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Identification of antitumour activity of novel derivatives of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-dione and 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo-[2,1-*c*][1,2,4]triazin-3(6*H*)-one

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Abstract—The series of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones (**11**–**20**) and 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-ones (**21**–**25**) were designed and their in vitro cytotoxic activities against human LS180, HeLa, T47D, A549 and RPMI 8226 carcinoma cells are presented. In the crystalline state molecule **12** exists as the predominant tautomeric 3-oxo form, whereas the second possible 3-hydroxy tautomer is not observed. Compound **19** revealed a strong affection to LS180 cancer cells at lower tested concentration (37.9 μM) and simultaneously was found to be non-toxic towards the normal cell line investigated—GMK cells. Furthermore, this compound was proved to possess the efficiency for DNA strand breakage of the examined cancer cell lines. However, imidazotriazin-3,4-dione **20** was able to cause significant viability decreases in human RPMI 8226 peripheral blood myeloma cells. Compound **22** has exhibited remarkable inhibitory effects against LS180 and A549 carcinoma cells, whereas **24** revealed the highest growth inhibition against A549 cell line. Simultaneously, at lower tested concentration these compounds were proved to be completely non-toxic for GMK cells. Moreover, cytotoxic and antibacterial properties of starting, tautomeric 1-aryl-2-hydrazonoimidazolidines (**1**–**6** and **8**–**9**) are presented. Six of them (**1**–**2**, **4**–**6** and **9**) proved active as antimicrobials. All these compounds revealed MIC values in the range of 15.0–78.6 μM. Their activities were compared to those of ampicillin and chloramphenicol.

1. Introduction

The tautomeric 1-aryl-2-hydrazinoimidazolines (1-aryl-2-hydrazonoimidazolidines) as monocyclic amidinohydrazines are useful building blocks for the synthesis of various heterobicyclic nitrogen systems of biological interest.[1–3] Moreover, the tautomeric amidinohydrazone group occurs in the molecules of the following anticancer drugs: bisanthrene, mitoguazone and ambazone.[4–8] Furthermore, intercalating antineoplastic bisanthrene, therapeutically used against breast cancer, adult acute non-lymphotic leukaemias and lymphomas,[8–10] is structurally based on the 2-hydrazino-Δ^2-imidazoline heterocyclic system. This drug was found to be less cardiotoxic than other intercalators such as mitoxantrone and adriamycin.[7,11] An imidazolidine ring occurs in the molecule of the tumour growth inhibiting 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine).[7,12] Previously reported 2-hydrazinoimidazolines with various substituents in the hydrazine residue showed anticancer activities with IC_{50} values in the range of 15.5–100 μM.[7]

Keywords: 8-Aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones; A novel DNA strand breaking agent; Crystal structure; 8-Aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-ones; Anticancer activity; 1-Aryl-2-hydrazonoimidazolidines; Cytotoxic activity; Antibacterial activity.
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The 1,2,4-triazine ring is a prominent structural motif found in numerous pharmacologically active compounds. For instance, certain azanucleosides (6-azacytosine and 6-azauracil), structurally based on the 1,2,4-triazine heterocyclic system, were proved to display antitumour,[13,14] antiviral[15,16] and antifungal[17] activities. 6-Azaisocytosine (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. Moreover, azaribine—the well-known antiviral drug is structurally associated with the 1,2,4-triazine moiety.[18] Condensed 1,2,4-triazines found application as pharmaceuticals, herbicides, pesticides and dyes.[19–24]

The synthesis of heterobicyclic systems, containing the 1,2,4-triazine moiety, has gained much attention because of their significant biological effects reported in the literature. Well-known, pyrrolo[2,1-*f*][1,2,4]triazines, congeners of substituted nucleic acid purines, showed an interesting broad spectrum of 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.[25,26] Some of pyrazolo[5,1-*c*][1,2,4]triazines exhibited antitumour and antifungal activity. It is noteworthy that many potential anticancer and antiviral drugs have been modelled on them.[27–32]

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 new bicyclic nucleosides related to 6-azaisocytosine,[33] while the others displayed the distinctly marked lower cytotoxicity towards normal cells and twofold higher against cancer cell lines.[3,34]

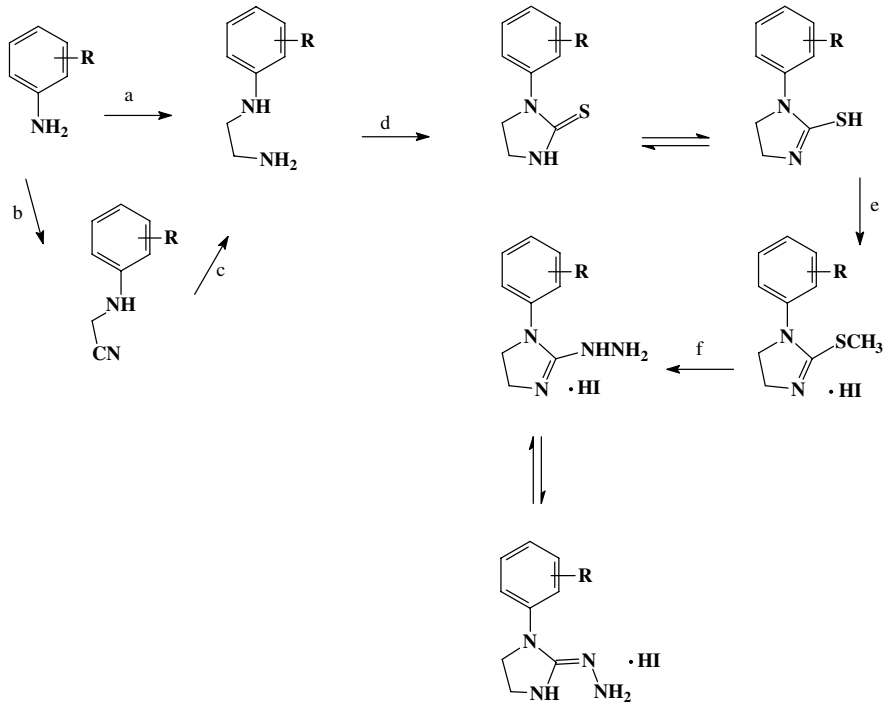
Prompted by these reports, in continuation of our search for bioactive molecules,[1–3,34–38] and having in mind the promising anticancer activities showed by previously obtained 1,2,4-triazine fused heterocycles,[3,25–32] it seemed worthwhile to synthesize some novel heterobicyclic derivatives incorporating the above-mentioned 1,2,4-triazine and imidazolidine moieties as expected pharmacophores.

In this paper we would like to present the results of X-ray crystal structure determination of **12** and in vitro antitumour activities of novel derivatives of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-dione (**11**–**20**) and 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (**21**–**25**). Furthermore, we would like to mention here the results of cytotoxicity studies of potentially active, monocyclic amidinohydrazines, in which the amidine moiety is incorporated into the imidazoline ring, that is, tautomeric 1-aryl-2-hydrazinoimidazolines without substituents in the hydrazine residue. These ones were used as building blocks and intermediates in the synthesis of final products. Moreover, six of them (**1**–**2**, **4**–**6** and **9**) proved active as antimicrobial agents.

2. Chemistry

The synthetic pathway used in the preparation of the title compounds was previously described by us[1,39] and is shown below (Schemes 1 and 2). The starting biologically active 1-aryl-2-hydrazonoimidazolidines (1-aryl-2-hydrazinoimidazolines) used in this study were prepared by patent pending according to Sztanke[40] from 1-aryl-2-methylthioimidazolines by a previously reported method.[1,41] The four-step synthetic pathway for the preparation of starting hydrazones was achieved by a sequence of reactions starting from the respective anilines and is outlined in Scheme 1. Thus in the first step, commercially available anilines were converted into *N*-arylethylenediamines by the Lehmann method[42] or by the classical Knoevenagel and Mercklin method with Takeda modification.[43,44] Their further condensation with carbon disulfide in the xylene medium led to the formation of intermediates—dithiocarbaminic acid derivatives, which could easily be cyclized in boiling solvent under reaction conditions to 1-arylimidazolidine-2-thiones (1-aryl-2-mercaptoimidazolines) with concomitant loss of hydrogen sulfide molecule. Because of existence of thiol–thione tautomerism, the alkylation of respective thiols with one equivalent methyl iodide was possible and afforded 1-aryl-2-methylthioimidazolines in 75–85% yields, which in turn were refluxed with hydrazine hydrate to obtain 1-aryl-2-hydrazinoimidazolines (1-aryl-2-hydrazonoimidazolidines) in good yields (65–76%).[2,34] Finally these compounds were used as the intermediates in the synthesis of reported here imidazotriazines (**11**–**25**) (Scheme 2).

Treatment of 1-aryl-2-hydrazonoimidazolidines with diethyl oxalate in refluxing *n*-butanol afforded the corresponding 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones (**11**–**20**). The course of reaction includes the formation of intermediate chain derivatives—products of the acylation of appropriate 1-aryl-2-hydrazonoimidazolidine by one ester group of the diethyl oxalate (with concomitant loss of ethanol molecule) and finally the reaction leads to formation of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-dione derivatives with loss of ethanol molecule. The condensation reaction can be carried out from free base (method i), or hydroiodide in the triethylamine presence (method ii). In the case of compounds investigated the amido-imido tautomerism is possible. The tautomeric equilibrium was dependent on the substituent present in the N-8 aromatic ring.[1,45] Molecular structure of the heterobicyclic system in the crystalline state has been confirmed by X-ray crystallography of the compound **12**. Perspective view of the molecule **12** with atom numbering is shown in Figure 1. Therefore, the bicyclic imidazo[2,1-*c*][1,2,4]triazine ring is the 2,6,7,8-tetrahydro-3,4-dioxo structure, the compound being 8-(2-methylphenyl)-



Scheme 1. Synthetic pathway for the preparation of starting 1-aryl-2-hydrazonoimidazolidines. Reagents and conditions: (a) aziridine, $AlCl_3$, dry toluene; (b) HCHO, $Na_2S_2O_5$, NaCN, water, reflux; (c) H_2, NiRa, MeOH/NH_3, 100 °C; (d) CS_2, xylene, rt, 20 min, reflux, 7 h; (e) CH_3I/MeOH, rt, 48 h, reflux, 6 h; (f) hydrazine hydrate/MeOH, reflux, 24 h.

2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3, 4-dione (**12**), and not tautomeric 3-hydroxy-8-(2-methylphenyl)-7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one.

New derivatives of 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (**21**–**25**) were obtained in the reaction of appropriate 1-aryl-2-hydrazonoimidazolidines with ethyl oxamate. The above-mentioned reaction can be carried out either by starting from 1-aryl-2-hydrazonoimidazolidine hydroiodide in the presence of triethylamine (method ii), or from free base (method i) with comparable yields. Both reactions were carried out by heating under reflux in alcoholic medium for 4–9 h. Reaction conditions were established experimentally. The scrutiny of ^{1}H NMR spectra confirms that under the reaction conditions, the formation of the fused imidazo[2,1-*c*][1,2,4]triazine system is accompanied with concomitant loss of ethanol and water molecule[39] as is illustrated in Scheme 2.

The ^{1}H NMR, ^{13}C NMR and MS spectral data of the final imidazotriazine-3,4-diones (**11**–**20**) and imidazotriazin-3(6*H*)-ones (**21**–**25**), respectively, obtained by the two above-mentioned methods were also identical.

In view of continuous and widespread interest in the design and the synthesis 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 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3, 4-diones (**11**–**20**) and 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin- 3(6*H*)-ones (**21**–**25**) might be considered as a useful method for preparation of these biologically active compounds because of the affordability of the starting materials, good yields obtained and straightforward product isolation.

The physicochemical and biological properties of the tested imidazotriazines (**11**–**25**) are presented in Table 1.

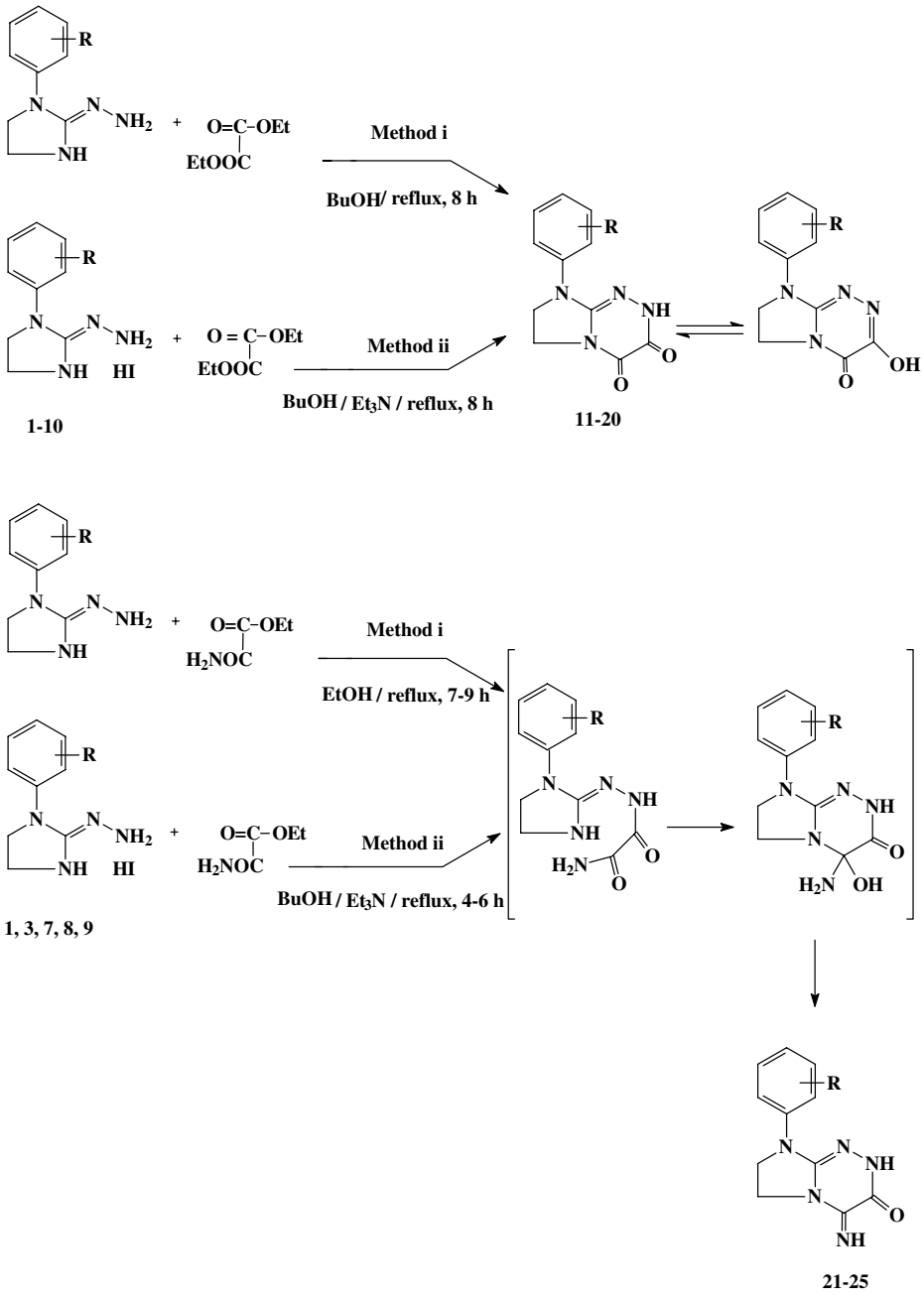
Antibacterially active intermediate tautomeric 1-aryl-2-hydrazonoimidazolidine hydroiodides (**1**–**2**, **4**–**6**, and **9**) were obtained by a previously reported method.[1,41]

Although in the case of the compounds investigated the hydrazone–hydrazine tautomerism is possible but in solution (DMSO-d_6) of **2** only the presence of the hydrazone tautomer was observed. Thus, in the ^{1}H, ^{1}H COSY spectrum of free base of **2** the correlation between endocyclic-NH proton signal derived from the imidazolidine ring at position 3 and the H-4 methylenic proton signals was 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 (–*NH*NH_2) from possible hydrazinic structure and the C-2 carbon. The HMBC and HMQC NMR spectroscopic data for compound **2** are presented in Table 2.

3. Results and discussion

3.1. Cytotoxic and antibacterial activities of the synthesized intermediates

The cytotoxic activities of the synthesized intermediates **1**, **3**, **5**–**8** were determined in vitro against several human



Scheme 2. Synthetic route to obtain final imidazotriazines. **1**: R = H; **2**: R = 2-CH_3; **3**: R = 4-CH_3; **4**: R = 2-CH_3O; **5**: R = 4-CH_3O; **6**: R = 2,3-$(CH_3)_2$; **7**: R = 2-Cl; **8**: R = 3-Cl; **9**: R = 4-Cl; **10**: R = 3,4-Cl_2; **11**: R = H; **12**: R = 2-CH_3; **13**: R = 4-CH_3; **14**: R = 2-CH_3O; **15**: R = 4-CH_3O; **16**: R = 2,3-$(CH_3)_2$; **17**: R = 2-Cl; **18**: R = 3-Cl; **19**: R = 4-Cl; **20**: R = 3,4-Cl_2; **21**: R = H; **22**: R = 4-CH_3; **23**: R = 2-Cl; **24**: R = 3-Cl; **25**: R = 4-Cl.

tumour cell lines such as: LS180 (colon), HeLa (cervix epitheloid) and T47D (breast). Moreover, one normal cell line—Vero (ECACC 88020401, African Green Monkey Kidney cell line, GMK clone) was included in the cytotoxicity study. Results for each test compound are reported as the growth inhibition percentage of the treated cells when compared to the untreated control ones. Compounds which reduced the growth are passed on for evaluation towards three human cancer and one normal cell lines. All the tested intermediates **1**, **3**, **5**–**8** revealed a certain degree of cytotoxicity against cancer cells. Simultaneously, at a lower concentration the investigated compounds, with exception of **6**, were found to be non-toxic towards the examined normal cell line—Green Monkey Kidney (GMK) cells. As shown in Table 3, the most sensitive tumour cell line was T47D human breast cancer cell line, against which the examined intermediates **3**, **5**–**7** showed 12–27% growth inhibition. Furthermore, their growth inhibitory potencies seemed to be concentration-dependent. Compounds **3**, **5**–**8** have shown 4–15% growth inhibitions against LS180 human colon adenocarcinoma cells. The most resistant tumour cell line was the HeLa cervix epitheloid carcinoma cell line, against which the examined compounds were proved to be quite ineffective (**5** and **8**) or nearly ineffective (**1**, **3**, **6**, and **7**).

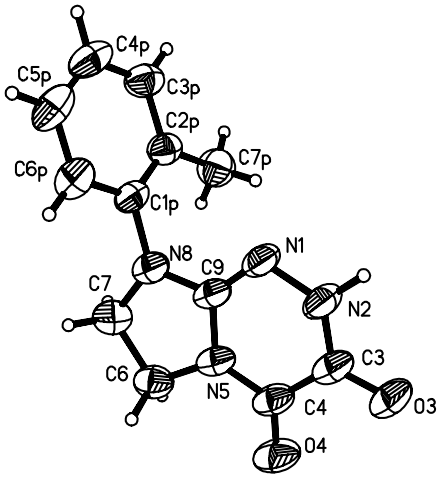
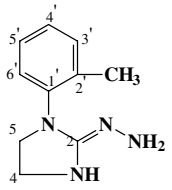


Figure 1. View of the molecule **12**. Bond distances within the heterocyclic ring system are (Å): N1–N2 1.397(3); N2–C3 1.340(3); C3–O3 1.243(3); C3–C4 1.487(4); C4–O4 1.216(3); C4–N5 1.369(3); N5–C6 1.457(3); C6–C7 1.528(4); C7–N8 1.449(3); C9–N8 1.358(3); N1–C9 1.284(3); C9–N5, 1.379(3).

On the basis of the obtained data, it is possible to conclude that the examined tautomeric intermediates, in which the amidine moiety of the amidinohydrazine group is incorporated into the imidazoline ring, display only very weak (**1** and **8**) or weak (**3** and **5**–**7**) cytotoxicity, in contrast with previously reported amidinohydrazines of imidazoline with various substituents in the hydrazine residue.[7]

Table 2. The HMBC and HMQC NMR spectroscopic data for free base of compound **2**



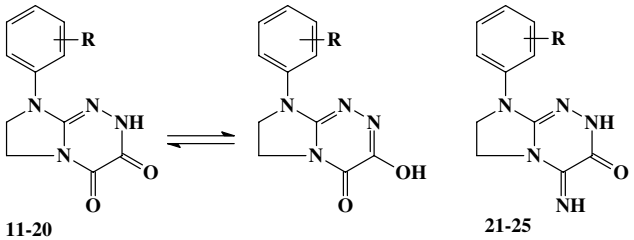
	HMBC correlations	HMQC correlations
C-2	H-4, H-5	—
C-4	H-4, H-5	H-4, H-6(w)
C-5	H-4, H-5	H-4(w), H-5
C-1′	H-4′(w), H-5′(w), H-6′	—
C-2′	H-3′	—
C-3′	H-4′, H-5′	—
C-4′	H-3′, H-6′	—
C-5′	H-4′(w), H-6′	H-5′
C-6′	H-4′, H-5′	H-6′

w, weak correlation.

On the contrary, the intermediates **1**–**2**, **4**–**6**, and **9** were proved to be antibacterially active. Determination of the in vitro antibacterial activity of the investigated intermediates was performed using the microdilution method, according to the National Committee for Clinical Laboratory Standards (NCCLS).[46,47]

The microdilution method for estimation of MIC values (the lowest concentration of compound required

Table 1. Physicochemical and biological properties of tested imidazotriazine-3,4-diones (**11**–**20**) and imidazotriazin-3(6*H*)-ones (**21**–**25**)



11-20 **21-25**

Compound	R	Yield (%)		Mp (°C)	Solubility[a] (mg L^{-1})	log *P*[b]	LD_{50}[1] (mg kg^{-1})
		(i)	(ii)				
11	H	62	61	311–316	6371	0.58	1400
12	2-CH_3	64	60	241–243	1821	0.74	nd
13	4-CH_3	67	61	330–332	1821	0.87	1800
14	2-CH_3O	60	58	248–251	3713	0.60	1100
15	4-CH_3O	68	63	302–303	3713	0.66	1500
16	2,3-$(CH_3)_2$	63	60	307–310	518.9	0.77	nd
17	2-Cl	58	54	307–309	1158	1.03	nd
18	3-Cl	59	57	316–318	1158	0.78	>2000
19	4-Cl	72	60	346–349	1158	1.28	>2000
20	3,4-Cl_2	62	58	323–324	206.8	1.67	nd
21	H	62	68	307–310	7562	0.51	nd
22	4-CH_3	67	73	297–299	2162	0.70	nd
23	2-Cl	64	65	215–218	1375	0.92	nd
24	3-Cl	56	60	253–256	1375	0.99	nd
25	4-Cl	57	63	282–284	1375	1.15	nd

LD_{50}, 50% lethal dose, the amount of a solid material, given all at once, which causes the death of 50% of a group of test animals; the acute toxicity of the examined imidazotriazine-3,4-diones was assessed in mice according to Litchfield and Wilcoxon.[63] nd, not determined.

[a] Water solubility values were calculated using EPI Suite for Windows v. 3.11. USEPA, Washington, DC, 2000.

[b] log *P* values were calculated using Pallas 3.1.1.2. software (distributed by CompuDrug) 2003.

Table 3. Inhibition of in vitro normal and tumour cell growth by the investigated intermediates

Cell lines	Cytotoxicity (growth inhibition in %)											
	1		**3**		**5**		**6**		**7**		**8**	
	I	II	I	II	I	II	I	II	I	II	I	II
Normal												
GMK	0	0	0	9	0	0	16	14	0	0	0	10
Cancer												
LS180	1	0	11	15	4	9	6	0	7	0	8	0
HeLa	6	3	3	0	0	0	8	0	2	0	0	0
T47D	0	0	12	17	17	23	21	25	22	27	5	0

Normal cell line: Vero (GMK, ECACC 88020401, African Green Monkey Kidney cells). Cancer cell lines: LS180 (ECACC 87021202)—human Caucasian colon adenocarcinoma cells. HeLa (ECACC 93021013)—human Negroid cervix epitheloid carcinoma. T47D (ECACC 85102201)—human breast carcinoma cells. I—concentration of 10 $\mu g\,mL^{-1}$, which corresponds to following concentration values: 32.9 μM (**1**), 31.4 μM (**3**), 29.9 μM (**5**), 30.1 μM (**6**), 29.5 μM (**7** and **8**). II—concentration of 50 $\mu g\,mL^{-1}$, which corresponds to following concentration values: 164.4 μM (**1**), 157.1 μM (**3**), 149.6 μM (**5**), 150.5 μM (**6**), 147.7 μM (**7** and **8**).

to inhibit the growth of the tested microorganism) was applied to evaluate their antibacterial activity. Four clinically isolated strains of bacteria—*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* (Gram-positive bacteria) and *Escherichia coli* (Gram-negative bacteria) were included in this study. The antibacterial potencies of the tested intermediates were compared with the activities of topical antibacterial drugs—ampicillin and chloramphenicol.

Six tested compounds in the present study were found to exert significant antibacterial activities against the microorganisms listed in Table 4. Thus, all the tested 1-aryl-2-hydrazonoimidazolidines (**1**–**2**, **4**–**6**, and **9**) showed high activity in relation to clinically isolated Gram-positive (*S. aureus*, *S. epidermidis*, *St. pyogenes*) and Gram-negative (*E. coli*) bacterial strains with MIC values from 15.0 to 78.6 μM. On the whole, the most potent in vitro antibacterial effect was demonstrated by compound **5** with 1-(4-methoxyphenyl) substituent at position 1 of the imidazolidine ring in relation to Gram-positive *S. aureus* and Gram-negative *E. coli* bacterial strains, with MIC values of 29.9 and 15.0 μM, respectively. Against *E. coli* strain tested, compound **5** demonstrated MIC value 2.4-fold higher than that of ampicillin and 1.2-fold lower than that of chloramphenicol. Against *S. aureus*, derivative **5** showed a MIC value 1.2-fold higher and 2.5-fold lower than that of ampicillin and chloramphenicol, respectively. Three tested compounds **5**, **6**, and **9** were found to demonstrate highly significant antibacterial effectiveness against *S. epidermidis* with comparable MIC values of 18.7, 18.8, and 18.4 μM, respectively. Their antibacterial potency was over 3.8-fold higher than that of ampicillin and over 3 times lower than that of chloramphenicol. The remaining compounds **1**, **2**, and **4** at the following MIC values of 41.1, 31.4 and 37.4 μM, respectively, inhibited the growth of *S. epidermidis*. These compounds showed superior potencies to ampicillin and inferior to chloramphenicol. All the tested compounds revealed in vitro effectiveness against *E. coli*. Furthermore, compounds **2** and **4**–**6** showed superior MIC values than that of ampicillin. All the investigated derivatives revealed effectiveness against *St. pyogenes* with MIC values from 59.1 to 78.6 μM. Among them the most potent antibacterial effect was demonstrated by compounds **5** and **9** in relation to above-mentioned bacterial strain, with MIC values 59.8 and 59.1 μM, respectively.

As a result, compound **5** was found to exhibit potent in vitro antibacterial activity with MIC value of 29.9, 18.7 and 15.0 μM against *S. aureus*, *S. epidermidis* and *E. coli*, respectively, and therefore may be considered promising for the development of new antibacterial agents.

3.2. Anticancer evaluation of the final imidazotriazines

The newly synthesized imidazo[2,1-*c*][1,2,4]triazine-3,4-diones of the type **11**–**16**, **18**–**19** and imidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-ones **21**–**25** were evaluated for their in vitro anticancer activity towards four human tumour cell lines derived from various cancer types: LS 180 (ECACC 87021202, human Caucasian colon adenocarcinoma cells), HeLa (ECACC 93021013, human Negroid epitheloid carcinoma), T47D (ECACC 85102201, human breast carcinoma cells) and A549 (ECACC 86012804, human Caucasian lung carcinoma). Moreover, one normal cell line—Vero (ECACC 88020401, African Green Monkey Kidney cell line, GMK clone) was included in the cytotoxicity study.

According to the data listed in Table 5, in the class of novel tested derivatives of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-dione the highest growth inhibitory potency was found for compound **19**, with a $\log P$ value of 1.28, having lipophilic electron-withdrawing Cl substituent at position 4 of the phenyl ring. It has shown 50% and 35% growth inhibition against LS180 colon adenocarcinoma cell line in both

Table 4. Antibacterial activity expressed as MIC (μM) of tested compounds

Microorganisms	Compound						Standard drugs	
	1	**2**	**4**	**5**	**6**	**9**	A	C
Staphylococcus aureus	41.1	39.3	37.4	29.9	37.6	29.5	35.8	12.1
Staphylococcus epidermidis	41.1	31.4	37.4	18.7	18.8	18.4	71.5	6.0
Streptococcus pyogenes	65.8	78.6	74.8	59.8	75.2	59.1	nd	nd
Escherichia coli	41.1	15.7	29.9	15.0	18.8	36.9	35.8	12.1

Standard drugs: A, ampicillin; C, chloramphenicol; nd, not determined.

Table 5. The inhibition ratio for normal and tumour cells growth by tested imidazotriazines

Compound	Cell lines									
	Normal		Cancer							
	GMK		LS180		HeLa		T47D		A549	
	I	II	I	II	I	II	I	II	I	II
11	7	30	0	12	0	0	0	0	0	17
12	0	0	0	0	0	0	0	0	24	29
13	0	0	2	0	0	11	14	22	20	31
14	26	17	30	29	5	8	1	9	0	39
15	0	10	0	46	6	0	7	21	0	0
16	18	18	0	0	0	0	21	22	0	0
18	11	24	0	0	27	38	0	0	0	0
19	0	7	50	35	2	26	5	1	0	15
21	26	30	0	1	30	34	0	3	7	38
22	0	0	32	45	0	0	0	0	37	38
23	0	2	6	2	0	0	0	0	6	16
24	0	0	0	1	6	2	3	31	36	50
25	0	0	0	5	8	0	16	27	25	27

Normal cell line: Vero (GMK, ECACC 88020401, African Green Monkey Kidney cells). Cancer cell lines: LS180 (ECACC 87021202)—human Caucasian colon adenocarcinoma cells. HeLa (ECACC 93021013)—human Negroid cervix epitheloid carcinoma. T47D (ECACC 85102201)—human breast carcinoma cells. A549 (ECACC 86012804)—human Caucasian lung carcinoma. I—concentration of 10 μg mL^{-1}, which corresponds to following concentration values: 43.5 μM (**11**), 41.0 μM (**12** and **13**), 38.4 μM (**14** and **15**), 38.7 μM (**16**), 37.9 μM (**18** and **19**), 43.6 μM (**21**), 41.1 μM (**22**), 37.9 μM (**23**–**25**). II—concentration of 50 μg mL^{-1}, which corresponds to following concentration values: 217.3 μM (**11**), 204.8 μM (**12** and **13**), 192.2 μM (**14** and **15**), 193.6 μM (**16**), 189.4 μM (**18** and **19**), 218.1 μM (**21**), 205.5 μM (**22**), 189.6 μM (**23**–**25**).

examined concentrations: 37.9 μM (10 μg mL^{-1}) and 189.4 μM (50 μg mL^{-1}), respectively. Moreover, this compound has exhibited 2% and 26% growth inhibition against HeLa cancer cells and 15% growth inhibition against A549 lung cancer cell line in higher tested concentration. Furthermore, the congener **18**, having a log*P* value of 0.78, with electron-withdrawing Cl substituent at position *meta*- of the phenyl ring, has shown 27% and 38% growth inhibition against HeLa cervix epitheloid carcinoma cell line, in both tested concentrations, respectively. Simultaneously, this compound has been found to be completely inactive against LS180 cancer cells. So, we can speculate that the presence of more lipophilic electron-withdrawing substituent, such as a chloro group at the 4-position of the phenyl ring (in **19**), is important for the growth inhibitory activity against LS180 carcinoma cells.

Similarly, compound **14**, with a log*P* value of 0.60, bearing strong electron-donating OCH_3 group at *ortho*-position of the phenyl ring, has exhibited 30% and 29% growth inhibition against LS180 colon adenocarcinoma cells in both the examined concentrations, respectively, and 39% growth inhibition against A549 lung carcinoma cell line in higher tested concentration. Besides, its slightly inhibitory effects towards HeLa and T47D cancer cells were noticed. In this case, the 2-OCH_3 position in the phenyl ring appears to be a factor that influenced growth inhibition since 4-OCH_3 derivative (**15**), with a log*P* value of 0.66, was proved to reveal lower growth inhibitory activity in comparison to **14**. Derivative **15** has exhibited potency against LS180 cancer cells (46% growth inhibition) but only at higher applied concentration. Furthermore, this compound has exhibited 7% and 21% growth inhibition against T47D breast cancer cell line in both examined concentrations, respectively, and 6% growth inhibition against HeLa cancer cells in a lower tested concentration.

The unsubstituted phenyl derivative **11**, having a log*P* value of 0.58 and the highest solubility profile, has exhibited lower activity than compounds with substitution of aryl ring (**12**–**19**). This compound has shown 17% and 12% growth inhibition against A549 and LS180 cancer cells, respectively, in higher tested concentration.

It should be noted that 2-methyl analogue (**12**), having lipophilic weak electron-donating substituent, such as a methyl group at position 2 of the phenyl ring, and a log*P* value of 0.74, has shown 24% and 29% growth inhibition against A549 lung carcinoma cell line. Furthermore, this compound was found to be completely inactive against all the other investigated cancer cells and simultaneously, non-toxic for normal cell line—GMK cells. It is noteworthy in this case that the replacement of *ortho*-CH_3 group in **12** (log*P* value of 0.74) with *para*-CH_3 in **13** rendered this compound a bit more lipophilic (log*P* value of 0.87). The position in the phenyl ring caused noticeable changes in its growth inhibitory properties against T47D and HeLa carcinoma cell lines and did not influence its toxicity towards A549 carcinoma cells. The introduction of a second methyl substituent to the 2-methyl derivative (**12**) (such as in case of 2,3-dimethyl derivative **16**, having a log*P* value of 0.77) manifested noticeable increase in activity against T47D breast cancer cells but also caused significant increase in toxicity towards GMK cells.

From the point of a structure–activity relationship in the class of the examined novel 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones (**11**–**16** and **18**–**19**), the results obtained reveal that the difference of activity is due to various substituents on the aromatic phenyl ring. An antiproliferative activity increased as a result of the replacement of hydrogen, 2-methyl, 4-methyl, 2-methoxy, 4-methoxy, 2,3-dimethyl and 3-chloro

substituents in **11**, **13**–**16**, **18**, respectively, with a 4-chloro substituent in **19**.

Furthermore, DNA damage in the examined cancer cell lines (T47D, HeLa, LS180) after incubation with the most potent imidazo[2,1-*c*][1,2,4]triazine-3,4-dione of the type **19** in a concentration of 37.9 μM was analyzed using pulsed field gel electrophoresis through a 1% agarose gel. This compound was found to be effective in inducing DNA damage. The additional DNA fragments of different number of base pairs appeared in the tested cancer cell lines in comparison to DNA of control cells, suggesting that compound **19** induced DNA cleavage (Fig. 2). 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.[48,49] In conclusion, the tested imidazotriazine-3,4-dione of the type **19** revealed the damaging influence on DNA molecules of cancer cells (Fig. 2). The examined heterobicycle was found to possess the efficiency for DNA strand breakage of cancer cell lines such as the cytotoxic antibiotic—bleomycin,[50] 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 **19** can have dramatic effects on higher-order chromatin structure because of its supercoiling and tight packaging within the nucleus.

As shown in Table 5, in the class of novel tested derivatives of 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin- 3(6*H*)-one, the highest growth inhibitory potency was proved for compound **24**, with a log *P* value of 0.99, having lipophilic electron-withdrawing substituent, such as a chloro group at position *meta*- of the phenyl ring. It has shown 36% and 50% growth inhibition against human A549 non-small lung carcinoma cell line at both examined concentrations: 37.9 and 189.6 μM, respectively. Furthermore, a significant level of activity revealed compound **22** with a log *P* value of 0.70, having lipophilic, weak electron-donating substituent such as a methyl group at position *para*- of the phenyl ring. It has shown 32% and 45% growth inhibition against LS180 human colon adenocarcinoma cell line and 37% and 38% growth inhibition against A549 lung carcinoma cells at both tested concentrations. Simultaneously, both these derivatives were proved to be completely non-toxic towards a normal cell line—GMK cells.

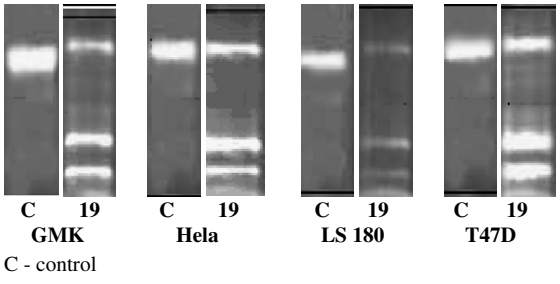


Figure 2. Induction of DNA damage in the tested cell lines after incubation with 8-(4-chlorophenyl)-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3, 4-dione (**19**) at concentration of 37.9 μM. Cells, prepared as agarose plugs, were lysed and subjected to pulsed field gel electrophoresis through a 1% agarose gel with propidium iodide.

Replacement of electron-withdrawing *meta*-Cl group in derivative **24** for either of similar lipophilicity and electron-withdrawing Cl substituents at positions *ortho*- (**23**) and *para*- (**25**) of the phenyl ring, respectively, results in reductions in activity against the investigated cancer cells (Tables 2 and 5). Imidazotriazinone **23**, having the lowest log *P* value of 0.92, revealed lower inhibitory effects than its slightly higher lipophilic congeners **24** and **25**. It is worth pointing out that compounds with substitution at phenyl ring (**22**–**25**) were practically non-toxic towards a normal cell line—GMK cells.

Unsubstituted analogue **21**, having the lowest log *P* value of 0.51 and the highest solubility profile (Table 2), was found to be active against HeLa cervix epitheloid carcinoma and A549 lung carcinoma. It has shown 30% and 34% growth inhibition against HeLa cancer cells, and 7% and 38% growth inhibition against A549 lung carcinoma cells in both tested concentrations, respectively. Similarly, this compound was found to express the distinctly marked toxicity towards GMK cells (Table 5).

It is noteworthy that in case of the tested 4-oxo and 4-imino derivatives of 8-phenyl- and 8-(4-methylphenyl)-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (**11**, **21** and **13**, **22**), growth inhibition increases appeared as a result of the conversion of the 4-oxo group present in the position 4 of heterobicycles **11**, **13** into the 4-imino one located in the same position (in compounds **21** and **22**). The 4-imino group present in **21**, **22** rendered these compounds highly soluble and hydrophilic. Probably, better water solubility profile, the decrease of lipophilicity and increasing percentage of nitrogen element, as observed in Table 6, could be a qualitative explanation of profitable changes in their growth inhibitory properties in relation to **11**, **13**.

We have also analyzed the percentage of viable normal human skin fibroblasts (HSF) and RPMI 8226 cancer cells following 24-h treatment with the tested concentrations (1, 50 and 100 μM) of imidazotriazines (**11**–**15**, **18**–**21** and **23**–**25**). We have found out that in this series an antitumour action is sensitive to minor structural modifications. Compounds with the best potency against RPMI 8226 peripheral blood myeloma cells possess two electron-withdrawing chlorine atoms at positions

Table 6. Calculated water solubility at 25 °C, log *P* values and percentages of nitrogen element for compounds **11**, **21** and **13**, **22**

Compound	Solubility[a] (mg L^{-1})	log *P*[b]	*N* (%)
11	6371	0.58	24.35
21	7562	0.51	30.55
13	1821	0.87	22.95
22	2162	0.70	28.79

[a] Water solubility values were calculated using EPI Suite for Windows v. 3.11. USEPA, Washington, DC, 2000.

[b] log *P* values were calculated using Pallas 3.1.1.2. software (distributed by CompuDrug) 2003.

3,4 of the phenyl ring (**20**) or one electron-withdrawing Cl substituent at positions 3 or 4 (compounds **18**, **19**, **25**). The introduction of a second Cl substituent significantly increased the antitumour activity. It has been proved for dichloro-substituted imidazo[2,1-*c*][1,2,4]triazine-3,4-dione **20** in relation to its monochloro-substituted congeners **18** and **19**. Thus, this compound in concentrations of 50 and 100 μM was the most potent of the series. It caused the highest viability decreases (to 35% and 25%, respectively) in human RPMI 8226 myeloma cell line after exposure for 24 h (Table 7). Moreover, based on performed examination, the distinctly marked lower cytotoxicity of the tested derivative against the normal cell line and almost threefold higher against RPMI 8226 myeloma cell line was ascertained. Taking into consideration the viability comparative study results concerning the influence of the tested imidazotriazine-3,4-dione **20** on the leukaemic and normal cell line the selective action of examined compound can be expected. Also the anticancer activity of the tested compound seemed to be dose-dependent.

Unexpectedly, the replacement of 4-chloro-substituent for 2-chloro-one that of the phenyl ring in the series of imidazotriazin-3(6*H*)-ones led to significant viability increase in RPMI 8226 cancer cells and reduction of antiproliferative action. It has been proved for 2-chloro-derivative (**23**) in comparison to 4-chloro one (**25**). In this case, the 4-position in the phenyl ring appears to be a factor that influences antiproliferative potency since *para*-substituted Cl derivative reveals better potency than *ortho*-substituted Cl congener. However, imidazotriazines without substituent and those containing methyl and methoxy substituents at the 2- and 4-position of the phenyl ring demonstrated only slightly antiproliferative or proliferative effects, for example, these compounds were proved to cause slight viability decreases (**11**, **13**–**15**) or increases (**12** and **21**) in human RPMI 8226 myeloma cells.

Furthermore, imidazotriazin-3(6*H*)-one (**22**) was proved to be potent against HT 29 human colon adenocarcinoma cell line and leukaemic Jurkat cells. Details of this test system have been published elsewhere.[51]

In conclusion, imidazotriazine-3,4-dione of the type **20** demonstrates antiproliferative properties justifying further investigation as the potential anticancer agent and may be used as a basis for the design of new non-toxic anticancer drugs.

Table 7. Percentage of viable normal human skin fibroblasts (HSF) and RPMI 8226 cancer cell line following 24 h treatment with the tested concentrations of the examined imidazotriazines

Compound	R	Concentration in μM	Cell viability in normal HSF cells	Cell viability in RPMI cancer cells
11	H	1	93 ± 2.9	96 ± 6.6
		50	95 ± 3.8	96 ± 6.1
		100	94 ± 6.3	86 ± 4.2
12	2-CH_3	1	104 ± 3.2	101 ± 9.9
		50	110 ± 2.9	121 ± 12.0
		100	108 ± 3.9	133 ± 6.9
13	4-CH_3	1	93 ± 2.4	95 ± 3.7
		50	97 ± 3.8	91 ± 2.6
		100	92 ± 3.5	83 ± 7.1
14	2-CH_3O	1	103 ± 4.5	90 ± 9.7
		50	101 ± 3.7	90 ± 8.5
		100	94 ± 4.4	85 ± 10.1
15	4-CH_3O	1	94 ± 5.3	98 ± 6.6
		50	92 ± 4.5	99 ± 7.5
		100	94 ± 7.1	99 ± 4.2
18	3-Cl	1	95 ± 6.6	89 ± 8.5
		50	91 ± 3.9	68 ± 6.3
		100	91 ± 4.9	62 ± 5.9
19	4-Cl	1	99 ± 5.2	104 ± 9.2
		50	95 ± 4.3	89 ± 4.2
		100	96 ± 4.1	61 ± 6.0
20	3,4-Cl_2	1	109 ± 4.2	98 ± 9.0
		50	90 ± 5.1	**35 ± 4.7**
		100	90 ± 4.7	**25 ± 3.2**
21	H	1	101 ± 2.9	95 ± 12.7
		50	103 ± 3.6	103 ± 7.8
		100	106 ± 4.1	106 ± 9.6
23	2-Cl	1	100 ± 5.5	93 ± 11.1
		50	104 ± 3.8	94 ± 9.7
		100	101 ± 4.4	93 ± 11.9
25	4-Cl	1	98 ± 3.6	94 ± 3.5
		50	84 ± 5.6	76 ± 3.1
		100	74 ± 1.6	64 ± 1.2

HSF—human skin fibroblast cells—primary cell line. RPMI 8226 (ECACC 87012702)—human peripheral blood myeloma.

4. Conclusion

In this report, an easy and useful method to obtain biologically active imidazo-triazine-3,4-dione and imidazotriazin-3(6*H*)-one aryl derivatives has been presented. It can be found that there is one imidazotriazine-3,4-dione (**19**) that has a strong affection to LS180 cancer cell line, revealing the efficiency for DNA strand breakage of cancer cells and there are two imidazotriazin-3(6*H*)-ones which exhibit strong effects on LS180, A549 (**22**) and A549 (**24**) carcinoma cell lines. Moreover, one imidazotriazine-3,4-dione (**20**) was proved to cause remarkable viability decreases in human RPMI 8226 peripheral blood myeloma cells. So both 8-(4-chlorophenyl)- and 8-(3,4-dichlorophenyl)-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones (**19**, **20**, respectively) and two 8-(4-methylphenyl)- and 8-(3-chlorophenyl)-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-ones (**22** and **24**, respectively) may have potential antitumour activities. Further optimization of these identified chemical leads can possibly lead to more active molecules. Since these imidazotriazines (**19**, **20**, **22** and **24**) 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

5.1. Instrumentations and general materials

Chemicals (ethyl oxalate, ethyl oxamate) were purchased from Merck and Sigma–Aldrich as 'synthesis grade' and used without further purification. Melting points (mp) were determined on a Boetius apparatus and are given uncorrected. The purity of each compound was established by thin-layer chromatography. TLC was carried out on commercial Merck SiO_2 60 F_{254} plates having fluorescence indicator. The spots were visualized in UV light at $\lambda = 254$ nm and 355 nm. ^{1}H NMR spectra for compounds **11**–**25** and ^{13}C NMR spectra for compounds **11**–**20** were studied on a Bruker 200 MHz spectrometer in DMSO-d_6 with TMS as an external standard at 295 K.[1,39] Besides ^{1}H ^{1}H COSY, HMBC and HMQC correlations were made for compound **2**. Mass spectroscopic analyses for compounds **11**–**20** were studied using a VG ZAB Spec (Manchester, United Kingdom) orthogonal acceleration time-of-flight instrument equipped with OPUS V.3.3 data system. The details of mass spectrometric studies have been published elsewhere.[45]

Diffraction data for **12** were measured at 295 K on a KM4 diffractometer using variable scan speed (ω-2θ scan mode) and graphite monochromated CuKα radiation ($\lambda = 1.54178$ Å). A single crystal of dimensions $0.22 \times 0.20 \times 0.17$ mm was used.

The structures of the compounds with their physicochemical data are shown in Table 2. The starting 1-aryl-2-hydrazonoimidazolidine hydroiodides[1,34,41] were obtained by earlier described method.[1,41]

5.1.1. General procedure for synthesis of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones (11–20) (method i). Free base of 1-aryl-2-hydrazinoimidazoline[41] (0.05 mol) was dissolved in 80 mL of *n*-butanol. The 0.05 mol (7.3 g) of diethyl oxalate was added and the mixture was heated under reflux for 8 h. During that time precipitation of the solid started. The mixture was refrigerated overnight and the precipitation yielded was collected and purified by crystallization from DMF/methanol (1:2) mixture.[1]

Full spectral characteristic of the obtained compounds is given in Ref. 1,45.

5.1.2. General procedure for synthesis of 8-aryl-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-diones (11–20) (method ii). Diethyl oxalate (7.3 g, 0.05 mol) was added to the suspension of appropriate 1-aryl-2-hydrazinoimidazoline hydroiodide[41] (0.05 mol) in 80 mL of *n*-butanol. The mixture was stirred and triethylamine (5 mL) was added. The reaction was carried out under reflux for 8 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF/methanol (1:2) mixture.[1]

Full spectral characteristic of the obtained compounds is given in Ref. 1,45. Their physicochemical and biological properties are presented in Table 1. The X-ray crystal structure determination that was made for compound **12** is for the first time presented in this paper.

5.1.2.1. Crystal data for 8-(2-methylphenyl)-2,6,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazine-3,4-dione (12). C_{12} H_{12} N_4 O_2, FW = 244.26, monoclinic, space group $P2_1/c$, unit cell parameters are: $a = 6.935(1)$ Å, $b = 7.654(2)$ Å, $c = 21.933(4)$ Å, $\beta = 96.27(3)°$, $V = 1157.3(4)$ Å^3, $Z = 4$, $d_{calc} = 1.402$ g cm^{-3}, μ(CuKα) = 0.825 mm^{-1}. In the θ range 6.13–70.30°, 2253 reflections were collected. The structure was solved by direct methods using SHELXS-97 program[52] and refined by the full-matrix least-squares on F^2 using SHELXL-97 program.[53] The non-hydrogen atoms were refined with anisotropic displacement parameters. H-atom positions were located from the geometry and were given isotropic factors of 1.2 U_{eq} of the bonded C-atoms; the C–H bond 'riding' model was used in the refinement. One humdred and sixty two parameters were refined, final discrepancy factors are $R1 = 0.0505$, $wR2 = 0.1361$ for $I > 2\sigma(I)$, and $R1 = 0.1228$, $wR2 = 0.1721$ for all data, $S = 0.998$. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as CCDC No. 625274. Copies of the data can be obtained on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44 1223 336 033; e-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk).

5.1.3. General procedure for synthesis of 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-ones (21–25) (method i). Free base of 1-aryl-2-hydrazonoimidazolidine[41] (0.05 mol) was dissolved in 60 mL of ethanol. Then 0.05 mol of ethyl oxamate was added

and the mixture was heated under reflux for 7–9 h. The mixture was kept overnight in a fridge, the precipitate yielded was collected by filtration and finally purified by recrystallization from DMF.[39]

5.1.4. General procedure for synthesis of 8-aryl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-ones (21–25) (method ii). Ethyl oxamate (0.05 mol) was added to the suspension of appropriate 1-aryl-2-hydrazonoimidazolidine hydroiodide[41] (0.05 mol) in 80 cm^3 of *n*-butanol. The reaction mixture was stirred, and triethylamine (5 mL) was added. Then the reaction was carried out under reflux for 4–6 h. During that time, the precipitation of a solid was observed. The crude product obtained after cooling was collected by filtration, washed with cold methanol and finally purified by recrystallization from DMF.[39]

5.1.4.1. 8-Phenyl-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (21). Mp: 307–310 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.20 (br s, 4H, $2CH_2$); 7.02–7.65 (m, 5H, ar-H), 7.89 (s, 1H, NH), 8.30 (s, 1H, NH); Anal. Calcd for $C_{11}H_{11}N_5O$: C, 57.63; H, 4.84; N, 30.55. Found: C, 57.82; H, 4.80; N, 30.41.

5.1.4.2. 8-(4-Methylphenyl)-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2, 4]triazin-3(6*H*)-one (22). Mp: 297–299 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 2.27 (s, 3H, CH_3), 4.38/4.54 (J = 9.1 Hz, J' = 7.6 Hz, 2 × dd, 4H, $2CH_2$); 7.21 (d, J = 8.3 Hz, 2H, ar-H: H3′ and H5′), 7.47 (d, J = 8.3 Hz, 2H, ar-H: H2′ and H6′), 7.68 (s, 1H, NH), 8.08 (s, 1H, NH); Anal. Calcd for $C_{12}H_{13}N_5O$: C, 59.25; H, 5.39; N, 28.79. Found: C, 59.11; H, 5.43; N, 28.70.

5.1.4.3. 8-(2-Chlorophenyl)-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (23). Mp: 215–218 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 3.96/4.17 (J = 9.0 Hz, J' = 7.6 Hz, 2 × dd, 4H, $2CH_2$); 7.30–7.61 (m, 4H, ar-H), 7.73 (s, 1H, NH), 8.13 (s, 1H, NH); Anal. Calcd for $C_{11}H_{10}ClN_5O$: C, 50.11; H, 3.82; Cl, 13.45; N, 26.56. Found: C, 49.90; H, 3.85; Cl, 13.54; N, 26.65.

5.1.4.4. 8-(3-Chlorophenyl)-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (24). Mp: 253–256 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.20 (br s, 4H, $2CH_2$); 7.08–7.80 (m, 4H, ar-H), 7.95 (s, 1H, NH), 8.36 (s, 1H, NH); Anal. Calcd for $C_{11}H_{10}ClN_5O$: C, 50.11; H, 3.82; Cl, 13.45; N, 26.56. Found: C, 50.25; H, 3.75; Cl, 13.35; N, 26.70.

5.1.4.5. 8-(4-Chlorophenyl)-4-imino-2,3,7,8-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3(6*H*)-one (25). Mp: 282–284 °C; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.18 (br s, 4H, $2CH_2$); 7.27 (d, J = 8.8 Hz, 2H, ar-H: H3′ and H5′), 7.53 (d, J = 8.8 Hz, 2H, ar-H: H2′ and H6′), 7.91 (s, 1H, NH), 8.31 (s, 1H, NH); Anal. Calcd for $C_{11}H_{10}ClN_5O$: C, 50.11; H, 3.82; Cl, 13.45; N, 26.56. Found: C, 50.22; H, 3.91; Cl, 13.31; N, 26.51.

5.2. Microbiology

5.2.1. Microdilution assays. The minimal inhibitory concentration (MIC) values for intermediates **1**–**2**, **4**–**6** and **9** defined as the lowest concentration of the compound preventing the visible growth were determined by using microdilution broth method according to NCCLS standards.[46] The inocula of microorganisms (10^6 cfu mL^{-1}) were prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The test compound dissolved in DMSO was first diluted to the highest concentration (500 μg mL^{-1}) to be tested. Then serial twofold dilutions were made in concentration ranging from 1.95 to 500 μg mL^{-1} in 10 mL sterile tubes. Prepared suspensions of the standard microorganisms were added to each dilution in a 1:1 ratio. Growth (or lack) of microorganisms was determined visually after incubation for 24 h at 37 °C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC.

The minimal inhibitory concentration (MIC) values were studied for following clinically isolated bacterial strains: *S. aureus*, *S. epidermidis*, *St. pyogenes* and *E. coli* towards compounds **1**–**2**, **4**–**6** and **9**. Ampicillin and chloramphenicol were used as standard drugs for comparison in the antibacterial study. Control experiments using DMSO were done for antibacterial activity studies. The presented results (Table 4) were obtained from three-independent measurements. The investigations were carried out in the Department of Medical Microbiology, Medical University, Lublin.

5.3. Inhibition of tumour cell growth assay

Intermediates **1**, **3**, **5**–**8** were tested for their in vitro antiproliferative potential towards three human cancer cell lines: human Caucasian colon adenocarcinoma (LS180), human cervix epitheloid carcinoma (HeLa) and human breast carcinoma (T47D). Moreover, one normal cell line—Vero (Green Monkey Kidney cells) was included in the cytotoxicity study.

The antiproliferative potential of the final imidazotriazines **11**–**16**, **18**–**19** and **21**–**25** was determined in vitro against four human tumour cell lines such as LS180 (colon), HeLa (cervix epitheloid), T47D (breast) and A549 (lung).

In the current protocol each cell line was inoculated at 10^4 cells per mL density and preincubated on a microtitre plate. Test agents were then added at two 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) labelling and detection kit III[54–57] on Elisa reader (Bio-Tec Instruments, USA).

The growth percentage was evaluated spectrophotometrically versus untreated controls using cell viability of growth assay. Results for each spectrophotometrical measure were reported as per cent of growth inhibition. All experiments were done in triplicate. The investigations were carried out in the Department of Biology and Genetics, Medical University, Lublin.

5.4. Detection of induction of DNA damage in the tested cell lines after incubation with the investigated imidazotriazine-3,4-dione (19)

Following treatment of heterocycle **19** with 32.6 μM, cells were washed twice with phosphate-buffered saline (PBS). In the next step the DNA was isolated using the lysing buffer and proteolytic enzyme—proteinase K.[58] After purification, DNA samples were spread over 1% agarose gel with addition of propidium iodide.[59] 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.

5.5. Action of novel imidazotriazines on the growth percentage of normal human skin fibroblasts (HSF) and human RPMI 8226 carcinoma cell line

Human peripheral blood myeloma—RPMI 8226 derived from the peripheral blood of a 61-year-old male with multiple myeloma, which is a recognized model for multiple myelomas.[60,61] Above-mentioned cancer cell line was obtained from the European Collection of Cell Cultures (ECACC 87012702). Normal cell line—human skin fibroblasts (HSF) were routinely grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 100 μg mL^{-1} of streptomycin, 100 U mL^{-1} of penicillin in plastic tissue culture flasks (Nunc, Denmark).

Cells were maintained in RPMI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine and 10 μg mL^{-1} gentamicin and 1 mM sodium pyruvate. Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2. These ones were passaged 3 times weekly and maintained at a density of 6×10^5 (leukaemia RPMI 8226), and 1×10^5 cells (HSF) 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 cells cultured in 96-well plates. All the tested compounds were dissolved in DMSO prior to dilution into the biological assay. The effect of the examined concentrations (1, 50 and 100 μM) of heterocycles **11**–**15**, **18**–**21**, **23**, **25** on the cell viability was estimated by a colorimetric assay MTT based (the succinate dehydrogenase inhibition, SDI test) described by Takenouchi and Munekata.[62] The metabolic activity was measured in cell populations via incubation with 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.

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