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Original article

Novel pentamidine derivatives: Synthesis, anti-tumor properties and polynucleotide-binding activities

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ABSTRACT

Novel amidino-substituted conformationally restricted derivatives of pentamidine were synthesized and their antiproliferative activity against several human cancer cell lines determined. It was found that introduction of furandicarboxamide core moiety (**9, 10**) increases antiproliferative activity as well as selectivity against certain tumor cell lines in comparison with amidino-substituted furan-mono-carboxamide (**5**, **6**). Unlike the furan series where *iso*-propyl substituted amidine (**10**) exhibits more potent overall antiproliferative activity and selectivity toward certain cell lines, the same was found for unsubstituted amidines in pyridine series. Amongst all tested compounds the compound **10** is the only one that possesses antiproliferative activity against SW 620 cell line (4 μM). Spectroscopic studies of the interactions of prepared diamidines with double-stranded DNA and RNA polynucleotides show that all compounds preferentially bind into the minor groove of DNA, while most of them intercalate into RNA. The structure-dependant biological activity and the lack of DNA/RNA selective binding suggest that the mechanism of action of the here-presented compounds is controlled not only by the interactions with cellular nucleic acids, but also with other more specific protein targets.

1. Introduction

The design of small molecules that target the minor groove of double helix of B-DNA has been of great interest for the development of new therapeutics for various diseases [1]. In the past few decades various minor groove binding dibenzamidines were found to possess antifungal, antleishmanial, antiplasmodial, antitrypanosomal and anti-*Pneumocystis carinii* activity [2,3]. In spite of the broad spectra of biological activities they exhibit, pentamidine is the only compound of this class that has been clinically used in humans. It is used against early stage human African trypanosomiasis, antimony-resistant leishmaniasis and for *P. jiroveci pneumonia* (PCP) in the treatment of AIDS patients [4]. However, despite its clinical usefulness it exhibits a number of serious adverse effects [5]. Results from previous studies suggest that the electronic and conformational nature of the linker may influence not only the chemical properties of the amidine moieties but also improve biological activity of the pentamidine derivatives [6]. Amongst those, furamidine, a bis-amidine diphenylfuran, displayed significant anti-PCP and antiprotozoal activities [7], its amidoxime prodrug currently being in Phase III trials against PCP.

In addition to their antimicrobial activities, pentamidine possess cytotoxic activity towards various human cancer cell lines [8] and is currently in Phase II clinical trials against refractory melanoma. A series of cyclic dibenzoamidines with triazine and carbazole linkers [9] as well as furamidine derivatives characterized by different structural features have also displayed activities against broad tumor cell line panels [10,11].

One of the studied linkers was poorly electrondonating, conformationally restricted amide group. Amidinophenyl substituted benzo and pyridine dicarboxamides were prepared and tested for their antimicrobial activity or cytotoxic activity against several human cancer cell lines [12–15]. Although they showed moderate activity against *P. carinii* and *Plasmodium falciparum*, their antitumor activity was poor [6,14,16].

In an attempt to investigate further the anti-tumor potential of carboxamide linked pentamidine derivatives we decided to prepare a series of novel dibenzamidines with various aromatic carboxamide linkers and amidino substituents. Amidino- and

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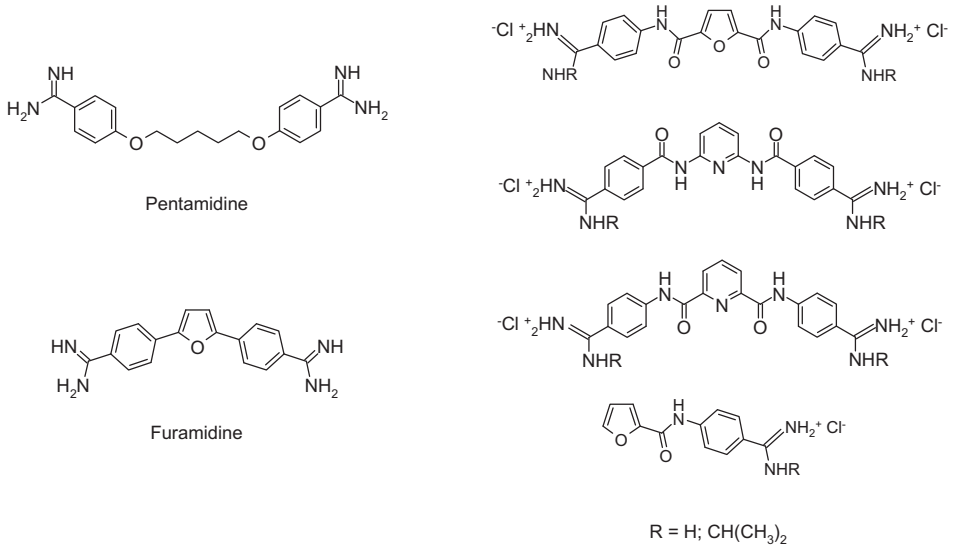


Fig. 1. Pentamidine, furamidine and new conformationally restricted derivatives.

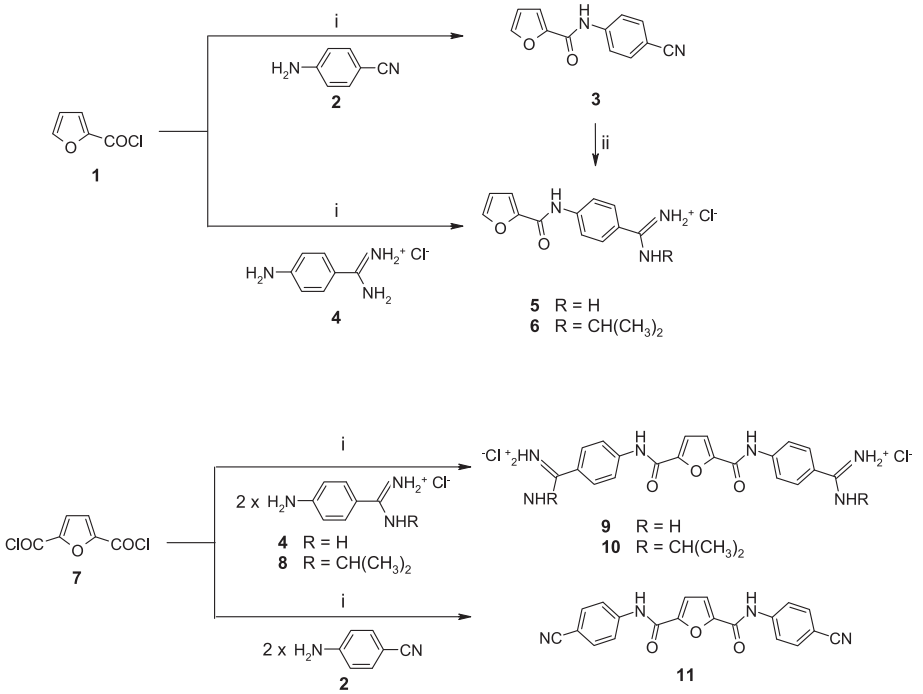
iso-propylamidino-substituted pentamidine derivatives with furan-2,5-di-yl-carboxamide and pyridine-2,6-di-yl-carboxamide linkers were prepared. In order to examine the influence of inverted amide bond pattern on biological activity, diamidinobenzoylpyridine-2,6-di-yl-amines were also synthesized (Fig. 1). Since it was proposed that pentamidine derivatives exert, at least partly, their biological activity through DNA binding, the *in vitro* antitumor activity of the newly prepared compounds was evaluated and polynucleotide-binding studies were conducted to determine whether they correlate to the *in vitro* biological activity.

Recently, working in the field of benzothieno and thienothienoquinolones, new intercalators with antiproliferative activity, we described the synthesis of a number of their precursors, cyano and amidino-substituted 3-chlorobenzo[*b*]thiophene- [17], thieno[2,3-*b*]- and thieno[3,2-*b*]thiophene-2-carboxanilides [18] which were found to exhibit interesting antiproliferative properties themselves. They were found to bind to polynucleotides in a weak nonintercalative way, and the mechanism of their antiproliferative activity is currently under investigation. In order to investigate the influence of different heterocyclic moieties of amidino and cyano-substituted carboxanilides on their biological activity, furan moiety was introduced instead of benzothiophene and thienothiophene groups (Fig. 1).

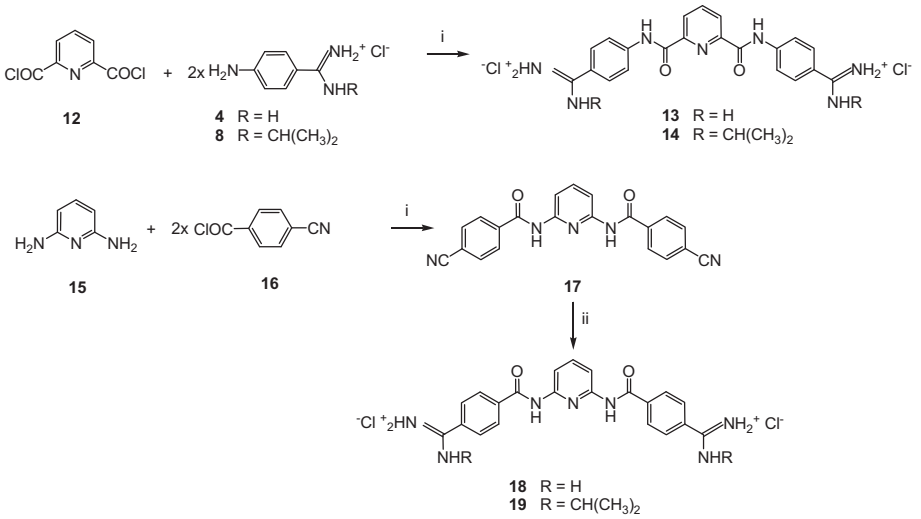
2. Results and discussion

2.1. Chemistry

Compounds **3**, **5**, **6** and **9**–**11** were synthesized according to the procedures shown in Scheme 1. Precursor **3** for the synthesis of compounds **5** and **6** was prepared by condensation of 2-furoyl-



Scheme 1. Reagents and conditions: i) dry DMF, dry THF, Et_3N (**9**); dry toluene, Et_3N (**3**, **5**, **10**, **11**); ii) 1. anhydrous EtOH, HCl(g), 2. anhydrous EtOH, RNH_2, 3. anhydrous EtOH, HCl(g).



Scheme 2. Reagents and conditions: i) dry DMF, dry THF, Et_3N (**13, 14**); dry toluene, dry THF, Et_3N (**17**); ii) 1. anhydrous EtOH, HCl(g), 2. anhydrous EtOH, RNH_2, 3. anhydrous EtOH, HCl(g).

chloride **1** and 4-amino-benzonitrile **2** in the presence of triethylamine. Modified procedure was used for the synthesis of the cyano-substituted amides **11** and **17**. Cyano-substituted carboxamide **3** was converted to the corresponding amidines under the Pinner conditions [17]. Treatment of **3** with dry HCl gas in anhydrous EtOH gave the imidate ester, which was then treated with dry ammonia gas to produce amidino-substituted 2-furan-carboxamide **5**, or with excess of *iso*-propylamine to give *iso*-propylamidino substituted carboxamide **6**. Crude compounds were, after crystallization, isolated as hydrochloride salts. Amidino compound **5** was also prepared by condensation of 2-furoyl-chloride **1** with corresponding 4-amino-benzamidine hydrochloride **4**. No significant difference in the yields was observed between linear and convergent method of the synthesis for compound **5**.

Since the solubility of dicyano-substituted furan-2,5-di-yl-carboxamide **11** was too low to use Pinner reaction, diamidines **9** and **10** were prepared by direct condensation of the corresponding diacylchloride **7** with appropriate amino-benzamidines (Scheme 1). The same approach was used for the synthesis of 4-amidino-substituted pyridine-2,6-di-yl-carboxamides **13** and **14** (Scheme 2), giving, in the case of **13**, yields similar to those reported by Tao *et al.* [6] for the synthesis of the same compound from the corresponding 4-cyano-phenyl substituted pyridine-2,6-di-yl-carboxamide. Attempts to synthesize amidino-substituted aminopyridines **18** and **19** by direct condensation of diaminopyridine **15** and amidino-substituted benzoyl chlorides failed and they were synthesized from cyano-substituted carboxamide **17** obtained by condensation of cyano substituted benzoylchloride **16** with diaminopyridine **15**. Compound **17** was converted to the corresponding amidines **18** and **19** under the Pinner conditions (Scheme 2).

2.2. Interactions of compounds *9*, *10*, *13*, *14*, *18* and *19* with double-stranded polynucleotides

Many aromatic diamidines are strong DNA minor groove binders, and some evidence suggests that groove binding is at least in part responsible for their biological activity [19]. Therefore, interactions of novel amidino-substituted dicarboxamides **9**, **10**, **13**, **14**, **18** and **19** with model double-stranded DNAs and RNAs were studied by a series of spectrophotometric methods under biologically relevant conditions (pH = 7, sodium cacodylate buffer, $I = 0.05$ M). For comparison, interactions of their shorter amidino-substituted analogues **5** and **6** were also studied.

Aqueous solutions of amidino-substituted compounds were initially studied by electronic absorption (UV/Vis). Under the experimental conditions used the absorbance intensities were proportional to their concentrations.

Introduction of the second *N*-4-amidino-substituted phenylcarbamoyl group to the position 5 of 2-furan-carboxamide system (**9**, **10**) resulted in bathochromic shift and increased ϵ values (approximately doubled) (Table 1). Substitution of furan core group with the pyridine one (**13**, **14**) caused a significant decrease of both λ_{max} and ϵ values, while reversing of an amide bond motif (**18**, **19**) resulted in the appearance of the second absorption band with lower λ_{max} (~235 nm).

Table 1
Electronic absorption data for compounds **5**, **6**, **9**, **10**, **13**, **14**, **18**, and **19**.[a]

Compd.	λ_{max}/nm	$\epsilon \times 10^3$/dm^3 mol^{-1} cm^{-1}
5	295	22.4
6	292	27.2
9	312	52.0
10	314	57.4
13	292	37.3
14	289	36.5
18	238	37.3
	300	21.3
19	236	58.3
	301	31.2

[a] Sodium cacodylate/HCl buffer. $I = 0.05$ mol dm^{-3}, pH = 7.

Table 2
ΔT_m values (°C)[a] of studied *ds*-polynucleotides at different ratios *r* at pH = 7 (sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}).

	r [b]	9	10	13	14	18	19
ct-DNA	0.3	7.5	5.6	6.7	8.5	13.5	13.1
	0.5	–[c]	6.6	–[c]	10.3	15	14.5
Poly A-poly U	0.3	1.6	4.5	~1.0	3.1	~1.0	3.7
	0.5	–[c]	20.1	–[c]	3.7	18.7	5.7
Poly dA-poly dT	0.3	30.2	29.5	20.4	24.1	29.3	30.5
	0.5	–[c]	31.7	–[c]	25.5	30.9	32.2

[a] Error in ΔT_m: ±0.5 °C.
[b] r = [compound]/[polynucleotide].
[c] Not possible to determine due to precipitation.

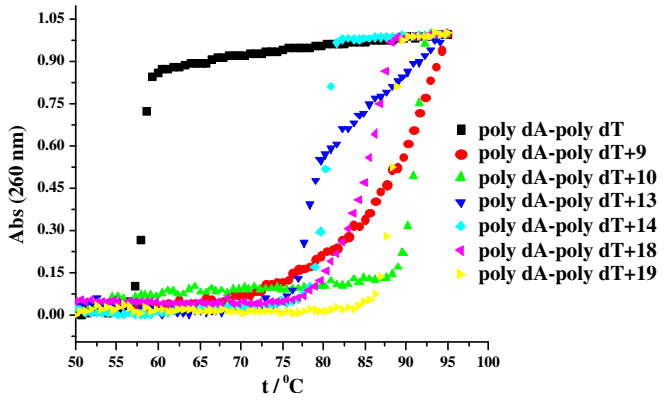


Fig. 2. The normalized thermal denaturation experiments with poly dA-poly dT in the presence of compounds **9, 10, 13, 14, 18** and **19** at the ratio $r_{([compound]/[polynucleotide])} = 0.3$ (sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}, pH 7).

2.2.1. Thermal denaturation experiments

Relative affinity of studied diamidines toward *ds*-DNA and *ds*-RNA were evaluated by thermal denaturation experiments at different ratios r ([compound]/[polynucleotide]). Melting temperatures (T_m) of polynucleotides were determined from melting curves (Table 2, Fig. 2 and Supp. Info).

Since amidino-substituted amides**5** and **6** did not stabilize double helix of *ct*-DNA, further experiments with those compounds were not conducted.

In general, diamidines moderately stabilize *ct*-DNA double helix at the ratio $r = 0.3$ with the ΔT_m values ranging from 5.6 to 13.5 °C, whereby the highest values were obtained for amidinobenzoyl pyridine-2,6-di-yl-carboxamides (**18**, **19**). Only slightly increased stabilization of *ct*-DNA at $r = 0.5$ points toward saturation of binding sites close to ratio $r = 0.3$. All studied compounds stabilized poly dA-poly dT significantly stronger than *ct*-DNA, which is consistent with the observation that pentamidine derivatives prefer AT-rich DNA sequences [20]. As in the case of *ct*-DNA increase of ratio *r* from 0.3 to 0.5 yielded only negligible increase of stabilization effect, suggesting saturation of binding sites close to $r = 0.3$.

Addition of diamidines to the poly A-poly U resulted in markedly weaker stabilization at $r = 0.3$ compared to *ct*-DNA, with the *iso*-propylamidine derivatives (**10, 14, 19**) exhibiting somewhat higher stabilization of RNA (poly A-poly U) in comparison to other tested compounds. Surprisingly, compounds **10** and **18**, and to some extent **19**, stabilized *ds*-RNA at ratio $r = 0.5$ significantly stronger than at ratio $r = 0.3$, which suggested change of binding pattern, possibly due to stacked dimers formation within the RNA major groove.

2.2.2. UV spectroscopy

In order to determine stability and stoichiometry of diamidine–polynucleotide complexes, UV titrations were performed. The UV spectra of compounds in the most cases resulted in marked changes upon addition of studied polynucleotides, exhibiting strong hypochromic effects and in some cases measurable bathochromic shifts of the UV maximum of a compound. However, spectroscopic changes were mostly observed at excess of a compound over polynucleotide concentrations and consequently significant deviations from isosbestic points were observed (Fig. 3a), suggesting mixed binding modes. Therefore it was not possible to process the titration data by means of the Scatchard equation [21] to determine the affinity of studied compounds toward polynucleotides.

An exception was the UV titration of **10** with *ct*-DNA (Fig. 3b), in which the isosbestic point was preserved and processing of the titration data by means of the Scatchard equation [21] gave binding constant of $K_s = 6.3 \times 10^4$ M^{-1}.

2.2.3. Ethidium bromide displacement assays

Since studied diamidines do not exhibit fluorescence, the affinities toward polynucleotides were determined by ethidium bromide (EB) displacement assay as previously described [22]. This technique, based on the quenching of the fluorescence of the ethidium bromide–polynucleotide complex upon EB displacement by a competitive compound, has also been used to estimate the affinity of ligands that bind to the DNA minor groove. Although EB displacement assay can be used for various polynucleotides, the obtained IC_{50} values cannot be directly compared since EB exhibits variable binding stoichiometries and affinities towards different nucleic acids.

Addition of all tested diamidines quenched the fluorescence of EB-polynucleotide complexes (Fig. 4, Table 3). Obtained IC_{50} values suggest that studied compounds possess similar capacity for displacing EB from its complex with *ct*-DNA, with the exception of compound **9** whose IC_{50} suggests somewhat stronger affinity towards *ct*-DNA. Of all tested polynucleotides, the highest IC_{50} values were obtained for displacement of EB from its poly dA-poly dT complex, which is in agreement with the highest ΔT_m values and the observation that pentamidine analogues prefer AT-rich DNA sequences.

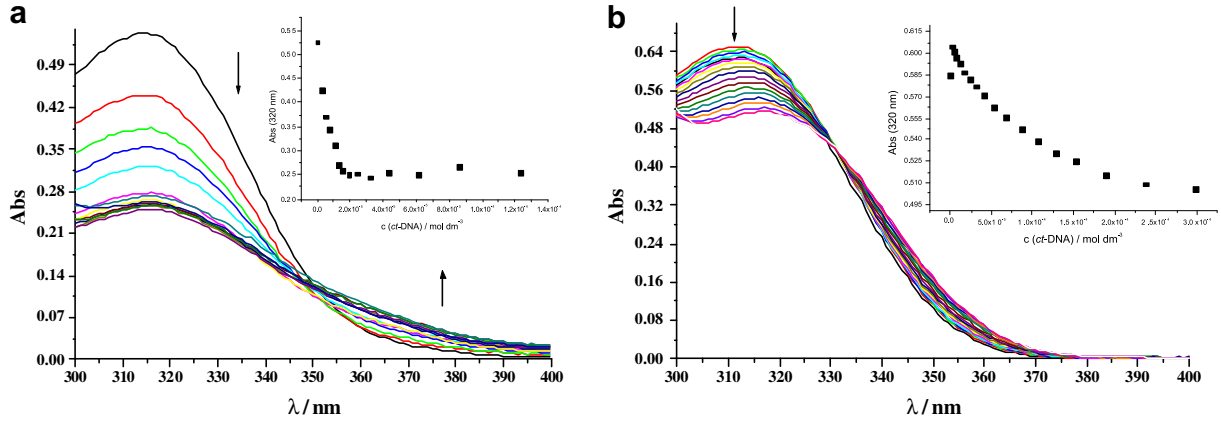


Fig. 3. UV/Vis spectral changes of a) **9** ($c = 1.0 \times 10^{-5}$ mol dm^{-3}) and b) **10** ($c = 1.2 \times 10^{-5}$ mol dm^{-3}) upon the addition of *ct*-DNA; (sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}, pH 7).

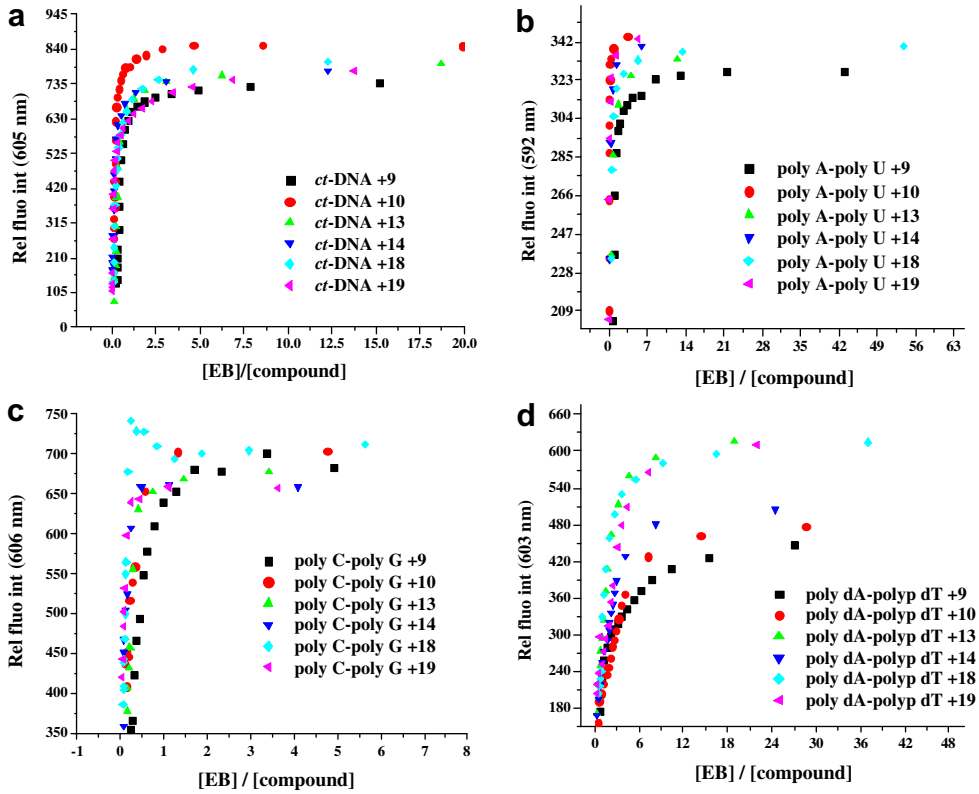


Fig. 4. Ethidium bromide (EB) assay: to EB solution, polynucleotides were added ([EB]/[polynucleotide] = 0.1), and quenching of EB-polynucleotide fluorescence emission was monitored as function of increasing [EB]/[compound]; Displacement of EB from its complexes with: a) *ct*-DNA, b) poly A-poly U; c) poly G-poly C, d) poly dA-poly dT; (sodium cacodylate/HCl buffer, I = 0.05 mol dm^{-3}, pH 7).

2.2.4. Circular dichroism spectroscopy

Circular dichroism (CD) spectropolarimetry offers useful information on conformational changes of the polynucleotide upon ligand binding as well as on the binding mode of studied ligands. Upon binding to asymmetric environment achiral compounds can show induced CD signals which are characteristic for the binding mode. For instance, DNA minor groove binders will exhibit large positive ICD signals in the ligand absorption region, while intercalators are characterized by weak ICD signal, which can be either positive or negative depending on the orientation of compounds longer axis in respect to the longer axes of adjacent basepairs [23]. The studied compounds are not chiral and therefore do not possess CD spectrum.

Experimental conditions used for CD experiments were similar to those used for UV titrations which showed mixed binding modes. Clear deviations from the isoelliptic point observed in CD spectra upon the subsequent additions of **9**, **10**, **13**, **14**, **18**, and **19** to different polynucleotides clearly support formation of several different types of complexes (Fig. 5 and Supp. Info.).

Under the conditions of a large excess of *ds*-DNA (*ct*-DNA and poly dA-poly dT) over compound (r = 0.1) a strong positive ICD band appeared at λ = 300–330 nm accompanied by significant decrease of intensity of *ds*-DNA CD bands (Fig. 5).

Further increase of compound concentration up to r = 0.3 did not induce significant changes, but at ratios r > 0.5 ICD bands strongly changed (ICD band maximum was blue shifted in the case of *ct*-DNAor in the experiments with poly dA-poly dT intensity of ICD band decreased) due to the formation of a different type of a complex with *ds*-DNAs (Fig. 5). Such a dependence of an ICD band on a ratio r suggests that at the excess of DNA over compound (up to r = 0.3) molecules predominantly bind into minor groove of DNA, while at the excess of compound over DNA, the surplus of small positively charged molecules is either stacked along negatively charged DNA backbone or forms dimers within DNA minor groove. The latter possibility is supported by strong bisignate exciton ICD bands at λ > 300 nm observed in several cases, which could be attributed to ligand–ligand stacked dimers positioned within DNA grooves [24,25].

At variance to the experiments with *ds*-DNAs, the addition of compounds **9, 10, 13, 14, 18,** and **19** to the solution of poly A-poly U at the ratio r <0.3 yielded appearance of a weak or moderate negative ICD band in the λ = 300–330 nm region. Weak negative ICD bands were observed for compounds **13** (303 nm) and **19** (299 nm), while **18** exhibits no observable ICD. At low [**10**]/[poly A-poly U] ratios (r = 0.1), the induced ICD signal in the region above 290 nm was small and negative, but at higher ratios (r = 0.2–0.3) it assumed bisignate pattern (positive at 306 nm and negative at 333 nm) which might result from exciton coupling (aggregation of

Table 3
IC_{50}-values[a] for EB displacement from different *ds*-polynucleotides by diamidines (Sodium cacodylate/HCl buffer, I = 0.05 mol dm^{-3}, pH = 7).

	9	10	13	14	18	19
ct-DNA	0.8	0.36	0.3	0.15	0.19	0.13
Poly A-poly U	1	0.03	0.47	0.17	0.5	0.07
Poly G-poly C	0.36	0.2	0.25	0.1	0.07	0.07
Poly dA-poly dT	2.1	2.23	1.6	1.37	1.7	2

[a] Ratio [EB]/[compound] at which 50% of EB fluorescence is quenched, meaning that approximately half of EB is displaced from its DNA complex. IC_{50} = [Int(EB-polyncleotide)-Int(EB_{free})]/2, where Int[EB-polynucleotide] is fluorescence intensity of EB-polynucleotide complex and Int(EB_{free}) fluorescence intensity of free EB before polynucleotide addition.

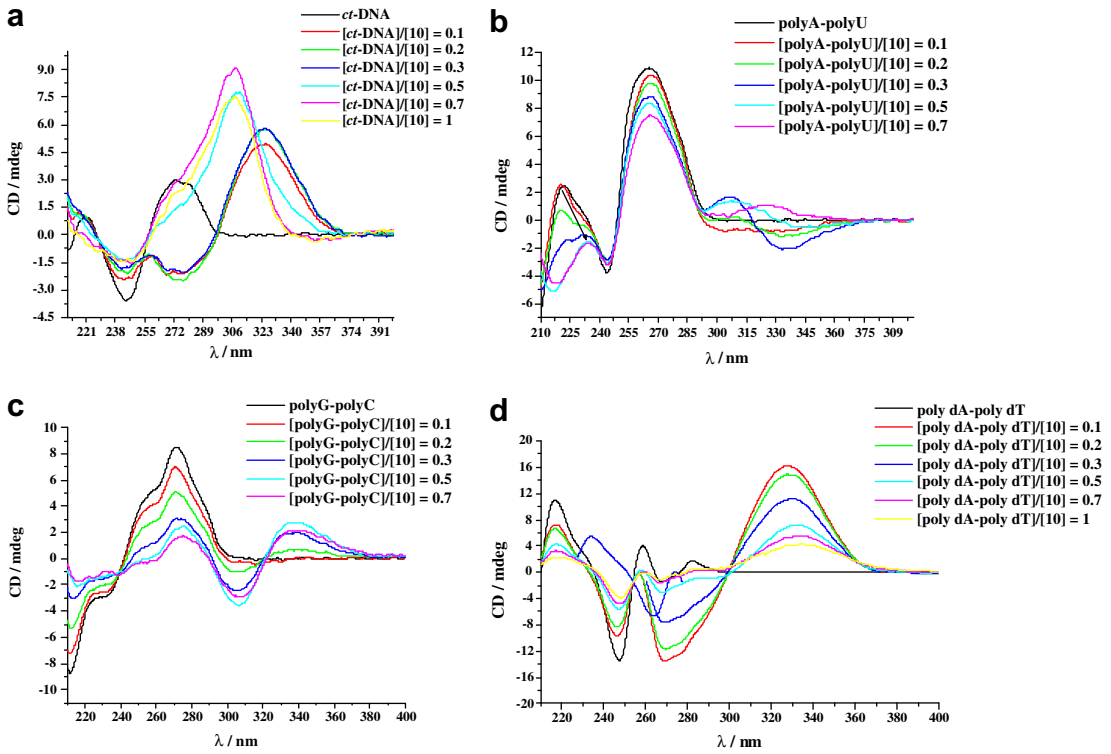


Fig. 5. CD spectra of *ds* polynucleotides in the presence of compound 10; a) *ct*-DNA, b) poly A-poly U; c) poly G-poly C, d) poly dA-poly dT; (sodium cacodylate/HCl buffer, $I = 0.05$ mol dm^{-3}, pH 7).

the ligand molecules and association of these aggregates with the polynucleotide) [24,25]. Intriguingly, compound **10** stabilized *ds*-RNA significantly stronger at $r = 0.5$ than at $r = 0.3$, which could also be attributed to stacked dimer formation within RNA major groove at $r > 0.3$. Negative signal, however, disappears with further addition of **10** and at the ratio of $r = 0.7$ gives positive ICD at 326 nm. There were marked changes in the CD signals of poly A-poly U as well. The positive CD bands (266 nm and 222 nm) decreased in intensity, while a negative band (244 nm) increased. Experiments with poly G-poly C resulted in the similar effects. Changes observed in CD signals of *ds*-RNA polynucleotides suggest that **9, 10, 13, 14, 18,** and **19** cause significant conformational changes in *ds*-RNA accompanied by weak negative ICD bands at low ratios r (excess of the RNA over compound), which is in the agreement with intercalative binding mode [23]. Again, at excess of compound over polynucleotide ($r > 0.3$) further changes in CD spectra were observed due to the agglomeration of small molecule surplus along polynucleotide.

2.3. Cytotoxic activity

Compounds **3**, **5**, **6**, **9**–**11**, **13**, **14**, **18** and **19** were tested for their potential antiproliferative effect on a panel of 5 human cell lines which were derived from 5 cancer types: Hep-2 (laryngeal carcinoma), HeLa (cervical carcinoma), MiaPaCa-2 (pancreatic carcinoma), SW 620 (colon carcinoma), and MCF-7 (breast carcinoma). Compounds **13**, **14**, **18** and **19** were additionally tested on MOLT-4 (lymphoblastic leukemia), HCT 116 (colon carcinoma), and H 460 (lung carcinoma) human cancer cells.

Tested compounds showed different antiproliferative effect (Table 4 and Table 5). Unlike their benzothiophene and thienothiophene anilide analogues, the cyano- and amidino- monosubstituted compounds **3**, **5** and **6** exhibited no antiproliferative activity producing only modest growth inhibition against HeLa or MCF-7 lines, predominantly at the highest tested concentration, pointing towards the importance of benzothiophene and thienothiophene moieties for the antiproliferative activity of that class of compounds [17,18].

On the other hand, prolongation of the amido-scaffold (**9**, **10**) resulted in markedly stronger antiproliferative effect towards certain cell lines, especially MCF-7. Isopropylamidino-substituted compound **10** exhibits somewhat stronger antiproliferative activity than its amidino-substituted analogue **9**. Compound **10** had the strongest antiproliferative effect towards the MCF-7 (4 μM) and SW620 (4 μM) cell lines. The weakest effect was observed for dicyano-substituted compound **11**, which produced no growth inhibition, even at the highest tested concentrations, indicating the influence of the amidino-substituents on antiproliferative activity in the series of the disubstituted furans. Unlike furan diamidines, pyridine diamidines (**13**, **14**, **18**, **19**) exhibited only weak antiproliferative activity to some of the tested cell lines.

Table 4
Inhibitory activities of compounds **3**, **5**, **6**, **9**, **10**, and **11** on the growth of human tumor cells.

	IC_{50}/μM[a]				
Compd.	Hep-2	HeLa	MiaPaCa-2	SW 620	MCF-7
3	≥100	47 ± 22	>100	>100	32 ± 9
5	≥100	≥100	>100	>100	33 ± 16
6	>100	60 ± 4	>100	>100	≥100
9	11 ± 10	>100	53 ± 39	>100	5 ± 4
10	5 ± 2	50 ± 30	79 ± 3	4 ± 3	4 ± 2
11	>100	>100	>100	>100	>100

[a] IC_{50}, the concentration that causes a 50% reduction of the cell growth.

Table 5
Inhibitory activities of compounds **13**, **14**, **18**, and **19** on the growth of human tumor cells.

	$IC_{50}/\mu M$[a]							
Compd.	Hep-2	HeLa	MiaPaCa-2	SW 620	MCF-7	MOLT-4	HCT 116	H 460
13	47 ± 2	18 ± 2	13 ± 1	>100	>100	>100	>100	19 ± 3
14	30 ± 14	53 ± 47	>100	>100	32 ± 0.2	>100	>100	>100
18	21 ± 11	19 ± 12	13 ± 0.06	>100	12 ± 0.6	>100	67 ± 30	17 ± 2
19	38 ± 38	56 ± 43	>100	>100	24 ± 4	>100	>100	>100

[a] IC_{50}, the concentration that causes a 50% reduction of the cell growth.

3. Conclusions

New conformationally restricted derivatives of pentamidine with arylcarboxamide linkers were synthesized and characterized. The cytostatic activities of newly prepared compounds were evaluated on several different human cancer cell lines. Compounds with furandicarboxamide moiety exhibited antiproliferative activity in low micromolar concentration range (**9** and **10**). At the same time introduction of furandicarboxamide linker strongly influenced the selectivity towards certain cell lines (Hep-2, SW 620 and MCF-7). The effect is less pronounced for pyridinedicarboxamide and diaminopyridine compounds (**13**, **14**, **18**, **19**) and they possess only moderate antiproliferative activities against some cell lines. Substitution of amidino groups by *iso*-propyl moiety in general increased the antiproliferative activities of furandicarboxamide derivatives. Unlike furan order, the antiproliferative activity and selectivity of the pyridine order is more pronounced for unsubstituted amidines (**13**, **18**). In addition, of all tested compounds only the compound **10** exerted antiproliferative activity against SW 620.

In contrast to the structure-sensitive biological activity, no significant differences in DNA/RNA binding were observed concerning the heterocylic core or the nature of amidino-substituents. Namely, spectroscopic analysis of compounds/*ds*-polynucleotides interactions showed that all tested compounds bind as groove binders to *ct*-DNA and poly dA-poly dT, while on the other hand they most probably intercalate into RNA double helices (poly A-poly U, poly G-poly C). Therefore, we assume that the mechanism of action of the here-presented compounds is controlled not only by the interaction with cellular nucleic acids, but also with other more specific protein target. Further experiments are needed in order to provide more details on the mechanism of their biological activity and selectivity towards different cell lines.

4. Experimental protocol

4.1. Chemistry

4.1.1. General methods

Melting points were determined on a Kofler hot-stage microscope and are uncorrected. IR spectra were recorded on a Nicolet Magna 760 spectrophotometer in KBr discs. ^1H and ^{13}C NMR spectra were recorded on either a Bruker Avance DPX 300 spectrometer using TMS as an internal standard in DMSO-d_6, $CDCl_3$ or CD_3OD. Elemental analysis for carbon, hydrogen and nitrogen were performed on a Perkin–Elmer 2400 elemental analyzer. All compounds were routinely checked by thin layer chromatography (TLC) with precoated Merck silica gel 60F-254 glass plates and the spots were detected under UV light.

4.1.2. General procedure A: synthesis of cyano-substituted carboxamides

To a solution of carbonyl chloride (dry toluene or dry DMF) was added dropwise a solution of appropriate amine (dry THF or dry toluene) or a solution of 4-aminobenzonitrile (toluene), followed by the addition of Et_3N. The mixture was refluxed for 1.5–14 h. After cooling, precipitated crystals were filtered off, washed with diluted HCl and water, and recrystallized.

4.1.3. General procedure B: synthesis of amidino-substituted carboxamides

To a solution (dry DMF) or a suspension (dry toluene) of *N*-isopropyl 4-amino-benzoamidine hydrochloride or 4-amino-benzoamidine hydrochloride was added dropwise a solution of appropriate carbonyl chloride (dry toluene or dry THF), followed by the addition of Et_3N. The mixture was refluxed for 2.5 h-2 days. After cooling, the solution was concentrated and precipitated crystals were filtered off and washed with diluted HCl and water. Dry HCl was bubbled into a suspension of a crude product in dry ethanol (40 ml) and stirred overnight. Precipitate was filtered off and recrystallized.

4.1.4. General procedure C: synthesis of amidines from the corresponding nitriles

Dry HCl gas was bubbled for 4 h into a cooled suspension (0 °C) of nitrile compound in anhydrous ethanol. The suspension was stirred at room temperature until the −CN band was undetectable (IR). After anhydrous diethyl ether was added, the corresponding imidate ester was collected by filtration. The product was suspended in anhydrous ethanol and the corresponding amine was added. For the synthesis of unsubstituted amidines, the suspension was treated with anhydrous ammonia gas and stirred for 3 days at room temperature. For the synthesis of isopropyl-substituted amidines, excess of isopropylamine (2 M equivalents) was added to a suspension of imidate ester. Solvent was removed by evaporation and dry diethyl ether was added. Obtained solid was treated with saturated HCl-EtOH solution (40 ml). Recrystallization from appropriate solvents gave pure amidines as hydrochloride salts.

4.1.5. N-(4-Cyanophenyl)-furan-2-yl-carboxamide (**3**)

Following the general procedure A, from **2** (9.96 g, 0.084 mol) in dry toluene (260 ml), **1** (7.54 ml, 0.076 mol) in dry toluene (10 ml), and Et_3N (10 ml, 0.17 mol), after refluxing for 1.5 h and recrystallization from chloroform: 14.3 g (88%) of light-brown powder was obtained, mp 176–178 °C; IR (KBr) (ν_{max}/cm^{-1}): 2220, 1670, 1580; ^1H NMR ($CDCl_3$) (δ ppm): 8.28 (s, 1H, H_{amide}) 7.79 (d, 2H, $J = 8.46$ Hz, $H_{arom.}$) 7.64 (d, 2H, $J = 8.71$ Hz, $H_{arom.}$) 7.54 (s, 1H, H_{furan5}) 7.29 (d, 1H, $J = 3.33$ Hz, H_{furan3}) 6.59 (dd, 1H, $J_1 = 3.33$ Hz, $J_2 = 1.54$ Hz, H_{furan4}); ^{13}C NMR ($CDCl_3$) (δ ppm): 158.9, 156.0, 147.9, 145.8, 145.4, 131.8, 125.9, 126.7, 121.1, 120.8, 117.5, 112.5; Anal. ($C_{12}H_8N_2O_2$) Calcd.: C, 59.00; H, 3.30; N, 11.47; Found: C, 59.06; H, 3.39; N, 11.46.

4.1.6. N-(4-Amidinophenyl)-furan-2-yl-carboxamide hydrochloride (**5**)

Method A.: following the general procedure B, from 4 (1 g, 0.0074 mol) in dry toluene (85 ml), 1 (0.58 ml, 0.0059 mol) in dry toluene (10 ml), and Et_3N (1.5 ml, 0.012 mol), after refluxing for 18 h and recrystallization from ethanol: 0.38 g (19%) of white powder was obtained,. mp 258–262 °C; IR (KBr) (ν_{max}/cm^{-1}): 3328, 3121, 1694; ^1H NMR (DMSO-d_6) (δ ppm): 10.76 (s, 1H, H_{amide}) 9.33 (s, 2H,

$H_{amidine}$) 9.12 (s, 2H, $H_{amidine}$) 8.03 (d, 2H, J = 8.87 Hz, $H_{arom.}$) 7.989–7.995 (m, 1H, H_{furan5}) 7.86 (d, 2H, J = 8.84 Hz, $H_{arom.}$) 7.56 (d, 1H, J = 3.46 Hz, H_{furan3}) 6.730–6.747 (m, 1H, H_{furan4}); ^{13}C NMR (DMSO-d_6) (δ ppm): 158.2, 149.6, 139.1, 137.8, 135.8, 120.6 (2C), 114.8, 112.1 (2C), 107.7, 104.1; Anal. ($C_{12}H_{12}ClN_3O_2 \times H_20$) Calcd.: C, 51.18; H, 4.97; N, 14.81; Found: C, 50.99; H,5.18; N,14.81. method b.: following the general procedure C, from 3 (1.51 g, 0.0071 mol) and after recrystallization from water: 0.263 g (14%) of white crystals.

4.1.7. N-[4–(N′-Isopropylamidino)phenyl]-furan-2-yl-carboxamide hydrochloride (**6**)

Following the general procedure C, from **3** (1.0 g, 0.0047 mol) and after recrystallization from methanol: 0.41 g (28%) of white crystals. was obtained,mp 292–296 °C; IR (KBr) (ν_{max}/cm^{-1}): 3117, 2969, 2854, 1678, 1666, 1621; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.72 (s, 1H, H_{amide}) 9.48 (d, 1H, J = 7.98 Hz, $H_{amidine}$) 9.37 (s, 1H, $H_{amidine}$) 9.04 (s, 1H, $H_{amidine}$) 8.01 (d, 2H, J = 8.31 Hz, $H_{arom.}$) 7.98 (s, 1H, H_{furan5}) 7.73 (d, 2H, J = 8.64 Hz, $H_{arom.}$) 7.54 (d, 1H, J = 3.33 Hz, H_{furan3}) 6.728–6.739 (m, 1H, H_{furan4}) 4.02–4.09 (m, 1H, $CH_{i\text{-}pr}$) 1.26 (d, 6H, J = 6.32, $2CH_{3\ i\text{-}pr}$); ^{13}C NMR (CD_3OD) (δ ppm): 164.4, 159.2, 148.8, 147.5, 144.8, 130.2 (2C), 125.9, 121.8 (2C), 117.3, 113.7; Anal. ($C_{15}H_{18}ClN_3O_2$) Calcd.: C, 58.53; H, 5.89; N, 13.65; Found: C, 58.23; H, 5.89; N, 13.41.

4.1.8. N,N′-di-(4-amidinophenyl)-(furan-2,5-di-yl)-carboxamide hydrochloride (**9**)

Following the general procedure B, from **4** (1.23 g, 0.0091 mol) in dry DMF (150 ml), **7** (0.8 g, 0.0041 mol) in dry THF (30 ml), and Et_3N (2.8 ml, 0.02 mol), after refluxing for 15 h and recrystallization from water: 0.37 g (19.6%) of white powder was obtained,. mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3300, 1660, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm): 11.81 (s, 2H, H_{amide}) 9.27 (s, 4H, $H_{amidine}$) 8.95 (s, 4H, $H_{amidine}$) 8.32 (d, 4H, J = 8.31 Hz, $H_{arom.}$) 7.83 (d, 4H, J = 8.31 Hz, $H_{arom.}$) 7.46 (s, 2H, H_{furan}); ^{13}C NMR (DMSO-d_6) (δ ppm): 164.9 (2C), 156.0 (2C), 148.2 (2C), 143.7 (2C), 129.0 (4C), 122.8 (2C), 120.1 (4C), 117.4 (2C); Anal. ($C_{20}H_{20}Cl_2N_6O_3 \times 3H_2O$) Calcd.: C, 46.43; H, 5.06; N, 16.34; Found: C, 46.75; H, 5.08; N, 16.70.

4.1.9. N,N′-di-[4–(N″-isopropylamidino)phenyl]-(furan-2,5-di-yl)-carboxamide hydrochloride (**10**)

Following the general procedure B, from **8** (2.21 g, 0.012 mol) in dry toluene (150 ml), **7** (1.0 g, 0.0052 mol) in dry toluene (50 ml), and Et_3N (0.9 ml, 0.006 mol), after refluxing for 14 h and recrystallization from ethanol: 0.52 g (18.3%) of white powder. was obtained,. mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3241, 3058, 2975, 2932, 1697, 1670, 1619; ^{1}H NMR (DMSO-d_6) (δ ppm): 11.87 (s, 2H, H_{amide}) 9.48 (d, 2H, J = 7.64 Hz, $H_{amidine}$) 9.47 (s, 2H, $H_{amidine}$) 9.01 (s, 2H, $H_{amidine}$) 8.33 (d, 2H, J = 7.64 Hz, $H_{arom.}$) 7.72 (d, 4H, J = 8.65 Hz, $H_{arom.}$) 7.44 (s, 2H, H_{furan}) 4.03–4.07 (m, 2H, J = 6.35 Hz, $CH_{i\text{-}pr}$) 1.25 (d, 12H, J = 6.32 Hz, $CH_{3\ i\text{-}pr}$); ^{13}C NMR (DMSO-d_6) (δ ppm): 161.4 (2C), 156.0 (2C), 148.3 (2C), 143.2 (2C), 129.1 (4C), 124.0 (2C), 120.1 (4C), 117.4 (2C), 45.0 (2C), 21.4 (4C); Anal. ($C_{26}H_{32}Cl_2N_6O_3 \times H_2O$) Calcd.: C, 55.22; H, 6.06; N, 14.86; Found: 55.41; H, 6.18; N, 14.98.

4.1.10. N,N′-di-(4-cyanophenyl)-(furan-2,5-di-yl)-carboxamide (**11**)

Following the general procedure A, from **7** (1.0 g, 0.0052 mol) in dry toluene (130 ml), **2** (1.22 g, 0.0103 mol) in dry toluene (30 ml), and Et_3N (3.5 ml, 0.025 mol), after refluxing for 14 h and recrystallization from the mixture of DMF and water (1:1): 0.24 g (12.7%) of white powder. was obtained,.mp >300 °C; IR (KBr) (ν_{max}/cm^{-1}): 2220, 1670, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.71 (s, 2H, H_{amide}) 7.985 (d, 4H, J = 8.75 Hz, $H_{arom.}$), 7.87 (d, 4H, J = 8.73 Hz, $H_{arom.}$) 7.55 (s, 2H, H_{furan}); ^{13}C NMR (DMSO-d_6) (δ ppm): 155.8 (2C), 148.1 (2C), 142.4 (2C), 133.3 (4C), 120.3 (4C), 118.9 (2C), 116.8 (2C), 105.9 (2C); Anal. ($C_{20}H_{12}N_4O_3$) Calcd.: C, 67.39; H, 3.39; N, 15.73; Found: C, 67.47; H, 3.52; N, 15.81.

4.1.11. N,N′-di-(4-amidinophenyl)-(pyridine-2,6-di-yl)-carboxamide hydrochloride (**13**)

Following the general procedure B, from 4 (0.8 g, 0.0059 mol) in dry DMF (100 ml), 12 (0.57 g, 0.0028 mol) in dry THF (40 ml), and Et_3N (1.4 ml, 0.010 mol), after refluxing for 48 h and recrystallization from methanol: 0.88 g (66%) of white powder. was obtained,.mp. >300 °C (lit.[6] = 318 °C); ^{1}H NMR (DMSO-d_6) (δ ppm): 11.79 (s, 2H, H_{amide}), 9.34 (s, 4H, $H_{amidine}$), 9.07 (s, 4H, $H_{amidine}$), 8.415 (d, 2H, H_{py}, J = 7.32 Hz), 8.33 (d, 5H, $4H_{arom.}$, $1H_{py}$, J = 8.64 Hz), 7.91 (d, 4H, $H_{arom.}$, J = 8.65 Hz).

4.1.12. N,N′-di-[4–(N″-isopropyl)amidinophenyl]-(pyridine-2,6-di-yl)-carboxamide hydrochloride (**14**)

Following the general procedure B, from 8 (1.5 g, 0.0085 mol) in dry DMF (100 ml), 12 (0.86 g, 0.0042 mol) in dry THF (50 ml), and Et_3N (3 ml, 0.022 mol), after refluxing for 48 h and recrystallization from ethanol: 1.25 g (53%) of white powder. was obtained mp 238–242 °C; IR (KBr) (ν_{max}/cm^{-1}): 3200, 3020, 1650, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm): 11.91 (s, 2H, H_{amide}), 9.54 (d, 2H, $H_{amidine}$, J = 7.68 Hz), 9.43 (s, 2H, $H_{amidine}$), 9.08 (s, 2H, $H_{amidine}$), 8.28–8.41 (m, 7H, $4H_{arom.}$, $3H_{py}$), 7.79 (d, 4H, $H_{arom.}$, J = 8.97 Hz), 4.05–4.07 (m, 2H, $CH_{i\text{-}pr}$), 1.27 (d, 12H, $CH_{3\ i\text{-}pr}$, J = 6.32 Hz); ^{13}C NMR (DMSO-d_6) (δ ppm): 162.8, 161.3, 149.1, 143.1, 139.9, 129.1, 126.0, 124.0, 120.6, 45.1, 21.4; Anal. ($C_{27}H_{33}Cl_2N_7O_2 \times 3H_2O$) Calcd.: C, 52.94; H, 6.41; N, 16.01. Found: C, 52.59; H, 6.85; N, 16.20.

4.1.13. N,N′-di-(4-cyanobenzoyl)-(pyridine-2,6-di-yl)-amine (**17**)

Following the general procedure A, from **16** (2.96 g, 0.0018 mol) in dry toluene (50 ml), **15** (0.72 g, 0.0071 mol) in dry THF (50 ml), and Et_3N (6.6 ml, 0.090 mol), after refluxing for 2.5 h and recrystallization from the mixture of DMF and ethanol (1:1): 1.77 g (68%) of white powder. was obtained mp 298-300 °C; IR (KBr) (ν_{max}/cm^{-1}): 3300, 2200, 1670, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.96 (s, 2H, H_{amide}), 8.11 (d, 4H, $H_{arom.}$, J = 8.24 Hz), 8.01 (d, 4H, $H_{arom.}$, J = 8.24 Hz), 7.91 (s, 3H, H_{py}); ^{13}C NMR (DMSO-d_6) (δ ppm): 164.9, 150.3, 140.4, 138.4, 132.6, 128.9, 118.4, 114.3, 111.7; Anal. ($C_{21}H_{13}N_5O_2$) Calcd.: C, 68.66; H, 3.57; N, 19.06. Found: C, 68.52; H, 3.66; N, 19.17.

4.1.14. N,N′-di-(4-amidinobenzoyl)-(pyridine-2,6-di-yl)-amine hydrochloride (**18**)

Following the general procedure C, from **17** (1.05 g, 0.0029 mol) and after recrystallization from methanol: 0.24 (22%) of white powder. was obtained, mp 224-228 °C; IR (KBr) (ν_{max}/cm^{-1}): 3200, 1670, 1590; ^{1}H NMR (DMSO-d_6) (δ ppm): 10.91 (s, 2H, H_{amide}), 9.56 (s, 4H, $H_{amidine}$), 9.33 (s, 4H, $H_{amidine}$), 8.16 (d, 4H, $H_{arom.}$, J = 8.31 Hz), 7.89–7.97 (m, 7H, $4H_{arom.}$, $3H_{py}$); ^{13}C NMR (DMSO-d_6) (δ ppm): Anal. ($C_{21}H_{21}Cl_2N_7O_2 \times 2H_2O$) Calcd.: C, 49.42; H, 4.94; N, 17.21. Found: C, 49.49; H, 5.30; N, 17.15.

4.1.15. N,N′-di-[4–(N″-isopropyl)amidinobenzoyl]-(pyridine-2,6-di-yl)-amine hydrochloride (**19**)

Following the general procedure C, from **17** (0.47 g, 0.0013 mol) and after recrystallization from the mixture of ethyl acetate and methanol (1:1): 0.16 g (28%) of white powder. was obtained, mp 230-234 °C; IR (KBr) (ν_{max}/cm^{-1}): 3380, 3040, 1650, 1620, 1600; ^{1}H NMR (DMSO-d_6) (δ ppm): 11.91 (s, 2H, H_{amide}), 9.54 (d, 2H, $H_{amidine}$, J = 7.68 Hz), 9.43 (s, 2H, $H_{amidine}$), 9.08 (s, 2H, $H_{amidine}$), 8.28–8.41 (m, 7H, $4H_{arom.}$, $3H_{py}$), 7.79 (d, 4H, $H_{arom.}$, J = 8.97 Hz), 4.05–4.07 (m, 2H, $CH_{i\text{-}pr}$), 1.27 (d, 12H, $CH_{3\ i\text{-}pr}$, J = 6.32 Hz); ^{13}C NMR (DMSO-d_6) (δ ppm): 162.8, 161.3, 149.1, 143.1, 139.9, 129.1, 126.0, 124.0, 120.6,

45.1, 21.4; Anal. ($C_{27}H_{33}Cl_2N_7O_2 \times 4H_2O$) Calcd.: C, 51.43; H, 6.55; N, 15.55. Found: C, 51.59; H, 6.85; N, 15.20.

4.2. Spectroscopy and study of interactions with DNA/RNA

The electronic absorption spectra were obtained on Varian Cary 100 Bio spectrometer, CD spectra on JASCO J815 spectrophotometer and fluorescence spectra on the Varian Eclipse fluorimeter, all in quartz cuvettes (1 cm). The spectroscopic studies were performed in aqueous buffer solution (pH = 7, sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}). Under the experimental conditions absorbance of studied compounds was proportional to their concentrations. Polynucleotides were purchased as noted: poly A-poly U, poly G-poly C, poly dA-poly dT (Sigma), calf thymus (*ct*-) DNA (Aldrich). Polynucleotides were dissolved in sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}, pH = 7. Calf thymus (*ct*-) DNA was additionally sonicated and filtered through a 0.45 μm filter [26,27]. Polynucleotide concentration was determined spectroscopically [25] as the concentration of phosphates. Spectroscopic titrations were performed by adding portions of polynucleotide solution into the solution of the studied compound.

Obtained data were corrected for dilution. Titration data were processed by Scatchard equation [21]. Values for K_s and n all have satisfactory correlation coefficients (>0.999). Thermal melting curves for DNA, RNA and their complexes with studied compounds were determined as previously described [27,28] by following the absorption change at 260 nm as a function of temperature. Absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalized. The T_m values are the midpoints of the transition curves, determined from the maximum of the first derivative and checked graphically by the tangent method [27]. ΔT_m values were calculated subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value here reported was the average of at least two measurements, the error in ΔT_m is ±0.5 °C.

Ethidium bromide (EB) displacement assay: to a polynucleotide solution ($c = 5 \times 10^{-5}$ mol dm^{-3}) ethidium bromide ($c = 5 \times 10^{-6}$ mol dm^{-3}) was added (r([EB]/[polynucleotide] = 0.1), and quenching of the EB-polynucleotide complex fluorescence emission (λ_{ex} = 520 nm, λ_{em} = 550–700 nm) was monitored as function of c(EB)/c(compound). The given IC_{50} values present the ratio c(EB)/c(compound) = [Int(EB/polynucleotide) − Int(EB_{free})]/2, where Int(EB/polynucleotide) is fluorescence intensity of EB/polynucleotide complex and Int(EB_{free}) is fluorescence intensity of the free ethidium bromide before polynucleotide is added. The compounds and their complexes with polynucleotides showed neither optical absorption nor fluorescence above 550 nm and did not interfere with the fluorescence of the free ethidium bromide.

4.3. Cytostatic activity evaluation

The HeLa (cervical carcinoma), MCF-7 (breast carcinoma), SW 620 and HCT116 (colon carcinoma), MiaPaCa-2 (pancreatic carcinoma), Hep-2 (laryngeal carcinoma) and H 460 (lung carcinoma) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM), while the MOLT-4 (lymphoblastic leukemia) cells were cultured in RPMI medium both supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

The growth inhibition activity was assessed as previously described [18]. The cells were inoculated onto standard 96-well microtiter plates on day 0. The cell concentrations were adjusted according to the cell population doubling time. Test agents were then added in five consecutive 10-fold dilutions (10^{-8} to 10^{-4} mol/l) and incubated for a further 72 h. Working dilutions were freshly prepared from DMSO stock solutions ($c = 1 \times 10^{-2}$ mol/l) on the day of testing. After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay, which detects dehydrogenase activity in viable cells. The absorbency (OD, optical density) was measured on a microplate reader at 570 nm.

Each test point was performed in quadruplicate in at least two individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound are calculated from dose-response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (*i.e.* 50%). If, however, for a given cell line all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g. PG value of 50), then the highest tested concentration is assigned as the default value, which is preceded by a ">" sign. Each result is a mean value from three separate experiments.

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