Bioorganic & Medicinal Chemistry Letters 19 (2009) 5095–5100

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Crystal structure, antitumour and antimetastatic activities of disubstituted fused 1,2,4-triazinones

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ARTICLE INFO

Article history:
Received 29 May 2009
Revised 2 July 2009
Accepted 2 July 2009
Available online 10 July 2009

Keywords:
Fused 1,2,4-triazine
Imidazo[2,1-*c*][1,2,4]triazine nucleus
Crystal structure
Antitumour activity
Antimetastatic potential

ABSTRACT

Molecular structure of 3,8-disubstituted 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-ones (**8**–**14**) was confirmed by X-ray crystallography of **14**. All the compounds were evaluated for their antitumour and antimetastatic activities in vitro. Furthermore, their cytotoxicities towards human normal cell line—HSF cells were established, allowing us to point out some structure–activity relationships. Among them, imidazotriazinone **12**, revealing remarkable dose-dependent viability decreases in human myeloma RPMI 8226 cells, was found to be completely non-toxic towards normal HSF cells. In addition, heterobicycles **8**–**12** were proved to exhibit significant antimetastatic potentials in the motility assay.

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The 1,2,4-triazine nucleus is a prominent structural core system present in numerous biologically active compounds. Azanucleosides, structurally based on the 1,2,4-triazine scaffold, such as 6-azacytosine and 6-azauracil, have been found to reveal antitumour,[1,2] antiviral,[3,4] and antifungal[5] activities. 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 biological interest due to its resistance to deaminase, while the well-known antiviral, antifungal and antipsoriatic drug, azaribine, is structurally associated with the 1,2,4-triazine moiety.[6]

Moreover, various condensed 1,2,4-triazines have been reported to be extremely potent and the majority of them found biological applications.[7–12] Pyrrolo[2,1-*f*][1,2,4]triazines as congeners of substituted nucleic acid purines have revealed an interesting broad spectrum of antiproliferative activity, as well as pronounced in vitro growth inhibitory activities against human leukaemic cell lines, comparable to that of 9-deazaadenosine.[13] Pyrrolo[2,1-*c*][1,2,4]triazines have also been found to possess 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.[14,15] Furthermore, some of pyrrazolo[5,1-*c*][1,2,4]triazines have been reported to reveal in vitro remarkable antitumoural and antifungal activities.[16–22]

Certain synthetic derivatives of the imidazo[2,1-*c*][1,2,4]triazin-4(1*H*)-one have been suggested as novel bicyclic nucleosides related to 6-azaisocytosine.[23] On the other hand, previously reported by us polyazaheterocycles, containing the dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one core system, have acquired considerable interest, because of their distinctly lower cytotoxicity towards normal cells and several-times higher against cancer cell lines;[24,25] a significant effect in tumour cells and simultaneously a lack of toxicity towards normal cells.[26,27] And so, our findings suggest that imidazotriazinones are able to selectively inhibit the growth of tumour cells, and therefore they might be promising drug candidates having a beneficial side-effect profile.

Prompted by these facts and in continuation of our attempt to obtain medicinally important heterocycles,[24–31] herein we report the molecular structure, antitumour and antimetastatic activities in vitro of 3,8-disubstituted 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-ones (**8–14**).

The bicyclic polyazaheterocycles (**8–13**), bearing the bridgehead nitrogen atom, have been generated in a straightforward manner via an alternative synthetic approach,[32] which obviate the use of 1-arylimidazolidin-2-one hydrazone and α-oxoacid, such as phenylglyoxylic acid (**B**), instead of its α-oxoester—ethyl phenylglyoxylate[34,35] previously used. In this new approach improved yields for the desired compounds **8–13** were obtained. The target imidazotriazinone **14** has been obtained via the condensation/cyclization reaction of 1-(2,5-dichlorophenyl)imidazolidin-2-one

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doi:10.1016/j.bmcl.2009.07.036

hydrazone with phenylglyoxylic acid, according to general procedure for the synthesis of heterobicycles **8–14**.[32,33] This novel heterobicycle (**14**) has not been previously described in the literature. The structural determination of this compound was achieved from its elemental analysis and spectral data (^{1}H NMR, IR).[36] The assigned structure of **14** was further validated by single-crystal X-ray analysis.

^{1}H NMR and IR spectra of all the imidazotriazinones **8–14** were considerably different in comparison to those of their starting materials (entries **1–7**). Above all, there were no signals of protons derived from the endocyclic imidazolidine-N*H* and exocyclic =N–N*H*$_2$ formations in the ^{1}H NMR spectra of heterobicycles **8–14**. Simultaneously, the number of aromatic protons had increased by five due to the additional unsubstituted phenyl ring present at the 3-position of the polyazaheterobicyclic structure of **8–14**. In the IR spectra the presence of absorption bands of 1687 and 1554 cm^{-1}, ascribable to the triazine-C=O group and the C=N bond at the ring junction confirmed the formation of the cyclic products. In the fully conjugated core system of the **8–14** type, where the double bond is contained within the triazinone ring, the difference in C=O and C=N frequencies was found to be in the order of 131–133 cm^{-1}. These data are in a full agreement with those provided by Le Count and Greer for other fully conjugated fused 1,2,4-triazin-5-ones.[37]

Biologically active 1-arylimidazolidin-2-one hydrazones[24,25] (entries **1–7**), useful as key building blocks for the synthesis of target core heterobicycles **8–14**, were prepared in gram quantities using reliable patent pending methodology proposed by Sztanke.[30] The four-step synthetic pathway,[26] leading to these compounds is depicted in Scheme 1. In the first step, commercially available substituted anilines were converted into *N*-arylethylenediamines by the Lehmann procedure[38] or by the classical Knoevenagel and Mercklin method using the modification of Takeda.[39,40] Their further condensation with carbon disulfide, in an inert organic solvent—xylene, led to the formation of 1-arylimidazolidine-2-thiones, followed by the cyclization of intermediate dithiocarbaminic acid derivatives, with concomitant loss of hydrogen sulfide molecule and the subsequent ring closure. Because of the existence of thiol–thione tautomerism, the alkylation of respective thiols with one equivalent of methyl iodide in methanolic medium was possible, to afford 1-aryl-2-methylthioimidazoline hydroiodides in 75–85% yields,[28] those in turn were refluxed with hydrazine hydrate in methanol to give 1-aryl-2-hydrazinoimidazoline hydroiodides in good yields (62–75%).[30]

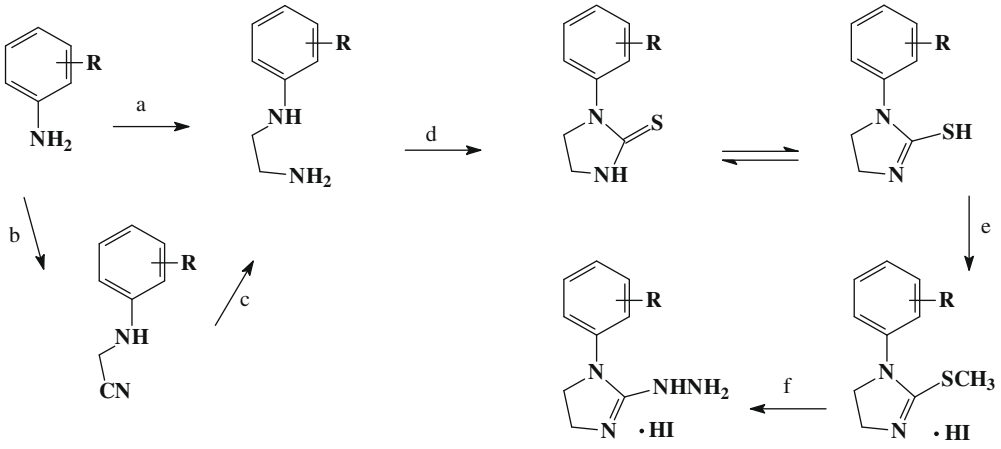
The target 3,8-disubstituted 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-ones (**8–14**) were obtained in good yields (Table 1) in one-pot synthetic approach by treating of hydrazones **1–7** with phenylglyoxylic acid in inert organic solvents under reflux for 5–7 h (Scheme 2). These polyazaheterocycles could be prepared starting both from hydroiodides of 1-arylimidazolidin-2-one hydrazone in the triethylamine presence (method i),[32] as well as from their free bases (method ii).[33] ^{1}H NMR and IR spectroscopic data of the target imidazotriazinones (**8–14**), obtained by the two above-mentioned methods, were also identical. Highest yields for imidazotriazinones of the **8–14** type were achieved, when appropriate 1-arylimidazolidin-2-one hydrazone hydroiodides, without conversion to their free bases, were used in the condensation with phenylglyoxylic acid (Table 1, method i).

Forming the dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one core of the molecules **8–14** is proposed to proceed by a stepwise mechanism. In the first step the condensation of an appropriate hydrazone of the **1–7** type with phenylglyoxalic acid (**B**) took place to afford an intermediate— the open-chain compound as the result of loss of a water molecule. Because of the presence of the hydrogen atom on the cyclic N-3 nitrogen of the imidazolidine ring, the subsequent cyclocondensation of this intermediate was attempted to give the bicyclic polyazaheterocycle of the **8–14** type, with concomitant loss of water and subsequent ring closure. The concurrent course of the cyclization reaction leading to the dihydroimidazo[2,1-*c*][1,2,4]triazole core system (with concomitant loss of formic acid molecule) was excluded, because spectral data

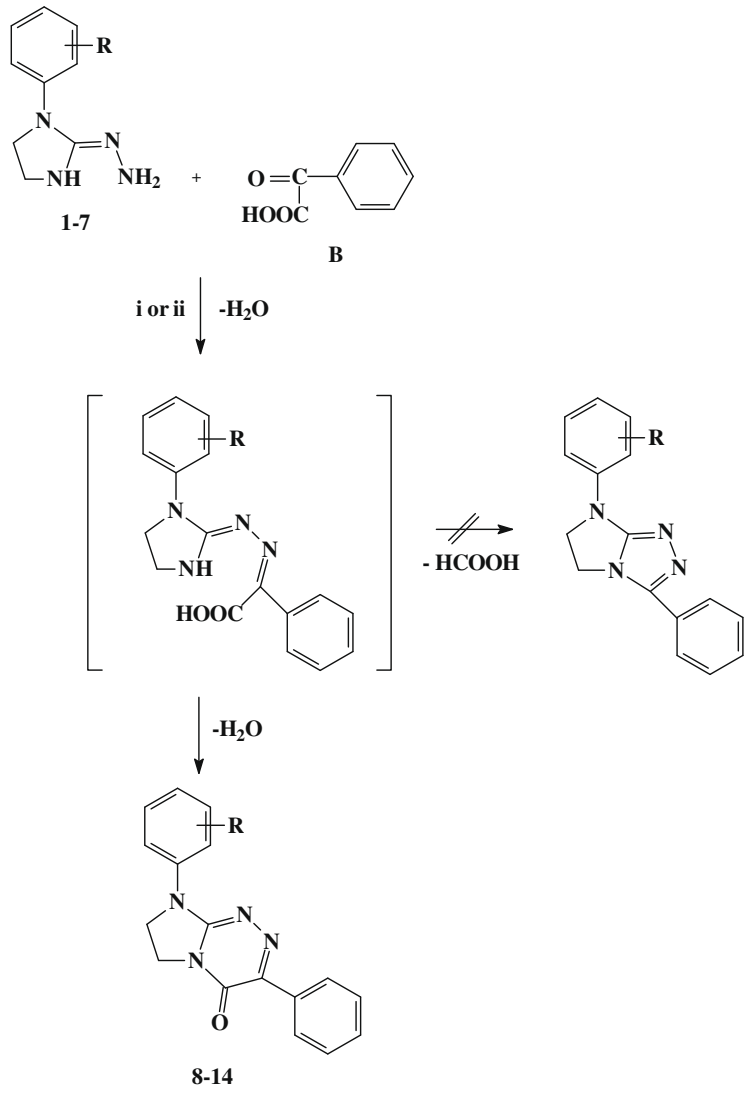
Table 1
Physicochemical properties of 3,8-disubstituted 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-ones (**8–14**)

Compound	R	Yield (%)		Mp (°C)	Molecular weight	log k_w
		(i)	(ii)			
8	4-CH_3O	75	63	249–251	320.35	3.01
9	3-CH_3	67	62	217–218	304.35	3.48
10	2-Cl	71	65	230–231	324.76	3.22
11	3-Cl	75	69	213–214	324.76	3.82
12	4-Cl	73	60	274–276	324.76	3.80
13	3,4-Cl_2	70	66	278–280	359.21	4.59
14	2,5-Cl_2	67	62	230–232	359.21	nd

Log k_W values (HPLC)[54] were determined on an octadecyl silica column (LC-18) with mobile phase of the type MeOH–H_2O according to the procedure[55] therein reported. nd—not determined.



Scheme 1. Synthetic pathway for the preparation of starting 1-aryl-2-hydrazinoimidazolines. 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.



Scheme 2. One-pot approach for the synthesis of imidazotriazinones **8**–**14**. **1**, **8**: R = 4-CH_3O; **2**, **9**: R = 3-CH_3; **3**, **10**: R = 2-Cl; **4**, **11**: R = 3-Cl; **5**, **12**: R = 4-Cl; **6**, **13**: R = 3,4-Cl_2; **7**, **14**: R = 2,5-Cl_2. Reagents and conditions: (i) (**1**–**7**) HI + B, triethylamine, *n*-butanol, reflux, 7 h; (ii) (**1**–**7**) + B, DMF, reflux, 5 h.

distinctly support the 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4-(6*H*)-one nucleus formation and argue against an alternative cyclization process.

Finally, the molecular structure of 3,8-disubstituted 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-ones was confirmed by X-ray crystallography of **14**.[41] Its structure was solved by direct methods using the SHELXS-97 program[42] and refined by full-matrix least-squares on F^2 using SHELXL-97 program.[43] A perspective view of the molecule **14** with atom numbering is shown in Figure 1.

Bond lengths in the dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one system indicate localized double bonds between C8a–N1 and N2–C3 atoms. The presence of an aryl substituent at the C3 atom does not allow for electron-delocalization in the C3–C4–O4 fragment, which is indicated by the bond length distribution. The endocyclic torsion-angle values (±4°) indicate planarity of the six-membered triazine ring, while the dihydroimidazole ring is considerable non-planar, adopting an envelope conformation. The phenyl substituents at the C3 and N8 atoms are not coplanar with the heterocyclic nucleus (*het*); observed interplanar angles are 29° and 64° for *het*/C3-phenyl and *het*/N8-phenyl, respectively. Due to the molecular structure of **14** the molecular packing in the solid state is stabilized by weak C–H···O, C–H···Cl and Cl···Cl contacts. Intermolecular C4p–H···Cl2 bonds link molecules to form a dimer while Cl1···Cl2[44] interactions connect adjacent dimers into a ribbon (Fig. 2). Associations of ribbons through the C–H···O=C hydro-

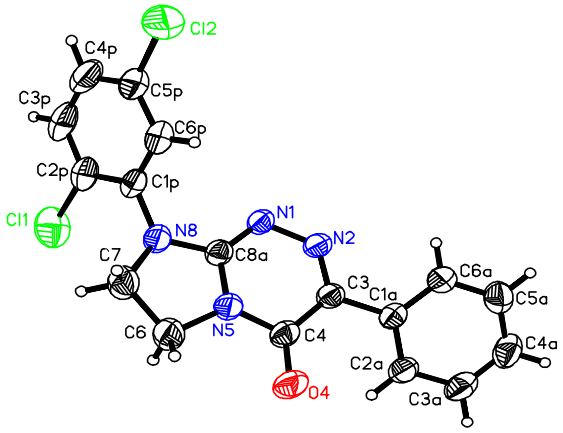


Figure 1. The molecular structure of heterobicycle **14**. Bond distances within 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one system are: N1–N2 1.381(2); N2–C3 1.306(3); C3–C4 1.472(3); C4–N5 1.372(3); N5–C6 1.459(3); C6–C7 1.520(4); C7–N8 1.473(3); N8–C8a 1.358(3); C8a–N1 1.307(3); C8a–N5 1.357(3); C4–O4 1.219(3) Å.

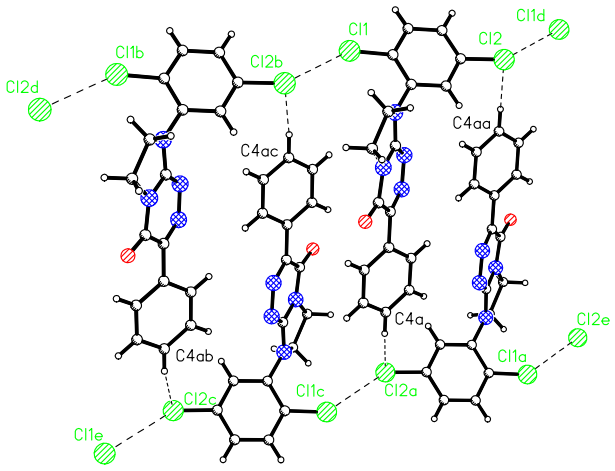


Figure 2. Packing diagram showing C–H···Cl and Cl···Cl[44] intermolecular contacts within the ribbon.

gen bonds and Cl1···Cl1 contacts completes three-dimensional crystal network.

Different arrangement of molecules is observed in the crystal of 2,6-dichlorophenyl-7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one derivative,[24] where O=C groups are involved in numerous C–H···O hydrogen bonds and orthogonal multipolar interactions to the heterocyclic system. In this case, antiparallel orientation of the C–Cl bonds of neighbouring molecules was observed.

Spectral (^{1}H NMR, IR) and analytical data of all the synthesized heterobicycles, given in References and notes and those provided in Supplementary data, were in full agreement with proposed structures (Table 1).

In view of current interest in the design of heterobicyclic derivatives containing the 1,2,4-triazine moiety, particularly on account of their promising biological properties an alternative synthetic approach leading to the formation of 3,8-disubstituted 7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-ones (**8–14**) 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.

An attempt was made to determine the action of target heterobicycles (**8–14**) on the viability of human peripheral blood myeloma cells (RPMI 8226 cells),[45] being a recognized model for multiple myelomas,[46,47] in order to select compounds having promising anticancer activity and a clean toxicity profile. In the experiment conducted RPMI 8226 cells and a normal cell line—HSF cells[48] were incubated in the presence of imidazotriazinones **8–14** and with culture medium alone. Antiproliferative activities in vitro of the tested compounds[49] were determined by a colourimetric assay MTT based, proposed by Takenouchi and Munekata.[50]

From the results of this biological assay (Table 2) certain structure–activity relationships can be derived. It is especially worth noting, that the presence of electron-withdrawing chlorine substituent at position-*para* of the phenyl ring was found to be essential for the imidazotriazinone to be active against human myeloma RPMI 8226 cells and to have selective action. Thus, the most interesting heterobicycle **12**, having a log k_w value of 3.80, with a *para*-chlorophenyl moiety at position 8 of heterobicyclic system, revealed remarkable dose-dependent viability decreases in myeloma RPMI 8226 cells. Simultaneously, this compound was found to be completely non-toxic for the normal cell line used—HSF cells, independently from the concentration applied. These experimental data suggest a beneficial toxicity profile of the imidazotriazinone **12**.

Replacement of the electron-withdrawing *para*-Cl substituent, that of the phenyl ring, in **12** for the electron-withdrawing *meta*-Cl group, leading to its positional analogue **11**, with a log k_w

Table 2
Percentage of viable human skin fibroblasts—HSF cells and human peripheral blood myeloma RPMI 8226 cells following 24 h treatment with the tested concentrations of heterobicycles **8–14**

Compound	R	Concentration in μM	Cell viability in normal HSF cells (in %)	Cell viability in cancer RPMI 8226 cells (in %)
8	4-CH_3O	1	88 ± 5.9	106 ± 6.2
		50	64 ± 5.7	57 ± 4.5
		100	73 ± 6.6	43 ± 1.8
9	3-CH_3	1	100 ± 4.1	107 ± 5.6
		50	94 ± 3.8	79 ± 4.7
		100	88 ± 1.4	71 ± 5.7
10	2-Cl	1	108 ± 3.1	103 ± 5.6
		50	104 ± 2.0	103 ± 6.2
		100	104 ± 3.9	101 ± 7.7
11	3-Cl	1	108 ± 3.8	105 ± 9.3
		50	85 ± 5.0	70 ± 4.1
		100	88 ± 5.0	60 ± 2.4
12	4-Cl	1	104 ± 3.3	101 ± 5.5
		50	96 ± 6.7	56 ± 1.2
		100	102 ± 6.5	39 ± 2.3
13	3,4-Cl_2	1	93 ± 1.7	108 ± 5.8
		50	48 ± 1.5	69 ± 5.3
		100	47 ± 1.4	58 ± 4.0
14	2,5-Cl_2	1	87 ± 1.6	109 ± 6.6
		50	31 ± 2.9	43 ± 4.1
		100	17 ± 3.2	41 ± 2.6

Cell viability after exposure for 24 h was evaluated by means of MTT (tetrazolium salt reduction) assay.
Cell viability in control—100%.
Data represent the mean ± SD of at least three independent measurements.
Human skin fibroblast cells—HSF cells—primary cell line.
Human peripheral blood myeloma cells—RPMI 8226 cells—cancer cell line.
The examined compounds **8–14** were dissolved in DMSO prior to dilution into the biological assay.

value of 3.82, led to the viability increase in RPMI 8226 myeloma cells, that is the reduction of antiproliferative action. Incorporation of an additional chlorine atom into the phenyl ring, leading to dichloro-substituted imidazotriazinone derivatives (**13** and **14**), was found to be responsible for the complete loss of their selectivity. An especially large cytotoxicity increase in normal cells was observed for the 2,5-dichloro derivative (**14**), which at a concentration of 100 μM caused over twofold greater viability decrease in normal HSF cells compared to cancer ones. This compound revealed remarkable dose-dependent viability decreases in normal cells. Replacement of the one chloro group, that of the phenyl ring, in the most promising 2,5-dichloro-substituted derivative (**14**), by a hydrogen atom leading to its 2-chloro analogue (**10**), having a log k_w value of 3.22, appeared to be unfavourable, because this structural change resulted in the complete loss of activity of **10**. As well, the replacement of electron-withdrawing *para*-chloro group at the phenyl ring in heterobicycle **12**, for either electron-withdrawing *ortho*-chloro one, leading to compound **10**, resulted in the complete disappearance of biological potency. Replacement of *para*-Cl substituent at the phenyl ring, with strong electron-donating *para*-OCH_3 group, leading to heterobicycle **8** (with a log k_w value of 3.01), had no influence on the viability in myeloma RPMI 8226 cells. Simultaneously, this structural change led to cytotoxicity increase towards normal cells. Furthermore, it has been noted, that replacement of electron-withdrawing 3-Cl substituent, that at the phenyl ring, in an imidazotriazinone **11** for a weakly electron-donating 3-CH_3 one, leading to the analogue **9**, with a log k_w value of 3.48, caused only slight viability changes in normal and cancer cell lines.

It may be concluded that the presence of the chlorine atom at *para*-position on the phenyl ring was the best choice of substitution in the series of imidazotriazinone compounds (**8–14**) giving rise to the most promising compound **12**.

In the next series of experiments, the effect of heterobicycles **8–14** on tumour cells was studied using the cell motility assay proposed by Yang et al.[51] In this assay, human cervix carcinoma cells (HeLa B cells),[52] were exposured to the culture medium alone and to 25 μM of an appropriate imidazotriazinone of the **8–14** type for 24 h. The results are presented as percentage of cell motility in comparison to control. Cell motility in the absence of the tested imidazotriazinone derivative was considered as 100%. This assay showed that five heterobicycles **8–12**, those bearing monosubstituted phenyl ring (Table 3 and Fig. 3) could significantly inhibit the migration ability of cervix carcinoma cells in vitro, inasmuch as a smaller percentage of these cells migrated to the wound area was found in HeLa B cells exposured to compounds **8–12** (Table 3 and Fig. 3). Introduction of an additional chlorine atom to the phenyl ring appeared to be unfavourable for the antimetastatic activity, because this structural change led to a dramatic decrease (see 3,4-dichloro derivative **13**) or even to the completely lack of biological activity (2,5-dichloro analogue **14**).

Table 3
Effect of 25 μM concentration of heterobicycles **8–14** on the motility of HeLa B cells

Compound	R	Cell motility (% of control)
8	4-CH_3O	59 ± 3.1
9	3-CH_3	68 ± 2.1
10	2-Cl	59 ± 3.1
11	3-Cl	52 ± 6.2
12	4-Cl	50 ± 9.3
13	3,4-Cl_2	90 ± 3.1
14	2,5-Cl_2	103 ± 15

Cell motility after 24 h in culture medium alone (control)–100%.
Data represent the mean ± SD of at least three independent measurements.
HeLa B (ECACC 85060701)—human Negroid cervix carcinoma cells.

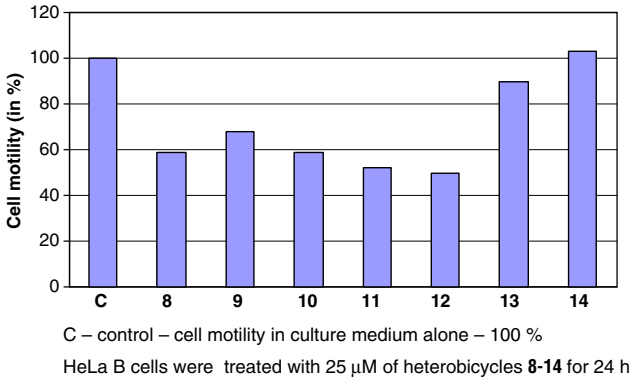


Figure 3. Antimetastatic activities of the examined imidazotriazinones **8**–**14**.

Because the ability of cancer cells to migrate is associated with their invasive metastatic potential,[53] these experimental data suggest that five imidazotriazinones **8–12** exert remarkable antimetastatic activities. According to the literature search, this is the first report related to antimetastatic activities of these imidazotriazinones.

In conclusion, our findings revealed that the incorporation of *para*-chlorophenyl moiety into position 8 of heterobicyclic scaffold, in the series of imidazotriazinone derivatives is essential and favourable for antitumour activity and selectivity. Taking into consideration the influence of the best compound (**12**) on human peripheral blood myeloma cells and normal ones, selective action can be expected. The anticancer effect of heterobicycle **12** is attributed to decreased cell division and inhibition of cell migration. Thus, imidazotriazinone **12** may be considered as a basis for the design of novel antitumour agents without affect normal cells. Since this compound is showing promising results in vitro, studies to establish its in vivo efficacy and safety are being planned. In addition, our findings suggest that imidazotriazinones **8–12** are able to inhibit or limit the metastatic process, and therefore these ones might be powerful candidates as preventive agents against cancer metastasis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.036.

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32. Synthesis of **8**–**14** (method i): Phenylglyoxylic acid (7.51 g, 0.05 mol) was added to the suspension of an appropriate 1-arylimidazolidin-2-one hydrazone hydroiodide (0.05 mol) in 60 cm^3 of *n*-butanol. The mixture was stirred vigorously, and triethylamine (5 mL) was added. The reaction was carried out under reflux for 7 h. The mixture was kept overnight in a refrigerator and during that time, precipitation of solid started. The crude product was collected, washed off with cold methanol, and finally purified by recrystallization from DMF. Physicochemical data of the synthesized compounds are collected in Table 1.
33. Synthesis of **8**–**14** (method ii): An appropriate 1-arylimidazolidin-2-one hydrazone (0.05 mol) was dissolved in 50 mL of DMF. Then phenylglyoxylic acid (7.51 g 0.05 mol) was added and the mixture was heated under reflux for 5 h. The mixture was kept overnight in a refrigerator, the precipitate yielded was collected by filtration, and finally purified by recrystallization from DMF. Physicochemical data of the synthesized compounds are collected in Table 1.
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36. 3-Phenyl-8-(2,5-dichlorophenyl)-7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one (**14**). Anal. Calcd. for $C_{17}H_{12}Cl_2N_4O$: C, 56.84; H, 3.37; Cl, 19.74; N, 15.60. Found: C, 56.77; H, 4.42; Cl, 19.66; N, 15.65. ^{1}H NMR (δ, ppm, DMSO-d_6, 300 MHz, TMS): 4.10 (dd, J = 8.9 Hz, J' = 3.3 Hz, 2H, CH_2), 4.32 (dd, J = 8.8 Hz, J' = 3.2 Hz, 2H, CH_2), 7.40–8.08 (m, 8H, $CH_{arom.}$); IR (KBr) (ν, cm^{-1}): 1686 (triazine-C=O), 1553 (C=N).
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41. Crystal data for 3-phenyl-8-(2,5-dichlorophenyl)-7,8-dihydroimidazo[2,1-*c*][1,2,4]triazin-4(6*H*)-one (**14**). $C_{17}H_{12}Cl_2N_4O$, FW = 359.21, monoclinic, *C*2/*c*, *a* = 24.459(6), *b* = 9.133(2), *c* = 14.388(3) Å, β = 100.37(3)°, the intensities were measured at room temperature, using CuKα radiation and a single crystal of dimensions 0.30 × 0.28 × 0.27 mm. 6814 Reflections were measured, of which 3469 were independent (R_{int} = 0.0693). Final discrepancy factors are R_1 = 0.0435, wR_2 = 0.1025 for $I > 2\sigma(I)$ and R_1 = 0.1044, wR_2 = 0.1225 for all data, S = 1.001. Full crystallographic details of **14** have been deposited with the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 728254. Copies of the data can be obtained, free of charge, 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). Single crystals of compound **14** were obtained from DMF/MeOH (4:1 v/v) mixture. All the crystals were grown by slow evaporation of solvents, at a temperature of about 293 K.
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45. Human peripheral blood myeloma cells—RPMI 8226 cells (ATCC CCL-155)—were obtained from the American Type Culture Collection. This cell line derived from the peripheral blood of a 61-year-old male with multiple myeloma. RPMI 8226 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. Cells were kept at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. Cells were maintained at a density of 3×10^5 cells per mL.
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49. The effect of the examined concentrations (1, 50 and 100 μM) of heterocycles (**8**–**14**) on the cell viability was estimated by a colorimetric assay MTT based (the succinate dehydrogenase inhibition, SDI test) proposed by Takenouchi and Munekata.[50] The cell proliferation was assessed after exposure for 24 h in cell populations via incubation with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), that was reduced into purple, water-insoluble formazan product by the mitochondrial dehydrogenases of viable cells. The production of formazan is measured spectrophotometrically following its solubilization. MTT reduction cell viability assay was performed using cells cultured in 96-well plates. The absorbance of each well was read using *E*-max microplate reader at 570 nm wavelength. The obtained results were presented as percentage of cell viability in comparison to control. The presented results were obtained from three independent measurements and provided with the standard deviation. The investigations were carried out in the Department of Virology and Immunology, Maria Curie-Skłodowska University, Lublin, Poland.
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