Synthesis, antioxidant and antiproliferative activities of 1,3,4-thiadiazoles derived from phenolic acids

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ABSTRACT

Two 2-amino-1,3,4-thiadiazoles containing phenolic hydroxyl groups were combined with different carboxylic acid chlorides giving sixteen amide derivatives with good antioxidant and antiproliferative potential. The compound 30c with an adamantane ring displayed excellent DPPH radical scavenging activity and good cytotoxic activity against human acute promyelocytic leukemia HL-60 cells, while 1,3,4-thiadiazole 30h with 4-chlorophenyl moiety was found to be the most effective in inhibition of survival of lung carcinoma A549 cells. All examined thiadiazoles except 3a and 30a exerted higher cytotoxic activities on A549 and HL-60 cancer cells when compared with normal fibroblasts MRC-5, pointing to selectivity in their antiproliferative action. Some of the most active novel compounds 3c, 30c, 30g and 30h induced significant increase in the percentage of HL-60 cells in the subG1 cell cycle phase in comparison with the control cells. The induction of cell death in HL-60 cells by these compounds was at least partially dependent on activation of caspase-3 and caspase-8. The compounds 3c and 30c exerted strong antiangiogenic activity. Furthermore, compounds 3c, 30c, 30g and 30h showed the ability to down-regulate the MMP2 and VEGFA expression levels in the treated HL-60 cells when compared with the control cell samples.

Antioxidants are compounds that, in low concentration, are able to delay or prevent the oxidation of biomolecules (proteins, nucleic acids, lipids, sugars) and inhibit oxidative stress, DNA mutations, malignant changes and other forms of cell damage.[1] Phenolic compounds are a large group of substances that have recently received much attention due to their antioxidant properties.

Numerous investigations relating to their radical-scavenging activity include structure-activity-relationship studies, reaction kinetics of polyphenols with radicals, substituent influence, number and arrangement of phenolic hydroxyl groups in the molecule and solvent effects.[2–5] Among phenolic compounds, the antioxidant activity of phenolic acids has attracted more attention because of their ubiquitous occurrence in nature and as potential models for the synthesis of new primary radical scavengers.[6] One of the most important phenolic acids, protocatechuic acid (3,4-dihydroxybenzoic acid), found in edible plants, vegetables and fruits, is known to exhibit potent antioxidant activity demonstrating the preventive effect on malignant diseases that are associated with radical species.[4,7–9]

Also, 2,3-dihydroxybenzoic acid, as a potent iron chelator, exerts an important protective effect against the cytotoxic action of H2O2 significantly increasing cell survival.[10] Moreover, the stable antioxidant molecules neutralize reactive oxygen species (ROS) by an electron transfer mechanism and diminish their DNA damaging ability and cancer formation. Thus, compounds exhibiting both antioxidant and antiproliferative potential are of great importance in discovery of new anticancer agents.

One of the well-known pharmacophores is 1,3,4-thiadiazole heterocyclic scaffold incorporated in many heterocyclic compounds with various grades of antiproliferative activity.[11–13] A series of 5-(2,5-dimethoxyphenyl)-1,3,4-thiadiazole-2-amino derivatives has been synthesized and screened for cytotoxic activity against HT-29 and MDA-MB-231 cancer cells.[14] New 2-arylamino- 5-aryl-1,3,4-thiadiazoles displayed potent anticancer potential against several cell lines with IC50 values from 4.3 to 9.2 μM.[15] Revelant et al. prepared novel 5-aryl-2-(3-thienylamino)- 1,3,4-thiadiazoles and tested them against a panel of six cancer cell lines with IC_{50} values from <10 μM in some experiments.[16] Finally, X.-H. Yang. et al. presented a series of 1,3,4-thiadiazole-2-amide derivatives as potential anticancer agents with good potential in inhibition of MCF-7 and B16-F10

cell proliferation.[17] These points prompted us to combine the bioactive functions of 1,3,4-thiadiazole with those of a phenol acid moiety with the intention of synthesizing novel conjugates possessing antioxidant and antiproliferative properties.

The title molecules were synthesized in two steps as shown in Scheme **1**. Heterocyclic 1,3,4-thiadiazole precursors 2 and 20 were obtained by reacting 3,4-dihydroxybenzoic 1 and 2,3-dihydroxybenzoic acid 1′ with thiosemicarbazide in the presence of phosphoryl chloride.[17] In the next step, a coupling reaction between 5-substituted-1,3,4-thiadiazol-2-amines 2 and 2′ and different carboxylic acid chlorides was performed in tetrahydrofuran or dioxane to give the final amide derivatives 3a–h and 3′a–h. The solid sodium hydrogencarbonate was used for neutralization of liberated hydrogen chloride. In some cases, the reaction of 2 and 2′ with RCOCl requires a long period of time (even at reflux conditions) and an excess of carboxylic acid chlorides was necessary for its completion. Except 3f and 3′f, all other compounds were prepared for the first time with satisfactory analytical and spectroscopic data (Supplementary Material).

To confirm whether the protocatechuic acid shows better scavenging activity than its derivatives, a series of esters[18] and amides[19] were synthesized and screened for their antioxidant potential. The obtained results suggest that the slow DPPH scavenging activity of the protocatechuic acid compared with its derivatives is due to the dissociation of the carboxyl group since it decreases the electron-withdrawing property of the substituent; this leads to low susceptibility of the formed quinone toward a nucleophilic attack by a solvent molecule.[20] Better radical scavenging activity of phenolic acid derivatives containing 1,2,4-triazole[21] and 1,3,4-oxadiazole[22] in comparison with parent acids was recently determined and attributed to the participation of the heterocyclic scaffold in resonance stabilization of the formed radical after homolytic cleavage of the O-H and N-H bonds by the DPPH radical, as it was previously demonstrated by DFT calculations for 1,2,4-triazole

derivatives.[21] Similarly, antioxidant capacity of 1,3,4-thiadiazoles derived from phenolic acids is related to their ability to release hydrogen atoms, either from nitrogen or oxygen; this leads to resonance stabilization of the obtained radical. The resulting phenoxyl or nitrogen radical can be highly stabilized through resonance since the unpaired electron may be additionally delocalized across 1,3,4-thiadiazole ring (Scheme 2).
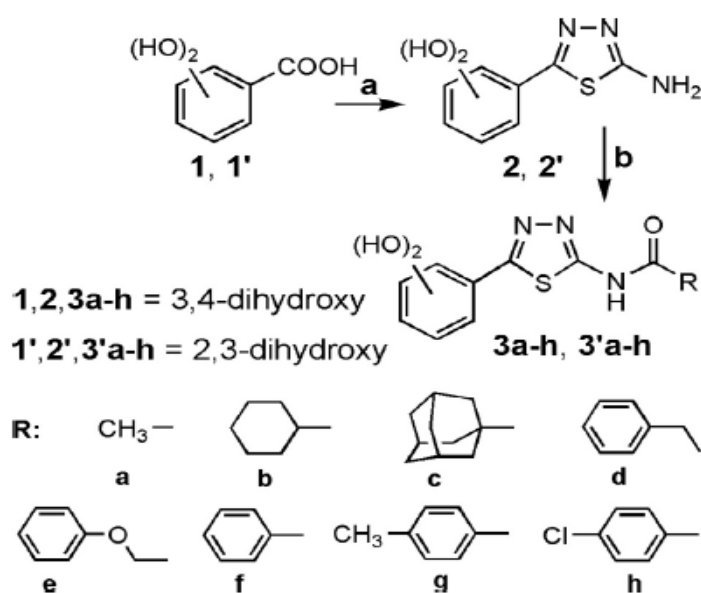
Scheme1. Reagents and conditions: a) POCI3, thiosemicarbazide, 1h, reflux; b) RCOCI, THF or dioxane, 24h r.t, or 12 h reflux
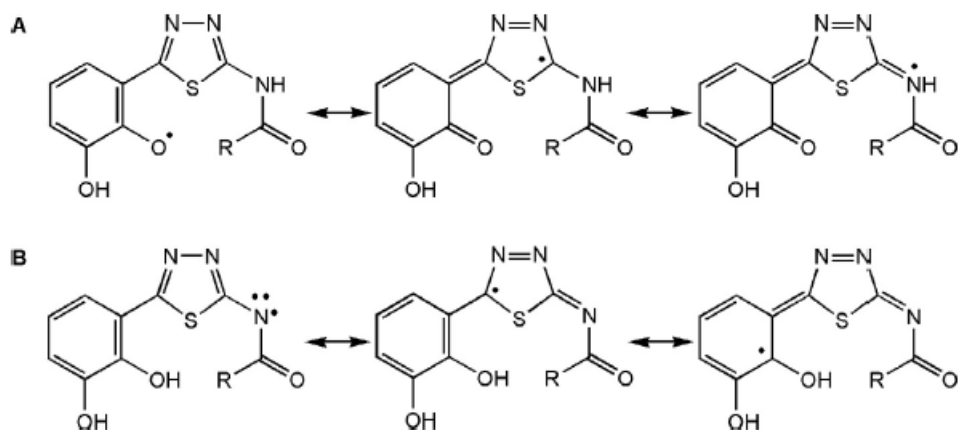
Scheme 2. Resonance stabilization of the radical after H atom abstraction from oxygen (A) and H atom abstraction from nitrogen (B).

Table 1.

DPPH scavenging activity of 1,3,4-thiadiazoles 3a-h and 3′a–h.

Compd.	$IC_{50}(\mu M)$	Compd.	$IC_{50}(\mu M)$
3a	23.36 ± 0.71	3′a	111.32 ± 2.22
3b	37.28 ± 0.92	3′b	19.88 ± 0.52
3c	17.85 ± 0.46	3′c	14.21 ± 0.34
3d	21.29 ± 0.64	3′d	20.89 ± 0.56
3e	36.05 ± 0.88	3′e	16.6 ± 0.39
3f	23.39 ± 0.74	3′f	22.28 ± 0.68
3g	33.97 ± 0.81	3′g	27.09 ± 0.77
3h	52.97 ± 1.43	3′h	30.17 ± 0.78
Ascorbic acid	38.02 ± 0.62		
NDGA	20.75 ± 0.48		

*Results are mean values ± SD of three measurements.

Table 2.

The *in vitro* cytotoxic activity of 1,3,4-thiadiazoles **3a–h** and **3′a–h** against three human cancer cell lines and normal human lung fibroblasts MRC-5.

Compd.	$IC_{50} \pm SD\ (\mu M)$			
	HL-60	HeLa	A549	MRC-5
3a	42.5 ± 0.2	124.7 ± 14.2	132.6 ± 19.4	58.6 ± 5.9
3b	8.8 ± 1.1	34.5 ± 2.2	16.3 ± 3.3	24.0 ± 0.3
3c	7.4 ± 0.3	16.1 ± 1.1	13.0 ± 3.0	20.1 ± 1.7
3d	12.0 ± 1.3	88.6 ± 9.7	15.4 ± 3.3	30.7 ± 4.4
3e	17.4 ± 2.4	51.3 ± 6.4	21.2 ± 1.3	30.9 ± 2.0
3f	17.6 ± 2.5	23.3 ± 3.0	13.0 ± 2.3	27.4 ± 4.0
3g	10.5 ± 1.1	67.1 ± 1.7	11.8 ± 0.5	27.1 ± 3.5
3h	14.7 ± 2.2	47.3 ± 3.4	14.6 ± 3.1	30.6 ± 4.0
3′a	49.0 ± 4.3	176.7 ± 21.3	200.0 ± 0.0	155.6 ± 13.5
3′b	7.9 ± 0.4	91.6 ± 7.0	11.0 ± 0.8	70.3 ± 14.2
3′c	7.3 ± 0.5	21.4 ± 4.2	12.8 ± 1.3	27.7 ± 4.3
3′d	13.9 ± 1.1	120.0 ± 18.9	11.8 ± 1.0	45.3 ± 4.1
3′e	11.2 ± 1.2	117.0 ± 14.5	10.5 ± 0.3	33.9 ± 6.1
3′f	20.7 ± 0.7	21.8 ± 2.3	12.3 ± 1.0	23.2 ± 2.3
3′g	10.6 ± 1.4	13.4 ± 2.5	11.3 ± 0.5	33.2 ± 3.0
3′h	9.6 ± 0.4	13.0 ± 2.2	9.5 ± 0.5	18.7 ± 1.9
Cisplatin	3.4 ± 0.1	2.2 ± 0.5	14.4 ± 0.7	9.2 ± 0.2

*Results are mean values ± SD of three independent experiments.

cytoprotective effects against generation of ROS induced by hydrogen peroxide in HL-60 cells. The HL-60 cells were treated for 24 h with sub-toxic IC20 concentrations of the tested compounds (6.5 μM for 3′c, 6 μM for 30c and 6.25 μM for 3′b and 3′e). Following the 24 h incubation, the cells were collected, washed and loaded with a 2′,7′-dichlorodihydrofluorescein diacetate.[22] Afterwards, the cells were exposed to 5 mM hydrogen peroxide solution (H_2O_2) for 30 min to induce generation of ROS. Fluorescence intensity of generated dichlorofluorescein was determined by flow cytometry.[22]
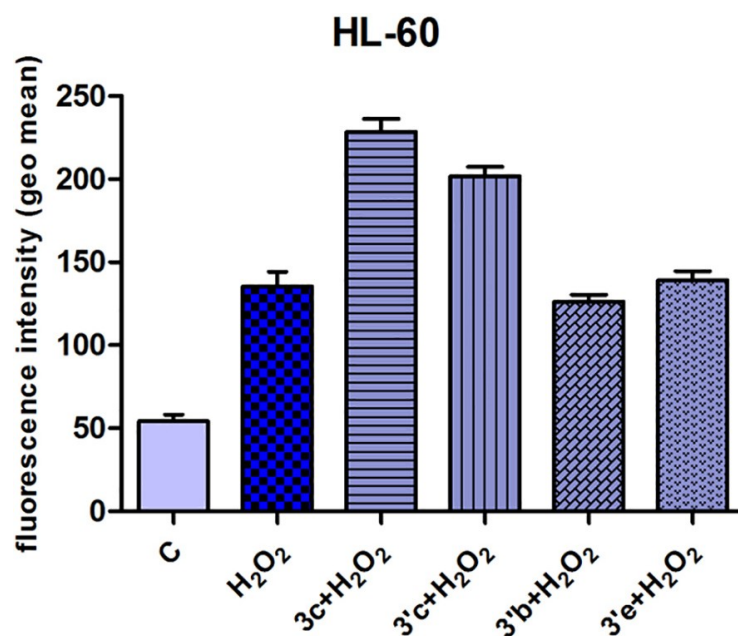

Fig. 1 Effects of 24 h pretreatment of HL-60 cells with IC20 concentrations of compounds 3c, 3 ′c, 3 ′b and 3 ′e on ROS production induced by 5 mM hydrogen peroxide. The results are presented as the mean ± SD of two independent experiments.

The pretreatment with compound 3′b slightly reduced the ROS levels induced by H_2O_2 in comparison with the HL-60 cells that were grown in the nutrient medium during 24 h and then exposed to H_2O_2 (Fig. 1). The ROS level in cells preincubated with compound 30e were comparable to those measured in the cells only exposed to H_2O_2, while compounds 3c and 3c led to remarkable increase of intracellular ROS levels.

The most active thiadiazoles 3c, 3´c, 3´g and 3´h were selected for further exploration of the mechanisms of their antiproliferative activity. Flow cytometric analysis of the cell cycle showed that each of the four examined compounds applied at IC_{50} and $2IC_{50}$ concentrations for 24 h led to a significant increase of human promyelocytic leukemia HL-60 cells in the subG1 cell cycle phases when compared with the control cell sample, as shown in Fig. 2. In addition to significantly increased percentage of subG1 cells, treatment with compound 3´c induced decrease of HL-60 cells within the S ($2IC_{50}$ concentration) and G2/M cell cycle phases (IC_{50} and $2IC_{50}$ concentrations). The compound 3´c induced the highest increase in the percentage of HL-60 cells in the subG1 cell cycle phase in comparison with the other compounds which were tested.

Morphological assessment of cell death type induced by the four selected 1,3,4 thiadiazoles in HL-60 cells was done by fluorescence microscopy using nucleic acid dyes acridine orange and ethidium bromide for cell staining. All examined compounds.
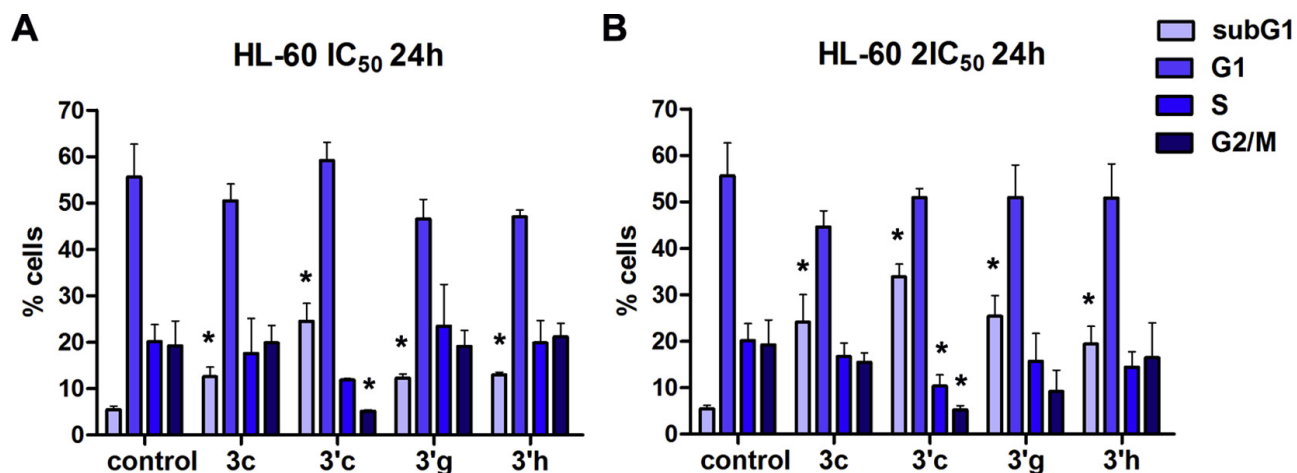

Fig. 2. Changes in the cell cycle phase distribution of HL-60 cells treated with IC_{50} (A) and $2IC_{50}$ concentrations (B) of the compounds 3c, 3´c, 3´g and 3´h after 24 h. The results are presented as the mean ± SD of three independent experiments. Statistically significant differences between control and treated cells are marked with * (p < 0.05).

applied at $2IC_{50}$ concentration for 24 h demonstrated the ability to induce apoptosis in HL-60 cells (Fig. 3). Typical morphological hallmarks of apoptotic cell death were observed in treated HL-60 cells, such as chromatin condensation and nuclear fragmentation as well as orange-red stained cells in the late stages of apoptosis or secondary necrosis.

To further elucidate the mechanisms of the cell death induced in HL-60 cells by the tested thiadazoles, we examined the effects of cell pretreatment with specific caspase inhibitors.
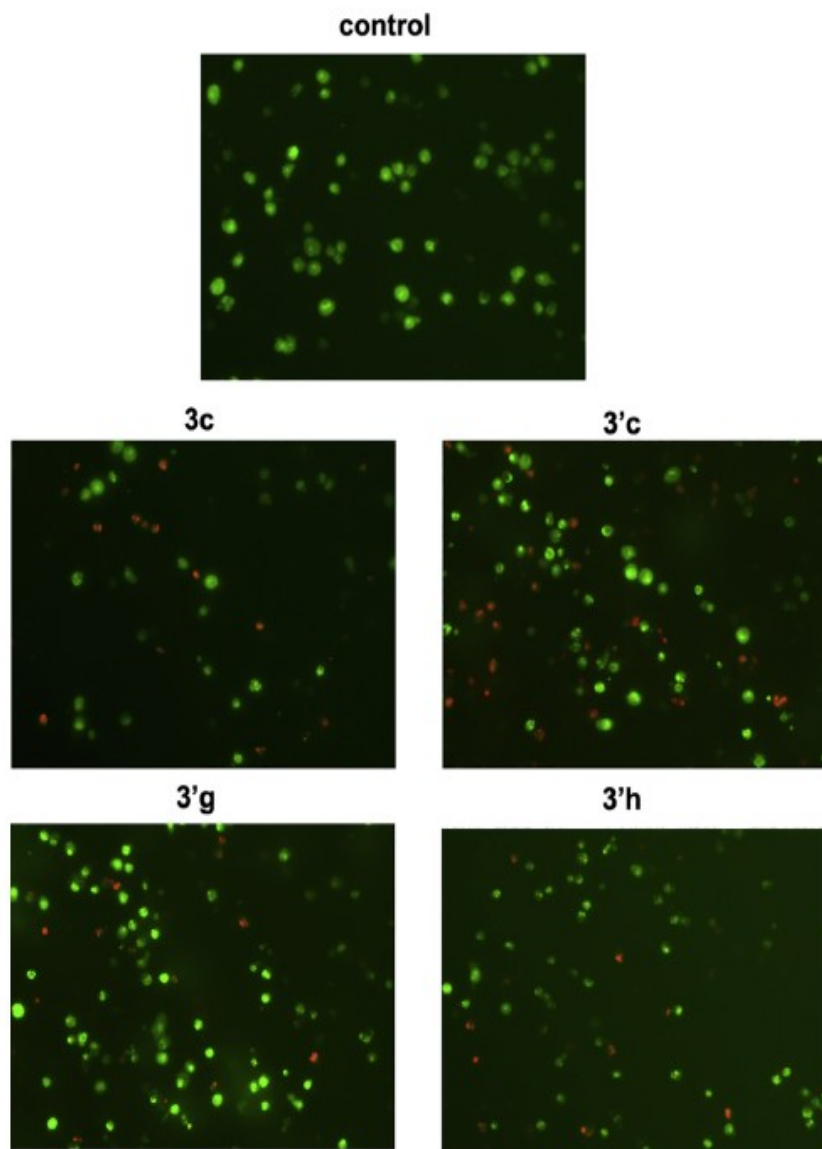
Fig 3. Photomicrographs of acridine orange/ethidium bromide control HL-60 cels exposed to $2IC_{50}$ conentration of the compounds **3c, 3´c, 3´g** and **3´h** for **24h** (20 X magnification)
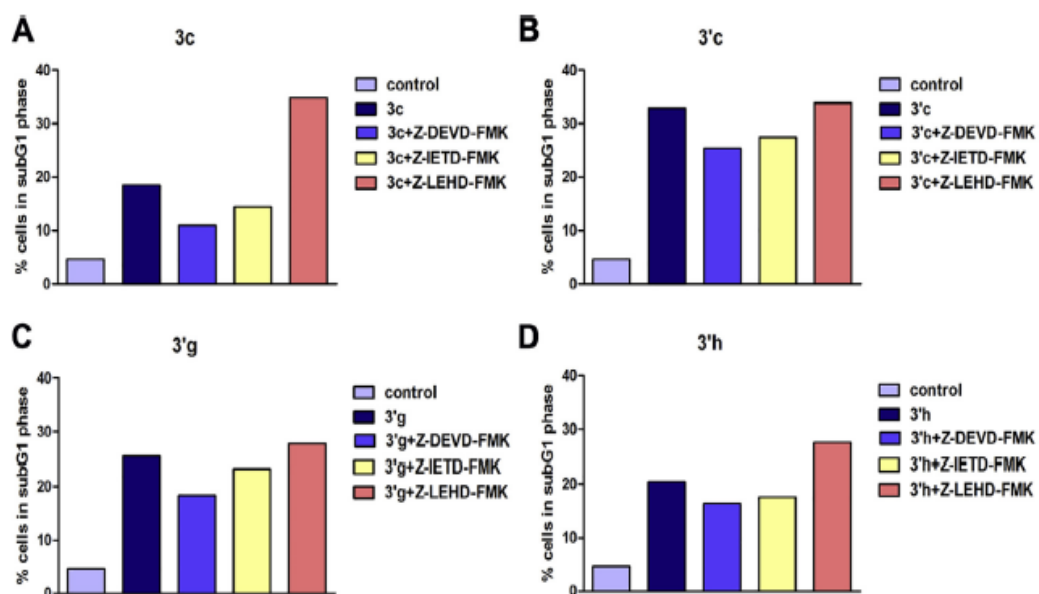
Fig. 4. Effects of the spcific caspase inhibitors (Z-DEVD-FMK-caspase-3 inhibitor, Z-IETD-FMK-caspase-8 inhibitor, Z-LEHD-FMK - capase-9 inhibitor) on the percentages of subG1HL-60 cells treated with 2IC$_{50}$ concentrations of the 3c (A), 3´ c (B) 3´ g (C) and 3´h (D)
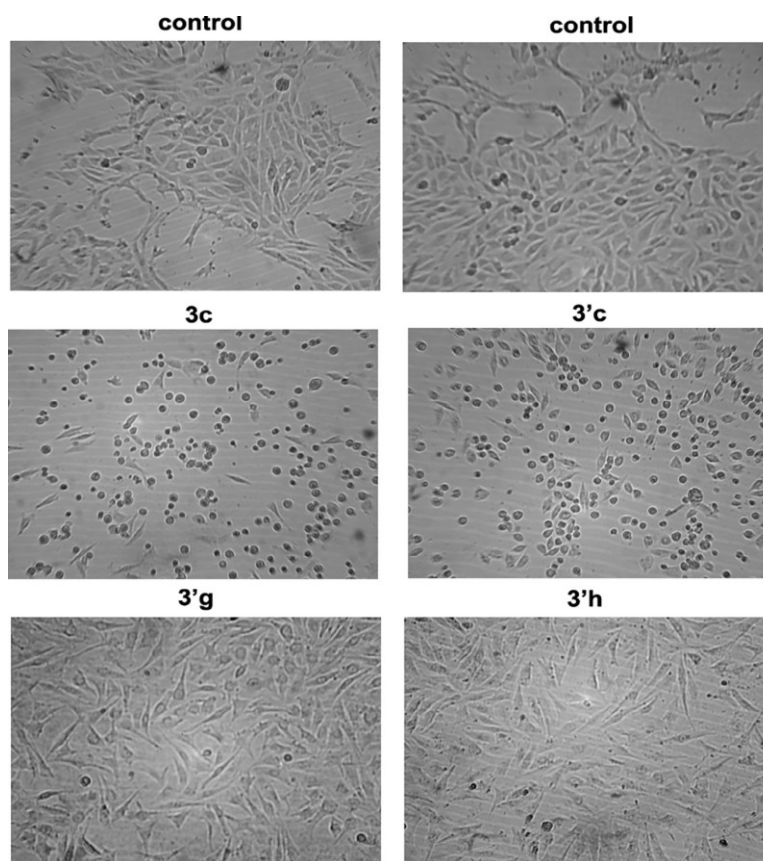
Fig.5. Photomicrographic of control Eahy926 cells and Eahy926 cells exposed to sub-toxic IC^{20} concentration of the compounds **3c**, **3´c**, **3´g** and **3´h** for 20h

The specific peptide caspase inhibitors applied at a concentration of 40 μM were: Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, a caspase-8 inhibitor and Z-LEHD-FMK, a caspase-9 inhibitor.[23] Pretreatment with a caspase-3 inhibitor of the HL-60 cells exposed to compounds 3c, 3´c, 3´g and 3´h led to decrease in the percentage of cells in the subG1 phase in comparison with the percentage of subG1 cells in the samples which were not preincubated with this inhibitor (Fig. 4). Furthermore, co-treatment with caspase-8 inhibitor was also shown to reduce the percentage of cells within the subG1 phase in the samples that were treated with each of the tested compounds, while pretreatment with caspase-9 inhibitor had no effect, or induced increase in the percentage of subG1 cells. The obtained results suggest that apoptosis execution in promyelocytic leukemia HL-60 cells by tested thiadiazoles is at least partially dependent on activation of caspase-3, the main effector caspase and caspase-8, implicated in the extrinsic apoptosis signaling pathway. In addition, these compounds may cause caspaseindependent apoptosis or other forms of cell death in HL-60 cells.

The possible antiangiogenic properties of the selected thiadiazoles were examined by endothelial cell tube formation assay.[24,25] As it could be seen in Fig. 5, the compounds 3c and 3´c applied at sub-toxic IC[20] concentrations (30 μM for both compounds) exerted strong antiangiogenic effects in vitro. Incubation with compounds 3c and 3´c of human endothelial EA.hy926 cells grown on the surface of matrigel asement membrane matrix effectively inhibited sprouting and elongation of EA.hy 926 cells their connecting and remodeling into capillary-like tube structures, which could be observed in the control cell samples. The compounds 3´g and 3´h applied at sub-toxic IC20 concentrations (50 mM for both compounds) exhibited quite weak antiangiogenic effects. These two compounds showed the ability to inhibit formation of the network of capillary-like tube structures. The in vitro antiangiogenic efficacy of novel thiadiazoles 3c and 3´c suggests

the significant cancerchemopreventive and cancer-therapeutic potential of these compounds, since inhibitors of angiogenesis represent an important treatment strategy for suppression of growth, invasion and metastasis of malignant tumors.[26]

We examined the effects of these compounds on the gene expression levels of matrix metalloproteinases 2 and 9 (MMP2 and MMP9), and vascular endothelial growth factor A (VEGFA) in human promyelocytic leukemia HL-60 cells. The *MMP2, MMP9* and VEGF secreted by malignant cells have been implicated in the cell growth, migration, invasion, metastasis and angiogenesis. [27,28] HL-60 cells were treated with sub-toxic IC_{20} concentrations of the compounds 3c, 3´c, 3´g and 3´h for 24 h (6.5 μM for compound 3c and 6 mM for 3´c, 3´g and 3´h). Measurement of mRNA expression levels was done by quantitative real time PCR (qPCR) and TaqMan[®] Gene Expression Assays.[29] Gene expression levels in the treated HL-60 cells were compared with the control, untreated HL-60 cells, as it could be seen in Fig. 6. Each of the tested compounds showed the ability to down-regulate the MMP2 expression levels in the treated HL-60 cells in comparison with the control cell samples, pointing to their antimetastatic properties. The compounds 3´c, 3´g and 3´h caused a remarkable decrease in the MMP2 expression levels, while compound 3c inhibited MMP2 expression to a lesser extent in comparison with these three compounds. However, all four tested compounds were shown to up-regulate MMP9 expression levels in HL-60 cells when compared with control cells. Furthermore, each of the examined compounds demonstrated the ability to decrease the expression of proangiogenic factor VEGFA in HL-60 cells. The compound 3´c exerted the most prominent suppressive effect.

In conclusion, we have synthesized two series of 1,3,4-thiadiazole-2-amides starting from 2,3-dihydroxybenzoic and 3,4-dihydroxybenzoic acid. Generally, the 3´ series prepared from 2,3-dihydroxybenzoic acid exhibited better antioxidant and antiproliferative activity.
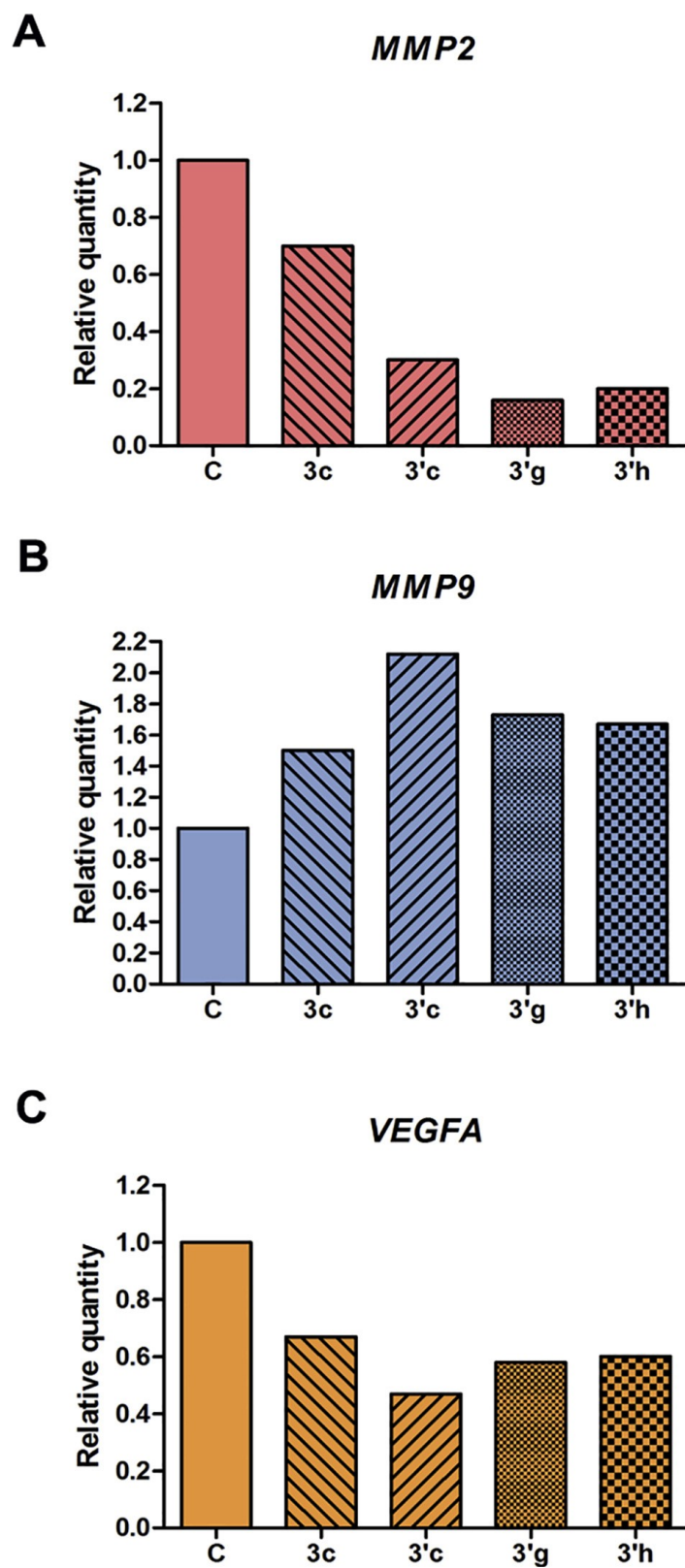
Fig. 6. Changes in gene expression levels of *MMP2* (A), *MMP9* (B) and *VEGFA* (C) in HL-60 cells treated with subtoxic IC$_{20}$ concentrations of compounds **3c**, **3´c**, **3´g** and **3´h** for 24 h.

Generally, the substituents attached to the amide bond had a stronger influence on radical scavenging properties and less pronounced effect on cytotoxic activity. The majority of the examined compounds (3b–h, 3´b–h) exerted the strongest cytotoxicity against human promyelocytic leukemia HL-60 and lung carcinoma A549 cells, while at the same time showing lower toxicity against normal human lung fibroblasts MRC-5, pointing to selectivity in their antiproliferative action. Exploration of the mechanisms of the antiproliferative effects of the **3c**, **3´c**, **3´g** and **3´h** revealed that these compounds caused statistically significant increase of HL-60 cells within the subG1 cell cycle phase when compared with untreated cells. The induction of cell death in HL-60 cells by the compounds **3c**, **3´c**, **3´g** and **3´h** was at least partially dependent on activation of caspase-3 and caspase-8. The compounds 3c and 3´c were the most effective in inhibition of angiogenesis in vitro. In addition, all four examined compounds decreased the MMP2 and VEGFA expression levels in HL-60 cells in comparison with those levels in the control cell samples. Our results point out the significant antiproliferative potential of novel thiadiazoles 3c, **3´c**, **3´g** and **3´h.**

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.07.003.

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