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# The 5-hydrazino-3-methylisothiazole-4-carboxylic acid, its new 5-substituted derivatives and their antiproliferative activity

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A B S T R A C T

Currently, the basic method of treatment of colon cancer is surgery. The range of anticancer drugs used in the treatment of colorectal cancer is small and is based mainly on systemic combination chemotherapy. As a result of the designed syntheses, we received new isothiazole derivatives with anticancer activity. The synthesized 5-hydrazino-3-methylisothiazole-4-carboxylic acid has never been obtained before. It is also a substrate for the synthesis of its innovative derivatives, i.e. compounds that are Schiff bases. The identification of the structure of new compounds was carried out using mass spectrometry (MS), proton nuclear magnetic resonance spectroscopy ($^{1}$H NMR), carbon nuclear magnetic resonance spectroscopy ($^{13}$C NMR) and infrared spectroscopy (IR). Potential antitumor activity was confirmed in antiproliferative MTT and SRB tests. The selected, most biologically active substances were characterized by high selectivity towards leukemia and colon cancer cell lines. They caused high inhibition of proliferation of human biphenotypic B cell myelomonocytic leukemia MV4-11 (13 compounds), human colon adenocarcinoma cell lines sensitive LoVo (8 compounds) and resistant to doxorubicin LoVo/DX (12 compounds). However, in the conducted studies, their activity against breast adenocarcinoma MCF-7 and normal non-tumorigenic epithelial cell line derived from mammary gland MCF-10A was substantially lower. The result of this work is claimed Polish patent application.

## 1. Introduction

Despite the major progress in biomedical research and the development of novel therapeutic strategies, cancer is one of the major causes of mortality worldwide [1]. Furthermore, the available drugs on the market have several side effects such as toxicity, poor tolerance, low selectivity and, what is more, people have developed drug resistance [2,3]. Therefore, identifying new targets involved in drug resistance may foster the development of new strategies for improving chemotherapy [4,5].

The substances containing isothiazole ring has been documented as distinguished pharmacological agents that show a broad range of biological action, mainly anticancer, [6-8] antiviral, [9-11] anti-inflammatory [12,13] and immunotropic [14]. An interesting panel of activities is also characterized by condensed heterocyclic compounds containing an isothiazole ring. A new series of 1,2-benzisothiazol-3-one derivatives showed moderate to high affinity to caspase-3, which could be used for the treatment of diseases such as atherosclerosis, cancer, autoimmune, neurodeneregative and cardiovascular diseases, including Alzheimer's, Parkinson's and Huntington's diseases [15]. In the isothiazole, fused pyrimidones series are potent PDE7 inhibitors, which has been extensively targeted for the treatment of a host of immunological and autoimmune conditions [16].

Our efforts to obtain new immunomodulatory compounds resulted in a synthesis of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1**. This compound has a very low antiproliferative activity on human biphenotypic B cell myelomonocytic leukemia MV4-11 and is intended for the preparation of new compounds, which also inhibit the proliferation of MV4-11 cells, as well human colon adenocarcinoma cell lines sensitive LoVo and resistant to doxorubicin LoVo/DX.

The new compound **1** is obtained in the nucleophilic substitution reaction 5-chloro-3-methylisothiazole-4-carboxylic acid with anhydrous hydrazine at room temperature for 5 days in a methanol solution (Scheme 1). The starting material, 5-chloro-3-methylisothiazole-4-carboxylic acid, was prepared according to the method described by Machoń [17].

The new 5-substituted derivatives (Table 1) were obtained in the nucleophilic addition reaction of 5-hydrazino-3-methylisothiazole-4-carboxylic acid with the corresponding carbonyl compounds in dimethylsulfoxide (DMSO). The mixture was stirred and heated in a temperature of 55–65 °C for 4 h.

## 2. Methods and materials

### 2.1. Chemistry

Progress of the reaction was controlled by thin layer chromatography (TLC) on Macherey-Nagel Pre-coated TLC sheets ALUGRAM SIL

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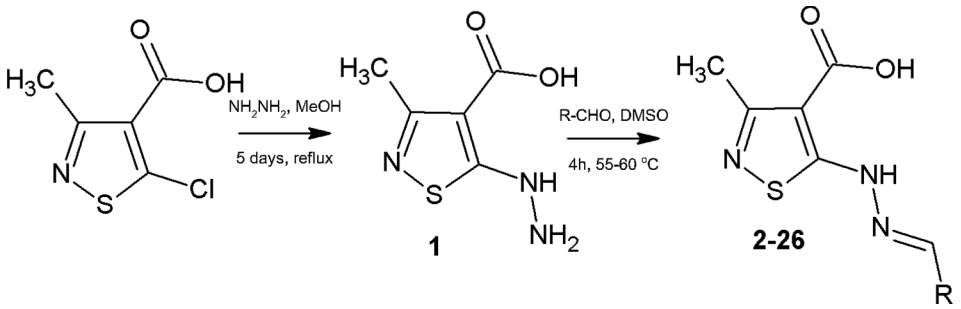



**Scheme 1.** Synthesis of 5-hydrazino-3-methylisothiazole-4-carboxylic acid and its 5-substituted derivatives.

G/UV and visualized by Fisher Bioblock Scientific 254 nm UV lamps. The melting points of all the new compounds were indicated on LLG uniMELT-2 100 V/AC-240 V/AC 1,3A IP20. The Thermo Scientific Nicolet iS50 FT-IR spectrometer was used to measure infrared spectra (IR). The samples were applied as solids and frequencies are given in $cm^{-1}$. The proton nuclear magnetic resonance ($^1$H NMR) and the carbon nuclear magnetic resonance ($^{13}$C NMR) spectra were measured in $d_6$-dimethylsulfoxide (DMSO-$d_6$) and received using Bruker ARX 300 MHz NMR spectrometer. Chemical Shift are reported in ppm units and signal multiplicities are collected by the abbreviations: s (singlet), d (doublet), t (triplet), m (multiplet). Mass spectrometry was performed on Bruker Daltonic ESI-Q-TOF apparatus. Monoisotopic mass calculated (calc.) by Compass DataAnalysis 4.2.

### *2.2. Procedures for the synthesis all the new compounds and their spectroscopic data (IR, $^1$H NMR, $^{13}$C NMR, MS spectroscopic)*

#### *2.2.1. 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1***

To 11.3 mmol of 5-chloro-3-methylisothiazole-4-carboxylic acid in 24 mL of methanol, 10 mL anhydrous hydrazine was added. The mixture was stirred at room temperature for 5 days. Then the mixture was filtered off and condensed to half the volume and set to crystallization (66%). The methanol was use to crystallize the product. M.p. = 144–145 °C. IR $cm^{-1}$: 1644 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.32 (s,3H, $CH_3$), 5.19 (s, 2H, $NH_2$), 8.54 (s, 1H, NH), 9.77 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.53 ($CH_3$), 103.51 (C3-isothiazole), 166.75 (C4-isothazole), 167.10 (C5-isothiazole), 183.66 (C═O). ESI-MS [M + H] *m/z*: 174.03, calc. *m/z*: 174.03.

#### *2.2.2. 5-{2[(3-methoxylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **2***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 2.4 mmol of 3-methoxylbenzladehyde was added to 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO. The mixture was stirred and heated in a temperature of 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (51%). The product was purified by wash in chloroform. M.p. = 169 °C. IR $cm^{-1}$: 1655 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.42 (s, 3H, $CH_3$), 3.78 (s, 3H, $OCH_3$), 7.01–6.98 (d, $J$ = 7.5 Hz, 1H, arH), 7.20–7.14 (t, $J$ = 7.8 Hz, 10.2 Hz, 2H, arH), 7.38–7.33 (t, $J$ = 7.8 Hz, 8.1 Hz, 1H, arH), 8.43 (s, 1H, N═CH), 11.20 (s, 1H, NH), 12.77 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.55 ($CH_3$), 55.69 ($OCH_3$), 104.40 (C3-isothiazole), 112.01 (N═CH), 116.34 (arC), 120.01 (arC), 130.60 (arC), 135.70 (arC), 146.47 (arC), 160.09 (arC), 165.14 (C4-isothiazole), 167.08 (C5-isothiazole), 176.58 (C═O). ESI-MS [M-H] *m/z*: 290.06, calc. *m/z*: 290.06.

#### *2.2.3. 5-{2[(3-chlorophenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **3***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 2.4 mmol 3-chlorobenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (43%). The product was purified by wash in chloroform. M.p. = 183 °C. IR $cm^{-1}$: 1633 (C═O). $^1$H NMR (300 MHz, DMSO-$d_6$) ppm: 2.43 (s, 3H, $CH_3$), 7.47–7.45 (d, $J$ = 4.5 Hz, 2H, arH) 7.62–7.55 (t, $J$ = 15 Hz, 4,2Hz, 1H, arH), 8.44 (s, 1H, N═CH), 11.29 (s, 1H, NH), 12.39 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.55 ($CH_3$), 104.74 (C3-isothiazole), 125.94 (N═CH), 126.47(arC), 130.15 (arC), 131.37 (arC), 134.25 (arC), 136.53 (arC), 144.95 (arC), 165.08 (C4-isothiazole), 167.15 (C5-isothiazole), 176.50 (C═O). ESI-MS [M-H] *m/z*: 294.01, calc. *m/z*: 294.01.

#### *2.2.4. 5-[2-(3-phenylprop-2-en-1-ylidene)hydrazin-1-yl]-3-methylisothiazole-4-carboxylic acid **4***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3.17 mmol cinnamic aldehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (66%). The product was purified by crystallizaton in chloroform. M.p. = 173–174 °C. IR $cm^{-1}$: 1720 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.41 (s, 3H, $CH_3$), 6.97–6.95 (d, $J$ = 4.8 Hz, 1H, arH), 7.39–7.30 (m, $J$ = 6.6 Hz, 7.5 Hz, 5.1 Hz, 6.9 Hz, 4H, arH), 7.63–7.60 (d, $J$ = 6.9 Hz, 2H, arH), 8.30–8.27 (t, $J$ = 4.2 Hz, 4.5 Hz, 1H, N═CH), 11.10 (s, 1H, NH),12.81 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.26 ($CH_3$), 109.22 (C3-isothiazole), 117.61 (N═CH), 125.01 (arC), 127.41 (2C, arC), 129.07 (2C, arC), 136.10 (CH═), 139.03 (arC), 149.83 (CH═), 164.89 (C4-isothiazole), 166.73 (C5-isothiazole), 175.76 (C═O). ESI-MS [M-H] *m/z*: 286.06, calc. *m/z*: 286.06.

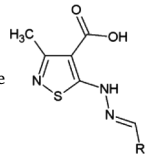
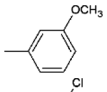
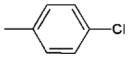
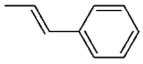
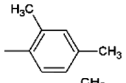
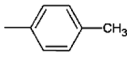
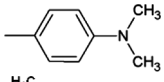
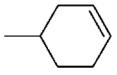
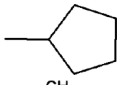
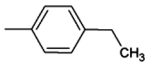
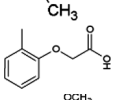
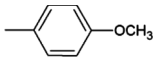
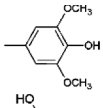
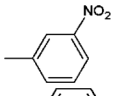
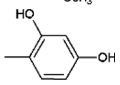
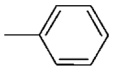
#### *2.2.5. 5-{2[(2,4-dimethylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **5***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 1.5 mL DMSO, 3 mmol 2,4-dimethylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (69%). The product was purified by wash in chloroform. M.p. = 170 = 171 °C. IR $cm^{-1}$: 1638 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.28 (s, 3H, $CH_3$), 2.38 (s, 3H, $CH_3$), 2.42 (s, 3H, $CH_3$), 7.08–7.05 (d, $J$ = 7.2 Hz, 2H,arH), 7.58–7.56 (d, $J$ = 8.4 Hz, 1H, arH), 8.72 (s, 1H, N═CH), 11.15 (s, 1H, NH), 12.79 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-$d_6$) ppm: 19.98 ($CH_3$), 21.46 ($CH_3$), 21.61 ($CH_3$), 103.94 (C3-isothiazole), 126.79 (N═CH), 127.51 (arC), 129.65 (arC), 132.16 (arC), 137.37 (arC), 139.96 (arC), 146.17 (arC), 165.21 (C4-isothiazole), 167.06 (C5-isothiazole), 176.58 (C═O). ESI-MS [M-H] *m/z*: 288.08, calc. *m/z*: 288.08.

#### *2.2.6. 5-{2[(3-methylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **6***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 4.24 mmol 3-methylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the

**Table 1**
Chemical structures of new isothiazole derivatives.

| Name of compound | Chemical structure | | |
|---|---|---|---|
| **1** |  | | |
| Chemical structure |  | | |
| Name of compound | -R | Name of compound | -R |
| **2** |  | **15** |  |
| **3** |  | **16** |  |
| **4** |  | **17** |  |
| **5** |  | **18** |  |
| **6** |  | **19** |  |
| **7** |  | **20** |  |
| **8** |  | **21** |  |
| **9** |  | **22** |  |
| **10** |  | **23** |  |
| **11** |  | **24** |  |
| **12** |  | **25** |  |
| **13** |  | **26** |  |
| **14** |  | | |

end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (64%). The product was purified by wash in chloroform. M.p. = 187 °C. IR $cm^{-1}$: 1657 (C═O). $^1$H NMR (300 MHz, DMSO-$d_6$) ppm: 2.33 (s, 3H, $CH_3$), 2.42 (s,3H, $CH_3$), 7.23–7.21 (d, $J$ = 7.5 Hz, 1H, arH), 7.34–7.29 (t, $J$ = 7.8 Hz, 7.8 Hz, 1H, arH), 7.42–7.40 (d, $J$ = 6.0 Hz, 2H, arH), 8.43 (s, 1H, N═CH), 11.17 (s, 1H, NH), 12.82 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-$d_6$) ppm: 21.19 ($CH_3$), 21.29 ($CH_3$), 104.00 (C3-isothiazole), 124.36 (N═CH), 127.39 (arC), 129.08 (arC), 131.05 (arC), 133.99 (arC), 138.42 (arC), 146.54 (arC), 164.89 (C4-isothiazole), 166.78 (C5-isothiazole), 176.30 (C═O). ESI-MS [M-H] $m/z$: 274.06, calc. $m/z$: 274.06.

### 2.2.7. 5-{2[4-(dimethylaminophenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **7**

To 2.6 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3 mmol 3-dimethylaminobenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (84%). The product was purified by wash in toluene. M.p. = 172 °C. IR $cm^{-1}$: 1695 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.40 (s,3H, $CH_3$), 2.99–2.96 (d, $J$ = 9.9 Hz, 6H, $N(CH_3)_2$), 6.75–6.72 (d, $J$ = 9 Hz, 2H, arH), 7.45–7.42 (d, $J$ = 9 Hz, 2H, arH), 8.30 (s, 1H, N═CH), 10.90 (s, 1H, NH), 12.71 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-$d_6$) ppm: 21.82 ($CH_3$), 40,35 ($NCH_3$), 40,90 ($NCH_3$), 103.63 (C3-isothiazole), 112.66 (N═CH), 121.81(2C, arC), 129.00 (arC), 147.90 (2C, arC), 152.33 (arC), 165.63 (C4-isothiazole), 167.13 (C5-isothiazole), 176.52 (C═O). ESI-MS [M-H] $m/z$: 303.10, calc. $m/z$: 303.09.

### 2.2.8. 5-{2[(2-methylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **8**

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3.4 mmol 2-methylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (91%). The product was purified by wash in chloroform. M.p. = 176 °C. IR $cm^{-1}$: 1640 (C═O). $^1$H NMR (300 MHz, DMSO-$d_6$) ppm: 2.42 (s, 6H, $CH_3$), 7.27–7.25 (d, $J$ = 6.3 Hz, 3H, arH), 7.69–7.67 (d, $J$ = 7.5 Hz, 1H, arH), 8.77 (s, 1H, N═CH), 11.22 (s, 1H, NH), 12.77 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-$d_6$) ppm: 19.42 ($CH_3$), 21.02 ($CH_3$), 103.59 (C3-isothiazole), 126.07 (N═CH), 126.21 (arC), 129.66 (arC), 130.98 (arC), 131.80 (arC), 136.88 (arC), 145,44 (arC), 164.61 (C4-isothiazole), 166.55 (C5-isothiazole), 176.06 (C═O). ESI-MS [M-H] $m/z$: 274.06, calc. $m/z$: 274.06.

### 2.2.9. 5-{2[(2-methoxylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **9**

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 4 mmol 2-methoxylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (73%). The product was purified by wash in chloroform. M.p. = 183–184 °C. IR $cm^{-1}$: 1652 (C═O). $^1$H NMR (300 MHz, DMSO-$d_6$) ppm: 2.42 (s, 3H, $CH_3$), 3.87–3.84 (d, $J$ = 10.5 Hz, 3H, $OCH_3$), 7.09–6.97 (m, $J$ = 8.1 Hz, 12 Hz, 7.5 Hz, 7.5 Hz, 2H, arH), 7.41–7.37 (t, $J$ = 6.9 Hz, 7.2 Hz, 1H, arH), 7.73–7.70 (d, $J$ = 6.3 Hz, 1H, arH), 8.78 (s, 1H, N═CH), 11.28 (s, 1H, NH), 12.74 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-$d_6$) ppm: 21.27 ($CH_3$), 56.00 ($OCH_3$), 103.80 (C3-isothiazole), 112.24 (N═CH), 121.07 (arC), 122.25 (arC), 125.54 (arC), 131.76 (arC), 141.94 (arC), 157.87 (arC), 164.75 (C4-isothiazole), 166.82 (C5-isothiazole), 176.31 (C═O). ESI-MS [M-H] $m/z$: 290.06, calc. $m/z$: 290.06.

### 2.2.10. 5-{2[(2-chlorophenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **10**

To 5.77 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 8.8 mmol 2-chlorobenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (31%). The product was purified by wash in chloroform. M.p. = 181 °C. IR $cm^{-1}$: 1663 (C═O). $^1$H NMR (300 MHz, DMSO-$d_6$) ppm: 2.43 (s, 3H, $CH_3$), 7.43–7.40 (t, $J$ = 3.3 Hz, 6 Hz, 2H, arH), 7.52–7.49 (t, $J$ = 6.3 Hz, 3 Hz, 1H, arH), 7.88–7.85 (t, $J$ = 5.7 Hz, 3.9 Hz, 1H, arH), 8.89 (s, 1H, N═CH), 11.54 (s, 1H, NH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.58 ($CH_3$), 104.78 (C3-isothiazole), 127.11 (N═CH), 128.19 (arH) 130.55 (arH), 131.83 (arH), 133.49 (arH), 142.53 (arH), 154.30 (arH), 164.91 (C4-isothiazole), 167.25 (C5-isothiazole), 176.59 (C═O). ESI-MS [M-H] $m/z$: 294.01, calc. $m/z$: 294.01.

*2.2.11. 5-{2[(4-ethylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **11***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3.6 mmol 4-ethylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (80%). The product was purified by crystallization in chloroform. M.p. = 162–163 °C. IR $cm^{-1}$: 1652 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 1.20–1.15 (t, *J* = 7.5 Hz, 7.8 Hz, 3H, $\underline{CH_3}CH_2$), 2.42 (s, 3H, $CH_3$), 2.66–2.58 (q, *J* = 7.5 Hz, 7.5 Hz, 7.5 Hz, 2H, $CH_3\underline{CH_2}$), 7.29–7.26 (d, *J* = 8.1 Hz, 2H, arH), 7.54–7.52 (d, *J* = 7.8 Hz, 2H, arH), 8.43 (s, 1H, N═CH), 11.13 (s, 1H, NH), 12.82 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-$d_6$) ppm: 15.61 ($CH_3\underline{CH_2}$), 21.28 ($CH_3$), 28.36 ($\underline{CH_3}CH_2$), 103.86 (C3-isothiazole), 127.11 (N═CH), 128.61 (2C, arC), 131.59 (2C, arC), 146.41 (arC), 146.41 (arC), 164.91 (C4-isothiazole), 166.76 (C5-isothiazole), 176.28 (C═O). ESI-MS [M-H] *m/z*: 288.08, calc. *m/z*: 288.08.

*2.2.12. 5-{2[(4-metoxylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **12***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 5 mmol 4-metoxylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (54%). The product was purified by crystallization in chloroform. M.p. = 163 °C. IR $cm^{-1}$: 1634 (C═O). $^1$H NMR (300 MHz, DMSO-$d_6$) ppm: 2.42 (s,3H, $CH_3$), 3.79 (s,3H, $OCH_3$), 7.02–6.99 (d, *J* = 8.4 Hz, 2H, arH), 7.57–7.55 (d, *J* = 8.4 Hz, 2H, arH), 8.40 (s, 1H, N═CH), 11.06 (s, 1H, NH), 12.80 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.29 ($CH_3$), 55.65 ($OCH_3$), 103.60 (C3-isothiazole), 114.66 (N═CH), 126.84 (arC), 128.67 (arC), 130.23 (arC), 146.40 (arC), 160.72 (arC), 161.93 (arC), 164.95 (C4-isothiazole), 166.70 (C5-isothiazole), 176.23 (C═O). ESI-MS [M-H] *m/z*: 290.06, calc. *m/z*: 290.06.

*2.2.13. 5-{2[(3-nitrophenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **13***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3.3 mmol 3-nitrobenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (53%). The product was purified by wash in chloroform. M.p. = 203–204 °C. IR

$cm^{-1}$: 1623 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.44 (s,3H, $CH_3$), 7.75–7.70 (t, *J* = 8.1 Hz, 7.8 Hz, 1H, arH), 8.05–8.02 (d, *J* = 7.5 Hz, 1H, arH), 8.24–8.22 (d, *J* = 7.8 Hz, 1H, arH), 8.37 (s, 1H, arH), 8.58 (s, N═CH), 11.38 (s, 1H, NH), 12.41 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.29 ($CH_3$), 104.77 (C3-isothiazole), 121.05 (N═CH), 124.42 (arC), 126.08 (arC), 132.95 (arC), 135, 83 (arC), 143.94 (arC), 148.53 (arC), 164.76 (C4-isothiazole), 166.97 (C5-isothiazole), 176.17 (C═O). ESI-MS [M-H] *m/z*: 305.03, calc. *m/z*: 305.03.

*2.2.14. 5-[2-(benzylidene)hydrazin-1-yl]-3-methylisothiazole-4-carboxylic acid **14***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 4.9 mmol benzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (86%). The product was purified by wash in chloroform. M.p. = 175–176 °C. IR $cm^{-1}$: 1647 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.42 (s, 3H, $CH_3$), 7.44–7.42 (d, *J* = 6.6 Hz, 3H, arH), 7.62–7.61 (d, *J* = 5.7 Hz, 2H, arH), 8.47 (s, 1H, N═CH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.57 ($CH_3$), 104.34 (C3-isothiazole), 127.31 (N═CH), 129.47 (2C, arC), 130.59 (2C, arC), 134.31 (arC), 146.69 (arC), 165.16 (C4-isothiazole), 167.09 (C5-isothiazole), 176.60 (C═O).ESI-MS [M-H] *m/z*: 250.05, calc. *m/z*: 250.04.

*2.2.15. 5-{2[(3-hydroxyphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **15***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 4.09 mmol 3-hydroxybenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (48%). The product was purified by crystallization in methanol. M.p. = 217 °C. IR $cm^{-1}$: 1643 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.42 (s, 3H, $CH_3$), 6.81–6.78 (d, *J* = 7.8 Hz, 1H, arH), 7.06–6.99 (t, *J* = 13.8 Hz, 7.5 Hz, 2H, arH), 7.25–7.20 (t, *J* = 7.8 Hz, 7.8 Hz, 1H, arH), 8.37 (s, 1H, N═CH), 9.63 (s, 1H, OH), 11.15 (s, 1H, NH), 12.70 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.01 ($CH_3$), 103.76 (C3-isothiazole), 112.47 (N═CH), 117.42 (arC), 118.41 (arC), 129.94 (arC), 135.01 (arC), 146.31 (arC), 157.67 (arC), 164.65 (C4-isothiazole), 166.54 (C5-isothiazole), 176.01 (C═O). ESI-MS [M-H] *m/z*: 276.04, calc. *m/z*: 276.04.

*2.2.16. 5-{2[(4-chlorophenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **16***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 2.4 mmol 4-chlorobenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (35%). The product was purified by wash in chloroform. M.p. = 172–173 °C. IR

$cm^{-1}$: 1624 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.42 (s, 1H, $CH_3$), 7.64–7.49 (m, *J* = 8.4 Hz, 8.4 Hz, 8.4 Hz, 12.3 Hz, 8.4 Hz, 4H, arH), 8.46 (s, 1H, N═CH), 11.25 (s, 1H, NH), 12.87 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.47 ($CH_3$), 104.49 (C3-isothiazole), 128.82 (N═CH), 129.56 (2C, arC), 130.49 (2C, arC), 133.18 (arC), 134.90 (arC), 145.30 (C4-isothiazole), 161.03 (C5-isothiazole), 165.04 (C═O). ESI-MS [M-H] *m/z*: 294.01, calc. *m/z*: 294.01.

*2.2.17. 5-{2[(2-hydroxyphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **17***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 2.4 mmol 4-hydroxybenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (38%). The product was purified by wash in toluene. M.p. = 183–185 °C. IR $cm^{-1}$: 1648 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.42 (s, 3H, $CH_3$), 6.89–6.84 (t, *J* = 8.7 Hz, 8.7 Hz, 2H, arH), 7.26–7.21 (t, *J* = 7.2 Hz, 7.8 Hz, 1H, arH), 7.60–7.58 (d, *J* = 7.5 Hz, arH), 8.73 (s, 1H, N═CH), 10.00 (s, 1H, OH), 11.23 (s, 1H, NH), 12.65 (s, 1H, OH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.55 ($CH_3$), 104.05 (C3-isothiazole), 116.78 (N═CH), 120.05 (arC), 120.38 (arC), 126.95 (arC), 131.86 (arC), 144.38 (arC), 156.96 (arC), 165.03 (C4-isothiazole), 167.19 (C5-isothiazole), 176.31 (C═O). ESI-MS [M-H] *m/z*: 276.04, calc. *m/z*: 276.04.

*2.2.18. 5-{2[(4-methylphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **18***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3.39 mmol 4-methylbenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (24%). The product was purified by crystallization in methanol. M.p. = 156–157 °C. IR $cm^{-1}$: 1619 (C═O). $^1$H NMR (300 MHz, DMSO-d6) ppm: 2.36 (s, 3H, $CH_3$), 2.49 (s, 3H, $CH_3$), 7.31–7.29 (d, *J* = 7.8 Hz, 2H, arH), 7.76–7.74 (d, *J* = 8.1 Hz, 2H, arH), 8.65 (s, 1H, N═CH). $^{13}$C NMR (300 MHz, DMSO-d6) ppm: 21.55 (2C, $CH_3$), 104.10 (C3-isothiazole), 128.72 (N═CH), 129.91 (2C, arC), 131.68 (2C, arC), 140.32 (arC), 141,69 (arC), 161.40 (C4-isothiazole), 165.05 (C5-isothiazole), 176.46 (C═O). ESI-MS [M-H] *m/z*: 274.06, calc. *m/z*: 274.06.

*2.2.19. 5-[2-(cyclohexylmetylidene)hydrazin-1-yl]-3-methylisothiazole-4-carboxylic acid **19***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 4.12 mmol cyclohexylcarboaldehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (59%). The product was purified by crystallization in methanol. M.p. = 166 °C. IR $cm^{-1}$: 1641 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 1.30–1.17 (q, *J* = 11.7 Hz, 15.9 Hz, 11.4 Hz, 5H, CH), 1.75–1.61 (q, *J* = 13.5 Hz, 13.2 Hz, 15.3 Hz, 5H, CH), 2.20 (s, 1H, CH), 2.38 (s, 3H, $CH_3$), 7.68–7.67 (d, *J* = 5.1 Hz, 1H, N═CH), 10.72 (s, 1H, NH), 12.65 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.51 ($CH_3$), 25.36 (2C, CH), 26.03 (3C, CH), 30.07 (CH), 103.33 (C3-isothiazole), 155.06 (N═CH), 165.24 (C4-isothiazole), 166.81 (C5-isothiazole), 176.85 (C═O). ESI-MS [M-H] *m/z*: 266.09, calc. *m/z*: 266.09.

*2.2.20. 5-{2[(pyridin-4-yl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **20***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 4.76 mmol 4-pyridinecarboxaldehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (31%). The product was purified by crystallization in methanol. M.p. = 226–228 °C. IR $cm^{-1}$: 1675 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 2.44 (s, 1H, $CH_3$), 7.55–7.53 (d, *J* = 5.4 Hz, 2H, pyrCH), 8.45 (s, 1H, N═CH), 8.63–8.61 (d, *J* = 5.4 Hz, 2H, pyrCH), 11.48 (s, 1H, NH), 12.97 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.26 ($CH_3$), 105.03 (C3-isothiazole), 120.82 (N═CH), 141.14 (2C, pyrH), 143.65 (pyrH), 150.60 (2C, pyrH), 164.68 (C4-isothiazole), 167.00 (C5-isothiazole), 176.19 (C═O). ESI-MS [M-H] *m/z*: 261.04, calc. *m/z*: 261.04.

*2.2.21. 5-{2[(cyclohex-3-en-1-yl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **21***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 3 mL DMSO, 3.4 mmol 3-cyclohexene-1-carboaldehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (48%). The product was purified by crystallization in methanol. M.p. = 161–163 °C. IR $cm^{-1}$: 1639 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 1.44 (s, 1H), 1.81 (s, 1H, CH), 2.04 (s, 5H, CH), 2.38 (s, 3H, $CH_3$), 5.67 (s, 2H, CH), 7.76–7.74 (d, *J* = 5.1 Hz, N═CH N═CH), 10.80 (s, 1H, NH), 12.69 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.52 ($CH_3$), 24.17 (CH), 26.08 (CH), 28.31 (CH), 36.44 (CH), 103.41 (C3-isothiazole), 125.82 (N═CH), 127.34 (2C, =CH), 154.34 (C4-isothiazole), 165.23 (C5-isothiazole), 166.84 (C═O). ESI-MS [M-H] *m/z*: 264.08, calc. *m/z*: 264.08.

*2.2.22. 5-{2[(cyclopentylmethylidene)hydrazin-1-yl]-3-methylisothiazole-4-carboxylic acid **22***

To 2.3 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3.7 mmol 3-cyclopenylcarboaldehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the clear mixture was poured into 50 mL of water and the product was filtered off and then washed with methanol (48%). The product was purified by crystallization in methanol. M.p. = 162–163 °C. IR $cm^{-1}$: 1638 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 1.59–1.52 (t, *J* = 7.8 Hz, 11.7 Hz, 7H, CH), 1.75 (s, 2H, CH), 2.18 (s, 1H), 2.38 (s,3H, $CH_3$), 2.68–2.63 (t, *J* = 7.2 Hz, 6.9 Hz, 1H, CH), 7.72–7.70 (d, *J* = 6 Hz, 1H, N═CH), 10.71 (s, 1H, NH), 12.71 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.52 ($CH_3$), 25.39 (2C, CH), 30.47 (2C, CH), 42.37 (CH), 103.25 (C3-isothiazole), 154.90 (N═CH), 165.24 (C4-isothiazole), 166.82 (C5-isothiazole), 176.73 (C═O). ESI-MS [M-H] *m/z*: 252.08, calc. *m/z*: 252.08.

*2.2.23. 5-[2-(propan-2-ylidene)hydrazin-1-yl]-3-methylisothiazole-4-carboxylic acid **23***

To 2 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 10 mL methonol, 10 mL acetone was added. The mixture was stirred and heated in a boiling temperature for 2 h. Then the mixture was filtered off and condensed to half the volume and set to crystallization (34%). The product was crystallized from methanol. M.p. = 166 °C. IR $cm^{-1}$: 1698 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 1.92 (s, 3H, $CH_3$), 1.98 (s, 3H, $CH_3$), 2.40 (s, 3H, $CH_3$), 10.08 (s, 1H, NH), 12.91 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 17.10 ($CH_3$), 21.38 ($CH_3$), 25.12 ($CH_3$), 103.93 (C3-isothiazole), 153.82 (C4-isothiazole), 166.06 (C5-isothiazole), 166.59 (C═O). 176.71. ESI-MS [M + H] *m/z*: 214.06, calc. *m/z*: 214.06.

*2.2.24. 5-{2[(2-carboxymethoxyl)phenoxymethylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **24***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 3 mmol 2-formylphenoxy acetic acid was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (69%). The product was purified by crystallization in methanol. M.p. = 180–182 °C. IR $cm^{-1}$: 1652 (C═O), 1600 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 2.41 (s, 3H, $CH_3$), 4.63 (s, 2H, $CH_2$), 7.09–6.92 (m, *J* = 9.3 Hz, 15.3 Hz, 7.5 Hz, 9.9 Hz, 9 Hz, 2H, arH), 7.34–7.29 (t, *J* = 7.8 Hz, 8.1 Hz, 1H, arH), 7.73–7.71 (d, *J* = 8.4 Hz, 1H, arH), 8.71 (s, 1H, N═CH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.49 ($CH_3$), 66.43 ($CH_2$), 105.06 (C3-isothiazole), 118.42 (N═CH), 121.52 (arC), 122.93 (arC), 128.58 (arC), 132.69 (arC), 151.02 (arC), 157.06 (arC), 165.55 (C4-isothiazole), 167.20 (C5-isothiazole), 170.88 (C═O), 176.30 (C═O). ESI-MS [M-H] *m/z*: 334.05, calc. *m/z*: 334.05.

*2.2.25. 5-{2[(4-hydroxy-3,5-dimethoxyphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **25***

To 2.2 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 1.5 mL DMSO, 2.19 mmol 4-hydroxy-3,5-dimetoxybenzladehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (59%). The product was purified by crystallization in methanol. M.p. = 194 °C. IR $cm^{-1}$: 1639 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 2.42 (s,3H, $CH_3$), 3.80 (s, 6H, $OCH_3$), 6.88 (s, 2H, arH), 8.31 (s, 1H, arH), 8.92 (s, 1H, N═CH), 11.09 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.57 $CH_3$, 56.56 (2C, $OCH_3$), 103.84 (C3-isothiazole), 105.01 (N═CH), 124.55 (2C, arC), 138.66 (2C, arC), 147.21 (arC), 148.74 (arC), 165.27 (C4-isothiazole), 166.98 (C5-isothiazole), 176.44 (C═O). ESI-MS [M-H] *m/z*: 336.06, calc. *m/z*: 336.06

*2.2.26. 5-{2[(2,4-dihydroxyphenyl)methylidene]hydrazin-1-yl}-3-methylisothiazole-4-carboxylic acid **26***

To 2.88 mmol of 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** in 0.5 mL DMSO, 5 mmol 2,4-dihydroxybenzaldehyde was added. The mixture was stirred and heated in temperature 55–65 °C for 4 h. At the end of the reaction (controlled in a TLC), the precipitate was filtered off and then washed with methanol (56%). The product was purified by crystallization in methanol. M.p. = 207 °C. IR $cm^{-1}$: 1624 (C═O). $^1H$ NMR (300 MHz, DMSO-d6) ppm: 2.41 (s,3H, $CH_3$), 6.33–6.31 (d, *J* = 7.2 Hz, 2H, arH), 7.39–7.36 (d, *J* = 9.3 Hz,1H, arH), 8.58 (s, 1H, N═CH), 9.85 (s, 1H, OH), 9.93 (s, 1H, OH), 11.05 (s, 1H, NH), 12.66 (s, 1H, OH). $^{13}C$ NMR (300 MHz, DMSO-d6) ppm: 21.56 ($CH_3$), 103.01 (C3-isothiazole), 103.51 (arC), 108.61 (arC), 111.80 (N═CH N═CH), 128.90 (arC), 145.70 (arC), 158.73 (arC), 161.28 (arC), 165.16 (C4-isothiazole), 167.14 (C5-isothiazole), 175.99 (C═O). ESI-MS [M-H] *m/z*: 292.03, calc. *m/z*: 292.03.

### 2.3. Pharmacology

#### 2.3.1. Cell culture

Five human cancer cell lines were used to evaluate the antiproliferative activity of the compounds: biphenotypic B cell myelomonocytic leukemia (MV4-11), human colon adenocarcinoma cell lines sensitive (LoVo) and resistant to doxorubicin (LoVo/DX), breast adenocarcinoma MCF-7 and normal non-tumorigenic epithelial cell line were derived from mammary gland MCF-10A. The MV-4-11, MCF-7 and MCF-10A cell lines were purchased from the American Type Culture Collection (ATCC Rockville, Maryland, USA), LoVo and LoVo/DX have been shared by Prof. Borowski from Technical University of Gdansk (Gdansk, Poland). Cell line collections belongs to the Institute of Immunology and Experimental Therapy (IIET). A medium RPMI 1640 (Gibco, Gaithersburg, USA) was used for culturing the human leukemia cells, supplemented with 10% FBS (fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, USA)). OptiMEM (Gibco, Gaithersburg, USA) and RPMI 1640 (PAA, Austria) mixed 1:1 were used for human colon adenocarcinoma cell lines. Medium was supplemented with 5% FBS (fetal bovine serum (PAA, Austria)) and 1 mM sodium puryvate, 2 mM L-glutamine (both Sigma-Aldrich, Germany). Antibiotics were added to culture media 100 mg/ml streptomycin with 100U/ml penicillin (both Polfa-Tarchomin, Poland).Cell lines were grown under standard conditions at 37 °C and 5% $CO_2$ in a humid atmosphere.

#### 2.3.2. Cells preparing for antiproliferative assays

All cell lines used in the research with the number $10^4$ cells per well were placed in 96-well plates (Sarstedt, Germany) in culture medium. The tested compounds were prepared in different concentrations (100 to 0.1 μg/ml) and the cells were exposed in them for 72 h. The reference material in the research was 5-fluorouracil (Ebewe, Austria). The DMSO as an solvent control was used (Sigma-Aldrich, Germany).

The evaluated antiproliferative activities have been expressed as an $IC_{50}$ value, which is the concentration of the tested compound (in μg/ml) that inhibits the proliferation of 50% of the cell population. The cytotoxicity data for each cell line have been collected from at least three independent experiments (conducted in triplicate) at each of the four different concentrations of the tested compounds. The resulting $IC_{50}$ values are presented as an arithmetic mean ± SD.

*2.3.2.1. MTT assay (for MV4-11 cell line)*. Twenty microliters of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich); stock solution: 5 mg/ml] was added to each well and incubated for 4 h. Then 80 μl of buffer (275 mL of distilled water (IIET, Wroclaw), 67.5 g sodium dodecyl sulfate (Sigma-Aldrich), 225 mL dimethylformamide (POCh, Poland) was placed in each well. The optical density at the 570 nm wavelength was read with Multiskan RC photometer (Labsystems, Finland).

*2.3.2.2. Sulforhodamine B assay (for other cell lines)*. The cells were attached to the bottom of plastic wells by fixing them *in situ* by gently adding 50 μl per well of cold 50% trichloroacetic acid TCA (POCh, Poland) and were incubated at 4 °C for 1 h. All wells were subjected to rinsing with water and left to dry. Then 50 μl of sulphorodamine B (SigmaAldrich, Germany) was added (0.4% in 1% acetic acid (POCh, Poland) for 30 min at room temperature. Last step was removing the SRB, by washing plates with 1% acetic acid. Then the stanning dye was dissolved in 150 μl buffer of 10 mMTris base (Sigma-Aldrich, Germany). Absorbance of each solution was read at Multiskan RC photometer (Labsystems, Finland) at the 540 nm wavelength.

## 3. Results

The biological results shown in Tables 2 and 3 indicate that the obtained isothiazole derivatives have strong anticancer properties. The series of new 26 isothiazole derivatives were tested for antiproliferative activity towards MV4-11. Compounds **2–13** caused the high inhibition of proliferation of this cell line (13 compounds). Poor antiproliferative activity exhibited 10 received derivatives (**15–22**), for which $IC_{50}$ values were designated, but due to the low activity compounds with these properties were not qualified for the next stage of research. For the next 4 compounds, $IC_{50}$ values were not determined (Table 1). The exchange phenyl ring in the core structure of isothiazole derivatives for propan-2-ylidene group (compound **23**) caused a decrease in antiproliferative activity. What is more, the substitution of the phenyl ring in position 2 with carboxymethoxyl (compound **24**), with the methoxy groups in position 3 and 5, the hydroxyl group in position 4 (compound **25**) and the hydroxyl groups in position 2 and 4 (compound **26**) also lead to a high decrease in the antiproliferative effect in comparision to the other derivatives.

**Table 2**
Results of examination of antiproliferative activity of all obtained compounds towards human biphenotypic B cell myelomonocytic leukemia (MV4-11). The substances selected for the consecutive research stage were marked.

| MV-4-11 Name of compound | $IC_{50}$ ± SD [μg/ml] | Name of compound | $IC_{50}$ ± SD [μg/ml] |
|---|---|---|---|
| **5-flourouracil** | 0.04 ± 0.01 | **14** | 7.80 ± 2.27 |
| **1** | 47.45 ± 1.54 | **15** | 18.91 ± 3.79 |
| **2** | 0.99 ± 0.11 | **16** | 19.03 ± 9.59 |
| **3** | 1.53 ± 0.73 | **17** | 20.05 ± 5.06 |
| **4** | 2.43 ± 1.47 | **18** | 22.97 ± 2.30 |
| **5** | 2.68 ± 1.18 | **19** | 23.38 ± 5.87 |
| **6** | 2.77 ± 1.39 | **20** | 38.41 ± 8.57 |
| **7** | 3.72 ± 1.05 | **21** | 46.03 ± 7.40 |
| **8** | 4.36 ± 0.95 | **22** | 68.32 ± 12.1 |
| **9** | 4.76 ± 1.01 | **23** | n.a. |
| **10** | 5.07 ± 3.47 | **24** | n.a. |
| **11** | 6.65 ± 3.63 | **25** | n.a. |
| **12** | 6.67 ± 3.10 | **26** | n.a. |
| **13** | 6.98 ± 0.79 | | |

n.a. – not active in the range of concentrations used.

During the next step, selected compounds (**2–13**) were evaluated in relation to human colon adenocarcinoma cell lines sensitive LoVo and resistant to doxorubicin LoVo/DX as well as in relation to human mammary gland cancer MCF-7 and normal non-tumorigenic epithelial cell line derived from mammary gland MCF-10A. The most active in this series compounds **2** and **3** were similar in action. Substitution of the phenyl ring with -$OCH_3$ (compound **2**) and -Cl (compound **3**) in position 3 gave the same type of strong activity towards human colon adenocarcinoma LoVo and multi-drug resistant LoVo/DX cell lines, but also with little antiproliferative activity towards MCF-7 and MCF-10A cell lines. Moreover, compound **2** revealed to some extent selectivity towards colon cancer cells, exhibiting ~10 times lower antiproliferative properties on MCF-10A normal epithelial cells than on LoVo cancer. Interestingly, obtained compounds break through the multidrug-resistance of LoVo/DX cells, which may suggest their possible high anticancer potential in relation to drug-resistant cancer cells.

Summarising, the eight substances were characterized by high antiproliferative activity ($IC_{50}$ below 10 μg/ml) against human colon adenocarcinoma cell line LoVo: **2**, **3**, **7**, **8**, **5**, **4**, **11**, **9** (accordance with decreasing activity). The other substances selected for testing showed significantly lower activity ($IC_{50}$ above 10 μg/ml). All examined compounds broke the resistance of LoVo/DX cells (resistance index (RI) below 1), whereas twelve substances achieved high activity against LoVo/Dx cells ($IC_{50}$ below 4.5 μg/ml): **11**, **4**, **7**, **2**, **3**, **8**, **9**, **5**, **6**, **14**, **10**, **12** (accordance with decreasing activity). MCF-7 cell line was the most insensitive amongst the tested cancer cell lines. Indeed, none of the compounds tested turned out to be more active with 5-fluorouracil, however all of the tested compounds cross the cell-resistance barrier and their activity on LoVo/DX cells is in many cases higher than the activity of doxorubicin.

**Table 3**
Result of examination of antiproliferative activity of 13 compounds towards human colon adenocarcinoma cell lines sensitive (LoVo) and multi-drug resistant (LoVo/DX), breast adenocarcinoma MCF-7 and normal non-tumorigenic epithelial cell line derived from mammary gland MCF-10A. $IC_{50}$ concentration of examined substances in μg/ml (average + standard deviation) and index of the resistance (RI, calculated as a result of dividing $IC_{50}$ values for a resistant line relative to a sensitive line).

| Name of compund | $IC_{50}$ ± SD[μg/ml] | | | | RI |
|---|---|---|---|---|---|
| | MCF-7 | MCF-10A | LoVo | LoVoDX | |
| **5-fluorouracil** | 1.22 ± 0.23 | 4.41 ± 1.84 | 0.42 ± 0.02 | 0.49 ± 1.84 | 1,16 |
| **2** | 10.74 ± 1.30 | 16.30 ± 7.47 | 1.55 ± 0.36 | 0.99 ± 0.35 | 0.64 |
| **3** | 10.83 ± 1.22 | 12.22 ± 5.27 | 2.26 ± 0.62 | 1.07 ± 0.52 | 0.47 |
| **4** | 23.41 ± 2.17 | 81.49 ± 0.67 | 5.52 ± 2.70 | 0.45 ± 0.04 | 0.08 |
| **5** | 23.97 ± 4.49 | 78.24 ± 4.56 | 4.70 ± 1.42 | 2.17 ± 1.93 | 0.46 |
| **6** | 28.69 ± 2.45 | 67.30 ± 5.09 | 11.60 ± 3.53 | 2.52 ± 1.01 | 0.22 |
| **7** | 33.90 ± 5.95 | 53.56 ± 1,09 | 3.93 ± 2.41 | 0.98 ± 0.98 | 0.25 |
| **8** | 24.53 ± 4.17 | 81.13 ± 5.71 | 4.42 ± 0.62 | 1.63 ± 0.78 | 0.37 |
| **9** | 28.29 ± 3.65 | 59.88 ± 10.59 | 9.41 ± 2.70 | 1.88 ± 0.97 | 0.20 |
| **10** | 66.08 ± 6.72 | 70.26 ± 1.82 | 14.08 ± 2.11 | 3.57 ± 1.19 | 0.25 |
| **11** | 34.48 ± 5.15 | 80.28 ± 5.66 | 5.90 ± 2.93 | 0.33 ± 0.15 | 0.05 |
| **12** | n.a. | 48.10 ± 7.50 | 51.43 ± 2.17 | 4.23 ± 3.98 | 0.08 |
| **13** | n.a. | n.a. | 37.26 ± 11.44 | 32.25 ± 6.89 | 0.86 |
| **14** | 32.98 ± 4.34 | 44.34 ± 7.18 | 24.65 ± 3.11 | 3.50 ± 0.38 | 0.14 |
| **Doxorubicini hydrochloride** | [N/T] | [N/T] | 0.14 ± 0.13 | 3.76 ± 1.37 | 27 |

N/T – no tested.
n.a. – not active in the range of concentrations used.

## 4. Discussion

Currently, new drugs are being sought, which are characterized by the specificity and selectivity of action, thus reducing the probability of side effects. In the design of isothiazole derivative structures with more favorable therapeutic properties than the lead structure, i.e. 5-hydrazine-3-methylisothiazole-4-carboxylic acid **1** of the entire series of derivatives were used different ways of designing new, effective and selective derivatives. The main method was the replacement of substituents on the aromatic ring, where the substituents were introduced using, among others, Topliss operating scheme **2,3**,**5-13**, **15-18**, **24**, **25**, **26**. The change of the benzene ring to the alkyl substituent **23** was also introduced, elongation of the chain connecting the two important moieties for binding to the site of action **4**, exchange of rings **19-22**. The most favorable strategy turned out to be replacement of substituents in the aromatic ring leading to the most active compounds of this series of derivatives. The most of the compounds exhibited very strong and directional action, which had potential therapeutic values. The activity of the tested compounds apparently depends on the type and location of substituents in the phenyl ring.

Among many physicochemical and structural parameters characterizing the molecule, the most common parameters are hydrophobic as well as electronic and steric properties. The hydrophobicity of a compound raises its biological activity due to the ease of overcoming cell membranes. However, hydrophobic drug substances are more susceptible to metabolic processes and elimination from the body. A positive value of π indicates the hydrophobicity of the substituent higher than hydrogen and a negative value for a smaller one. The electron properties of substituents have a significant impact on the degree of ionization and polarity of drugs, which affects the ability of drugs to pass through cell membranes and the strength of binding to the receptor. The electron parameters for the substituents on the aromatic rings are referred to as the Hammet constant σ, which is characterized by the electronodonor or electronoacceptor properties of the substituent. There are few drugs whose action depend only on the effects of electron substituents and their hydrophobic, but also the activity is affected by the volume, size and shape of the molecule by conditioning interaction with the receptor [18].

First, we obtained a 4-isothiazole carboxylic acid derivative containing an unsubstituted phenyl ring **14**. Then, a 4-Cl derivative **16** was obtained. It is a more hydrophobic and electron acceptor derivative than a derivative with a monosubstituted benzene ring. This derivative **16** is characterized by lower activity, such as benzene mono-derivative **14**, which suggests that negative π (hydrophobicity constant) and/or σ (Hammet constant) values are important for their activity, and the *para*-substituent is sterically unfavorable. Next, we follow the branch of the Topliss scheme that indicates the synthesis of 4-OMe **12**, which caused an increase in activity compared to the monogenic derivative. However, derivatives with the −OH group **15**, **17**, **25**, **26** about lower activities are characterized by a higher negative solid hydrophobic value than derivatives with the -OMe group **2**, **9**,**12**. The Topliss scheme in this branch suggests the 4-$NMe_2$ derivative **7** has been obtained, which had a higher activity relative to the monogenic derivative **14**, but also to 4-OMe **12**. Changing the electron acceptor substituent -Cl **16** in the para position increases activity compared to substituents **7**, **12** with an electron donor character with lower hydrophobic.

The derivatives from the other two branches of the Topliss scheme were deliberately obtained, although the scheme suggested that we not direct the synthesis to the remaining branches. Interestingly, 3-Cl **3** with the electronoaceptor substituent with significant hydrophobicity is associated with increased activity compared to monosubstituent benzene derivative **14**, as well as in comparison with 4-OMe **12**, 4-NMe **7** and 4-Cl **16**. This may suggest a significant effect of the induction effect in the meta position on the activity. From the middle branching of the Topliss regimen, 4-Me **18** was characterized by the lowest activity from the previously discussed derivatives, while the derivative with the substituent -Et in the same position **11** belongs to the most active compounds. The activity of this group of compounds is probably influenced by steric factors and electron effects because of the higher activity of derivatives with $NMe_2$ **7** than benzene **14** and -OMe **12** compounds. The derivative 3-$NO_2$ **13** is associated with a lower activity from 3-Cl **3**. Both substituents in compounds **3** and **13** are located in the electronoacceptor meta position. On the other hand, other nitro derivatives show very poor activity and were not presented in this work.

The presence of two disubstituted by –Me **5** derivatives at position 2 and 4 increases potency as compared to the parent derivative **14**, but is also the most active monosubstituted derivative with -Me group, i.e. **6**, **8**, **18**. The compounds that substitution of aromatic rings with methoxy group **2**, **9**, **12** were exhibited higher antiproliferative activity. An interesting observation was that introduction in the metha position of the same functional groups -Cl, -Me, which in the ortho position of the phenyl ring enhance biological activity. Biological experiments revealed that introduction of electronegative substituents -Cl, -Et into the phenyl ring enhanced the activity of compounds [19]. The most active

compounds were with –OMe, -Me, -Cl substituent in the phenyl ring in position 3 of the isothiazole ring. Halogen substituents on the ring benzene **3**, **10**, **16** increase the hydrophobic and electronoacceptor properties, while the −OH group had hydrophilic and electronodonor properties. Compounds containing the −OH group **15**, **17**, **25**, **26** had lower activity compared to those previously discussed. The cyclopentyl substituent **22** has the highest hydrophobic properties. The reduction in activity in this compound can be explained by the omission of the optimum hydrophobic or the action of electron effects. The next substituents were checked to see if the assumptions were correct. The change of the benzene ring **14** to another ring like cyclohexane **19**, pyridine **20**, cyclohexene **21** is associated with decreased antiproliferative activity. Also, the exchange phenyl ring for monoaliphatic chain **23** decreased anticancer activity, so even a minor modification of the isothiazole structure may lead to a significant change in activity. Selected compounds, 4-isothiazole carboxylic acid derivatives, particulary those containing hydrazone function C-5 position cycle of isothiazole, did not show any interesting anticancer activity in 10 and 100 mg/ml concetrations [20].

The obtained results indicated the important role of the aromatic ring for the activity of isothiazole derivatives with selective anti-tumor activity. Aromatic systems usually take part in van der Waals interactions with flat, hydrophobic fragments of binding sites. On the other hand, reducing the activity of the derivative **19** with cyclohexane ring indicates that the structure devoid of a flat fragment and is characterized by a weakened interaction at the binding site of this series of compounds. Introducing benzene ring to the 5-hydrazino-3-methylisothiazole-4-carboxylic acid **1** causes a 6-fold increase in activity. What is more, the activity is increased 43 times due to benzene ring with the 3-OMe **2** substituent compared to compound **1**. Compounds of this series with a favorable biological activity are characterized by a rather positive hydrophobic constant (except for compounds with the –OMe substituent). However, compounds with high hydrophobic are found outside the optimum hydrophobicity. Hammel's constant for the most active group of compounds **2–14** is assumed mainly negative values -OMe, $NMe_2$, Me, Et, but also though to a lesser extent positive values -Cl, $NO_2$.

The other isothiazole derivatives with antineoplastic activity are characterized by a different chemical structure. The 3-(4-phenylisothiazol-5-yl)-2H-chromen-2-one derivatives have been found to possess moderate antiproliferative activity against different cancerous cell lines such as A549 (lung carcinoma), PC3 (human prostate cancer cells leukemia), SKOV3 (human ovary adenocarcinoma cells) and B16F10 (murine melanoma) [6]. The investigated drug CP-547,632 belongs to (3-aryl-4-carboxamidoisothiazol-3-yl)carbamides has undergone trials in the USA as a promising tyrosine kinase inhibitor with anticancer activity [8]. The isothiazole carboxamidine compounds were synthesized by Larson et al., which are a series of novel and selective Chk2 inhibitors [21]. Varaprasad et al. discovered 3-hydroxy-4-carboxyalkylamidino-5-arylamino-isothiazoles as potent in vitro MEK1 inhibitors [7]. 3D-QSAR studies have proved anticancer activity of isothiazole derivatives [22,23].

A series of 3,7-diaryl-6,7-dihydroisothiazolo[4,5-*b*]pyridin-5(4H)-ones showed effective growth inhibition of human non-drug-resistant parental A2780 ovarian and MOR lung adenocarcinoma cell lines [24]. Different substituted benzo[*d*]isothiazole derivatives had antitumor activity against human mammary carcinoma cell line [25].

Previously, our department has synthesized the ethyl esters of 5-hydrazino-3-methyl-isothiazol-4-carboxylic acid of the immunomodulating activity, but no acid derivatives were obtained. However, these compounds had no anticancer activity [26]. The benzo[*d*]isothiazole hydrazones obtained by the Vicini group had antiproliferative activity against human haematological and solid tumours cell lines [27].

Biological examination of Shiff base-like compounds confirmed their strong antineoplastic activity [18]. Very important is fact that the Tian research group had obtained 4-aminoacridone Schiff bases, which had selective antiproliferative activity for the HeLa and Raji cell lines. What is more, docking studies of these compounds revealed their predicting multidrug resistance modulatory behavior [28]. Furthermore, heterocyclic Schiff bases are compounds with anticancer, antiviral, antibacterial and anti-Alzheimer activity [29-31]. What is more, modification of Schiff bases has provided higher potency and reduction of toxicity [32]. Therefore, it is necessary to perform a study of the mechanism of action of the obtained 5-substituted isothiazole derivatives.

## 5. Conclusions

Of the 26 compounds obtained, 13 compounds were qualificated for further biological examinations due to their high activity on the MV4-11 line. Some of these 13 compounds are characterized by their small anti-cancer selective properties towards colorectal cancer cells such as compounds **2**, **4**, **5**, **6**, **7**, **8** and **11**. It may be concluded that activity is associated mainly with the presence of −CH═N− (Schiff's base) moiety with substituent of the proper size and shape in position 5 of the isothiazole ring in 3-methyl-4-isothiazole carboxylic acid derivatives.

Cytostatic drugs are characterized by a narrow therapeutic index and low selectivity in relation to actively dividing cells (they act on healthy and cancerous cells), which is a frequent cause of side effects of these drugs. The advantage of the obtained isothiazole derivatives is their low toxicity on healthy cells, and at the same time the high activity toward colorectal cancer cells, which is even greater in relation to multi-drug resistant cells.

5-fluorouracil, which is used in combination chemotherapy of colorectal cancer, was selected as a control drug in the abovementioned studies. Much like the compounds obtained in the project, 5-fluorouracil is characterized by low molecular weight (about 130,077 g/mol). The obtained compounds had lower antiploriferative activity than 5-fluorouracil, but they had the ability to overcome the resistance of the tumor to the cytostatics. Often not only the most active compounds from the series become medicine. Some weaker compounds of the series may have a smaller range of potential side effects. In addition, they can be potentially one of the components of chemotherapeutic systems as a factor preventing the development of drug resistance during chemotherapy.

Perhaps in further research, the compounds obtained will prove to be the highest-generation medicinal substances targeted at multi-drug resistant colorectal cancers and will be free from onerous adverse effects which occur as a result of all currently used cytostatics in a cancer therapy. Certainly, further studies on the compounds obtained are necessary. The described compounds have recently been protected by patent application.

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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.2019.103082.

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