The \( N' \)-Substituted Derivatives of 5-Chloro-3-Methylisothiazole-4-Carboxylic Acid Hydrazide with Antiproliferative Activity

Izabela Jęskowiak 1,* 1, Stanislaw Ryng 1, Marta Świtalska 2, Joanna Wietrzyk 2, Iwona Bryndal 3, Tadeusz Lis 4 and Marcin Mączyński 1

1 Department of Organic Chemistry, Faculty of Pharmacy, Wrocław Medical University, 211A Borowska Str, 50-556 Wrocław, Poland; stanislaw.ryng@umed.wroc.pl (S.R.); marcin.macynski@umed.wroc.pl (M.M.)
2 Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wrocław, Poland; martaswitalska@hirszfeld.pl (M.S.); joanna.wietrzyk@hirszfeld.pl (J.W.)
3 Department of Drug Technology, Faculty of Pharmacy, Wrocław Medical University, 211A Borowska Str, 50-556 Wrocław, Poland; iwona.bryndal@umed.wroc.pl
4 Faculty of Chemistry, University of Wrocław, 14 Joliot-Curie, 50-383 Wrocław, Poland; tadeusz.lis@chem.uni.wroc.pl
* Correspondence: izabela.jeskowiak@student.umed.wroc.pl

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Abstract: Thanks to the progress in oncology, pharmacological treatment of cancer is gaining in importance and in the near future anti-cancer chemotherapeutics are expected to be the main method of treatment for cancer diseases. What is more, the search for new anti-cancer compounds with the desired application properties is constantly underway. As a result of designed syntheses, we obtained some new \( N' \)-substituted 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide derivatives with anticancer activity. The structure of new compounds was determined by mass spectrometry (MS), elemental analysis, proton nuclear magnetic resonance spectroscopy (\(^1\)H-NMR), carbon nuclear magnetic resonance spectroscopy (\(^{13}\)C-NMR), \(^1\)H-\(^{13}\)C NMR correlations and infrared spectroscopy (IR). Moreover, the structures of the compounds were confirmed by crystallographic examination. The antiproliferative MTT tests for 11 prepared compounds was conducted towards human biphenotypic B cell myelomonocytic leukemia MV4-11. SRB test was used to examine their potential anticancer activity towards human colon adenocarcinoma cell lines sensitive LoVo, resistant to doxorubicin LoVo/DX, breast adenocarcinoma MCF-7 and normal non-tumorigenic epithelial cell line derived from mammary gland MCF-10A. The most active compound was 5-chloro-3-methyl-\( N' \)-(1E,2E)-(3-phenylprop-2-en-1-ylidene)isothiazole-4-carbohydrazide, which showed the highest antiproliferative activity against all tested cell lines.

Keywords: 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide derivatives; antiproliferative activity; isothiazole

1. Introduction

Chemotherapy is a method of systemic treatment of malignant tumors using cytostatic drugs, which stop the division and spread of cancer cells [1]. The basis of modern chemotherapy is the pairing of several cytostatics belonging to different classes. The simultaneous administration of several anticancer drugs reduces the risk of developing resistance to the treatment and leads to enhanced cytostatic activity. Cancer chemotherapy is difficult for many reasons. Due to the not fully understood causes of cancer, minimal biochemical differences between the cancer cell and the normal one, no
specificity of antitumor drug action of cytostatic agents, their low therapeutic factor and toxicity to normal tissues [2–6], we decided to design new compounds with potential anticancer activity.

Many studies have indicated the antitumor potential of isothiazole derivatives. CP-547.632 39, which belongs to the (3-aryl-4-carboxamido-isothiazol-3-yl)-carbamides, is a promising tyrosine kinase inhibitor with antineoplastic activity [7,8] used in the treatment of non-small cell lung cancer. CP-547.632 39 is an anti-angiogenic drug, which is effective only in combination with other cytostatics [9,10].

Another biological target of various isothiazole derivatives are MEK1 and MEK2 kinases [11–14], checkpoint kinases (Chk1 and Chk2) [15,16], tropomyosin receptor kinase A, TrkA [17] and a histone acetyltransferase (HAT enzyme)—Tip60 (KAT5) [18], whose overexpression is responsible for the induction of malignancy. This is a direction of intensive research, which addresses the synthesis of new isothiazole derivatives with the activity of tumor suppression inhibitors.

2. Results

The aim of our synthesis was to obtain new isothiazole derivatives with anticancer activity. The modification of the 4-position of isothiazole was characterized by the formation of N'-substituted 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazides with the chlorine substitution of the isothiazole ring in the 5-position. 5-Chloro-3-methyl-isothiazole-4-carbohydrazide 2 has already been synthetized by Kuczyński et al. [19], whereas in this work we used another method of synthesizing hydrazides through an intermediate azide product (Scheme 1). The new method for preparing hydrazide 1 is more efficient, as the use of 2-PrOH as the solvent enables the product to crystallize out from the reaction mixture while cooling and can be obtained pure after a methanol wash. Moreover, we describe the spectral properties of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide 2, because these data have not been reported so far. Scheme 1 presents the synthesis of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide 2 and the 4-substituted derivatives 3–11.

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\text{Scheme 1. Synthesis of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide derivatives.}
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5-Chloro-3-methylisothiazole-4-carboxylic acid 1 was prepared according to the method described by Machoń [20]. Substrate 1 and 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide 2 were also examined in terms of their antiproliferative activity.

The N’-substituted 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide derivatives 3–11 (Scheme 1) were obtained by the nucleophilic addition reaction in yields ranging from 50% to 86%. The new derivatives were obtained in the reaction 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide 2 with the corresponding carbonyl compounds heated at a temperature of 78 °C while being stirring vigorously for 4 h in ethanol. The synthesis took place according to the nucleophilic addition mechanism consisting of the attachment of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide 2 to the carbon bond of the C=O carbonyl group to form a carbanion. The next step was the elimination of a water molecule and creation of an imine bond.

In the IR spectra of all the compounds, an absorption band was observed in the range of 1643–668 cm⁻¹ corresponding to the C=O carbonyl bond, at 1551–1594 cm⁻¹ corresponding to the N=CH azamethine bond and in the 3164–3271 cm⁻¹ range for the amine NH group. The structures of new compounds were also determined by mass spectrometry (Figures S1–S10). In the ¹H-NMR (Figures S11–S30) and ¹³C-NMR (Figure S31–S40) spectra, double signals for each of the protons were present.

In the present study, the spectral data were measured in a DMSO-d₆ solution. For these compounds, we observed in ¹H-NMR and ¹³C-NMR signals belonging to Z and E geometrical isomers about the C=N double bond, which is characteristic of arylidene-hydrazide structure [21–25]. In addition, the compounds with this structure may exist as cis–trans amide conformers [26–28]. What is more, we have done ¹H–¹³C NMR correlations (figures S41–S50) for all compounds, which confirmed the presence of isomers. The correlation spectrum of the proton and carbon in the most active compound 3 (Figure 1) allows us to determine the presence of double signals from geometric isomers.

![Figure 1. The correlation spectrum of proton and carbon of the most active compound 3 from this series.](image-url)
Additionally, three isothiazole derivative compounds, denoted as 3, 4 and 8, were crystallized and X-ray crystallography confirmed their chemical structure with the expected \textit{trans} (E) configuration in the solid form. The asymmetric unit of 3 consists of two independent molecules, denoted as A (and D in the case of disordered part attached to the atom C41A) and B (and C in the case of disordered part attached to the atom C41B), respectively (Figure 2a), whereas compounds 4 (Figure 2b) and 8 (Figure 2c) crystallize with one molecule in the asymmetric unit.

![Figure 2. X-ray structures of 3 (a) (the dotted line indicates N-H...O hydrogen bond which linking together both independent molecules—denoted as A and B), 4 (b) and 8 (c), with atom-numbering scheme. Displacement ellipsoids are drawn at the 50% probability level. H atoms are shown as small spheres of arbitrary radii. For compounds 2 and 3, atoms and bonds from a disordered isothiazole ring with a lower occupancy factor were marked with a transparency of 0.6.](image)

In the first stage of biological research, 11 prepared compounds were tested for their antiproliferative activity towards human leukemia MV4-11 cells (Table 1). The highest activity was revealed by compound 3 with IC\textsubscript{50} 4.4 µg/mL. Other tested compounds had lower activity, with 3-8 times smaller IC\textsubscript{50} values than compound 3. Three compounds (2, 9 and 10) had very low antiproliferative activity with IC\textsubscript{50} > 80 µg/mL, and two compounds 1 and 11 had no antiproliferative activity against leukemia cells. The MV4-11 leukemia line is often used in screening for antiproliferative activity as this cell line is very sensitive to the antiproliferative effect of various groups of compounds.
By using it, inactive compounds can be eliminated from further research. On the other hand, we can be quite sure that no potentially active molecules are missed in this way.

Table 1. Results of examination of IC$_{50}$ of the all obtained compounds against the human biphenotypic B cell myelomonoctytic leukemia (MV4-11).

| Name of Compound | IC$_{50}$ ± SD [µg/mL] | µM | Name of Compound | IC$_{50}$ ± SD [µg/mL] | µM |
|-----------------|------------------------|----|-----------------|------------------------|----|
| Cisplatin       | 0.38 ± 0.14            | 1.28 ± 0.45 | 6               | 21.3 ± 8.2             | 69.4 ± 26.8 |
| 1               | n.a.                   | n.a.         | 7               | 22.4 ± 8.6             | 72.3 ± 27.8 |
| 2               | n.a. [45%] *           | n.a.         | 8               | 36.6 ± 11.6            | 131.2 ± 41.7 |
| 3               | 4.3 ± 1.9              | 14 ± 6.4     | 9               | n.a. [42.5%] *         | n.a.         |
| 4               | 15.2 ± 2.4             | 48.4 ± 7.8   | 10              | n.a. [47%] *           | n.a.         |
| 5               | 18.7 ± 1.7             | 57.7 ± 5.3   | 11              | n.a.                   | n.a.         |

DMSO (0.5%) 9% **

n.a.—no activity in a concentration of 1–80 µg/mL. IC$_{50}$—compound concentration leading to 50% inhibition of cell proliferation. Data are presented as mean ± SD of 3-5 independent experiments. *—inhibition of cell proliferation by compounds in a concentration of 80 µg/mL. **—inhibition of cell proliferation by DMSO in a concentration of 0.5%.

In the next stage, five compounds with IC$_{50}$ values on MV4-11 cells lower than 30 µg/mL were tested for their antiproliferative activity against MCF-7 (breast cancer), LoVo (colon cancer) human cell lines and also against Doxorubicin-resistant colon cancer LoVo/DX (P-gp-dependent, MRP-, LRP-dependent multidrug resistance). The cytotoxicity experiments were also performed towards the MCF-10A cell line (normal breast epithelial).

The data for the in vitro anticancer activity (Tables 1 and 2) were expressed as the IC$_{50}$—concentration of the compound (in µg/mL and µM) that inhibits proliferation of cells by 50% compared to the untreated control cells. Cisplatin and DMSO (in a concentration comparable to the one which was used at the highest concentration of compounds) were used as a positive control.

Table 2. Antiproliferative activity of the most active compounds 9 against selected cell lines (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).

| Name of Compound | IC$_{50}$ ± SD [µg/mL] | µM | MCF-7 | MCF-10A | LoVo | LoVo/DX |
|-----------------|------------------------|----|-------|---------|------|---------|
| Cisplatin       | 1.56 ± 0.3             | 5.2 ± 1.0 | 2.9 ± 0.4 | 9.7 ± 1.4 | 1.7 ± 0.8 | 5.6 ± 2.6 | 0.84 ± 0.17 | 2.8 ± 0.6 | 0.49 |
| 3               | 12 ± 1.9               | 39.3 ± 6.3 | 20.9 ± 2.2 | 68.6 ± 7.3 | 7.64 ± 1.7 | 25 ± 5.5 | 10.4 ± 0.7 | 34.2 ± 2.3 | 1.37 |
| 4               | 15.2 ± 1.8             | 48.6 ± 5.7 | 24.6 ± 2.2 | 78.5 ± 7.1 | 18.6 ± 1.1 | 59.4 ± 3.4 | 15 ± 1.1 | 47.9 ± 3.7 | 0.81 |
| 5               | 20.1 ± 2.6             | 65.1 ± 8.4 | 60.8 ± 5.1 | 196.6 ± 16.6 | 22.7 ± 0.3 | 73.4 ± 0.8 | 29.2 ± 5.3 | 94.6 ± 17.3 | 1.29 |
| 6               | 13.9 ± 1.9             | 42.9 ± 5.9 | 40.6 ± 3.7 | 125.4 ± 11.3 | 31.7 ± 6.7 | 97.7 ± 20.8 | 28.8 ± 2.7 | 88.8 ± 8.4 | 0.91 |
| 7               | 17.8 ± 3.1             | 58 ± 10.2 | 56 ± 14.1 | 182.5 ± 48.9 | 27.8 ± 10.4 | 180.5 ± 34 | 20 ± 1.1 | 65 ± 3.5 | 0.72 |
| DMSO (0.5%)     | 16% **                 | 5.3% ** | 10% **   | 2.7% ** | - | ** inhibition of cell proliferation by DMSO in a concentration of 0.5%. RI* was calculated according to the formula RI = (IC$_{50}$ estimated against resistant cell line)/IC$_{50}$ estimated against non-resistant cell line); values range: 0 < RI < 2—indicate that the tested compound is able to overcome drug resistance; 2 < RI < 10—defines the moderate ability of the compound to overcome drug resistance; RI > 10—defines no influence on the drug resistance phenomenon.

Compound 3 also had the highest activity against breast MCF-7, colon LoVo and LoVo/DX cancer cells (IC$_{50}$ < 15 µg/mL). A similar activity was revealed by compound 4 with IC$_{50}$ < 20 µg/mL. Compound 7 had the lowest antiproliferative activity against colon cancer cell lines (non-resistant and resistant) with IC$_{50}$ > 30 µg/mL (Table 2).

The cytotoxicity study of compounds towards MCF-10A cell line showed that compounds 3 and 4 also had the highest activity against normal cells. Compounds 5, 8 and 9 were about three times less cytotoxic against normal MCF-10A cells than against cancer MCF-7 cells (Table 2).
We also calculated the resistance indexes (RI) by dividing the IC\textsubscript{50} values of the compounds tested against the cells of drug resistant cell LoVo/DX by respective values obtained against the drug sensitive LoVo cell line (Table 2). All tested compounds were able to overcome the barrier of P-gp-dependent resistance. Compound 7 had the highest ability to overcome the barrier of resistance (RI = 0.72), and compound 3 had a lower ability (RI = 1.36).

3. Discussion

The main purpose of this work was to develop synthesis methods to obtain isothiazole derivatives with antitumor activity, in order to demonstrate the influence of the structure of the compounds obtained on antitumor activity as well as to determine the leading structure. Earlier, we prepared 5-hydrazino-3-methylisothiazole-4-carboxylic acid and its new 5-substituted derivatives, among which 13 compounds displayed strong antiproliferative activity. In this scientific work we obtained 3-methylisothiazole derivatives with modifications of position 4, which contained the same substituent as the most active 5-substituted derivatives of 3-methylisothiazole [29]. The same tests and cell lines as in the previously published work [29] were used. We chose different reference drugs to compare the results. In the previous studies, the reference medicine was 5-Fluorouracil, which is a small molecule therapeutic substance used in the treatment of colorectal cancer [29]. However, in this work, we chose Cisplatin as a reference medicine, which is also a small-molecule drug. It is the basis of many combination treatment regimens of various types of cancers, including breast cancer. The synthesized derivatives in position at 4 of the isothiazole moiety, including hydrazide groups, were selected on the basis of the most active derivatives from a series of derivatives substituted in the 5 position of the isothiazole containing the CH=N-Schiff’s base group [29]. The activity of the substituent change in position at 4 of the isothiazole was assessed by preserving the 5-chloro-3-methylisothiazole fragment.

The highest activity towards all examined cancer and normal cell lines was demonstrated by compound 3, which contains a hydrazide group with a -3-phenylprop-2-en-1-ylidene substituent. Other compounds qualified for the second stage of studies are 5–7 times less active than compound 3 for all tested cell lines, except for the MCF-7 breast cancer line. Compounds 4–7 have similar IC\textsubscript{50} values for all tumor lines and a desirable weak antiproliferative activity relative to the normal line, except for compound 4, which has the 3-Cl substituent in the meta position. In this group of derivatives with lower IC\textsubscript{50} values, 4–7 dominated the compounds, which contained the substituent in the meta position, such as 4 (3-Cl), 5 (3-NO\textsubscript{2}) and 7 (3-OMe). Compounds 2 (substrate for the synthesis of compounds 3–11), 9 (two Me groups, i.e., 2-Me and 4-Me) and 10 (2-Me) are characterized by very poor activity. IC\textsubscript{50} values were not determined, but only the inhibition of cell proliferation at a concentration of 80 \( \mu \text{g/mL} \). Compound 1, the substrate for the synthesis of hydrazide 2 and 11, containing the ortho (2-Cl) substituted phenyl ring shows no antiproliferative activity. The compound that substitute aromatic rings with methoxy group 7 (3-OMe) exhibits 1.5–2 times higher antiproliferative activity than phenyl derivative 8. The ability of the obtained compounds to overcome drug resistance of the studied cancer cells was confirmed by low values of the resistance index, RI. RI values from 0 to 2 indicate the sensitivity of the cells tested to the compound used. RI values from 2 to 10 indicate moderate drug resistance of the cells in question to the test compound, and RI values > 10 indicate strong drug resistance. The activity against the LoVo/DX drug-resistant cell line and its equivalent LoVo sensitive line was calculated and compared. All compounds showed RI below 2. Compound 3, which is the most active, has over 2-fold higher RI index (1.37). Compound 7 showed the lowest RI index (0.72).

The most active compound of this series is 5-chloro-N’-[(1E, 2E)-3-phenylprop-2-en-1-ylidene]-3-methylisothiazole-4-carboxyhydrazide 3, but it is less activity than compounds from the 5-substituted isothiazole Schiff base series, Cisplatin and 5-Fluorouracil towards sensitive (LoVo) and multi-drug resistant (LoVo/DX) human colon adenocarcinoma cell lines [29]. However, the activity of this series is relatively higher towards breast adenocarcinoma MCF-7 and comparable to the normal non-tumorigenic epithelial cell line derived from mammary gland MCF-10A. What is more, in both series 3-Cl (compound 4) with a monosubstituted benzene ring is in the group of most active compounds. In addition to this, we
also examined the synthesis substrates 1 and 2, which are devoid of any anticancer activity. The results indicated that the activity of the synthesized derivatives 5-hydrazino-3-methylisothiazole-4-carboxylic acids [29] and new 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide derivatives 3–11 is mainly associated with the presence of the \(-\text{N}_{\equiv}\text{CH}\) group with a suitable size and shape substituent in 3-methyl-4-isothiazole derivatives.

The application possibilities of hydrazides were confirmed after isonicotinic acid hydrazide (INH) was obtained. The unusual clinical value of INH has substituted the synthesis of other heterocyclic hydrazides [30]. Also, a series of 7-azaindolyl hydrazones are characterized as antiproliferative against MCF-7 breast carcinoma cell line [25]. N-methyl and \(N,N\)-dimethyl bis(indolyl)hydrazide hydrazone analog derivatives had anti-proliferative activity against cervical (HeLa) and breast cancer (MCF-7) [31]. The series of (\(E\))-\(N\)-((2-chloro-7-methoxyquinolin-3-yl)methylene)-3-(phenylthio)propanehydrazide derivatives had a greater cytotoxic effect on the neuroblastoma cells (SH-SY5Y and Kelly) compared to the breast cancer cell lines (MCF-7 and MDA-MB-231) [32].

The advantage of the obtained isothiazole derivatives is their low toxicity on healthy cells, and at the same time the selectivity in relation to colorectal cancer cells by the most active compound of this series. In addition, these compounds can be potentially one of the components of chemotherapeutic systems as a factor preventing the development of drug resistance during chemotherapy. Indeed, none of the compounds tested turned out to be more active with 5-Fluorouracil and Cisplatin. 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.

Based on the results of biological studies of the 5-substituted 5-hydrazine-3-methylisothiazole-4-carboxylic acid derivatives [29] and compounds from this work—\(N\)’-substituted derivatives of 5-chloro-3-methylisothiazole-4-carboxylic acid the lead structure of anti-cancer isothiazole derivatives was determined. The designated leading structure contains structural elements with anti-tumor effect, i.e., methylisothiazole, carboxyl and azomethine group, as well as the phenylprop-2-ene group located near the azomethine group of the 5-methylisothiazole derivatives, gives them probably potential selectivity towards tumor lines. In this scientific work the most active compound against all cancer cell lines is 5-chloro-\(N\)’-[(\(1E,2E\))-(3-phenylprop-2-en-1-ylidene)]-3-methylisothiazole-4-carboxhydrazide 3, which possesses almost two times lower activity towards the MCF-10A normal cell line than against cancer cells. This indicates the potential selectivity of the compound with regards to cancer cells such as leukemia, breast and colon cancer cell lines. In addition, the most active compounds in both series contain substituents at the \(meta\) position of the phenyl ring near the azomethine group. In our opinion, the research on low-molecular weight of isothiazole derivatives with antiproliferative activity is very desirable because of the demand for oncological drugs that break the increasing resistance of tumors to cytostatics currently used in therapy.

4. Materials and Methods

4.1. General Information

Commercially available reagents were used without further purification. Progress of the reaction was controlled by thin layer chromatography (TLC) on ALUGRAM SIL G/UV pre-coated TLC sheets (Macherey-Nagel, Dylan, Germany) and visualized by ultraviolet (UV) light at 254 nm (Bioblock Scientific lamp, Fisher, Hampton, NH, USA). Melting points of all new compounds were measured by a LLG uniMELT-2 apparatus (LLG). A Thermo Scientific Nicolet iS50 FT-IR spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to record infrared spectra (IR). The samples were applied as solids and frequencies are given in \(\text{cm}^{-1}\). Proton nuclear magnetic resonance (\(^1\text{H-NMR}\)), carbon nuclear magnetic resonance (\(^{13}\text{C-NMR}\)) and 2D \(^1\text{H-}^{13}\text{C}\) NMR correlation spectra were recorded in deuterated dimethyl sulfoxide (DMSO-\(d_6\)) using a Bruker ARX-300 spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany). Chemical shifts are reported in in parts per million (ppm) units and signal multiplicities were collected by the abbreviations: s (singlet), d (doublet), t
(triplet), m (multiplet). The values of coupling constant are reported as $J$ in Hz. Elemental analysis was obtained on NA 1500 equipment (Carlo Erba, Sabadell, Barcelona, Spain). Mass spectrometry (MS) was performed on a compact$^\text{TM}$ Electrospray Ionisation-Quadrupole-Time of Flight (ESI-Q-TOF) apparatus (Bruker Daltonics, Billerica, MA, USA). The samples for ESI-MS experiments were dissolved in methanol. Monoisotopic mass was calculated (calc.) by Compass Data Analysis 4.2.

4.2. Procedures for the Synthesis All the New Compounds and Their Spectroscopic Data (IR, $^1$H-NMR, $^{13}$C-NMR, 2D $^1$H-$^{13}$C NMR, ESI-MS)

4.2.1. 5-Chloro-3-Methylisothiazole-4-Carboxyhydrazide 2

A 45% solution of thionyl chloride dissolved in benzene (90 mL) was added to 33.8 mmol of 5-chloro-3-methylisothiazole-4-carboxylic acid $1$. The mixture was heating under reflux for 3 h to obtain a clear solution. After cooling, the solution was distilled in an evaporator to give an oily residue. Thereafter benzene (30 mL) was added twice and distilled off in each case to remove residual thionyl chloride.

In an ice-cold water bath cooled to 8 °C with a magnetic stirrer, 5-chloro-3-methylisothiazole-4-carboxylic acid chloride (30 mmol, 5981.2 mg) was dissolved in acetone (240 mL). The reaction mixture was kept at a temperature of up to 8 °C at all times. At the same time, sodium azide (83 mmol, 539.5 mg) was dissolved in distilled water (18 mL) and then slowly added to the reaction mixture. After addition of the sodium azide solution, stirring was continued for 30 min. The separated salt was filtered off. The solution was distilled from the reaction mixture until the semi-liquid form. The suspension was cooled in an ice-cold water bath. The precipitate was filtered off and washed with 2-propanol.

To azide (9.8 mmol, 1980.0 mg), 2-propanol (40 mL) and anhydrous hydrazine (2.0 mL, 2000.0 mg, 63.7 mmol) was added. Then, the reaction mixture was heated at a temperature of 83 °C for 45 min under reflux. The course of the reaction was monitored by TLC eluting with ethyl acetate. The solid precipitated in the course of the reaction was then filtered off through a paper filter. After cooling the filtrate, colorless crystals fell out of the mixture, which were washed with cold methanol (yield 64%, 450 mg). The separated product was precipitated from the reaction mixture to give yellow crystals, mp 106.4 °C.

4.2.2. 5-Chloro-N’-[(1E,2E)-3-phenylprop-2-en-1-ylidene]-3-methylisothiazole-4-carbonyhydrazide 3

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 439.2 mg, 2.3 mmol) and cinnamaldehyde (303.9 mg, 2.3 mmol) in EtOH (2.0 mL) was heated at 78 °C for 4 h. At the end of the reaction (monitored by TLC chloroform–ethyl acetate 7:3), reaction mixture was cooled. Thereafter benzene (30 mL) was added. Then, the reaction mixture was heated at a temperature of 83 °C for 4 h. At the same time, sodium azide (83 mmol, 539.5 mg) was added. Then, the reaction mixture was heated at a temperature of 83 °C for 4 h. Thereafter benzene (30 mL) was added twice and distilled off in each case to remove residual thionyl chloride.

The solution was distilled from the reaction mixture until the semi-liquid form. The suspension was cooled in an ice-cold water bath. The precipitate was filtered off and washed with 2-propanol.

To azide (9.8 mmol, 1980.0 mg), 2-propanol (40 mL) and anhydrous hydrazine (2.0 mL, 2000.0 mg, 63.7 mmol) was added. Then, the reaction mixture was heated at a temperature of 83 °C for 45 min under reflux. The course of the reaction was monitored by TLC eluting with ethyl acetate. The solid precipitated in the course of the reaction was then filtered off through a paper filter. After cooling the filtrate, colorless crystals fell out of the mixture, which were washed with cold methanol (yield 64%, 450 mg). The separated product was precipitated from the reaction mixture to give yellow crystals, mp 106.4 °C.

The solution was distilled from the reaction mixture until the semi-liquid form. The suspension was cooled in an ice-cold water bath. The precipitate was filtered off and washed with 2-propanol.

To azide (9.8 mmol, 1980.0 mg), 2-propanol (40 mL) and anhydrous hydrazine (2.0 mL, 2000.0 mg, 63.7 mmol) was added. Then, the reaction mixture was heated at a temperature of 83 °C for 45 min under reflux. The course of the reaction was monitored by TLC eluting with ethyl acetate. The solid precipitated in the course of the reaction was then filtered off through a paper filter. After cooling the filtrate, colorless crystals fell out of the mixture, which were washed with cold methanol (yield 64%, 450 mg). The separated product was precipitated from the reaction mixture to give yellow crystals, mp 106.4 °C.
4.2.3. 5-Chloro-N’-(E)-(3-chlorophenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 4

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 496.57, 2.6 mmol) and 3-chlorobenzaldehyde (365.4 mg, 2.6 mmol) in EtOH (1.5 mL) was heated at 78 °C for 4 h. At the end of the reaction (controlled in a TLC chloroform–ethyl acetate 9:1), the reaction mixture was cooled. The separated product was precipitated and washed with cold methanol (yield 67%, 545 mg). The product was purified by crystallization from acetonitrile to give yellow crystals, mp = 174–175 °C. IR ν max 1557, 1648, 3216 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.39 and 2.46′ (3H, s, CH₃), 7.44 (3H, s, arH) and 7.51′ (3H, dd, J = 3.0 Hz, 3.0 Hz, arH), 7.70–7.74 (1H, m, arH) and 7.81′ (1H, s, arH), 8.10 and 8.28′ (1H, s, N=CH), 12.18 and 12.34′ (1H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 18.1 and 18.8 (CH₃), 121.1 and 121.4′ (isothiazole-C3), 124.3 and 124.6′ (isothiazole-C4), 129.5 and 129.8′ (arb), 130.5 and 131.4′ (arb), 133.3 and 133.4′ (arb), 135.7 and 135.7′ (arb), 143.4 and 146.8′ (arb), 151.0 and 151.1′ (isothiazole-C4), 157.4 and 163.5′ (isothiazole-C5), 165.2 and 165.7′ (C=O); anal. C 54.40, H 4.12%, N 13.37%, calcd for C₁₄H₉ClN₂O₃S, 322.9810 (calcd for C₁₄H₉ClN₂O₃S, 323.0011).

4.2.4. 5-Chloro-N’-(E)-(3-nitrophenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 5

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 286.4 mg, 1.5 mmol) and 3-nitrobenzaldehyde (226.6 mg, 1.5 mmol) in EtOH (1.5 mL) was heated at 78 °C for 4 h. During the reaction, the resulting suspension thickens. At the end of the reaction (controlled by TLC, chloroform–ethyl acetate 1:3), the separated product was filtered and washed with cold methanol (yield 53%, 258 mg). The product was purified by crystallization from 70% ethanol to give yellow crystals, mp = 174–175 °C. IR ν max 1557, 1644, 3271 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.40 and 2.47′ (3H, s, CH₃), 7.68–7.77 (2H, m, arH), 7.90 (1H, d, J = 9.0 Hz, arH), 8.18–8.24′ (3H, m, arH), 8.29′ (2H, d, J = 9.0 Hz, arH), 8.42 and 8.58′ (1H, s, N=CH), 12.39 (2H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 18.1 and 18.8′ (CH₃), 121.1 and 121.4′ (iso-thiazole-C3), 124.3 and 124.6′ (isothiazole-C4), 130.5 and 131.6′ (arb), 132.6 and 133.5′ (arb), 135.6 and 135.7′ (arb), 143.0 (arb), 146.4 and 148.2′ (arb), 150.5 and 151.4′ (arb), 157.8 and 158.5′ (iso-thiazole-C4), 163.9 (iso-thiazole-C5), 165.5 and 166.0′ (C=O); anal. C 44.37, H 2.60, N 17.13%, calcd for C₁₂H₉ClN₂O₃S, 34.48, H 2.79, N 17.25%; ESI-MS m/z 311.9750 (calcd for C₁₂H₉ClN₂O₃S, 311.9771).

4.2.5. 5-Chloro-N’-(E)-(4-ethylphenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 6

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 226.6 mg, 1.5 mmol) and 4-ethylbenzaldehyde (134.1 mg, 1.0 mmol) in EtOH (1.5 mL) was heated at 78 °C for 4 h. During the reaction, the resulting suspension thickens. At the end of the reaction (controlled by TLC chloroform–ethyl acetate 7:3), the separated product was filtered and washed with cold methanol (yield 65%, 200 mg). The product was purified by crystallization from acetonitrile to give colorless crystals, mp = 152–154 °C. IR ν max 1551, 1668, 3195 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 1.12 (1H, s, CH₃), 2.14 (1H, s, CH₃CH₂), 1.14 (1H, s, CH₃CH₂), 1.17 (2H, s, CH₃CH₂), 1.20 (1H, s, CH₃CH₂), 2.29 and 2.46′ (3H, s, CH₃), 2.57′ (1H, s, CH₃CH₂), 2.60′ (1H, s, CH₃CH₂), 2.64′ (1H, s, CH₃CH₂), 2.68′ (1H, d, J = 9.0 Hz, CH₃CH₂), 7.23 (2H, d, J = 6.0 Hz, arH), 7.23′ (1H, d, J = 6.0 Hz, arH), 7.39 (2H, d, J = 9.0 Hz, arH), 7.67′ (2H, d, J = 9.0 Hz, arH), 8.09 and 8.25′ (1H, s, N=CH), 11.99 and 12.17′ (1H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 15.2 (CH₃), 18.8 (CH₃), 28.0 (CH₃), 126.8 (isothiazole-C3), 127.4 and 128.3′ (N=CH), 131.3 and 132.0′ (arb), 145.3 (arb), 146.2 and 146.6′ (arb), 148.7 (arb), 150.2 and 151.1′ (arb), 157.4 (isothiazole-C4), 163.5 (isothiazole-C5), 165.5 and 166.0′ (C=O); anal. C 54.40, H 4.31, 13.53%, calcd for C₁₄H₁₄ClN₃O₃S 54.63, H 4.58, N 13.65%; ESI-MS m/z 306.0346 (calcd for C₁₄H₁₄ClN₃O₃S, 306.0473).

4.2.6. 5-Chloro-N’-(E)-(3-methoxyphenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 7

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 458.3 mg, 2.4 mmol) and 3-methoxybenzaldehyde (326.7 mg, 2.4 mmol) in EtOH (1.5 mL) was heated at 78 °C
for 4 h. At the end of the reaction (controlled by TLC chloroform–ethyl acetate 7:3), the formed clear mixture was poured into water. The separated product fell out, and was washed with cold methanol (yield 80%, 590 mg). The product was purified by crystallization from 70% ethanol to give colorless crystals, mp = 139–140 °C; IR ν_max 1580, 1666, 3181 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.40 and 2.47' (3H, Is, CH₃), 3.73 and 3.82' (3H, 1s, OCH₃), 6.96–7.05 (4H, m, arH), and 7.30' (3H, d, J = 9.0 Hz, arH), 7.39' (1H, d, J = 9 Hz, arH), 8.08 and 8.27' (1H, s, N=CH), 12.07 and 12.26' (1H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 18.7 (CH₃), 55.0 (OCH₃), 111.5 (isothiazole-C3), 115.9 (N=CH), 119.3 (arC), 120.3 (arC), 130.0 (arC), 131.8 (arC), 135.2 (arC), 144.8 (arC), 149.8 (arC), 157.5 (isothiazole-C4), 157.7 (isothiazole-C5), 159.4 (C=O); anal. C 50.84, H 3.91, N 13.06%; calc. for C₁₂H₁₁ClN₂O₅S C 51.52, H 3.60, N 15.02%; ESI-MS m/z 278.0187 (calcd for C₁₃H₁₂ClN₂O₅S, 278.0160).

### 4.2.7. 5-Chloro-N’-(E)-(2-methylphenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 8

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 362.8 mg, 1.9 mmol) and benzaldehyde (201.6 mg, 1.9 mmol) in EtOH (1.5 mL) was heated at 78 °C for 4 h. At the end of the reaction (controlled by TLC, chloroform–ethyl acetate 7:3), the reaction mixture was cooled. The separated product was precipitated and then washed with cold methanol (yield 50%, 265 mg). The product was purified by crystallization from acetonitrile to give colorless crystals, mp = 149.7 and 151.1 °C; IR ν_max 1557, 1648, 3192 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.39 and 2.47° (3H, Is, CH₃), 3.78–7.40 (3H, m, arH), 7.46–7.48° (5H, m, arH), 7.74 (1H, d, J = 3.0 Hz, arH), 7.76 (1H, d, J = 6.0 Hz, arH) 8.12 and 8.29° (1H, s, N=CH), 12.09 and 12.21° (1H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 18.3 (CH₃), 126.7 (isothiazole-C3), 127.3 and 128.9° (arC), 130.2 and 130.5° (N=CH), 131.8 (arC), 133.7 (arC), 145.2 (arC), 148.7 (arC), 150.3 and 151.2° (arC), 157.5 (isothiazole-C4), 163.7 (isothiazole-C5), 165.5 and 166.0° (C=O); anal. C 51.67, H 3.35, N 14.92%; calc. for C₁₂H₁₁ClN₂O₅S C 51.52, H 3.60, N 15.02%; ESI-MS m/z 278.0187 (calcd for C₁₃H₁₂ClN₂O₅S, 278.0160).

### 4.2.8. 5-Chloro-N’-(E)-(2,4-dimethylphenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 9

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 401.0 mg, 2.1 mmol) and 2,4-dimethylbenzaldehyde (281.7 mg, 2.1 mmol) in EtOH (2.0 mL) was heated at 78 °C for 4 h. At the end of the reaction (controlled by TLC, chloroform–ethyl acetate 9:1), the reaction mixture was cooled. The separated product was precipitated and then washed with cold methanol (yield 86%, 554 mg). The product was purified by crystallization from acetonitrile to give colorless crystals, mp = 232–234 °C; IR ν_max 1556, 1667, 3182 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.23° (6H, s, CH₃), 2.25 (3H, s, CH₃), 2.38° (6H, s, CH₃), 2.46 (3H, s, CH₃), 7.00 (2H, d, J = 9.0 Hz, arH) and 7.10° (2H, d, J = 6.0 Hz, arH), 7.31 (1H, d, J = 6.0 Hz, arH) and 7.76° (1H, d, J = 9.0 Hz, arH) 8.32 and 8.52° (1H, s, N=CH), 11.96 and 12.09° (1H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 18.7 and 18.8° (CH₃), 18.9 and 19.2° (CH₃), 20.9 (CH₃), 126.2 (C3-isothiazole) 126.7 and 126.9° (N=CH), 127.0 and 128.9° (arC), 131.6 and 131.7° (arC), 131.9 and 132.3° (arC), 136.7 and 137.1° (arC), 139.5 and 140.0° (arC), 144.9 and 147.4° (arC), 149.7 and 151.1° (arC), 157.3 and 163.6° (isothiazole-C4), 165.3 and 166.0° (C=O); anal. C 54.72, H 4.55, N 13.67%; calc. for C₁₄H₁₃ClN₂O₅S C 54.63, H 4.58, N 13.65%; ESI-MS m/z 306.0506 (calcd for C₁₄H₁₃ClN₂O₅S, 306.0473).

### 4.2.9. 5-Chloro-N’-(E)-(2-methylphenyl)methylidene]-3-methylisothiazole-4-carbohydrazide 10

A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 477.4 mg, 2.5 mmol) and 2-methylbenzaldehyde (300.3 mg, 2.5 mmol) in EtOH (5.0 mL) was heated at 78 °C for 4 h. At the end of the reaction (controlled by TLC, chloroform–ethyl acetate 7:3), the reaction mixture was cooled. The separated product was precipitated and then washed with cold methanol (yield 66%, 480 mg). The product was purified by crystallization from acetonitrile to give colorless crystals, mp = 226–228 °C; IR ν_max 1594, 1643, 3173 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.22 and 2.38° (3H, s, CH₃), 2.43 and 2.46° (3H, s, CH₃), 7.19 (1H, s, arH), 7.21 (1H, s, arH), 7.25° (1H, s, arH), 7.27 (2H, d, J = 6.0 Hz, arH), 7.31° (1H, d, J = 6.0 Hz, arH), 7.42° (1H, d, J = 9.0 Hz, arH), 7.84° (1H, d, J = 6.0 Hz,
A stirred mixture of 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide (2, 324.6 mg, 1.7 mmol) and 2-chlorobenzaldehyde (238.9 mg, 1.7 mmol) in EtOH (1.5 mL) was heated at 78 °C for 4 h. During the reaction, the resulting suspension thickens. At the end of the reaction (controlled by TLC, chloroform–ethyl acetate 9:1), the separated product was filtered and washed with cold methanol (yield 62%, 0.329 mg). The product was purified by crystallization from 70% ethanol to give yellow crystals, mp = 232–233 °C; IR ν_max 1590, 1644, 3167 cm⁻¹; ¹H-NMR (DMSO-d₆, 300 MHz) δ 2.39 and 2.47” (3H, s, CH₃), 7.33 (1H, d, J = 6.0 Hz, arH), 7.36’ (1H, dd, J = 6.0 Hz, 6Hz, arH), 7.43–7.49 (3H, m, arH), 7.52’ (1H, d, J = 6.0 Hz, arH), 7.56’ (1H, s, arH), 8.04’ (1H, d, J = 9.0 Hz, arH), 8.51 and 8.70’ (1H, s, N=CH), 12.27 and 12.39’ (1H, s, NH); ¹³C-NMR (DMSO-d₆, 75.4 MHz) δ 19.1 (CH₃), 126.5 (isothiazole-C₃), 127.3 and 128.0 (C=CH), 130.3 and 131.2’ (arC), 131.8 and 132.2’ (arC), 133.4 and 133.7’ (arC), 141.7 (arC), 144.9 (arC), 151.7 (arC), 157.9 (isothiazole-C₄), 164.1 (isothiazole-C₅), 165.8 and 166.3’ (C=O); anal. C 53.41, H 4.04, N 14.21%, calcd for C₁₃H₁₂ClN₃OS C 53.15, H 4.12, N 14.30%; ESI-MS m/z 292.0337 (calcd for C₁₃H₁₂ClN₃OS, 292.0317).

4.2.10. 5-Chloro-N’-[(E)-2-(2-chlorophenyl)methylene]-3-methylisothiazole-4-carboxyhydrazide 11

Crystals of 3, 4 and 8 suitable for single-crystal X-ray diffraction analysis were obtained by dissolution in acetonitrile/2-propanol followed by slow evaporation of the solvent at room temperature. Crystallographic measurements for 3, 4 and 8 were collected with K-correction diffractometers: Xcalibur R (Agilent Technologies, city, state abbrev if USA, country) with a Ruby CCD camera (3 and 4) and a Kuma KM-4 CCD with a Sapphire2 CCD camera (8), with graphite monochromatized Mo-Kα radiation (λ = 0.7073 Å) at 100(2) K, using an Oxford Cryosystems cooler. Data collection, cell refinement, data reduction and analysis were carried out with CrysAlisPro [33]. Analytical absorption correction was applied to data with the use of CrysAlisPro. The crystal structures were solved using SHELXS [34] and refined on F² by a full-matrix least squares technique with SHELXL-2014 [35] with anisotropic thermal parameters for all the ordered non-H atoms. In the final refinement cycles, H atoms were repositioned in their calculated positions and treated as riding atoms, with C-H = 0.95–0.98 Å, and N-H = 0.88 Å and with U_{iso}(H) = 1.2U_{eq}(C, N) for CH and NH or 1.5U_{eq}(C) for CH₃. All figures were made using DIAMOND program [36].

**Compound 3:** C₁₄H₁₂ClN₃OS, Mᵣ = 305.78 g mol⁻¹, colorless plates, size 0.50 × 0.21 × 0.02 mm, triclinic, space group P1, a = 7.282 (2) Å, b = 7.465 (2) Å, c = 14.381 (4) Å, α = 83.85 (5)^°, β = 81.97 (5)^°, γ = 68.47 (5)^°, V = 718.8 (4) Å³, Z = 2, D_{calc} = 1.413 g cm⁻³, F(000) = 316, μ = 0.41 mm⁻¹, 6066 measured reflections, 6066 independent reflections, 3909 reflections with l > 2σ(l), 420 parameters (99 restraints), R[F² > 2σ(F²)] = 0.091, wR(F²) = 0.214, S = 1.04, largest diff. peak and hole = 1.29 e Å⁻³/−0.45 e Å⁻³. The diffraction experiment was performed with a twinned crystal [21]. The asymmetric unit of 3 consists of two independent molecules and in both molecules (denoted as A and B), the isothiazole ring was found to be disordered over two sites and refined with the occupancy factors of 0.749 (11) and 0.251 (11) in molecule A, and 0.686 (10) and 0.314 (10) in molecule B. The atoms of lower occupancy were denoted as D (in case of disordered part attached to C41A) and C (in case of disordered part attached to C41B). Disordered atoms were refined with anisotropic displacement parameter using EADP instruction of SHELXL [35].

**Compound 4:** C₁₂H₁₀Cl₂N₃OS, Mᵣ = 314.18 g mol⁻¹, colorless needles, size 0.25 × 0.14 × 0.08 mm, monoclinic, space group P2₁/c, a = 11.593 (4) Å, b = 13.972 (5) Å, c = 8.238 (2) Å, β = 90.51 (5)^°, V = 1334.3 (7) Å³, Z = 4, D_{calc} = 1.564 g cm⁻³, F(000) = 640, μ = 0.64 mm⁻¹, 5794 measured
1.0 mM sodium pyruvate, 2 mM L-glutamine and 10% FBS (all from Sigma Aldrich, Steinheim, Germany) and 0.1 µg/mL penicillin (Polfa Tarchomin S.A. Poland), and 100 µg/mL streptomycin (Sigma Aldrich). Afterwards, the tested compounds were diluted in a culture medium to reach the final concentrations at the range of 1-80 µg/mL. Twenty-four hours prior to the addition of the tested compounds, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 1 × 10^4 or 0.75 × 10^4 (MCF-7) cells per well. The assay was performed after 72 h of exposure to varying concentrations of the tested agents. The in vitro cytotoxic effect of all agents was examined using the MTT (MV4-11) or SRB assay [37]. The results
were calculated as IC\textsubscript{50} (inhibitory concentration 50%) the concentration of tested agent, which is cytotoxic for 50% of the cancer cells. IC\textsubscript{50} values were calculated for each experiment separately and mean values ± SD are presented in Tables 1 and 2. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated 3–4 times.

The resistance indexes (RI) were calculated by dividing the IC\textsubscript{50} values of the compounds tested against the cells of drug resistant cell LoVo/DX line by respective values obtained against the cells of drug sensitive LoVo line (Table 2). According to Harker et al. [38], three categories of the cells could be distinguished: (a) the cells are drug-sensitive if the ratio approaches 0–2; (b) the cells are moderately drug-resistant if the ratio ranges from 2 to 10; (c) the cells are markedly drug-resistant if the ratio is higher than 10.

**MTT Assay (for MV4-11 Cell Line)**

This technique was applied for cells growing in suspension culture. An assay was performed after 72 h exposure to varying concentrations of the tested agents. For the last 4 h of incubation, 20 µL of MTT solution was added to each well (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; stock solution: 5 mg/mL, Sigma Aldrich). When the incubation time was completed, 80 µL of the lysing mixture was added to each well (lysing mixture: 225 mL dimethylformamide, POCh, Gliwice, Poland, 67.5 g sodium dodecyl sulfate, Sigma Aldrich, and 275 mL of distilled water). After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on Synergy H4 photometer (BioTek Instruments, USA) at 570 nm wavelength. The background optical density was measured in the wells filled with culture medium, without the cells.

**Sulforhodamine B Assay (for Other Cell Lines)**

This technique was applied for the cytotoxicity screening against cells growing in adherent culture. The details of this technique were described by Skehan et al. [39]. The cytotoxicity assay was performed after 72 h exposure of the cultured cells to varying concentrations of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, Sigma Aldrich) on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The cellular material fixed with TCA was stained with 0.1% sulforhodamine B (SRB, Sigma Aldrich) dissolved in 1% acetic acid (POCh) for 30 min. Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (Sigma Aldrich) for determination of optical density (at 540 nm) on Synergy H4 photometer (BioTek Instruments). The background optical density was measured in the wells filled with culture medium, without the cells.

5. Conclusions

In recent decades, there has been great progress in designing and obtaining modern oncology drugs [40]. In this study we obtained nine compounds, which are the 5-chloro-3-methylisothiazole-4-carboxylic acid hydrazide derivatives 3–11. All the new compounds 3–11 and substrates 1 and 2 were tested for activity against the MV4-11 cell line. The substrates had no anticancer activity. The antiproliferative activity of the five most active compounds was examined against breast MCF-7, colon LoVo and LoVo/DX cancer cells and normal MCF-10A cells. 5-Chloro-N’-[(1\textsubscript{E},2\textsubscript{E})-3-phenylprop-2-en-1-ylidene]-3-methylisothiazole-4-carboxyhydrazide 3, which is the most active compound of this series, possessed almost two times lower activity (IC\textsubscript{50} value) towards the MCF-10A normal cell line than against cancer cells. This compound is characterized by potential selectivity toward cancer cells such as leukemia, breast and colon cancer cell lines and low toxicity against healthy cells. Other tested compounds displayed slightly weaker antiproliferative activity against tumor cells, but in the case of normal MCF-10A cells, their activity was about three times lower. On the basis of the conducted research, it can be concluded that the antiproliferative activity of this group of derivatives corresponds to the hydrazide moiety. The position of the azomethine group -N=CH at the 5-position
of the isothiazole in Schiff bases [36] is more favorable than in the 4-position of the isothiazole ring on hydrazide derivatives 3-11.

Supplementary Materials: The following are available online at http://www.mdpi.com/1420-3049/25/1/88/s1, Figure S1: ESI-MS spectrum of compound 2, Figure S2: ESI-MS spectrum of compound 3, Figure S3: ESI-MS spectrum of compound 4, Figure S4: ESI-MS spectrum of compound 5, Figure S5: ESI-MS spectrum of compound 6, Figure S6: ESI-MS spectrum of compound 7, Figure S7: ESI-MS spectrum of compound 8, Figure S8: ESI-MS spectrum of compound 9, Figure S9: ESI-MS spectrum of compound 10, Figure S10: ESI-MS spectrum of compound 11, Figure S11: 1H NMR spectrum of compound 2, Figure S12: 1H NMR spectrum of compound 11, Figure S13: 1H NMR spectrum of compound 4, Figure S14: 1H NMR spectrum of compound 5, Figure S15: 1H NMR spectrum of compound 6, Figure S16: 1H NMR spectrum of compound 7, Figure S17: 1H NMR spectrum of compound 8, Figure S18: 1H NMR spectrum of compound 9, Figure S19: 1H NMR spectrum of compound 10, Figure S20: 1H NMR spectrum of compound 11, Figure S21: 13C NMR spectrum of compound 2, Figure S22: 13C NMR spectrum of compound 3, Figure S23: 13C NMR spectrum of compound 4, Figure S24: 13C NMR spectrum of compound 5, Figure S25: 13C NMR spectrum of compound 6, Figure S26: 13C NMR spectrum of compound 7, Figure S27: 13C NMR spectrum of compound 8, Figure S28: 13C NMR spectrum of compound 9, Figure S29: 13C NMR spectrum of compound 10, Figure S30: 13C NMR spectrum of compound 11, Figure S31: 1H-13C NMR spectrum of compound 2, Figure S32: 1H-13C NMR spectrum of compound 3, Figure S33: 1H-13C NMR spectrum of compound 4, Figure S34: 1H-13C NMR spectrum of compound 5, Figure S35: 1H-13C NMR spectrum of compound 6, Figure S36: 1H-13C NMR spectrum of compound 7, Figure S37: 1H-13C NMR spectrum of compound 8, Figure S38: 1H-13C NMR spectrum of compound 9, Figure S39: 1H-13C NMR spectrum of compound 10, Figure S40: 1H-13C NMR spectrum of compound 11.

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