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Green synthesis and biological evaluation of 6-substituted-2-(2-hydroxy/methoxy phenyl)benzothiazole derivatives as potential antioxidant, antibacterial and antitumor agents

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ABSTRACT

We present a new efficient green synthetic protocol for introduction of substituents to the C-6 position of 2-arylbenzothiazole nuclei. Newly synthesized compounds were designed to study the influence of the hydroxy and methoxy groups on the 2-arylbenzothiazole scaffold, as well as the influence of the type of substituents placed on the C-6 position of benzothiazole moiety on biological activity, including antibacterial, antitumor and antioxidant activity. Modest activity was observed against the tested Gram-positive and Gram-negative bacterial strains for only amidino derivatives **5d** and **6d**. The tested compounds exhibited moderate to strong antiproliferative activity towards the tumor cell lines tested. The SAR study revealed that the introduction of substituents into the benzene ring of the benzothiazole nuclei is essential for antiproliferative activity, while introduction of the hydroxy group into the 2-aryl moiety of the 2-arybenzothiazole scaffold significantly improved selectivity against tumor cell lines. The observed results revealed several novel 6-substituted-2-arylbenzothiazole compounds, **5b**, **5c**, **5f** and **6f,** with strong and selective antiproliferative activity towards HeLa cells in micro and submicromolar concentrations, with the most selective compounds being 6-ammonium-2-(2-hydroxy/methoxyphenyl)benzothiazoles **5f** and **6f**. The compound **5f** bearing the hydroxy group on the 2-arylbenzothiazole core showed the most promising antioxidative activity evaluated by DPPH, ABTS and FRAP *in vitro* assays. The presence of the amino protonated group attached at the benzothiazole moiety was essential for the antiproliferative and antioxidant activity observed, exerted through a change in the levels of the reactive oxygen species-modulated HIF-1 protein.

1. Introduction

Over the past decade, benzothiazole derivatives have been recognized as ideal scaffolds with a high degree of structural diversity that might be useful for the design, optimization and synthesis of new pharmaceutical agents [1]. The benzothiazole nuclei is already a structural part of some drugs used in clinical applications, such as for example *zopolrestat* for the treatment of diabetes, *riluzole* for the treatment of amyotrophic lateral sclerosis, or *frentizole* used as an antiviral or immunosuppressive agent (Fig. 1) [2].

Structurally simple 2-arylbenzothiazole derivatives have been extensively studied, particularly for their antitumor activities [1,2], due to the interesting antitumor profile of their activity and the unique mechanism of their biological action [3]. A literature review revealed some recent examples of their broad spectrum of biological activities, including antitumor [4,5], antimicrobial [6,7], antiparasitic [8], anti-inflammatory [9,10], and antioxidant [11] activities.

It has already been proven that an excess of reactive oxygen species (ROS) has a crucial role in the pathogenesis of various chronic diseases, such as inflammation, cancer, neurodegenerative diseases or diabetes, by damaging the biomacromolecules such as proteins, lipids or DNA/RNA [12–15]. Therefore, development and evaluation of novel potent

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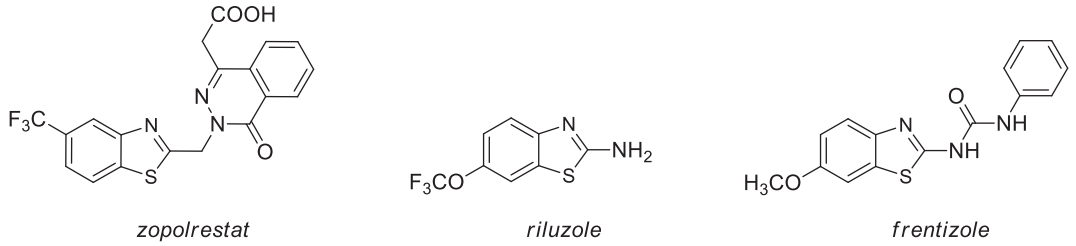
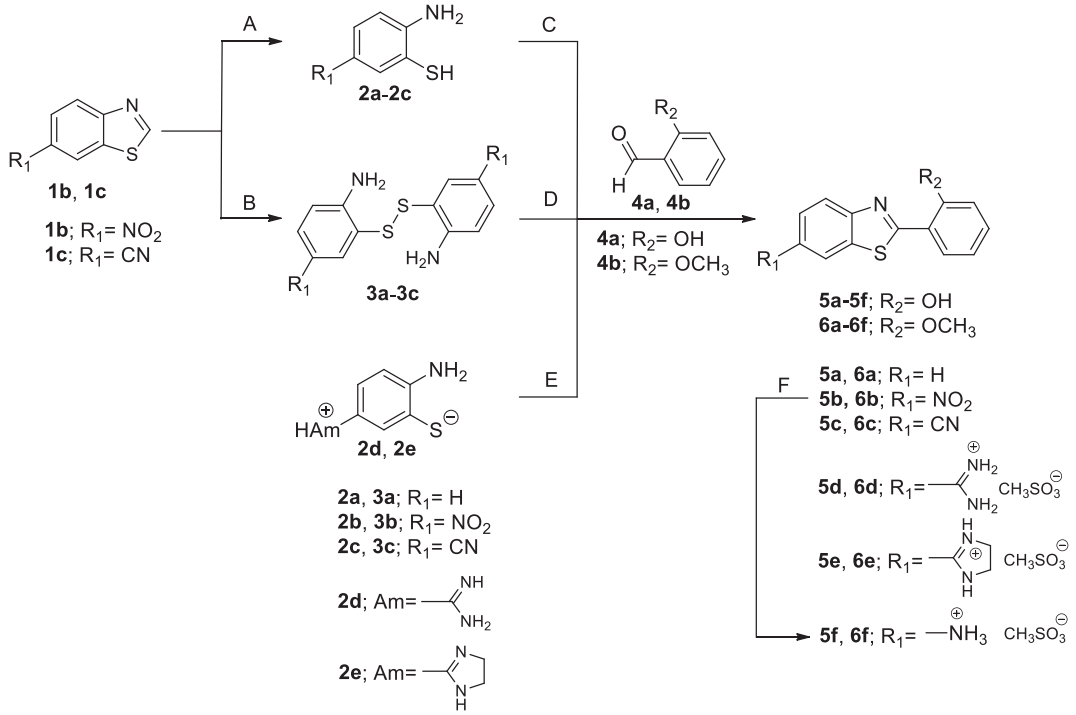


Fig. 1. Structures of clinically used benzothiazole derivatives.



Reagents and conditions:
A: glycerol / EDA, N_2, 100 °C 15 min, then H_2O / N_2, HCl pH 2-3
B: glycerol / EDA, N_2, 100 °C 15 min, then H_2O / H_2O_2 5-10 °C 15-30 min
C: glycerol, 110 °C 2-24 h or 170 °C 1 h
D: glycerol, 160-175 °C 30-60 min
E: (i) HOAc, reflux 3 h, then H_2O/NaOH pH 12; (ii) 2-PrOH/CH_3SO_3H, rt 2 h
F: (i) $SnCl_2$/HCl-MeOH, reflux 30 min, then H_2O/NaOH pH 12; (ii) 2-PrOH/CH_3SO_3H, rt 2 h

Scheme 1. Synthesis of 6-substituted 2-(2-hydroxyphenyl)- and 2-(2-methoxyphenyl)- benzothiazoles **5a**-**5f** and **6a**-**6f.**

antioxidative molecules is of great importance as these may be used in the treatment of various diseases. Several publications provide evidence of the antioxidative activity and potential of benzothiazole derivatives as antioxidative agents [16,17]. In a study of chrysin-benzothiazole conjugates as potential antioxidant and anticancer agents, it was found that a variation of the substituent at the C-6-position of benzothiazole moiety significantly modifies antitumor activity in the cancer cells investigated [18]. Our previous results regarding the antiproliferative [19–22] and antioxidative [23] activities of amino, cyano, nitro, and amidino substituted 2-aryl benzothiazole derivatives also show the influence on biological activity of the type and position of the substituent placed at the benzothiazole nuclei in the 2-arylbenzothiazole skeleton. The results of the antioxidative effects indicated that the variable number of hydroxy groups strongly influenced the antioxidative activity observed, and reduced the potency of the tested compounds. Concerning the type of the amidino substituent placed on the heteroaromatic scaffold, it could be noticed that, in general, the unsubstituted amidino group had a greater impact on increased antioxidative activity [23].

On the other hand, for chemistry-driven drug discovery, development of new green and efficient organic processes is one of the main goals. To a synthetic organic chemist, the challenge is indeed posed to substitute efficiently the volatile, flammable, or toxic solvents conventionally used with green and inexpensive solvents. For example, glycerol has recently been recognized as a valuable green solvent that meets numerous criteria of green chemistry [24,25]. Recently, we have developed two complementary eco-friendly methods for condensation of 2-amino-5-(2-imidazolinium)benzothiolate (**2e**) with various aryl/heteroaryl carbaldehydes, using acetic acid and glycerol, without the use of a catalyst or any additional oxidation step [26]. We found glycerol appropriate for condensation reactions of thermally and acid-sensitive heteroaryl carboxaldehydes, and as part of this research we wanted to extended our investigation to other reactions relevant for the synthesis of 6-substituted-2-arylbenzothiazoles using sustainable solvents as well.

In continuation of our previous research, we present here the design, green synthesis and structure-activity relationships of a series of novel 2-hydroxyphenyl- and 2-methoxyphenylbenzothiazole

compounds bearing different substituents at the C-6 position. The antiproliferative, antibacterial and antioxidative properties of the newly prepared compounds were evaluated. For compounds that showed selective antiproliferative and strong antioxidant activities, we analyzed the ROS modulated HIF-1 protein expression, having in mind the role of hypoxia-induced ROS on tumorigenesis. HIF-1 protein has already been recognized as an important target in antitumor therapy, because in hypoxia it binds to hypoxia-response elements in the promoter regions of genes involved in tumorigenesis [27–29].

2. Results and discussion

2.1. Chemistry

Synthesis of 2-hydroxyphenyl- and 2-methoxyphenylbenzothiazole derivatives **5a-5f** and **6a-6f** was carried out by the reactions outlined in Scheme 1. Previously, for preparation of versatile 6-nitro- and 6-cyano-2-arylbenzothiazole derivatives we used the condensation method of 2-amino-5-nitrothiophenol (**2b**) and 2-amino-5-cyanothiophenol (**2c**) with substituted benzaldehydes in pyridine, followed by oxidation of the intermediate benzothiazoline with $FeCl_3$ [19,20]. Unfortunately, this method suffers from a few drawbacks such as prolonged reaction time, one additional oxidation step, and the usage of the toxic solvent pyridine. This prompted us to develop a new synthetic route for condensation of aldehydes and substituted 2-aminothiophenols. Although there is a large number of condensation methods reported [30,31] only a few are efficient using a simple thermal reaction without any catalyst or oxidant. Furthermore, the majority of reported reactions for the formation of 2-arylbenzothiazoles from aldehydes use unsubstituted 2-aminothiphenol in the condensation reactions. There are only a few reports about the condensation of substituted 2-aminophenyl disulfide with aryl aldehydes, where the initial formation of the disulfide is achieved by the basic hydrolysis of the corresponding 2-aminobenzothiazoles [32–34].

Initially, we investigated the synthesis of 2-amino-5-nitrothiophenol (**2b**) and 2-amino-5-cyanothiophenol (**2c**) by basic cleavage of 6-nitrobenzothiazole (**1b**) and 6-cyanobenzothiazole (**1c**) with ethylenediamine (EDA), as we previously found it to be the selective reagent for ring opening of benzothiazoles bearing hydrolytically unstable substituents [35]. Reaction of the corresponding 6-substituted benzothiazole **2b** and **2c** with EDA in glycerol was carried out at 90–100 °C for 10–15 min, and subsequent acidification gave the corresponding thiophenols **2b** and **2c** in almost quantitative yield and purity higher than 98% (Method A). Using the same reaction conditions for benzothiazole ring opening and by subsequent oxidation with H_2O_2, bis(2-amino-5-nitrophenyl) disulfide (**3b**) and bis(2-amino-5-cyanophenyl) disulfide (**3c**) were obtained in a yield of 97% and 92% without the need for additional product purification (Method B).

Although there is a method described in the literature for preparation of 2-(2-hydroxyphenyl)benzothiazole (**5a**) in glycerol by condensation of 2-aminothiophenol (**2a**) and salicylaldehyde (**4a**) at room temperature, using this methodology we obtained benzothiazole **5a** in a moderate yield of 40% [36]. Furthermore, this condensation method was unsuccessful when applied using salicylaldehyde (**4a**) and 2-amino-5-nitrothiophenol (**2b**), even with prolonged reaction time (5 days), suggesting that the strong electron withdrawing nature of the nitro substituent significantly affected the rate of the reaction. However, benzothiazole **5a** and 6-cyano-2-(2-hydroxyphenyl)benzothiazole (**5c**) were prepared by heating the reaction mixture of thiophenols **2a** and **2c** with aldehyde **4a** in glycerol at 110 °C, while 2-(2-hydroxyphenyl)-6-nitrobenzothiazole (**5b**) was synthesized by condensation of thiophenol **2b** and aldehyde **4a** at 170 °C. Quenching the reaction with water and crystallization afforded the product **5a-5c** in a moderate yield (Method C). During our experiments with thiophenoles **2b** and **2c** we observed their tendency towards spontaneous dimerization in the solid state, which meant they had to be used within a few days before dimerization started. Consequently, we turned our attention to the synthesis of our 2-arylbezothiazole derivatives from the stable disulfide **3a-3c**. To the best of our knowledge, there is no report of synthesis of benzothiazole derivatives from 2-aminophenyl disulfide and aldehydes by simple thermal reaction, without the need for a catalyst, or using an environmentally friendly solvent, such as glycerol. We optimized condensation reactions of disulfides **3a-3c** with aldehydes **4a** and **4b**, and found that formation of the benzothiazole product was completed at a temperature of 160–175 °C within 60 min (Method D). Most importantly, the products **5a-5c** and **6a-6c** were obtained in a very good yield of 70–80% with purity higher than 97% by quenching the reaction with diluted ethanol and filtering the resulting precipitate, where no further purification was required, as indicated by LC-MS and ^{1}H NMR analyses.

For condensation of 2-amino-5-amidiniumbenzenethiolate (**2d**) and 2-amino-5-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)-benzenethiolate (**2e**) with aldehydes **4a** and **4b**, we used our recently developed method in acetic acid [37] (Method E). The corresponding novel 6-amidino-substituted compounds **5d**, **5e**, **6d** and **6e** were isolated as mesylate salts by two simple acid-base reaction steps, with an overall good yield. Finally, reduction of 6-nitro- derivatives **5b** and **6b** with $SnCl_2$ in methanol/HCl mixture (Method F) afforded novel 6-amino- derivatives **5f** and **6f**, which were also isolated as mesylate salts in a very good yield. The structures of the previously reported compounds **2a**, **2b**, **3a**, **3b**, **5a** and **6a** were confirmed by ^{1}H NMR and LC-MS data, as well as by comparing our experimental melting points with published values. The novel benzothiazole derivatives **5b-5f** and **6b-6f** were fully characterized by ^{1}H and ^{13}C NMR spectroscopy, UPLC-MS spectrometry and elemental analysis.

2.2. Antibacterial activity

The *in vitro* antibacterial activity of 2-arylbenzothiazole derivatives was tested against the Gram-negative bacteria *Escherichia coli* (TolC-) and *Moraxella catarrhalis* (ATCC 23246) and Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC29212). The minimum inhibitory concentration (MICs) values for all compounds **5a-5f** and **6a-6f** were evaluated and Azithromycin (AZT) was used as the standard reference antibiotic. The compounds having MICs values higher than 128 μg/ml were considered inactive. In general, the majority of the evaluated compounds did not show antibacterial activity towards tested Gram-positive and Gram-negative bacteria. The exceptions were compounds **5a, 6a, 5d, 6d** and **5e** (Table 1) which exhibited good activity against *Moraxella catarrhalis* but lower than Azithromycin (MICs 0.06 μg/ml). From the observed results, the presence of hydroxy or methoxy substituent at the C-2 position of phenyl group was shown to have no influence on the antibacterial activity of the tested 2-phenyl substituted benzothiazole derivatives. In addition, the incorporation of the different amidino group at the C-6 position of 2-(2-hydroxy/methoxyphenyl)benzothiazole **5a** and **6a**, 2-(2-hydroxy/methoxyphenyl)-benzothiazole-6-amidinium

Table 1
Antibacterial activity (MICs) of benzothiazole compounds.

Comp.	MICs[a] (μg/ml)			
	S. aureus	*M. catarrhalis*	*E. faecalis*	*E. coli*
5a	> 256	4	> 256	> 256
6a	> 256	< 16	> 256	> 256
5d	32	4	64	32
6d	128	4	128	64
5e	256	8	256	256
AZT[b]	1	0.06	2	0.5

[a] Minimal inhibitory concentrations.
[b] Azithromycin.

Table 2
In vitro antiproliferative activity (IC_{50}) of benzothiazole compounds **5a-5f** and **6a-6f.**

Comp.	IC_{50}[a] (μM) *Cell lines*				
	A549	HeLa	MCF-7	SW 620	HFF
5a	**84.4 ± 2.1**	**42.2 ± 2.2**	**85.9 ± 13.0**	**> 100**	**70.5 ± 13.4**
6a	49.1 ± 4.8	21.2 ± 4.3	54.9 ± 22.1	69.9 ± 16.2	36.0 ± 4.9
5b	> 100	5.9 ± 0.7	5.0 ± 5.4	> 100	> 100
6b	> 100	33.8 ± 4.9	> 100	> 100	> 100
5c	90.0 ± 9.0	0.2 ± 0.0	65.6 ± 15.1	> 100	> 100
6c	5.7 ± 0.2	2.3 ± 2.1	14.9 ± 7.3	> 100	6.7 ± 3.7
5d	6.8 ± 0.3	3.0 ± 0.8	3.8 ± 2.1	4.7 ± 0.5	23.9 ± 1.6
6d	4.7 ± 0.3	0.8 ± 0.1	4.4 ± 2.1	5.7 ± 0.2	4.4 ± 1.4
5e	6.9 ± 1.7	21.8 ± 6.2	4.2 ± 3.3	> 100	> 100
6e	5.2 ± 0.6	2.0 ± 0.3	3.9 ± 0.7	5.9 ± 0.4	3.7 ± 0.5
5f	> 100	9.5 ± 0.5	78.2 ± 7.4	> 100	> 100
6f	> 100	14.6 ± 7.1	> 100	> 100	> 100
5-FU	2.8 ± 0.1	8.8 ± 1.0	0.1 ± 0.0	0.1 ± 0.0	0.9 ± 0.1

[a] IC_{50} values are the concentrations that cause 50% inhibition of cancer cell growth (μM).

methanesulfonate **5d** and **6d,** as well as 6-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)-2-(2-hydroxyphenyl)benzothiazole methanesulfonate (**5e**), enhanced the anti-*Moraxella catarrhalis* activity (MICs 4, < 16, 4, 4 and 8 μg/ml, respectively).

2.3. Antiproliferative activity

Antiproliferative activities of the 2-arylbenzothiazole derivatives were evaluated against four human tumor cell lines: cervical carcinoma (HeLa), colorectal metastatic adenocarcinoma (SW620), breast metastatic epithelial adenocarcinoma (MCF-7), lung carcinoma (A549) and against cell lines of human skin fibroblasts (HFF). As a positive control, 5-fluorouracil (5-FU), was used. The results obtained are summarized in Table 2.

The tested compounds exhibited moderate to strong antiproliferative activity towards tumor cell lines. Generally, introduction of substituents to the C-6 position of the 2-arylbenzothiazole scaffold led to an increase in antiproliferative activity compared to the C-6 unsubstituted 2-(2-hydroxyphenyl)benzothiazole (**5a**) and 2-(2-methoxyphenyl)benzothiazole (**6a**), which exhibited modest inhibitory effects on the tested cell lines. Low micromolar potency (IC_{50} < 5 μM) of cationic 6-amidino-substituted 2-arylbenzothiazoles **5d** and **6d**, and 6-imidazolinyl-(2-methoxyphenyl)benzothiazole (**6e**) was observed on almost all tested cell lines, which is in agreement with our previous data on the antiproliferative activity of 6-amidino and 6-imidazolinyl-2-arylbenzothiazole derivatives [19,21,22]. Interestingly, 6-imidazolinyl-2-(2-hydroxyphenyl)benzothiazole (**5e**) exerted a strong inhibitory effect on A549 (IC_{50} = 6.9 μM) and MCF-7 (IC_{50} = 4.2 μM), with no inhibitory effect against human skin fibroblasts. This selectivity of 6-imidazolinyl-substituted benzothiazole **5e** was observed for the first time within the previously tested 6-imidazolinyl-2-aryl/heteroarylbenzothiazole derivatives, which showed strong antiproliferative activity but with a lack of more substantial selectivity [19,21,22,26,37]. Additionally, with the introduction of the nitro and cyano group to the C-6 position of benzothiazole moiety, the antiproliferative activity was significantly improved, with clear selectivity towards HeLa and MCF-7 cells. Compound 2-(2-hydroxyphenyl)-6-nitrobenzothiazole (**5b**) showed strong activity towards HeLa (IC_{50} = 5.9 μM) and MCF-7 (IC_{50} = 5.0 μM) while 2-(2-hydroxyphenyl)-6-cyanobenzothiazole (**5c**) exerted activity only towards HeLa (IC_{50} = 0.2 μM) cell lines. Interestingly, these derivatives did not show any activity against human skin fibroblasts. Replacement of the cationic amidino moiety at the C-6 position of benzothiazole with an ammonium group, as in 6-ammonium-2-(2-hydroxy/methoxyphenyl)benzothiazoles **5f** and **6f,** led to the enhancement of selectivity towards HeLa cells with no activity against other tumor cell lines and normal human skin fibroblasts. The benzothiazole derivatives **6c**, **5d**, **6d**, **5e** and **6e** exhibited antiproliferative activity on A549 cell lines comparable to 5-fluorouracil, used as the standard antitumor drug, while the activity of compounds **5b**, **5c**, **6c**, **5d**, **6d** and **6e** on HeLa cell lines was even better than the standard drug.

From the SAR study, it could be concluded that the introduction of substituents into the benzene part of benzothiazole is essential for antiproliferative activity, while the introduction of the hydroxy group into the 2-aryl moiety of the 2-arybenzothiazole scaffold significantly improved selectivity against tumor cell lines. The observed results reveal several novel 6-substituted 2-arylbenzothiazole compounds, **5b**, **5c**, **5f** and **6f,** with strong and selective antiproliferative activity towards HeLa cells.

2.4. Antioxidative activity

The antioxidant properties of benzothiazole derivatives **5a-5f** and **6a-6f** were evaluated by DPPH and ABTS stable radicals as well as by FRAP *in vitro* assays. The radical scavenging method using the DPPH stable radical, indicates the ability of the tested species to donate a proton/electron to an iron-free medium [38]. The working concentration of DPPH was 100 μM and the results were read after 30 min (Table 3). The results were presented as IC_{50} values with the exception of compounds **6b**, **5c, 6c, 5d**, **5e** and **6e**. They did not react under assay conditions, while compounds **5a, 6a**, **5b** and **6d** showed poor antioxidant activity, with very high IC_{50} (from 500 μM to 1 mM). The best activity was measured for two compounds **5f** and **6f**, which showed

Table 3
Antioxidant activity of compounds **5a-5f** and **6a-6f** by DPPH, FRAP and ABTS method.

Comp.	DPPH IC_{50} μM	FRAP $mmolFe^{2+}/mmol_C$	ABTS IC_{50} μM
5a	> 100	317.9 ± 7.5	38.3 ± 3.6
6a	> 100	78.8 ± 3.5	> 200
5b	> 100	299.6 ± 2.7	117.1 ± 16.0
6b	NE[a]	342.5 ± 4.6	> 200
5c	NE	174.2 ± 2.7	61.3 ± 5.0
6c	NE	325.6 ± 2.6	> 200
5d	NE	34.7 ± 0.4	55.4 ± 3.1
6d	> 100	6.3 ± 1.4	> 200
5e	NE	16.9 ± 1.0	47.8 ± 0.0
6e	NE	2.8 ± 0.6	> 200
5f	4.8 ± 1.0	553.5 ± 4.2	42.2 ± 1.3
6f	51.37 ± 0.9	244.4 ± 7.7	45.3 ± 0.0
BHT	25.0 ± 4.2	2089.3 ± 56.0	–

[a] NE: No effect.

lower IC_{50} values (4.8 μM and 51.4 μM). The most prominent antioxidative capacity was shown by the compound **5f** (4.8 μM), whose antioxidative activity was more pronounced than the butylated hydroxytoluene (BHT) used as a control. The antioxidant capacity of compounds was tested as their ability to decrease the color reacting directly with the $ABTS^{\bullet+}$, which is more reactive than the DPPH radical, and could be used to evaluate hydrophilic and lipophilic compounds [39]. The results were expressed as IC_{50} values, as presented in Table 3. The compounds **6a**, **6b**, **6c**, **6d** and **6e** showed a very low ability for stabilization of $ABTS^{\bullet+}$ radical (> 200 μM) while all the other tested compounds exhibited good antioxidant activity, ranging from 38 to 117 μM concentration. The best results were observed for compounds **5a**, **5f** and **6f,** with values of 38.3, 42.2 and 45.3 μM, respectively. The reducing ability of the compounds tested by the FRAP assay refers to their antioxidant properties and is expressed as the $mmolFe^{2+}/mmol_{Compound}$ value. The best activity among the tested compounds was observed for compound **5f** (553.5 $mmolFe^{2+}/mmol_C$). However, the antioxidative activity obtained is still quite low in comparison to the control BHT compound (2089.3 $mmolFe^{2+}/mmol_C$).

From the structure of the activity relationship, it is clear that presence of phenolic and amino protonated groups attached to the benzothiazole moiety is essential for antioxidant activity.

2.5. Western blot analysis of HIF-1 protein expression of HeLa treated with compounds 5c, 5f, 6c and 6f

Compounds **5c**, **5f**, **6c** and **6f** that exerted the most promising antiproliferative and antioxidative properties were evaluated as potential suppressors of the hypoxia-inducible factor-1 (HIF-1) protein. The HIF-1 protein consists of two subunits, HIF-1α and HIF-1β, which are both constitutively expressed in cells. The HIF-1α subunit is sensitive to changes in cell oxygenation, and under normoxia, when oxygen is abundant in cells, HIF-1α is hydroxylated at proline 564 and 402 residues by prolyl hydroxylases. This hydroxylated protein is rapidly degraded in the proteasome through the von Hippel-Lindau protein [40]. HIF-1 protein levels are, however, stabilized in the hypoxic environment in tumor cells through the ROS, and the HIF-1α subunit escapes degradation, binds to HIF-1β in the nucleus and initiates transcription of the genes involved in tumor cell proliferation, metastases or resistance to cancer therapy [27]. This is why regulation of HIF-1 levels or its activity in cancer cells has already been acknowledged in cancer therapy [41,42].

In our research, we showed that the HIF-1α hydroxylated protein tagged for proteosomal degradation was significantly up-regulated ($p < 0.05$) by treatment of HeLa cells with compounds **5f**, **6c** and **6f** at concentrations of IC_{50} and 2 × IC_{50}, for 48 h respectively (Fig. 2). This observation is indicative of a mechanism of action that increases proteosomal degradation of the HIF-1α subunit of the HIF-1 protein complex, a mechanism that usually occurs under normoxic conditions. Indeed, hydroxylation of HIF-1α on proline 564 residue enhances its ubiquitin-mediated degradation [43]. In the end, a non-significant increase of HIF-1α relative expression was observed after 48 h treatment of HeLa cells with compound **5c** at IC_{50} and 2 × IC_{50} concentration respectively (Fig. 2).

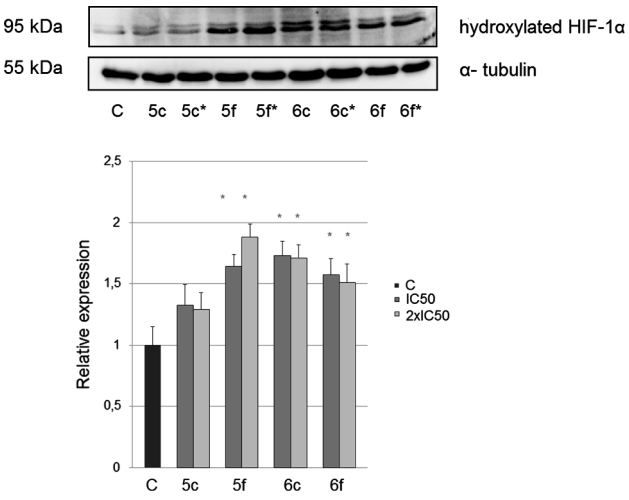


Fig. 2. Representative Western blot and summary representation of hydroxylated HIF-1α relative expression in HeLa cells treated with compounds **5c, 5f, 6c** and **6f** at concentrations of IC_{50} and 2 × IC_{50} for 48 h respectively. The results are presented as average relative expression values + SEM of chemiluminescent signals obtained in three replicate experiments. Statistically significant changes (ANOVA, $p < 0.05$) are marked with an asterisk. Abbreviations: C- control cells; * on the compound code designates the 2 × IC_{50} treatment.

3. Conclusions

We found a new, practical and green synthetic protocol for introduction of substituents to the C-6 position of the 2-arylbenzothiazole scaffold. Modest activity against tested Gram-positive and Gram-negative bacterial strains was observed for amidino derivatives **5d** and **6d**, while all other compounds have not shown any antibacterial activity in our testing. The observed results reveal several novel 6-substituted 2-benzothiazole compounds, **5b**, **5c**, **5e**, **5f** and **6f**, with strong and selective antiproliferative activity towards tumor cells, and no activity against human skin fibroblasts. The most selective compounds that inhibited the growth of HeLa cell lines were 6-ammonium-2-(2-hydroxy/methoxyphenyl)benzothiazole **5f** and **6f**. The compound **5f** having the hydroxy group on the 2-arylbenzothiazole moiety showed the most promising radical scavenging activity. HIF-1α hydroxylated protein was significantly upregulated by treatment of HeLa cells with compounds **5f**, **6c** and **6f**, so they were marked as potential suppressors of the hypoxia-induced HIF-1 protein. Based on these results, compounds **5f** and **6f** were chosen as the leading compounds for further rationalized design of the benzothiazole skeleton.

4. Experimental

4.1. Chemistry

Melting points were determined by means of Original Kofler Mikroheitztisch apparatus (Reichert, Wien). ^1H NMR and ^{13}C NMR spectra were recorded with the Bruker Avance DPX-300 or Bruker AV-600 using TMS as internal standard. Chemical shifts are reported in parts per million (ppm) relative to TMS. UPLC-MS spectra were recorded with Agilent 1290 Infiniti II/6120 Qudropole LC/MS spectrometers using electrospray ionization (ESI). Elemental analyses for carbon, hydrogen and nitrogen were performed on Perkin-Elmer 2400 elemental analyser. Analyses are indicated as symbols of elements, and analytical results obtained are within 0.4% of the theoretical value. Synthesis of 6-cyanobenzothiazole (**1c**) was carried out according to the literature [44]. Synthesis of 2-amino-5-amidiniumbenzenethiolate (**2d**) and 2-amino-5-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)-benzenethiolate hydrate (**2e**) were carried out according to the literature [35] while the 6-nitrobenzothiazole (**1b**), 2-aminothiphenole (**2a**), bis(2-aminophenyl) disulfide (**3a**), 2-hydroxybenzaldehyde (**4a**) and 2-methoxybenzaldehide (**4b**) were commercially available.

4.1.1. General synthetic procedures

Method A: To a stirred suspension of 6-nitrobenzothiazole (**1b**) or 6-cyanobenzothiazole (**1c**) (5.0 mmol) in glycerol (2.5–3.0 g) under nitrogen, ethylenediamine (12.5 mmol) was added and heated at 90–100 °C for 10–15 min. The reaction mixture was diluted with deoxygenated water, cooled under nitrogen to 5–10 °C and made acidic (pH 2–3) with concd. HCl. The resulting precipitate was filtered,

washed with cold water, and dried under vacuum over KOH giving pure thiophenoles **2b** and **2c**.

Method B: To a stirred suspension of 6-nitrobenzothiazole (**1b**) or 6-cyanobenzothiazole (**1c**) (10 mmol) in glycerol (5 g) under nitrogen, ethylenediamine (25 mmol) was added and heated at 90–100 °C for 10–15 min. The reaction mixture was poured onto ice-water mixture (200 ml), 1.0 ml of 30% H_2O_2 was added dropwise and stirred at 5 °C for 30 min. The resulting precipitate was filtered, washed with water, and air-dried giving pure disulfides **3b** and **3c**.

Method C: To a stirred suspension of the corresponding thiophenol **2a**-**2c** (1.0 mmol) in glycerol (1.5–2.0 g), 2-hydroxybenzaldehyde (**4a**) (1.0 mmol) was added and heated at 110–170 °C for 1–24 h. The reaction mixture was quenched with water and cooled in the refrigerator overnight. The resulting precipitate was collected by filtration and washed with diluted ethanol giving compounds **5a**-**5c**.

Method D: To a stirred suspension of the corresponding disulfide **3a**-**3c** (0.5 mmol) in glycerol (1.5–2.0 g), 2-hydroxybenzaldehyde (**4a**) or 2-methoxybenzaldehide (**4b**) (1.0 mmol) was added and heated at 160–175 °C for 30–60 min. The reaction mixture was cooled below 100 °C, quenched with 75% ethanol and cooled in the refrigerator overnight. The resulting precipitate was collected by filtration, washed with diluted ethanol and air-dried giving compounds **5a**-**5c** and **6a**-**6c**.

Method E: To a stirred solution of amidino-substituted 2-aminobenzenethiolate **2d** or **2e** (1.0 mmol) in glacial acetic acid (5 ml), a corresponding 2-substituted benzaldehyde (1.0 mmol) **4a** or **4b** (1.0 mmol) was added. The reaction mixture was stirred and heated under nitrogen for 4 h, then poured onto ice and made alkaline (pH 10–11) with 20% NaOH. Resulting free base was filtered, washed with water and dried. The free base was suspended in 2-propanol (10 ml), methanesulfonic acid (0.065 ml, 1.0 mmol) was added and stirred at room temperature for 2 h. After cooling overnight, the resulting precipitate was filtered, washed with acetone and dried at 75 °C giving pure compound **5d**, **6d**, **5e** and **6e**.

Method F: To a stirred solution of tin(II) chloride dihydrate (1.28 g, 5.0 mmol) in concd. hydrochloric acid (2.5 ml) and methanol (2.5 ml), a corresponding 6-nitro-substituted benzothiazole **5b** or **6b** (1.0 mmol) was added and heated to reflux for 30 min. The reaction mixture was poured onto ice, made slightly basic (pH 8–9) with 20% NaOH and the product was extracted with diethyl-ether. The solvent was removed under reduced pressure and resulting crude amine was converted into methanesulfonate salt as described in **5f** and **6f**.

*4.1.1.1. 2-Amino-5-nitrothiophenol (**2b**).* According to **Method A**, 6-nitrobenzothiazole (**1b**) (0.900 g, 5.0 mmol) and ethylenediamine (0.840 ml, 12.5 mmol) were used and heated at 100 °C for 10 min, giving 0.812 g (95.4%) of yellow solid; mp = 90–91 °C (dec.) (lit [45] mp = 89–90 °C). UPLC (254 nm): 99.4 area %; MS (ESI) *m/z*: 171.1 ($M+H^+$). ^{1}H NMR (300 MHz, $CDCl_3$) (δ ppm): 8.34 (d, 1H, *J* = 2.4 Hz, Ar-*H*), 8.02 (dd, 1H, *J* = 2.4 Hz, *J* = 8.9 Hz, Ar-*H*), 6.71 (d, 1H, *J* = 8.9 Hz, Ar-*H*), 4.94 (s, 2H, −N*H*$_2$), 3.01 (s, 1H, −S*H*).

*4.1.1.2. 2-Amino-5-cyanothiophenol (**2c**).* According to **Method A**, 6-cyanobenzothiazole (**1c**) (0.800 g, 5.0 mmol) and ethylenediamine (0.840 ml, 12.5 mmol) were used and heated at 90 °C for 15 min, giving 0.704 g (93.7%) of colourless solid; mp = 88–91 °C (dec.) (lit [46] mp = 85–89 °C). UPLC (254 nm): 98.1 area %; MS (ESI) *m/z*: 151.1 ($M+H^+$). ^{1}H NMR (600 MHz, $CDCl_3$) (δ ppm): 7.66 (d, 1H, *J* = 1.7 Hz, Ar-*H*), 7.36 (dd, 1H, *J* = 1.7 Hz, *J* = 8.4 Hz, Ar-*H*), 6.71 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 4.72 (s, 2H, −N*H*$_2$), 2.93 (s, 1H, −S*H*).

4.1.1.3. Bis(2-amino-5-nitrophenyl) disulfide (3b). According to **Method B**, 6-nitrobenzothiazole (**1b**) (1.80 g, 10 mmol) and ethylenediamine (1.68 ml, 25.0 mmol) were used and heated at 90 °C for 10 min, giving 1.64 g (97.0%) of yellow solid; mp = 237–239 °C (lit [45] mp = 237–238 °C). UPLC (254 nm): 100 area %; MS (ESI) *m/z*: 339.1 ($M+H^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 8.00 (dd, 1H, *J* = 2.6 Hz, *J* = 9.1 Hz, Ar-*H*), 7.52 (d, 1H, *J* = 2.6 Hz, Ar-*H*), 7.21 (s, 2H, −N*H*$_2$), 6.86 (d, 1H, *J* = 9.2 Hz, Ar-*H*).

*4.1.1.4. Bis(2-amino-5-cyanophenyl) disulfide (**3c**).* According to **Method B**, 6-cyanobenzothiazole (**1c**) (1.60 g, 10 mmol) and ethylenediamine (1.68 ml, 25.0 mmol) were used and heated at 95 °C for 15 min, giving 1.37 g (91.9%) of pale yellow solid; mp = 195–197 °C (lit [44] mp = 188 °C). UPLC (254 nm): 98.1 area %; MS (ESI) *m/z*: 299.1 ($M+H^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 7.48 (dd, 1H, *J* = 1.9 Hz, *J* = 8.6 Hz, Ar-*H*), 7.10 (d, 1H, *J* = 1.8 Hz, Ar-*H*), 6.82 (d, 1H, *J* = 8.6 Hz, Ar-*H*), 6.63 (s, 2H, −N*H*$_2$).

4.1.1.5. 2-(2-Hydroxyphenyl)benzothiazole (5a). According to **Method C**, 2-aminothiophenol (**2a**) (0.125 g, 1.0 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used and heated at 110 °C for 2 h. Crystallization from ethanol gave 0.142 g (62.5%) of colorless solid; mp = 131–132 °C (lit [47] mp = 131 °C). UPLC (254 nm): 100 area %; MS (ESI) *m/z*: 228.1 ($M+H^+$). ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 11.60 (s, 1H, −O*H*), 8.17 (dd, 1H, *J* = 1.7 Hz, *J* = 7.8 Hz, Ar-*H*), 8.17 (d, 1H, *J* = 7.8 Hz, Ar-*H*), 8.07 (d, 1H, *J* = 8.1 Hz, Ar-*H*), 7.55 (m, 1H, Ar-*H*), 7.47–7.41 (m, 2H, Ar-*H*), 7.09 (dd, 1H, *J* = 1.0 Hz, *J* = 8.2 Hz, Ar-*H*), 7.03 (m, 1H, Ar-*H*).

According to **Method D**, bis(2-aminophenyl) disulfide (**3a**) (0.125 g, 0.5 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used and heated at 175 °C for 45 min, giving 0.184 g (81.1%) of colourless solid. UPLC (254 nm): 98.9 area %; MS (ESI) *m/z*: 228.1 ($M+H^+$).

4.1.1.6. 2-(2-Hydroxyphenyl)-6-nitrobenzothiazole (5b). According to **Method C**, 2-Amino-5-nitrothiophenol (**2b**) (0.170 g, 1.0 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used and heated at 170 °C for 1 h. Crystallization from toluene gave 0.161 g (59.2%) of beige solid; mp = 239–241 °C. UPLC (254 nm): 100 area %; MS (ESI) *m/z*: 273.1 ($M+H^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 11.58 (s, 1H, −O*H*), 9.18 (d, 1H, *J* = 2.2 Hz, Ar-*H*), 8.36–8.32 (m, 2H, Ar-*H*), 8.20 (d, 1H, *J* = 9.0 Hz, Ar-*H*), 7.48 (m, 1H, Ar-*H*), 7.12 (dd, 1H, *J* = 8.3 Hz, *J* = 0.9 Hz, Ar-*H*), 7.05 (m, 1H, Ar-*H*). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 170.0 (s), 156.8 (s), 155.5 (s), 143.8 (s), 135.7 (s), 133.4 (d), 128.6 (d), 122.4 (d), 121.5 (d), 119.8 (d), 119.1 (d), 118.7 (s), 116.9 (d). Analysis calcd for $C_{13}H_8N_2O_3S$ (272.28): C, 57.35; H, 2.96; N, 10.29. Found: C, 57.48; H, 2.90; N, 10.18.

According to **Method D**, bis(2-amino-5-nitrophenyl) disulfide (**3b**) (0.170 g, 0.5 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used and heated at 170–175 °C for 30 min, giving 0.219 g (80.5%) of beige solid. UPLC (254 nm): 97.1 area %; MS (ESI) *m/z*: 273.1 ($M+H^+$).

4.1.1.7. 6-Cyano-2-(2-hydroxyphenyl)benzothiazole (5c). According to **Method C**, 2-Amino-5-cyanothiophenol (**2c**) (0.150 g, 1.0 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used and heated at 110 °C for 24 h. Crystallization from toluene gave 0.178 g (70.6%) of pale yellow solid; mp = 201–205 °C. UPLC (230 nm): 97.1 area %; MS (ESI) *m/z*: 253.1 ($M+H^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 11.58 (bs, 1H, −O*H*), 8.74 (d, 1H, *J* = 1.6 Hz, Ar-*H*), 8.33 (dd, 1H, *J* = 7.7 Hz, *J* = 1.6 Hz, Ar-*H*), 8.18 (d, 1H, *J* = 8.5 Hz, Ar-*H*), 7.91 (dd, 1H, *J* = 8.5 Hz, *J* = 1.6 Hz, Ar-*H*), 7.46 (m, 1H, Ar-*H*), 7.12 (d, 1H, *J* = 8.2 Hz, Ar-*H*), 7.04 (m, 1H, Ar-*H*). ^{13}C NMR (150 MHz, DMSO-d_6) (δ ppm): 168.3 (s), 156.5 (s), 154.0 (s), 135.5 (s), 133.2 (d), 129.3 (d), 128.6 (d), 127.3 (d), 122.9 (d), 119.8 (d), 119.0 (s), 118.5 (s), 116.8 (d), 106.6 (s). Analysis calcd for $C_{14}H_8N_2OS$ (252.29): C, 66.65; H, 3.20; N, 11.10. Found: C, 66.38; H, 3.42; N, 11.32.

According to **Method D**, bis(2-amino-5-cyanophenyl) disulfide (**3c**) (0.150 g, 0.5 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used and heated at 160–165 °C for 30 min, giving 0.188 g (74.6%) of pale yellow solid. UPLC (254 nm): 97.4 area %; MS (ESI) *m/z*: 253.1 ($M+H^+$).

*4.1.1.8. 6-Amidinium-2-(2-hydroxyphenyl)benzothiazole methanesulfonate (**5d**)*. According to **Method E**, 2-amino-5-amidiniumbenzenethiolate (**2d**) (0.167 g, 1.0 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used giving 0.240 g (65.8%) of pale yellow solid; mp = 287–289 °C. UPLC (254 nm): 99.4 area %; MS (ESI) *m/z*: 270.2 [(M + H$^+$) calcd for free base $C_{14}H_{11}N_3OS$ (269.06)]. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 11.57 (bs, 1H, −O*H*), 9.39 (s, 2H, −C(N*H*$_2$)$_2^+$), 9.06 (s, 2H, −C(N*H*$_2$)$_2^+$), 8.65 (s, 1H, Ar-*H*), 8.34 (d, 1H, *J* = 7.0 Hz, Ar-*H*), 8.24 (d, 1H, *J* = 7.8 Hz, Ar-*H*), 7.80 (d, 1H, *J* = 8.6 Hz, Ar-*H*), 7.47 (m, 1H, Ar-*H*), 7.13 (d, 1H, *J* = 8.6 Hz, Ar-*H*), 7.05 (m, 1H, Ar-*H*), 2.36 (s, 3H, C*H*$_3$SO$_3^-$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 168.0 (s), 165.5 (s), 156.6 (s), 154.7 (s), 135.2 (s), 133.2 (d), 128.6 (d), 126.0 (d), 124.2 (s), 123.0 (d), 122.3 (d), 119.8 (d), 118.6 (s), 116.9 (d), 39.8. Analysis calcd for $C_{15}H_{15}N_3O_4S_2$ (365.43): C, 49.30; H, 4.14; N, 11.50. Found: C, 49.28; H, 4.18; N, 11.47.

*4.1.1.9. 6-(4,5-Dihydro-1H-imidazol-3-ium-2-yl)-2-(2-hydroxyphenyl) benzothiazole methanesulfonate (**5e**)*. According to **Method E**, 2-amino-5-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)benzenethiolate hydrate (**2e**) (0.211 g, 1.0 mmol) and 2-hydroxybenzaldehyde (**4a**) (0.122 g, 1.0 mmol) were used giving 0.245 g (62.7%) of colourless solid; mp = 279–282 °C. UPLC (254 nm): 100 area %; MS (ESI) *m/z*: 296.2 [(M + H$^+$) calcd for free base $C_{16}H_{13}N_3OS$ (295.08)]. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 11.60 (bs, 1H, −O*H*), 10.57 (bs, 2H, −C(N*H*−)$_2^+$), 8.75 (d, 1H, *J* = 1.5 Hz, Ar-*H*), 8.35 (dd, 1H, *J* = 7.8 Hz, *J* = 1.5 Hz, Ar-*H*), 8.28 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 8.02 (dd, 1H, *J* = 8.6 Hz, *J* = 1.8 Hz, Ar-*H*), 7.48 (m, 1H, Ar-*H*), 7.14 (d, 1H, *J* = 8.0 Hz, Ar-*H*), 7.06 (m, 1H, Ar-*H*), 4.06 (s, 4H, −C*H*$_2$C*H*$_2$−), 2.35 (s, 3H, C*H*$_3$SO$_3^-$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 168.4 (s), 164.9 (s), 156.6 (s), 155.0 (s), 135.5 (s), 133.3 (d), 128.6 (d), 126.0 (d), 123.3 (d), 122.6 (d), 119.8 (d), 118.6 (s), 118.0 (s), 116.9 (d), 44.5 (t, 2C), 39.7 (q). Analysis calcd for $C_{17}H_{17}N_3O_4S_2$ (391.46): C, 52.16; H, 4.38; N, 10.73. Found: C, 52.32; H, 4.49; N, 10.57.

*4.1.1.10. 2-(2-Hydroxyphenyl)benzothiazole-6-ammonium methanesulfonate (**5f**)*. According to **Method F**, 2-(2-hydroxyphenyl)-6-nitrobenzothiazole (**5b**) (0.272 g, 1.0 mmol) was used and heated to reflux for 30 min. Afterwards, the obtained crude amine was suspended in 2-propanol (10 ml), methanesulfonic acid (0.065 ml, 1.0 mmol) was added and stirred at room temperature for 2 h. After cooling overnight, the resulting precipitate was filtered, washed with diethyl-ether and dried at 75 °C giving 0.302 g (89.3%) of colorless solid; mp = 287–290 °C (dec.). UPLC (230 nm): 100 area %; MS (ESI) *m/z*: 243.1 [(M + H$^+$) calcd for free amine $C_{13}H_{10}N_2OS$, 242.05]. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 11.53 (bs, 1H, −O*H*), 8.15 (dd, 1H, *J* = 8.0 Hz, *J* = 1.4 Hz, Ar-*H*), 8.03 (d, 1H, *J* = 8.5 Hz, Ar-*H*), 7.87 (s, 1H, Ar-*H*), 7.41 (m, 1H, Ar-*H*), 7.31 (dd, 1H, *J* = 8.5 Hz, *J* = 1.8 Hz, Ar-*H*), 7.09 (d, 1H, *J* = 8.0 Hz, Ar-*H*), 7.02 (m, 1H, Ar-*H*), 2.35 (s, 3H, C*H*$_3$SO$_3^-$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 165.1 (s), 156.2 (s), 149.8 (s), 135.7 (s), 132.5 (d), 131.0 (s), 128.4 (d), 123.0 (d), 120.9 (d), 119.8 (d), 118.5 (s), 116.9 (d), 115.0 (d), 39.8 (q). Analysis calcd for $C_{14}H_{14}N_2O_4S_2$ (338.40): C, 49.69; H, 4.17; N, 8.28. Found: C, 49.91; H, 4.03; N, 8.11.

*4.1.1.11. 2-(2-Methoxyphenyl)benzothiazole (**6a**)*. According to **Method D**, bis(2-aminophenyl) disulfide (**3a**) (0.125 g, 0.5 mmol) and 2-methoxybenzaldehide (**4b**) (0.136 g, 1.0 mmol) were used and heated at 160–165 °C for 60 min, giving 0.169 g (70.4%) of colorless solid; mp = 105–107 °C (lit [48] mp = 103–105 °C). UPLC (230 nm): 100 area %; MS (ESI) *m/z*: 242.1 (M + H$^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 8.45 (dd, 1H, *J* = 7.9 Hz, *J* = 1.7 Hz, Ar-*H*), 8.13 (bd, 1H, *J* = 7.4 Hz, Ar-*H*), 8.06 (d, 1H, *J* = 8.0 Hz, Ar-*H*), 7.60–7.51 (m, 2H, Ar-*H*), 7.44 (ddd, 1H, *J* = 8.0 Hz, *J* = 7.4 Hz, *J* = 1.0 Hz, Ar-*H*), 7.32 (bd, 1H, *J* = 8.0 Hz, Ar-*H*), 7.18 (ddd, 1H, *J* = 7.9 Hz, *J* = 7.4 Hz, *J* = 0.8 Hz, Ar-*H*), 4.07 (s, 3H, −OC*H*$_3$).

*4.1.1.12. 2-(2-Methoxyphenyl)-6-nitrobenzothiazole (**6b**)*. According to **Method D**, bis(2-amino-5-nitrophenyl) disulfide (**3b**) (0.170 g, 0.5 mmol) and 2-methoxybenzaldehide (**4b**) (0.136 g, 1.0 mmol) were used and heated at 170–175 °C for 30 min, giving 0.223 g (77.9%) of beige solid; mp = 193–195 °C. UPLC (254 nm): 98.7 area %; MS (ESI) *m/z*: 287.1 (M + H$^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 9.19 (d, 1H, *J* = 2.3 Hz, Ar-*H*), 8.49 (dd, 1H, *J* = 7.8 Hz, *J* = 1.6 Hz, Ar-*H*), 8.35 (dd, 1H, *J* = 9.0 Hz, *J* = 2.4 Hz, Ar-*H*), 8.21 (d, 1H, *J* = 8.9 Hz, Ar-*H*), 7.65 (m, 1H, Ar-*H*), 7.37 (d, 1H, *J* = 8.2 Hz, Ar-*H*), 7.21 (m, 1H, Ar-*H*), 4.11 (s, 3H, −OC*H*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 168.3 (s), 157.4 (s), 156.1 (s), 143.8 (s), 135.7 (s), 133.3 (d), 128.6 (d), 122.4 (d), 121.5 (d), 120.0 (d), 120.5 (s), 118.6 (d), 112.6 (d), 56.0 (q). Analysis calcd for $C_{14}H_{10}N_2O_3S$ (286.31): C, 58.73; H, 3.52; N, 9.78. Found: C, 58.90; H, 3.34; N, 9.74.

*4.1.1.13. 6-Cyano-2-(2-methoxyphenyl)benzothiazole (**6c**)*. According to **Method D**, bis(2-amino-5-cyanophenyl) disulfide (**3c**) (0.150 g, 0.5 mmol) and 2-methoxybenzaldehide (**4b**) (0.136 g, 1.0 mmol) were used and heated at 160–165 °C for 60 min, giving 0.210 g (78.8%) of pale yellow solid; mp = 177–179 °C. UPLC (230 nm): 98.4 area %; MS (ESI) *m/z*: 267.2 (M + H$^+$). ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 8.75 (d, 1H, *J* = 1.7 Hz, Ar-*H*), 8.47 (dd, 1H, *J* = 8.0 Hz, *J* = 1.6 Hz, Ar-*H*), 8.19 (d, 1H, *J* = 8.5 Hz, Ar-*H*), 7.92 (dd, 1H, *J* = 8.5 Hz, *J* = 1.7 Hz, Ar-*H*), 7.63 (m, 1H, Ar-*H*), 7.36 (d, 1H, *J* = 8.2 Hz, Ar-*H*), 7.21 (dd, 1H, *J* = 8.0 Hz, *J* = 7.4 Hz, Ar-*H*), 4.10 (s, 3H, −OC*H*$_3$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 166.6 (s), 157.4 (s), 153.9 (s), 135.8 (s), 133.4 (d), 129.3 (d), 128.8 (d), 127.3 (d), 123.1 (d), 121.2 (d), 120.4 (s), 119.0 (s), 112.7 (d), 106.7 (s), 56.2 (q). Analysis calcd for $C_{15}H_{10}N_2OS$ (266.32): C, 67.65; H, 3.78; N, 10.52. Found: C, 67.49; H, 3.84; N, 10.51.

*4.1.1.14. 6-Amidinium-(2-methoxyphenyl)benzothiazole methanesulfonate (**6d**)*. According to **Method E**, 2-amino-5-amidiniumbenzenethiolate (**2d**) (0.167 g, 1.0 mmol) and 2-methoxybenzaldehide (**4b**) (0.136 g, 1.0 mmol) were used giving 0.282 g (74.5%) of colorless solid; mp = 265–267 °C. UPLC (254 nm): 99.3 area %; MS (ESI) *m/z*: 284.2 [(M + H$^+$) calcd for free base $C_{15}H_{13}N_3OS$ (283.08)]. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 9.26 (bs, 4H, −C(N*H*$_2$)$_2^+$), 8.65 (s, 1H, Ar-*H*), 8.49 (d, 1H, *J* = 7.7 Hz, Ar-*H*), 8.25 (d, 1H, *J* = 8.9 Hz, Ar-*H*), 7.91 (d, 1H, *J* = 8.9 Hz, Ar-*H*), 7.64 (m, 1H, Ar-*H*), 7.37 (d, 1H, *J* = 8.2 Hz, Ar-*H*), 7.22 (m, 1H, Ar-*H*), 4.11 (s, 3H, −OC*H*$_3$), 2.37 (s, 3H, C*H*$_3$SO$_3^-$). ^{13}C NMR (150 MHz, DMSO-d_6) (δ ppm): 166.3 (s), 165.5 (s), 157.3 (s), 154.5 (s), 135.5 (s), 133.3 (d), 128.7 (d), 125.8 (d), 124.3 (s), 122.8 (d), 122.4 (d), 121.2 (d), 120.5 (s), 112.7 (d), 56.2 (q), 39.7 (q). Analysis calcd for $C_{16}H_{17}N_3O_4S_2$ (379.45): C, 50.64; H, 4.52; N, 11.07. Found: C, 50.39; H, 4.71; N, 11.21.

*4.1.1.15. 6-(4,5-Dihydro-1H-imidazol-3-ium-2-yl)-2-(2-methoxyphenyl) benzothiazole methanesulfonate (**6e**)*. According to **Method E**, 2-amino-5-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)benzenethiolate hydrate (**2e**) (0.211 g, 1.0 mmol) and 2-methoxybenzaldehide (**4b**) (0.136 g, 1.0 mmol) were used giving 0.336 g (83.0%) of colorless solid; mp = 285–289 °C. UPLC (254 nm): 99.3 area %; MS (ESI) *m/z*: 310.2 [(M + H$^+$) calcd for free base $C_{17}H_{15}N_3OS$ (309.39)]. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 10.57 (s, 2H, −C(N*H*−)$_2^+$), 8.74 (d, 1H, *J* = 1.2 Hz, Ar-*H*), 8.49 (dd, 1H, *J* = 8.0 Hz, *J* = 1.2 Hz, Ar-*H*), 8.30 (d, 1H, *J* = 8.6 Hz, Ar-*H*), 8.03 (dd, 1H, *J* = 8.6 Hz, *J* = 1.4 Hz, Ar-*H*), 7.65 (m, 1H, Ar-*H*), 7.37 (d, 1H, *J* = 8.4 Hz, Ar-*H*), 7.22 (m, 1H, Ar-*H*), 4.12 (s, 3H, −OC*H*$_3$), 4.07 (s, 4H, −C*H*$_2$C*H*$_2$−), 2.32 (s, 3H, C*H*$_3$SO$_3^-$). ^{13}C NMR (150 MHz, DMSO-d_6) (δ ppm): 166.9 (s), 164.9 (s), 157.5 (s), 154.9 (s), 135.8 (s), 133.6 (d), 128.8 (d), 126.0 (d), 123.2 (d), 122.9 (d), 121.2 (d), 120.4 (s), 118.1 (s), 112.8 (d), 56.2 (q), 44.5 (t, 2C), 39.7 (q). Analysis calcd for $C_{18}H_{19}N_3O_4S_2$ (405.49): C, 53.32; H, 4.72; N, 10.36. Found: C, 53.33; H, 4.81; N, 10.29.

*4.1.1.16. 2-(2-Methoxyphenyl)benzothiazole-6-ammonium methanesulfonate (**6f**).* According to **Method F**, 2-(2-methoxyphenyl)-6-nitrobenzothiazole (**6b**) (0.286 g 1.0 mmol) was used and heated to reflux for 30 min. Afterwards, the obtained crude amine was suspended in 2-propanol (10 ml), methanesulfonic acid (0.065 ml, 1.0 mmol) was added and stirred at room temperature for 2 h. After cooling overnight, the resulting precipitate was filtered, washed with diethyl-ether and dried at 75 °C giving 0.288 g (81.6%) of colorless solid; mp = 229–232 °C (dec.). UPLC (230 nm): 100 area %; MS (ESI) *m/z*: 257.2 [(M+H$^+$) calcd for free amine $C_{14}H_{12}N_2OS$, 256.07]. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 8.42 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, Ar-*H*), 8.08 (d, 1H, *J* = 8.7 Hz, Ar-*H*), 7.97 (d, 1H, *J* = 2.0 Hz, Ar-*H*), 7.58 (m, 1H, Ar-*H*), 7.38 (dd, 1H, *J* = 8.7 Hz, *J* = 2.1 Hz, Ar-*H*), 7.33 (d, 1H, *J* = 8.3 Hz, Ar-*H*), 7.18 (m, 1H, Ar-*H*), 4.07 (s, 3H, −OCH_3), 2.35 (s, 3H, C$H_3SO_3^-$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ ppm): 162.9 (s), 157.0 (s), 150.0 (s), 136.4 (s), 132.7 (d), 130.6 (s), 128.5 (d), 123.3(d), 121.2 (d), 121.1 (d), 120.8 (s), 115.1 (d), 112.7 (d), 56.1 (q), 39.8 (q). Analysis calcd for $C_{15}H_{16}N_2O_4S_2$ (352.43): C, 51.12; H, 4.58; N, 7.95. Found: C, 51.04; H, 4.67; N, 8.09.

4.2. Antibacterial activity

Determination of minimal inhibitory concentrations (MICs) was performed according to guidelines of the Clinical Laboratory Standards Institute [49]. Testing was performed by the standard broth microdilution method with azithromycin as the reference antibiotic. Bacterial strains used as the primary screening panel included two strains of Gram-negative species, *Esherichia coli* (TolC-) (efflux pump deficient strain ECM1556 tolC:Tn10) and *Moraxella catarrhalis* (ATCC 23246), and two fully sensitive strains of Gram-positive species *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212). Bacteria were grown on appropriate agar plates: Mueller-Hinton agar with 5% sheep blood for enterococci and *M. catarrhalis* and Mueller Hinton agar for staphylococci and *E. coli*. Compounds were tested as double dilutions (concentration range 128–0.25 μg/ml) in 96-well microtiter plates. Bacterial inocula were prepared by direct colony suspension method and plates inoculated with 5 × 10^4 cfu/well. Results were determined by visual read-out after overnight incubation at 37 °C in ambient air.

4.3. Antiproliferative activity

4.3.1. Cell culturing

The cell lines HeLa (cervical carcinoma), SW 620 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A549 (lung carcinoma) and HFF (human skin fibroblasts), were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

4.3.2. Proliferation assays

The panel cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at 5000 cells per well according to the doubling times of specific cell line. Test compounds and control compounds (5-fluorouracile) were then added in five 10-fold dilutions (0.01–100 μM) followed by a 72-h incubation. Working dilutions were freshly prepared on the day of testing in the growth medium. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in the working concentrations (DMSO concentration never exceeded 0.1%). After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay: experimentally determined absorbance values were transformed into a cell percentage growth (PG) using the formulas proposed by NIH and described previously [50]. This method directly relies on comparison with the control cells number at the day of assay because it compares the growth of treated cells with the growth of untreated cells in control wells on the same plate − the results are therefore a percentile difference from the calculated expected value. The IC_{50} values for each compound were calculated from dose-response curves using linear regression analysis by fitting the mean test concentrations that give PG values above and below the reference value. If, however, all of the tested concentrations produce PGs exceeding the respective reference level of effect (*e.g.* PG value of 50) for a given cell line, the highest tested concentration is assigned as the default value (in the screening data report that default value is preceded by a " > " sign). Each test point was performed in quadruplicate in three individual experiments. Finally, the effects of the tested substances were evaluated by plotting the mean percentage growth for each cell type in comparison to control on dose response graphs.

4.4. Antioxidative activity

4.4.1. Determination of the reducing activity of the stable 1,1-diphenylpicrylhydrazyl radical (DPPH)

Reducing activity was measured according to previously reported procedure with minor modification [51]. To solution of DPPH (final concentration 100 μM) in absolute ethanol was added equal volume of tested compounds various concentrations dissolved in DMSO. The compounds were dissolved in DMSO and tested as double dilutions (concentration range 10 mM – 0.6 μM) in 96-well microtiter plates in triplicates Ethanol and DMSO was also tested by adjusting the concentration and used as control and sample blank was also performed. Absorbance was recorded after 30 min in dark at room temperature at 517 nm on microplate reader μQuant (Biotec Inc.). All measurements were done in triplicate. The results were averaged and are presented in Table 3.

The free radical scavenging activity was calculated using the following formula:

[(Ac − As)/Ac] × 100, where *As* is absorbance of DPPH with sample and *Ac* is absorbance of DPPH without sample containing only control substances. The IC_{50} values for each compound were calculated from dose-response curves using nonlinear regression analysis by using GraphPad Prism 8 Ink. program.

4.4.2. Determination of ferric reducing/antioxidant power (FRAP assay)

The previously reported FRAP assay procedure on a 96-well microplate was applied with minor modifications [52], where antioxidant capacity of the tested compounds represent their power to reduce a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to the ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex at low pH, which is simple, fast and reproducible. A solution of 10 mM Fe^{3+}-TPTZ and 20 mM ferric chloride was diluted in 300 mM sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Solution of tested compound (20 μl) was added to the 96-well microplate followed by working FRAP solution (280 μl). The mixture was shaken and incubated for 30 min at 37 °C in the dark. Final concentrations of tested compound were 0.005 mM. The absorbance at 593 nm was recorded using microplate reader μQuant (Biotec Inc.). Ferrous sulphate ($FeSO_4{\cdot}7H_2O$) was used to develop a 20–2000 μmol/l standard curve for FRAP assay. All results were then expressed as Fe^{2+} equivalents (Fe^{2+} μmol). All tests were done in triplicate. The results were averaged and are presented in Table 3.

4.4.3. Free radical scavenging ability by the use of a stable ABTS radical cation (2,20 azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)

The total antioxidant activity was measured by Trolox Equivalent Antioxidant Capacity (TEAC) method [39] and was modified for microplate reader. For the standard TEAC assay, $ABTS^{\bullet+}$ was prepared by mixing an ABTS stock solution (7 mM in water) with 2.45 mM potassium persulphate. The mixture was allowed to stand for 16 h at room temperature in the dark until reaching a stable oxidative state. On the day of analysis, the $ABTS^{\bullet+}$ solution was diluted with methanol to an

absorbance of 0.70 ± 0.0 at 734 nm. The radical was stable in this form for more than two days when stored in the dark at room temperature. Standards and solutions of tested compounds (20 μl) were mixed with working $ABTS^{\cdot+}$ radical cation solution (280 μl) in each well of the microplate, shaken and incubated at room temperature for 5 min. The decrease of absorbance at 734 nm was recorded by μQuant (Biotec Inc.). All measurements were done in triplicate. The results were averaged and are presented in Table 3.

The free radical scavenging ability was calculated using the following formula:

[(Ac − As)/Ac] × 100, where *As* is absorbance of $ABTS^{\cdot+}$ with sample and *Ac* is absorbance of $ABTS^{\cdot+}$ without sample containing only control substances. The IC_{50} values for each compound were calculated from dose-response curves using nonlinear regression analysis by using GraphPad Prism 8 Ink. program.

4.5. Western blot analysis

The HeLa cells were seeded in six well plate, 3×10^5 cells/well, and treated with compounds **5c**, **5f, 6c** and **6f** at concentrations of IC_{50} and $2 \times IC_{50}$ values for 48 h respectively. Protein lysates were prepared using a buffer containing 50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease and phosphatase inhibitor cocktail (Roche, Switzerland). A total of 50 μg of proteins were resolved on 10% SDS polyacrylamide gels using the Mini-protean cell (Bio-Rad, USA). The membranes were incubated with primary antibodies raised against hydroxy-HIF-1α (Pro564) (HIF-1α, 1:1000, rabbit mAb, Cell Signaling Technology, NL) at 4 °C overnight. Secondary antibody linked to anti-mouse (1:1000, Dako, USA) was used. The signal was visualized by Western Lightening Chemiluminescence Reagent Plus Kit (Perkin Elmer, USA) on the ImageQuant LAS500 (GE Healthcare, USA) and α- tubulin (1:1000, mouse mAb, Sigma, USA) was used as a loading control. The signal intensities of particular bands were normalized with the intensity of the loading control and compared in Quantity One software (Bio-Rad, USA). The values are expressed as the average ± SEM. Differences in protein relative expression status obtained by Western blot analysis were analyzed by single One-way ANOVA ($p < 0.05$) in Microsoft Office Excel.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103537.

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