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Short communication

Synthesis, structure elucidation and identification of antitumoural properties of novel fused 1,2,4-triazine aryl derivatives

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Abstract

Synthesis, structure elucidation and anticancer activities of novel fused 1,2,4-triazine aryl derivatives containing the ethoxycarbonyl (**6**–**10**) and carbohydrazide formations (**11**–**15**) are presented. Molecular structures of the synthesized compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR, EI-MS spectra and elemental analyses. Antitumour activities *in vitro* for heterobicyclic hydrazides of the type **11**–**14** were evaluated by BrdU method for human LS180, SiHa and T47D carcinoma cells. Amongst them, hydrazide **14** has exhibited remarkable inhibitory effect against SiHa and LS180 tumour cells, and simultaneously was found to be non-toxic towards the human normal cell line – HSF cells. Furthermore, the pulse field gel electrophoresis experiment was performed for characterizing DNA-cleaving activity of heterobicycle **14**. The DNA fragments of 2500, 2000 and 500 kilobase pairs (kbp) were commonly detected in the cancer cell lines (SiHa, LS180 and T47D) treated with compound **14**. DNA fragmentation pattern, since three types of fragments induced by the tested hydrazide of the type **14** were detected, suggesting a way of interaction with DNA. It is worth pointing out, that DNA strand breaks were also produced in human breast cancer (T47D) cells, a cell line where the induction of DNA fragmentation is very difficult. Moreover, the statistically significant apoptotic activity in T47D human breast cancer cells for the tested heterobicycle **14** was proved using the annexin V – binding assay. The antiproliferative properties *in vitro* for compounds **6**–**14** were evaluated by MTT method for human leukaemic Jurkat cells. Significant viability decreases in Jurkat cells treated with different concentrations of compounds **10** and **11** were observed, suggesting that these derivatives have antiproliferative activities. Their acute toxicities were established. For these compounds the influence on the central nervous system of mice in behavioural tests was examined. Molecular structure for free base of the intermediate **4** was confirmed by ^{1}H–^{1}H COSY, HMBC and HMQC correlations.

Keywords: Fused 1,2,4-triazines; Imidazo[2,1-*c*][1,2,4]triazine nucleus; Ethoxycarbonyl group; Carbohydrazide formation; A novel DNA strand breaking agent; Anticancer activity; Apoptotic activity

1. Introduction

The 1,2,4-triazine ring is a prominent structural core system found in numerous biologically active compounds. For instance certain azanucleosides (6-azacytosine, 6-azauracil), structurally based on the 1,2,4-triazine nucleus, have displayed an impressive array of biological activities, among which antitumour [1,2], antiviral [3,4], and antifungal [5] properties have been cited in the scientific literature.

In addition, 6-azaisocytosine (e.g., 3-amino-1,2,4-triazin-5(2*H*)-one), an isosteric isomer of 6-azacytosine and 6-azauracil, is of great biological interest due to its resistance to deaminase, while azaribine – the well-known antiviral drug is structurally associated with the 1,2,4-triazine moiety [6].

Various condensed 1,2,4-triazines found application as pharmaceuticals, herbicides, pesticides and dyes [7–12]. Pyrrolo

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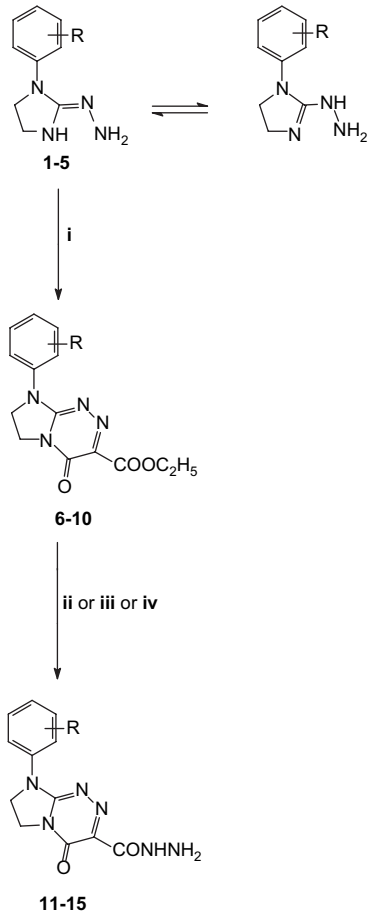
[2,1-*f*][1,2,4]triazines, congeners of substituted nucleic acid purines, have been synthesized and screened *in vitro*, revealing an interesting broad spectrum antiproliferative activity and a pronounced *in vitro* growth inhibitory activity against leukaemic cell lines, comparable to that of 9-deazaadenosine. Pyrrolo [2,1-*c*][1,2,4]triazines demonstrated inhibitory effects on the growth of a wide range of cancer cells generally at 10^{-5} M level, and in some cases, even at micromolar concentrations [13,14]. Some of pyrazolo[5,1-*c*][1,2,4]triazines have acquired considerable importance because of their remarkable antitumour and antifungal activities. It is noteworthy that many potential anticancer and antiviral drugs have been modelled on them [15–20].

Recently, there is a widespread interest in the synthesis and design of novel imidazo[2,1-*c*][1,2,4]triazine derivatives because of their potential biological activities associated with their skeleton. For instance certain synthetic derivatives of the imidazo[2,1-*c*][1,2,4]triazin-4(1*H*)-one have been designed and obtained as novel bicyclic nucleosides related to 6-azaisocytosine [21], while the others displayed the distinctly marked lower cytotoxicity towards normal cells (HSF, GMK) and several-times higher against cancer cell lines [22,23]. Another previously obtained derivatives containing the imidazo[2,1-*c*][1,2,4]triazine nucleus revealed a strong affection to tumour cells and simultaneously were found to be non-toxic towards the normal cell line investigated – GMK cells [24].

In continuation of our attempt to achieve medicinally important organic molecules [22–28], and having in mind the promising anticancer activities showed by previously obtained 1,2,4-triazine fused heterocycles [13–20,22–24], it seemed worthwhile to synthesize some novel heterobicyclic derivatives bearing the 1,2,4-triazine moiety as an expected pharmacophore. The presented herein novel fused 1,2,4-triazine aryl derivatives containing the ester (**6–10**) and hydrazide formations (**11–15**) hold the aza skeleton of the known pyrrolo[2,1-*c*][1,2,4]triazine scaffold, the derivatives of which showed good antiproliferative activities with IC_{50} values in the range of 5.5–88 μM [13]. In an attempt to achieve new heterobicycles with possible anticancer properties, we designed and synthesized the above-mentioned fused 1,2,4-triazines to evaluate their anticancer activities. Furthermore, some behavioural pharmacological tests (acute toxicity and "writhing syndrome") were performed to check the influence of the compounds on the central nervous system of the laboratory animals.

2. Chemical part

The synthetic pathway used in the preparation of the compounds is outlined in Scheme 1. Biologically active starting materials 1-aryl-2-hydrazonoimidazolidines (1-aryl-2-hydrazinoimidazolines) [22,23] (**1–5**) were prepared by patent pending and according to Sztanke [29]. Treatment of hydrazones **1–5** with diethyl 2-oxomalonate in refluxing *n*-butanol afforded the corresponding ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formates (**6–10**) (method i), which in turn were refluxed with hydrazine hydrate to obtain 8-aryl-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3-carbohydrazides (**11–15**). In an attempt to prepare the final



1, 6, 11: R=H; **2, 7, 12**: R=4-CH_3; **3, 8, 13**: R=4-CH_3O; **4, 9, 14**: R=3-Cl; **5, 10, 15**: R=3,4-Cl_2

Scheme 1. Synthetic route to obtained heterobicycles. Reagents and conditions: i – (**1–5**) + diethyl 2-oxomalonate, *n*-butanol, reflux, 7 h; ii – (**6–10**) + hydrazine hydrate (100%), *n*-butanol, reflux, 5 h; iii – (**6–10**) + hydrazine hydrate (100%), etanol, reflux, 6 h; iv – (**6–10**) + hydrazine hydrate (100%), the alloy bath, 90 °C, 7 h.

products (**11–15**) in the highest yield, three different synthetic methods were applied. Thus, the condensation of above-mentioned esters (**6–10**) with hydrazine hydrate was carried out: (a) in *n*-butanol at the temperature of boiling for 5 h (method ii), (b) in ethanolic medium under reflux for 6 h (method iii), and (c) without any solvent by heating in the alloy bath in the temperature of ca. 90 °C for 7 h (method iv). Substrates were mixed together in molar ratio 1:1 (method ii), 1:3 (method iii) and 1:10 (method iv) (Scheme 1). It was found that all these methods led directly to the formation of the desired hydrazides **11–15** (with concomitant loss of ethanol molecule). Highest yields (on an average 74%) for hydrazides **11–15** were achieved when the reaction is performed in ethanolic medium in excess of hydrazine hydrate (method iii). Mixed melting points of the final products (**11–15**) obtained by three above-mentioned methods have not shown any depression. The IR, ^{1}H NMR, ^{13}C NMR and MS spectral data of these compounds were also identical.

In view of continuous and widespread interest in the design of novel heterobicyclic derivatives containing the 1,2,4-triazine moiety, particularly on account of their pharmacological properties the synthetic approach leading to the formation of novel fused 1,2,4-triazine aryl derivatives containing the ester (**6**–**10**) and hydrazide formations (**11**–**15**), described by us, might be considered as a useful method for the preparation of these biologically active compounds because of the affordability of the starting materials, good yields obtained and straightforward product isolation.

NMR spectral characteristic of the synthesized heterobicyclic hydrazides (**11**–**15**) revealed in their ^{1}H NMR spectra signals of the H-7 and H-6 as a broad singlet at ca 4.19 ppm (**11**–**14**) or as two double-doublet signals at 3.60 and 4.03 ppm with the coupling constants of $J = 8.5$ Hz, $J' = 7.5$ Hz and $J = 8.6$ Hz, $J' = 7.5$ Hz, respectively (**15**). Furthermore, the ^{1}H NMR spectra of hydrazides **11**–**15** displayed no signals belonging to the $-OCH_2CH_3$ group; instead new signals derived from hydrazide structure appeared between 4.40–4.63 ppm ($-NHNH_2$) and 9.40–9.70 ppm ($-NHNH_2$) integrating for two protons and one proton, respectively (controlled by changing with D_2O). Their further ^{1}H NMR spectroscopic details are included in the Section 5.

The ^{13}C NMR chemical shift values of the C-7 and C-6 carbon atoms confirmed unequal character of both methylene groups as well (ca. 45.8 ppm for C-7 and 40.5 ppm for C-6). Moreover, in the ^{13}C NMR spectra of hydrazides of the type **11**–**15**, the quaternary carbon signals derived from the exocyclic hydrazide group ($-CONHNH_2$) were recorded in the range of 152.4–152.5 ppm. Additionally the endocyclic triazine-C=O (triazine-C-4), triazine-C-3 and C-8a signals were seen at about 143.8, 150.8 and 161.0 ppm, respectively.

In the IR spectra of carbohydrazides **11**–**15**, the presence of absorption bands in the range of 1685–1690 and 1553–1562 cm^{-1}, attainable to the endocyclic triazine-C=O group at position 4 and the C=N bond at the ring junction, respectively, confirmed the formation of final products. Also, the IR spectra of compounds **11**–**15** revealed an additional peak at about 1740 cm^{-1} due to the exocyclic-carbonyl function derived from the hydrazide structure. From the IR spectral data there is a clear relationship between the frequency of the ring carbonyl group –C=O and the frequency of the C=N bond at the ring junction and the difference in their frequencies is of the order of 124–132 cm^{-1}. These data are in complete agreement with the literature data [30].

EI-MS of heterobicyclic hydrazides (**11**–**15**) provided molecular ion peaks at *m*/*z* 272, 286, 302, 306, 340, respectively, with determined intensities confirming their molecular weights. The most typical fragmentation pattern involved the cleavage of the 31 molecular unit fragment of the side chain that most probably was N_2H_3. Further details are presented in Section 5.

All the chemical and physical data for compounds **11**–**15** are given in Section 5.

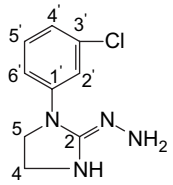
The intermediates – tautomeric 1-aryl-2-hydrazonoimidazolidine hydroiodides (**1**–**5**) were obtained by a previously reported method [24,29,31]. However, the HMBC, HMQC correlations and ^{13}C NMR spectroscopic data of free base of intermediate **4** are presented in this paper for the first time. Further physicochemical, experimental and spectroscopic details are reported in the Section 5. Although in the case of the compound investigated the hydrazone–hydrazine tautomerism is possible but in solution (DMSO-d_6) only the presence of the hydrazone tautomer was observed. In the ^{1}H–^{1}H COSY spectrum of free base of **4** the correlation between endocyclic-NH proton signal derived from the imidazolidine ring at position 3 and the H-4 methylenic proton signals were observed as expected for the hydrazone tautomer. Also in the HMBC NMR spectrum of this compound there is a lack of correlation between the exocyclic-NH proton ($-NHNH_2$) from possible hydrazinic structure and the C-2 carbon. The HMBC and HMQC NMR spectroscopic data for compound **4** are presented in Table 1. NMR spectral characteristic of compound **4** is given in the Section 5. The chemical shift values of the C-5 and C-4 carbon atoms exhibit unequal character as well (51.3 ppm for the C-5 and 40.9 ppm for the C-4) as was seen in the ^{13}C NMR spectrum.

3. Results and discussion

The series of heterobicyclic hydrazides of the type **11**–**14** were evaluated for their antitumour activities. Results for each test compound are reported as the growth inhibition percentage of the treated cells when compared to the untreated control ones. Compounds **11**–**14** were tested against three human tumour cell lines derived from various cancer types (colon, uterus, breast): LS 180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), SiHa (ECACC 85060701, uterus cancer cells), T47D (ECACC 85102201, human breast carcinoma cells). Moreover, two normal cell lines – HSF (human skin fibroblast) cells and Vero (ECACC 88020401, African Green Monkey Kidney cell line, GMK clone) were included in the cytotoxicity study.

According to the data summarized in Table 2, the final heterobicyclic hydrazide of the type **14**, with electron-withdrawing

Table 1
The HMBC and HMQC NMR spectroscopic data for free base of intermediate **4**



	HMBC correlations	HMQC correlations
C-2	H-4, H-5	–
C-4	H-4, H-5	H-4, H-5 (w)
C-5	H-4, H-5	H-5, H-4 (w)
C-1′	H-2′, H-5′, H-6′	–
C-2′	H-4′, H-6′	H-2′
C-3′	H-2′, H-4′, H-6′	–
C-4′	H-5′, H-6′	H-4′
C-5′	H-6′, H-2′	H-5′
C-6′	H-2′, H-4′	H-6′

w – Weak correlation.

substituent, such as a chloro group at position 3 of the phenyl ring, was found to be the most active of the series. It has shown 45 and 63% growth inhibition, respectively, against uterus cancer (SiHa) cells in both examined concentrations: 32.6 μM (10 μg mL^{-1}) and 163.0 μM (50 μg mL^{-1}). Similarly, this heterobicycle has exhibited 42 and 54% growth inhibition against human colon adenocarcinoma (LS180) cells in both tested concentrations, respectively. However, the remaining human breast cancer (T47D) cell line has shown the least susceptibility level to this compound (the following growth inhibition values were analysed: 26 and 40% for both its tested concentrations).

It is worth pointing out that heterobicycle **14** was found to be non-toxic towards the human cell line investigated – HSF cells. Furthermore, the distinctly marked lower cytotoxicity of compound **14** towards one monkey normal cell line – GMK cells and almost two-times higher against SiHa and LS180 cancer cells was ascertained.

Taking into consideration the growth inhibition comparative study results concerning the influence of the tested heterobicycle (**14**) on cancer and normal cell lines, the selective action of the examined compound can be expected. Also the anticancer activity of the investigated heterobicycle seemed to be dose-dependent.

From the point of a structure–activity relationship in the class of novel tested 8-aryl-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3-carbohydrazides (**11–14**), the results obtained clearly indicated that a chloro substituent at position 3 in the phenyl ring played a vital role for exerting cytotoxic effect on human tumour cell lines. Generally, an antitumour property increases as a result of the replacement of hydrogen, 4-methyl and 4-methoxy substituents in **11–13** with a 3-chloro substituent in **14**. Thus, the highest growth inhibitory activity was observed for compound **14**, having a 3-chlorophenyl substituent at position 8 of the heterobicycle. Compounds **11–13** containing phenyl, 4-methylphenyl and 4-methoxyphenyl groups in position 8 of the heterobicycle demonstrated only slightly antiproliferative effects against the tested cancer cell lines. Simultaneously, heterobicycles **12** and **13** were strictly speaking non-toxic for normal cell lines investigated – HSF and GMK cells, whereas hydrazide **11** was found to be non-toxic for GMK cells.

Furthermore, DNA damage in the examined cancer cells after incubation with the most potent hydrazide **14** at a concentration of 32.6 μM was analyzed using field gel electrophoresis through a 1% agarose gel. This method is applied for the analysis of DNA-fragmenting activity of various antitumour agents [32–34]. Compound **14** was found to be effective in inducing DNA damage. The additional DNA fragments of 2500, 2000 and 500 kilobase pairs (kbp) appeared in the cancer cell lines (SiHa, LS180, T47D) treated with compound **14** in comparison to DNA of control cells, suggesting strongly that the examined heterobicycle induced DNA cleavage (see Fig. 1). DNA fragmentation pattern, since three types of fragments induced by the tested hydrazide of the type **14** were detected, suggesting a way of interaction with DNA. Endonucleases that mediate this DNA cleavage may be classified by substrate specificity. Upregulation of endonucleases, their intranuclear and intracellular redistribution and primary changes of chromatin structure are three major pathways of DNA fragmentation.

It is worth pointing out that DNA strand breaks were also produced in human breast cancer cells, a cell line where the induction of DNA fragmentation is very difficult [35,36]. In conclusion the tested hydrazide of type **14** revealed the damaging influence on DNA molecules of cancer cells. The examined heterobicycle was found to possess the efficiency for DNA strand breakage of cancer cell lines such as the cytotoxic antibiotic – bleomycin [37], isolated from *Streptomyces verticillus* and effective in combination therapies against certain types of skin cancer, testicular carcinoma and lymphomas. DNA strand breakage caused by compound **14** can have dramatic effects on higher-order chromatin structure because of its supercoiling and tight packaging within the nucleus.

In the next series of experiments, the apoptotic activity of the heterobicyclic hydrazide **14** in human breast cancer (T47D) cells was proved using the apoptosis assay based on the annexin V/propidium iodide staining. When T47D human breast cancer cells were grown in the presence of compound **14** (32.6 μM), a substantial percentage of apoptosis became evident (8.5%, compared to 2% in the control) (Table 3 and Photo 1). On the contrary, when the normal cells – human skin fibroblast (HSF) cells were grown in the presence of derivative **14** (32.6 μM), the

Table 2
Inhibition of *in vitro* normal and tumour cells growth by heterobicycles **9** and **11–14**

Cell line	Cytotoxicity (growth inhibition in %)									
	9 [22]		**11**		**12**		**13**		**14**	
	I	II	I	II	I	II	I	II	I	II
Normal cell lines										
HSF	16	17	16	20	0	0	0	0	0	0
GMK	20	25	2	0	2	0	0	0	20	29
Cancer cell lines										
LS180	30	35	0	15	0	14	1	1	42	54
SiHa	41	52	0	0	11	9	19	18	45	63
T47D	26	41	4	9	5	8	1	0	26	40

HSF – human skin fibroblast cells – primary cell line; Vero (GMK, ECACC 88020401, African Green Monkey Kidney cells); LS180 (ECACC 87021202) – human Caucasian colon adenocarcinoma cells; SiHa (ECACC 85060701) – uterus cancer cells; T47D (ECACC 85102201) – human breast carcinoma cells; I – concentration of 10 μg mL^{-1}, which corresponds to following concentration values: 31.2 μM (**9**), 36.7 μM (**11**), 34.9 μM (**12**), 33.1 μM (**13**), 32.6 μM (**14**); II – concentration of 50 μg mL^{-1}, which corresponds to following concentration values: 155.9 μM (**9**), 183.6 μM (**11**), 174.6 μM (**12**), 165.4 μM (**13**), 163.0 μM (**14**); compound **15** was not tested due to its incomplete solubility.

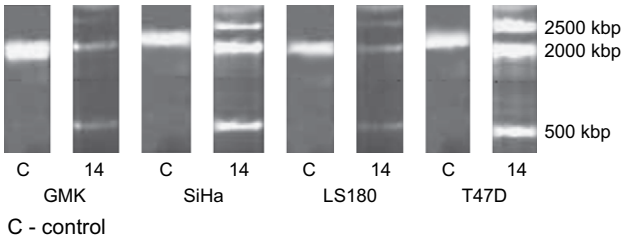


Fig. 1. Induction of DNA damage in the tested cell lines after incubation with heterobicycle **14** in concentration of 32.6 μM. Cells, prepared as agarose plugs, were lysed and subjected to pulsed field gel electrophoresis through a 1% agarose gel with propidium iodide.

quantity of apoptotic cells was found to be 1%, while in the control 0.6% (see Table 3 and Photo 2). Based on the performed examination, statistically significant apoptotic activity of the tested hydrazide of the type **14** in human breast cancer cells was ascertained.

Although breast cancer is most often treated with conventional cytotoxic agents, it was proved difficult to induce apoptosis in breast cancer cells [36]. Therefore, search for new agents that are effective in activating apoptosis, such as **14**, seemed to be of great interest. Finally, our present data suggest that a novel heterobicyclic hydrazide of the type **14** could be useful in stimulating apoptosis in human breast cancer cells.

Owing to above-mentioned biological activity, compound **14** may be promising for the development of novel agents that induce the DNA strand breakage, and therefore may be used as the basis for the design of novel non-toxic anticancer drugs.

However, the results obtained from the tested ester and hydrazide derivative of 8-(3-chlorophenyl)-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine (**9** and **14**), showed that an improved antitumour activity against LS180 and SiHa cancer cells is due to the conversion of the ethoxycarbonyl formation present in the position 3 of heterobicycle **9** into the carbohydrazide group located in the same position (in compound **14**). The carbohydrazide formation present in **14** renders this compound highly soluble and hydrophilic. Probably, the decrease in lipophilicity, better water solubility profile and increasing percentage of nitrogen element could be a qualitative explanation of profitable changes in its growth inhibitory properties in relation to **9** as observed in Table 4.

Table 3
The effect of 32.6 μM heterocycle **14** on the level of apoptotic cells in the tested normal and cancer cells

Cell lines	The amount of apoptotic cells in %
Normal cell line	
HSF in control	0.6 ± 0.05
HSF + compound **14**	1.0 ± 0.1
Cancer cell line	
T47D in control	2.0 ± 0.1
T47D + compound **14**	8.5* ± 0.5

HSF – human skin fibroblast cells – primary cell line; T47D (ECACC 85102201) – human breast carcinoma cells; *statistical significance ($p < 0.001$); apoptosis was determined using an annexin V-based assay; all experiments were conducted in triplicate and gave similar results; the data are presented as a mean ± SD of three independent experiments.

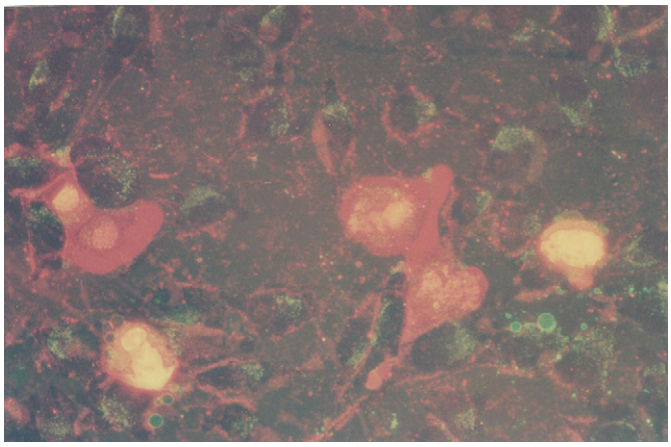


Photo 1. The apoptotic effects of compound **14** (32.6 μM) in T47D human breast cancer cells after 72 h of exposure. The assay is based on annexin V/propidium iodide staining. Apoptotic cells: cells with nuclei stained yellow (early stage of apoptosis) or red (late stage of apoptosis). The remaining unstained cells represent the living cells. Magnification 400×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the next series of experiments, we have also investigated the action of the designed heterobicycles (**6**–**14**) on the viability of human leukaemic Jurkat cells in order to select compounds having promising antiproliferative activities. Jurkat cells are a human leukaemic cell line, which express CD4 clusters and has the ability to produce several cytokines in response to stimuli. It is a well-established model for the study of apoptotic death pathways of cancer cells [38,39]. Therefore, in the conducted experiment Jurkat cells were incubated in the presence of heterobicycles **6**–**14**. Compounds **6**–**9** and **12**–**14** in three different concentrations of the compound solutions (1, 50, 100 μM) nearly did not change the viability of human leukaemic Jurkat cells. Simultaneously, so slight viability changes seemed to be mainly concentration-independent (Table 5). However, the results summarized in Table 5 pointed out that two of the examined derivatives: **10** and **11** exhibit evident antiproliferative potencies. The highest viability decreases (to 28 and 23%, respectively, after exposure

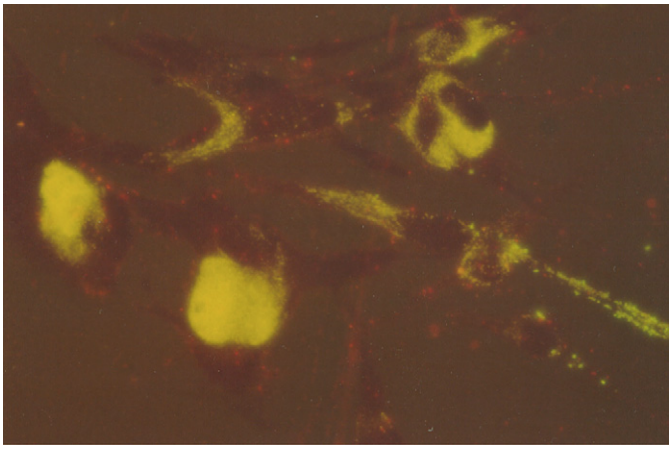


Photo 2. The effect of the examined heterobicyclic hydrazide of the type **14** (32.6 μM) on human skin fibroblast cells stained with annexin and propidium iodide. Apoptosis was measured at 72 h of incubation. Magnification 400×.

Table 4
Calculated water solubility at 25 °C, log *P* values and percentages of nitrogen element for compounds **9** and **14**

Compound	Solubility[a] (mg L^{-1})	log *P*[b]	N (%)
9	13.62	2.39	17.47
14	265.70	0.73	27.40

[a] Water solubility values were calculated using EPI Suite for Windows v. 3.11. USEPA, Washington, DC, 2000.
[b] The log *P* values were calculated using Pallas 3.1.1.2. software (distributed by CompuDrug) 2003.

for 24 h) in Jurkat cells treated with 50 and 100 μM heterobicycle **10** were observed. The toxic effect of the investigated compound seemed to be dose-dependent.

In our previous studies compound **10** has shown 50 and 46% growth inhibition against LS180 and SiHa cancer cell lines, respectively, in concentration of 50 μg mL^{-1}. Moreover, its distinctly marked lower cytotoxicity against the examined normal cell lines (HSF, GMK) and almost two-times higher against cancer cells was ascertained. Full details of the method of testing have been described elsewhere [22].

Also, compound **11** applied in a concentration of 100 μM evoked a significant viability decrease (to 33%) in leukaemic Jurkat cells after exposure for 24 h. Based on performed examination, it can be found from Table 5, that derivatives **10** and **11** were proved to reveal strong effects against the examined Jurkat cells. Simultaneously, these heterobicycles demonstrated slight toxic effects towards human skin fibroblast (HSF) cells.

Table 5
Percentage of viable normal human skin fibroblast (HSF) cells and human leukaemic Jurkat cells following 24 h treatment with the tested concentrations of heterobicycles **6**–**14**

Compound	Concentration in μM	Cell viability in normal HSF cells (in %)	Cell viability in cancer Jurkat cells (in %)
6	1	91.6 ± 4.7	82 ± 6.8
	50	98.1 ± 3.8	103 ± 8.0
	100	77.1 ± 5.4	105 ± 7.6
7	1	91 ± 2.9	83 ± 5.2
	50	87 ± 2.8	89 ± 4.5
	100	82 ± 3.0	93 ± 6.2
8	1	103.5 ± 5.6	118 ± 4.3
	50	100 ± 5.8	110 ± 2.9
	100	97 ± 4.0	110 ± 4.7
9	1	104 ± 5.0	114 ± 3.9
	50	103 ± 3.6	111 ± 3.0
	100	98 ± 3.9	107 ± 3.2
10	1	102 ± 4.5	91 ± 6.4
	50	84 ± 1.8	**28 ± 3.8**
	100	85 ± 1.9	**23 ± 2.4**
11	1	82 ± 1.4	89 ± 7.0
	50	83 ± 3.2	82 ± 4.2
	100	82 ± 1.4	**33 ± 4.0**
12	1	109 ± 7.3	93 ± 6.7
	50	111 ± 10.9	87 ± 9.3
	100	95 ± 7.4	79 ± 10.6
13	1	98 ± 3.0	97 ± 11.3
	50	102 ± 4.4	97 ± 12.7
	100	100 ± 5.3	100 ± 10.4
14	1	108 ± 9.6	100 ± 10.7
	50	113 ± 5.9	116 ± 10.8
	100	105 ± 6.8	115 ± 11.1

Cell viability after exposure for 24 h was evaluated by means of MTT (tetrazolium salt reduction) assay; cell viability in control – 100%; compound **15** was not tested due to its incomplete solubility.

Table 6
Tumor cell growth inhibition expressed as IC_{50} (μM) in the presence of the most active compounds (**10**, **11** and **14**)

Cell line	IC_{50} (in μM)		
	10	**11**	**14**
Adherent			
LS180	>100	>100	>100
SiHa	>100	>100	**75.4**
T47D	>100	>100	>100
Leukaemic			
Jurkat	**32.2**	**85.5**	>100

IC_{50} – the molar concentration that inhibits tumour cell growth to 50%; Vero (GMK, ECACC 88020401, African Green Monkey Kidney cells); LS180 (ECACC 87021202) – human Caucasian colon adenocarcinoma cells; SiHa (ECACC 85060701) – uterus cancer cells; T47D (ECACC 85102201) – human breast carcinoma cells; Jurkat (ECACC 88042803) – human leukaemic T cell lymphoblast.

Taking into consideration the influence of both investigated compounds (**10** and **11**) on leukaemic Jurkat cells and a normal cell line – human skin fibroblast (HSF) cells, the selective action of the examined derivatives can be expected.

IC_{50} values for the most active compounds (**10**, **11** and **14**) calculated from the dose–survival curves obtained after 72 h agent treatment (**14**) from 5-bromo-2′-deoxy-uridine (BrdU) test and from the dose–survival curves obtained after 24 h drug treatment (**10** and **11**) from MTT test, are shown in Table 6. Heterobicycle **10** was found to be effective against leukaemic Jurkat cells with the best IC_{50} value of 32.2 μM. Also, derivative **11** with an IC_{50} value of 85.5 μM, was found to be moderately active against the above-mentioned Jurkat cells, but it was almost 2.7-fold less potent than **10**. Heterobicyclic hydrazide of the type **14** exhibited moderate activity against SiHa cancer cells with an IC_{50} value of 75.4 μM, but it was completely non-toxic towards human normal cell line – HSF cells.

Due to effectiveness of the tested heterobicycles (**10** and **11**) against leukaemic Jurkat cells, some behavioural tests were performed to exclude or confirm their influence on the central nervous system. It has been proved that compound **10** showed no effect on the central nervous system of mice in behavioural tests applied. This one given in a dose of 0.1 LD_{50} did not produce the decrease in number of animals exhibiting pain reactivity in the ''writhing syndrome'' test. However, the heterobicyclic hydrazide of the type **11** exhibited only weak analgesic activity in the ''writhing test''. The acute toxicities of both compounds were relatively low (over 1000 mg kg^{-1} i.p.). Furthermore, two investigated heterobicycles revealed no neurotoxic properties, because, given in a dose of 0.1 LD_{50}, did not impair the motor coordination of mice in the rota-rod test.

4. Conclusion

In this report, easy and useful methods to obtain biologically active fused 1,2,4-triazine aryl derivatives containing the ester or hydrazide formations have been presented. It can be found

from Tables 2–6 that there is one compound (**14**) that possesses efficiency for DNA strand breakage of the examined cancer cells and has a strong affinity to SiHa cancer cells and reveals statistically significant apoptotic activity in T47D carcinoma cells and two heterobicycles (**10** and **11**) that exhibit strong effects to human leukaemic Jurkat cells. So both 8-phenyl and 8-(3-chlorophenyl)-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]-triazine-3-carbohydrazides (**11** and **14**) and one ethyl 1-(4-oxo-8-(3,4-dichlorophenyl)-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate (**10**) may have potential antitumoural activities. Further optimisation of these identified chemical leads can possibly lead to more active molecules. Since above-mentioned heterobicycles (**10**, **11** and **14**) are showing promising results, studies to establish their *in vivo* efficacy and safety are being planned for their further development. Further studies are in progress to define the important mechanisms of action of the above-mentioned compounds.

5. Experimental protocols

5.1. Instrumentations and general materials

Chemicals (hydrazine hydrate, diethyl 2-oxomalonate) were purchased from Merck and Sigma-Aldrich as 'synthesis grade' and used without further purification. Melting points (m.p.) were determined on a Boetius apparatus and are given uncorrected. The IR spectra were measured as potassium bromide pellets using a Perkin-Elmer 1725X spectrometer. ^{1}H NMR spectra for compounds **11**–**14** were recorded on a Bruker 200 MHz spectrometer in DMSO-d_6 with TMS as an external standard at 295 K. ^{1}H NMR spectrum for compound **15** was recorded on a Bruker 300 MHz spectrometer in DMSO-d_6 with TMS as an external standard at 295 K. ^{13}C NMR spectra for compounds **4**, **11**–**15** were recorded on a Bruker AC 200F instrument. Besides ^{1}H–^{1}H COSY, HMBC and HMQC correlations were made for free base of intermediate **4**. Mass spectroscopic analyses for compounds **4** and **11**–**15** were performed at 70 eV on an AMD-402 and a Trace DSQ (Thermo Finnigan) mass spectrometers, respectively, for molecular ion peaks. The molecular ion for compounds **14** and **15** was calculated according to ^{35}Cl isotope.

Thin-layer chromatography was carried out on commercial Merck SiO_2 60 F_{254} plates having fluorescence indicator. The spots were visualized in UV light $\lambda = 254$ and 355 nm. Elemental analyses were performed on a Perkin-Elmer analyzer and were in range of ±0.4% for each element analyzed (C, H, N, I, Cl). The starting 1-(3-chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**) was obtained by earlier described method [29]. Its physicochemical and spectral data are the following:

*1-(3-Chlorophenyl)-2-hydrazonoimidazolidine hydroiodide (**4**)* [23]: recrystallized from propan-2-ol, yield 62%, m.p. 55–56 °C. Spectroscopic data for $C_9H_{12}ClIN_4$: IR (KBr) (ν, cm^{-1}): 1509 (N^+H), 1571 (C=N), 3460–3315 ($NH + NH_2$); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 3.95 (dd, $J = 9.3$ Hz, $J' = 8.1$ Hz, 2H, CH_2), 4.22 (dd, $J = 9.3$ Hz, $J' = 8.1$ Hz, 2H, CH_2), 5.22 (s, 2H, NH_2), 7.25–7.53 (m, 4H, $CH_{arom.}$), 8.65 (s, 1H, NH), 9.26 (s, 1H, N^+H); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS) 40.9 (imidazolidine-C-4), 51.3 (imidazolidine-C-5), 122.9 (C-4′), 124.4 (C-6′), 126.8 (C-2′), 131.2 (C-5′), 132.2 (C-3′), 141.8 (C-1′), 158.5 (imidazolidine-C-2); *m/z*: 338[M^+].

5.1.1. General procedure for synthesis of ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazin-3-yl)formates (**6**–**10**) (method i)

Free base of 1-aryl-2-hydrazonoimidazolidine [23] (0.05 mol) was dissolved in 80 mL of *n*-butanol. Diethyl 2-oxomalonate (8.71 g, 0.05 mol) was added and the mixture was refluxed for 7 h. During that time precipitation of the solid started. The mixture was cooled overnight, the precipitate yielded was collected and purified by recrystallization from DMF or DMF/methanol mixture in the proportion indicated [22]. Physicochemical, analytical and spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) characteristics of the obtained compounds, given in Ref. [22], are in full agreement with proposed structures.

5.1.2. General procedure for synthesis of 8-aryl-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazine-3-carbohydrazides (**11**–**15**) (method ii)

A solution of respective ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate (**6**–**10**) [22] (0.03 mol) and 0.03 mol of hydrazine hydrate (100%) in 50 mL of *n*-butanol were refluxed for 5 h. The excess of solvent was distilled under reduced pressure. The mixture was cooled overnight, the precipitate yielded was collected and finally purified by recrystallization from DMF.

5.1.3. General procedure for synthesis of 8-aryl-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazine-3-carbohydrazides (**11**–**15**) (method iii)

A solution of the corresponding ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate (**6**–**10**) [22] (0.05 mol) and 0.15 mol of hydrazine hydrate (100%) in 80 mL of ethanol was refluxed for 6 h. The excess of solvent was removed by evaporation. Upon cooling the precipitate was collected, washed with 2 × 10 mL cold ethanol and finally purified by recrystallization from DMF.

5.1.4. General procedure for synthesis of 8-aryl-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazine-3-carbohydrazides (**11**–**15**) (method iv)

A solution of the corresponding ethyl 1-(4-oxo-8-aryl-4,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)formate (**6**–**10**) [22] (0.05 mol) and 0.5 mol of hydrazine hydrate (100%) were heated on the alloy bath in the temperature of ca. 90 °C for 7 h. The excess of hydrazine hydrate was distilled under reduced pressure. The reaction mixture was cooled, formed precipitate was collected, washed with 2 × 20 mL cold ethanol and finally purified by recrystallization from DMF.

*5.1.4.1. 8-Phenyl-4-oxo-4,6,7,8-tetrahydroimidazo[2,1-c][1,2,4]triazine-3-carbohydrazide (**11**).* Recrystallization from DMF; yield 71% (method ii)/79% (method iii)/66% (method iv), m.p. 293–294 °C. Spectroscopic data for $C_{12}H_{12}N_6O_2$: IR (KBr) (ν, cm^{-1}): 1744 (hydrazide-C=O), 1685 (triazine-C=O), 1561

(C=N); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 4.21 (br s, 4H, $2CH_2$), 4.59 (s, 2H, NH_2), 7.17–7.86 (m, 5H, ar-H), 9.68 (s, 1H, NH); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS): 40.5 (imidazolidine-C-6), 45.4 (imidazolidine-C-7), ar C: [119.3 (CH), 124.2 (2CH), 129.0 (2CH), 138.3 (C)], 143.7 (triazine-C-4), 151.0 (triazine-C-3), 152.4 (hydrazide-C=O), 160.9 (C-8a); EI-MS [70 eV, *m/z* (%)]: 273 (8.5), 272 (M^+, 55.0), 244 (11.9), 243 (14.6), 242 (100.0), 241 (85.4), 215 (5.5), 214 (17.9), 213 (4.9), 211 (9.1), 187 (4.2), 186 (27.5), 185 (31.2), 170 (4.2), 160 (4.5), 159 (16.3), 158 (56.1), 145 (11.3), 144 (22.7), 143 (5.3), 133 (3.4), 132 (30.1), 131 (41.0), 129 (3.2), 128 (5.0), 119 (4.7), 118 (13.8), 117 (15.9), 116 (4.0), 107 (4.6), 106 (19.9), 105 (16.6), 104 (40.9), 92 (3.3), 91 (14.9), 90 (4.8), 81 (6.4), 78 (7.9), 77 (78.3), 70 (27.0), 69 (4.5), 65 (15.6), 64 (4.1), 63 (3.8), 54 (5.0), 51 (17.1), 50 (3.5), 42 (25.3), 41 (3.6), 39 (4.9).

*5.1.4.2. 8-(4-Methylphenyl)-4-oxo-4,6,7,8-tetrahydroimidazo [2,1-c][1,2,4]triazine-3-carbohydrazide (**12**).* Recrystallization from DMF; yield 67% (method ii)/74% (method iii)/68% (method iv), m.p. 270–272 °C. Spectroscopic data for $C_{13}H_{14}N_6O_2$: IR (KBr) (ν, cm^{-1}): 1738 (hydrazide-C=O), 1690 (triazine-C=O), 1562 (C=N); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 2.31 (s, 1H, CH_3), 4.19 (br s, 4H, $2CH_2$), 4.63 (s, 2H, NH_2), 7.26 (d, $J = 8.5$ Hz, 2H, ar: H-2′ and H-6′), 7.71 (d, $J = 8.4$ Hz, 2H, ar: H-3′ and H-5′), 9.67 (s, 1H, NH); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS): 20.4 (-CH$_3$), 40.5 (imidazolidine-C-6), 45.5 (imidazolidine-C-7), ar C: [119.4 (2CH), 129.4 (2CH), 133.4 (C), 135.8 (C)], 143.4 (triazine-C-4), 151.0 (triazine-C-3), 152.4 (hydrazide-C=O), 161.0 (C-8a); EI-MS [70 eV, *m/z* (%)]: 287 (11.5), 286 (M^+, 72.8), 271 (6.1), 258 (12.5), 257 (15.8), 256 (98.4), 255 (100.0), 229 (6.7), 228 (14.6), 227 (5.2), 211 (8.4), 201 (7.9), 200 (43.7), 199 (26.9), 184 (4.6), 174 (5.6), 173 (24.7), 172 (76.7), 159 (8.5), 158 (18.8), 157 (7.1), 147 (4.5), 146 (42.0), 145 (46.3), 144 (6.2), 143 (5.2), 132 (12.6), 131 (19.9), 130 (5.5), 121 (4.4), 120 (20.5), 119 (19.2), 118 (39.7), 117 (13.8), 116 (6.7), 105 (8.9), 104 (6.7), 92 (6.9), 91 (75.4), 90 (9.5), 89 (11.8), 81 (4.4), 79 (7.0), 78 (6.9), 77 (16.1), 70 (16.9), 65 (25.3), 51 (4.6), 42 (17.4), 41 (4.8).

*5.1.4.3. 8-(4-Methoxyphenyl)-4-oxo-4,6,7,8-tetrahydroimidazo [2,1-c][1,2,4]triazine-3-carbohydrazide (**13**).* Recrystallization from DMF; yield 65% (method ii)/70% (method iii)/63% (method iv), m.p. 301–302 °C. Spectroscopic data for $C_{13}H_{14}N_6O_3$: IR (KBr) (ν, cm^{-1}): 1742 (hydrazide-C=O), 1688 (triazine-C=O), 1556 (C=N); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 3.78 (s, 3H, $-OCH_3$), 4.17 (br s, 4H, $2CH_2$), 4.58 (s, 2H, NH_2), 7.02 (d, $J = 8.5$ Hz, 2H, ar: H-2′ and H-6′), 7.73 (d, $J = 8.5$ Hz, 2H, ar: H-3′ and H-5′), 9.67 (s, 1H, NH); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS): 40.5 (imidazolidine-C-6), 46.0 (imidazolidine-C-7), 55.3 (-CH$_3$O), ar C: [114.1 (2CH), 121.5 (2CH), 131.2 (C), 156.2 (C)], 143.0 (triazine-C-4), 151.1 (triazine-C-3), 152.4 (hydrazide-C=O), 161.0 (C-8a); EI-MS [70 eV, *m/z* (%)]: 303 (11.7), 302 (M^+, 74.6), 287 (10.8), 274 (12.1), 273 (15.6), 272 (98.1), 271 (100.0), 245 (5.8), 244 (10.2), 243 (5.7), 228 (6.0), 217 (12.8), 216 (41.8), 215 (15.3), 189 (25.5), 188 (44.2), 175 (5.4), 174 (18.6), 173 (7.3), 162 (37.0), 161 (34.8), 160 (6.3), 158 (9.4), 149 (6.8), 148 (10.4), 147 (20.1), 146 (7.2), 136 (13.5), 135 (28.6), 134 (22.4), 133 (18.3), 132 (8.2), 121 (10.8), 120 (48.1), 118 (5.5), 117 (6.1), 108 (9.5), 107 (7.1), 105 (7.2), 104 (6.5), 92 (18.8), 91 (6.3), 78 (8.4), 77 (20.6), 70 (9.8), 65 (9.8), 64 (8.9), 63 (6.3), 54 (5.4), 42 (13.4).

*5.1.4.4. 8-(3-Chlorophenyl)-4-oxo-4,6,7,8-tetrahydroimidazo [2,1-c][1,2,4]triazine-3-carbohydrazide (**14**).* Recrystallization from DMF; yield 68% (method ii)/73% (method iii)/64% (method iv), m.p. 239–241 °C. Spectroscopic data for $C_{12}H_{11}ClN_6O_2$: IR (KBr) (ν, cm^{-1}): 1739 (hydrazide-C=O), 1685 (triazine-C=O), 1553 (C=N); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 4.20 (br s, 4H, $2CH_2$), 4.59 (s, 2H, NH_2), 7.24–7.73 (m, 4H, ar-H), 9.70 (s, 1H, NH); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS): 40.5 (imidazolidine-C-6), 45.3 (imidazolidine-C-7), ar C: [117.3 (CH), 118.8 (CH), 123.7 (CH), 130.6 (CH), 133.4 (C), 139.8 (C)], 144.3 (triazine-C-4), 150.8 (triazine-C-3), 152.4 (hydrazide-C=O), 160.8 (C-8a); EI-MS [70 eV, *m/z* (%)]: 308 (18.8), 307 (9.9), 306 (M^+, 59.7), 279 (6.2), 278 (43.2), 277 (39.0) 276 (100.0), 275 (86.2), 250 (8.3), 249 (8.6), 248 (24.0), 222 (10.8), 221 (12.8), 220 (32.2), 219 (29.2), 212 (8.8), 211 (33.4), 194 (14.2), 193 (9.2), 192 (35.1), 184 (9.2), 179 (7.1), 178 (14.5), 168 (6.4), 167 (10.1), 166 (19.5), 165 (27.3), 158 (9.7), 157 (8.8), 152 (7.4), 142 (7.8), 141 (6.2), 140 (29.9), 139 (15.0), 138 (36.4), 125 (10.9), 117 (10.9), 113 (20.2), 112 (8.2), 111 (63.8), 99 (12.0), 90 (7.8), 81 (12.4), 77 (8.1), 76 (7.3), 75 (29.2), 70 (39.0), 63 (6.6), 54 (7.7), 42 (33.8).

*5.1.4.5. 8-(3,4-Dichlorophenyl)-4-oxo-4,6,7,8-tetrahydroimidazo [2,1-c][1,2,4]triazine-3-carbohydrazide (**15**).* Recrystallization from DMF; yield 70% (method ii)/66% (method iii)/61% (method iv), m.p. 157–158 °C. Spectroscopic data for $C_{12}H_{10}Cl_2N_6O_2$: IR (KBr) (ν, cm^{-1}): 1739 (hydrazide-C=O), 1683 (triazine-C=O), 1554 (C=N); ^{1}H NMR (δ, ppm, DMSO-d_6, TMS): 3.60 ($J = 8.5$ Hz, $J' = 7.5$ Hz, dd, 2H, CH_2), 4.03 ($J = 8.6$ Hz, $J' = 7.5$ Hz, dd, 2H, CH_2), 4.40 (s, 2H, NH_2), 7.46–8.26 (m, 3H, ar-H), 9.40 (s, 1H, NH); ^{13}C NMR (δ, ppm, DMSO-d_6, TMS): 40.3 (imidazolidine-C-6), 47.0 (imidazolidine-C-7), ar C: [118.2 (CH), 120.2 (C), 123.2 (CH), 129.9 (CH), 130.8 (C), 140.2 (C)], 144.8 (triazine-C-4), 150.2 (triazine-C-3), 152.5 (hydrazide-C=O), 161.2 (C-8a); EI-MS [70 eV, *m/z* (%)]: 342 (11.3), 340 (M^+, 18.4), 312 (25.7), 311 (23.2), 310 (35.4), 309 (31.8), 256 (12.1), 255 (11.2), 254 (16.5), 253 (10.6), 245 (11.7), 230 (13.7), 228 (23.6), 226 (11.0), 189 (11.0), 187 (18.7), 186 (18.5), 178 (11.6), 177 (13.7), 176 (53.1), 175 (26.6), 174 (96.3), 173 (15.4), 172 (20.8), 165 (12.0), 163 (56.6), 162 (11.5), 161 (100.0), 159 (12.5), 151 (11.1), 147 (20.0), 145 (32.0), 143 (13.2), 139 (15.0), 133 (11.8), 126 (14.8), 125 (14.2), 111 (17.8), 109 (25.9), 99 (18.7), 90 (18.6), 75 (14.5), 74 (10.9), 70 (22.7), 69 (13.4), 63 (18.9), 62 (10.6), 43 (15.8), 42 (32.8), 41 (11.1).

5.2. Inhibition of tumour cell growth assay

The newly synthesized heterobicyclic hydrazides of the type **11**–**14**, with exception of **15**, due to its incomplete solubility, were evaluated for their antitumoural properties *in vitro* towards three human cancer cell lines: human Caucasian colon

adenocarcinoma (LS180), human uterus cancer (SiHa) and human breast carcinoma (T47D). Moreover, two normal cell lines – HSF (human skin fibroblast) cells and Vero (Green Monkey Kidney cells) were included in the cytotoxicity study.

In the current protocol each cell line was inoculated at 10^4 cells per mL density and preincubated on a microtiter plate. Test agents were then added at double examined concentrations (10 and 50 μg mL^{-1}) and culture incubated for 72 h. End-point determinations were made with 5-bromo-2′-deoxy-uridine (BrdU) labeling and detection kit III [40–43] on Elisa reader (BIO-TEC Instruments USA).

The growth percentage was evaluated spectrophotometrically versus untreated controls with used cell viability of growth assay. Results for each spectrophotometrical measure were noticed as per cent of growth inhibition. All the investigated compounds were dissolved in DMSO prior to dilution into the biological assay. All experiments were done in triplicates. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin, Poland.

5.3. Detection of induction of DNA damage in the tested cell lines after incubation with heterobicycle **14**

Following treatment of heterobicyclic hydrazide **14** (32.6 μM), cells were washed twice with phosphate buffered saline (PBS). In the next step the DNA was isolated by using the lysing buffer and proteolytic enzyme – proteinase K [44]. After purification, DNA samples were spread over 1% agarose gel with addition of propidium iodide. Electrophoresis was performed at 120 V for 1.5 h at the room temperature. All experiments were repeated three times. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin, Poland.

5.4. Apoptosis assay

To estimate the apoptotic effect of compound **14** on the normal cell line – HSF cells and human breast carcinoma (T47D) cells, the annexin V–FITC apoptosis detection kit (Sigma) was used. The procedure consists of the binding of annexin V, having a strong Ca^{2+}-dependent affinity for phosphatidylserine (PS) in the membrane of cells, which is the beginning the apoptotic process and the binding of propidium iodide to the cellular DNA in cells where the cell membrane is totally compromised [45–49]. For apoptosis quantitation, the amount of apoptotic cells could be observed under a confocal microscope Nicon Eclipse (Zeiss, Germany) by using a filter $\lambda = 460–490$ nm. A total number of 1000 cells in three independent measurements were subjected to analysis. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin, Poland.

5.5. Action of novel heterobicycles (**6–14**) on the viability of human leukaemic Jurkat cells

Human peripheral blood leukaemia, T cell line (Jurkat) was obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław. The experiments were conducted according to the previously described procedure [38] and are the following. Cells were maintained in RPMI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine and 10 μg mL^{-1} gentamycin. Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2. Cells were passaged three times weekly and maintained at a density of 2×10^5 cells per mL. Cells used in the experiment were in logarithmic growth phase. The medium used for experiment had the same constituents as that used for cell passage, unless otherwise indicated.

MTT reduction cell viability assay was performed using Jurkat cells cultured in 96-well plates. Compounds **6–14** were dissolved in DMSO prior to dilution into the biological assay. Heterobicycle **15** was not tested due to its incomplete solubility. The effect of the examined concentrations of heterocycles **6–14** on the cell viability was estimated by a MTT based colorimetric assay (the succinate dehydrogenase inhibition, SDI test) described by Takenouchi and Munekata [50]. The metabolic activity was measured in cell populations via incubation with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) that was reduced into a coloured, water-insoluble formazan salt by viable cells. Additions were made to the culture medium for 24 h. Cells were washed twice with HEPES-buffered incubation medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5.5 mM glucose and 20 mM HEPES, pH 7.4), and incubated for 45 min at 37 °C in HBM containing MTT (0.5 mg mL^{-1}). After this period, the HBM was carefully removed and the blue formazan product was solubilized in 300 μL of 100% DMSO. The absorbance of each well was read in an ELISA microplate reader at 570 nm. The obtained results were presented as percentage of cell viability in comparison to control. The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland.

5.6. Behavioural experiments

Behavioural experiments were performed on male Albino Swiss mice (body weights of 20–25 g) purchased from licensed dealer, Górzkowska, Warsaw, Poland. Eight to ten animals were kept in a cage, at room temperature of 20 ± 1 °C, on a 12/12 h light–dark cycle (light on from 7 a.m. to 7 p.m.). Standard food (Bacutil, Motycz, Poland) and water were available ad libitum. Experimental and control groups consisting of 10 animals each were selected by means of a randomized schedule. The experiments were performed between 8 a.m. and 3 p.m. The investigated compounds (**10** and **11**) were administered intraperitoneally (i.p.) as suspension in 3% Tween 80 in a constant volume of 10 mL kg^{-1}. Control animals received the equivalent volume of solvent. Each experimental group consisted of ten animals.

The investigations were carried out in the Department of Toxicology, Medical University, Lublin, Poland.

5.6.1. Acute toxicity in mice for compounds **10** and **11**

Acute toxicity was assessed according to Litchfield and Wilcoxon methods [51] and presented as LD_{50} calculated from the mortality of mice after 24 h.

*5.6.2. The influence of heterobicycles **10** and **11** on the motor impairment in mice*

Motor coordination was evaluated in the rota-rod test [52].

*5.6.3. Pain reactivity in the ''writhing syndrome'' test in mice for compounds **10** and **11***

Pain reactivity was measured in mice by the ''writhing syndrome'' test of Witkin et al. [53]. The test was performed in mice by the intraperitoneal injection of 0.6% solution of acetic acid in a volume of 10 mL kg^{-1} 30 min after the administration of the investigated compounds **10** and **11** at a dose of 0.1 LD_{50}. The number of writhing episodes was counted for 30 min after the injection of acetic acid.

References

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