Accepted Manuscript

Allylic isothiouronium salts: The discovery of a novel class of thiourea analogues with antitumor activity

Misael Ferreira, Laura Sartori Assunção, Adny Henrique Silva, Fabíola Branco Filippin-Monteiro, Tânia Beatriz Creczynski-Pasa, Marcus Mandolesi Sá

PII: S0223-5234(17)30078-8

DOI: 10.1016/j.ejmech.2017.02.013

Reference: EJMECH 9209

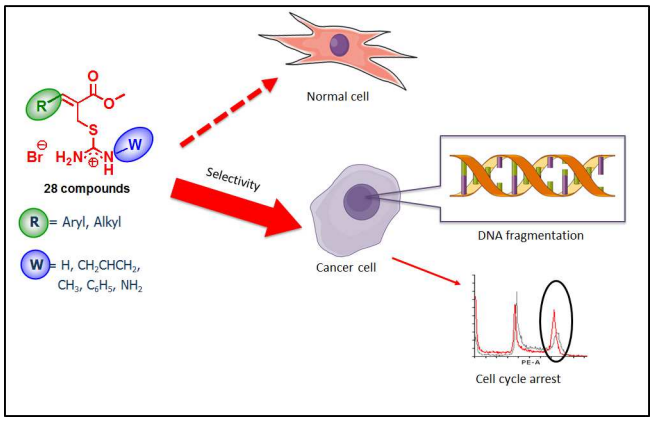
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 30 November 2016

Revised Date: 19 January 2017

Accepted Date: 6 February 2017

Please cite this article as: M. Ferreira, L.S. Assunção, A.H. Silva, F.B. Filippin-Monteiro, T.B. Creczynski-Pasa, M.M. Sá, Allylic isothiouronium salts: The discovery of a novel class of thiourea analogues with antitumor activity, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.02.013.



Allylic isothiouronium salts: the discovery of a novel class of thiourea analogues with antitumor activity

Misael Ferreira[a,+], Laura Sartori Assunção[b,+], Adny Henrique Silva[c], Fabíola Branco Filippin-Monteiro[d], Tânia Beatriz Creczynski-Pasa[b], Marcus Mandolesi Sá[a,*]

[a] *Departamento de Química, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

[b] *Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

[c] *Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

[d] *Departamento de Análises Clínicas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

* Corresponding Author: Prof. Dr. M. M. Sá
MESOLab (Laboratory of Methodology and Organic Synthesis)
Departamento de Química, Universidade Federal de Santa Catarina
Florianópolis, SC 88040-900, Brazil
Tel.: +55-48-37213642; fax: +55-48-37216850
E-mail: marcus.sa@ufsc.br

[+] As first authors, M. Ferreira and L. S. Assunção contributed equally in the studies related to chemistry and biology, respectively.

Allylic isothiouronium salts: the discovery of a novel class of thiourea analogues with antitumor activity

Abstract

A series of 28 aryl- and alkyl-substituted isothiouronium salts were readily synthesized in high yields through the reaction of allylic bromides with thiourea, *N*-monosubstituted thioureas or thiosemicarbazide. The *S*-allylic isothiouronium salts substituted with aliphatic groups were found to be the most effective against leukemia cells. These compounds combine high antitumor activity and low toxicity toward non-tumoral cells, with selectivity index higher than 20 in some cases. Furthermore, the selected isothiouronium salts induced G2/M cell cycle arrest and cell death, possibly by apoptosis. Therefore, these compounds can be considered as a promising class of antitumor agents due to the potent cytostatic activity associated with high selectivity.

Keywords

isothiouronium salts; allylic bromides; antitumor activity; DNA fragmentation; leukemia

1. Introduction

Cancer is the leading cause of death worldwide and the development and discovery of new anticancer agents are the key focus of several research groups.[1] Leukemia, a type of cancer characterized by the clonal proliferation of malignant white blood cell precursors, can be subdivided based on the predominant cell of origin (lymphoid or myeloid) and the rate of progression (acute or chronic) [2-4]. Different forms of therapy have been used to treat leukemia, such as radiotherapy, chemotherapy, immunotherapy, and bone marrow transplant, with chemotherapy being the most frequently used [5-7]. The existing chemotherapeutic treatments are known to display a wide range of side effects, caused by the low selectivity and high toxicity of the drugs employed. Furthermore, a large number of anticancer agents promote the death of cancer cells by cell cycle arrest, induction of apoptosis and differentiation, as well as through inhibition of cell growth and proliferation, or a combination of two or more of these mechanisms [8-10].

The treatment selected for a leukemia patient is dependent on many factors, such as prognostic features and the stage and type of leukemia [11]. Despite the remarkable progress achieved in the treatment of acute and chronic leukemia, truly effective therapies are absent for the majority of the patients affected by this disease. The most common reasons for failure include drug resistance and treatment-related toxicity associated with intensive therapies [12]. A common example is the anthracycline antibiotic doxorubicin, a widely used anticancer agent that, due to its low bioavailability, requires high doses for therapeutic response, which increases the cumulative risk of cardiotoxicity, myelosuppression and nephrotoxicity [13].

Therefore, the search for more efficient chemotherapies for leukemia, which minimize the toxicity to normal tissues, is of great importance. One of the ways to

achieve this goal is the development of new chemical entities that are more active, more selective, less toxic, and readily available through resourceful methods.

One of the classes of interest in the search for new chemotherapeutic agents is the group of isothiouronium salts. These compounds can exert biological effects and have attracted the attention of researchers due to their diverse activities, such as inhibitors of protein kinase C [14] as well as agonists of the GABA-type [15, 16] and histamine-H_3 receptors [17]. Furthermore, they also exhibit a variety of pharmacological properties, such as local anesthetic [18] and antimicrobial [19-22] activity. Recently, we reported the moderate activity of selected *S*-allylic isothiouronium salts against *Mycobacterium tuberculosis* [23]. However, despite displaying these biological properties, the antitumor activity of isothiouronium salts has been scarcely explored [24, 25].

Therefore, the aim of this study was to evaluate whether this class of compounds could also exhibit antitumor activity. Specifically, this Communication reports the chemical synthesis of twenty-eight *S*-allylic isothiouronium salts and the assessment of their cytotoxicity profile, including an evaluation of possible cell cycle alterations as well as the potential selectivity against leukemia cells.

2. Results and discussion

2.1. Chemistry

A variety of isothiouronium salts **1**, **2**, and **3** were readily synthesized in two steps from α-methylene-β-hydroxy esters **4** (Morita-Baylis-Hillman adducts) [26]. The synthetic strategy involves the preparation of the corresponding allylic bromide **5**, a key precursor obtained through the reaction of α-methylene-β-hydroxyesters **4** with LiBr in acidic medium at 25 °C [27] (Scheme 1). This two-step protocol to obtain

allylic bromides **5** was adapted for the multigram scale, with overall yields of 65-85%, thus constituting a practical route to access these structurally versatile starting materials.

Insert Scheme 1.

The subsequent treatment of the allylic bromides **5** with thiourea (**6a**) as well as *N*-monosubstituted thioureas (**6b-e**) led to the formation of the corresponding isothiouronium salts [28] **1a-k**, **2a-g**, and **3a-j** in nearly quantitative yields (Scheme 1). The choice of the solvent was shown to be crucial to achieving successful transformations. While the *N*-unsubstituted isothiouronium salts **1**, originating from thiourea (**6a**), could be formed smoothly in aqueous acetone as an insoluble crystalline solid, the preparation of *N*-substituted isothiouronium salts **2a,b** and **3a-j** could not be achieved under similar conditions. However, running the reaction in acetonitrile at 25 °C furnished the expected *N*-substituted isothiouronium salts **2** and **3** as crystalline products that precipitated out and were readily collected by filtration, thus facilitating the work-up and isolation steps. After purification, the isothiouronium salts were analyzed by IR, $^{1}H/^{13}C$ NMR, and HRMS. Stretching bands related to C=O and C=C groups can be observed on the IR spectrum at 1704-1730 cm^{-1} and 1634-1658 cm^{-1}, respectively. Furthermore, ^{1}H NMR analysis revealed the presence of the expected peak for the olefinic hydrogen (=CH) at 7.5-8.0 ppm, in the case of aryl-substituted derivatives (**1a**-**h**, **2a**-**e**, **3a**-**i**), or at 6.9-7.1 ppm, in the alkyl-derived salts (**1i-k**, **2f**,**g**, **3j**). This observation unequivocally supports the stereochemistry of the double bond being *Z* for all products, which is also in line with the analysis of the decoupled ^{13}C NMR spectra [28] (see the Experimental section in the Supplementary Material).

For the synthesis of the *N*-amino substituted salts **2c-e** derived from thiosemicarbazide (**6d**), the conditions using acetonitrile as the solvent were also successfully employed, but in these particular cases the products were characterized by NMR as mixtures of isomers in solution, possibly due to the fast equilibrium between tautomeric forms of the allylic isothioureido N-C=N moiety [29].

2.2. Biological activity

2.2.1. Cytotoxicity and selectivity index of S-allylic isothiouronium salts related to leukemia and non-tumoral cell lines

To evaluate the cytotoxicity potential of the *S*-allylic isothiouronium salts, the MTT assay was performed with a murine leukemia cell line (L1210) and a murine non-tumoral cell line (NIH-3T3). The IC_{50} concentration (concentration required to reduce cell viability by 50%) [30] was determined by non-linear regression and the selectivity index (SI) was calculated. A selectivity index higher than 1 indicates that cytotoxicity on tumoral cells surpassed healthy non-tumoral ones [31]. To compare the selectivity of the compounds studied here, we classified them as remarkable (SI above 12), moderate (from 6 to 12) and weak (from 1 to 5) selectivity. The antineoplasic agent mitoxantrone was used as positive control. Mitoxantrone is a synthetic molecule structurally similar to the anthracyclines. This drug was developed to reduce the cardiototoxicity presented by the naturally occurred anthracycline doxorubicin. It is used in breast and prostate cancer, acute myeloid leukemia, and lymphoma treatment [32, 33], also presenting improvements in the survival rate of children suffering from acute lymphoblastic leukemia relapse [34].

The results for the cytotoxicity are summarized in Tables 1 and 2. The effect of substitution on both the allylic chain (R = aryl [Table 1] or alkyl [Table 2] groups) and

the isothiourea moiety (*N*-unsubstituted or substituted by a variety of R^1 groups) was evaluated and both were significant to the activity. Table 1 shows the results for a diversity of aromatic-derived allylic salts (R = aryl) **1a-h**, **2a-e**, and **3a-i** with different substitution patterns at R^1 originating from the thiourea portion. A relatively low IC_{50} (4-18 μM) was observed for the *N*-unsubstituted analogues **1a-h** (Table 1, entries 1-8). Likewise, mitoxantrone presented cytotoxicity toward leukemia cell line, with an IC_{50} of 15 μM, however with low selectivity (SI = 2.8). As previously mentioned, the low selectivity of an anticancer agent might represent a risk of toxicity to patients in cumulative doses. Conversely, three of the *N*-unsubstituted analogues (**1a**, **1e**, **1h**) showed remarkable selectivity (SI = 17.5, > 6.3, and 10.2, respectively), which in turns could present less risk of toxic effects in cancer treatment eventually

Insert Table 1.

Insert Table 2.

On the other hand, substitution of a hydrogen at one of the nitrogen atoms of the isothioureido portion by hydrophobic methyl or allyl groups (**2a,b**, where $R^1 \neq H$) was deleterious for both the activity and selectivity (Table 1, entries 9 and 10). Conversely, the introduction of the more polar and hydrophilic amino group, as in the thiosemicarbazide derivatives **2c-e** (logP ~ 1 [35]), caused an increment in the activity (entries 11-13).

Remarkably, *N*-substitution by a more lipophilic phenyl group as in *N*-phenyl isothioureas **3a-i** (logP ~ 4-5 [35]; see Table 1, entries 14-22) was found to improve both the antitumor activity as well as the selectivity. Besides the high SI found for the phenyl-substituted salt **3a** (SI = 9.0, entry 14), the analogues **3c-g** containing electron-withdrawing groups at the phenyl ring showed SI values ranging from *c.a.* 3 to 13 (Table 1, entries 16-20). In the particular case of the nitro-substituted

derivatives **3e** and **3g** (Table 1, entries 18 and 20), the very low cytotoxicity toward non-tumoral cells is worth noting, as nitro groups are usually related to a variety of biological effects [36, 37]. Thus, the *N*-phenyl-substituted isothioureas **3a,c-g** appear as promising leads for the development of selective antitumor agents, not only because they show good SI values but also due to their relatively high lipophilicity, which may improve the penetration of these compounds into cells.

Another attractive class of potential antileukemic leads was found to be derived from the aliphatic allylic salts **1i-k**, **2f,g**, and **3j** (R = alkyl, Table 2), wherein high antitumor activity (IC_{50} = 3-14 μM) as well as selectivity (SI = 5.3-23.7) were observed for the *N*-unsubstituted analogues **1i-k** (Table 2, entries 1-3). When the selectivity index was analyzed, the allylic salts substituted with alkyl groups, such as ethyl (R = CH_3CH_2; $SI_{(\mathbf{1i})}$ = 23.7) and methyl (R = CH_3; $SI_{(\mathbf{1j})}$ > 20), exerted a greater toxic effect against leukemia (L1210) cells with a lower concentration of each salt (3 and 5 μM, respectively). The relative low lipophilicity can be modulated by adding or removing carbon atoms from the R chain (logP [35] varying from 0.61 for **1j** to 1.91 for **1k**), and the optimum chain size was determined as being the two-carbon moiety as in **1i** (Table 2, entry 1).

As expected, based on the results for the aromatic series (see Table 1, entries 9 and 10), the *N*-methyl- and *N*-allyl-substituted salts **2f,g** of the alkyl series (Table 2, entries 4 and 5) are comparatively less cytotoxic and less selective than their *N*-unsubstituted analogues **1i-k** (entries 1-3). A different trend was observed for **3j** (Table 2, entry 6), the least lipophilic *N*-phenyl-substituted salt tested (logP = 3.18 [35]), where its high antitumor activity was partially obfuscated by the pronounced cytotoxicity toward non-tumoral cells, thus giving a low SI value of 2.8. Therefore, the alkyl-substituted isothiouronium salts **1i** and **1j** were also considered as promising

leads for further development as selective antileukemic agents.

2.2.2. Effect of S-allylic isothiouronium salts on DNA content of leukemia cells in cell cycle distribution

Cell division is controlled by checkpoints that avoid genomic instability and ensure the proper cell cycle progression. The checkpoints act during the transition from the G1 to the S phase (DNA replication) and from the G2 to the M phase (mitosis). Chemicals and other agents can either delay replication or cause irreparable DNA damage that is recognized by the checkpoints, leading to a cell cycle arrest and/or death [38, 39].

In order to investigate whether the effects of the *S*-allylic isothiouronium salts are associated with cell cycle alterations, the leukemia cells were 24-h exposed with the corresponding IC_{50} of each tested compound. Flow cytometry analysis of cell cycle, using propidium iodide (PI) as DNA label, was performed to observe the distribution of cells in the specific phases of the cycle for replication. It was observed that the IC_{50} of the *N*-unsubstituted salts **1a-h** (data not shown for **1g)** induced DNA fragmentation, represented by an increase of approximately 20% in the number of events in the sub-G1 compared to the control group (Figure 1C). Our previous study demonstrated similar results for melanoma cells exposed to compounds **1a** and **1e** [29]. In that case, compound **1c** showed greater activity against melanoma cells, leading to DNA fragmentation of more than 50% of cell population under evaluation [29]. However, DNA fragmentation in leukemia cells induced by **1c** was observed in about 20% of cell population. This difference observed in both studies might be explained by the biological distinctions between the two cancer cell types, which reflect in specific responses to the stimulus [9].

Insert Figure 1.

The *N*-methyl salt **2a** induced cell cycle arrest in the G2/M phase with an increase of 14% in the number of events, while *N*-phenyl salt **3b** induced a significant DNA fragmentation in the sub-G1 when compared to cells without treatment (Figures 1D and 1E, respectively). The aliphatic allylic salts **1i** (data not shown), **1k** and **2f** induced cell cycle arrest in the G2/M phase leading to cell death, represented by an increase in the DNA fragmentation in the sub-G1 phase (Figure 1F). This G2/M arrest might be explained by a phenomenon in the cell cycle progression due to an extensive damage in the DNA content or chromosome segregation caused by these compounds, which make it impossible for the cell to continue its replication and, as a result, it enters into the death state [40]. Furthermore, compounds **1j** and **2g** also induced DNA fragmentation, increasing by 27% the number of events in sub-G1 without affecting the G2/M phase significantly. In terms of the mechanism of action, this observation suggests that compounds **1i**, **1k**, and **2f** differ from the others in the group.

Other isothiouronium salts showed no significant alteration in the cell cycle distribution. This might be due to the fact that the IC_{50} of the compounds was determined by a mitochondrial activity assay, mainly using dehydrogenases. Since changes in mitochondrial metabolism and mitochondrial damages are earlier noticed in cell death process, changes in DNA content might not be observed in flow cytometry due to a time-dependent response [41-44].

The G2/M cell cycle arrest observed in L1210 cells caused by the exposure to compounds **1i**, **1k** and **2f** might be an interesting approach in cancer therapy, since they increase the sensitivity when combined with other anticancer agents and

eventually augment the cell death rates [45, 46]. Also, effective treatment of cancer with agents that induce G2/M arrest can be carried out with lower drug concentrations, thus reducing the side effects [47, 48].

In order to investigate the cell death caused by the *S*-allylic isothiouronium salts, leukemia cells were exposed for 24 h to compounds that presented SI higher than 6 (**1a**, **1e**, **1h**, **3a**, **3g**, and **1j**) and were subsequently stained with Annexin-V-FITC and PI for further evaluation through flow cytometry (Figure 2). It was observed that compound **3g** presented an increase of events stained with annexin-V-FITC, which is suggestive of an apoptotic process, since annexin conjugates to phosphatidylserine once it is externalized in cell membrane during apoptotic events. Moreover, cells exposed to salts **1a** and **1e** presented a portion of its population double stained, suggesting late apoptotic or necrotic process. Furthermore, another fraction of cell population exposed to these same compounds also presented PI labeling, suggestive of a necrotic process characterized by loss of extracellular membrane integrity, allowing PI penetration [49-51].

Insert Figure 2.

3. Conclusions

In summary, a diverse set of *S*-allylic isothiouronium salts were readily prepared in high yields and screened against leukemia cells. A representative group of *N*-unsubstituted isothiouronium salts not only showed high potent activity against tumoral cells but also displayed remarkable selectivity. Similarly, the externalization of phosphatidylserine caused by the nitro-substituted derivative **3g** suggests an apoptotic process, being an interesting approach to cancer treatment, once this mechanism of death does not induce an inflammatory process, unlike necrosis.

Furthermore, the alkyl-substituted derivatives are more potent than the aryl-substituted analogues, inducing G2/M cell cycle arrest and, possibly, apoptosis. However, further studies should be done to characterize better the cell death in all cases.

4. Experimental section

4.1. Chemistry

4.1.1. General considerations

All chemicals were of reagent grade and were used as received. Melting points were determined using a Microquímica MQPF301 hot-plate apparatus and are uncorrected. Infrared spectra were acquired with a Perkin-Elmer FT-IR 1600 spectrometer (range 4000-400 cm^{-1}) using KBr for all samples. ^{1}H NMR spectra were recorded at 400 MHz or 200 MHz and ^{13}C NMR spectra (fully decoupled) were recorded at 100 MHz or 50 MHz. Samples were prepared in an appropriate deuterated solvent ($CDCl_3$, D_2O, DMSO-d_6). Chemical shifts are reported in parts per million (ppm, δ) relative to TMS at 0.00 ppm or solvent ($CDCl_3$ at 7.26 ppm or D_2O at 4.64 ppm or DMSO-d_6 at 2.50 ppm for ^{1}H NMR, and $CDCl_3$ at 77.16 ppm or DMSO-d_6 at 39.52 ppm for ^{13}C NMR) as the internal standard. Coupling constants (*J*) are measured in Hertz (Hz) and coupling patterns are designated as s (singlet); d (doublet); dd (doublet of doublets); t (triplet); appt (apparent triplet); dq (doublet of quartet); m (multiplet); brs (broad signal). Elemental analyses were conducted in a CHNS-O Carlo Erba EA-1110 analyzer. The ESI-QTOF mass spectrometer was operated in the positive ion mode at 4.5 kV and at a desolvation temperature of 180 °C. The standard electrospray ion (ESI) source was used to generate the ions. The instrument was calibrated in the range *m/z* 50-3000 using a calibration standard (low

concentration tuning mix solution) and data were processed with the aid of computer software. The isothiouronium bromides **1a,c,e-j**, isothiosemicarbazide bromides **2d,e** and *N*-phenylisothiouronium bromides **3a,i** were prepared and purified according to the previously described methods and showed physical and spectral data in accordance with their expected structure and by comparison with spectral data in literature [23, 28, 29].

*4.1.2. Typical procedure for the synthesis of isothiouronium salts **1***

To a stirred solution of allylic bromide **5** (1.0 mmol) in 5.0 mL of acetone:H_2O (4:1 v/v) at 25 °C was added 0.95 mmol of thiourea (**6a**). After stirring for 1 h, the organic salt was concentrated under reduced pressure and the resulting solid was crushed with ethyl ether/CH_2Cl_2 (4:1) and filtered to give pure isothiouronium salts **1b,d**. For **1k**, the resulting product was washed with ethyl ether to give pure isothiouronium salt as oil.

*4.1.2.1. Methyl (Z)-2-(isothioureidomethyl)-3-(4-methylphenyl)-2-propenoate hydrobromide (**1b**)*

Yield 96% (348 mg); white solid, mp 172.0-174.0 °C; ^{1}H NMR (400 MHz, D_2O): δ 2.17 (s, 3H), 3.66 (s, 3H), 4.01 (s, 2H), 7.10 (s, 4H), 7.61 (s, 1H); ^{13}C NMR (100 MHz, D_2O, DMSO-d_6 as internal standard): δ 22.2 (CH_3), 30.6 (CH_2), 54.5 (OCH_3), 124.4 (C), 131.3 (2 × CH), 131.4 (2 × CH), 131.9 (C), 142.8 (=CH), 146.9 (C), 170.1 (C), 172.1 (C); IR (KBr) ν_{max}/cm^{-1}: 3292, 3253, 3081, 2949, 1709, 1636, 1437, 1322, 1271; Anal. Calcd for $C_{13}H_{17}BrN_2O_2S$ (%): C, 45.22; H, 4.96; N, 8.11; found: C, 45.38; H, 5.35; N, 8.19.

*4.1.2.2. Methyl (Z)-3-(4-fluorophenyl)-2-(isothioureidomethyl)-2-propenoate hydrobromide (**1d**)*

Yield 94% (602 mg); white solid, mp 177.0-179.0 °C; ^{1}H NMR (200 MHz, D_2O): δ 3.70 (s, 3H), 4.08 (s, 2H), 7.07 (t, *J* = 8.5 Hz, 2H), 7.31 (dd, *J* = 5.6, 8.5 Hz, 2H), 7.78 (s, 1H); ^{13}C NMR (100 MHz, D_2O, DMSO-d_6 as internal standard): δ 30.4 (CH_2), 54.5 (OCH_3), 117.7 (d, *J* = 24.9 Hz, 2 × CH), 125.6 (C), 131.2 (C), 133.4 (d, *J* = 7.3 Hz, 2 × CH), 145.8 (=CH), 164.8 (d, *J* = 247.7 Hz, C), 169.9 (C), 171.9 (C); IR (KBr) ν_{max}/cm^{-1}: 3368, 3213, 3106, 3037, 2954, 1704, 1650, 1597, 1510, 1438, 1233, 1204, 1162; Anal. Calcd for $C_{12}H_{14}BrFN_2O_2S$ (%): C, 41.27; H, 4.04; N, 8.02; found: C, 41.16; H, 3.52; N, 8.08.

*4.1.2.3. Methyl (Z)-2-isothioureidomethyl-2-hexenoate hydrobromide (**1k**)*

Yield 98% (293 mg); clear yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.90 (t, *J* = 7.4 Hz, 3H), 1.37-1.56 (m, 2H), 2.19-2.30 (m, 2H), 3.60 (brs, 2H), 3.75 (s, 3H), 4.07 (s, 2H), 7.02 (t, *J* = 7.6 Hz, 1H), 8.70-8.90 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.0 (CH_3), 21.8 (CH_2), 28.0 (CH_2), 31.5 (CH_2), 53.2 (OCH_3), 126.1 (C), 151.1 (=CH), 168.5 (C), 172.0 (C); IR (neat) ν_{max}/cm^{-1}: 3289, 3189, 3064, 2961, 2874, 2735, 1712, 1646, 1440, 1298, 1198, 1071; HRMS (ESI+): *m*/*z* calcd for $C_9H_{17}N_2O_2S$ $[M]^+$: 217.1005; found: 217.1002.

*4.1.3. Typical procedure for the synthesis of N-substituted isothiouronium salts **2** and **3***

To a stirred solution of allylic bromide **5** (1.0 mmol) in 5.0 mL of acetonitrile at 25 °C was added 0.95 mmol of *N*-substituted thiourea (**5b** or **5c** or **5e**) or thiosemicarbazide (**5d**). After stirring for 1 h, the organic salt was concentrated under reduced pressure and the resulting solid was crushed with acetonitrile and filtered to give pure isothiouronium salts **2a-c**. For **2f**,**g** and **3j**, the resulting crude product was triturated with ethyl ether to give pure isothiouronium salt as oil.

*4.1.3.1. Methyl (Z)-2-[(N-methylisothioureido)methyl]-3-(4-methoxyphenyl)-2-propenoate hydrobromide (**2a**)*

Yield 89% (232 mg); white solid, mp 126.0-128.0 °C; ^{1}H NMR (400 MHz, D_2O): δ 2.69 (s, 3H), 3.64 (s, 6H), 4.01 (s, 2H), 6.81 (d, *J* = 8.4 Hz, 2H), 7.14 (d, *J* = 8.4 Hz, 2H), 7.49 (s, 1H); ^{13}C NMR (50 MHz, D_2O, DMSO-d_6 as internal standard): δ 31.2 (CH_2), 31.7 (CH_3), 54.4 (OCH_3), 57.0 (OCH_3), 116.1 (2 × CH), 123.7 (C), 127.5 (C), 133.4 (2 × CH), 145.8 (=CH), 162.0 (C), 168.1 (C), 170.1 (C); IR (KBr) ν_{max}/cm^{-1}: 3168, 3072, 2991, 2947, 2908, 1717, 1702, 1646, 1601, 1511, 1439, 1259, 1181; Anal. Calcd for $C_{14}H_{19}BrN_2O_3S$ (%): C, 44.81; H, 5.10; N, 7.46; found: C, 45.16; H, 5.53; N, 7.53.

*4.1.3.2. Methyl (Z)-2-[(N-allylisothioureido)methyl]-3-(4-methoxyphenyl)-2-propenoate hydrobromide (**2b**)*

Yield 98% (242 mg); white solid, mp 162.0-163.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.75 (s, 3H), 3.78 (s, 3H), 4.02 (s, 2H), 4.38 (s, 2H), 5.19-5.26 (m, 2H), 5.76-5.85 (m, 1H), 7.04 (d, *J* = 8.4 Hz, 2H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.79 (s, 1H), 9.33 (brs, 1H), 9.52 (brs, 1H), 9.95 (brs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.0 (CH_2), 45.7 (CH_2), 52.6 (OCH_3), 55.5 (OCH_3), 114.7 (2 × CH), 117.5 (=CH_2), 121.3 (C), 125.7 (C), 131.4 (=CH), 132.1 (2 × CH), 143.5 (=CH), 160.8 (C), 165.7 (C), 166.6 (C); IR (KBr) ν_{max}/cm^{-1}: 3294, 3189, 3026, 2839, 1714, 1638, 1598, 1512, 1440, 1281, 1261, 1175; Anal. Calcd for $C_{16}H_{21}BrN_2O_3S$ (%): C, 47.88; H, 5.27; N, 6.98; found: C, 47.98; H, 5.03; N, 7.06.

*4.1.3.3. Methyl (Z)-2-[(N-aminoisothioureido)methyl]-3-phenyl-2-propenoate hydrobromide (**2c**)*

Yield 92% (856 mg); white solid, mp 157.0-159.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6): mixture of isomers (92:8) coexisting in solution (data for the major isomer)

δ 3.77 (s, 3H), 4.20 (s, 2H), 7.43-7.53 (m, 5H), 7.84 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 28.8 (CH_2), 52.6 (OCH_3), 125.0 (C), 129.1 (2 × CH), 129.6 (2 × CH), 130.0 (CH), 133.6 (C), 143.4 (=CH), 166.3 (C), 166.4 (C); IR (KBr) ν_{max}/cm^{-1}: 3345, 3311, 3103, 2950, 2779, 1721, 1654, 1626, 1545, 1436, 1284, 1205, 1166, 949; Anal. Calcd. for $C_{12}H_{16}BrN_3O_2S$ (%): C, 41.63; H, 4.66; N, 12.14; found: C, 41.89; H, 4.69; N, 12.22.

*4.1.3.4. Methyl (Z)-2-[(N-methylisothioureido)methyl]-2-pentenoate hydrobromide (**2f**)*

Yield 98% (341 mg); clear yellow oil; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.00 (t, J = 7.6 Hz, 3H), 2.27 (dq, J = 7.6, 7.8 Hz, 2H), 2.88 (d, J = 4.0 Hz, 3H), 3.69 (s, 3H), 4.12 (s, 2H), 6.92 (t, J = 7.8 Hz, 1H), 9.12 (brs, 1H), 9.42 (brs, 1H), 9.71 (brs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.1 (CH_3), 22.0 (CH_2), 28.1 (CH_2), 30.8 (CH_3), 52.3 (OCH_3), 124.8 (C), 150.2 (=CH), 165.8 (C), 165.9 (C); IR (neat) ν_{max}/cm^{-1}: 3155, 3060, 2972, 1714, 1653, 1604, 1438, 1315, 1287, 1199; HRMS (ESI+): *m*/*z* calcd for $C_9H_{17}N_2O_2S$ $[M]^+$: 217.1005; found: 217.1006.

*4.1.3.5. Methyl (Z)-2-[(N-allylisothioureido)methyl]-2-pentenoate hydrobromide (**2g**)*

Yield 96% (373 mg); clear yellow oil; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.00 (t, J = 7.4 Hz, 3H), 2.28 (dq, J = 7.4, 7.6 Hz, 2H), 3.69 (s, 3H), 3.94-3.98 (m, 2H), 4.15 (s, 2H), 5.20-5.24 (m, 2H), 5.76-5.85 (m, 1H), 6.92 (t, J = 7.6 Hz, 1H), 9.21 (brs, 1H), 9.48 (brs, 1H), 9.92 (brs, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.1 (CH_3), 22.1 (CH_2), 28.2 (CH_2), 45.7 (CH_2), 52.3 (OCH_3), 117.5 (=CH_2), 124.9 (C), 131.4 (=CH), 150.1 (=CH), 165.4 (C), 165.9 (C); IR (neat) ν_{max}/cm^{-1}: 3143, 3070, 2975, 1717, 1644, 1594, 1438, 1315, 1287, 1251, 1199; HRMS (ESI+): *m*/*z* calcd for $C_{11}H_{19}N_2O_2S$ $[M]^+$: 243.1162; found: 243.1165.

*4.1.3.6. Methyl (Z)-2-[(N-phenylisothioureido)methyl]-2-pentenoate hydrobromide (**3j**)*

Yield 98% (391 mg); clear yellow oil; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.00 (t,

J = 7.6 Hz, 3H), 2.31 (dq, *J* = 7.6, 7.8 Hz, 2H), 3.71 (s, 3H), 4.24 (s, 2H), 6.97 (t, *J* = 7.8 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 1H), 7.51 (appt, *J* = 7.6 Hz, 2H), 11.42 (brs, 1H); ^{13}C NMR (50 MHz, DMSO-d_6):δ 13.1 (CH_3), 22.1 (CH_3), 28.4 (CH_2), 52.4 (OCH_3), 124.7 (C), 125.2 (2 × CH), 128.3 (CH), 130.0 (2 × CH), 135.0 (=CH), 150.7 (C), 166.0 (C), 167.3 (C); IR (neat) ν_{max}/cm^{-1}: 3266, 3063, 2971, 2877, 2801, 1713, 1638, 1578, 1494, 1439, 1286, 1199, 1146; HRMS (ESI+): *m*/*z* calcd for $C_{14}H_{19}N_2O_2S$ $[M]^+$: 279.1162; found: 279.1167.

4.2. Biological activity

4.2.1. Materials

The cell culture media, fetal bovine serum and antibiotics (penicillin/streptomycin) were purchased from GIBCO (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) and ethylene diaminetetraacetic acid disodium salt (EDTA) were purchased from Merck (Darmstadt, Germany). Binding buffer was purchased from Millipore (Billerica, MA, USA) and other reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA).

4.2.2. Cell lines and culture conditions

The cell lines L1210 (murine lymphocytic leukemia) and NIH-3T3 (murine fibroblast) were purchased from the Rio de Janeiro Cell Bank. The cells were cultured in RPMI-1640 (L1210) or DEMEM (NIH-3T3) medium, supplemented with 1.5 g/L sodium bicarbonate, 10 mM HEPES, pH 7.4, 100 U/mL penicillin G, 100 μg/mL streptomycin and 10% fetal calf serum at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. Cells were passaged 3 times a week and cultures with greater than 95% of viable cells in trypan-blue exclusion tests were used for the

experiments.

4.2.3. Viability assay

The MTT method was used to determine the cell viability [52]. NIH-3T3 cells (1 × 10^4 cells/well) and L1210 cells (1 × 10^5 cells/well) were seeded in 96-well plates and incubated for 24 h with increasing concentrations of the compounds, ranging from 1 to 100 μM. For the control group, cells were incubated without treatment. After incubation, the old culture medium was replaced by fresh culture medium with 5 mg/mL of MTT, followed by incubation for 1-4 h at 37 °C. MTT-formazan crystals were dissolved in 100 μL of DMSO and the absorbance was measured at 540 nm using a micro-well system reader. The IC_{50} values were calculated using the software Prism 5.0 (GraphPad Software) through non-linear regression analysis of the logarithm concentration as a function of the normalized response (percentage of viable cells after 24 h incubation).

The activity of the compounds in tumoral cells was classified according to IC_{50} as:

High: < 5 μM; Good: 5 to 10 μM; Moderate: 11 to 25 μM; Low: > 26 μM

4.2.4. Selectivity Index

The selectivity index was calculated as the ratio of the IC_{50} of the non-tumoral cell line to the IC_{50} of the tumoral cell line, as presented in the following formula. An SI value higher than 1 indicates that the cytotoxicity against tumoral cells surpassed that toward non-tumoral cells [31].

$$SI = \frac{IC_{50}\ non-tumoral\ cells}{IC_{50}\ tumoral\ cells}$$

4.2.5. Flow cytometry analysis

To analyze the cell cycle of cells treated with isothiouronium salts, flow cytometry was used following a previously described method [53]. Leukemia cells (1 × 10^6/well) were incubated with the IC_{50} concentrations of each compound for 24 h, in a 12-well plate. After incubation, the cells were centrifuged at 400 × g at room temperature for 10 min. The cells were then washed with 1 mL of PBS and centrifuged again. The supernatant was discarded and cells were fixed with 200 μL of 70% ethanol for 30 min at 4 °C. PBS (1 mL) with 2% of BSA was then added and the cells were centrifuged for 10 min at 400 × g. The supernatants were removed and the cells were permeabilized with lysis buffer (0.1% Triton-X in PBS) and 0.5 μL of RNase (100 μg/mL). The DNA content was stained with propidium iodide (PI) (20 μg/mL) and analyzed using a FACSCanto flow cytometer (Becton Dickinson). The DNA content of the cell population in each phase of the cell cycle was determined using Flowing 2.5.1 software (University of Turku, Turku, Finland).

To evaluate cell death a flow cytometric assay using Annexin-V-FITC and propidium iodide was performed as previously described, with minor modifications [51]. Briefly, 1 × 10^6 cells/well were exposed to the IC_{50} of each compound for 24 h, in a 12-well plate, centrifuged for 10 minutes at 400 g and 4 °C , washed twice with cold PBS (1 mL), resuspended in binding buffer 1× and incubated with 2.5 μL Annexin-V-FITC for 15 min. After, 5 μL of PI solution (20 μg/mL) was added in the moment of cells acquisition. Cell death was analyzed using FACSCanto flow cytometry equipment (Becton Dickinson) and Flowing 2.5.1 software (University of Turku, Turku, Finland). The percentage of apoptotic and/or necrotic cells induced by the compounds exposure was compared to cells without treatment (control group).

Acknowledgements

The authors wish to thank the Central de Análises (Departamento de Química, UFSC, Florianópolis) for spectroscopic analysis. Special thanks to CEBIME (Laboratório Central de Biologia Molecular e Estrutural), for obtaining the mass spectra, and LAMEB I (Laboratório Multiusuário de Estudos em Biologia I) for the analysis carried out on the Flow Cythometer BD FACSCanto II. M.F. and L.S.A. thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for fellowships. M.M.S. is grateful to CNPq for a research fellowship. Financial support from INCT-Catalysis/CNPq is also gratefully acknowledged.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article (selected NMR spectra for novel compounds) can be found, in the online version, at http://

References

[1] A.S. Narang, D.S. Desai, Anticancer Drug Development. Unique Aspects of Pharmaceutical Development, Pharm. Perspect. Cancer Ther. (2009) 49–92.

[2] S.J. McKenna, Leukemia, Oral Surg. Oral Med. Oral Pathol. 89 (2000) 137–139.

[3] S.A. Wander, M.J. Levis, A.T. Fathi, The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond, Ther. Adv. Hematol. 5 (2014) 65–77.

[4] C. Capron, K. Jondeau, L. Casetti, V. Jalbert, C. Costa, E. Verhoyen, J.M. Massé, P. Coppo, M.C. Béné, P. Bourdoncle, E. Cramer-Bordé, I. Dusanter-Fourt, Viability and

stress protection of chronic lymphoid leukemia cells involves overactivation of mitochondrial phosphoSTAT3Ser$_{727}$, Cell Death Dis. 5 (2014) e1451.

[5] H. Greim, D.A. Kaden, R.A. Larson, C.M. Palermo, J.M. Rice, D. Ross, R. Snyder, The bone marrow niche, stem cells, and leukemia: impact of drugs, chemicals, and the environment, Ann. N.Y. Acad. Sci. 1310 (2014) 7–31.

[6] M.F.C. Cardoso, P.C. Rodrigues, M.E.I.M. Oliveira, I.L. Gama, I.M.C.B. da Silva, I.O. Santos, D.R. Rocha, R.T. Pinho, V.F. Ferreira, M.C.B.V. Souza, F.C. Silva, F.P. Silva-Jr, Synthesis and evaluation of the cytotoxic activity of 1,2-furanonaphthoquinones tethered to 1,2,3-1*H*-triazoles in myeloid and lymphoid leukemia cell lines, Eur. J. Med. Chem. 84 (2014) 708–717.

[7] J. Golab, R. Zagozdzon, R. Kaminski, K. Kozar, K. Gryska, D. Izycki, A. Mackiewicz, T. Stoklosa, A. Giermasz, W. Lasek, M. Jakobisiak, Potentiatied antitumor effectiveness of combined chemo-immunotherapy with Interleukin-12 and 5-fluorouracil of L1210 leukemia *in vivo,* Leukemia 15 (2001) 613–620.

[8] K. Suppipat, H.D. Lacorazza, From the Table to the Bedside: Can Food-Derived Sulforaphane be used as a Novel Agent to Treat Leukemia?, Curr. Cancer Drug Targ. 14 (2014) 434–445.

[9] M. Ferreira, L.S. Assunção, F.B. Filippin-Monteiro, T.B. Creczynski-Pasa, M.M. Sá, Synthesis of 1,3-thiazine-2,4-diones with potential anticancer activity, Eur. J. Med. Chem. 70 (2013) 411–418.

[10] K. Li, C. Hu, C. Mei, Z. Ren, J.C. Vera, Z. Zhuang, J. Jin, H. Tong, Sequential combination of decitabine and idarubicin synergistically enhances anti-leukemia effect followed by demethylating Wnt pathway inhibitor promoters and downregulating Wnt pathway nuclear target, J. Transl. Med. 12 (2014) 167.

[11] C.-H. Pui, C.G. Mullighan, W.E. Evans, M.V. Relling, Pediatric acute lymphoblastic leukemia: where are we going and how do we get there?, Blood 120 (2012) 1165-1174.

[12] S.R. Cataland, M. Lucas, J.C. Byrd, Antibody Therapy of Acute and Chronic Leukemias, Curr. Pharm. Biotechnol. 2 (2001) 357–367.

[13] B.S. Chhikara, N.S. Jean, D. Mandal, A. Kumar, K. Parang, Fatty acyl amide derivatives of doxorubicin: Synthesis and *in vitro* anticancer activities, Eur. J. Med. Chem. 46 (2011) 2037–2042.

[14] R.T. Hendricks, D. Sherman, B. Strulovici, C.A. Broka, 2-Aryl-indolyl maleimides - novel and potent inhibitors of protein kinase C, Bioorg. Med. Chem. Lett. 5 (1995) 67-72.

[15] R.D. Allan, H.W. Dickenson, G.A.R. Johnston, R. Kazlauskas, K.N. Mewett, Structural analogues of ZAPA as $GABA_A$ agonists, Neurochem. Int. 30 (1997) 583–591.

[16] R.D. Allan, H.W. Dickenson, B.P. Hiern, G.A.R. Johnston, R. Kazlauskas, Isothiouronium compounds as γ-aminobutyric acid agonists, Br. J. Pharmac. 88 (1986) 379–387.

[17] C.R. Ganellin, B. Bang-Andersen, Y.S. Khalaf, W. Tertiuk, J.M. Arrang, M. Garbarg, X. Ligneau, A. Rouleau, J.C. Schwartz, Imetit and *N*-methyl derivatives. The transition from potent agonist to antagonist at histamine H_3 receptors, Bioorg. Med. Chem. Lett. 2 (1992) 1231–1234.

[18] B.M. Regan, F.T. Galysh, R.N. Morris, The 2-Thiopseudourea Moiety, a New Local Anesthesiophore, J. Med. Chem. 10 (1967) 649–652.

[19] N. Iwai, T. Fujii, H. Nagura, M. Wachi, T. Kitazume, Structure-activity relationship study of the bacterial actin-like protein MreB inhibitors: Effects of substitution of benzyl group in *S*-benzyliothiourea, Biosci. Biotechnol. Biochem. 71 (2007) 246–248.

[20] G.J. Bean, S.T. Flickinger, W.M. Westler, M.E. McCully, D. Sept, D.B. Weibel, K.J. Amann, A22 disrupts the bacterial actin cytoskeleton by directly binding and inducing a low-affinity state in MreB, Biochemistry 48 (2009) 4852–4857.

[21] A. Trani, P. Ferrari, R. Pallanza, R. Ciabatti, Thioureas and Isothiouronium Salts of the Aglycone of Teicoplanin. I. Synthesis and Biological Activity, J. Antibiot. 42 (1989) 1268–1275.

[22] A.M. Badawi, E.M.S. Azzam, S.M.I. Morsy, Surface and biocidal activity of some synthesized metallo azobenzene isothiouronium salts, Bioorg. Med. Chem. 14 (2006) 8661–8665.

[23] G.P. Silveira, M. Ferreira, L. Fernandes, G.C. Moraski, S. Cho, C. Hwang, S.G. Franzblau, M.M. Sá, Allylic thiocyanates as a new class of antitubercular agents, Bioorg. Med. Chem. Lett. 22 (2012) 6486–6489.

[24] C. Vértesi, A. Molnár, L. Guczoghy, Isothiouronium Salts, PCT WO 85/04399 (Chem. Abstr. (1985) 105:97026).

[25] M. Koronkiewicz, Z. Chilmonczyk, Z. Kazimierczuk, Proapoptotic effects of novel pentabromobenzylisothioureas in human leukemia cell lines, Med. Chem. Res. 21 (2012) 3111–3118.

[26] D. Basavaiah, B.S. Reddy, S.S. Badsara, Recent Contributions from the Baylis-Hillman Reaction to Organic Chemistry, Chem. Rev. 110 (2010) 5447–5674.

[27] M. Ferreira, L. Fernandes, M.M. Sá, A highly efficient and general method for the preparation of (*Z*)-allylic bromides derived from Morita-Baylis-Hillman adducts, J. Braz. Chem. Soc. 20 (2009) 564–568.

[28] M.M. Sá, M. Ferreira, A.J. Bortoluzzi, L. Fernandes, S. Cunha, Exploring the reaction of multifunctional allylic bromides with *N*,*S*-dinucleophiles: isothiuronium salts and analogs as useful motifs to assemble the 1,3-thiazine core, Arkivoc xi (2010) 303–321.

[29] J. Cisilotto, M. Ferreira, F.B. Filippin-Monteiro, A.J. Bortoluzzi, M.M. Sá, T.B. Creczynski-Pasa, Isothiouronium Salts Reduce NRAS Expression, Induce Apoptosis and Decrease Invasion of Melanoma Cells, Anticancer Agents Med. Chem. 15 (2015) 353–362.

[30] Food and Drug Administration, Guidance for Industry Antiviral Product Development - Conducting and Submitting Virology Studies to the Agency, US (2006).

[31] D. Callacondo-Riva, A. Quispe-Mauricio, S. Lindo-Gamarra, A.J. Vaisberg, Cytotoxic activity of an ethanolic extract of *gnaphalium spicatum keto keto* on human tumor cell lines, Rev. Peru Med. Exp. Salud Publica 25 (2008) 380–385.

[32] N. Li, Y. Ma, C. Yang, L. Guo, X. Yang, Interaction of anticancer drug mitoxantrone with DNA analyzed by electrochemical and spectroscopic methods, Biophys. Chem. 116 (2005) 199–205.

[33] Z. Hajihassan, A. Rabbani-Chadegani, Studies on the binding affinity of anticancer drug mitoxantrone to chromatin, DNA and histone proteins, J. Biomed. Sci. 16:31 (2009).

[34] C. Parker, R. Waters, C. Leighton, J. Hancock, R. Sutton, A.V. Moorman, P. Ancliff, M. Morgan, A. Masurekar, N. Goulden, N. Green, T. Révész, P. Darbyshire, S. Love, V. Saha, Effect of mitoxantrone on outcome of children with first relapse of acute lymphoblastic leukaemia (ALL R3): an open-label randomised trial, Lancet 376 (2010) 2009–2017.

[35] Values for logP were calculated using Molinspiration software - www.molinspiration.com

[36] J.H. Tocher, Reductive Activation of Nitroheterocyclic Compounds, Gen. Pharmac. 28 (1997) 485–487.

[37] F.R. Paula, S.H.P. Serrano, L.C. Tavares, Aspects of Bioactivity and Toxicity of Nitrocompounds, Quim. Nova 32 (2009) 1013–1020.

[38] A. Murray, Cell cycle checkpoints, Curr. Opin. Cell Biol. 6 (1994) 872–876.

[39] N.C. Walworth, Cell-cycle checkpoint kinases: checking in on the cell cycle, Curr. Opin. Cell Biol. 12 (2000) 697–704.

[40] R.S. DiPaola, To Arrest or Not To G_2-M Cell-Cycle Arrest, Clin. Cancer Res. 8 (2002) 3311–3314.

[41] D.R. Green, G. Kroemer, The pathophysiology of mitochondrial cell death, Science 305 (2004) 626–629.

[42] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.

[43] C. Mammucari, R. Rizzuto, Signaling pathways in mitochondrial dysfunction and aging, Mech. Ageing Dev. 131 (2010) 536–543.

[44] R. Scherz-Shouval, Z. Elazar, ROS, mitochondria and the regulation of autophagy, Trends Cell Biol. 17 (2007) 422–427.

[45] J.A. Pietenpol, Z.A. Stewart, Cell cycle checkpoint signaling: Cell cycle arrest versus apoptosis, Toxicology 181-182 (2002) 475–481.

[46] Y.-S. Ho, J.-S. Duh, J.-H. Jeng, Y.-J. Wang, Y.-C. Liang, C.-H. Lin, C.-J. Tseng, C.-F. Yu, R.-J. Chen, J.-K. Lin, Griseofulvin Potentiates Antitumorigenesis Effects of Nocodazole Through Induction of Apoptosis and G2/M Cell Cycle Arrest in Human Colorectal Cancer Cells, Int. J. Cancer 91 (2001) 393–401.

[47] R.J. Sleiman, D.R. Catchpoole, B.W. Stewart, Drug-induced death of leukaemic cells after G2/M arrest: higher order DNA fragmentation as an indicator of mechanism, Br. J. Cancer 77 (1998) 40–50.

[48] T. Kawabe, G2 checkpoint abrogators as anticancer drugs, Mol. Cancer Ther. (2004) 513–519.

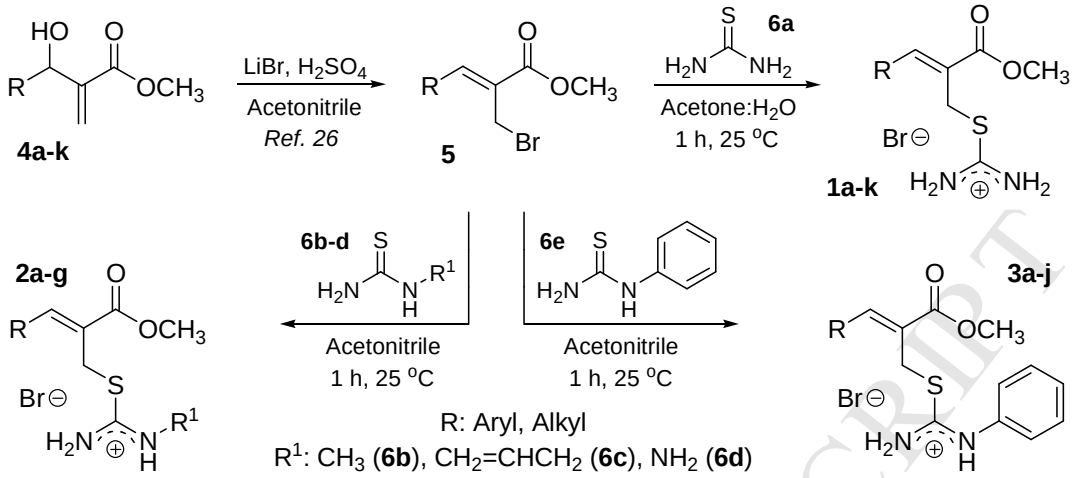
[49] V. Jurišić, V. Bumbaširević, In vitro assays for cell death determination, Arch. Oncol. 16 (2008) 49–54.

[50] L.F.R. Span, H.M. Pennings, G. Vierwinden, J.B.M. Boezeman, R.P. Raymakers, T. de Witte, The dynamic process of apoptosis analyzed by flow cytometry using Annexin-V/propidium iodide and a modified in situ end labeling technique, Cytometry 47 (2002) 24–31.

[51] I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsperger, A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, J. Immunol. Methods 184 (1995) 39–51.

[52] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.

[53] L. Yang, S. Wu, Q. Zhang, F. Liu, P. Wu, 23,24-Dihydrocucurbitacin B induces G2/M cell-cycle arrest and mitochondria-dependent apoptosis in human breast cancer cells (Bcap37), Cancer Lett. 256 (2007) 267–278.



Scheme 1. Synthesis of *S*-allylic isothiouronium salts **1-3** from allylic bromides **5** and thioureas **6**.

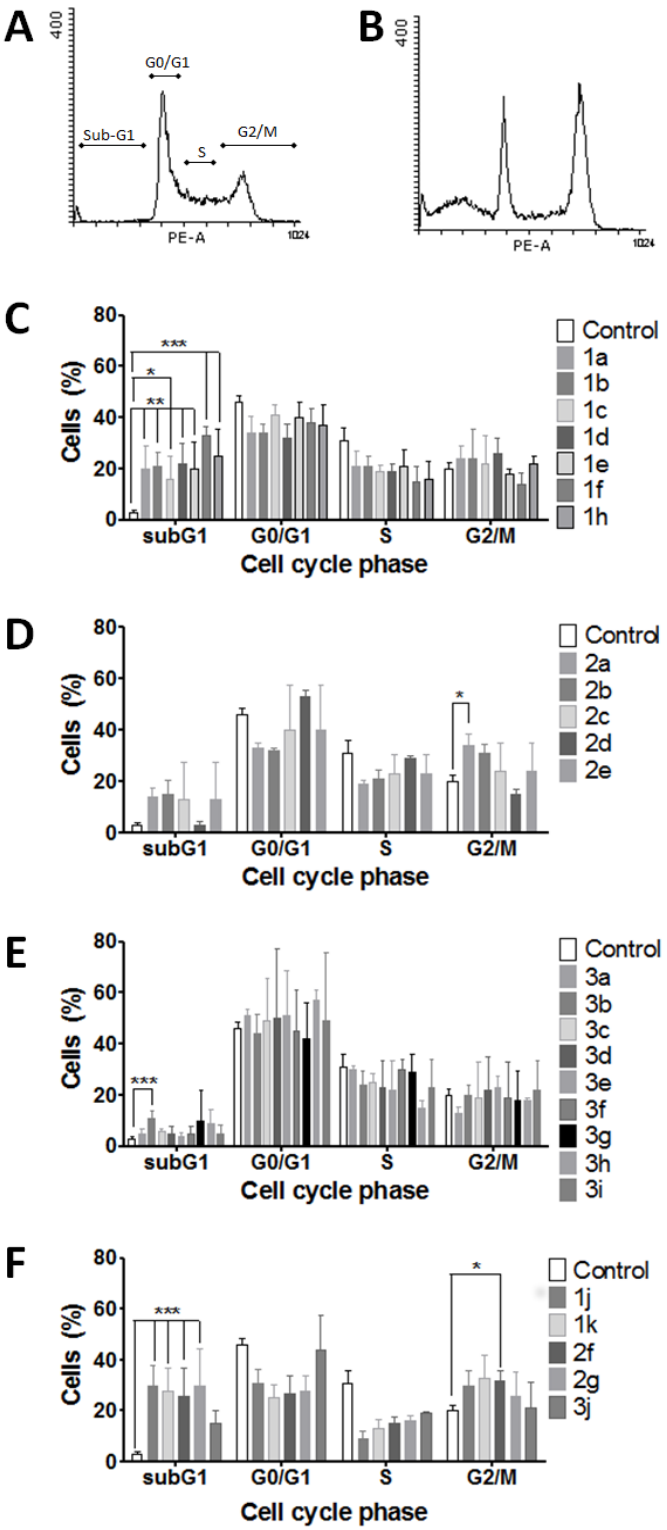


Figure 1. Flow cytometry analysis of cell cycle after 24-h exposure with IC_{50} of the tested compounds. (**A**) Representative histogram of undamaged DNA content

distribution in the cell cycle. (**B**) Representative histogram of DNA fragmentation in sub-G1 and G2/M arrest induced by treatment with compound **1k**. (**C**) Cell cycle distribution of L1210 cells exposed to *N*-unsubstituted salts, (**D** and **E**) to *N*-substituted salts, and (**F**) to aliphatic allylic salts. G0/G1, S, and G2/M represent cell cycle phases and sub-G1 indicates DNA fragmentation. Each phase was calculated using the Flowing 2.5.1 software and compared to the control group (without treatment). Data represent mean ± SD of three independent experiments. ***p<0.0001, **p<0.001, *p<0.005.

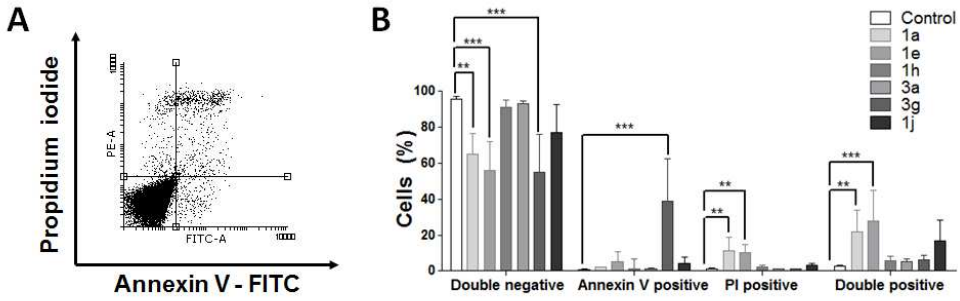


Figure 2. Cell death after 24-h exposure with IC_{50} of the tested compounds. Flow cytometry with double staining annexin V-FITC and propidium iodide. (A) Representative dotplot of L1210 cells labeled with Annexin V/FITC and PI. Alive cells are double negative for Annexin V/PI, Annexin V positive indicates phosphatidylserine externalization, PI positive indicates cell membrane disruption and double positive indicates both events at the same cell. (B) Quantification of L1210 cells labeled with Annevin V/FITC and PI after compounds exposure for 24 h. Percentage of cell death was calculated using the Flowing 2.5.1 program followed by GraphPad Prism 5 through one-way ANOVA analysis followed by Dunnet *post hoc* test. Data represent mean ± SD of four independent experiments. ***p < 0.0001, **p < 0.001.

Table 1. IC_{50} values for aryl-substituted compounds against L1210 and NIH-3T3 cell lines, selectivity index (SI) and logP values.

#	Compound	R	R^1	IC_{50} (μM)[a]		SI[b]	logP[c]
				L1210	NIH-3T3		
1	**1a**	C_6H_5	H	4 ± 0	70 ± 9	17.5	2.07
2	**1b**	4-$CH_3C_6H_4$	H	18 ± 2	45 ± 12	2.5	2.51
3	**1c**	2-$C_{10}H_7$	H	7 ± 3	11 ± 5	1.4	3.25
4	**1d**	4-FC_6H_4	H	13 ± 3	56 ± 8	4.3	2.23
5	**1e**	2-ClC_6H_4	H	16 ± 1	>100	> 6.3	2.52
6	**1f**	4-$NO_2C_6H_4$	H	12 ± 5	21± 14	1.8	2.02
7	**1g**	4-$CH_3OC_6H_4$	H	16 ± 6	73 ± 17	4.5	2.12
8	**1h**	3,4-$(OCH_2O)C_6H_3$	H	9 ± 1	92 ± 18	10.2	1.96
9	**2a**	4-$CH_3OC_6H_4$	CH_3	75 ± 2	>100	>1.3	2.50
10	**2b**	4-$CH_3OC_6H_4$	CH_2=$CHCH_2$	60 ± 1	>100	>1.7	3.14
11	**2c**	C_6H_5	NH_2	48 ±43	>100	>2.0	0.99
12	**2d**	4-$CH_3OC_6H_4$	NH_2	41 ± 17	>100	>2.4	1.05
13	**2e**	4-$NO_2C_6H_4$	NH_2	25 ± 9	>100	>4.0	0.95
14	**3a**	C_6H_5	C_6H_5	11 ± 3	>100	9.0	4.14
15	**3b**	4-$CH_3C_6H_4$	C_6H_5	120 ± 9	>100	>0.8	4.59
16	**3c**	4-FC_6H_4	C_6H_5	22 ± 9	>100	>4.5	4.30
17	**3d**	4-ClC_6H_4	C_6H_5	18 ± 2	69 ± 15	3.5	4.82
18	**3e**	2-$NO_2C_6H_4$	C_6H_5	22 ± 6	>100	>4.5	3.87
19	**3f**	3-$NO_2C_6H_4$	C_6H_5	13 ± 4	38 ± 17	2.9	4.07
20	**3g**	4-$NO_2C_6H_4$	C_6H_5	6 ± 3	76 ± 29	12.6	5.00
21	**3h**	4-$CH_3OC_6H_4$	C_6H_5	19 ± 8	61 ± 2	3.2	4.19
22	**3i**	3,4-$(OCH_2O)C_6H_3$	C_6H_5	79 ± 9	>100	>1.3	4.03
23	**Mitoxantrone**	-	-	15 ± 5	42 ± 8	2.8	0.36

[a] Data were expressed as mean ± SD (n = 3).

[b] Defined as the ratio between IC_{50} of the non-tumoral cell and tumoral cell.

[c] Reference [35].

[d] NS = non-significant.

Table 2. IC_{50} values for alkyl-substituted compounds against L1210 and NIH-3T3 cell lines, selectivity index (SI) and logP values.[a]

#	Compound	R	R^1	IC_{50} (μM)		SI	logP
				L1210	NIH-3T3		
1	**1i**	CH_3CH_2	H	3 ± 1	71 ± 26	23.7	1.11
2	**1j**	CH_3	H	5 ± 3	>100	>20.0	0.61
3	**1k**	$CH_3CH_2CH_2$	H	14 ± 2	75 ±4	5.3	1.91
4	**2f**	CH_3CH_2	CH_3	20 ± 1	>100	>5.0	1.49
5	**2g**	CH_3CH_2	CH_2=$CHCH_2$	15 ± 1	35 ± 1	2.3	2.13
6	**3j**	CH_3CH_2	C_6H_5	3 ± 1	8 ± 4	2.8	3.18

[a] See Table 1 for details.

Allylic isothiouronium salts: the discovery of a novel class of thiourea analogues with antitumor activity

Misael Ferreira[a], Laura Sartori Assunção[b], Adny Henrique Silva[c], Fabíola Branco Filippin-Monteiro[d], Tânia Beatriz Creczynski-Pasa[b], Marcus Mandolesi Sá[a,*]

[a] *Departamento de Química, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

[b] *Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

[c] *Departamento de Bioquímica, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

[d] *Departamento de Análises Clínicas, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil*

Highlights

→ Isothiouronium salts are readily prepared from simple reagents under mild conditions

→ Isothiouronium salts possess selective antitumor activity against leukemia cells

→ Alkyl-substituted derivatives are more potent than the aryl ones

→ High selectivity index for leukemia cells was achieved in some cases

→ The mechanism of action underlying allylic isothiouronium salts is apoptosis