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Novel amidino substituted 2-phenylbenzothiazoles: Synthesis, antitumor evaluation in vitro and acute toxicity testing in vivo

Livio Racané [b], Marijeta Kralj [c], Lidija Šuman [c], Ranko Stojković [c], Vesna Tralić-Kulenović [b], Grace Karminski-Zamola [a,*]

[a] Department of Organic Chemistry, Faculty of Chemical Engineering and Technology, University of Zagreb, Marulićev trg 20, PO Box 177, HR-10000 Zagreb, Croatia
[b] Department of Applied Chemistry, Faculty of Textile Technology, University of Zagreb, baruna Filipovića 10000 Zagreb, Croatia
[c] Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, PO Box 180, HR-10000 Zagreb, Croatia

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ABSTRACT

The efficient synthesis of new bis-substituted nitro-amidino, amino-amidino (**10a**, **10b**–**13a**, **13b**) and previously prepared diamidino 2-phenyl-benzothiazoles (**9a**, **9b**) is described. The compounds **11a** and **11b** were prepared by recently developed methodology of the key precursors in zwitterionic form **8a** and **8b** with 4-nitrobenzoylchloride in a very good yield (70%). All compounds except diamidino-substituted 2-phenylbenzothiazole **9a** show exceptionally prominent tumor cell-growth inhibitory activity and cytotoxicity, whereby the special selectivity of amino-amidine 2-phenylbenzothiazole **12a** towards MCF-7 and H 460 cells makes this compound a prospective lead compound that should be further evaluated in animal models. All in vivo tested compounds (**12a**, **12b**, **13a** and **13b**) are absorbed from mice gastrointestinal system. LD_{50} are between 67.33 and 696.2 mg/kg body weight (OECD/EPA toxicity categories 2–3).

1. Introduction

In the last two decades, many heterocyclic compounds from the benzothiazole series were synthesized and their biological and pharmacological activity investigated. They were studied extensively for their antiallergic,[1] anti-inflammatory,[1,2] antitumor[3–7] and analgesic[8,9] activity. Considering the mechanism of action, it was shown that benzothiazole derivatives act as tyrosine kinase[10–13] and topoisomerase I and II inhibitors.[14,15] Therefore, various benzothiazole compounds are further of considerable interest for their diverse pharmaceutical uses. It was reported recently about a novel series of optically active 2-aminobenzothiazole derivatives and their in vitro cytotoxicity against mouse Ehrlich Ascites Carcinoma (EAC) and two human cancer cell lines MCF-7 and HeLa. In alkaline comet assay some compounds showed dose-dependent DNA damaging activity.[16] Newly prepared 2-acetyl-3-(6-methoxybenzothiazo)-2-yl-amino-acrylonitrile showed significant antiproliferative activity and is a potent inducer of programed cell death in leukemia cells.[17] The most recent article describing the synthesis of corresponding aminophenyl- substituted benzothiazoles with different adenine mimic which resulted in the identification of promising scaffolds, giving rise to the inhibition of different kinases with the IC_{50} values in the nanomolar range.[18]

Our previously obtained results showed that antiproliferative activity of cyano, amidino[19] and amino[20] substituted 2-phenylbenzothiazole derivatives strongly depends on the position of the substituent on 2-phenylbenzothiazole skeleton, as well as on the type of amidino substituent attached. We found that, in a series of unsubstituted, *N*-isopropyl substituted, as well as 2-imidazolinyl mono and bisamidino derivatives of 2-phenylbenzothiazole, *N*-isopropyl substituted amidine posses less pronounced antiproliferative activity on tested tumor cells.

In relation with the above considerations, we designed and efficiently synthesized new nitro-amidino, amino-amidino and diamidino-substituted 2-phenylbenzothiazole derivatives and tested their antitumor activity in vitro and determinate acute oral toxicity in mice of the most active compounds.

2. Results and discussion

2.1. Chemistry

An efficient synthesis of different amidino substituted 2-phenylbenzothiazole derivatives was carried out by reactions outlined in Scheme 1.

Bis-nitrile and nitro-nitrile **5–7** derivatives were prepared by condensation reaction of cyano or nitro-substituted 2-aminobenzothiole **1** or **2**[21] with commercially available 4-cyano or 4-nitrobenzoylchloride **3** and **4** (77–84%), respectively and were used as

* Corresponding author. Tel.: +385 14597297; fax: +385 14597224.
E-mail address: gzamola@fkit.hr (G. Karminski-Zamola).

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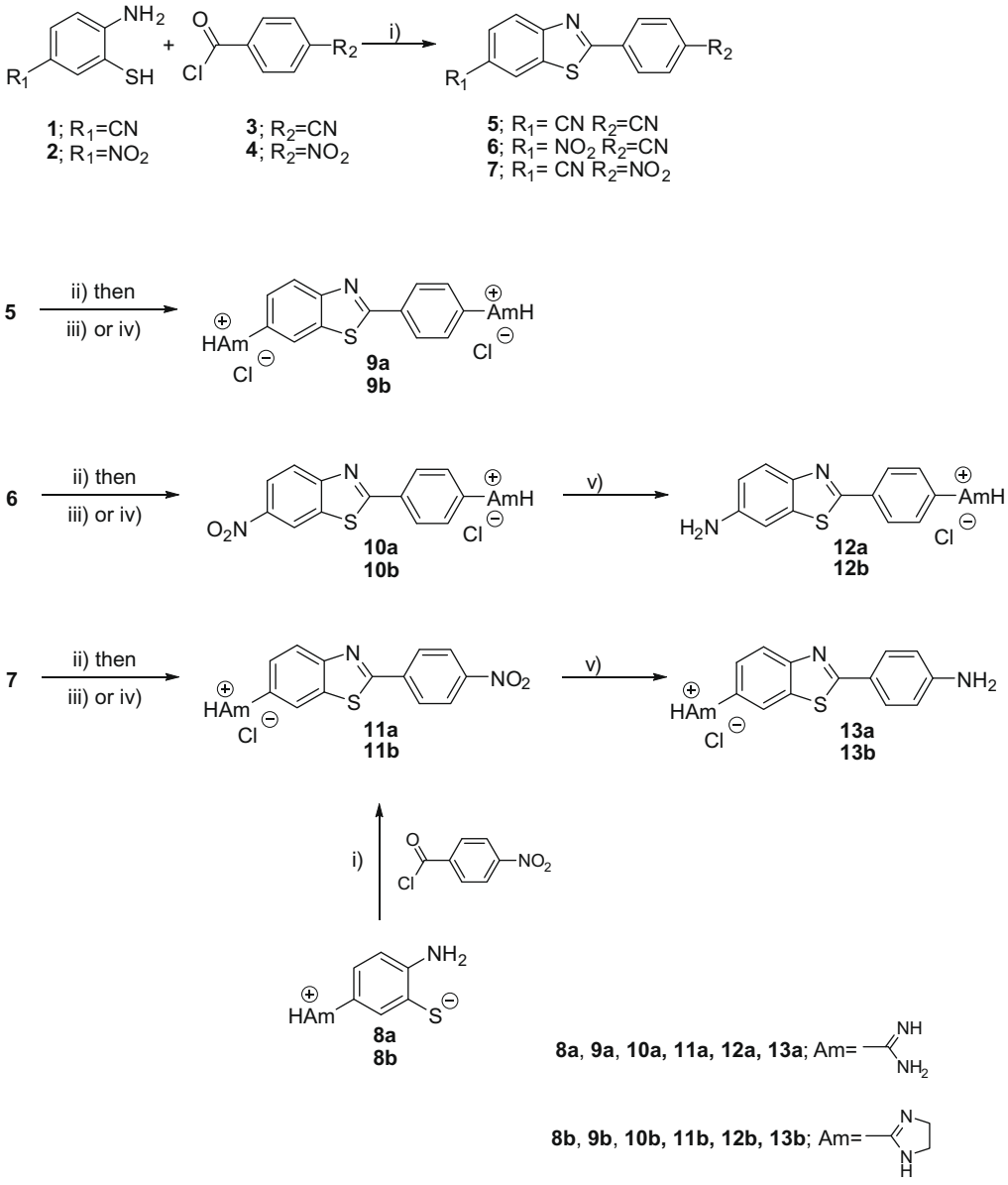

Scheme 1. Reagents and conditions: (i) HOAc, 4 h, reflux; (ii) 2-(2-ethoxyethoxy)ethanol/HCl(g), 5–7 days, rt; (iii) abs EtOH/NH_3 (g), two days rt, then 1 h 60 °C; (iv) abs EtOH/$NH_2(CH_2)_2NH_2$, 4 h, reflux; (v) $SnCl_2$/MeOH/HCl, 15 min, reflux.

starting compounds for the preparation of targeted amidino substituted 2-phenylbenzothiazole by Pinner reaction. Compounds **5** and **9a** were previously prepared by method different from ours, but the authors did not report neither the yield of pure product nor spectroscopic characteristics.[22] For the conversion of nitrile **5–7** into corresponding imidoyl ether hydrochlorides, in the first step of Pinner reaction, abs 2-(2-ethoxyethoxy)ethanol was used instead of abs ethanol due to very low solubility of compounds.

The conversion of nitriles into imidoyl ether hydrochlorides was quantitative followed by subsequent reaction with gaseous ammonia or ethylenediamine affording amidine **9a**, **9b**–**11a**, **11b** in 43–79% yield. Recently, we described an efficient method for preparation of amidino substituted 2-aminobenzothiole[23] thus opening a possibility of another synthetic approach for preparation of 6-amidino substituted 2-phenyl or 2-heteroarylbenzothiazolyl molecules. Condensation reaction of these key precursors in zwitterionic form **8a** and **8b**[23] with 4-nitrobenzoylchloride in acetic acid afforded compounds **11a** and **11b** in a very good yield (70%), and this approach is much better in term of time consuming. Finally, the reduction of nitro derivatives **10a**, **10b**–**11a**, **11b** was accomplished applying the reaction with tin(II) chloride as reducens in hydrochloric acid–methanol mixture in a good yield (55–80%). The amino-amidino derivatives **12a**, **12b**–**13a**, **13b** were only isolated from reaction as mono hydrochloride salt in spite of strong acidic reaction conditions. The structure of compounds was determined by IR, ^1H and ^{13}C NMR spectroscopy as well as elemental analysis. All amidino compounds **9a**, **9b**–**13a**, **13b** were isolated as hydrochloride salts to improve their aqueous solubility but amidino-nitro compounds **10a**, **10b**–**11a**, **11b** were sparingly water-soluble and being the problem for in vitro testing.

2.2. Biological results

2.2.1. Antiproliferative activity

Compounds **9a**, **9b**–**13a**, **13b** were screened for their potential antiproliferative effects on a panel of five human cell lines, which were derived from different cancer types including MCF-7 (breast carcinoma), SW 620 and HCT 116 (colon carcinoma), H 460 (lung

carcinoma) and MOLT-4 (acute lymphoblastic leukemia) (Table 1 and Fig. 1). All compounds except diamidino-substituted 2-phenylbenzothiazole (**9a**) show exceptionally prominent tumor cell-growth inhibitory activity and cytotoxicity. Interestingly, when both phenyl and benzothiazole sides of the molecule are substituted with imidazoline (**9b**) the activity is dramatically stronger, but not selective.

In general, nitro-amidino substituted 2-phenylbenzothiazole (**10** and **11**) derivatives showed similar activity as their amino-amidino substituted analogues (**12** and **13**). Still, nitro-imidazolinyl substituted compounds (**10b**, **11b**) exert somewhat lower activity compared to nitro-amidino substituted analogues (**10a**, **11a**) which are nonselective cytotoxic. On the contrary, amino-imidazolinyl substituted compounds (**12b** and **13b**) are to some extent more potent compared to amino-amidino substituted 2-phenylbenzothiazoles (**12a** and **13a**). Moreover, although the differences are not marked, it appears that the position of the amidine moiety on the phenyl ring results in a better activity compared to its position on the benzothiazole ring.

In general, benzothiazoles were demonstrated to be potent aryl hydrocarbon receptor (AhR) agonists, binding to AhR results in induction of CYP1A1, causes generation of electrophilic reactive species which forms DNA adduct, ultimately resulting in cell death by activation of apoptotic machinery. Previously described 2-(4-amino-3-methylphenyl)benzothiazole showed superb sensitivity towards breast cancer MCF-7 cell line. This selectivity is assigned to differences in metabolic activation and inactivation between cell lines, whereby CYP1A1 and CYP1B1, activated via AhR (which was shown to be highly expressed in breast cancer cells) are shown to be the major determinants of this metabolic conversion.[24,25] According to these data, we could speculate that the same mechanism of action could be responsible for the here-presented benzothiazole derivatives. Moreover, the cell line MCF-7 showed superior sensitivity to **9a** and **12a**, compared to other cell lines. However, compounds **13a** and **13b**, which are close analogues of previously described 2-(4-amino-3-methylphenyl)benzothiazole did not show any selectivity to MCF-7 cell line.

Still, prominent and selective activity of **12a** towards MCF-7 and H 460 cells makes this compound a prospective lead compound that should be further evaluated in animal models. Therefore, preliminary acute toxicity testing was performed.

Table 1
In vitro inhibition of compounds **9a**, **9b–13a**, **13b** on the growth of tumor cells

Compd	IC_{50}[a] (μM)				
	MOLT-4	HCT 116	SW 620	MCF-7	H 460
9a	>100[b]	64 ± 35[b]	>100[b]	11 ± 1	78 ± 20[b]
9b	3 ± 2	2 ± 0.3	0.8 ± 0.4	1 ± 0.1	2 ± 0.2
11a	2 ± 0.1	2 ± 0.04	2 ± 0.1	2 ± 0.3	1 ± 0.2
11b	17 ± 1	8 ± 5	7 ± 5	11 ± 0.6	4 ± 0.1
10a	1 ± 0.2	1 ± 0.1	2 ± 0.3	1 ± 0.3	1 ± 0.2
10b	2 ± 0.7	3 ± 1	2 ± 0.5	3 ± 0.4	3 ± 0.2
12a	2 ± 0.8	3 ± 0.6	4 ± 0.2	0.2 ± 0.01	0.2 ± 0.08
12b	1 ± 0.2	1 ± 0.03	0.3 ± 0.1	1 ± 0.04	0.3 ± 0.05
13a	3 ± 0.7	10 ± 3	7 ± 2	9 ± 0.7	4 ± 0.6
13b	1 ± 0.2	1 ± 0.1	0.7 ± 0.04	1.7 ± 0.2	0.4 ± 0.05

[a] IC_{50}; the concentration that causes a 50% reduction of the cell growth.
[b] These IC_{50} measurements were not significantly smaller than 100 μM at $p < 0.001$ (one-tailed Z-test). For all other measurements, IC_{50} is significantly smaller than 100 μM, indicating that the compounds have cytostatic/cytotoxic activity.

2.2.2. Acute toxicity testing in vivo

The aim of the in vivo studies was to determine acute oral Toxicity LD_{50} dose of the test substances on murine model using OECD Guideline for the testing of chemicals, Section 4: Health Effects Test No. 425, Acute Oral Toxicity: Up-and-Down Procedure.[26] Although all in vitro tested compounds except **9a** showed similar activities only the amino-amidinic derivative **12a**, **12b**, **13a**, and **13b** were sufficiently water soluble for in vivo testing. The estimated LD_{50} for each of tested substances (based on maximum likelihood) of long term results is shown in Table 2.

From the obtained data it can be seen that all tested substances absorbed in gastrointestinal tract of animals after intragastric gavage caused biological/toxicological effect in animals. Substances **12a**, **12b**, **13a** and **13b** can be classified as category 2 and substance **13b** can be classified as category 3 according to OECD/EPA toxicity categories.[27]

Interestingly, compound **13b** showed the lowest acute oral toxicity, although it exerted the most pronounced toxicity to tumor cells. To check whether the differences in the toxicity could be a consequence of different intestinal absorption, we utilized a web-based application called PreADMET, which has been developed for rapid prediction of drug-likeness and ADME/Tox data.[28] However, according to these data the percentage of human intestinal absorption prediction, defined as the sum of bioavailability and absorption evaluated from ratio of excretion or cumulative excretion in urine, bile and feces,[29] for all compounds are very similar (86% for **12a** and **13a** and 88% for **12b** and **13b**). Therefore, the

Table 2
Estimated LD_{50} (in mg/kg) in vivo

Compound	Estimated LD_{50}[a] (mg/kg)
12a	249.8
12b	249.8
13a	67.33
13b	696.2

[a] The LD_{50} is calculated with AOT425StatPgm based on maximum likelihood for long term results at 95% confidence interval.

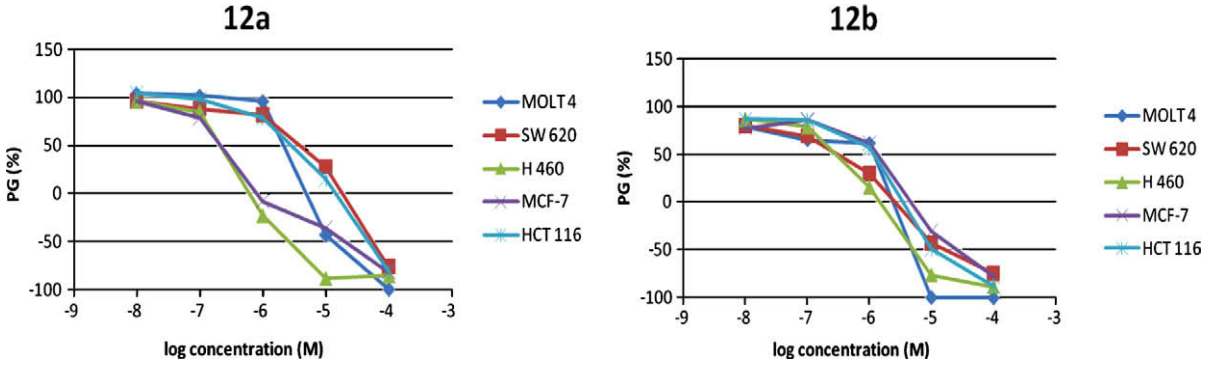

Figure 1. Dose–response profiles for compounds **12a** and **12b** tested in vitro. PG = percentage of growth.

differences in the toxicity data are probably consequences of the compounds activity itself. The encouraging result pointing to lower toxicity for the most active compound will be further evaluated in in vivo antitumor studies, whereby the data attained herein will be used to determine starting doses of tested substances.

3. Conclusion

The series of new amidino-nitro and amidino-amino substituted benzothiazoles was efficiently prepared by our recently postulated synthetic methodology, and the new convergent synthesis of amidino-substituted benzothiazoles form zwitterions **8a** and **8b** was developed.

All compounds except diamidino-substituted 2-phenylbenzothiazole **9a** show exceptionally prominent tumor cell-growth inhibitory activity and cytotoxicity (IC_{50} were in the micromolar range), whereby the special selectivity of amino-amidine 2-phenylbenzothiazole **12a** towards MCF-7 and H 460 cells makes this compound a prospective lead compound that should be further evaluated in animal models. All in vivo tested compounds (**12a**, **12b**, **13a** and **13b**) are absorbed from mice gastrointestinal system. LD_{50} are between 67.33 and 696.2 mg/kg body weight (OECD/EPA toxicity categories 2–3). Compound **13b** was least toxic, albeit very active in antiproliferative study, which makes it exceptionally interesting for further in vivo studies.

4. Experimental

4.1. Chemistry

Melting points were determined on an Original Kofler Mikroheitztisch apparatus (Reichert, Wien). The ^{1}H NMR and the ^{13}C NMR spectra were recorded with a Brucker Avance DPX-300 or Brucker AV-600, the deuterated solvents indicated were used. Chemical shifts are reported in parts per million (ppm) relative to TMS. IR spectra were recorded with Bruker Vertex 70 FTIR spectrophotometer with an ATR sampling accessory and the signal are given by wave numbers (cm^{-1}). Mass spectra were recorded with an Agilent 1100 Series LC/MSD Trap SL spectrometer using electrospray ionization (ESI). Elemental analyses were performed at the microanalytical laboratories of the 'Rugjer Boskovic' Institute. All chemicals and solvents were purchased from Aldrich Chemical or Acros Organics and dried by standard procedure. The 4-amino-3-sulfanylbenzonitrile **1** and 2-amino-5-nitrobenzothiole **2** were freshly prepared by alkaline hydrolysis of 6-cyano or 6-nitrobenzothiazole according to the literature.[21] The 5-amidinium-2-aminobenzothiolate **8a** and 5-(imidazolinium-2-yl)-2-aminobenzothiolate hydrate **8b** were prepared according to the literature.[23]

4.1.1. Synthesis; general procedures

(i) *Condensation reaction of corresponding 6-substituted-2-aminothiophenole with 4-substituted benzoylchloride*: To a solution of corresponding 6-substituted-2-aminothiophenole (1.1 equiv) in glacial acetic acid 4-substituted-benzoylchloride (1.0 equiv) was added in one portion and the reaction mixture was refluxed under nitrogen for 4 h. After cooling to rt, the resulting precipitate was collected by filtration, washed with diethyl ether and dried under vacuum over KOH. The crude product was purified by crystallization giving pure compound (**5**, **6**, **7**, **11a**, and **11b**).

(ii) *Conversion of corresponding nitrile into imidoyl ether hydrochloride*: A suspension of corresponding nitrile (1 equiv) in 2-(2-ethoxyethoxy)ethanol was saturated with dry gaseous HCl at 5 °C. The flask was stoppered, and stirred at rt until IR spectra indicated the disappearance of the nitrile peak. The excess HCl was removed from the suspension with a stream of nitrogen. The reaction mixture was poured into dry ether (500 mL) and the resulting crystals of imidoyl ether hydrochloride filtered off, washed with abs ether, and dried under vacuum over KOH. The yield of corresponding white hygroscopic crystalline solid of imidoyl ether hydrochloride was quantitative and has been prepared prior to use without purification in reaction with ammonia (iii) or ethylenediamine (iv).

(iii) *Reaction of imidoyl ether hydrochloride with ammonia*: The corresponding imidoyl ether hydrochloride (1 equiv) in abs ethanol was cooled to 5–10 °C and saturated with dry gaseous ammonia. The flask was stoppered and content was stirred at room temperature for three days. The reaction mixture was heated with stirring at 60 °C for 1 h, cooled over night, and the resulting precipitate filtered off, washed with diethyl ether, and dried under vacuum. The crude product was crystallized from appropriate solvent (charcoal) several times, until compound was analytically pure (**9a**, **10a**, and **11a**).

(iv) *Reaction of imidate hydrochloride with ethylenediamine*: To the suspension of corresponding imidoyl ether hydrochloride (1 equiv) in abs EtOH freshly distilled ethylenediamine (5 equiv) was added in nitrogen atmosphere. The reaction mixture was refluxed for 4 h under nitrogen, cooled to rt and acidified with concd HCl. After standing in refrigerator over night, the resulting precipitate was collected by filtration, washed with diethyl ether and dried under vacuum. The crude product was purified by crystallization giving pure compound (**9b**, **10b**, and **11b**).

(v) *Reduction of corresponding nitro-substituted compound*: A solution of tin(II) chloride dihydrate (10 equiv) in concd HCl and methanol was added to the corresponding nitro compound (1 equiv). The reaction mixture was stirred and refluxed for 15 min. The precipitate was filtered off, washed with methanol, diethyl ether, and dried under vacuum. The crude product was purified by crystallization giving pure compound (**12a**, **12b**, **13a** and **13b**).

4.1.1.1. 6-Cyano-2(4-cyanophenyl)benzothiazole (5). According to (i), 4-amino-3-sulfanylbenzonitrile **1** (3.30 g, 22 mmol), 4-cyanobenzoylchloride **3** (3.31 g, 20 mmol), and 100 mL of glacial acetic acid were used. Crystallization from toluene afforded 3.56 g (77.7%) of pale yellow solid; mp 284–286 °C (lit.[22], mp 288–289 °C). IR (ATR): ν = 2227 (CN) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.93 (dd, 1H, J = 1.7 Hz, J = 8.5 Hz, H-Bt), 8.02 (d, 2H, J = 8.4 Hz, H-Ph), 8.24 (d, 1H, J = 8.5 Hz, H-Bt), 8.28 (d, 2H, J = 8.4 Hz, H-Ph), 8.76 (d, 1H, J = 1.6 Hz, H-Bt). MS (ESI) m/z: 262.2 ($M+H^+$). Anal. Calcd for $C_{15}H_7N_3S$ (261.30): C, 68.95; H, 2.70; N, 16.08. Found: C, 69.03; H, 2.71; N, 16.01.

4.1.1.2. 6-Nitro-2-(4-cyanophenyl)benzothiazole (6). According to (i), 2-amino-5-nitrobenzothiole **2** (3.78 g, 22 mmol), 4-cyanobenzoylchloride **3** (3.31 g, 20 mmol) and 100 mL of glacial acetic acid were used. Crystallization from DMF afforded 4.44 g (79.0%) of yellow solid; mp 219–220 °C. IR (ATR): ν = 2229 (CN), 1523 (NO_2) cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.84 (d, 2H, J = 8.6 Hz, H-Ph), 8.20 (d, 1H, J = 8.9 Hz, H-Bt), 8.25 (d, 2H, J = 8.5 Hz, H-Ph), 8.41 (dd, 1H, J = 2.2 Hz, J = 8.9 Hz, H-Bt), 8.89 (d, 1H, J = 2.1 Hz, H-Bt). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 114.9 (s), 117.4 (s), 117.9 (d), 121.8 (d), 123.6 (d), 127.9 (d, 2C), 132.5 (d, 2C), 135.1 (s), 135.9 (s), 145.0 (s), 157.0 (s), 170.5 (s). MS (ESI) m/z: 282.0 ($M+H^+$). Anal. Calcd for $C_{14}H_7N_3O_2S$ (281.29): C, 59.78; H, 2.51; N, 14.94. Found: C, 59.83; H, 2.48; N, 15.03.

4.1.1.3. 6-Cyano-2-(4-nitrophenyl)benzothiazole (7). According to (i); 4-amino-3-sulfanylbenzonitrile **1** (3.30 g, 22 mmol), 4-nitrobenzoylchloride **4** (3.71 g, 20 mmol) and 100 mL of glacial acetic acid were used. Crystallization from xylene afforded 4.74 g (84.3%) of yellow solid; mp 284–285 °C. IR (ATR): ν = 2229 (CN), 1512 (NO_2) cm^{-1}. ^{1}H NMR (600 MHz, DMSO-d_6): δ = 7.92 (d, 1H,

J = 7.7 Hz, H-Bt), 8.22 (d, 1H, J = 7.9 Hz, H-Bt), 8.33 (s, 4H, H-Ph), 8.77 (s, 1H, H-Bt). MS (ESI) m/z: 282.0 ($M+H^+$). Anal. Calcd for $C_{14}H_7N_3O_2S$ (281.29): C, 59.78; H, 2.51; N, 14.94. Found: C, 59.69; H, 2.55; N, 14.91.

4.1.1.4. 2-(4-Amidinophenyl)-6-amidinobenzothiazole dihydrochloride (9a). According to (ii), 6-cyano-2(4-cyanophenyl)benzothiazole **5** (1.0 g, 3.83 mmol) and 50 mL of 2-(2-ethoxyethoxy)ethanol were used, reaction time seven days. Afterwards, the obtained diimidoyl ether dihydrochloride of **5** and 50 mL of abs ethanol were used according to (iii). Crystallization from water–acetone afforded 0.680 g (43.9%) of colorless solid; mp >300 °C (lit.[22] mp >300 °C). IR (ATR): ν = 3030 ($C(NH_2)NH_2^+$), 1666 ($C(NH_2)NH_2^+$) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 8.02 (dd, 1H, J = 2.2 Hz, J = 8.4 Hz, H-Bt), 8.09 (d, 2H, J = 8.3 Hz, H-Ph), 8.32 (d, 1H, J = 8.7 Hz, H-Bt), 8.37 (d, 2H, J = 8.3 Hz, H-Ph), 8.82 (d, 1H, J = 1.2 Hz, H-Bt), 9.58 (br s, 8H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 123.9 (d), 124.2 (d), 125.9 (s), 127.1 (d), 128.3 (d, 2C), 129.9 (d, 2C), 131.3 (s), 135.5 (s), 136.9 (s), 156.8 (s), 165.4 (s), 165.9 (s), 170.3 (s). MS (ESI) m/z: 295.8 [($M+H^+$) calcd for free base $C_{15}H_{13}N_5S$, 295.09]. Anal. Calcd for $C_{15}H_{15}Cl_2N_5S{\cdot}2H_2O$ (404.31): C, 44.56; H, 4.74; N, 17.32. Found: C, 44.45; H, 4.82; N, 17.18.

4.1.1.5. 2-[4-(Imidazolin-2-yl)phenyl]-6-(imidazolin-2-yl)benzothiazole dihydrochloride (9b). According to (ii), 6-cyano-2(4-cyanophenyl)benzothiazole **5** (1.0 g, 3.83 mmol) and 50 mL of 2-(2-ethoxyethoxy)ethanol were used, reaction time seven days. Afterwards, the obtained diimidoyl ether dihydrochloride of **5**, ethylenediamine (2.57 mL, 38.3 mmol), and 50 mL of abs ethanol were used according to (iv). Crystallization from water–acetone afforded 1.125 g (64.8%) of pale yellow solid; mp >300 °C (lit.[19] mp >300 °C). IR (ATR): ν = 3081 ($NHCNH^+$), 2962 ($NHCNH^+$) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 4.06 (s, 8H, H-CH_2), 8.22 (d, 1H, J = 8.6 Hz, H-Bt), 8.29 (d, 2H, J = 8.4 Hz, H-Ph), 8.33 (d, 1H, J = 8.6 Hz, H-Bt), 8.40 (d, 2H, J = 8.4 Hz, H-Ph), 9.02 (s, 1H, H-Bt), 10.98 (br s, 4H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 45.1 (t, 4C), 120.2 (d), 124.3 (d), 124.8 (s), 125.7 (s), 127.5 (d), 128.7 (d, 2C), 130.4 (d, 2C), 135.9 (s), 137.6 (s), 157.2 (s), 164.8 (s), 165.2 (s), 170.7 (s). MS (ESI) m/z: 347.8 [($M+H^+$) calcd for free base $C_{19}H_{17}N_5S$, 347.12]. Anal. Calcd for $C_{19}H_{19}Cl_2N_5S{\cdot}2H_2O$ (456.39): C, 50.00; H, 5.08; N, 15.35. Found: C, 50.12; H, 4.92; N, 15.50.

4.1.1.6. 2-(4-Amidinophenyl)-6-nitrobenzothiazole hydrochloride (10a). According to (ii), 6-nitro-2-(4-cyanophenyl)benzothiazole **6** (1.0 g, 3.55 mmol) and 50 mL of 2-(2-ethoxyethoxy)-ethanol were used, reaction time five days. Afterwards, the obtained imidoyl ether hydrochloride of **6** and 50 mL of abs ethanol were used according to (iii). Crystallization from 2 M HCl afforded 0.956 g (76.5%) of colorless solid; mp 292–295 °C (decomp.). IR (ATR): ν = 2999 ($C(NH_2)NH_2^+$), 1663 ($C(NH_2)NH_2^+$), 1504 (NO_2) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 8.06 (d, 2H, J = 8.4 Hz, H-Ph), 8.25 (d, 1H, J = 9.0 Hz, H-Bt), 8.31–8.37 (m, 3H, 2H-Ph and H-Bt), 9.25 (d, 1H, J = 2.2 Hz, H-Bt), 9.59 (br s, 4H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 120.3 (d), 122.6 (d), 124.2 (d), 128.3 (d, 2C), 129.9 (d, 2C), 128.3 (s), 136.0 (s), 136.8 (s), 145.2 (s), 157.5 (s), 165.4 (s), 172.4 (s). MS (ESI) m/z: 298.7 [($M+H^+$) calcd for free base $C_{14}H_{10}N_4O_2S$, 298.05]. Anal. Calcd for $C_{14}H_{11}ClN_4O_2S{\cdot}H_2O$ (352.80): C, 47.66; H, 3.71; N, 15.88. Found: C, 47.50; H, 3.88; N, 15.91.

4.1.1.7. 2-[4-(Imidazolin-2-yl)phenyl]-6-nitrobenzothiazole hydrochloride (10b). According to (ii), 6-nitro-2-(4-cyanophenyl)benzothiazole **6** (1.0 g, 3.55 mmol) and 50 mL of 2-(2-ethoxyethoxy)ethanol were used, reaction time five days. Afterwards, the obtained imidoyl ether hydrochloride of **6**, ethylenediamine (1.18 mL, 17.7 mmol) and 50 mL of abs ethanol were used according to (iv). Crystallization from 2 M HCl afforded 0.893 g (66.6%) of pale yellow solid; mp 292–294 °C (decomp.). IR (ATR): ν = 3053 ($NHCNH^+$), 2946 ($NHCNH^+$), 1512 (NO_2) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 4.06 (s, 4H, H-CH_2), 8.26 (d, 2H, J = 9.0 Hz, H-Ph), 8.34–8.38 (m, 4H, 2H-Ph and 2H-Bt), 9.17 (d, 1H, J = 2.2 Hz, H-Bt), 11.18 (br s, 2H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 45.1 (t), 119.9 (d), 122.4 (d), 124.1 (d), 125.6 (s), 128.5 (d, 2C), 130.6 (d, 2C), 136.2 (s), 137.6 (s), 145.7 (s), 157.6 (s), 164.8 (s), 172.1 (s). MS (ESI) m/z: 324.7 [($M+H^+$) calcd for free base $C_{16}H_{12}N_4O_2S$, 324.07]. Anal. Calcd for $C_{16}H_{13}ClN_4O_2S{\cdot}H_2O$ (378.83): C, 50.73; H, 3.99; N, 14.79. Found: C, 50.70; H, 4.08; N, 14.81.

4.1.1.8. 2-(4-Nitrophenyl)-6-amidinobenzothiazole hydrochloride (11a). According to (i), 5-amidinium-2-aminobenzothiolate **8a** (0.50 g, 3 mmol), 4-nitrobenzoylchloride **4** (0.614 g, 3.3 mmol) and 25 mL of glacial acetic acid were used. Crystallization from 2 M HCl afforded 0.747 g (70.6%) of colorless solid of **11a**. According to (ii), 6-cyano-2-(4-nitrophenyl)benzothiazole **7** (1.0 g, 3.55 mmol) and 50 mL of 2-(2-ethoxyethoxy)ethanol were used, reaction time seven days. Afterwards, the obtained imidoyl ether hydrochloride of **7** and 50 mL of abs ethanol were used according to (iii). Crystallization from 2 M HCl afforded 0.924 g (73.8%) of colorless solid of **11a**; mp >300 °C. IR (ATR): ν = 3023 ($C(NH_2)NH_2^+$), 1666 ($C(NH_2)NH_2^+$), 1500 (NO_2) cm^{-1}. ^1H NMR (600 MHz, DMSO-d_6): δ = 7.90 (dd, 1H, J = 2.4 Hz, J = 8.6 Hz, H-Bt), 8.27 (d, 1H, J = 8.6 Hz, H-Bt), 8.36 (m, 4H, H-Ph), 8.70 (d, 1H, J = 2.4 Hz, H-Bt), 9.27 (br s, 2H, H-Am), 9.51 (br s, 2H, H-Am). ^{13}C NMR (150 MHz, DMSO-d_6): δ = 124.0 (d), 124.3 (d), 125.1 (d, 2C), 126.2 (s), 127.2 (d), 129.3 (d, 2C), 135.7 (s), 138.1 (s), 149.7 (s), 156.8 (s), 165.9 (s), 169.5 (s). MS (ESI) m/z: 298.7 [($M+H^+$) calcd for free base $C_{14}H_{10}N_4O_2S$, 298.05]. Anal. Calcd for $C_{14}H_{11}ClN_4O_2S{\cdot}H_2O$ (352.80): C, 47.66; H, 3.71; N, 15.88. Found: C, 47.61; H, 3.78; N, 15.93.

4.1.1.9. 2-(4-Nitrophenyl)-6-(imidazolin-2-yl)benzothiazole hydrochloride (11b). According to (i), 5-(imidazolinium-2-yl)-2-aminobenzothiolate hydrate **8b** (0.634 g, 3 mmol), 4-nitrobenzoylchloride **4** (0.614 g, 3.3 mmol) and 25 mL of glacial acetic acid were used. Crystallization from 2 M HCl afforded 0.809 g (71.2%) of pale yellow solid of **11b**.

According to (ii), 6-cyano-2-(4-nitrophenyl)benzothiazole **7** (1.0 g, 3.55 mmol) and 50 mL of 2-(2-ethoxyethoxy)ethanol were used, reaction time seven days. Afterwards, the obtained imidoyl ether hydrochloride of **7**, ethylenediamine (1.18 mL, 17.7 mmol) and 50 mL of abs ethanol were used according to (iv). Crystallization from 2 M HCl afforded 1.061 g (78.9%) of pale yellow solid of **11b**; mp >300 °C; IR (ATR): ν = 3058 ($NHCNH^+$), 2964 ($NHCNH^+$), 1519 (NO_2) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 4.07 (s, 4H, H-CH_2), 8.17 (dd, 1H, J = 1.7 Hz, J = 8.7 Hz, H-Bt), 8.40 (d, 1H, J = 8.7 Hz, H-Bt), 8.44 (s, 4H, H-Ph), 8.95 (d, 1H, J = 1.4 Hz, H-Bt), 10.89 (br s, 2H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): 45.0 (t, 2C), 120.3 (s), 124.4 (d), 124.8 (d), 125.0 (d, 2C), 127.7 (d), 129.5 (d, 2C), 136.2 (s), 165.2 (s), 169.3 (s). MS (ESI) m/z: 324.7 [($M+H^+$) calcd for free base $C_{16}H_{12}N_4O_2S$, 324.07]. Anal. Calcd for $C_{16}H_{13}ClN_4O_2S{\cdot}H_2O$ (378.83): C, 50.73; H, 3.99; N, 14.79. Found: C, 50.59; H, 3.83; N, 15.08.

4.1.1.10. 2-(4-Amidinophenyl)-6-aminobenzothiazole hydrochloride (12a). According to (v), 2-(4-amidinophenyl)-6-nitrobenzothiazole hydrochloride hydrate **10a** (1.0 g, 2.83 mmol), tin(II) chloride dihydrate (6.4 g, 28 mmol), 12 mL of methanol and 12 mL of concd HCl were used. Crystallization from water afforded 0.628 g (68.7%) of yellow solid; mp 295–297 °C (decomp.). IR (ATR): ν = 3193 (NH_2), 3042 ($C(NH_2)NH_2^+$), 1667 ($C(NH_2)NH_2^+$) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ = 5.68 (s, 2H, H-NH_2), 6.86 (dd, 1H, J = 1.9 Hz, J = 8.8 Hz, H-Bt), 7.14 (d, 1H, J = 1.9 Hz, H-Bt),

7.75 (d, 1H, J = 8.8 Hz, H-Bt), 7.97 (d, 2H, J = 8.4 Hz, H-Ph), 8.15 (d, 2H, J = 8.4 Hz, H-Ph), 9.43 (br s, 4H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 103.9 (d), 116.2 (d), 124.2 (d), 127.0 (d, 2C), 129.3 (s), 129.6 (d, 2C), 137.5 (s), 138.4 (s), 145.6 (s), 148.7 (s), 158.8 (s), 165.5 (s). MS (ESI) m/z: 268.7 [(M+H$^+$) calcd for free base $C_{14}H_{12}N_4S$, 268.08]. Anal. Calcd for $C_{14}H_{13}ClN_4S{\cdot}H_2O$ (322.81): C, 52.09; H, 4.68; N, 17.36. Found: C, 52.21; H, 4.69; N, 17.23.

4.1.1.11. 2-[4-(Imidazolin-2-yl)phenyl]-6-aminobenzothiazole hydrochloride (12b). According to (v), 2-[4-(imidazolin-2-yl)-phenyl]-6-nitrobenzothiazole hydrochloride hydrate **10b** (1.0 g, 2.64 mmol), tin(II) chloride dihydrate (6.0 g, 26 mmol), 12 mL of methanol and 12 mL of concd HCl were used. Crystallization from water–acetone afforded 0.505 g (54.8%) of orange solid; mp 292–295 °C (decomp.). IR (ATR): ν = 3360 (NH_2), 3304 (NH_2), 3198 (NHCNH$^+$), 2963 (NHCNH$^+$) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.03 (s, 4H, H-CH_2), 5.80 (br s, 2H, H-NH_2), 6.87 (dd, 1H, J = 1.9 Hz, J = 8.8 Hz, H-Bt), 7.15 (d, 1H, J = 1.9 Hz, H-Bt), 7.76 (d, 1H, J = 8.8 Hz, H-Bt), 8.12 (d, 2H, J = 8.5 Hz, H-Ph), 8.21 (d, 2H, J = 8.5 Hz, H-Ph), 10.8 (br s, 2H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 44.9 (t, 2C), 104.1 (d), 116.4 (d), 123.3 (s), 124.4 (d), 127.3 (d, 2C), 130.0 (d, 2C), 137.6 (s), 139.0 (s), 145.7 (s), 148.5 (s), 158.6 (s), 164.7 (s). MS (ESI) m/z: 294.8 [(M+H$^+$) calcd for free base $C_{16}H_{14}N_4S$, 294.09]. Anal. Calcd for $C_{16}H_{15}ClN_4S{\cdot}H_2O$ (348.85): C, 55.09; H, 4.91; N, 16.06. Found: C, 55.18; H, 4.88; N, 16.12.

4.1.1.12. 2-(4-Aminophenyl)-6-amidinobenzothiazole hydrochloride (13a). According to (v), 2-(4-nitrophenyl)-6-amidinobenzothiazole hydrochloride hydrate **11a** (1.0 g, 2.83 mmol), tin(II) chloride dihydrate (6.4 g, 28 mmol), 12 mL of methanol and 12 mL of concd HCl were used. Crystallization from water afforded 0.689 g (75.4%) of yellow solid; mp 260–262 °C (decomp.). IR (ATR): ν = 3180 (NH_2), 3030 (C(NH_2)NH_2^+), 1662 (C(NH_2)NH_2^+) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.11 (s, 2H, H-NH_2), 6.69 (d, 2H, J = 8.7 Hz, H-Ph), 7.81–7.86 (m, 3H, H-Bt + 2H-Ph), 8.07 (d, 1H, J = 8.5 Hz, H-Bt), 8.53 (s, 1H, H-Bt), 9.00 (s, 2H, H-Am), 9.37 (s, 2H, H-Am). ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 116.7 (d, 2C), 122.4 (d), 122.6 (s), 123.3 (d), 124.2 (s), 126.7 (d), 129.8 (d, 2C), 134.6 (s), 149.4 (s), 157.6 (s), 165.8 (s), 172.4 (s). MS (ESI) m/z: 268.7 [(M+H$^+$) calcd for free base $C_{14}H_{12}N_4S$, 268.08]. Anal. Calcd for $C_{14}H_{13}ClN_4S{\cdot}H_2O$ (322.81): C, 52.09; H, 4.68; N, 17.36. Found: C, 52.31; H, 4.72; N, 17.13.

4.1.1.13. 2-(4-Aminophenyl)-6-(imidazolin-2-yl)benzothiazole hydrochloride (13b). According to (v), 2-(4-nitrophenyl)-6-(imidazolin-2-yl)benzothiazole hydrochloride hydrate **11b** (1.0 g, 2.64 mmol), tin(II) chloride dihydrate (6.0 g, 26 mmol), 12 mL of methanol and 12 mL of concd HCl were used. Crystallization from water afforded 0.758 g (82.3%) of yellow solid; mp 278–282 °C (decomp.). IR (ATR): ν = 3374 (NH_2), 3310 (NH_2), 3203 (NHCNH$^+$), 2980 (NHCNH$^+$) cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 4.03 (s, 4H, H-CH_2), 6.17 (br s, 2H, H-NH_2), 6.70 (d, 2H, J = 8.7 Hz, H-Ph), 7.83 (d, 2H, J = 8.7 Hz, H-Ph), 8.07 (m, 2H, H-Bt), 8.75 (s, 1H, H-Bt), 10.77 (br s, 2H, H-Am); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ = 44.6 (t, 2C), 113.9 (d, 2C), 117.5 (s), 119.4 (s), 122.1 (d), 123.2 (d), 126.7 (d), 129.7 (d, 2C), 134.5 (s), 153.4 (s), 157.8 (s), 164.8 (s), 173.2 (s). MS (ESI) m/z: 294.8 [(M+H$^+$) calcd for free base $C_{16}H_{14}N_4S$, 294.09]. Anal. Calcd for $C_{16}H_{15}ClN_4S{\cdot}H_2O$ (348.85): C, 55.09; H, 4.91; N, 16.06. Found: C, 55.01; H, 4.95; N, 16.11.

4.2. Biological evaluation

4.2.1. Antiproliferative activity

The experiments were carried out on five human cell lines (obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), which are derived from four cancer types. MCF-7 (breast carcinoma), SW 620 (colon carcinoma), HCT 116 (colon carcinoma), H 460 (lung carcinoma) were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM), while MOLT-4 (acute lymphoblastic leukemia) were cultured in suspension in RPMI medium, both 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. The growth inhibition activity was assessed as described previously, according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program. The cells were inoculated onto a series of standard 96-well microtiter plates on day 0, at 1–3 × 10^4 cells/mL, depending on the doubling times of specific cell line. Test agents (dissolved in DMSO, $c = 4 \times 10^{-2}$ M) were then diluted with the cell culture medium in five serial 10-fold dilutions (10^{-8} to 10^{-4} M), added to cells and incubated for a further 72 h. The cell growth rate was evaluated by performing the MTT assay after 72 h of incubation, which detects mitochondrial dehydrogenase activity in viable cells. Each test was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from concentration–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (i.e., 50%). If however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g., PG value of 50), then the highest tested concentration is assigned as the default measurement value, which is preceded by a '>' sign preceding the number.

4.2.2. Acute oral toxicity test in vivo

4.2.2.1. Animals. Healthy nulliparous and non-pregnant BALB/C female mice, 12 weeks of age at the initiation of the experiment, were used. Mice were obtained from Rudjer Boskovic Institute's breeding colony. During experimental period single animal was kept per cage. Bottom of cage was covered with sawdust (Allspan®, Germany). Standard food for laboratory mice (4RF 21 GLP® Mucedola srl, Italy) was used. All animals were fastening prior to dosing by withholding food (not water) for 3–4 h and 1–2 h after dosing. After that period access to food and water was *ad libitum*. Animals were kept in conventional circumstances: light/dark rhythms 12/12 h, temperature 22 °C, and humidity 55%. All experiments were performed according to the OECD 425 Guideline for the testing of chemicals—Acute oral Toxicity: Up-and-down procedure,[26] ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (86/609/EEC) and Croatian animal protection law (NN 135/2006).

Altogether 35 animals were used in experiments to determine LD_{50} for all four tested substances. Animals are dosed, one at a time, at 24 h intervals. The first animal receives a dose at the level of the best estimate of the LD_{50}. Depending on the outcome for the previous animal, the dose for the next animal is adjusted up or down. If an animal survives, the dose for the next animal is increased; if it dies, the dose for the next animal is decreased. After reaching the reversal of the initial outcome, that is, the point where an increasing (or decreasing) dose pattern is reversed by giving a smaller (or a higher) dose, four additional animals are dosed following the same UDP.[26]

4.2.2.2. Drugs. All test substances were stored at +4 °C. All test substances were dissolved and diluted in physiological saline immediately prior to injection by using ultrasonic water bath Bransonic® 2510 for 15 min at the temperature of 69 °C. Following the period of fasting the animals were weighed and the test substances were administered. Doses of substances required by OECD 425

Guideline[26] were adjusted to be contained in 0.5 mL/25 g of mouse body mass. The test substances were administered in a single dose by gavage using a suitable intubation canula.

4.2.2.3. Statistical analysis. Statistical analyses were conducted by the 'Acute Oral Toxicity (Guideline 425) Statistical Program' (AOT425StatPgm) which is developed by Westat for the US EPA and designed to be used with the acute oral toxicity testing procedure presented in OECD Guideline for the testing of chemicals, Section 4: Health Effects Test No. 425, Acute Oral Toxicity: Up-and-Down Procedure.[26]

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