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Synthesis and antiproliferative activity of N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles

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Abstract—A number of N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles were synthesized and evaluated for their antiproliferative activities. The panel substitution included alkyl, aryl, and morpholinoalkyl derivatives. The structures of compounds were identified from elemental, IR, ^{1}H NMR, ^{13}C NMR and MS spectra analyses. The cytotoxicity in vitro against the four human cell lines: SW707 (rectal), HCV29T (bladder), A549 (lung), and T47D (breast) was determined. Alkyl and morpholinoalkyl derivatives exhibited significantly lower effect than phenyl ones. The highest antiproliferative activity was found for 2-(2,4-dichlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole, with ID_{50} two times lower (SW707, T47D) than for cisplatin studied comparatively as the control compound.

1. Introduction

The antitumor activities of 2-amino-1,3,4-thiadiazole (ATDA, NSC-4728) and the related compounds: 2-ethylamino-1,3,4-thiadiazole (EATDA), 2,2′-(methylenediamino)bis-1,3,4-thiadiazole (NSC-143019) were found in several experimental tumor systems about 50 years ago.[1] SAR studies showed that substitution of the amino group by alkyl or acetyl in ATDA led to less active compounds in in vitro conditions; the corresponding phenyl compound was inactive.[2] 5-Hydroxyderivative was active only against some tumors but the mercapto- and 5-chloro-analogues were not.[3]

Inosine 5′-phosphate (IMP) dehydrogenase is a site of action of aminothiadiazole metabolites. They block the formation of guanine nucleotides from IMP which is connected with equal and concomitant inhibition of DNA and RNA syntheses not affecting the protein synthesis significantly. Inhibition is reversed completely by nicotinamide, guanosine, deoxyguanosine, and partially by xanthosine. Other nucleosides did not influence the inhibition.[4]

2-Amino-1,3,4-thiadiazole (ATDA), as the most promising compound, was used in phase II clinical trial in patients with different tumors: renal,[5] colon,[6] ovarian,[7] and others.[8] However, due to marked hyperuricemia as well as painful stomatitis, its clinical applicability was limited.[9] Nicotinamide was found both to prevent these clinically limiting toxic effects and to suppress antitumor properties.[4b] Recently new derivatives with 1,3,4-thiadiazole nucleus[10] as well as Fe(II)/Fe(III) complexes of 2-amino-1,3,4-thiadiazoles[11] have been synthesized and evaluated for their antiproliferative activity against the panel of human cancer cell lines.

A wide spectrum of antiproliferative activity of compounds with the thiadiazole ring, modified by the type of substituent, switched our search to new, not reported in the literature, differently N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles. The early studies of compounds with 2,4-dihydroxyphenyl moiety of strong biological activity[12] pointed to relatively low toxicity of the compounds substituted in this way in relation to the analogues, presented in the literature (acute toxicity, cytotoxicity).[13] This may indicate low toxicity of the presented group of compounds and

Keywords: 2-Amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles; Sulfinylbis(2,4-dihydroxythiobenzoyl); Synthesis; Antiproliferative activity; ID_{50} values.
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secure successful subsequent stages of biological investigations.

The aim of this paper was the synthesis of N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles and the investigation of their antiproliferative effect in vitro against the cells of various human tumor cell lines.

2. Results

N-Substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles (**1**–**17**) were obtained according to Fig. 1. They were prepared by the reaction of sulfinylbis(2,4-dihydroxythiobenzoyl) (**STB**) with commercially available thiosemicarbazides in the endocyclizing process.[14,15] Purity of the compounds was monitored by reversed-phase (RP-18) HPLC chromatography (methanol-water).

All these derivatives were characterized by spectral and elemental analysis data which confirmed their structures. The data show a band in the range 7.6–10.0 ppm characteristic of NH proton (lower values for aliphatic N-substituents, higher for aryl ones), at about 9.5–10.6 and 10.2–11.0 ppm of 4C–OH and 2C–OH protons in the resorcinol moiety, respectively. There are characteristic signals at 163–168 and 155–156 ppm for C-2 and C-5 of 2-aminothiadiazole ring in ^{13}C NMR.[15,16] Mass spectra of compounds gave molecular ion peaks but with different intensities. The major fragmentation pathway in most derivatives involved the cleavage of the S–C_5 and N–N bonds of the 1,3,4-thiadiazole ring with elimination of 135 *m/z* ion and ions of mass depending on the substitution type of N-aryl ring (168 *m/z* for compound **8**), similar to that proposed by Karakuş and Rollas.[17] The cleavage of C_5–N_4 and S–C_2 bonds was also observed (153 *m/z*). The mass fragmentation pathway of compound **8** is shown in Figure 2.

The antiproliferative activity of synthesized compounds has been evaluated against four different human cancer cell lines (Table 1). Cisplatin was used as a reference drug. The cytotoxic activity in vitro was expressed as ID_{50} (μg/mL), the concentration of compound that inhibits proliferation rate of the tumor cells by 50% as

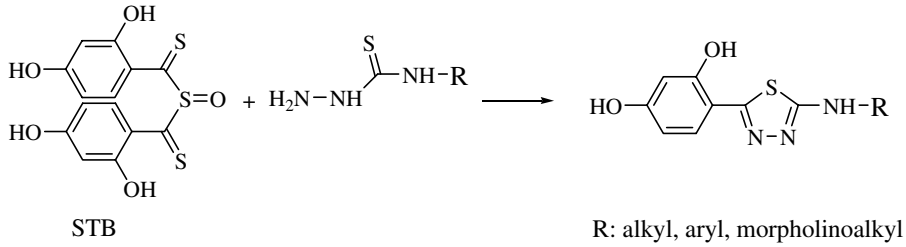


Figure 1. Synthesis scheme of N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles.

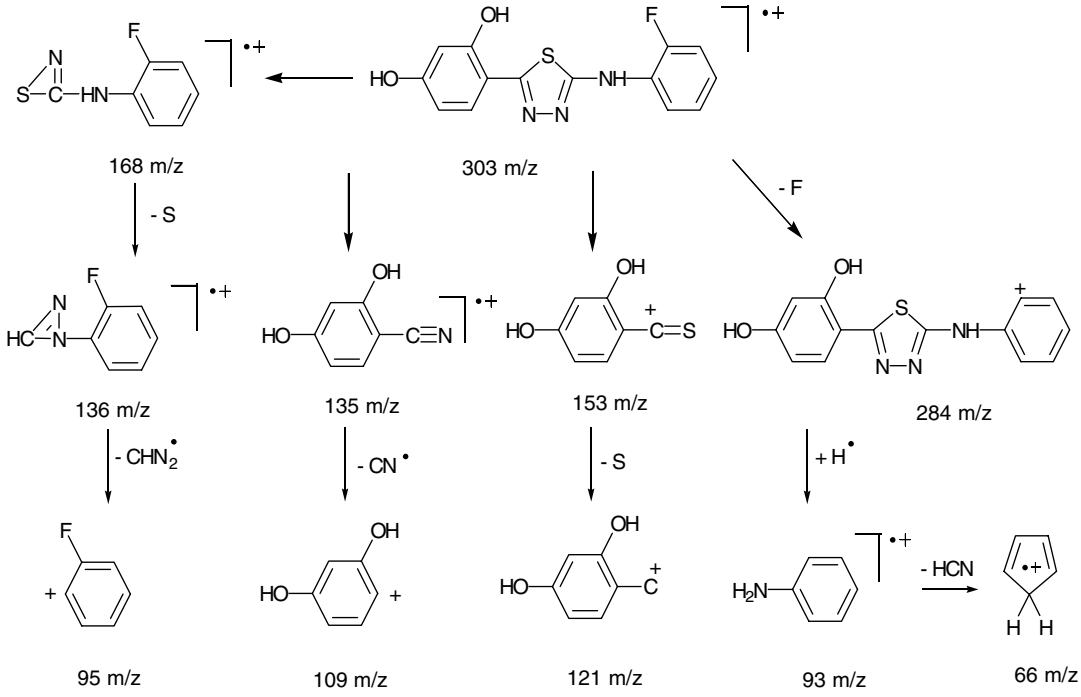
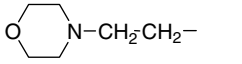
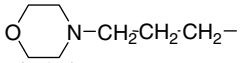


Figure 2. The mass fragmentation pathway of N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles (compound **8**).

Table 1. Structure and antiproliferative activity of N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles

Compound	Substituent	Cell line/ ID_{50}[a] (μg/mL)			
		HCV29T	A549	SW707	T47D
1	CH_3–	54.7 ± 1.1	40.0 ± 8.6	34.7 ± 5.9	32.79 ± 6.8
2	$(CH_3)_3C$–	23.8 ± 7.0	44.1 ± 12.7	33.2 ± 0.7	43.5 ± 23.6
3	CH_2=$CHCH_2$–	58.8 ± 1.1	47.9 ± 14.4	41.6 ± 3.5	27.2 ± 1.1
4	C_6H_5–	4.2 ± 1.2	8.9 ± 1.3	4.3 ± 1.2	3.8 ± 1.2
5	2-CH_3–C_6H_4–	27.9 ± 2.2	19.2 ± 5.2	16.1 ± 1.4	6.0 ± 0.7
6	2,6-CH_3–C_6H_3–	32.8 ± 4.8	33.5 ± 7.1	28.4 ± 2.7	16.9 ± 4.1
7	4-C_2H_5–C_6H_4–	7.6 ± 0.1	12.2 ± 2.6	5.8 ± 1.1	4.9 ± 0.2
8	2-F–C_6H_4–	10.9 ± 4.6	6.35 ± 1.5	17.54 ± 8.8	5.78 ± 1.1
9	3-F–C_6H_4–	6.9 ± 1.6	5.6 ± 1.0	8.5 ± 1.3	5.2 ± 1.8
10	4-F–C_6H_4–	6.2 ± 1.4	NEG[b]	3.6 ± 1.1	4.2 ± 1.2
11	2-Cl–C_6H_4–	27.8 ± 8.3	25.0 ± 6.8	11.8 ± 2.4	5.6 ± 0.5
12	3-Cl–C_6H_4–	5.4 ± 1.8	24.1 ± 7.5	3.7 ± 1.1	3.9 ± 1.1
13	4-Cl–C_6H_4–	19.1 ± 6.2	6.9 ± 1.6	5.4 ± 0.3	4.1 ± 0.5
14	2,4-Cl–C_6H_3–	22.8 ± 8.1	5.3 ± 2.7	2.8 ± 2.6	1.5 ± 1.3
15	4-C_6H_5–O–C_6H_4–	9.4 ± 3.2	20.2 ± 4.5	16.1 ± 2.6	5.4 ± 1.1
16		25.4 ± 6.4	38.3 ± 2.5	33.0 ± 1.4	18.2 ± 7.1
17		19.6 ± 10.9	39.1 ± 2.5	27.5 ± 1.9	19.5 ± 3.4
18	Cisplatin	0.7 ± 1.5	3.3 ± 1.4	4.9 ± 1.5	6.2 ± 1.5

[a] ID_{50} (μg/mL) indicates the compound concentration that inhibits the proliferation rate of tumor cells by 50% as compared to the control untreated cells. The values are means ± SD of nine independent experiments.
[b] NEG, negative in the studied concentrations (up to 100 μg/mL).

compared to the control untreated cells. The results of substance screening are summarized in Table 1. Antiproliferative activity in vitro of the presented compounds is varied. Derivatives **4**, **10**, **12**, and **14** proved to be the most active. Some of them meet the cytotoxic activity criterion ($ID_{50} \leqslant 4$ μg/mL), namely compounds **12** and **14** against T47D and SW707 cells, compound **4** against T47D, and **10** against SW707 cells.[18] Their activity is higher or comparable to that of referential cisplatin. Compound **10** is characterized by some selectivity. With high activity against SW707 and T47D cell lines no activity was observed for A549 cell line with the studied concentrations.

3. Discussion

In terms of structure–activity relationships, we observed poor activity of N-aliphatic derivatives (**1**–**3**), independent of saturation extent of the chain or its branching. Antiproliferative properties of phenyl derivatives (**4**–**15**) are relatively higher, though they are dependent on N-aromatic ring-substitution degree. The parent compound (**4**) revealed high antiproliferative activity of all lines applied. Substitution of phenyl ring with the lipophilic electron-donating substituents (**5**, **6**, **7**) does not affect favorably the activity in comparison with starting system **4**. However, fluoro- and chloro-derivatives (**8**–**14**) are the most interesting analogues, that is, the systems with the hydrophobic substituents ($\pi > 0$) of electron-withdrawing character ($\sigma > 0$). Substitution of the ring with a fluorine atom in the *para*-position (**10**), chlorine in the *meta*-position (**12**) or two chlorine atoms in positions 2 and 4 (**14**) seems to be particularly interesting. The presence of electron-acceptor substituents in the ring causes formation of electron-deficient N-aryl ring which gives probably a favorable effect in the ligand and receptor interactions. 4-Phenoxy derivative (**15**) exhibits a medium level of activity. However, N-substitution by morpholinoalkyl groups (**16**, **17**) does not promote antiproliferative activity.

The presented results indicate 2-amino-1,3,4-thiadiazole functions as pharmacophore of antiproliferative activity. However, 2,4-dihydroxyphenyl moiety in position 5 also plays a significant role. Literature reports lack the information of antitumor activity of 2-phenylamino-1,3,4-thiadiazole.[2] However, additional substitution in position 5 with 2,4-dihydroxyphenyl provides antiproliferative activity in vitro comparable to that of cisplatin. Probably, besides a favorable hydrophobic–hydrophilic character shown by molecules due to the presence of this substituent, it significantly affects electronic properties important in compound biological target(s) interactions responsible for antiproliferative effect. The effects of hydrogen bonds, both intra- and intermolecular, cannot be excluded.

The presented results indicate high level of antiproliferative activity of some N-substituted 2-amino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazoles. Taking into account relatively low toxicity of compounds with 2,4-dihydroxyphenyl moiety compared to the analogues presented in the literature, this group of compounds seems to be very interesting. Therefore, the investigations including design, synthesis, and analysis of subsequent derivatives as well as extension of antitumor activity studies will be continued.

4. Materials and methods

4.1. Analytical studies

The melting point was determined on a Sanyo melting point apparatus. The elemental analysis was performed in order to determine C, H, and N contents (Perkin-Elmer 2400). The analyses (C, H, and N) were within ±0.4% of the theoretical values. The vibrational spectra were recorded with a Perkin-Elmer FT-IR 1725X spectrophotometer using KBr pellets in the range of 600–4000 cm^{-1}. ^1H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Varian–Gemini 200, a Varian Mercury 400 or a Bruker DRX 500 instrument. Chemical shifts (δ, ppm) were given with tetramethylsilane (TMS). The MS spectra (EI, 70 eV) were run on a AMD-604 apparatus.

4.2. Synthesis

Sulfinylbis(2,4-dihydroxythiobenzoyl) (**STB**) as the starting reagent was obtained from 2,4-dihydroxybenzenecarbodithioic acid and $SOCl_2$ in diethyl ether.[14]

A mixture of **STB** (0.01 mol) and 3-thiosemicarbazide (0.02 mol) in methanol (50 mL) was refluxed for 3 h. The reaction mixture was hot filtered. The further procedure for individual compounds is described in detail.

4.2.1. 2-Methylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (1). Reaction of 4-methyl-3-thiosemicarbazide (Lancaster). The filtrate was concentrated to dryness. The resulted product was crystallized from aqueous (5:3) methanol (85 mL). Yield: 74%; mp: 224–226 °C. ^1H NMR (500 MHz, DMSO-d_6, δ): 2.90–2.91 (d, 3H, CH_3), 6.35–6.37 (dd, J = 8.5 and 2.3 Hz, 1H, C_5–H), 6.38 (d, J = 2.3 Hz, 1H, C_3–H), 7.52–7.54 (d, J = 8.5 Hz, 1H, C_6–H), 7.64 (s, 1H, NH), 9.81 (s, 1H, C_4–OH), 11.04 (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3387 (OH, NH), 1631 (C=N, C=C), 1182 (C–OH), 1045 (N=C–S–C=N), 658 (C–S–C); EI-MS (*m*/*z*, %): 223 (M^+, 100), 194 (5), 153 (13), 136 (9), 135 (8), 121 (4), 94 (8), 88 (52), 74 (10), 69 (7), 52 (5). Anal. Calcd for $C_9H_9N_3O_2S$ (223.25): C, 48.42; H, 4.06; N, 18.82. Found: C, 48.65; H, 4.04; N, 18.74.

4.2.2. 2-*tert*-Butylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (2). Reaction of 4-*tert*-butyl-3-thiosemicarbazide (Lancaster). During the reflux there was precipitated a product which was filtered off, washed with water, and crystallized from aqueous (1:1) methanol (60 mL). Yield: 64%; mp: 109–111 °C. ^1H NMR (500 MHz, DMSO-d_6, δ): 1.40 (s, 9H, CH_3), 6.37–6.39 (dd, J = 8.6 and 2.3 Hz, 1H, C_5–H), 6.42 (d, J = 2.3 Hz, 1H, C_3–H), 7.53–7.54 (d, J = 8.6 Hz, 1H, C_6–H), 7.95 (s, 1H, NH), 9.48 (s, 1H, C_4–OH), 11.1 (br band, 1H, C_2–OH); IR (KBr, cm^{-1}): 3433, 3308 (OH, NH), 2920, 2860 (CH_3), 1665 (C=N), 1506 (C=C), 1219 (C–OH), 675 (C–S–C); EI-MS (*m*/*z*, %): 265 (M^+, 40), 250 (4), 209 (100), 153 (15), 136 (40), 74 (14), 57 (10). Anal. Calcd for $C_{12}H_{15}N_3O_2S$ (265.34): C, 54.32; H, 5.70; N, 15.84. Found: C, 54.12; H, 5.72; N, 15.80.

4.2.3. 2-Allylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (3). Reaction of 4-allyl-3-thiosemicarbazide (Lancaster). Water (100 mL) was added to the filtrate. The precipitated product was filtered off and crystallized from methanol (50 mL). Yield: 61%; mp: 104–106 °C. ^1H NMR (400 MHz, DMSO-d_6, δ): 3.57–4.42 (m, 2H, CH_2), 5.03–5.41 (m, 2H, CH_2CH), 5.80–5.98 (m, 1H, CH_2CH), 6.42–6.44 (dd, J = 8.6 and 2.2 Hz, 1H, C_5–H), 6.57 (d, J = 2.2 Hz, 1H, C-3), 7.72–7.74 (d, J = 8.6 Hz, 1H, C_6–H), 8.20 (s, 1H, NH), 9.96 (s, 1H, C_4–OH), 10.23 (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3249 (OH, NH), 2924 (C_{alif}–H), 1613 (C=N, C=C), 1179 (C–OH), 1040 (N=C–S–C=N), 666 (C–S–C); EI-MS (*m*/*z*, %) 249 (M^+, 99), 234 (17), 153 (52), 135 (30), 131 (41), 121 (7), 114 (72), 109 (5), 97 (14), 81 (58), 69 (21), 56 (88), 45 (22), 41 (100), 39, 36. Anal. Calcd for $C_{11}H_{11}N_3O_2S$ (249.29): C, 53.00; H, 4.45; N, 16.86. Found: C, 53.21; H, 4.47; N, 16.90.

4.2.4. 2-Phenylamino-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (4). The compound was described previously.[15]

4.2.5. 2-(2-Methylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (5). Reaction of 4-(2-methylphenyl)-3-thiosemicarbazide (Lancaster). Water (50 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from aqueous (2:1) methanol (60 mL). Yield: 75%; mp: 219–220 °C. ^1H NMR (500 MHz, DMSO-d_6, δ): 2.29 (s, 3H, CH_3), 6.38–6.40 (dd, J = 8.5 and 2.3 Hz, 1H, C_5–H), 6.42 (d, J = 2.3 Hz, 1H, C_3–H), 7.01–7.04 (m, 1H, J = 7.7 Hz, Ar′-H), 7.20–7.23 (t, 2H, J = 7.8 Hz, Ar′-H), 7.71–7.73 (d, J = 8.5 Hz, 1H, C_6–H), 7.88–7.90 (d, J = 7.8 Hz, 1H, Ar′-H), 9.33 (s, 1H, NH), 9.86 (s, 1H, C_4–OH), 10.84 (s, 1H, C_2–OH); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 17.94 (CH_3), 102.48 (C-3), 108.04 (C-5), 108.54 (C-1), 121.08 (C-6′), 123.58 (C-4′), 126.56 (C-3′), 128.60 (C-6), 128.88 (C-2′), 130.60 (C-5′), 139.36 (C-1′), 155.30 (C_{thia}-5), 155.78 (C-4), 160.10 (C-2), 165.48 (C_{thia}-2); IR (KBr, cm^{-1}): 3361 (OH, NH), 2932 (CH_3), 1629 (C=N), 1591 (C=C), 1183 (C-OH), 1093 (N=C–S–C=N), 672 (C–S–C); EI-MS (*m*/*z*, %): 299 (M^+, 100), 284 (11), 266 (6), 164 (46), 153 (8), 136 (6), 135 (4), 131 (24), 121 (2), 117 (3), 105 (5), 91 (4), 39 (3). Anal. Calcd for $C_{15}H_{13}N_3O_2S$ (299.35): C, 60.18; H, 4.38; N, 14.04. Found: C, 59.90; H, 4.40; N, 13.98.

4.2.6. 2-(2,6-Dimethylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (6). Reaction of 4-(2,6-dimethylphenyl)-3-thiosemicarbazide. Water (50 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from aqueous (2:1) methanol (60 mL). Yield: 77%; mp: 198–200 °C. ^1H NMR (400 MHz, DMSO-d_6, δ): 2.23 (s, 6H, CH_3), 6.37–6.40 (dd, J = 8.7 and 2.4 Hz, 1H, C_5–H), 6.46 (d, J = 2.4 Hz, 1H, C_3–H), 7.04–7.11 (m, 1H, $C_{4'}$–H), 7.17–7.18 (m, 2H, $C_{3',5'}$–H), 7.67–7.69 (d, J = 8.7 Hz, 1H, C_6–H), 9.50 (m, 1H, NH), 10.04 (br band, 1H, C_4–OH), 10.53 (s, 1H, C_2–OH); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 17.58 (2C, CH_3), 102.94 (C-3), 108.29 (C-5), 108.75 (C-1), 127.49 (C-4′), 128.36 (C-6), 128.61 (C-3′, 5′), 135.52 (C-2′, 6′), 136.48 (C-1′),

154.39 (C_{thia}-5), 156.08 (C-4), 160.61 (C-2), 167.61 (C_{thia}-2); IR (KBr, cm^{-1}): 3686 (OH, NH), 2919 (CH_3), 1601 (C=N, C=C), 1206 (C–OH), 651 (C–S–C); EI-MS (*m*/*z*, %): 313 (M^+, 64), 298 (20), 284 (4), 280 (6), 237 (12), 187 (3), 178 (100), 163 (25), 151 (21), 145 (78), 131 (20), 119 (40), 103 (14), 91 (25), 79 (18), 77 (34), 65 (15), 51 (14), 36 (23). Anal. Calcd for $C_{16}H_{15}N_3O_2S$ (313.38): C, 61.32; H, 4.82; N, 13.41. Found: C, 61.15; H, 4.80; N, 13.47.

4.2.7. 2-(4-Ethylphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (7). Reaction of 4-(4-ethylphenyl)-3-thiosemicarbazide (Lancaster). Water (50 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from aqueous (2:1) methanol (45 mL). Yield: 82%; mp: 234–236 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 1.14-1.19 (t, J = 7.5 Hz, 3H, CH_3), 2.54–2.59 (q, J = 7.5 Hz, 2H, CH_2), 6.39–6.41 (dd, J = 8.6 and 2.3 Hz, 1H, C_5–H), 6.43 (d, J = 2.2 Hz, 1H, C_3–H), 7.17–7.19 (d, J = 8.6 Hz, 2H, $C_{2',6'}$-H), 7.53–7.55 (d, J = 8.6 Hz, 2H, $C_{3',5'}$–H), 7.77–7.79 (d, J = 8.6 Hz, 1H, C_6–H), 9.90 (s, 1H, NH), 10.17 (s, 1H, C_4–OH), 10.88 (s, 1H, C_2–OH); ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 15.29 (CH_3), 27.01 (CH_2), 101.97 (C-3), 107.60 (C-5), 108.01 (C-1), 116.93 (C-2′,6′), 127.76 (C-3′,5′), 128.11 (C-6), 136.50 (C-4′), 138.20 (C-1′), 154.13 (C_{thia}-5), 155.22 (C-4), 159.70 (C-2), 163.19 (C_{thia}-2); IR (KBr, cm^{-1}): 3404, 3252, 3192 (OH, NH), 2963 (C_{alif}–H), 1623 (C=N, C=C), 1217 (C-OH), 1133, 1018 (N=C–S–C=N), 677 (C–S–C); EI-MS (*m*/*z*, %): 313 (M^+, 100), 298 (27), 178 (6), 163 (18), 150 (5), 131 (5), 94 (4). Anal. Calcd for $C_{16}H_{15}N_3O_2S$ (313.38): C, 61.32; H, 4.82; N, 13.41. Found: C, 60.03; H, 4.80; N, 13.47.

4.2.8. 2-(2-Fluorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4- thiadiazole (8). Reaction of 4-(2-fluorophenyl)-3-thiosemicarbazide (Lancaster). Water (120 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from aqueous (1:1) methanol (50 mL). Yield: 79%; mp: 230–232 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 6.39–6.42 (dd, J = 8.5 Hz and 2.3 Hz, 1H, C_5–H), 6.44 (d, J = 2.2 Hz, 1H, C_3–H), 7.00–7.06 (m, 1H, Ar′-H), 7.12–7.29 (m, 2H, Ar′-H), 7.79–7.81 (d, J = 8.5 Hz, 1H, C_6–H), 8.42–8.44 (m, J = 8.4 and 1.3 Hz, 1H, Ar′-H), 9.91 (s, 1H, NH), 10.05 (s, 1H, C_4–OH), 10.88 (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3409, 3358, 3264 (OH, NH), 1622 (C=N), 1600 (C=C), 1246 (C–OH), 1034 (N=C–S–C=N), 675 (C–S–C); EI-MS (*m*/*z*, %): 303 (M^+, 100), 284 (40), 244 (8), 168 (39), 153 (15), 141 (9), 135 (16), 136 (24), 121 (8), 109 (2), 110 (13), 95 (8), 94 (18), 83 (14), 66 (11), 65 (6), 52 (8), 39 (11). Anal. Calcd for $C_{14}H_{10}FN_3O_2S$ (303.32): C, 55.44; H, 3.32; N, 13.85. Found: C, 55.18; H, 3.33; N, 13.79.

4.2.9. 2-(3-Fluorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (9). Reaction of 4-(3-fluorophenyl)-3-thiosemicarbazide (Lancaster). Water (100 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from methanol (80 mL). Yield: 76%; mp: 245–247 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 6.40–6.42 (dd, J = 8.7 and 2.4 Hz, 1H, C_5–H), 6.48 (d, J = 2.3 Hz, 1H, C_3–H), 6.74–6.81 (m, 1H, Ar′-H), 7.33–7.34 (m, 2H, Ar′-H), 7.71–7.74 (m, 1H, Ar′-H), 7.81–7.83 (d, J = 8.7 Hz, 1H, C_6–H), 9.99 (s, 1H, NH), 10.64 (s, 1H, C_4–OH), 11.04 (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3447, 3203 (OH, NH), 1623 (C=N, C=C), 1238 (C–OH), 1132 (C–F), 672 (C–S–C); EI-MS (*m*/*z*, %): 303 (M^+, 100), 274 (3), 168 (50), 153 (13), 141 (13), 135 (10), 136 (20), 121 (7), 109 (8), 95 (15), 94 (20), 83 (10), 66 (11), 52 (6), 39 (8). Anal. Calcd for $C_{14}H_{10}FN_3O_2S$ (303.32): C, 55.44; H, 3.32; N, 13.85. Found: C, 55.29; H, 3.31; N, 13.80.

4.2.10. 2-(4-Fluorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (10). Reaction of 4-(4-fluorophenyl)-3-thiosemicarbazide (Lancaster). Water was added to the filtrate (100 mL). The precipitated product was filtered off, washed with water, and crystallized from methanol (70 mL). Yield: 69%; mp: 279–280 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ): 6.42–6.43 (d, 1H, C-5), 6.44–6.45 (s, 1H, C_3–H), 7.00–7.08 (m, 1H, $C_{4'}$–H), 7.12–7.26 (m, 2H, $C_{2',6'}$–H), 7.61–7.70 (m, 2H, $C_{3',5'}$–H), 7.82–7.84 (d, 1H, C_6–H), 9.92 (s, 1H, NH), 10.27 (s, 1H, C_4–OH), 10.85 (s, 1H, C_2–OH); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 102.40 (C-3), 108.74 (C-5), 108.45 (C-1), 115.50 (C-2′, 6′), 118.83 (C-3′, 5′), 128.55 (C-6), 137.36 (C-1′), 154.78 (C_{thia}-5), 155.66 (C-4), 157.94 (C-4′), 160.23 (C-2), 163.57 (C_{thia}-2); IR (KBr, cm^{-1}): 3400, 3259, 3216 (OH, NH), 1629 (C=N), 1590 (C=C), 1231 (C–OH), 1134 (C–F), 1052 (N=C–S–C=N), 677 (C–S–C); EI-MS (*m*/*z*, %): 303 (M^+, 100), 168 (39), 135 (11), 136 (14), 121 (4), 109 (3), 110 (8), 95 (5), 94 (12), 83 (6), 66 (6). Anal. Calcd for $C_{14}H_{10}FN_3O_2S$ (303.32): C, 55.44; H, 3.22; N, 13.85. Found: C, 55.61; H, 3.23; N, 13.79.

4.2.11. 2-(2-Chlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (11). Reaction of 4-(2-chlorophenyl)-3-thiosemicarbazide (Alfa Aesar). The precipitated product was filtered off, washed with water, and crystallized from methanol (50 mL). Yield: 81%; mp: 217–218 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 6.39–6.41 (dd, J = 8.6 and 2.3 Hz, 1H, C_5–H), 6.43 (d, J = 2.3 Hz, 1H, C_3–H), 7.06–7.09 (t, J = 7.8 Hz, 1H, Ar′-H), 7.34–7.38 (t, J = 7.7 Hz, 1H, Ar′-H), 7.47–7.49 (dd, J = 7.9 and 1.4 Hz, 1H, Ar′-H), 7.77–7.78 (d, J = 8.6 Hz, 1H, C_6–H), 8.30–8.32 (d, J = 7.9 Hz, 1H, Ar′-H), 9.65 (s, 1H, NH), 9.88 (s, 1H, C_4–OH), 10.85 (s, 1H, C_2–OH); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 102.42 (C-3), 108.08 (C-5), 108.51 (C-1), 121.41 (C-6′), 122.63 (C′), 123.70 (C′), 127.80 (C′), 128.49 (C-6), 129.59 (C′), 137.49 (C′), 155.74 (C_{thia}-5), 156.17 (C-4), 160.30 (C-2), 164.08 (C_{thia}-2); IR (KBr, cm^{-1}): 3340, 3173 (OH, NH), 1600 (C=N, C=C), 1255 (C–OH), 1102 (C–Cl), 1041 (N=C–S–C=N), 680 (C–S–C); EI-MS (*m*/*z*, %): 319 (M^+, 100), 302 (4), 284 (95), 184 (28), 170 (4), 153 (13), 149 (50), 142 (9), 135 (10), 122 (9), 121 (5), 111 (5), 108 (5), 94 (11), 90 (6), 75 (6), 39 (9). Anal. Calcd for $C_{14}H_{10}ClN_3O_2S$ (319.77): C, 52.59; H, 3.15; N, 13.14. Found: C, 52.76; H, 3.16; N, 13.09.

4.2.12. 2-(3-Chlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (12). Reaction of 4-(3-chlorophenyl)-3-thiosemicarbazide (Lancaster). Water (100 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from aqueous (4:1) methanol (50 mL). Yield: 77%; mp: 265–266 °C. ^{1}H NMR (200 MHz, DMSO-d_6, δ): 6.42 (s, 1H, C_5–H), 6.52 (s, 1H, C_3–H), 6.97–7.05 (m, 1H, Ar′-H), 7.31–7.55 (m, 2H, Ar′-H), 7.81–7.84 (d, 1H, C_6–H), 7.92 (s, 1H, Ar′-H), 9.80–11.02 (br band, 3H, $C_{2,4}$–OH, NH); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 102.42 (C-3), 108.15 (C-5), 108.43 (C-1), 115.65 (C-6′), 116.57 (C-2′), 120.94 (C-4′), 128.50 (C-6), 130.55 (C-5′), 133.44 (C-3′), 142.16 (C-1′), 155.09 (C_{thia}-5), 155.66 (C-4), 160.40 (C-2), 163.10 (C_{thia}-2); IR (KBr, cm^{-1}): 3245 (OH, NH), 1626 (C=N), 1598 (C=C), 1225 (C–OH), 1110 (C–Cl), 677 (C–S–C); EI-MS (*m*/*z*, %): 319 (M^+, 100), 184 (36), 167 (5), 152 (5), 153 (10), 149 (16), 136 (8), 121 (5), 111 (6), 94 (10), 66 (5), 52 (3), 39 (4). Anal. Calcd for $C_{14}H_{10}ClN_3O_2S$ (319.77): C, 52.59; H, 3.15; N, 13.14. Found: C, 52.39; H, 3.13; N, 13.19.

4.2.13. 2-(4-Chlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (13). Reaction of 4-(4-chlorophenyl)-3-thiosemicarbazide (Lancaster). Water (100 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from aqueous (4:1) methanol (50 mL). Yield: 69%; mp: 249–251 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 6.40–6.42 (dd, J = 8.5 Hz and 2.3 Hz, 1H, C_5–H), 6.44 (d, J = 2.3 Hz, 1H, C_3–H), 7.37–7.39 (d, J = 8.9 Hz, 2H, $C_{2',6'}$–H), 7.67–7.70 (d, J = 8.9 Hz, 2H, $C_{3',5'}$–H), 7.79–7.80 (d, J = 8.6 Hz, 1H, C_6–H), 9.89 (s, 1H, NH), 10.37 (s, 1H, C_4–OH), 10.85 (s, 1H, C_2–OH); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 102.43 (C-3), 108.12 (C-5), 108.45 (C-1), 118.72 (C-2′,6′), 124.84 (C-4′), 128.55 (C-6), 128.81 (C-3′,5′), 139.77 (C-1′), 155.01 (C_{thia}-5), 155.68 (C-4), 160.32 (C-2), 163.21 (C_{thia}-2); IR (KBr, cm^{-1}): 3314, 3250 (OH, NH), 1625 (C=N), 1601 (C=C), 1221 (C–OH), 1105 (C–Cl), 1011 (N=C–S–C=N), 673 (C–S–C); EI-MS (*m*/*z*, %): 319 (M^+, 100), 184 (30), 167 (5), 152 (6), 153 (8), 149 (13), 135 (7), 121 (3), 111 (5), 94 (9), 75 (4), 66 (4), 39 (3). Anal. Calcd for $C_{14}H_{10}ClN_3O_2S$ (319.77): C, 52.59; H, 3.15; N, 13.14. Found: C, 52.38; H, 3.14; N, 13.20.

4.2.14. 2-(2,4-Dichlorophenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (14). Reaction of 4-(2,4-dichlorophenyl)-3-thiosemicarbazide (Lancaster). Water (100 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from methanol (70 mL). Yield: 77%; mp: 206–208 °C. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 6.39–6.42 (dd, J = 8.6 and 2.3 Hz, 1H, C_5–H), 6.47 (d, J = 2.2 Hz, 1H, C_3–H), 7.39–7.45 (m, 1H, Ar′-H), 7.60–7.63 (m, 1H, Ar′-H), 7.81–7.83 (d, J = 8.6 Hz, 1H, C_6–H), 8.41–8.45 (t, J = 8.8 Hz, 1H, Ar′-H), 9.50 (s, 1H, NH), 9.87 (m, 1H, C_4–OH), 10.93 (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3262, 3178, (OH, NH), 1620 (C=N), 1593 (C=C), 1240 (C–OH), 1097 (C–Cl), 1053 (N=C–S–C=N), 665 (C–S–C); EI-MS (*m*/*z*, %): 354 (M^+, 19), 353 (100), 321 (6), 318 (93), 302 (8), 218 (42), 219 (5), 207 (16), 187 (8), 183 (66), 172 (17), 161 (50), 153 (32), 146 (3), 137 (18), 135 (23), 121 (10), 109 (14), 94 (23), 90 (12), 63 (19), 52 (14), 39 (16); Anal. Calcd for $C_{14}H_9Cl_2N_3O_2S$ (354.22): C, 47.47; H, 2.56; N, 11.86. Found: C, 47.28; H, 2.57; N, 11.81.

4.2.15. 2-(4-Phenoxyphenylamino)-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (15). Reaction of 4-(4-phenoxyphenyl)-3-thiosemicarbazide (Aldrich). Water (100 mL) was added to the filtrate. The precipitated product was filtered off, washed with water, and crystallized from methanol (70 mL). Yield: 65%; mp: 87 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 6.38–6.41 (dd, J = 8.6 and 2.3 Hz, 1H, C_5–H), 6.42 (d, J = 2.2 Hz, 1H, C_3–H), 6.94–7.14 (m, 5H, Ar″-H), 7.34–7.40 (m, 2H, Ar′-H), 7.55–7.68 (m, 2H, Ar′-H), 7.76–7.78 (d, J = 8.6 Hz, 1H, C_6–H), 9.90 (s, 1H, NH), 10.26 (s, 1H, C_4–OH), 10.86 (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3198, 3307 (OH, NH), 1665 (C=N), 1583 (C=C), 1270 (C–O–C), 1162 (C–OH), 1021 (N=C–S–C=N), 693 (C–S–C); EI-MS (*m*/*z*, %): 377 (M^+, 100), 302 (9), 284 (6), 242 (16), 227 (8), 210 (9), 185 (6), 165 (5), 153 (5), 150 (8), 135 (7), 121 (3), 94 (10), 77 (15), 51 (6). Anal. Calcd for $C_{20}H_{15}N_3O_3S$ (377.42): C, 63.65; H, 4.01; N, 11.13. Found: C, 63.86; H, 4.00; N, 11.08.

4.2.16. 2-[2-(4-Morpholino)ethylamino]-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (16). The compound was described previously.[15]

4.2.17. 2-[3-(4-Morpholino)propylamino]-5-(2,4-dihydroxyphenyl)-1,3,4-thiadiazole (17). Reaction of 4-[3-(4-morpholino)propyl]-3-thiosemicarbazide (Lancaster). The product was precipitated after cooling off the filtrate. The precipitate was filtered off, washed with water, and crystallized from aqueous (1:1) methanol (40 mL). Yield: 61%; mp: 218–220 °C. ^{1}H NMR (500 MHz, DMSO-d_6, δ): 1.83–1.91 (m, 2H, CH_2), 3.26–3.28 (m, 8H, CH_2NH, CH_2N), 3.55–3.75 (m, 4H, OCH_2), 6.34–6.37 (dd, J = 8.5 and 2.3 Hz, 1H, C_5–H), 6.39 (d, J = 2.2 Hz, 1H, C-3), 7.55–7.56 (d, J = 8.4 Hz, 1H, C_6–H), 9.81 (s, 1H, NH), 9.97 (s, 1H, C_4–OH), 10.84. (s, 1H, C_2–OH); IR (KBr, cm^{-1}): 3395 (OH, NH), 2923 (CH_2), 1605 (C=N, C=C), 1258 (C–OH); EI-MS (*m*/*z*, %): 336 (M^+, 4), 302 (100), 250 (6), 236 (6), 223 (6), 209 (8), 167 (50), 153 (18), 135 (17), 136 (20), 127 (7), 112 (7), 109 (4), 54 (100), 86 (10), 76 (18), 64 (10), 56 (17), 44 (16). Anal. Calcd for $C_{15}H_{20}N_4O_3S$ (336.42): C, 53.55; H, 5.99; N, 16.65. Found: C, 53.77; H, 5.96; N, 16.69.

4.3. Antiproliferative assay in vitro

The following established in vitro human cell lines were applied: T47D (breast cancer), SW707 (rectal adenocarcinoma), and A549 (non-small cell lung carcinoma) from the American Type Culture Collection (Rockville, Maryland, USA) and HCV29T (bladder cancer) from Fibiger Institute, Copenhagen, Denmark. Twenty-four hours before the addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, USA) at a density of 10^4 cells/well. All cell lines were maintained in the

opti-MEM supplemented with 2 mM glutamine (Gibco, Warsaw, Poland), streptomycin (50 μg/mL), penicillin (50 U/mL) (Polfa, Tarchomin, Poland), and 5% fetal calf serum (Gibco, Grand Island, USA). The cells were incubated at 37 °C in the humid atmosphere saturated with 5% CO_2. The solutions of compounds (1 mg/mL) were prepared ex tempore by dissolving the substance in 100 μL DMSO completed with 900 μL of tissue culture medium. Afterwards, the compounds were diluted in the culture medium to reach the final concentrations ranging from 0.1 to 100 μg/mL. The solvent (DMSO) in the highest concentration used in the test did not reveal any cytotoxic activity. Cisplatin was applied as a test referential agent. The cytotoxicity assay was performed after 72 h exposure of the cultured cells at the concentration ranging from 0.1 to 100 μg/mL of the tested agents. The SRB test measuring the cell proliferation inhibition in in vitro culture was applied.[19] The cells attached to the plastic were fixed with cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) added on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 min. The unbound dye was removed by rinsing (four times) with 1% acetic acid, and the protein-bound dye was extracted with 10 mM unbuffered Tris base (tris(hydroxymethyl)aminomethane, POCh, Gliwice, Poland) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Uniskan II (Labsystems, Helsinki, Finland). The compounds were tested in triplicate per experiment. The experiments were repeated at least three times.

4.4. Statistical analysis

Statistical analyses were performed using a personal computer with a commercially available statistics program Statistica 5.0.

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