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FULL PAPER

Synthesis and Cytotoxic Activity of Triterpenoid Thiazoles Derived from Allobetulin, Methyl Betulonate, Methyl Oleanonate, and Oleanonic Acid

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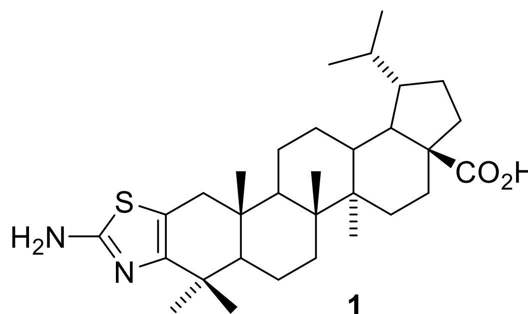
Supporting information for this article is given via a link at the end of the document. It contains experimental data for methyl betulonate derivatives **3b – 3l**, methyl oleanonate derivatives **4b – 4l**, and oleanonic acid derivatives **5b – 5l**, and pictures of ^{1}H and ^{13}C NMR spectra of all new compounds.

Abstract: 41 New triterpenoids were prepared from allobetulon, methyl betulonate, methyl oleanonate, and oleanonic acid to study their influence on cancer cells. Each 3-oxo triterpene was brominated at C-2, substituted with thiocyanate; following cyclization with appropriate ammonium salts gave N-substituted thiazoles. All compounds were tested for their in vitro cytotoxic activity on eight cancer cell lines and two non-cancer fibroblasts. 2-Bromoallobetulon (**2b**) methyl 2-bromobetulonate (**3b**), 2-bromooleanonic acid (**5b**), and 2-thiocyanooleanonic acid (**5c**) were best with IC_{50} < 10 μM to CCRF-CEM cells (e.g. **3b**, IC_{50} =2.9 μM) as well as 2'-(diethylamino)olean-12(13)-eno[2,3-*d*]thiazole-28-oic acid (**5f**, IC_{50} 9.7 μM) and 2'-(N-methylpiperazino)olean-12(13)-eno[2,3-*d*]thiazole-28-oic acid (**5k**, IC_{50} 11.4 μM). Compound **5c** leads to accumulation of cells in the G2 phase of the cell cycle and inhibits RNA/DNA synthesis significantly at 1 × IC_{50}. The G2/M cell cycle arrest probably corresponds to the DNA/RNA synthesis inhibition, similarly to actinomycin D. Compound **5c** is new, active, non-toxic, therefore it is the most promising for future drug development. Methyl 2-bromobetulonate (**3b**) and methyl 2-thiocyanometulonate (**3c**) inhibited nucleic acid synthesis only at 5 × IC_{50}. We assume that in **3b** and **3c** (unlike in **5c**) DNA/RNA inhibition is a non-specific event and unknown primary cytotoxic target is activated at 1 × IC_{50} or lower concentration.

Introduction

Pentacyclic triterpenes are natural compounds that may be found in almost all living organisms, they are particularly common in plants. They often have many interesting biological activities such as anti-inflammatory,[1,2] antibacterial,[3,4] antiviral,[5,6] antiprotozoal,[7,8] cytotoxic, [9,10] and anti-hypoglycemic.[11,12] However, in many cases the effective concentrations of most of the natural triterpenoids are not low enough for their therapeutic use. In addition, their solubility in water is low, which causes insufficient bioavailability. As a result, it is necessary to focus on the preparation of triterpenoid derivatives with higher activity, improved pharmacological properties, and more favorable therapeutic index.[13]



Recently, many cytotoxic derivatives were found among triterpenes that contain a heterocycle fused to the A-ring of the triterpenoid skeleton. The most active compounds were indoles,[14,15] pyrazoles,[14,16-22] isoxazoles,[23,24] triazoles,[25] pyrazines,[14,20,26-30] quinoxalines,[14] pyrimidines,[23] and triazines.[31,32] Previously, we synthesised a set of triterpenes modified with five membered heterocycles and among them aminothiazole derivative **1** was the most active on multiple cancer cell lines,[19] which sparked our interest in such compounds.

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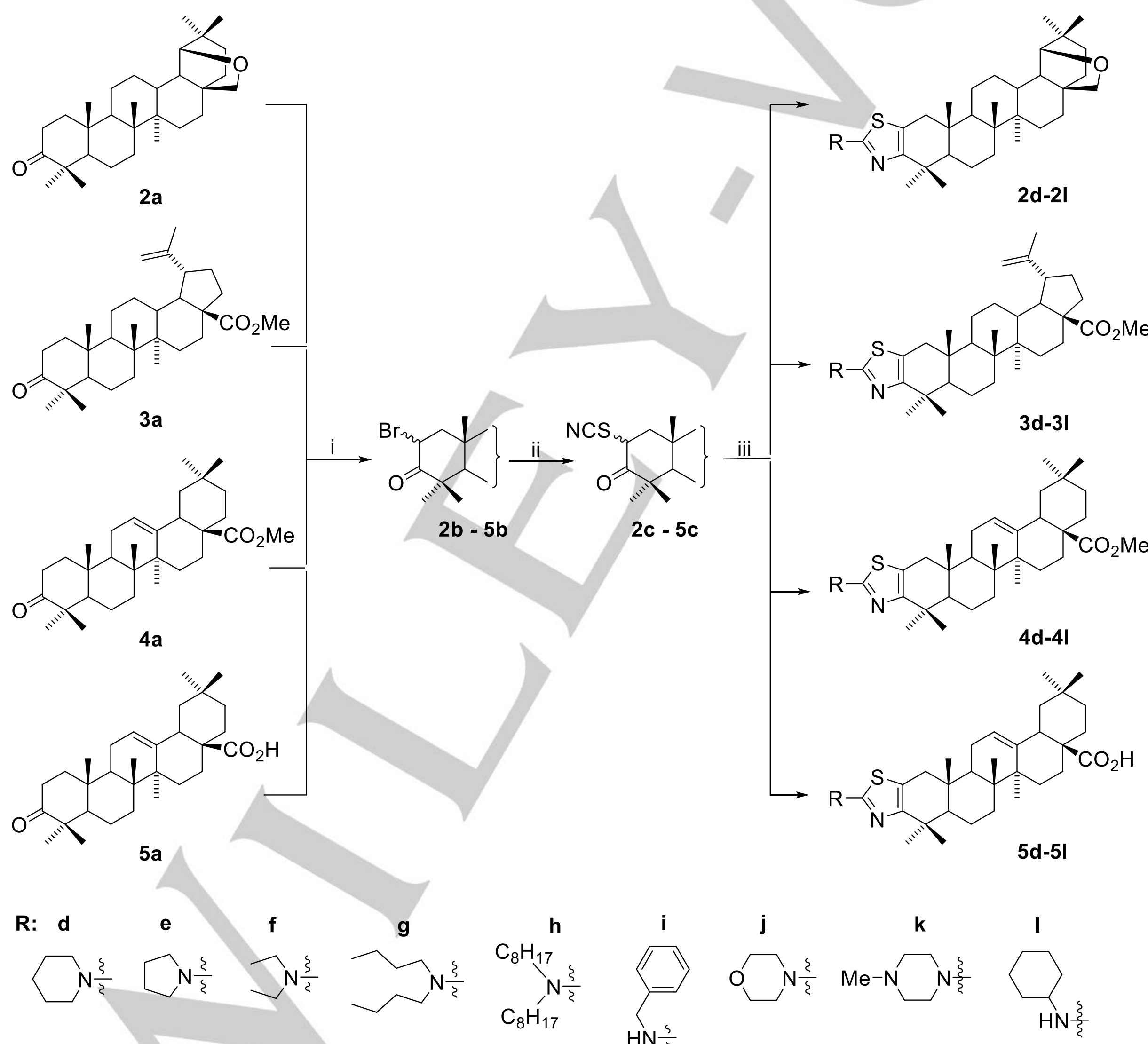
We also reported the synthesis of cytotoxic triterpenoids conjugated to biotin which would allow us to search for their molecular targets which may afford more information about the mechanism of action.[33] Consequently, we have seen hints in ongoing pull down assays that many of the potential targets are enzymes dealing with lipophilic substrates or membrane proteins that contain significant lipophilic surface areas. In order to improve the ligand-target binding, we decided to add a variety of rather lipophilic substituent(s) onto the polar amino group in triterpenoid aminothiazoles similar to **1**. To further explore the structure-activity relationships (SARs) among substituted triterpenoid aminothiazoles, we choose to add substituents of various size and shape to the NH_2 group. In order to get more data about the influence of the terpenic part of the molecule on the cytotoxic activity, we prepared four analogous sets of *N*-substituted aminothiazoles from four different commercially available triterpenes, allobetulon (**2a**), methyl betulonate (**3a**), methyl oleanonate (**4a**), and oleanonic acid (**5a**). Compounds **2a**, **3a**, **4a**, and **5a** are derivatives of triterpenes betulinic acid, oleanolic acid, and 18α-oleanane, derivatives of which are commonly studied for their high cytotoxic activities.[34-37] Comparing activities in derivatives prepared from methyl oleanonate (**4a**) and from free oleanonic acid (**5a**) would allow us to estimate, if methyl esters are less active than free acids, as it is usual in triterpenoids.[20,27,38] The classical Hantzsch synthesis of aminothiazoles does not give good yields in the case of triterpenoids and steroids because of harsh reaction conditions[39,40] and the reaction does not allow to simply obtain N-substituted compounds. Therefore, we used an alternative approach[41-43] involving the reaction of 3-oxo-2-thiocyanotriterpenoids with various alkyl ammonium acetates. This method allowed us to obtain a variety of *N*-substituted triterpenoid aminothiazoles and was used the first time to prepare terpenoid aminothiazoles.



Scheme 1. Reagents and conditions: (i) $CuBr_2$, $CHCl_3$, EtOAc, MeOH, r.t., 18 h; (ii) NH_4SCN, N-methylpyrrolidone, 50 °C, 4 h; (iii) piperidinium acetate (for **d**), pyrrolidinium acetate (for **e**), diethylammonium acetate (for **f**), dibutylammonium acetate (for **g**), dioctylammonium acetate (for **h**), benzylammonium acetate (for **i**), morpholinium acetate (for **j**), N-methylpiperazinium acetate (for **k**), or cyclohexylammonium acetate (for **l**), $CHCl_3$, r.t., 24 h.

Results and Discussion

Chemistry

In our work,[19] the aminothiazole derivative **1** was obtained in rather low yield which was probably caused by a presence of the

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free 28-carboxyl group which is lowering the solubility and causes problems during purification. Allobetulon (**2a**), methyl betulonate (**3a**), and methyl oleanonate (**4a**) do not contain carboxyl group, therefore we expected simple purification and higher yields. The starting 3-oxotriterpenoids **2a**, **3a**, **4a**, and **5a** were brominated by $CuBr_2$ in a mixture of $CHCl_3$, EtOAc, and MeOH at r.t. according to the known procedure.[23,44-48] The crude 2-bromo-3-oxoderivatives **2b**, **3b**, **4b**, and **5b** were used without further purification for the synthesis of 3-oxo-2-thiocyano derivatives **2c**, **3c**, **4c**, and **5c** by the nucleophilic substitution of bromide by the ammonium thiocyanate in *N*-methylpyrrolidone at 50 °C. 2-Thiocyano-3-oxo derivatives **2c**, **3c**, **4c**, and **5c** were obtained as mixtures of $2\alpha/2\beta$ epimers, which were not separated because the final cyclization and aromatization of each epimer leads to the same flat, aromatic system (Scheme 1). Thiazoles **2d** – **2l**, **3d** – **3l**, **4d** – **4l** and **5d** – **5l** were prepared by a procedure[23] in which each thiocyanate was stirred with five equivalents of the corresponding alkyl ammonium acetate at r.t. for various periods of time. The yields of cyclization were usually moderate to high depending on the solubility of the starting terpene in organic solvents, which influenced the work-up procedures and chromatography.

Biological assays - cytotoxicity

The cytotoxic activity of all synthesized compounds was investigated in vitro against eight human cancer cell lines and two non-tumor fibroblasts by using the standard MTS test (Table 1). The cancer cell lines were derived from T-lymphoblastic leukemia CCRF-CEM, leukemia K562 and their multiresistant counterparts (CEM-DNR, K562-TAX), solid tumors including lung (A549) and colon (HCT116, HCT116p53-/-) carcinomas, osteosarcoma cell line (U2OS), and for comparison, on two human non-cancer fibroblast lines (BJ, MRC-5). In general, the CCRF-CEM line was the most sensitive cancer cell line to the prepared compounds with only a few exceptions. Therefore SARs assumption were mostly based on the activities in CCRF-CEM cells.

Table 1. Cytotoxic activities of selected compounds on eight tumor (including resistant) and two normal fibroblast cell lines.[a]

	IC$_{50}$ (μM)[b]										
Comp.	CCRF-CEM	CEM-DNR	HCT116	HCT116 p53$^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	TI[c]
1	3.5	11.2	5.1	4.3	4.8	6.9	7.0	-	24.9	15.7	5.8
2a[d]	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	-	-
3a[d]	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	-	-
4a[d]	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	-	-
5a[d]	15.1	17.4	45.1	>50.0	>50.0	21.5	49.5	48.5	>50.0	>50.0	5.3
2b	**5.2**	>50.0	21.5	22.8	11.7	**7.5**	>50.0	47.5	>50.0	>50.0	>9.6
2c	43.0	>50.0	35.6	>50.0	41.1	>50.0	>50.0	>50.0	>50.0	>50.0	>1.2
2l	42.8	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.2
3b	**2.9**	15.0	14.9	12.4	**9.0**	11.0	>50.0	13.5	22.9	26.3	8.5
3c	10.8	>50.0	34.6	29.9	15.5	>50.0	46.2	>50.0	32.3	36.1	3.2
4b	14.0	22.2	>50.0	>50.0	19.9	**7.2**	>50.0	>50.0	>50.0	48.2	>3.5
4c	29.0	48.5	>50.0	38.5	46.9	>50.0	>50.0	47.8	>50.0	45.7	>1.7
4f	41.8	42.6	44.9	43.6	42.6	42.3	44.2	43.2	>50.0	42.4	>1.1
5b	**4.5**	14.8	**4.1**	**8.2**	**2.1**	13.5	15.8	29.3	27.7	20.0	5.0
5c	**6.4**	31.1	23.6	16.3	28.6	>50.0	21.7	46.1	>50.0	48.0	>7.7
5f	**9.7**	>50.0	>50.0	48.7	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>5.2
5i	23.5	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>2.1
5j	25.6	48.5	>50.0	21.1	46.1	>50.0	>50.0	48.7	>50.0	>50.0	>2.0
5k	11.4	31.1	>50.0	>50.0	>50.0	14.4	>50.0	>50.0	>50.0	>50.0	>4.4
5l	42.8	>50.0	>50.0	>50.0	>50.0	14.3	>50.0	>50.0	>50.0	>50.0	>1.2

[a]All other compounds prepared in this work were also tested but their activities on these 10 cell lines were higher than 50 μM and may be found in Supporting data file. [b]The lowest concentration that kills 50 % of cells. The standard deviation in cytotoxicity assays is typically up to 15 % of the average value. [c]Therapeutic index is calculated for IC$_{50}$ of CCRF-CEM line vs average of both fibroblasts (BJ and MRC-5). [d]Parent compounds used as standards. Compounds with IC$_{50}$ > 50 μM are considered inactive.

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Among the starting material and intermediates, 2-bromo-3-oxoderivatives **2b**, **3b**, and **5b** were cytotoxic against the CCRF-CEM line in a low micromolar range of 3-5 μM. This is surprising because compound **2b** is derivative of allobetulon **2a**, analogues of which are often inactive.[27,33] In addition, the active compound **3b** is a methyl ester and triterpenic methyl esters are also usually not active.[27,33] Even bromo derivative **4b**, also a methyl ester, had a moderate IC_{50} of 14 μM. 2-Thiocyano derivatives **3c** and **5c** had

Table 2. Influence of **2b**, **3b**, **3c**, **5b**, **5c**, and **5f** on cell cycle and DNA and RNA synthesis inhibition at 1 × and 5 × IC_{50}.

	Used conc. (μM)	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)	$pH3^{Ser10}$ (%)	DNA synthesis	RNA synthesis
Control		3.38	41.63	44.18	14.19	1.62	58.76	48.80
2b	5.2[a]	5.84	44.78	40.95	14.27	0.99	52.18	41.37
2b	21.2[b]	43.57	44.30	37.92	17.77	1.74	37.24	31.77
3b	2.9[a]	7.76	40.23	38.24	21.53	2.79	25.98	49.31
3b	14.5[b]	32.49	45.58	31.06	23.35	2.44	**13.34**	**1.37**
3c	10.8[a]	3.42	36.14	47.92	15.93	2.09	41.05	47.46
3c	54.0[b]	58.50	30.90	41.29	27.81	1.02	**13.82**	**0.97**
5b	4.5[a]	5.96	42.73	42.51	14.77	1.11	61.70	53.54
5b	22.5[b]	57.69	45.40	39.88	14.71	1.55	36.20	24.60
5c	6.4[a]	41.55	56.86	22.77	**20.37**	1.11	**10.52**	**5.44**
5c	32.0[b]	83.11	34.90	25.32	**39.78**	0.26	**2.86**	**0.64**
5f	9.7[a]	4.27	36.59	48.14	15.27	1.18	40.16	67.11
5f	48.5[b]	7.79	40.35	49.43	10.22	1.87	32.95	58.95

[a]The values were obtained at 1 × IC_{50}. [b]The values were obtained at 5 × IC_{50}. Control are cells treated with vehicle.

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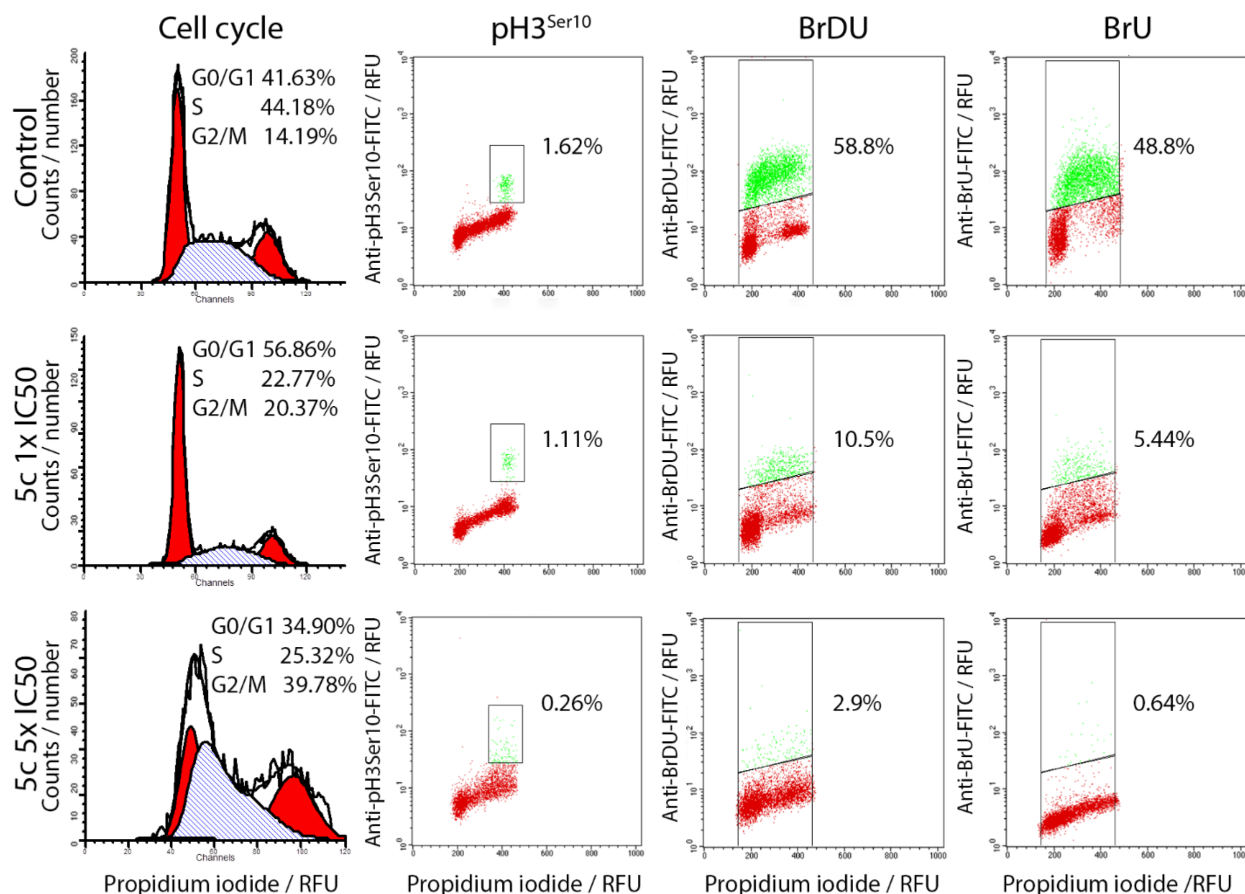

Figure 1. Graphs and dot plots of flow cytometry analysis are showing the cell cycle inhibition in G2/M phase and almost complete DNA, RNA synthesis inhibition by the best compound **5c**, monitored by incorporation of BrDU/BrU into the DNA.

IC_{50} values of 6 – 10 µM. In contrast, compounds **2c** and **4c** had IC_{50} values in higher micromolar ranges (Table 1). The cytotoxicity of most of the substituted aminothiazoles was below the detection limit with two exceptions, **5f** and **5k** with cytotoxicity of 9.7 and 11.3 µM (CCRF-CEM), respectively, which we consider enough active to be interesting for further studies towards the mechanism of action. The activity of **2l**, **4f**, **5i**, **5j** and **5l** was higher than the detection limit, however, not sufficient for more tests. The activity of almost all derivatives with IC_{50} < 50 µM on the resistant cell lines CEM-DNR and K562-TAX is worse in comparison to parental cell lines CCRF-CEM and K562 and indicates the possible mechanism of resistance by MDR transporter proteins.
To sum up, within this study, 8 compounds (**2b**, **3b**, **3c**, **4b**, **5b**, **5c**, **5f**, **5k**) had higher cytotoxic activity than their parent compounds **2a**, **3a**, **4a**, and **5a**. This is significant improvement. Six compounds (**2b**, **3b**, **3c**, **5b**, **5c**, **5f)** with IC_{50} < 10 µM were further investigated for their mechanisms of action and therefore the analysis of apoptosis, cell cycle, DNA and RNA synthesis in CCRF-CEM cells was done.

Biological assays – analysis of apoptosis, cell cycle, and DNA/RNA synthesis

All of those promising compounds with an IC_{50} below or around 10 µM in CCRF-CEM cell line (**2b**, **3b**, **3c**, **5b**, **5c**, **5f**) were further investigated for their mechanisms of action and therefore the analysis of apoptosis, cell cycle, DNA and RNA synthesis in CCRF-CEM cells at 1 × or 5 × IC_{50} was done (Table 2). These concentrations were used in concordance with our previous publications.[49-51] Interestingly, highly active compound **5c** led to the accumulation of cells in G2 phase of the cell cycle and had no toxicity in non-cancer fibroblasts. Nucleic acid synthesis monitored by BrU/BrDU incorporation was almost completely inhibited by **5c** at both tested concentrations, pointing to a possible mechanism of action (Figure 1) somewhere within the regulatory mechanisms of cell cycle proliferation. Therefore, **5c** is the most promising candidate for further cell biology studies and drug development. Similar phenomenon (inhibition of DNA/RNA synthesis) but at higher concentrations (5 × IC_{50}) was detected for compounds **3b** and **3c**. However, in this case the interpretation of why the DNA/RNA synthesis is inhibited at higher concentration

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than the IC_{50} is more difficult. It can, for example, be simply a secondary effect of the cytotoxicity in general or a result of other processes in dying cells. Other tested derivatives had no significant effect on the cell cycle and DNA/RNA synthesis.

Conclusions

We have described the synthesis and biological activities of 41 new triterpenoid derivatives. From known 3-oxocompounds **2a**, **3a**, **4a**, and **5a**, we prepared 2-bromo-3-oxocompounds, then 2-thiocyano-3-oxocompounds and finally, the cyclization with alkyl ammonium acetates afforded N-substituted aminothiazoles. We used nine different types of substituents at the amino group to be able to evaluate their influence on cytotoxicity. Substituents were chosen rather lipophilic, because we expected to improve interactions of the terpenes with lipophilic areas on potential protein targets. Although the majority of the aminothiazoles were active in high micromolar ranges, there were two exceptions, **5f** and **5k**, with cytotoxicity around 10 μM. Cells treated with **5f** and **5k** were used for cell cycle and DNA/RNA synthesis analysis. However, no significant effect on the cell cycle and nucleic acid synthesis was detected. Both compounds are derivatives of oleanonic acid with free 28-carboxy groups. This suggests that future research on aminothiazoles should be more oriented towards free terpenic acids but such studies should also take problems with isolation of the products, yields, and solubility into account. Of the substituents at the thiazole part, diethylamino and *N*-methylpiperazino substituents were the best. Within this study, however, the most active compounds were the intermediate 2-bromo-3-oxo derivatives **2b**, **3b**, **5b**, and thiocyanates **3c**, and **5c** and studies to better understand the mechanism of action were also performed with them. Thiocyanates **3c** and **5c** were identified to modulate the cell cycle and it is highly possible that observed S phase alteration and G2/M cell cycle arrest is a response to the DNA/RNA inhibition which in the case of compound **5c** is significant already at 1 × IC_{50}. Similar response of the cells was observed when cancer cells were treated with low doses of actinomycin D which is commonly used antineoplastic drug and well-know inhibitor of RNA/DNA synthesis.[52] We can speculate if such mechanism is due to the direct interference with some specific targets like cyclin-dependent kinases, RNA/DNA polymerases[53] or in general it is a dose depentent effect of the drug which is atenuating nucleic acid synthesis and therefore abrogates check point signaling. Further biological studies will be needed to answer such questions. Since **5c** has also no toxicity on non-cancer fibroblasts, it is the most promising candidate for further drug development.

Experimental Section

General experimental procedures
Materials and instruments
Melting points were determined using a Büchi B-545 apparatus and are uncorrected. Optical rotations were measured on an Autopol III (Rudolph Research, Flanders, USA) polarimeter in MeOH at 25 °C and are in [10^{-1} deg cm^2 g^{-1}]. IR spectra were recorded on a Nicolet Avatar 370 FTIR. DRIFT stands for Diffuse Reflectance Infrared Fourier Transform. ^1H and ^{13}C NMR spectra were recorded on Varian^{UNITY} Inova 400 (400 MHz for ^1H) or Varian^{UNITY} Inova 300 (300 MHz for ^1H) or Jeol ECX-500SS (500 MHz for ^1H) instruments, using $CDCl_3$, D_6-DMSO or CD_3OD as solvents (25°C). Chemical shifts were eider referenced to the residual signal of the solvent ($CDCl_3$, D_6-DMSO) or to TMS added as an internal standard. ^{13}C NMR spectra were eider referenced to $CDCl_3$ (77.00 ppm) or D_6-DMSO (39.51 ppm) or to TMS added as an internal standard. EI-MS spectra were recorded on an INCOS 50 (Finnigan MAT) spectrometer at 70 eV and an ion source temperature of 150 °C. The samples were introduced from a direct exposure probe at a heating rate of 10 mA/s. Relative abundances stated are related to the most abundant ion in the region of $m/z > 180$. HRMS analysis was performed using LC-MS an Orbitrap high-resolution mass spectrometer (Dionex Ultimate 3000, Thermo Exactive plus, MA, USA) operating at positive full scan mode in the range of 100–1000 m/z. The settings for electrospray ionization were as follows: oven temperature of 150 °C, source voltage of 3.6 kV. The acquired data were internally calibrated with phthalate as a contaminant in methanol (m/z 297.15909). Samples were diluted to a final concentration of 0.1 mg/mL in methanol. The samples were injected to mass spectrometer over autosampler after HPLC separation: precolumn phenomenex 2.6μm C18. Mobile phase isokrat MeCN/IPA/ammonium acetate 0.01 M 80/10/10, flow 0,3 mL/min and MeCN/IPA/water/HCOOH 80/10/10/0.1 for compounds **3g**, **3h**, **4g**, and **4h**. TLC was carried out on Kieselgel 60 F_{254} plates (Merck) detected by spraying with 10% aqueous H_2SO_4 and heating to 150 – 200 °C. Column chromatography was performed using silica gel 60 (Merck 7734). Work-up refers to pouring the reaction mixture into water, extracting the product into organic solvent, washing the organic layer successively in this order with water, diluted aqueous HCl, water, saturated aqueous sodium hydrogencarbonate, and again water. Then the organic phase was dried over magnesium sulfate, filtered, and the solvents evaporated under reduced pressure. Analytical samples were dried over P_2O_5 under diminished pressure. Allobetulon (**2a**), methyl betulonate (**3a**), methyl oleanonate (**4a**) and oleanonic acid (**5a**) were obtained from company Betulinines (www.betulinines.com), which manufactures them from betulin, betulinic acid and oleanolic acid in bulk scale. Scheme and references about their preparation were added to the supporting data file. All other chemicals and solvents were obtained from Sigma-Aldrich.

Synthetic procedures
General procedure for synthesis of 2-bromoderivatives
Each starting oxoderivative **2a**, **3a**, **4a**, and **5a** (23 mmol) was dissolved in a mixture of solvents (chloroform, ethylacetate and methanol, ratio is at each experiment). Anhydrous $CuBr_2$ (50 mmol) was added, and reaction mixture was stirred for 18 hours at r.t. until the starting material was fully consumed, (controlled by TLC in toluene/diethylether 5:1 v/v). Then, the precipitate of copper(I) bromide was filtered off, organic solvents were washed with water, dried over $MgSO_4$, and crude product was purified by chromatography on silica gel (mobile phase at each experiment). Evaporation of solvents gave each 2-bromoderivative as a mixture of 2α/2β epimeres.

General procedure for synthesis of 2-thiocyano derivatives
Each bromo derivative **2b**, **3b**, **4b**, and **5b** (19 mmol) was dissolved in *N*-methylpyrrolidone (100 mL). Ammonium

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thiocyanate (96 mmol) was added, and reaction mixture was stirred for 4 hours at 50 °C until the reaction was finished (controlled by TLC, mobile phase at each experiment). Then, water was added, resulting solid precipitate was filtered off and organic phase was washed with water. Crude product was purified by chromatography on silica gel (mobile phase at each experiment), which provided each 2-thiocyano derivative as a mixture of 2α/2β epimeres.

General procedure for synthesis of triterpenoid thiazole-derivatives

Alkylammonium acetate of each corresponding amine (5 mmol) was added to the solution of 2-thiocyano derivative (1 mmol) in chloroform (20 mL). Reaction mixture was left for 24 hours at r.t., monitored by TLC (mobile phase at each experiment). Reaction mixture was then washed with water several times, organic phase was dried with $MgSO_4$ and the solvents were removed under vacuo. Crude product was purified by chromatography on silica gel (mobile phase at each experiment) unless otherwise stated at the experiment. Collected fractions were evaporated to give respective thiazole-derivatives.

2α/β-bromoallobetulone (**2b**)

Epimeric mixture of 2-bromoallobetulons **2b** was prepared according to the general procedure from allobetulon **2a** (10 g, 22.7 mmol) and $CuBr_2$ (11.2 g, 50 mmol) in mixture of chloroform (50 mL), ethyl acetate (150 mL) and methanol (20 mL). After standard work up without further purification compound **2b** was obtained as white crystals (11.3 g, 96%): R_f=0.50 (toluene/diethylether 20:1); mp: 222–224 °C (hexane/MeOH); $[\alpha]_D^{20}$=+68 (*c*=0.55 in $CHCl_3$); (lit.[44,51] mp: 216–225 °C; $[\alpha]_D^{20}$=+74).

2α/β-thiocyanoallobetulone (**2c**)

Epimeric mixture of **2c** was prepared according to the general procedure from compound **2b** (10 g; 19.2 mmol) and ammonium thiocyanate (7.3 g; 96 mmol) in *N*-methylpyrrolidone (100 mL) after 4 hours at 50 °C, controlled by TLC (mobile phase toluene/diethylether 5:1). After standard work up and purification (gradient elution: toluene → toluene/diethylether 5:1) compound **2c** was obtained as a mixture of epimers in the ratio 0.3:0.7 determined by the intensity of signals in NMR, white microcrystals (7.3 g; 76%): R_f=0.20 (toluene/diethylether 5:1); mp: 150–156 °C (toluene/diethylether); $[\alpha]_D^{20}$=+5 (*c*=1.5 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ=0.81, 0.82, 0.91, 0.95, 0.96, 0.97, 1.05, 1.12, 1.13, 1.15, 1.17, 1.20 (all s, 21H, 7×CH_3 from both epimers), 2.02 (dd, J_1 = 13.5 Hz, J_2 = 8.3 Hz, 0.7H, H-1a from one epimer), 2.52 (t, *J* = 12.9 Hz, 0.7H, H-1b from one epimer), 2.75 (dd, J_1 = 12.9 Hz, J_2 = 6.0 Hz, 0.3H, H-1b from the other epimer), 3.46 (d, *J* = 7.7 Hz, 1H, H-28a from both epimers), 3.54 (s, 1H, H-19 from both epimers), 3.78 (d, *J* = 7.5 Hz, 1H, H-28b from both epimers), 4.71 – 4.76 ppm (m, 1H, H-2 from both epimers); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.39, 15.02, 15.85, 16.43, 18.81, 18.98, 19.56, 19.85, 21.22, 21.31, 22.07, 24.50, 24.52, 24.76, 26.08, 26.14, 26.17, 26.26, 26.36, 26.38, 28.74, 29.14, 32.24, 32.66, 33.46, 34.02, 34.36, 36.24, 36.68, 38.53, 39.16, 40.45, 40.79, 40.82, 40.92, 41.40, 41.43, 46.67, 46.72, 47.34, 49.51, 49.53, 50.24, 50.68, 51.05, 52.23, 52.35, 53.98, 57.41, 71.18, 71.20, 87.87, 87.88, 112.21, 112.46, 209.35, 211.62 ppm; IR (film): ν=2930, 2860, 2152 (SCN), 1700 (C=O), 1457(-O-), 1033 cm^{-1}; MS (ESI) m/z (%): 498.4 (100) [M+H]$^+$; Anal. calcd for $C_{31}H_{47}NO_2S$: C 74.80, H 9.52, S 6.44, N 2.81; found C 74.80, H 9.64, S 6.02, N 2.57.

Thiazole derivative **2d**

Compound **2d** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and piperidinium acetate (732 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtred through a short pad of silica gel. Evaporation of solvent furnished **2d** (536 mg; 95 %); R_f=0.35 (toluene/diethylether 5:1); mp: 152–155 °C (toluene/diethylether); $[\alpha]_D^{20}$=+50 (*c*=0.79 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.91 (s, 3H), 0.94 (s, 6H), 1.03 (s, 3H), 1.13 (s, 3H), 1.23 (s, 3H, 7×CH_3), 1.58 – 1.73 (m, 6H, H-piperidine), 2.15 (d, *J* = 15.2 Hz, 1H, H-1a), 2.61 (d, *J* = 14.9 Hz, 1H, H-1b), 3.30 – 3.43 (m, 4H, H-piperidine), 3.46 (d, *J* = 7.9 Hz, 1H, H-28a), 3.55 (s, 1H, H-19), 3.80 ppm (d, *J* = 7.6 Hz, 1H, H-28b); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.49, 15.41, 16.51, 19.51, 21.45, 21.91, 24.25, 24.55, 25.15, 26.22, 26.45, 28.79, 29.68, 30.24, 32.68, 33.03, 34.25, 36.25, 36.70, 37.13, 38.56, 39.28, 40.59, 40.70, 41.48, 46.75, 49.41, 49.66, 52.92, 71.25, 87.88, 112.25, 143.65, 153.22, 166.55 ppm; IR (film): ν=2935, 2858, 1553 (thiazole), 1524 (thiazole), 1415 (thiazole), 1035 cm^{-1}; MS (ESI) m/z (%): 565.4 (100) [M+H]$^+$; Anal. calcd for $C_{36}H_{56}N_2OS$: C 76.54, H 9.99, N 4.96, S 5.68; found C 76.70, H 9.81, N 4.42, S 5.44.

Thiazole derivative **2e**

Compound **2e** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and pyrrolidinium acetate (655 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtred through a short pad of silica gel. Evaporation of solvent furnished **2e** (501 mg; 91 %): R_f=0.20 (toluene/diethylether 5:1); mp: 151–155 °C (toluene/diethylether); $[\alpha]_D^{20}$=+58 (*c*=1.26 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.82 (s, 3H), 0.92 (s, 3H), 0.95 (s, 6H), 1.03 (s, 3H), 1.17 (s, 3H), 1.27 (s, 3H, 7×CH_3), 1.96 – 2.04 (m, 4H, H-pyrrolidine), 2.16 (d, *J* = 15.2 Hz, 1H, H-1a), 2.61 (d, *J* = 15.3 Hz, 1H, H-1b), 3.36 – 3.51 (m, 5H, H-28a, H-pyrrolidine), 3.56 (s, 1H, H-19), 3.81 ppm (d, *J* = 7.9 Hz, H-28b); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.50, 15.42, 16.49, 19.52, 21.45, 21.84, 24.55, 25.65, 26.22, 26.46, 28.80, 30.22, 32.69, 33.03, 34.26, 36.26, 36.71, 37.22, 38.57, 39.41, 40.59, 40.70, 41.48, 46.75, 49.66, 53.00, 71.25, 87. 89, 113.01, 143.31, 153.14, 164.67; IR (film): ν=2944, 2865, 1546 (thiazole), 1453 (thiazole), 1034 cm^{-1}; MS (ESI): m/z (%) = 551.4 (100) [M+H]$^+$; Anal. calcd for $C_{35}H_{54}N_2OS$: C 76.31, H 9.88, N 5.09, S 5.82; found C 76.53, H 9.91, N 4.12, S 5.64.

Thiazole derivative **2f**

Compound **2f** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and diethylammonium acetate (665 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtred through a short pad of silica gel. Evaporation of solvent furnished **2f** (520 mg; 94 %): R_f=0.35 (toluene/diethylether 5:1); mp: 152–156 °C (toluene/diethylether); $[\alpha]_D^{20}$=+57 (*c*=0.43 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.93 (s, 3H), 0.94 (s, 6H), 1.03 (s, 3H), 1.12 (s, 3H), 1.23 (s, 3H, 7×CH_3), 1.20 (t, *J* = 7.0 Hz, 6H, ($\underline{CH_3}CH_2)_2$N), 2.14 (d, *J* = 15.3 Hz, 1H, H-1a), 2.59 (d, *J* = 15.0 Hz, 1H, H-1b), 3.35 – 3.47 (m, 5H, H-28a, ($CH_3\underline{CH_2})_2$N), 3.56 (s, 1H, H-19), 3.81 ppm (d, *J* = 7.6 Hz, 1H, H-28b); ^{13}C NMR (75

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MHz, $CDCl_3$): δ=12.72, 13.51, 15.44, 16.56, 19.55, 21.44, 21.92, 24.55, 26.23, 26.47, 28.80, 30.25, 32.70, 33.96, 34.26, 36.26, 36.72, 37.14, 38.61, 39.31, 40.60, 40.70, 41.48, 44.98, 46.77, 49.69, 52.93, 71.27, 87.90, 112.48, 143.17, 153.11, 166.19 ppm; IR (film): v=2925, 2865, 1535 (thiazole), 1468 (thiazole), 1035 cm^{-1}; MS (ESI) m/z (%): 553.4 (100) $[M+H]^+$; Anal. calcd for $C_{35}H_{56}N_2OS$: C 76.03, H 10.21, N 5.07, S 5.80; found C 75.54, H 10.36, N 4.64, S 5.57.

Thiazole derivative **2g**

Compound **2g** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and dibutylammonium acetate (947 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtered through a short pad of silica gel. Evaporation of solvent furnished **2g** (463 mg; 76 %): R_f=0.40 (toluene/diethylether 5:1); mp: 148–152 °C (toluene/diethylether); $[\alpha]_D^{20}$=+55 (*c*=0.51 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.92 – 0.97 (m, 15 H), 1.03 (s, 3H), 1.11 (s, 3H), 1.21 (s, 3H, 7×CH_3, ($\underline{CH_3}CH_2CH_2CH_2)_2N$), 1.28 -1.40 (m, 8H, ($CH_3\underline{CH_2CH_2}CH_2)_2N$), 2.13 (d, *J* = 15.2 Hz, 1H, H-1a), 2.58 (d, *J* = 15.0 Hz, 1H, H-1b), 3.24 – 3.43 (m, 4H, ($CH_3CH_2CH_2\underline{CH_2})_2N$), 3.46 (d, *J* = 7.6 Hz, 1H, H-28a), 3.55 (s, 1H, H-19), 3.80 ppm (d, *J* = 7.6 Hz, 1H, H-28b); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.51, 13.92, 15.44, 16.58, 19.55, 20.16, 21.42, 21.92, 24.55, 26.23, 26.48, 27.41, 28.80, 29.62, 30.23, 32.70, 33.07, 34.26, 36.26, 36.72, 37.14, 38.61, 39.31, 40.60, 40.70, 41.48, 46.77, 49.69, 50.81, 52.89, 71.28, 87.90, 112.27, 143.78, 153.01, 166.74 ppm; IR (film): v=2925, 2864, 1538 (thiazole), 1465 (thiazole), 1035 cm^{-1}; MS (ESI) m/z (%): 609.4 (100) $[M+H]^+$; Anal. calcd for $C_{39}H_{64}N_2OS$: C 76.92, H 10.59, N 4.60, S 5.27; found C 76.92, H 10.61, N 4.33, S 5.24.

Thiazole derivative **2h**

Compound **2h** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and dioctylammonium acetate (1.50 g; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtered through a short pad of silica gel. Evaporation of solvent furnished **2h** (562 mg; 78 %): R_f=0.45 (toluene/diethylether 5:1); mp: 146–152 °C (toluene/diethylether); $[\alpha]_D^{20}$=+51 (*c*=1.18 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.86 – 0.91 (m, 6H, H-dioctyl), 0.93 (s, 3H), 0.95 (s, 6H), 1.03 (s, 3H), 1.11 (s, 3H), 1.22 (s, 3H, 7×CH_3), 1.25 – 1.33 (m, 24H, ($CH_3\underline{(CH_2)_6}CH_2)_2N$), 2.14 (d, *J* = 15.2 Hz, 1H, H-1a), 2.59 (d, *J* = 14.9 Hz, 1H, H-1b), 3.23 – 3.41 (m, 4H, ($CH_3(CH_2)_6\underline{CH_2})_2N$), 3.46 (d, *J* = 7.9 Hz, 1H, H-28a), 3.56 (s, 1H, H-19), 3.81 ppm (d, *J* = 7.9 Hz, 1H, H-28b); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.52, 14.11, 15.44, 16.58, 19.57, 21.43, 21.93, 22.65, 24.55, 26.24, 26.49, 26.98, 27.42, 27.46, 28.80, 29.24, 29.34, 30.25, 31.82, 32.70, 33.08, 34.27, 36.27, 36.73, 37.15, 38.62, 39.33, 40.61, 40.71, 41.49, 46.78, 49.70, 51.19, 52.90, 71.28, 87.91, 112.27, 143.09, 153.02, 166.72 ppm; IR (film): v=2925, 2856, 1539 (thiazole), 1453 (thiazole), 1036 cm^{-1}; MS (ESI) m/z (%): 721.6 (100) $[M+H]^+$; Anal. calcd for $C_{47}H_{80}N_2OS$: C 78.27, H 11.18, N 3.88, S 4.45; found C 77.65, H 11.09, N 3.53, S 4.43.

Thiazole derivative **2i**

Compound **2i** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and benzylammonium acetate (836 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtered through a short pad of silica gel. Evaporation of solvent furnished **2i** (522 mg; 89 %): R_f=0.10 (toluene/diethylether 5:1); mp: 148–154 °C (toluene/diethylether); $[\alpha]_D^{20}$=+56 (*c*=0.61 in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.92 (s, 3H), 0.95 (s, 6H), 1.03 (s, 3H), 1.14 (s, 3H), 1.24 (s, 3H, 7×CH_3), 2.15 (d, *J* = 15.5 Hz, 1H, H-1a), 2.59 (d, *J* = 15.2 Hz, 1h, H-1b), 3.46 (d, *J* = 7.9 Hz, 1H, H-28a), 3.55 (s, 1H, H-19), 3.80 (d, *J* = 7.9 Hz, 1H, H-28b), 4.39 (s, 2H, $Ph\underline{CH_2}$), 5.59 (bs, 1H, NH), 7.29 – 7.44 ppm (m, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.49, 15.43, 16.51, 19.45, 21.45, 21.99, 24.55, 26.22, 26.43, 26.47, 28.80, 30.23, 32.69, 33.00, 34.25, 36.26, 36.71, 37.00, 38.66, 39.28, 40.60, 40.71, 41.48, 46.75, 49.68, 50.13, 52.80, 71.25, 87.89, 114.31, 127.63, 127.72, 128.62, 137.71, 142.82, 151.94, 166.77 ppm; IR (film): v=3520 (NH), 2934, 2865, 1542 (thiazole), 1463 (thiazole), 1035 cm^{-1}; MS (ESI) m/z (%): 587.4 (100) $[M+H]^+$; Anal. calcd for $C_{38}H_{54}N_2OS$: C 77.76, H 9.27, N 4.77, S 5.46; found C 77.69, H 9.28, N 4.55, S 4.93.

Thiazole derivative **2j**

Compound **2j** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and morpholinium acetate (736 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtered through a short pad of silica gel. Evaporation of solvent furnished **2j** (510 mg; 90 %): R_f=0.20 (toluene/diethylether5:1); mp: 151–155 °C (toluene/diethylether); $[\alpha]_D^{20}$=+65 (*c*=0.95 in $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.91 (s, 3H), 0.95 (s, 6H), 1.03 (s, 3H), 1.12 (s, 3H), 1.22 (s, 3H, 7×CH_3), 2.17 (d, *J* = 15.2 Hz, 1H, H-1a), 2.63 (d, *J* = 15.2 Hz, 1H, H-1b), 3.39 (t, *J* = 4.9 Hz, 4H, $O(CH_2\underline{CH_2})_2N$), 3.46 (d, *J* = 8.1 Hz, 1H, H-28a), 3.55 (s, 1H, H-19), 3.79 – 3.81 ppm (m, 5H, H-28b, $O(\underline{CH_2}CH_2)_2N$); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.49, 15.43, 16.49, 19.52, 21.47, 21.98, 24.55, 26.23, 26.45, 26.47, 28.79, 30.29, 32.71, 33.04, 34.27, 36.27, 36.73, 37.22, 38.63, 39.30, 40.62, 40.72, 41.49, 46.78, 48.57, 49.69, 52.89, 66.28, 71.27, 87.90, 115.21, 153.34, 168.39 ppm; IR (film): v=2924, 2856, 1529 (thiazole), 1452 (thiazole), 1036 cm^{-1}; MS (ESI) m/z (%): 567.4 (100) $[M+H]^+$; Anal. calcd for $C_{35}H_{54}N_2O_2S$: C 74.16, H 9.60, N 4.94, S 5.66; found C 73.43, H 9.63, N 4.59, S 6.03.

Thiazole derivative **2k**

Compound **2k** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and *N*-methylpiperazinium acetate (802 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtered through a short pad of silica gel. Evaporation of solvent furnished **2k** (557 mg; 96 %): R_f=0.15 (toluene/diethylether 5:1); mp: 147–154 °C (toluene/diethylether); $[\alpha]_D^{20}$=+61 (*c*=0.99, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.80 (s, 3H), 0.90 (s, 3H), 0.94 (s, 6H), 1.02 (s, 3H), 1.11 (s, 3H), 1.21 (s, 3H, 7×CH_3), 2.15 (d, *J* = 15.2 Hz, 1H, H-1a), 2.35 (s, 3H, *N*-CH_3), 2.52 – 2.55 (m, 4H, $CH_3N(\underline{CH_2}CH_2)_2N$), 2.61 (d, *J* = 15.2 Hz, 1H, H-1b), 3.40 – 3.48 (m, 5H, H-28a, $CH_3N(CH_2\underline{CH_2})_2N$), 3.54 (s, 1H, H-19), 3.79 ppm (d, *J* = 7.6 Hz, 1H, H-28b); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.50, 15.42, 16.51, 19.51, 21.45, 21.98, 24.55, 26.22, 26.44, 28.79, 30.28, 32.69, 33.03, 34.25, 36.26, 36.71, 37.19, 38.59, 39.28,

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40.60, 40.71, 41.48, 46.12, 46.75, 48.07, 49.66, 52.87, 54.27, 71.25, 87.88, 114.97, 147.97, 153.28, 168.03 ppm; IR (film): ν=2940, 2865, 1529 (thiazole), 1453 (thiazole), 1035 cm^{-1}; MS (ESI) m/z (%): 580.4 (100) $[M+H]^+$; Anal. calcd for $C_{36}H_{57}N_3OS$: C 74.56, H 9.91, N 7.25, S 5.53; found C 73.13, H 9.44, N 6.58, S 5.23.

Thiazole derivative **2l**

Compound **2l** was prepared according to the general procedure from 2α/β-thiocyano allobetulone (**2c**) (500 mg; 1.0 mmol) and cyclohexylammonium acetate (797 mg; 5.0 mmol) in chloroform (20 mL) after 24 hours controlled by TLC (mobile phase toluene/diethylether 5:1). Dissolved crude product was filtered through a short pad of silica gel. Evaporation of solvent furnished **2l** (504 mg; 87 %): R_f=0.10 (toluene/diethylether 5:1); mp: 14–151 °C (toluene/diethylether); $[\alpha]_D^{20}$=+60 (*c*=0.92 in $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ=0.81 (s, 3H), 0.92 (s, 3H), 0.94 (s, 6H), 1.03 (s, 3H), 1.12 (s, 3H), 1.22 (s, 3H, 7×CH_3), 2.14 (d, *J* = 15.2 Hz, 1H, H-1a), 2.58 (d, *J* = 15.3 Hz, 1H, H-1b), 3.08 – 3.20 (m, 1H, H-cyclohexyl), 3.46 (d, *J* = 7.9 Hz, 1H, H-28a), 3.55 (s, 1H, H-19), 3.80 (d, *J* = 7.6 Hz, 1H, H-28b), 5.30 ppm (bs, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.49, 15.42, 16.50, 19.44, 21.45, 21.94, 24.54, 24.64, 25.52, 26.21, 26.44, 26.46, 28.80, 30.20, 32.68, 33.00, 33.04, 33.08, 34.24, 36.26, 36.70, 36.89, 38.64, 39.30, 40.59, 40.71, 41.48, 46.75, 49.68, 52.81, 55.25, 71.25, 87.89, 113.27, 145.26, 151.93, 166.25 ppm; IR (film): ν=3500 (NH), 2927, 2856, 1538 (thiazole), 1452 (thiazole), 1032 cm^{-1}; MS (ESI) m/z (%): 578.4 (100) $[M+H]^+$; Anal. calcd for $C_{37}H_{58}N_2OS$: C 76.76, H 10.10, N 4.84, S 5.54; found C 76.23, H 10.06, N 4.87, S 5.40.

Cell lines

Cell lines were chosen and used the same way as in our previous work.[49,51,54] All cells (if not indicated otherwise) were purchased from the American Tissue Culture Collection (ATCC). The CCRF-CEM line is derived from T lymphoblastic leukemia, evincing high chemosensitivity, K562 represent cells from an acute myeloid leukemia patient sample with bcr-abl translocation, U2OS line is derived from osteosarcoma, HCT116 is colorectal tumor cell line and its p53 gene knock-down counterpart (HCT116p53-/-, Horizon Discovery Ltd, UK) is a model of human cancers with p53 mutation frequently associated with poor prognosis, A549 line is lung adenocarcinoma. The daunorubicin resistant subline of CCRF-CEM cells (CEM-DNR bulk) and paclitaxel resistant subline K562-TAX were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicine or paclitaxel, respectively. The CEM-DNR bulk cells overexpress MRP-1 and P-glycoprotein protein, while K562-TAX cells overexpress P-glycoprotein only. Both proteins belong to the family of ABC transporters and are involved in the primary and/or acquired multidrug resistance phenomenon.[49] MRC-5 and BJ cell lines were used as a non-tumor control and represent human fibroblasts. The cells were maintained in nunc/corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium according to ATCC or Horizon recommendations (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

Cytotoxic MTS assay

MTS assays were performed as described earlier[49-51,54] The assays were carried out at the Institute of Molecular and Translational Medicine by robotic platform (HighResBiosolutions). Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25000 – 35000 cells/mL based on cell growth characteristics). Cells were added by automatic pipetor (30 µL) into 384 well microtiter plates. All tested compounds were dissolved in 100% DMSO and four-fold dilutions of the intended test concentration were added in 0.15 µL aliquots at time zero to the microtiter plate wells by the echoacustic non-contact liquid handler Echo550 (Labcyte). The experiments were performed in technical duplicates and three biological replicates at least. The cells were incubated with the tested compounds for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed by using the MTS test. Aliquots (5 µL) of the MTS stock solution were pipetted into each well and incubated for additional 1–4 h. After this incubation period, the optical density (OD) was measured at 490 nm with an Envision reader (Perkin Elmer). Tumor cell survival (TCS) was calculated by using the following equation: $TCS = (OD_{drug\text{-}exposed\ well}/mean\ OD_{control\ wells}) \times 100\%$. The IC_{50} value, the drug concentration that is lethal to 50% of the tumor cells, was calculated from the appropriate dose-response curves in Dotmatics software.

Cell Cycle and Apoptosis Analysis

Analysis of the cell cycle and apoptosis was done in concordance with our previus research.[50,51,54] Suspension of CCRF-CEM cells, seeded at a density of 1.10^6 cells/mL in 6-well panels, were cultivated with the 1 or 5 × IC_{50} of tested compound in a humidified CO_2 incubator at 37 °C in RPMI 1640 cell culture medium containing 10% fetal calf serum, 10 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Together with the treated cells, control sample containing vehicle was harvested at the same time point after 24 h. After another 24 hours, cells were then washed with cold PBS and fixed in 70% ethanol added dropwise and stored overnight at -20 °C. Afterwards, cells were washed in hypotonic citrate buffer, treated with RNAse (50 µg/mL) and stained with propidium iodide. Flow cytometer using a 488 nm single beam laser (Becton Dickinson) was used for measurement. Cell cycle was analyzed in the program ModFitLT (Verity), and apoptosis was measured in logarithmic model expressing percentage of the particles with propidium content lower than cells in G0/G1 phase (<G1) of the cell cycle. Half of the sample was used for $pH3^{Ser10}$ antibody (Sigma) labeling and subsequent flow cytometry analysis of mitotic cells.[50]

BrDU Incorporation Analysis (DNA synthesis)

For this analysis, the same procedure of cultivation as previously[50,51,54] was used. Before harvesting, 10 µM 5-bromo-2-deoxyuridine (BrDU), was added to the cells for puls-labeling for 30 min. Cells were fixed with ice-cold 70% ethanol and stored overnight. Before the analysis, cells were washed with PBS, and resuspended in 2 M HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$ (Borax), cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. Staining with primary anti-BrDU antibody (Exbio) for 30 min at room temperature in the dark followed. Cells were then washed with PBS and stained with secondary antimouse-FITC antibody (Sigma). Cells were then washed with PBS again and incubated with propidium iodide (0.1 mg/mL) and RNAse A (0.5

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mg/mL) for 1 h at room temperature in the dark and afterwards analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).[50]

BrU Incorporation Analysis (RNA synthesis)
For this analysis, the same procedure of cultivation as previously[50,51,54] was used. Cells were cultured and treated as above. Before harvesting, pulse-labeling with 1 mM 5-bromouridine (BrU) for 30 min followed. The cells were then fixed in 1% buffered paraformaldehyde with 0.05 % of NP-40 in room temperature for 15 min, and then stored in 4°C overnight. Before measurement, they werewashed in PBS with 1% glycin, washed in PBS again, and stained by primary anti-BrDU antibody crossreacting to BrU (Exbio) for 30 min at room temperature in the dark. After another washing step in PBS cells were stained by secondary antimouse-FITC antibody (Sigma). Following the staining, cells were washed with PBS and fixed with 1% PBS buffered paraformaldehyde with 0.05% of NP-40 for 1 hour. Cells were washed by PBS, incubated with propidium iodide (0.1 mg/mL) and RNAse A (0.5 mg/mL) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACS Calibur, Becton Dickinson).[50]

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Keywords: thiazoles • cytotoxicity • cell cycle • triterpene • heterocycles

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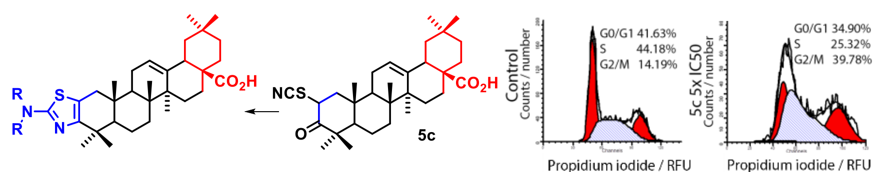

Cytotoxic triterpenes (SAR & MOA): Aminothiazoles were prepared from four triterpenic scaffolds. Diethylamino- and N-methylpiperidinothiazoles of oleanonic acid were best, however, much better candidate is intermediate thiocyanate **5c** as it is new, active, non-toxic, inducing apoptosis selectively in cancer cells via cell cycle arrest in G2/M phase and inhibits DNA/RNA synthesis.

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