20} concentrations of the examined compounds (Fig. 4).

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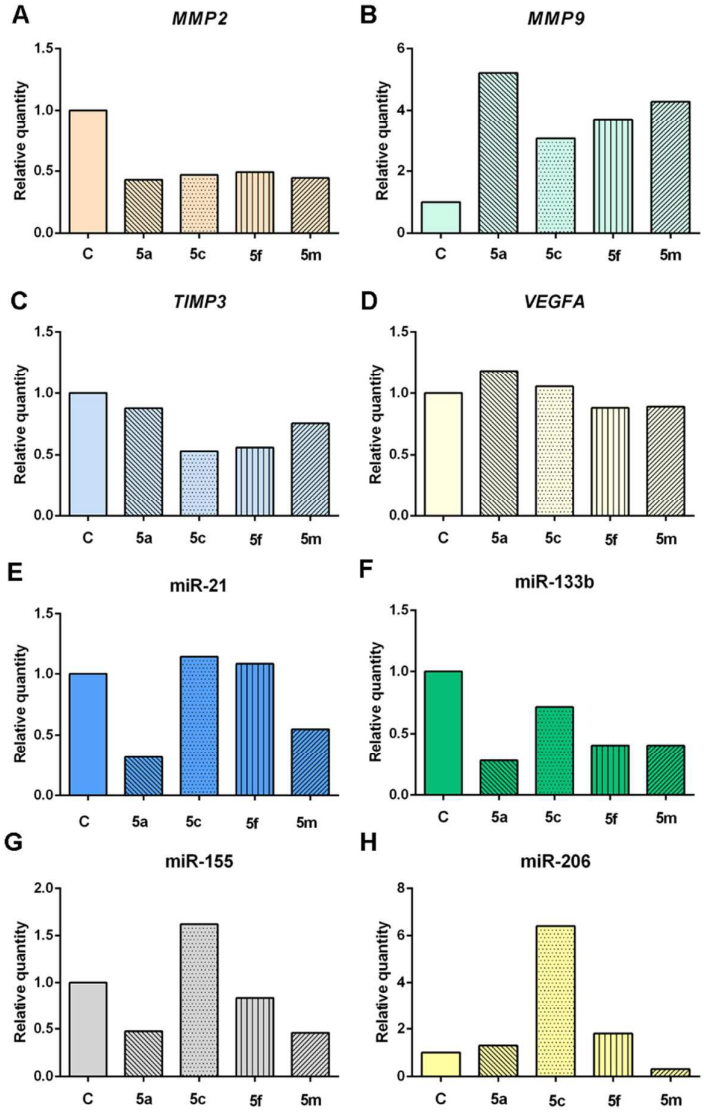

Fig. 4. Changes in expression levels of *MMP2* gene(A), *MMP9* gene (B), *TIMP3* gene (C), *VEGFA* gene (D), miR-21 (E), miR-133b (F), miR-155 (G) and miR-206 (H) in HeLa cells exposed to subtoxic IC_{20} concentrations of the compounds **5a**, **5c**, **5f** and **5 m** (10 μM for each compound) for 24 h. Representative graphs are shown.

All of the examined compounds (**5a**, **5c**, **5f**, and **5m**) decreased expression levels of *MMP2* and *TIMP3*, but increased expression levels of *MMP9*, when compared with those levels in untreated, control HeLa cells. In addition, compound **5a** slightly increased the *VEGFA* expression level in HeLa cells, while compounds **5f** and **5m** slightly decreased the expression of *VEGFA* in these cells. The observed downregulation of *VEGFA* expression in HeLa cells and inhibition of formation of tubular structures by EA.hy926 cells exerted by compounds **5f** and **5m** indicate the mild antiangiogenic activities of these compounds (Fig. 5).

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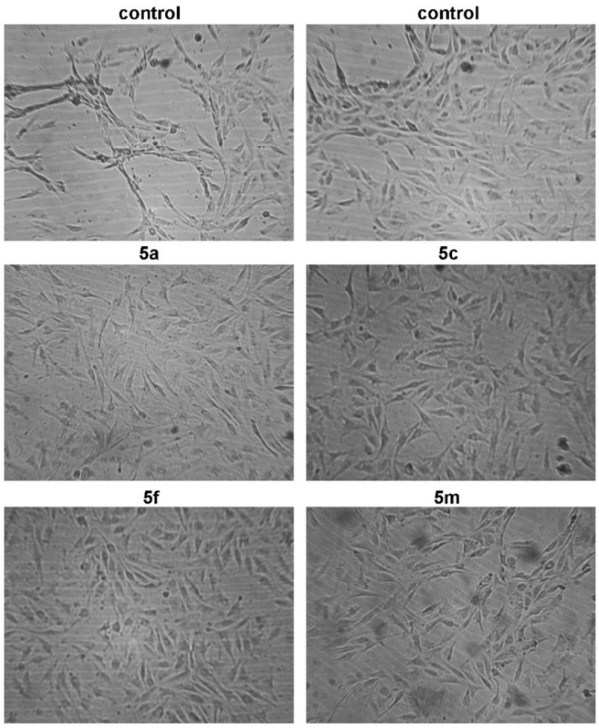

Fig. 5. Photomicrographs of control EA.hy926 cells and EA.hy926 cells incubated with subtoxic IC_{20} concentrations of the compounds **5a**, **5c**, **5f** (6.5 μM for each compound) and **5m** (8 μM) for 20 h.

The ability of the examined 1,3,4-thiadiazole-chalcone hybrids to downregulate expression levels of *MMP2* in HeLa cells might suggest their suppressive effects on cervical cancer cell invasion and establishment of metastasis. The investigation of Kato et al. suggested that expression levels of *MMP2* may be closely associated with invasion ability of cervical cancer cells.[35] We observed that all tested compounds remarkably increased *MMP9* expression levels in HeLa cells. MMP9 is connected with increased invasion and metastasis, but besides this protumorigenic role, there is experimental evidence that MMP9 may exert protective effects on cancer progression and metastasis in addition to anti-angiogenic effect.[36,37] The study in a mouse model of multistage skin carcinogenesis caused by HPV16 oncogene demonstrated that *MMP9* deficient transgenic mice had decreased proliferation rate of keratinocytes and a lower incidence of invasive tumors, which had more aggressive phenotype, while mice expressing *MMP9* developed a larger number of tumors, which had less aggressive phenotype.[37] Furthermore, MMP9 may suppress further progression of malignant tumors through generation of various anti-angiogenic peptides, such as angiostatin and tumstatin.[36] Taken together, these findings might suggest that upregulation of *MMP9* expression levels in tumor stroma which could be induced by our compounds**,** might contribute to suppression of tumor growth and progression.

Considering the effects of the compounds on microRNA expression levels in HeLa cells, the compound **5a** decreased levels of three crucial oncogenic miRNAs-miR-21/155 and protumorigenic miR-133b in cervical cancer, while it slightly increased level of tumor suppressive miR-206, when

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compared with those levels measured in control cells. The compound **5c** remarkably increased expression levels of miR-206 and miR-155 in comparison to control HeLa cells, and decreased expression of protumorigenic miR-133b. Treatment with compounds **5c** and **5f** caused mild increase in miR-21 expression in HeLa cells. Exposure to compound **5f** caused decrease in the expression of miR-133b and miR-155 in addition to increase in the expression level of miR-206. The compound **5m** lowered the expression levels of all four examined miRNAs when compared with controls cells. Our research showed that compounds **5a** and **5m** induced remarkable decrease in the expression level of oncogenic miR-21, that could result in reduced proliferation, migration and invasion of cervical carcinoma cells. These tumor-suppressive effects had shown to be regulated through phosphatase and tensin homolog (*PTEN*).[38] The antiproliferative potential of our four compounds is further proved by their ability to reduce levels of oncogenic miR-133b, which had been reported to be involved in enhanced proliferation and colony formation of cervical cancer cells, as well as in further progression and metastasis of cervical cancers, affecting AKT1 and ERK signaling pathways.[39] Moreover, the compounds **5a**, **5f** and **5m** lowered the expression levels of miR-155, which is overexpressed in cervical cancer tissue, stimulates cell proliferation and exerts oncogenic activity, confirming their cancer-suppressive properties.[40] The pro-apoptotic effects of compounds **5a**, **5c** and **5f** could be, at least in part, attributed to the increased expression of tumor-suppressor miR-206 in treated HeLa cells. The miR-206 had been reported as an inducer of apoptosis in HeLa cells, that was associated with inhibition of neurogenic locus notch homolog protein 3 (Notch 3).[34]

The investigated compounds and miRNAs changed the examined gene expression levels. *TIMP3* is a proven target of miR-21, *MMP9* is a target of miR-133b, while *VEGF* mRNA is target of miR-206 according to TargetScan Human 7.0 (http://www.targetscan.org/).[41]

2.2.6. DNA binding study

As it is well known, the interaction of DNA with a small molecule can give rise to changes in the absorbance and in the peak position in the absorption spectra. Hyperchromism and hypochromism are regarded as spectral evidence for DNA double-helix structural change when DNA reacts with other molecules. Hyperchromism originates from the disruption of the DNA duplex secondary structure and is indicative of the partial or non-intercalative modes[42] and hypochromism originates from the stabilization of the DNA duplex by either the intercalation binding mode or the electrostatic effect of small molecules.[43,44] Electronic absorption spectra of biologically most active 1,3,4-thiadiazole–chalcone hybrids recorded at different concentrations without or with fixed concentration of CT-DNA are shown in Fig. 6A. UV–vis spectra of all compounds displayed similar absorption bands. It was found that the maximum absorption of **5a**, **5c**, **5f** and **5m** was centered at 309 nm, 310 nm, 305 nm and 323 nm, respectively. Upon interaction with CT-DNA, the formation of a compound─CT-DNA occured with no shift of absorption maximum at 259 nm. Detailed absorption

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changes induced by binding of the compounds to CT-DNA were further calculated from the experimental data, Fig. 6B. The value of the sum of absorbances at 259 nm of a free compound and free CT-DNA was a different from the absorption value of a compound─CT-DNA.

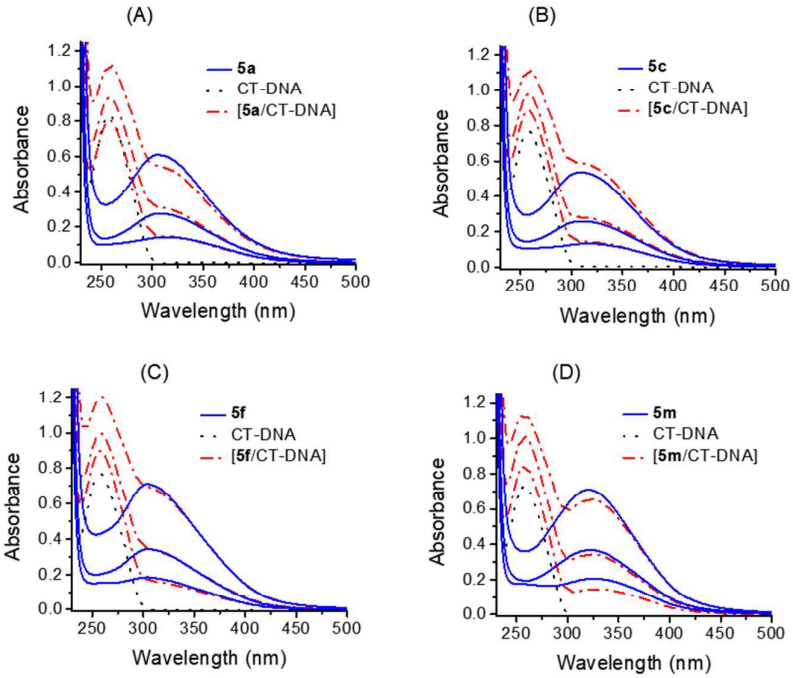

Fig. 6A. Changes in UV–Vis absorption spectra of CT-DNA (95 µM) after interaction with different concentrations of 1,3,4-thiadiazole-chalcone hybrids. Panels A, B, C and D: UV–Vis absorption spectra of **5a** (10µM, 20µM and 40µM), **5c** (10µM, 20µM and 40µM), **5f** (10µM, 20µM and 40µM) and **5m** (10µM, 20µM and 40µM), respectively before and after interaction with CT-DNA.

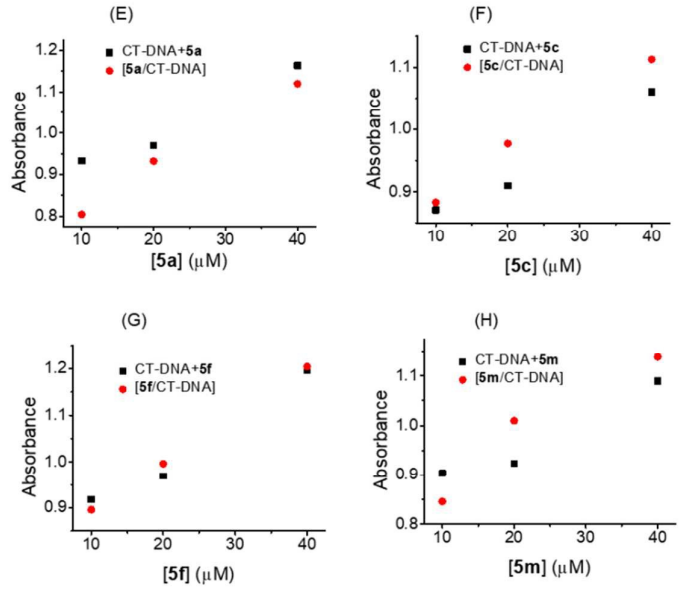

Fig. 6B. Changes in UV–Vis absorption spectra of CT-DNA (95 µM) after interaction with different concentrations of 1,3,4-thiadiazole-chalcone hybrids. Panels E, F, G and H: comparison of absorption

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at 259 nm between the CT-DNA─1,3,4-thiadiazole–chalcone compounds and the sum values of CT-DNA and 1,3,4-thiadiazole–chalcone compounds. The representative absorption assay curves are shown. Triplicate assays were applied to all compounds and control CT-DNA.

The hypochromism of about 13% was observed with lower concentrations of **5a**, Fig. 6B (Panel E) while the decrease in absorption intensity at 259 nm was less pronounced at higher concentrations. In case of **5c**, Fig. 6B (Panel F) the hyperchromism was observed (-13.7%, -7.36% and -4.9% at 10 μM, 20 μM and 40 μM, respectively) and with **5m**, Fig. 6B (Panel H), hyperchromism was also observed, more pronounced at lower concentrations probably. The DNA showed the least changes after interaction with **5f**, Fig. 6B (Panel G) (this weak hypochromism was calculated as 2.4% at the lowest concentration of the compound). Previous studies demonstrated that a significant hypochromic effect with a concomitant red shift upon ligand binding to DNA was the typical characteristic of the intercalating mode.[45,46] In this work, the obtained hypochromic effects of **5a** and **5f** and hyperchromic effects of **5c** and **5m** with no red shift probably reflect changes in the conformation and structure of CT-DNA upon binding of the compounds in the minor groove, *via* formation of hydrogen bonds between hydroxyl groups of 1,3,4-thiadiazole─chalcone parts of the compounds and exposed AT base pairs.

The stability of binding between CT-DNA and 1,3,4-thiadiazole–chalcone compounds was determined by a spectroscopic titration. Absorption spectra of **5a**, **5c**, **5f** and **5m** without and with CT-DNA at different concentrations are shown in Fig. 7, Panels A – D). The absorbance at 259 nm was monitored for each concentration of DNA (insets in Fig. 7, show plots after linearization). Binding constant K_b of **5a**, **5c**, **5f** and **5m** were calculated using equation (2) and values of 1.09×10^3 M^{-1}, 0.74×10^3 M^{-1}, 0.71×10^3 M^{-1} and 1.01×10^3 M^{-1}, respectively, are obtained. These values are comparable with ones previously published for anthraquinone-chalcone hybrids[12] and other structurally different compounds.[47,48] However, relatively low correlation coefficients, indicate low binding strength of interaction of 1,3,4-thiadiazole-chalcone hybrids with helix of CT-DNA.

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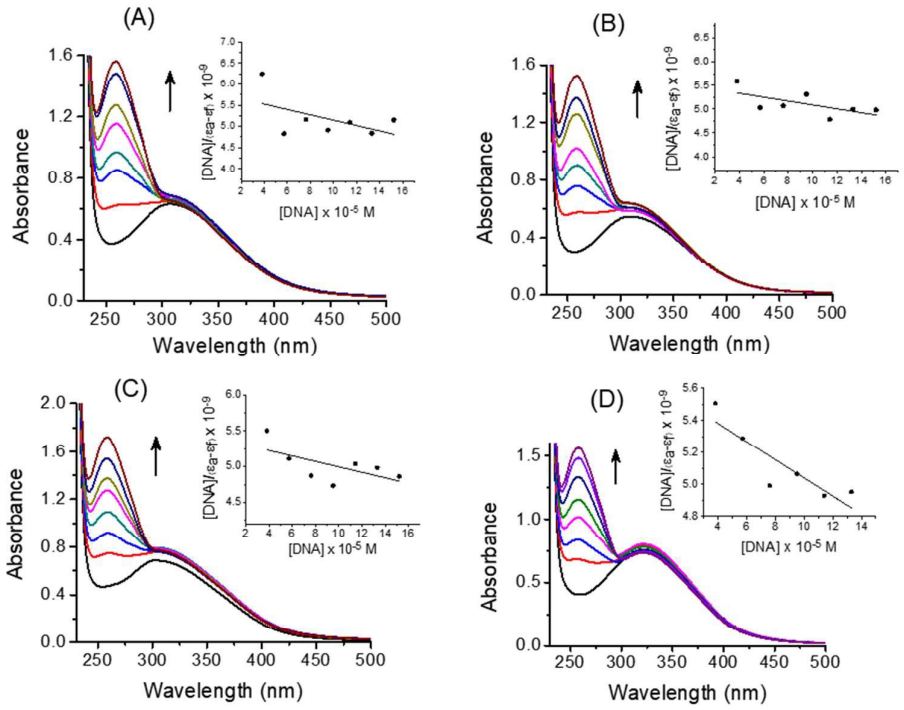

Fig. 7. The representative absorption titration curves of **5a** (A), **5c** (B), **5f** (C) and **5m** (D) at fixed concentration (40 μM) with increasing concentrations of CT-DNA (2.2, 4.4, 6.6, 8.8, 11 and 13 × 10^{-5} M); *Insets*: determination of binding constant (K_b) by plot of [DNA]/($\varepsilon_a - \varepsilon_f$) *vs.* [DNA]; The arrow show the changes in absorbance upon increasing amounts of CT-DNA. Triplicate assays were applied to all compounds and control CT-DNA.

In order to provide additional insight into the interactions between the DNA and 1,3,4-thiadiazole─chalcone hybrids, the study with the minor groove binder Hoechst 33258 was performed. Hoechst 33258 (H) binds strongly and selectively with high affinity to double-stranded B-DNA structure and like other minor groove binders, it recognizes at least four AT base pairs. It binds by combination of hydrogen bonding, van der Waals contacts with the walls of the minor groove, and electrostatic interactions between its cationic structure and the DNA.[49] Binding of Hoechst 33258 to CT-DNA was followed by excitation at 350 nm with maximum in fluorescence at 444 nm. The fluorescence intensity of the band at 444 nm of the Hoechst−CT-DNA system decreased remarkably with the increasing concentration of the compounds (Fig. 8). The fluorescence intensities of H−CT-DNA−**compound** system were 58%, 60%, 55% and 51% of H−CT-DNA system at maximal applied concentration of **5a**, **5c**, **5f** and **5m**, respectively. The observed reduction of fluorescence indicated the propensity of the 1,3,4-thiadiazole–chalcone hybrids to bind to DNA minor groove. *Insets* in Fig. 8 show the quenching plots demonstrating that quenching of H bound to CT-DNA by these compounds are in agreement with the linear Stern-Volmer Eq. (3) for the investigated concentration ranges of the compounds. The corresponding quenching constants of H–CT-DNA system for **5a**, **5c**, **5f** and **5m**

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were calculated by linear regression of the plot I_0/I *versus* [**compound**]/[CT-DNA] as K = 3.30, 2.95, 3.95 and 4.66, respectively.

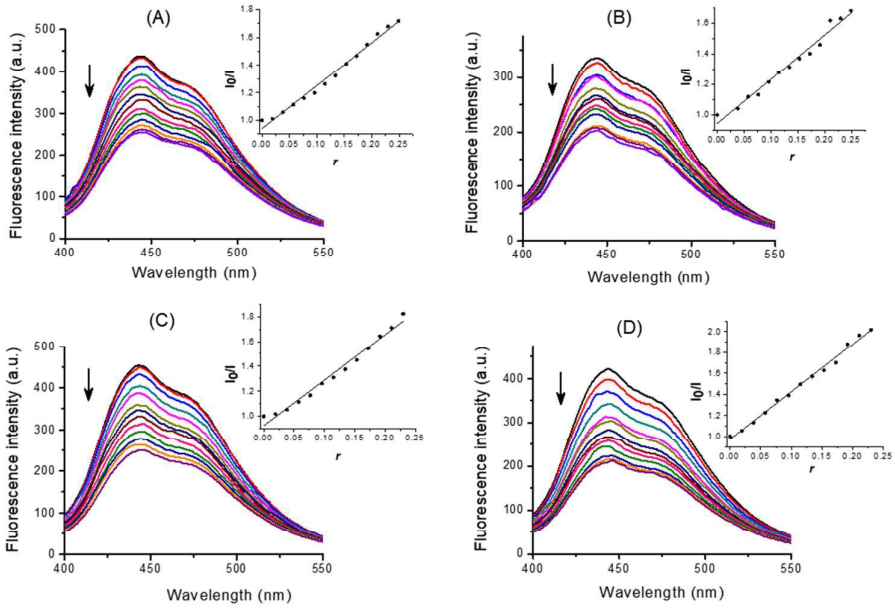

Fig. 8. Displacement of DNA-bound Hoechst 33258 (H) by **5a** (A), **5c** (B), **5f** (C) and **5m** (D)**.** Emission spectra (λ_{ex} = 350 nm) of H (2.8 x 10^{-5} M) bound to CT-DNA (1 x 10^{-4} M, top line) and quenching of H—CT-DNA system by the compounds at increasing concentrations (0 – 2.2 x 10^{-5} M, curves from top to bottom). *Insets*: Fluorescence curves of EB bound to CT-DNA at λ_{max} = 444 nm by 1,3,4-thiadiazole-chalcone compounds; ***r*** = [**compound**]/[CT-DNA]. The arrow shows that fluorescence intensity decreased with increasing concentration of the complex. The representative fluorescence assay curves are shown. Triplicate assays were applied to all compounds and control CT-DNA.

Comparing K values for the quenching fluorescence intensity obtained by the displacement experiments, it may be concluded that **5m** with thiophene ring was more efficient as a groove binder than other tested 1,3,4-thiadiazole–chalcone hybrids with benzene rings. However, considering the extent of the interaction of the compounds with CT-DNA, it can be concluded that these compounds binds to DNA exhibiting minor groove mode with low binding strength, Also, the results suggested that the differences in benzoic moiety contribute to binding, as well as the binding in the groove most likely occurs *via* forming hydrogen bonds among hydroxyl groups of 1,3,4-thiadiazole─chalcone parts of the compounds and exposed AT base pairs.

2.2.7. Comet assay

Comet assay[50] is a versatile and sensitive method for measuring DNA damage in terms of single and double-strand breaks in DNA. In order to evaluate the extent of DNA damage induced by selected compounds comet assay was performed on MRC-5 cells which were treated for 24h with

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different concentration of compounds **5a**, **5c**, **5f** and **5m**. From the results presented in Fig. 9 it can be seen that the compounds caused DNA damage, evidenced by the increase in % of DNA in the comet tail. Each of the tested compounds exhibited as concentration-dependent DNA damage in MRC-5 cells. At the highest concentration tested (25 μM) DNA damage detected was 45% in case of **5a** compound, while in the case of **5c** DNA damage was 38%. Lower degree of DNA damage was detected in case of compounds **5m** and **5f**, 25% and 20%, respectively.

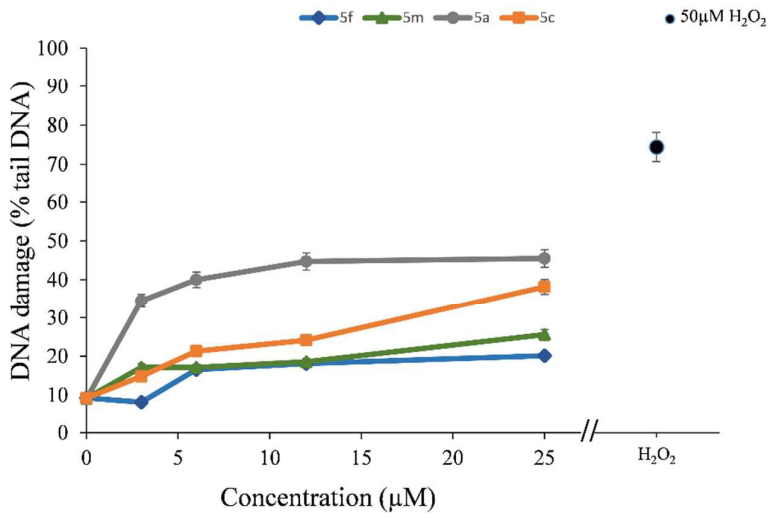

Fig. 9. Detection of DNA damage (% tail DNA) using the Comet assay with MRC-5 cells after 24h exposure with compounds **5f**, **5m**, **5a** and **5c** (3.125 – 25 μM). PBS was used as negative control, and H_2O_2 (50 μM, 5min *in-gel* exposure) was used as positive control. The y-axis shows the mean ± SD of DNA damage measured through tail intensity parameter. The experiments were conducted in triplicate and repeated twice.

2.2.8. pUC19 DNA cleavage study

Since a weak DNA damaging activity was defected in cell culture, the abilities of most potent 1,3,4-thiadiazole–chalcone hybrids to cleave double-stranded plasmid DNA were investigated using an agarose electrophoretic assay and DMSO as solvent of compounds in the performed range of concentration (from 0.5 – 20%) had no effects on conformation of plasmid DNA (results not shown). As shown by a representative agarose gel electrophoresis in Fig. 10 (A), lane 1, control plasmid pUC19 consisted mainly of supercoiled form FI and nicked form FII, with also a small quantity of linear form FIII. All of the compounds were tested and the results showed that there were no significant changes in band mobility, and no strand scission under applied range of concentrations. In Fig. 10 (A) results obtained with compound **5a** are shown. Very similar results were obtained with compounds **5c**, **5f** and **5m** (not shown). Further, strand scission was tested under reducing conditions, in presence of iron(II). Iron(II) per se is involved in the damage of DNA[51] the results of cleavage pUC19 by iron(II) shown in Fig. 10 (B), lanes P+Fe(II) (1:1) and P+Fe(II) (1:2). When the compound

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and iron were used in mole ratio 1:1, (Fig. 10 (B), lanes P+**5a**+Fe(II) (1:1), P+**5c**Fe(II) (1:1), P+**5f**+Fe(II) (1:1) and P+**5m**+Fe(II) (1:1)) and mole ratio 1:2 ((Fig. 10 (B), lanes P+**5a**+Fe(II) (1:2), P+**5c**Fe(II) (1:2), P+**5f**+Fe(II) (1:2) and P+**5m**+Fe(II) (1:2)), 1,3,4-thiadiazole–chalcone hybrids abolished the effects of iron, probably by complex formation and/or by anti-oxidative action of catechol moiety. Iron Fe(III) had no effect under any conditions, Fig. 10 (B), lanes P+**5m**+Fe(III) (1:2) and P+Fe(III) (1:2). These results indicate that DNA cleavage could not be the cause of cytotoxicity. The obtained protective activities of 1,3,4-thiadiazole–chalcone hybrids are comparable to those published for anthraquinone-chalcone hybrids.[12]

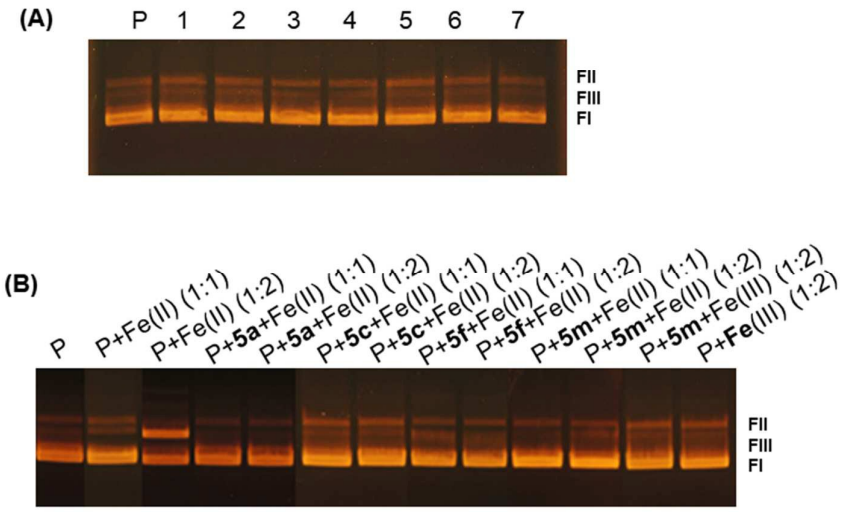

Fig. 10. The representative agarose gel electrophoresis of plasmid pUC19 treated with 1,3,4-thiadiazole–chalcone hybrid: (A) plasmid pUC19 (12 nM) without (lane P) and with **5a** at concentration of 0.05 mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.25 mM, 0.3 mM and 0.4 mM, lanes 1, 2, 3, 4, 5, 6, and 7, respectively; (B) effects of **5a**, **5c**, **5f** and **5m** on Fe(II) induced pUC19 damage with **compound**–iron mole ratio 1:1 and 1:2.

3. Conclusion

Combining two pharmacophores, 1,3,4-thiadiazole derived from protocatechuic acid with antioxidant properties and a series of chalcones, thirteen novel hybrid compounds were prepared and evaluated for radical scavenging, cytotoxic and DNA-binding potential. The synthesized 1,3,4-thiadiazole-chalcone hybrids containing phenolic moiety exerted a strong antioxidant and moderate to good cytotoxic activity against HL-60 and HeLa cells. The selected compounds **5a**, **5c**, **5f** and **5m** caused G2/M cell cycle arrest in HeLa cells and showed the ability to trigger apoptosis in HeLa cells through activation of caspase-3, caspase-8, and caspase-9. All examined compounds remarkably downregulated the expression levels of *MMP2* in treated HeLa cells, suggesting their suppressive

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effects on cervical cancer cell invasion and metastasis. The compounds **5a** and **5m** induced remarkable decrease in the expression level of oncogenic miR-21 in HeLa cells while all four examined compounds lowered levels of protumorigenic miR-133b. In addition, the compounds **5a**, **5f** and **5m** decreased the expression levels of oncogenic miR-155. The treatment of HeLa cells with compounds **5a**, **5c** and **5f** elevated expression of tumor-suppressor miR-206. These effects of compounds on expression levels of four examined miRNAs point to their prominent cancer-suppressive properties. 1,3,4-Thiadiazole-chalcone hybrids bind weakly to the helix of CT-DNA in the minor groove, induce weak DNA damage in cell culture, and protect plasmid DNA from iron(II)–induced DNA damage, but DNA displacement of fluorescent probes and plasmid DNA damage activity as well as damage DNA by comet assay indicated their DNA damaging potential. Taken together, the results of our research indicate the promising antiproliferative properties of four newly synthesized 1,3,4-thiadiazole-chalcone hybrids containing phenolic moiety.

4. Experimental section

4.1. Chemistry

4.1.1. Physical measurements and methods

Melting points were determined on a Mel-Temp capillary melting points apparatus, model 1001 and are uncorrected. Elemental (C, H, N, S) analysis of the samples was carried out in the Center for Instrumental Analysis, Faculty of Chemistry, Belgrade. UV spectra were recorded using an Agilent Technologies, Cary 300 Series UV-Vis Spectrophotometer. IR spectra were obtained on a Perkin Elmer Spectrum One FT-IR spectrometer with a KBr disc. ^{1}H and ^{13}C-NMR spectra were taken on a Varian Gemini 200 MHz spectrometer.

4.1.2. Procedure for the preparation of 5a-m

*Procedure for the synthesis of **2**.* A mixture of 3,4-dihydroxybenzoic acid, **1** (2.00 mmol, 0.308 g,) and $POCl_3$ (1.0 mL) was stirred for 20 minutes at the room temperature. Then, thiosemicarbazide (2.50 mmol, 0.228 g) was added and the resulting suspension was refluxed for 1 hour. After cooling the flask in an ice bath, 3.0 ml of distilled water was added carefully, and reflux was continued for 1 hour. The mixture was then cooled to the room temperature, saturated aqueous solution of NaOH was added until pH 8.5 was reached and the suspension was stirred for 1 hour at the room temperature. The formed precipitate of the corresponding 1,3,4-thiadiazole derivative (**2**) was then filtrated, dried over $CaCl_2$ and recrystallized from hot 50 % aqueous EtOH.

*Procedure for the synthesis of **4**.* A mixture of 4-formylbenzoic acid (2.3 mmol, 0.345 g), a corresponding substituted acetophenone (2.00 mmol) and solid NaOH (4.6 mmol, 0.184 g) in dry methanol (10.0 ml) was refluxed for 2 hours. After completion, the mixture was cooled to the room temperature, 5.0 ml of distilled water was added and pH of the solution was adjusted to 4.5 with HCl aqueous solution (2 M), upon which a precipitate was formed. The precipitate (**4a-m**) was stirred for 30 minutes and then subsequently collected by suction filtration, dried over $CaCl_2$ and recrystallized from hot MeOH.

*Procedure for the synthesis of **5a-m**.* To the mixture of **4** (1.00 mmol) in dry dichloromethane (4.0 ml), $SOCl_2$ (4.00 mmol, 0.3 mL) was slowly added, followed by two drops of dimethylformamide. The resulting mixture was then stirred for 2 h at room temperature. Afterwards, the solvent was evaporated under reduced pressure, and the excess of $SOCl_2$ was removed by

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azeotropic distillation with toluene. 2-Amino-1,3,4-thiadiazole, **2** (1.00 mmol, 0.227 g) and dry dioxane were added to the formed acid chloride, without its previous isolation, and the mixture was then refluxed for 12 hours. After cooling, 20.0 ml of distilled water was slowly added to the solution with vigorous stirring at room temperature, followed with a formation of precipitate (**5a-m**). Stirring was continued for 1 hour and precipitate was then filtrated, washed with water and dried over $CaCl_2$. All compounds (except **5e**) contained a smaller quantity of dioxane which was not removed even after drying at 110 °C for 12 h. To remove dioxane, final compounds **5j** and **5l** were dissolved in *N*,*N*-dimethylformamide and **5k** was dissolved in dimethyl sulfoxide, after which distilled water was added, followed with a formation of precipitate. The formed precipitate was then filtrated, washed with water and dried over $CaCl_2$. All other final compounds (**5a-d**, **5f-i** and **5m**) were dissolved in tetrahydrofuran and solvent was evaporated to dryness under reduced pressure.

4.1.2.1. (E)-4-(3-Oxo-3-phenylprop-1-en-1-yl)benzoic acid (**4a**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.55-7.69, (m, 3H, Ar-H); 7.78, (d, 1H, J_{AB} = 15.8 Hz, CH=); 8.00, (s, 4H, Ar-H); 8.06, (d, 1H, J_{BA} = 15.8 Hz, CH=); 8.18, (d, 2H, J = 7.4 Hz, Ar-H); 13.16, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 124.44, 128.71 (2C), 128.95 (4C), 129.83 (2C), 132.26, 133.40, 137.51, 138.88, 142.63, 166.93, 189.32.

4.1.2.2. (Z)-4-(3-Oxo-3-(o-tolyl)prop-1-en-1-yl)benzoic acid (**4b**): ^{1}H NMR (200 MHz, DMSO-d_6): 2.39, (s, 3H, CH_3); 7.31-7.38, (m, 2H, Ar-H); 7.44, (d, 1H, J_{AB} = 7.0 Hz, CH=); 7.53, (s, 1H, Ar-H); 7.68, (d, 1H, J_{BA} = 7.0 Hz, CH=); 7.90, (d, 2H, J_{AB} = 8.2 Hz, Ar-H); 7.97, (d, 2H, J_{BA} = 8.2 Hz, Ar-H); 13.13, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 20.13, 125.88, 128.35, 128.60, 128.85 (2C), 129.84 (2C), 131.03, 131.41, 132.32, 136.76, 138.55, 138.62, 143.24, 166.89, 194.77.

4.1.2.3. (E)-4-(3-Oxo-3-(m-tolyl)prop-1-en-1-yl)benzoic acid (**4c**): ^{1}H NMR (200 MHz, DMSO-d_6): 2.42, (s, 3H, CH_3); 7.42-7.51, (m, 2H, Ar-H); 7.77, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.96-8.04, (m, 6H, Ar-H); 8.05, (d, 1H, J_{BA} = 7.0 Hz, CH=); 13.81, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 20.99, 124.50, 125.92, 128.79, 128.95 (2C), 129.11, 129.81 (2C), 132.22, 134.01, 137.55, 138.37, 138.91, 142.45, 166.93, 189.30.

4.1.2.4. (E)-4-(3-Oxo-3-(p-tolyl)prop-1-en-1-yl)benzoic acid (**4d**): ^{1}H NMR (200 MHz, DMSO-d_6): 2.40, (s, 3H, CH_3); 7.38, (d, 2H, J_{AB} = 7.8 Hz, Ar-H); 7.78, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.99, (s, 4H, Ar-H); 8.03, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.09, (d, 2H, J_{BA} = 7.8 Hz, Ar-H); 13.09, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 21.36, 124.42, 128.92 (2C), 128.98 (2C), 129.55 (2C), 129.88 (2C), 132.21, 135.04, 139.00, 142.32, 143.96, 167.02, 188.70.

4.1.2.5. (Z)-4-(3-(2-Methoxyphenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4e**): ^{1}H NMR (200 MHz, DMSO-d_6): 3.88, (s, 3H, OCH_3); 7.07, (t, 1H, J = 7.2 Hz, Ar-H); 7.20, (d, 1H, J_{AB} = 8.2 Hz, CH=); 7.46-7.63, (m, 3H, Ar-H); 7.53, (d, 1H, J_{BA} = 8.2 Hz, CH=); 7.85, (d, 2H, J_{AB} = 8.2 Hz, Ar-H); 7.97, (d, 2H, J_{BA} = 8.2 Hz, Ar-H); 13.09, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 56.07, 112.60, 120.79, 128.70 (3C), 129.14, 129.85, 130.00 (2C), 132.18, 133.52, 138.94, 141.05, 158.11, 167.00, 192.05.

4.1.2.6. (E)-4-(3-(3-Methoxyphenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4f**): ^{1}H NMR (200 MHz, DMSO-d_6): 3.85, (s, 3H, OCH_3); 7.25, (dd, 1H, J = 8.0 and 2.2 Hz, Ar-H); 7.50, (t, 1H, J = 8.0 Hz, Ar-H); 7.63, (s, 1H, Ar-H); 7.77, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.79, (d, 1H, J = 8.0 Hz, Ar-H); 7.98, (d, 2H, J_{AB} = 8.6 Hz, Ar-H); 8.02, (d, 2H, J_{BA} = 8.6 Hz, Ar-H); 8.04, (d, 1H, J_{BA} = 15.6 Hz, CH=); 13.09, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 55.56, 113.29, 119.53, 121.33, 124.43, 129.09 (2C), 129.89 (2C), 130.12, 132.31, 138.92, 138.97, 142.78, 159.78, 167.03, 189.04.

4.1.2.7. (E)-4-(3-(4-Methoxyphenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4g**): ^{1}H NMR (200 MHz, DMSO-d_6): 3.87, (s, 3H, OCH_3); 7.09, (d, 2H, J = 9.0 Hz, Ar-H); 7.74, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.99, (s, 4H, Ar-H); 8.06, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.19, (d, 2H, J_{BA} = 9.0 Hz, Ar-H); 13.11, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 55.75, 114.25 (2C), 124.43, 128.93 (2C), 129.87 (2C), 130.45, 131.21 (2C), 132.10, 139.12, 141.85, 163.56, 167.03, 187.46.

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4.1.2.8. (Z)-4-(3-(2-Fluorophenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4h**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.36, (d, 1H, J_{AB} = 7.6 Hz, CH=); 7.38-7.44, (m, 1H, Ar-H); 7.52-7.74, (m, 2H, Ar-H); 7.68, (d, 1H, J_{BA} = 7.6 Hz, CH=); 7.81, (t, 1H, J = 7.6 Hz, Ar-H); 7.91, (d, 2H, J_{AB} = 8.2 Hz, Ar-H); 7.99, (d, 2H, J_{BA} = 8.2 Hz, Ar-H); 13.07, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 116.85 (J_{CF} = 22.2 Hz), 125.05 (J_{CF} = 3.3 Hz), 126.79 (J_{CF} = 12.9 Hz), 127.70 (J_{CF} = 3.7 Hz), 128.99 (2C), 129.98 (2C), 130.74, 132.53, 134.67 (J_{CF} = 8.8 Hz), 138.50, 143.20, 160.50 (J_{CF} = 250.6 Hz), 166.94, 188.91.

4.1.2.9. (E)-4-(3-(4-Fluorophenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4i**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.42, (t, 2H, J = 8.8 Hz, Ar-H); 7.78, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.96-8.01, (m, 4H, Ar-H); 8.08, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.28, (m, 2H, Ar-H); 13.15, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 115.99 (2C, J_{CF} = 21.8 Hz), 124.16, 129.08 (2C), 129.87 (2C), 131.80 (2C, J_{CF} = 9.3 Hz), 132.33, 134.21 (J_{CF} = 2.4 Hz), 138.88, 142.83, 165.33 (J_{CF} = 250.8 Hz), 167.00, 187.80.

4.1.2.10. (E)-4-(3-(4-Bromophenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4j**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.79, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.80, (d, 2H, J_{AB} = 8.4 Hz, Ar-H); 7.96-8.05, (m, 4H, Ar-H); 8.05, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.12, (d, 2H, J_{BA} = 8.4 Hz, Ar-H); 13.17, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 124.01, 127.67, 129.10 (2C), 129.84 (2C), 130.77 (2C), 132.02 (2C), 132.45, 136.47, 138.77, 143.15, 166.97, 188.40.

4.1.2.11. (E)-4-(3-(4-Nitrophenyl)-3-oxoprop-1-en-1-yl)benzoic acid (**4k**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.82, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.00, (s, 4H, Ar-H); 8.06, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.37, (s, 4H, Ar-H); 13.18, (bs, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 123.97 (3C), 129.22 (2C), 129.85 (2C), 130.09 (2C), 132.64, 138.54, 142.17, 144.09, 150.04, 166.93, 188.39.

4.1.2.12. 4-(3-(Anthracen-1-yl)-3-oxoprop-1-en-1-yl)benzoic acid (**4l**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.54-7.68, (m, 3H, Ar-$H_{anthracene}$); 7.74, (d, 1H, J_{AB} = 16.0 Hz, CH=); 7.86, (d, 1H, J_{BA} = 16.0 Hz, CH=); 7.96, (d, 2H, J_{AB} = 8.4 Hz, Ar-H); 8.00, (d, 2H, J_{BA} = 8.4 Hz, Ar-H); 8.10-8.18, (m, 3H, Ar-$H_{anthracene}$); 8.35, (d, 1H, J = 8.6 Hz, Ar-$H_{anthracene}$); 8.71, (s, 1H, Ar-$H_{anthracene}$); 9.13, (s, 1H, Ar-$H_{anthracene}$); 13.17, (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 124.32, 124.76, 126.43 (2C), 127.25, 127.76, 128.03, 128.54, 128.79, 128.98 (2C), 129.30, 129.92 (2C), 131.31, 131.61, 132.22, 132.35, 133.10, 135.62, 138.80, 143.30, 166.99, 193.52.

4.1.2.13. (E)-4-(3-Oxo-3-(thiophen-2-yl)prop-1-en-1-yl)benzoic acid (**4m**): ^{1}H NMR (200 MHz, DMSO-d_6): 7.33, (t, 1H, J = 4.4 Hz, Ar-$H_{thiophene}$); 7.76, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.00, (s, 4H, Ar-H); 8.01, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.09, (d, 1H, J = 4.4 Hz, Ar-$H_{thiophene}$); 8.38, (d, 1H, J = 4.4 Hz, Ar-$H_{thiophene}$); 13.15, (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6): 124.24, 129.06 (3C), 129.88 (2C), 132.31, 134.20, 136.02, 138.77, 141.87, 145.44, 166.99, 181.66.

4.1.2.14. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-oxo-3-phenylprop-1-en-1-yl)benz-amide × H_2O (**5a**): Beige powder; yield: 0.31 g (68%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.87, (d, 1H, J = 8.2 Hz, H-$5_{phenolic}$); 7.24, (dd, 1H, J = 8.2 and 1.6 Hz, H-$6_{phenolic}$); 7.39, (d, 1H, J = 1.6 Hz, H-$2_{phenolic}$); 7.55-7.73, (m, 3H, Ar-H); 7.80, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.09, (d, 2H, J = 7.4 Hz, Ar-H); 8.12, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.18-8.22, (m, 4H, Ar-H); 9.45, (s, 1H, OH); 9.61, (s, 1H, OH); 13.13, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.86, 116.34, 119.21, 121.58, 124.57, 128.74 (2C), 128.93 (2C), 129.00 (4C), 133.05, 133.41, 137.50, 138.94, 142.48, 146.00, 148.33, 158.58, 162.53, 164.76, 189.26; IR (KBr, cm^{-1}): 3435; 2925; 1666; 1655; 1601; 1533; 1309; 1296; 1219, 747; Anal. Calcd. For $C_{24}H_{17}N_3O_4S \times H_2O$ (461.50 g/mol): C, 62.46; H, 4.15; N, 9.10; S, 6.95; Found: C, 62.54; H, 4.14; N, 9.07; S, 6.97.

4.1.2.15. (Z)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-oxo-3-(o-tolyl)prop-1-en-1-yl)benz-amide × 1.5 H_2O (**5b**): Light yellow powder; yield: 0.41 g (85%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 2.41, (s, 3H, CH_3); 6.86, (d, 1H, J = 8.0 Hz, H-$5_{phenolic}$); 7.24, (d, 1H, J = 8.0 Hz, H-$6_{phenolic}$); 7.33-7.38, (m, 2H, Ar-H and 1H, H-$2_{phenolic}$); 7.45, (d, 1H, J_{AB} = 7.0 Hz, CH=); 7.58, (s, 2H, Ar-H); 7.72, (d, 1H, J_{BA} = 7.0 Hz, CH=); 7.98, (d, 2H, J_{AB} = 8.0 Hz, Ar-H); 8.17, (d, 2H, J_{BA} =

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8.0 Hz, Ar-H); 9.45, (s, 1H, OH); 9.61, (s, 1H, OH); 13.11, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 20.18, 113.87, 116.34, 119.21, 121.58, 125.87, 128.47, 128.68, 128.88 (2C), 129.04 (2C), 131.06, 131.43, 133.14, 136.83, 138.52, 138.69, 143.04, 146.00, 148.33, 158.60, 162.54, 164.81, 194.68; IR (KBr, cm^{-1}): 3466; 3173; 1667; 1630; 1606; 1532; 1450; 1301; 1275; 807; Anal. Calcd. For $C_{25}H_{19}N_3O_4S\times1.5H_2O$ (484.53 g/mol): C, 61.97; H, 4.58; N, 8.67; S, 6.62; Found: C, 61.99; H, 4.59; N, 8.65; S, 6.63.

4.1.2.16. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-oxo-3-(m-tolyl)prop-1-en-1-yl)benz-amide (**5c**): Yellow powder; yield: 0.38 g (82%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 2.44, (s, 3H, CH_3); 6.88, (d, 1H, J = 8.0 Hz, H-5$_{phenolic}$); 7.26, (dd, 1H, J = 8.0 and 2.0 Hz, H-6$_{phenolic}$); 7.40, (d, 1H, J = 2.0 Hz, H-2$_{phenolic}$); 7.44-7.53, (m, 2H, Ar-H); 7.81, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.96-8.24, (m, 6H, Ar-H); 8.12, (d, 1H, J_{BA} = 15.6 Hz, CH=), 9.46, (s, 1H, OH); 9.62, (s, 1H, OH); 13.14, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 20.98, 113.84, 116.31, 119.17, 121.54, 124.67, 125.94, 128.79, 128.97 (4C), 129.12, 133.01, 134.03, 137.52, 138.37, 138.97, 142.29, 145.97, 148.30, 158.52, 162.50, 164.74, 189.26; IR (KBr, cm^{-1}): 3426; 3169; 1644; 1653; 1607; 1532; 1309; 1297; 1254; 758; Anal. Calcd. For $C_{25}H_{19}N_3O_4S$ (457.51 g/mol): C, 65.63; H, 4.19; N, 9.18; S, 7.01; Found: C, 65.61; H, 4.18; N, 9.20; S, 7.02.

4.1.2.17. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-oxo-3-(p-tolyl)prop-1-en-1-yl)benz-amide (**5d**): Yellow powder; yield: 0.38 g (84%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 2.71, (s, 3H, CH_3); 6.87, (d, 1H, J = 8.2 Hz, H-5$_{phenolic}$); 7.24, (dd, 1H, J = 8.2 and 1.8 Hz, H-6$_{phenolic}$); 7.39, (d, 1H, J = 1.8 Hz, H-2$_{phenolic}$); 7.39, (d, 2H, J_{AB} = 7.8 Hz, Ar-H); 7.78, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.06-8.13, (m, 4H, Ar-H); 8.11, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.20, (d, 2H, J_{BA} = 7.8 Hz, Ar-H); 9.46, (s, 1H, OH); 9.61, (s, 1H, OH); 13.13, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 21.31, 113.88, 116.34, 119.20, 121.59, 124.61, 128.96 (6C), 129.49 (2C), 132.97, 135.01, 139.02, 142.09, 143.92, 146.00, 148.33, 158.59, 162.53, 164.78, 188.67; IR (KBr, cm^{-1}): 3434; 3192; 2920; 1668; 1654; 1609; 1533; 1308; 1296; 1210; 759; Anal. Calcd. For $C_{25}H_{19}N_3O_4S$ (457.51 g/mol): C, 65.63; H, 4.19; N, 9.18; S, 7.01; Found: C, 65.60; H, 4.20; N, 9.17; S, 7.00.

4.1.2.18. (Z)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(2-methoxyphenyl)-3-oxoprop-1-en-1-yl)benzamide × 2 H_2O (**5e**): Yellow-green powder; yield: 0.36 g (70%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 3.89, (s, 3H, OCH_3); 6.86, (d, 1H, J = 8.2 Hz, H-5$_{phenolic}$); 7.08, (t, 1H, J = 7.4 Hz, Ar-H); 7.22, (d, 1H, J_{AB} = 8.2 Hz, CH=); 7.23, (d, 1H, J = 8.2 Hz, H-6$_{phenolic}$); 7.38, (s, 1H, H-2$_{phenolic}$); 7.54, (d, 1H, J_{BA} = 8.2 Hz, CH=); 7.58, (s, 3H, Ar-H); 7.93, (d, 2H, J_{AB} = 8.2 Hz, Ar-H); 8.17, (d, 2H, J_{BA} = 8.2 Hz, Ar-H); 9.45, (s, 1H, OH); 9.61, (s, 1H, OH); 13.11, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 56.03, 112.56, 113.87, 116.34, 119.22, 120.72, 121.60, 128.62 (2C), 128.73, 129.10 (2C), 129.28, 129.76, 132.99, 133.41, 138.95, 140.84, 146.00, 148.33, 158.06, 158.59, 162.51, 164.81, 192.00; IR (KBr, cm^{-1}): 3434; 3236; 2934; 1661; 1607; 1536; 1448; 1315; 1295; 1245; 747; Anal. Calcd. For $C_{25}H_{19}N_3O_5S\times2H_2O$ (509.54 g/mol): C, 58.93; H, 4.55; N, 8.25; S, 6.29; Found: C, 58.95; H, 4.57; N, 8.26; S, 6.27.

4.1.2.19. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(3-methoxyphenyl)-3-oxoprop-1-en-1-yl)benzamide × H_2O (**5f**): Dark yellow powder; yield: 0.38 g (78%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 3.86, (s, 3H, OCH_3); 6.87, (d, 1H, J = 8.0 Hz, H-5$_{phenolic}$); 7.23-7.27, (m, 1H, Ar-H and 1H, H-6$_{phenolic}$); 7.39, (s, 1H, H-2$_{phenolic}$); 7.51, (t, 1H, J = 8.0 Hz, Ar-H); 7.65, (s, 1H, Ar-H); 7.80, (d, 1H, J_{AB} = 15.6 Hz, CH=); 7.82, (d, 1H, J = 8.0 Hz, Ar-H); 8.09, (d, 2H, J_{AB} = 8.6 Hz, Ar-H); 8.10, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.20, (d, 2H, J_{BA} = 8.6 Hz, Ar-H); 9.45, (s, 1H, OH); 9.61, (s, 1H, OH); 13.13, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 55.54, 113.32, 113.88, 116.35, 119.23, 119.46, 121.30, 121.61, 124.59, 129.04 (4C), 130.06, 133.06, 138.94 (2C), 142.56, 146.01, 148.34, 158.63, 159.74, 162.55, 164.78, 189.00; IR (KBr, cm^{-1}): 3434; 2937; 1663; 1607; 1538; 1448; 1317; 1293; 1259; 759; Anal. Calcd. For $C_{25}H_{19}N_3O_5S\times H_2O$ (491.52 g/mol): C, 61.09; H, 4.31; N, 8.55; S, 6.52; Found: C, 61.11; H, 4.29; N, 8.57; S, 6.53.

4.1.2.20. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(4-methoxyphenyl)-3-oxoprop-1-en-1-yl)benzamide × 0.5 H_2O (**5g**): Light yellow powder; yield: 0.34 g (70%); mp: > 250ºC; ^{1}H

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NMR (200 MHz, DMSO-d_6): 3.88, (s, 3H, OCH_3); 6.87, (d, 1H, J = 8.2 Hz, H-$5_{phenolic}$); 7.10, (d, 2H, J_{AB} = 9.0 Hz Ar-H); 7.24, (dd, 1H, J = 8.2 and 2.0 Hz, H-$6_{phenolic}$); 7.39, (d, 1H, J = 2.0 Hz, H-$2_{phenolic}$); 7.76, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.08, (d, 2H, J_{AB} = 8.4 Hz, Ar-H); 8.13, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.20, (d, 2H, J_{BA} = 8.4 Hz, Ar-H); 8.21, (d, 2H, J_{BA} = 9.0 Hz, Ar-H); 9.46, (s, 1H, OH); 9.61, (s, 1H, OH); 13.12, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 55.71, 113.84, 114.20 (2C), 116.32, 119.18, 121.56, 124.62, 128.46, 128.89 (2C), 128.98 (2C), 130.42, 131.17 (2C), 139.14, 141.61, 145.98, 148.31, 158.48, 162.52, 163.53, 164.78, 187.40; IR (KBr, cm^{-1}): 3406; 3173; 2940; 1670; 1654; 1605; 1593; 1533; 1307; 1263; 1172; 765; Anal. Calcd. For $C_{25}H_{19}N_3O_5S\times0.5H_2O$ (482.52 g/mol): C, 62.23; H, 4.18; N, 8.71; S, 6.64; Found: C, 62.25; H, 4.19; N, 8.72; S, 6.65.

4.1.2.21. (Z)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(2-fluorophenyl)-3-oxoprop-1-en-1-yl)benzamide × H_2O (**5h**): Yellow powder; yield: 0.26 g (54%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.86, (d, 1H, J = 8.2 Hz, H-$5_{phenolic}$); 7.24, (dd, 1H, J = 8.2 and 1.6 Hz, H-$6_{phenolic}$); 7.37, (d, 1H, J_{AB} = 7.6 Hz, CH=); 7.39-7.44, (m, 1H, Ar-H and 1H, H-$2_{phenolic}$); 7.58-7.80 (m, 2H, Ar-H and 1H, CH=), 7.83 (t, 1H, J = 7.6 Hz, Ar-H); 7.99, (d, 2H, J_{AB} = 8.2 Hz, Ar-H); 8.18, (d, 1H, J_{BA} = 8.2 Hz, Ar-H); 9.46, (s, 1H, OH); 9.61, (s, 1H, OH); 13.14, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.86, 116.33, 116.78 (J_{CF} = 22.2 Hz), 119.21, 121.57, 124.97 (J_{CF} = 3.3 Hz), 126.76 (J_{CF} = 12.8 Hz), 127.81 (J_{CF} = 3.7 Hz), 128.92 (2C), 129.10 (2C), 130.70 (J_{CF} = 2.1 Hz), 133.34, 134.59 (J_{CF} = 8.8 Hz), 138.51, 142.97, 146.00, 148.33, 158.64, 160.46 (J_{CF} = 250.8 Hz), 162.51, 164.79, 188.84; IR (KBr, cm^{-1}): 3405; 2928; 1671; 1662; 1611; 1539; 1451; 1317; 1293; 1268; 755; Anal. Calcd. For $C_{24}H_{16}N_3O_4SF\times H_2O$ (479.49 g/mol): C, 60.11; H, 3.96; N, 8.76; S, 6.69; Found: C, 60.13; H, 3.95; N, 8.78; S, 6.70.

4.1.2.22. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(4-fluorophenyl)-3-oxoprop-1-en-1-yl)benzamide × 2 H_2O (**5i**): Yellow powder; yield: 0.31 g (63%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.87, (d, 1H, J = 8.2 Hz, H-$5_{phenolic}$); 7.24, (dd, 1H, J = 8.2 and 1.6 Hz, H-$6_{phenolic}$); 7.38, (d, 1H, J = 1.6 Hz, H-$2_{phenolic}$); 7.42, (t, 2H, J = 8.8 Hz, Ar-H); 7.81, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.09, (d, 2H, J_{AB} = 8.4 Hz, Ar-H); 8.14, (d, 1H, J_{BA} =15.6 Hz, CH=); 8.20, (d, 2H, J_{BA} = 8.4 Hz, Ar-H); 8.31, (m, 2H, Ar-H); 9.45, (s, 1H, OH); 9.61, (s, 1H, OH); 13.13, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.84, 115.93 (2C, J_{CF} = 21.6 Hz), 116.31, 119.18, 121.55, 124.32, 128.99 (2C), 129.02 (2C), 131.76 (2C, J_{CF} = 9.4 Hz), 133.06, 134.17 (J_{CF} = 2.6 Hz), 138.88, 142.61, 145.98, 148.31, 158.50 (J_{CF} = 6.7 Hz), 162.51, 164.73, 165.28 (J_{CF} = 250.8 Hz), 187.75; IR (KBr, cm^{-1}): 3225; 3071; 2951; 1668; 1654; 1603; 1538; 1508; 1308; 1220; 831; Anal. Calcd. For $C_{24}H_{16}N_3O_4SF\times2H_2O$ (497.50 g/mol): C, 57.94; H, 4.05; N, 8.45; S, 6.44; Found: C, 57.92; H, 4.06; N, 8.43; S, 6.45.

4.1.2.23. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(4-bromophenyl)-3-oxoprop-1-en-1-yl)benzamide × 2 H_2O (**5j**): Yellow-green powder; yield: 0.49 g (87%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.87, (d, 1H, J = 7.6 Hz, H-$5_{phenolic}$); 7.24, (d, 1H, J = 7.6 Hz, H-$6_{phenolic}$); 7.39, (s, 1H, H-$2_{phenolic}$); 7.79, (d, 2H, J = 8.4 Hz, Ar-H); 7.80, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.05-8.21, (m, 6H, Ar-H and 1H, CH=); 9.46, (s, 1H, OH); 9.61, (s, 1H, OH); 13.13, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.85, 116.32, 119.19, 121.55, 124.20, 127.63, 129.00 (2C), 129.08 (2C), 130.75 (2C), 131.99 (2C), 133.14, 136.46, 138.83, 142.95, 145.99, 148.32, 158.59, 162.53, 164.69, 188.37; IR (KBr, cm^{-1}): 3406; 3033; 2923; 1662; 1655; 1600; 1584; 1534; 1305; 1214; 759; Anal. Calcd. For $C_{24}H_{16}N_3O_4SBr\times2H_2O$ (558.41 g/mol): C, 51.62; H, 3.61; N, 7.52; S, 5.74; Found: C, 51.64; H, 3.60; N, 7.54; S, 5.75.

4.1.2.24. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-(4-nitrophenyl)-3-oxoprop-1-en-1-yl)benzamide x 4 H_2O (**5k**): Brown powder; yield: 0.30 g (53%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.86, (d, 1H, J = 6.4 Hz, H-$5_{phenolic}$); 7.24, (d, 1H, J = 6.4 Hz, H-$6_{phenolic}$); 7.38, (s, 1H, H-$2_{phenolic}$); 7.86, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.09-8.19, (m, 4H, Ar-H and 1H, CH=); 8.40, (s, 4H, Ar-H); 9.45, (s, 1H, OH); 9.61, (s, 1H, OH); 13.16, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.84, 116.31, 119.18, 121.54, 123.95 (2C), 124.22, 129.02 (2C), 129.23 (2C), 130.09 (2C), 133.36, 138.61, 142.18, 143.91, 145.98, 148.33, 150.07, 158.59, 162.51, 164.71, 188.42; IR (KBr, cm^{-1}): 3404; 3165;

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2930; 1664; 1655; 1607; 1590; 1523; 1306; 1298; 1212; 747; Anal. Calcd. For $C_{24}H_{16}N_4O_6S\times4H_2O$ (560.54 g/mol): C, 51.43; H, 4.32; N, 10.00; S, 5.72; Found: C, 51.45; H, 4.31; N, 9.98; S, 5.73.

4.1.2.25. (E)-4-(3-(Anthracen-1-yl)-3-oxoprop-1-en-1-yl)-N-(5-(3,4-dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)benzamide × H_2O (**5l**): Dark orange powder; yield: 0.43 g (76%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.87, (d, 1H, J = 8.2 Hz, H-5$_{phenolic}$); 7.25, (d, 1H, J = 8.2 Hz, H-6$_{phenolic}$); 7.39, (s, 1H, H-2$_{phenolic}$); 7.55-7.70, (m, 3H, Ar-H$_{anthracene}$), 7.78, (d, 1H, J_{AB} = 15.8 Hz, CH=); 7.92, (d, 1H, J_{BA} = 15.8 Hz, CH=); 7.98-8.22, (m, 4H, Ar-H and 3H, Ar-H$_{anthracene}$); 8.37, (d, 1H, J = 8.4 Hz, Ar-H$_{anthracene}$); 8.73, (s, 1H, Ar-H$_{anthracene}$); 9.16, (s, 1H, Ar-H$_{anthracene}$); 9.46, (s, 1H, OH); 9.62, (s, 1H, OH); 13.14, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.90, 116.36, 119.24, 121.62, 124.27, 124.72, 126.38 (2C), 127.21, 127.72, 127.99, 128.65, 128.74 (2C), 128.93 (2C), 129.05, 131.28, 131.58, 132.19, 133.10, 135.57, 138.82, 143.06, 146.02, 148.35, 158.63, 162.54, 164.80, 193.43; IR (KBr, cm^{-1}): 3405; 3047; 2930; 1655; 1607; 1538; 1510; 1302; 1290; 1255; 1201; 749; Anal. Calcd. For $C_{32}H_{21}N_3O_4S\times H_2O$ (561.62 g/mol): C, 68.43; H, 4.13; N, 7.48; S, 5.71; Found: C, 68.40; H, 4.14; N, 7.50; S, 5.73.

4.1.2.26. (E)-N-(5-(3,4-Dihydroxyphenyl)-1,3,4-thiadiazol-2-yl)-4-(3-oxo-3-(thiophen-2-yl)prop-1-en-1-yl)benzamide (**5m**): Yellow powder; yield: 0.28 g (62%); mp: > 250ºC; ^{1}H NMR (200 MHz, DMSO-d_6): 6.87, (d, 1H, J = 8.2 Hz, H-5$_{phenolic}$); 7.24, (dd, 1H, J = 8.2 and 1.8 Hz, H-6$_{phenolic}$); 7.34, (t, 1H, J = 4.4 Hz, Ar-H$_{thiophene}$); 7.39, (d, 1H, J = 1.8 Hz, H-2$_{phenolic}$); 7.79, (d, 1H, J_{AB} = 15.6 Hz, CH=); 8.06, (d, 1H, J_{BA} = 15.6 Hz, CH=); 8.08, (d, 2H, J_{AB} = 8.4 Hz, Ar-H); 8.10, (d, 1H, J = 4.4 Hz, Ar-H$_{thiophene}$); 8.20, (d, 2H, J_{BA} = 8.4 Hz, Ar-H); 8.41, (d, 1H, J = 4.4 Hz, Ar-H$_{thiophene}$); 9.47, (s, 1H, OH); 9.62, (s, 1H, OH); 13.13, (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6): 113.85, 116.33, 119.20, 121.55, 124.44, 129.01 (5C), 133.07, 134.18, 135.99, 138.80, 141.66, 145.39, 145.99, 148.32, 158.55, 162.53, 164.81, 181.63; IR (KBr, cm^{-1}): 3406; 2927; 1671; 1654; 1595; 1540; 1413; 1317; 1292; 1189; 760; Anal. Calcd. For $C_{22}H_{15}N_3O_4S_2$ (449.51 g/mol): C, 58.78; H, 3.36; N, 9.35; S, 14.26; Found: C, 58.80; H, 3.34; N, 9.36; S, 14.21.

4.2. Biology

4.2.1. Cytotoxic activity

The cytotoxic activity of the newly synthesized 1,3,4-thiadiazole-chalcone hybrids containing antioxidant phenolic moiety was evaluated on three human malignant cell lines: cervical adenocarcinoma HeLa, acute promyelocytic leukemia HL-60, lung carcinoma A549, and human normal lung fibroblasts MRC-5, as described in our previous studies.[20] All tested cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa (2,000 cells per well), A549 (5,000 cells per well) and MRC-5 cells (5,000 cells per well) were seeded into 96-well microtiter plates and after 20 h the five different concentrations of the compounds (ranging from 6.25 μM to 100 μM or 3.125 μM to 50 μM) were added to the cells. The control cell samples were incubated in nutrient medium only. HL-60 cells (7,000 cells per well), were seeded 2 h before the addition of solutions of the compounds. Stock solutions of the compounds were made in DMSO at a concentration of 5 mM. The cell survival was measured by MTT assay after treatment that lasted 72h, according to the standard procedure firstly described by Mosmann,[52] and which was modified by Ohno and Abe,[53] as described previously in more detail.[20] The concentrations of compounds dissolved in DMSO and further diluted in nutrient medium were carefully chosen in order to eliminate any toxic side effect of DMSO on all tested human cell lines. The maximum final concentrations of DMSO did not exceed 1%.
The chemotherapy drug cisplatin was used as a positive control. Each of the three independent experiment was performed in triplicate.

4.2.2. DPPH free radical scavenging assay

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The determination of the DPPH free radical scavenging activity of the studied compounds was conducted according to method described by Kontogiorgis et al.[54] Briefly, 1 mL (0.05 mM) of DPPH solution in methanol was mixed with an equivalent volume of the tested compound (20 μL of compound solution in DMSO and 980 μL of methanol). The sample was stored in dark at room temperature. The absorbance was acquired at 517 nm after incubation period of 30 min. Methanol was taken as control. IC_{50} values represent the concentration necessary to obtain 50% of a maximum scavenging activity. Ascorbic acid was used as positive control. The results are presented as mean ± SD calculated from the independent triplicate experiments using Microsoft Excel software.

4.2.3. Determination of intracellular ROS levels

Human HeLa cells were incubated with subtoxic IC_{20} concentrations of the selected compounds **5a**, **5c**, **5f** and **5m** (applied concentration was 10 μM for each compound) for 24 h. Afterwards, the HeLa cells were collected, washed with phosphate buffered saline (PBS) and incubated in a solution of 30 μM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma Aldrich) in PBS for 45 min at 37°C, according to standard experimental procedure which we described previously.[55] The cells were then washed with PBS. The part of treated cell samples was analysed immediately, while the other part of treated cells samples was exposed to 10 mM hydrogen peroxide solution (H_2O_2) for 30 min at 37°C. After 30 min, these cells were washed with PBS and collected. The intensity of green fluorescence emitted by the dichlorofluorescein was determined on a FACSCalibur flow cytometer (BD Biosciences Franklin Lakes, NJ, USA). The data (20,000 events acquired for each cell sample) were analysed using CELLQuest software (BD Biosciences).

4.2.4. Cell cycle analysis by flow cytometry

Human cervical carcinoma HeLa cells were incubated with compounds **5a**, **5c**, **5f** and **5m** for 24 h (applied concentrations corresponded to IC_{50} and $2IC_{50}$ concentrations which were determined after 72 h treatment). Following 24 h incubation, the cells were collected, washed with PBS and fixed in 70% ethanol, according to standard protocol.[56] Cell samples were stored at -20°C for at least one week before staining. Afterwards, HeLa cells were collected by centrifugation, washed, resuspended in PBS containing RNase A and incubated for 30 min at 37°C. Subsequently the propidium iodide staining solution was added to the cells. Percentages of HeLa cells within specific phases of the cell cycle were assessed using a BD FACSCalibur flow cytometer. The analyses of acquired data (10,000 events collected for each gated cell sample) were performed using a CELLQuest software. Cell cycle distribution data are presented as mean±S.D. of three independent experiments. The statistical significance of differences between the control and cell samples exposed to tested compounds was evaluated using one-way ANOVA with Dunnett's post test. *p* values below 0.05 were considered statistically significant.

4.2.5. Identification of target caspases

To investigate whether the examined compounds could induce apoptosis in treated HeLa cells the percentages of subG1 cells in the samples pretreated with specific caspase inhibitors were detyermined, as previously described in our previous research.[20] The HeLa cells were pretreated for 2 h with 40 μM concentrations of the specific peptide caspase inhibitors: Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, a caspase-8 inhibitor and Z-LEHD-FMK, a caspase-9 inhibitor (R&D Systems, Minneapolis, USA).

4.2.6. Gene and microRNA expression analyses

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HeLa cells were seeded into 75 cm^2 cell culture flasks (4×10^6 cells/flask). After 2 h, the cells were treated with low subtoxic IC_{20} concentrations of the compounds **5a**, **5c**, **5f** and **5m** for 24 h (10 μM for each compound). Control cells were grown in nutrient medium only. After 24h incubation, the cells were collected, washed and the cell samples were stored at -80°C until further analyses. Total RNA was extracted with TriReagent (Sigma), according to manufacturer's protocol. Gene expression was quantified by two-step reverse transcription reaction followed by real-time quantitative PCR (RT-qPCR) with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania). Reaction of cDNA amplification was performed with *MMP2* (Hs01548727_m1), *MMP9* (Hs00957562_m1), *VEGFA* (Hs00900055_m1), and *TIMP3* (Hs00165949_m1) assays, using TaqMan technology.
Stem-loop reverse transcription and RT-qPCR were used for miR-21 (ID 000397), miR-133b (ID 002247), miR-155 (ID 002623), and miR-206 (ID 000510) expression analysis.
Gene and microRNA expression values were normalized to *GAPDH* (Hs02758991_g1), and small nuclear RNA-RNU6 B (ID001093) and calculated by comparative ΔΔCt method, with 7500 System SDS software (Applied Biosystems, Foster City, California, USA).

4.2.7. Endothelial cell tube formation assay

To explore whether antiangiogenic effects of the compounds were explored on human umbilical vein endothelial EA.hy926 cells.[57,58] The EA.hy926 cells seeded on Corning® Matrigel® basement membrane matrix (Corning: cat. number 356234) were exposed to suboxic IC_{20} concentrations of the four tested compounds (the compounds **5a**, **5c**, **5f** (6.5 μM for each compound) and **5m** (8 μM) for 20 h. After treatment that lasted 20 h, the photomicrographs of EA.hy926 cells were captured under the inverted phase-contrast microscope.

4.2.8. Interaction with DNA

4.2.8.1. Absorption spectral measurements

Calf thymus DNA (lyophilized, highly polymerized, obtained from Serva, Heidelberg) (CT-DNA) was used for the experiments. The stock solution was prepared by dissolving the DNA in Tris buffer (10 mM Tris-HCl pH 7.9) overnight at 4 °C. This stock solution was stored at 4 °C and was stable for several days. A solution of CT-DNA in water gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} of 1.89–2.01, indicating that DNA was sufficiently free of protein. The concentration of DNA (3.16 mg mL^{-1}) was determined from the UV absorbance at 260 nm using the extinction coefficient ε_{260}=6600 M^{-1} cm^{-1}.[59] 1,3,4–thiadiazole-chalcone hybrids **5a**, **5c**, **5f** and **5m** were dissolved in dimethyl sulfoxide in concentration of 2 mM. These solutions were used as stock solutions.
For an UV-Vis measurement, a small volume of a stock solution of the compound (20 μL) was added to DNA solution (10 μL of CT-DNA) and the volume was adjusted up to 1 mL with 40 mM bicarbonate buffer, pH 8.4. Reaction mixtures were incubated at 37 °C for 90 min with occasional vortexing. UV–Vis spectra were recorded on a UV-1800 Shimadzu UV/Visible spectrophotometer operating from 200 to 800 nm in 1.0 cm quartz cells. Spectra of the compounds of the same concentrations were also recorded, as well as spectra of CT-DNA.
The percentage of hyperchromism or hypocromism was determined from

$$\{[(\varepsilon_{DNA} + \varepsilon_{COMP}) - \varepsilon_B]/ (\varepsilon_{DNA} + \varepsilon_{COMP})\} \times 100 \quad (1)$$

where ε_{DNA} is the extinction coefficient of CT-DNA, ε_{COMP} is the extinction coefficient of free compound and ε_B is the extinction coefficient of the bound compound complex.

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The absorbance titrations were performed at a fixed concentration of the compound and gradually increasing concentration of double stranded CT-DNA. The absorbance at 259 nm was monitored for each concentration of DNA. The binding constant K_b was determined using the equation (2):[60]

$$[DNA] \times (\varepsilon_a - \varepsilon_f)^{-1} = [DNA] \times (\varepsilon_b - \varepsilon_f)^{-1} + K_b^{-1} \times (\varepsilon_b - \varepsilon_f)^{-1}, \qquad (2)$$

where ε_a, ε_f, ε_b are absorbance/[compound], extinction coefficient of the free compound and the extinction coefficient of the bound compound, respectively. A plot of [DNA]/($\varepsilon_a - \varepsilon_f$) *versus* [DNA] gave a slope and an intercept equal to $1/(\varepsilon_a - \varepsilon_f)$ and $(1/K_b)(1/(\varepsilon_b - \varepsilon_f))$, respectively. The binding constant K_b is calculated from the ratio of the slope to the intercept.

4.2.8.2. Fluorescence measurements

The competitive interactions of 1,3,4-thiadiazole-chalcone hybrids **5a**, **5c**, **5f** and **5m** and the fluorescence probe Hoechst 33258 (H), with CT-DNA have been studied by measuring the change of fluorescence intensity of H−DNA solution after addition of the compound. Reaction mixtures containing 100 μM of CT-DNA (calculated per phosphate) in 1 mL of 40 mM bicarbonate solution (pH 8.4) were pretreated with 1.5 μL of 1% H probe solution (28 μM final concentration) (in separate experiments) for 20 min and the mixture was analyzed by fluorescence measurement. Then the increasing concentrations of the compounds were successively added and the changes in the fluorescence intensity were measured using a Thermo Scientific Lumina Fluorescence spectrometer (Finland) equipped with a 150 W Xenon lamp. The slits on the excitation and emission beams were fixed at 10 nm. All measurements were performed by excitation at 350 nm in the range of 390−550 nm. The control was H−CT-DNA solution. Compounds **5a**, **5c**, **5f** and **5m**, did not have fluorescence under applied conditions. The obtained fluorescence quenching data were analyzed according to the Stern–Volmer equation (3):[61]

$$I_0/I = 1 + K\boldsymbol{r} \qquad (3)$$

where I_0 and I represent the fluorescence intensities of H−CT-DNA in absence and presence of the compounds, respectively, K is quenching constant and $\boldsymbol{r}$ is ratio of the bound concentration of a probe to the bound concentration of DNA ($\boldsymbol{r}$ = [**compound**]/[CT-DNA]. The K value is calculated from the ratio of the slope to the intercept from the plot of I_0/I *versus* $\boldsymbol{r}$.

Primary spectra of all spectrometric measurements were imported into OriginPro 9.0 and were processed by this software package.

4.2.8.3. Experiments with plasmid DNA

Plasmid pUC19 (2686 bp in length, purchased from Sigma-Aldrich, USA) was prepared by its transformation in chemically competent cells *Escherichia coli* (*E. coli*) strain XL1 blue. Amplification of the clone was done according to the protocol for growing *E. coli* culture overnight in LB medium at 37 °C[62] and purification was performed using Qiagen Plasmid plus Maxi kit. Finally, DNA was eluted in 10 mM Tris-HCl buffer and stored at −20 °C. The concentration of plasmid DNA (0.460 μg/μL) was determined by measuring the absorbance of the DNA-containing solution at 260 nm. One optical unit corresponds to 50 μg mL^{-1} of double stranded DNA.

Plasmid DNA (1 μL, 460 ng/ μL) was incubated with increasing volumes of 2 mM stock solution in DMSO of the compound (0.5, 1, 1.5, 2, 2.5, 3 and 4 μL) in a 20 μL reaction mixture in 40 mM bicarbonate buffer (pH 8.4) at 37 °C, for 90 minutes. DMSO had no efects on DNA conformation

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under aplied concentration. The reaction mixtures were vortexed from time to time. The reaction was terminated by short centrifugation at 10000 rpm and addition of 5 μL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in TAE buffer, pH 8.24 (40 mM Tris-acetate, 1 mM EDTA)), and analyzed by an agarose electrophoresis.

Interactions of the tested compounds with pUC19 in presence of Fe(II) and Fe(III) were done as follows. Solutions of 20 μL volume consisted of 1 μL or 2 μL $FeSO_4$ (8 mM, freshly prepared in a sterile water) or 1 μL or 2 μL $FeCl_3$ (8 mM, freshly prepared in a sterile water) with 4 μL of 2 mM solution of 1,3,4-thiadiazole-chalcone hybrids **5a**, **5c**, **5f** and **5m** in bicarbonate buffer were incubated at 37°C, for 60 minutes, then 1 μL of pUC19 were added to reaction mixtures and incubated further for 90 minutes. The reaction mixtures were vortexed from time to time and terminated by short centrifugation at 10000 rpm and addition of 5 μL of loading buffer and analyzed by agarose electrophoresis.

4.2.8.4. Agarose electrophoresis

The samples were subjected to electrophoresis on 1% agarose gel (Amersham Pharmacia-Biotech, Inc) prepared in TAE buffer pH 8.24. The electrophoresis was performed at a constant voltage (80 V) until bromophenol blue had passed through 75% of the gel. A Submarine Mini-gel Electrophoresis Unit (Hoeffer HE 33) with an EPS 300 power supply was used. After electrophoresis, the gel was stained for 30 min by soaking it in an aqueous ethidium bromide solution (0.5 μg mL^{-1}). The stained gel was illuminated under a UV transilluminator Vilber-Lourmat (France) at 312 nm and photographed with a Nikon Coolpix P340 Digital Camera through filter DEEP YELLOW 15 (TIFFEN, USA).

4.2.8.5. Comet assay

Human normal MRC-5 cells were treated with four concentrations of selected compounds **5a**, **5c**, **5f** and **5m** (applied concentrations were 25 μM, 12.5 μM, 6.25 μM and 3.125 μM for each compound) for 24 h. The IC_{20} subtoxic concentration was 25 μM for all four compounds, as determined by MTT test after 24 h treatment. After incubation, the cells were collected, washed with phosphate buffered saline (PBS), suspended in freezing medium (RPMI with 10% DMSO and 20 % FCS) and the cell samples were frozen at -80°C.

The medium-throughput version of the single-cell gel electrophoresis assay was used to evaluate DNA damage as previously described[63] with some modifications. Frozen MRC-5 cells were thawed by addition of 1 ml of PBS to a frozen 0.5 mL aliquot and as soon as the sample was thawed the suspended cells were centrifuged for 10 min at 2000 rpm at 4°C. The pellet was suspended in PBS and the washing step was repeated. The number of cells was adjusted to $2.5x10^5$ /ml with PBS and 30μL of cell suspension were mixed with 140 μL of 1% LMP agarose at 37°C. Twelve drops (10 μL) of agarose-cell suspension were placed on an NMP agarose coated slide using template provided on the metal base of the 12 gel chamber. Cells were lysed by placing the slides in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris with 1% Triton X-100 pH 10 for 1 h at 4°C. The slides were placed in horizontal gel electrophoresis tank in electrophoresis solution (0.3 M NaOH, 0.001 M Na_2EDTA) for 20 min. Electrophoresis was carried out for 30 min at voltage gradient of 1V/cm across the platform at 4° C. Neutralization of the slides was performed by washing them for 10 min in PBS in a staining jar at 4°C. Slides were than fixed by placing them in 70% ethanol for 10 min followed by 10 min incubation in absolute ethanol. DNA was stained by immersing the slides in a staining jar with SYBR Gold (Invitrogen) for 30 min in the dark. Staining solution was prepared according to the manufacturer's instructions in 10 mM Tris, 1 mM EDTA buffer, pH 8. Stained slides were rinsed

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twice with water and left to dry in the dark. Scoring of the comets was carried out using semi-automated image analysis system (Comet Assay IV; Perceptive Instruments). On each gel 50 nucleoids were analyzed and the results were expressed as percentage of tail intensity (% of DNA in tail). All experiments were performed in triplicate. PBS was used as negative control. The median % tail DNA for 50 comets was calculated for each of the duplicate gel in the experiments; the mean of the two median values was then calculated. The mean percentage of DNA in the tail is calculated from the independent triplicate experiments using Microsoft Excel software.

Conflicts of interest

There are no conflicts to declare.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/

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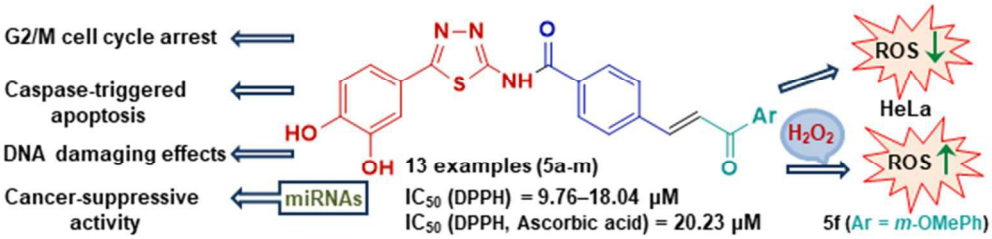

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