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Original Scientific Article

Synthesis, DNA Interactions and Anticancer Evaluation of Novel Diamidine Derivatives of 3,4-Ethylenedioxythiophene†

Ivana Stolić,[a] Monika Avdičević,[b] Nikola Bregović,[c] Ivo Piantanida,[d] Ljubica Glavaš-Obrovac,[b,e] and Miroslav Bajić[a,*]

[a]*Department of Chemistry and Biochemistry, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, HR-10000 Zagreb, Croatia*
[b]*University Hospital Centre Osijek, Huttlerova 4, HR-31000 Osijek, Croatia*
[c]*Division of Physical Chemistry, Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, HR-10000 Zagreb, Croatia*
[d]*Division of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, P.O. Box 180, HR-10000 Zagreb, Croatia*
[e]*Faculty of Medicine, J. J. Strossmayer University of Osijek, Huttlerova 4, HR-31000 Osijek, Croatia*

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Abstract. Eight novel diamidino 3,4-ethylenedioxythiophene-2,5-dicarboxanilides (**5a–h**), obtained by condensation reaction of 3,4-ethylenedioxythiophene-2,5-dicarbonyl chloride and corresponding 3- or 4-aminobenzamidines, were evaluated for interactions with double-stranded DNA and RNA, and their cytotoxicity was assayed against the panel of human cancer cell lines. All compounds preferentially bind into the minor groove of DNA and had higher affinity for DNA than for RNA. Compounds **5a–h** showed a moderate antiproliferative effect toward the panel of seven carcinoma cells line, whereby the highest inhibitory potential was displayed by compound **5a** with unsubstituted amidino moieties in *para* position. (doi: 10.5562/cca2141)

Keywords: amide-amidines, 3,4-ethylenedioxythiophene, diarylamidine, DNA/RNA binding, antitumor activity

INTRODUCTION

Cancer is one of the leading causes of death in the world. The toll taken in human cost and health care economy is enormous. Deaths from cancer worldwide are projected to continue rising from 7.6 million in 2008 to an estimated 13.1 million in 2030.[1] The majority of the currently available drugs have well established shortcomings, such as poor efficiency, non-selectivity, and high toxicity in non-cancerous cells.[2] Considering this, the development of new anticancer agents that selectively act on the target, with high potency and less toxicity, is urgently needed.

Recent developments in genomic and molecular biology have provided new information about the genes from cancer cells. Despite this new information, DNA-targeted chemotherapy of cancer is still largely based on the application of drugs that have been in use for some time.[3–5] Among different structures, notable attention has been given to rational design, synthesis and characterisation of the structure-activity relationship of diarylamidines, small molecules that bind through noncovalent interaction to the minor groove of B-DNA.[6–8]

Diarylamidines have a wide range of potential therapeutic applications, such as ACIS inhibitors,[9] antiparasitic,[10,11] antifungal,[12] antibacterial,[13,14] antiviral[15] and anticancer agents.[4,6,16,17] Until now, pentamidine, 1,5-bis(4-amidinophenoxy)pentane, is the only one of this class with significant human clinical use. The development of pentamidine as a therapeutic drug is limited due to its known toxic side effects as a consequence of parent drug metabolism.[18] Unfortunately, metabolic breakup of the parent molecule may be a problem encountered with many of direct pentamidine analogues, especially those containing an ether bond in the bridge between the cationic moieties.[12]

It is well established that varying the central linker, substitutive group or substituent position can create a significant difference in the space configuration and distribution of electron density within the molecule, and thus influence the DNA-binding mode.[19,20] This stimulate us to design, synthesize and test the new

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* Author to whom correspondence should be addressed. (E-mail: miroslav.bajic@vef.hr)

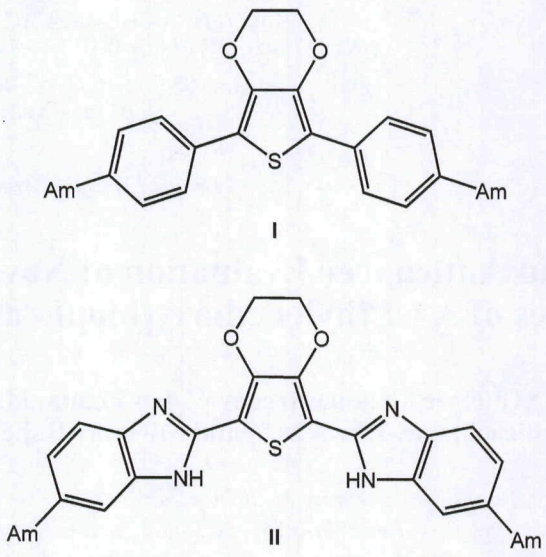


Figure 1.

pentamidine analogues with a robust 3,4-ethylenedioxythiophene (EDOT) linker instead of an unstable alkyl chain[18] or unstable benzo[*c*]thiophene ring.[21] Diarylamidine derivatives of EDOT previously synthesized in our laboratory (Figure 1) have shown significant antitumor activity.[22,23] The obtained results show that small changes in molecular structure have profound effects on DNA recognition and biological activity. It was found that tested compounds prefer binding into minor groove of ds-DNA over ds-RNA.

To obtain further information on the DNA binding modes of this type of compounds, we have synthesized new derivatives of EDOT, namely, 2,5-bis[(amidinophenyl)carboxamide]-3,4-ethylenedioxythiophene **5a–h** with the terminal amidine group in *para-* or *meta-* position. Two types of structural modifications were tested, the nature of the central linker and the position of amidine groups and thereby the influence of electron density and geometric shape on interactions with DNA and biological activity of newly synthesized compounds. A few series of similar compounds, in which two phenylamidines are linked by different bridges containing carboxamide moieties, their DNA/RNA binding affinities and broad spectra of biological activity have been described earlier.[7,24–26]

To test the flexibility and structural characteristics of the linker, comprising a poorly electron-donating amide moiety in addition to thiophene with an electron releasing cyclic ether, we prepared *N*-alkyl or *N*-phenyl derivatives of 3,4-ethylenedioxythiophene-2,5-dicarboxamide and studied the stability of prepared compounds, amide bond isomerisation and twisting of the dioxane ring by experimental methods (tandem mass spectrometry, X-ray structure analysis) and theoretical DFT calculations.[27,28] Mass spectrometry was used as the most widespread method for detection of metabolites and fragments produced by degradation of parent molecules, because early identification of potential metabolic liabilities provides a better perspective in the design of new drug candidates.

EXPERIMENTAL

Compounds

The aminobenzamidine compounds **4a**, **4e, 4f** and **4g** are commercially available, and synthesis of compounds **2** and **4b** were published earlier.[23,29] The synthesis and physical properties of the others compounds are given as follows.

Solvents were distilled from appropriate drying agents shortly before use. TLC was carried out on DC-plastikfolien Kieselgel 60 F254, Merck. Melting points were determined on a Büchi 510 melting point apparatus and were uncorrected. IR spectra [ν_{max} / cm^{-1}] were obtained on a Bruker Vertex 70 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer. Chemical shifts (δ / ppm) were referred to TMS. Mass spectra were recorded on a Waters Micromass Q-ToF micro. Elemental analyses were performed by the Applied Laboratory Research Department at INA d.d., Research and Development Sector, Central Analytical Laboratory.

General Method for the Synthesis of 3- or 4-Aminobenzamidines

A solution of 3- or 4-aminobenzonitrile (**3a** or **3b**) (9.13 g, 77.4 mmol) in anhydrous CH_3OH (230 mL) was cooled in an ice bath and saturated with dry HCl gas. The suspension was stirred at room temperature until IR spectra indicated the absence of the cyano peak (8 days). Anhydrous diethyl ether was added to the suspension and the solid was collected by filtration, washed with anhydrous diethyl ether and dried under reduced pressure over KOH to yield 13.77 g (95.4 %) of the corresponding imidate ester hydrochloride. The resulting salt was used in the next step without additional purification.

The crude imidate ester hydrochloride (2.98 g, 16.0 mmol) was suspended in anhydrous methanol (50 mL) and the corresponding amine was added. The suspension was stirred at room temperature for 4 days under nitrogen atmosphere. The solvent was removed under reduced pressure and the residue was recrystallized from ethanol-diethyl ether.

*4-Amino-*N*-isobutylbenzamidine Hydrochloride (**4c**)*

Amine: isobutylamine (2.58 g, 35.28 mmol); yield 3.12 g (86.2 %) of white powder; m.p. > 250 °C; IR (ν_{max} / cm^{-1}): 3143, 1598, 1500, 1313, 1050, 665, 497; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.31 (s, 1H, NH), 9.00 (s, 1H, NH), 8.65 (s, 1H, NH), 7.53 (d, 2H, J = 8.64 Hz,

ArH), 6.64 (d, 2H, J = 8.70 Hz, ArH), 6.17 (s, 2H, NH_2), 3.20 (t, 2H, J = 6.52 Hz, CH_2) 1.98 (m, 1H, J = 6.77 Hz, CH), 0.93 (d, 6H, J = 6.67 Hz, CH_3);

*4-Amino-*N*-cyclopentylbenzamidine Hydrochloride (**4d**)*
Amine: cyclopentylamine (7.24 mL, 6.16 g, 73.12 mmol); yield 3.79 g (98.9 %) of white powder; m.p. > 250 °C; IR (ν_{max} / cm^{-1}): 3570, 3194, 1589, 1515, 1326, 1095, 834, 675; ^{1}H NMR (DMSO-d_6) δ/ppm: 7.42 (d, 2H, J = 8.58 Hz, ArH), 6.55 (d, 2H, J = 8.61 Hz, ArH), 5.59 (s, 2H, NH_2), 3.97 (q, 1H, J = 6.51 Hz, CH) 1.90 (m, 2H, CH_2), 1.68 (br s, 2H, CH_2), 1.51 (m, 4H, CH_2);

*2-(3-Aminophenyl)-4,5-dihydro-1*H*-imidazol Hydrochloride (**4h**)*
Amine: 1,2-ethylenediamine (7.0 mL, 6.3 g, 10.48 mmol); yield 2.01 g (53.6 %) of white powder; m.p. > 250 °C; IR (ν_{max} / cm^{-1}): 3215, 1630, 1594, 1576, 1493, 1374, 1290, 864, 785, 781, 713, 698, 543, 528; ^{1}H NMR (DMSO-d_6) δ/ppm: 9,89 (s, 2H, NH), 7.24 (t, 1H, J = 7.79 Hz, ArH), 7.04 (td, 1H, J_1 = 7.60 Hz, J_2 = 0.83 Hz, ArH), 6.99 (t, 1H, J = 1.83 Hz, ArH), 6.91 (ddd, 1H, J_1 = 8.15 Hz, J_2 = 2.18 Hz, J_3 = 0.84 Hz, ArH), 5.44 (s, 2H, NH_2), 3.96 (s, 4H, $-CH_2CH_2-$).

*2,5-Bis[*N*-(4-amidinophenylcarboxamide)]-3,4-ethylenedioxythiophene Dihydrochloride (**5a**)*
A suspension of 3,4-ethylenedioxythiophene-2,5-dicarbonyl chloride (**2**) (2.65 g, 9.9 mmol) and compound **4a** (3.63 g, 17.5 mmol) in dry chloroform (150 mL) was stirred under reflux for 3 days under nitrogen atmosphere. The solvent was removed under reduce pressure, and the residue was suspended in water, filtered off and washed with 10 % HCl, 10 % $NaHCO_3$ and water. Recrystallization from chloroform-diethyl ether give 1.62 g (30.4 %) of pale brown powder, m.p. > 250 °C; IR (ν_{max} / cm^{-1}): 3345, 1487, 1326, 1092, 747, 497; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.72 (s, 2H, CONH), 9.32 (br s, 4H, NH), 9.08 (br s, 4H, NH), 7.94 (d, 4H, J = 8.90 Hz, ArH), 7.88 (d, 4H, J = 9.05 Hz, ArH) 4.59 (s, 4H, OCH_2CH_2O); ^{13}C NMR (DMSO-d_6) δ/ppm: 165.2, 159.2, 143.3, 141.9, 129.7, 123.3, 120.3, 116.6, 65.9; HRMS: calcd. for $C_{22}H_{21}N_6O_4S$ $(M+H)^+$, 465.1345; found: 465.1332; *Anal. Calcd.* mass fractions of elements, w / %, for $C_{22}H_{20}N_6O_4S \times 2HCl \times 4H_2O$ (M_r = 609.49): C 43.35, H 4.96, N 13.79, S 5.26; found: C 43.39, H 4.72, N 13.56, S 5.29.

*2,5-Bis[*N*-(4-*N*′-isopropylamidinophenyl)carboxamide]-3,4-ethylenedioxythiophene Dihydrochloride (**5b**)*
Compound **4b** (1.40 g, 6.6 mmol) was added to a stirred suspension of 3,4-ethylenedioxythiophene-2,5-dicarbonyl chloride (**2**) (0.94 g, 3.5 mmol) in dry chloroform (30 mL) and the mixture was stirred for 2 days at room temperature under nitrogen atmosphere. The solvent was removed under reduce pressure, and the residue was suspended in water and precipitated with acetone to yield 0.51 g (23.3 %) of white powder, m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 3606, 3028, 1519, 1089, 597; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.66 (s, 2H, CONH), 9.49+9.46 (s+s, 2H, NH), 9.32 (br s, 2H, NH), 8.92 (br s, 2H, NH), 7.92 (d, 4H, J = 8.64 Hz, ArH), 7.75 (d, 4H, J = 8.77 Hz, ArH), 4.59 (s, 4H, OCH_2CH_2O), 4.02 (m, 2H, CH), 1.28 (d, 12H, J = 6.33 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ/ppm: 161.3, 158.6, 142.3, 141.3, 129.2, 124.2, 119.8, 116.3, 65.5, 45.0, 21.2; HRMS: calcd. for $C_{28}H_{33}N_6O_4S$ $(M+H)^+$, 549.2284; found: 549.2268. *Anal. Calcd.* mass fractions of elements, w / %, for $C_{28}H_{32}N_6O_4S \times 2HCl$ (M_r = 621.59): C 54.10, H 5.51, N 13.52, S 5.16; found: C 54.10, H 5.72, N 13.31, S 4.97.

*2,5-Bis[*N*-(4-*N*′-isobutylamidinophenyl)carboxamide]-3,4-ethylenedioxythiophene Dihydrochloride (**5c**)*
To a suspension of dicarbonyl chloride (**2**) (0.54 g, 2.0 mmol) in dry chloroform (120 mL), compound **4c** (0.98 g, 4.3 mmol) was added and the mixture was stirred for 3 days at room temperature under nitrogen atmosphere. The solvent was removed under reduced pressure, and the residue was suspended in water and precipitated with acetone to yield 0.23 g (17.2 %) of white powder; m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 3026, 1662, 1517, 1321, 1244, 1095, 813, 649; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.67 (s, 4H, NH), 9.35 (s, 2H, NH), 8.94 (s, 2H, NH), 7.93 (d, 4H, J = 8.77 Hz, ArH), 7.77 (d, 4H, J = 8.74 Hz, ArH), 4.59 (s, 4H, OCH_2CH_2O), 3.22 (d, 4H, J = 6.98 Hz, CH_2) 2.00 (m, 1H, J = 6.78 Hz, CH), 0.98 (d, 12H, J = 6.66 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ/ppm: 162, 141, 136, 129, 126, 125, 114, 65, 49, 27, 19; HRMS: calcd. for $C_{30}H_{37}N_6O_4S$ $(M+H)^+$, 577.2597; found: 577.2582. *Anal. Calcd.* mass fractions of elements, w / %, for $C_{30}H_{36}N_6O_4S \times 2HCl \times 2.5H_2O$ (M_r = 694.68): C 51.87, H 6.24, N 12.10, S 4.62; found: C 51.98, H 5.88, N 11.81, S 4.55.

*2,5-Bis[*N*-(4-*N*′-cyclopentylamidinophenyl)carboxamide]-3,4-ethylenedioxythiophene Dihydrochloride (**5d**)*
To a suspension of dicarbonyl chloride (**2**) (0.25 g, 0.9 mmol) in dry chloroform (70 mL), compound **4d** (0.39 g, 1.6 mmol) was added and the mixture was stirred under reflux for 3 days under nitrogen atmosphere. The solvent was removed under reduce pressure, and the residue was suspended in water and precipitated with acetone to yield 0.22 g (38.6 %) of white powder; m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 3004, 1522, 1320, 1097, 743, 646; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.68 (s, 2H, CO-NH), 9.62+9.59 (br s+s, 2H, NH), 9.39 (br s, 2H, NH), 8.97 (br s, 2H, NH) 7.92 (d, 4H, J = 8.83 Hz, ArH), 7.77 (d, 4H, J = 8.78 Hz, ArH), 4.59 (s, 4H, OCH_2CH_2O), 4.12 (m, 2H, CH) 2.05 (m, 4H, CH_2), 1.75–1.57 (m, 12H, CH_2); ^{13}C NMR (DMSO-d_6) δ/ppm: 161.9, 158.6, 142.2, 141.4, 129.3, 124.1, 119.7, 116.2, 65.4, 54.1, 31.4, 23.6; HRMS: calcd. for $C_{32}H_{37}N_6O_4S$ $(M+H)^+$, 601.2597; found: 601.2586. *Anal. Calcd.* mass

fractions of elements, *w* / %, for $C_{32}H_{36}N_6O_4S \times 2HCl \times 5H_2O$ (M_r = 763.74): C 50.33, H 6.33, N 11.00, S 4.20; found: C 50.55, H 6.15, N 10.79, S 4.52.

*2,5-Bis[*N*-(4-(2-imidazolinyl)phenyl)carboxamide]-3,4-ethylenedioxythiophene Dihydrochloride (**5e**)*

To a suspension of dicarbonyl chloride (**2**) (0.44 g, 1.7 mmol) in dry chloroform (120 mL), compound **4e** (0.72 g, 3.7 mmol) was added and the mixture was stirred under reflux for 2 days under nitrogen atmosphere. The solvent was removed under reduced pressure, and the residue was suspended in water and precipitated with acetone to yield 0.27 g (25 %) of pale brown powder; m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 3360, 1606, 1527, 1380, 1087, 660, 595; ^{1}H NMR (DMSO-d_6) δ/ppm: 10.55 (s, 4H, NH), 9.77 (s, 2H, NH), 8.03 (d, 4H, J = 9.02 Hz, ArH 7.98 (d, 4H, J = 9.07 Hz, ArH), 4.59 (s, 4H, OCH_2CH_2O), 4.00 (s, 8H, CH_2); ^{13}C NMR (DMSO-d_6) δ/ppm: 164.7, 159.2, 143.8, 142.0, 130.1, 120.5, 117.6, 116.7, 65.9, 44.9. HRMS: calcd. for $C_{26}H_{25}N_6O_4S$ $(M+H)^+$, 517.1658; found: 517.1641. *Anal. Calcd.* mass fractions of elements, *w* / %, for $C_{26}H_{24}N_6O_4S \times 2HCl \times 4H_2O$ (M_r = 661.56): C 47.20, H 5.18, N 12.70, S 4.85; found: C 47.07, H 4.93, N 12.44, S 4.90.

*2,5-Bis[*N*-(4-(1,4,5,6-tetrahydropyrimidin-2-yl)phenyl)-carboxamide]-3,4-ethylenedioxythiophene Dihydrochloride (**5f**)*

To a suspension of dicarbonyl chloride (**2**) (0.0.39 g, 1.5 mmol) in dry chloroform (100 mL), compound **4f** (0.63 g, 3 mmol) was added and the mixture was stirred at 50 °C for 4 days under nitrogen atmosphere. The solvent was removed under reduce pressure, and the residue was suspended in water and precipitated with acetone to yield 0.16 g (17.4 %) of white powder; m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 3253, 1641, 1535, 1307, 1157,1093, 847, 671; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.93 (s, 4H, NH), 9.67 (s, 2H, NH), 7.94 (d, 4H, J = 8.7 Hz, ArH), 7.76 (d, 4H, J = 8.7 Hz, ArH), 4.58 (s, 4H, OCH_2CH_2O), 3.49 (t, 8H, J = 5.22 Hz, CH_2), 1.98 (m, 4H, J = 4.95 Hz, CH_2); ^{13}C NMR (DMSO-d_6) δ/ppm: 159.1, 158.9, 142.7, 141.9, 129.0, 123.9, 120.4, 116.7, 65.9, 18.3; HRMS: calcd. for $C_{28}H_{29}N_6O_4S$ $(M+H)^+$, 545.1971; found: 545.1935. *Anal. Calcd.* mass fractions of elements, *w* / %, for $C_{28}H_{28}N_6O_4S \times 2HCl \times 4H_2O$ (M_r = 689.62): C 48.77, H 5.55, N 12.19, S 4.65; found: C 49.15, H 5.82, N 11.90, S 4.72.

*2,5-Bis[*N*-(3-amidinophenylcarboxamide)]-3,4-ethylene-dioxythiophene Dihydrochloride (**5g**)*

Suspension of 3,4-ethylenedioxythiophene-2,5-dicarbonyl chloride (**2**) (0.47 g, 1.77 mmol) and compound **4g** (0.61 g, 3.56 mmol) in dry chloroform (100 mL) was stirred under reflux for 2 days under nitrogen atmosphere. The solvent was removed under reduce pressure and the residue was suspended in water and precipitated with acetone to yield 0.69 g (72.6 %) of pale brown powder; m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 2971, 1668, 1517, 1319, 1094, 820, 644; ^{1}H NMR (DMSO-d_6) δ/ppm: 9.58 (s, 2H, CONH), 9.41 (s, 4H, NH), 9.13 (s, 4H, NH), 8.22 (s, 2H, ArH), 8.02 (d, 2H, J = 8.30 Hz, ArH), 7.64 (t, 2H, J = 7.92 Hz, ArH), 7.56 (d, 2H, J = 7.89 Hz, ArH), 4.61 (s, 4H, OCH_2CH_2O); ^{13}C NMR (DMSO-d_6) δ/ppm: 166.2, 159.1, 141.7, 138.8, 130.1, 129.3, 125.7, 124.2, 120.2, 116.5, 65.9. HRMS: calcd. for $C_{22}H_{21}N_6O_4S$ $(M+H)^+$, 465.1345; found: 465.1348. *Anal. Calcd.* mass fractions of elements, *w* / %, for $C_{22}H_{20}N_6O_4S \times 2HCl \times 2.5H_2O$ (M_r = 582.46): C 45.37, H 4.67, N 14.43, S 5.51; found: C 45.04, H 4.55, N 13.89, S 5.58.

*2,5-Bis[N-(3-(2-imidazolinyl)phenyl)carboxamide]-3,4-ethylenedioxythiophene Dihydrochloride (**5h**)*

To a suspension of dicarbonyl chloride (**2**) (0.45 g, 1.7 mmol) in dry chloroform (100 mL), compound **4h** (0.68 g, 3.4 mmol) was added and the mixture was stirred under reflux for 7 days under nitrogen atmosphere. The solvent was removed under reduced pressure, and the residue was suspended in water and precipitated with acetone to yield 0.14 g (14,5 %) of pale brown powder; m.p. > 250 °C, IR (ν_{max} / cm^{-1}): 3346, 3078, 2958, 1663, 1425, 1251, 1160, 1035, 932, 877, 842, 801, 749, 644, 587, 541, 467 ; ^{1}H NMR (DMSO-d_6) δ/ppm: 10.67 (s, 4H, NH), 9.58 (s, 2H, NH), 8.40 (s, 2H, ArH), 7.98 (d, 2H, J = 8.35 Hz, ArH), 7.71 (d, 2H, J = 7.80 Hz, ArH), 7.66 (t, 2H, J_1 = 7.97 Hz, ArH), 4.60 (s, 4H, OCH_2CH_2O), 4.01 (s, 8H, CH_2); ^{13}C NMR (DMSO-d_6) δ/ppm: 164.7, 158.5, 141.3, 138.5, 129.9, 126.0, 124.2, 122.8, 120.4, 116.0, 65.5, 44.3; HRMS: calcd. for $C_{26}H_{25}N_6O_4S$ $(M+H)^+$, 517.1658; found: 517.1631. *Anal. Calcd.* mass fractions of elements, *w* / %, for $C_{26}H_{24}N_6O_4S \times 2HCl \times 5H_2O$ (M_r = 679.58): C 45.95, H 5.34, N 12.37, S 4.72; found: C 46.12, H 5.50, N 12.33, S 4.53.

Mercurimetric Chloride Determination

General Method

A solution of **5a** (1.5 mg in 5 ml of water) was prepared. 0.2 mL of nitric acid (c = 4.99 × 10^{-2} mol dm^{-3}) and 0.04 mL of diphenylcarbazone ethanol solution (c = 4.16 × 10^{-3} mol dm^{-3}) were added and the mixture was titrated with standard $Hg(NO_3)_2$ (c = 5.11 × 10^{-3} mol dm^{-3}). Intensive purple coloration (complex of diphenylcarbazone with mercuric ions) occurred at the equivalence point where the amount of Hg^{2+} corresponds to 0.5 $n(Cl^-)$.

Potentiometric Measurements

General Method

A solution of **5a** for pH measurement was prepared using redistilled water. The electrode used for pH measurements was a combined glass electrode, Metrohm LL

Micro glass electrode 6.0234.100. with a Methrohm 827 pH meter. The temperature was kept constant at (25.0 ± 0.1) °C during pH measurements. Three standard buffer solutions (pH = 3, 6 and 9) were used for electrode calibration.

Spectroscopy

Electronic absorption spectra were recorded on a Varian Cary 100 Bio and PerkinElmer Lambda 25 spectrometer, fluorescence emission spectra were recorded on a Varian Eclipse fluorimeter, and CD spectra on a Jasco J815, in all cases using quartz cuvettes (1 cm). Measurements were performed in an aqueous buffer solution (pH = 7; sodium cacodylate buffer, I_c = 0.05 mol dm^{-3}). Under the experimental conditions used, the absorbance and fluorescence intensities of the studied compounds were proportional to their concentration. Polynucleotides were purchased from Sigma and Aldrich and were dissolved in sodium cacodylate buffer (I_c = 0.05 mol dm^{-3}, pH = 7). Calf thymus DNA (ct-DNA) was additionally sonicated and filtered through a 0.45 μm filter. Their concentration was determined spectroscopically as the concentration of phosphates.[30] Spectroscopic titrations were performed by adding portions of polynucleotide solution into the solution of the studied compound. Obtained data were corrected for dilution.

DNA binding experiments were performed at (25.0 ± 0.2) °C. The UV-Vis titration of diamidines **5a–h** was performed in a buffer (sodium cacodylate buffer, I_c = 0.05 mol dm^{-3}, pH = 7) medium using a fixed complex concentration, to which increments of the ct-DNA stock solutions (0.0–3.5 × 10^{-4} mol dm^{-3}) were added. The resulting solutions were incubated for 10 min before the absorption spectra were recorded.

DNA-melting experiments were carried out by monitoring the absorbance of ct-DNA and poly A–poly U at λ = 260 nm at varying temperature in the absence and presence of diamidines **5a–h**, at r = 0.1, 0.2, 0.3 and 0.5 compound to polynucleotide ratio with a ramp rate of 0.5 °C min^{-1} in the buffer (pH = 7) using a Peltier system attached to the UV-Vis spectrophotometer. Absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalized. Melting temperature (T_m) values are the midpoints of transition curves, determined from the maximum of the first derivative and checked graphically by the tangent method.[23] 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 was ± 0.5 °C.

Ethidium bromide (**EB**) displacement assay: to polynucleotide solution (c = 5 × 10^{-5} mol dm^{-3}), ethidium bromide (c = 5 × 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} = 601 nm) was monitored as a function of c(**EB**)/c(compound). The given IC_{50} values represent the ratio c(**EB**)/c(compound) = [Int(**EB**/polynucleotide) – Int(**EB**$_{free}$)]/2, where Int(**EB**/polynucleotide) is the fluorescence intensity of the **EB**/polynucleotide complex and Int(**EB**$_{free}$) is the fluorescence intensity of the free ethidium bromide before polynucleotide was added. The apparent binding constants were calculated from: K_{EtBr} × [EtBr] = K_{app} × [drug], where [drug] = concentration of the tested compound at a 50 % reduction of fluorescence. The binding constant of ethidium bromide to calf thymus DNA under comparable conditions is 1.0 × 10^6 mol^{-1} dm^3.[30]

Cell Culturing and MTT Test

Five tumour cell lines, colorectal adenocarcinomas (CaCo2, HT29), larynx carcinoma (HEp2), cervix adenocarcinoma (HeLa), pancreatic adenocarcinoma (MIAPaCa2) and Madine-Darby canine kidney (MDCKI) normal cells were grown in DME medium (Gibco, EU). The bronchioalveolar carcinoma (NCI H358) and gastric adenocarcinoma (AGS) tumour cell lines were grown in RPMI 1640 medium (Gibco, EU). Both media were supplemented with 10 % heat-inactivated foetal bovine serum-FBS (Gibco, EU), 2 × 10^{-3} mol dm^{-3} glutamine (Gibco, EU), 1 × 10^{-3} mol dm^{-3} sodium pyruvate (Gibco, EU), 1 × 10^{-2} mol dm^{-3} HEPES (Sigma-Aldrich, USA) and 100 U / 0.1 mg antibiotic/antimycotic (Gibco, EU).

Cells were grown at t = 37 °C, with 5 % CO_2 gas in humidified CO_2 incubator (ShelLab, Sheldon Mfg. Inc., USA). The trypan blue dye exclusion method was used to assess cell viability. Tested compounds were dissolved in high-purity water as a 1 × 10^{-2} mol dm^{-3} stock solution. Working dilutions were prepared in a concentration range 10^{-3}–10^{-6} mol dm^{-3}.

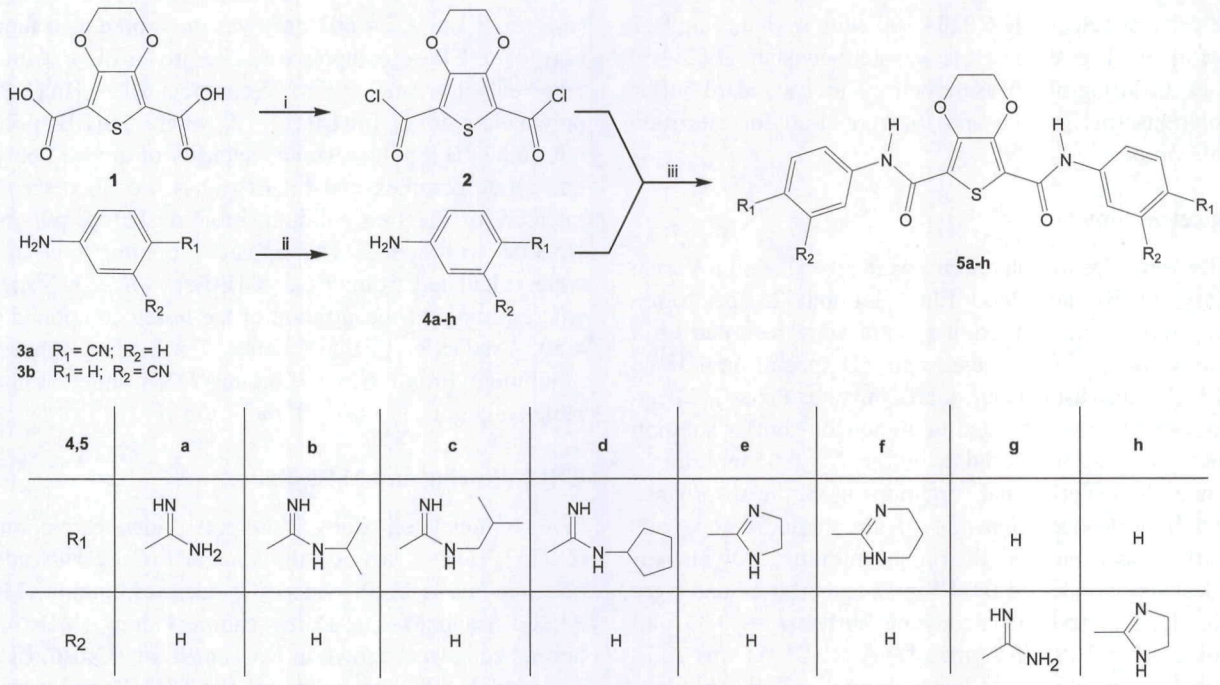
For the MTT[31] test, cells were seeded in 96 microwell flat bottom plates (Greiner, Austria) at 2 × 10^4 cells/mL. After 72 hours of incubation with the tested compounds MTT (Merck, Germany) was added. DMSO (Merck, Germany) was used to dissolve the MTT-formazane crystals formed. Absorbance was measured at 570 nm on Stat fax 2100 plate reader (Awareness Technology Inc., USA).

All experiments were performed three times in triplicates. The IC_{50} value, defined as the concentration of compound achieving 50 % of cell growth inhibition, was calculated and used to compare cytotoxicity among the compounds.

RESULTS AND DISCUSSION

Chemistry

The target amido-amidines **5a–h** were synthesized according to the procedure outlined in Scheme 1 by the



4,5	a	b	c	d	e	f	g	h
R_1	HN, NH_2	NH, N, H	NH, N, H	NH, N, H	N, N, H	N, HN	H	H
R_2	H	H	H	H	H	H	HN, NH_2	N, N, H

Scheme 1. Reagents: (i) $SOCl_2$, DMF, benzene; (ii) 1. HCl/CH_3OH, 2. R–NH_2, CH_3OH; (iii) $CHCl_3$.

condensation reaction of 3,4-ethylenedioxythiophene-2,5-dicarbonyl chloride (**2**) and corresponding 3- or 4-aminobenzamidines **4a–h**. Dicarbonyl chloride **2** was prepared in good yield (89 %) by reaction of 3,4-ethylenedioxythiophene-2,5-dicarboxylic acid (**1**) with thionyl chloride and DMF in dry benzene.[23] 3- or 4-aminobenzamidines **4a–h** were purchased (**4a**, **4e**, **4f** and **4g**) or synthesized from commercially available aminobenzonitrile by means of the Pinner reaction (**4b**, **4c**, **4d** and **4h**).

The number and nature of positively charged groups in small molecules that bind in the minor groove of DNA play an important role in complex formation.[32] In general, these molecules should have a minimum two cationic groups to show significant binding and biological activity. Due to the high pK values of the amine moieties and low solubility of the compounds, the number of protons per molecule present in compounds **5a–h** and the corresponding pK values could not be determined by standard potentiometric pH titration. Therefore, the concentration of the chloride anion in solutions of studied compounds was determined by mercurimetric titration using diphenylcarbazone as indicator.[33] In all cases, the Cl^- concentration was two times higher than the concentration of the compound, which confirms the presumption that all investigated compounds were isolated as dihydrochlorides. The measured pH values of **5a** and **5b** solutions ($c = 5 \times 10^{-4}$ mol dm^{-3}) were 8.454 and 8.357, respectively, suggesting that no dissociation occurred after **5a** and **5b** were dissolved in water. These findings indicate that pK values of the investigated compounds are quite high (> 10) which is in agreement with previous studies.[34] It can be therefore concluded that under physiological conditions (pH = 7) all prepared compounds exist as dications.

UV-Vis and Fluorescence Spectroscopic Characteristics of Compounds 5a–h

Based on the position of terminal amidine moieties, compounds **5a–h** are divided into two groups: *para* derivatives (**5a–f**, Scheme 1) and *meta* derivatives (**5g** and **5h**, Scheme 1). Since DNA binding studies are conducted by spectrophotometric methods, the spectroscopic behaviour of aqueous solutions of investigates compounds **5a–h** was studied by UV-Vis (Figure 2, Table 1) and fluorescence spectrophotometry (Figure 3) performed at room temperature.

Linear dependence of UV-Vis spectra on the concentration of all studied compounds in the range c (**5a–h**) = 1.97 – 4.92 × 10^{-5} mol dm^{-3} as well as negligible temperature dependent changes (25–90 °C) and excellent reproducibility upon cooling to 25 °C indicated the absence of intermolecular interactions. Aqueous solutions of all studied compounds were stable over several weeks.

UV-Vis spectroscopic properties of the synthesized compounds are strongly dependent on the nature and position of the amidine group. UV-Vis spectra of *para* derivatives **5a–f** showed two absorption maxima, one in the 246–261 nm range, and the other, more intense, in the 327–335 nm range (Figure 2, Table 1). The *meta* derivatives **5g** and **5h** showed only one absorption maximum at λ = 318 nm, of lower molar extinction

Table 1. Electronic absorption maxima and corresponding molar extinction coefficients of studied compounds in water

Compound	λ_{max} / nm	$10^3 \times \varepsilon$ / dm^3 mol^{-1} cm^{-1}
5a	252 330	16.9 57.6
5b	246 328	16.9 47.6
5c	246 327	13.8 40.5
5d	246 329	20.0 53.6
5e	261 335	22.1 63.2
5f	246 328	19.8 49.3
5g	318	29.1
5h	318	33.3

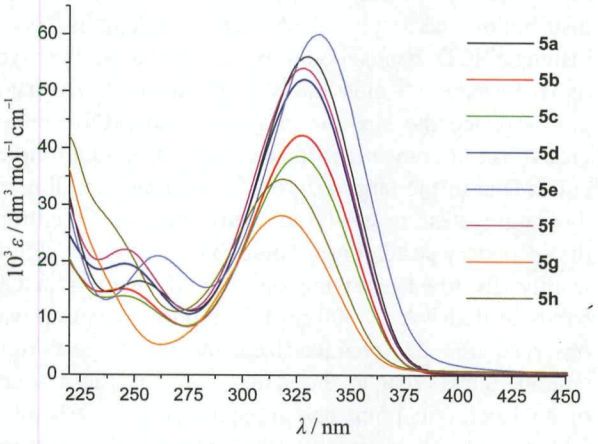


Figure 2. UV-Vis spectra of **5a–h** at c = 1.97–4.92 × 10^{-5} mol dm^{-3}.

values (ε) and blue shifted in comparison to their *para* analogues. These differences resulted from the position of amidine groups within molecules.

Within *para* derivatives, alkylation of parent compound **5a** or introduction of 1,4,5,6-tetrahydropyrimidine as amidine moiety resulted in a decrease of molar extinction values (ε) while retaining the position of absorption maxima. On the other hand, introduction of imidazoline, a cyclic amidine moiety, resulted in an increase of molar extinction values (ε) along with a red shift of maxima by about 10 nm. The same result was also obtained in *meta* derivatives.

Fluorescence emission of compounds **5a–f** (Supplement: Figure S1) was proportional to their concentration up to 3 × 10^{-6} mol dm^{-3}, the relative fluorescence intensity varying with compound structure.

Interactions with Double Stranded (ds-) DNA and RNA

Low fluorescence emission of all studied compounds even at 5 × 10^{-6} mol dm^{-3} concentrations and maximum instrument sensitivity hampered the application of fluorimetric titrations in studies of interactions with DNA/RNA.

UV-Vis Titrations with Polynucleotides

UV-Vis spectroscopy is an effective tool to study the interactions of small molecules with polynucleotides. Here, UV-Vis titrations were performed in a buffered medium (sodium cacodylate buffer, I_c = 0.05 mol dm^{-3}, pH = 7) at room temperature. Addition of ct-DNA to a solution of compounds **5a–h** resulted in a pronounced hypochromic effect of their absorption spectra > 300 nm, while no significant shift of maxima was observed (Figure 3, Table 2, Supplement: Figure S2).

Addition of ct-DNA induced changes in UV-Vis spectra of all tested compounds up to the ratio $r_{[compd]/[ctDNA]}$ = 0.2–0.5, while further additions of the

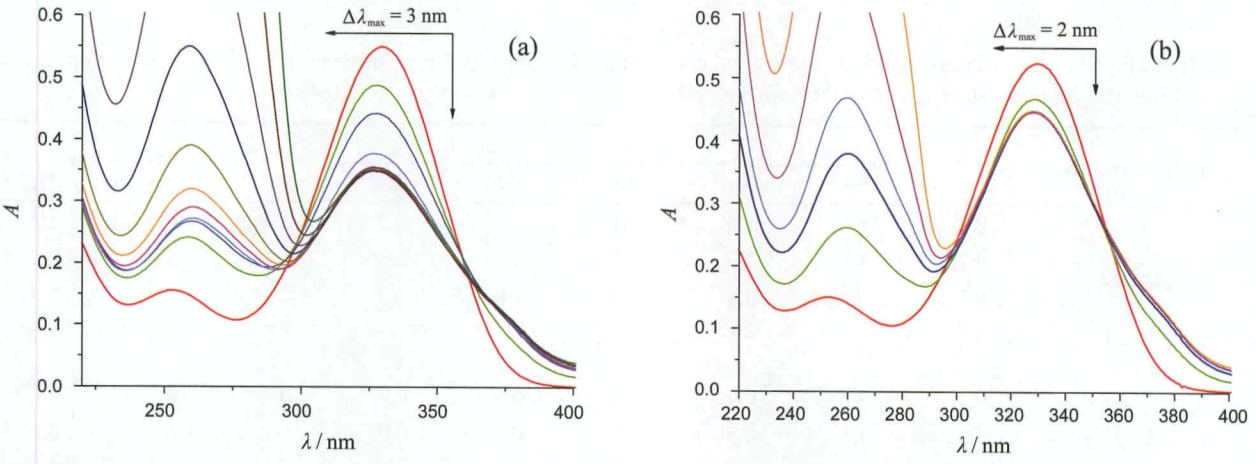


Figure 3. Changes in UV-Vis spectrum of **5a** (c = 1.08 × 10^{-5} mol dm^{-3}) upon titration with ct-DNA (a) and poly A–poly U (b); at pH = 7, Na cacodylate buffer, I_c = 0.05 mol dm^{-3}.

Table 2. Changes in the UV-Vis spectra of **5a–h** upon titration with ct-DNA and poly A–poly U (pH = 7, Na cacodylate buffer, I_c = 0.05 mol dm^{-3})

Compound	ct-DNA H / %[a]	poly A–poly U H / %[a]	λ_{max} / nm
5a	36	15	330
5b	43	22	328
5c	25	17	327
5d	31	11	329
5e	44	37	335
5f	23	21	328
5g	[b]	[c]	318
5h	11	7	318

[a] Hypochromic effect; $H = ((A_{compd.} - A_{complex}) / A_{compd.}) \cdot 100$.
[b] Absorbance changes in opposite direction (Figure 3): A decreases for r = 4–0.88, followed by increase of A for r > 0.88.
[c] Absorbance changes in opposite direction (Figure 3): A decreases for r = 4–0.66, followed by increase of A for r > 0.66.

mentioned polynucleotide did not produce any additional spectral changes, suggesting that at the mentioned ratio r, tested compounds were completely bound to ct-DNA.

Changes observed in the UV-Vis spectra of compounds **5a–h** in ct-DNA titrations, along with a clear deviation from the isosbestic points > 300 nm, suggested formation of at least two different types of complexes with ct-DNA.

Addition of ds-RNA (poly A–poly U) to diamidines **5a–h** induced smaller changes in the UV-Vis spectra of tested compounds in comparison with DNA titrations (Figure 3b, Table 2).

Thermal Melting Experiments

Changes in the melting point (T_m) of ct-DNA and ds-RNA upon the addition of compounds **5a–h** were measured in the range of $r_{[compound]/[polynucleotide]}$ = 0–0.5. While all studied compounds had a considerable stabilisation impact on the thermal denaturation of ct-DNA, their addition did not stabilize ds-RNA (poly A–poly U) (Table 3).

Circular Dichroism (CD) Experiments

Thus far, non-covalent interactions at temperature of 25 °C were studied by monitoring the spectroscopic properties of studied compounds upon addition of polynucleotides. To get an insight into the changes of polynucleotide properties induced by small molecule binding, we have chosen CD spectroscopy as a highly sensitive method for conformational changes in the secondary structure of polynucleotides.[35] In addition, achiral small molecules can eventually acquire an induced CD spectrum (ICD) upon binding to polynucleotides, which could give useful information about the modes of interaction.[35–39] Amidine derivatives **5a–h** are not chiral and therefore do not possess an intrinsic CD spectrum, but when added to ct-DNA, they acquire induced CD (ICD) bands > 300 nm (Supplement: Figure S3), agreeing well with the corresponding UV spectra (Figure 3).

Addition of most of the studied compounds to ds-RNA (poly A–poly U) resulted in similar, bisignate ICD bands > 300 nm, characterised by an isoelliptic point (only one type of complex) and negative/positive band distribution taken from shorter wavelengths. Such bisignate ICD bands could be attributed to the dye-dimer formation,[35] most likely within the ds-RNA major groove since the shallow and very wide RNA minor groove is not convenient for the binding of small molecules. Due to the large size of the binding site, dimeric dye aggregates are easily accommodated and therefore the secondary structure of the double helix is not significantly disturbed, showing only small changes in CD bands of ds-RNA (< 300 nm). The only exception was observed for **5f**, characterized by the largest rigid amidine substituent in the series, whereby the absence of an isoelliptic point and un-symmetric ICD band > 300 nm of the reversed order (weak positive/strong negative band taken from shorter wavelengths) suggested the presence of several different forms of dye aggregates along the ds-RNA.

Table 3. The ΔT_m / °C[a] values of studied ct-DNA and poly A–poly U upon addition of different ratios r[b] of tested compounds at pH = 7.0 (buffer sodium cacodylate, I_c = 0.05 mol dm^{-3})

polynucleotide	r[b]	ΔT_m / °C[a]							
		5a	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**
ct-DNA	0.1	1.3	1.6	2.1	2.5	1.2	1.4	0.8	1.8
	0.2	3.3	2.9	3.1	4.7	2.3	4	1.9	2.5
	0.3	6	3.8	3.4	7.0	–	4.5	2.7	3.4
	0.5	–	6.4	9.2	–	–	8.9	4.4	6.5
poly A–poly U	0.1	0.1	0.4	0.3	0.3	0.2	0.2	0.3	0.3
	0.2	0.1	0.5	0.5	0.8	0.9	–	0.5	0.6
	0.3	–	0.5	0.5	–	–	–	0.6	–

[a] Error in ΔT_m: ± 0.5 °C.
[b] r = [compound] / [polynucleotide].

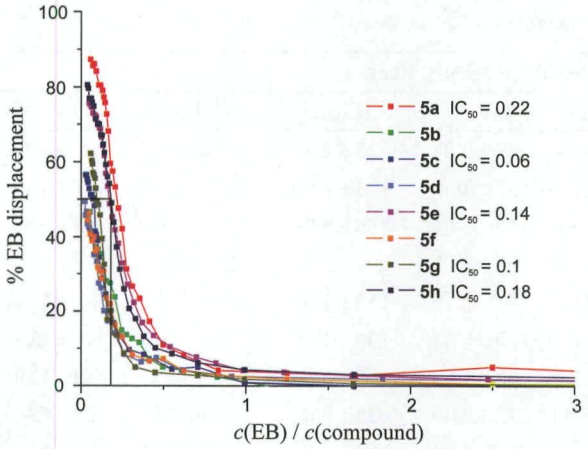


Figure 4. Ethidium bromide (**EB**) displacement assay: to ct-DNA solution ($c = 5 \times 10^{-5}$ mol dm^{-3}) ethidium bromide ($c = 5 \times 10^{-6}$ mol dm^{-3}) was added (r ([**EB**]/[ct-DNA]) = 0.1), and quenching of the **EB**/DNA complex fluorescence emission (λ_{ex} = 520 nm, λ_{em} = 601 nm) was monitored as function of c(**EB**)/c(compound). The given IC_{50} values present the ratio c(**EB**)/c(compound) = [Int(**EB**/DNA)–Int($\mathbf{EB}_{free}$)]/2, where Int(**EB**/DNA) is fluorescence intensity of **EB**/DNA complex and Int($\mathbf{EB}_{free}$) is fluorescence intensity of the free ethidium bromide before DNA is added.

Table 4. Association constants of investigated compounds with ct-DNA

	$10^{-5} \times K_{app}$ / dm^3 mol^{-1}					
Compound	**EB**[(a)]	**5a**	**5c**	**5e**	**5g**	**5h**
	10.0	2.18	0.60	1.41	0.98	1.82

[(a)] Ref. 30.

Changes in the CD spectrum of ct-DNA were dependent on the size and flexibility of amidine substituents attached to **5a–h**. Addition of **5a**, **5b**, **5g** and **5h** characterised by small acyclic amidines resulted in a strong negative ICD band > 300 nm, combined with various changes in CD spectra < 300 nm. It should be noted that at 300 nm it is not possible to distinguish between the possible ICD bands of dye and changes in the CD spectrum of ct-DNA caused by small molecule distorting the double helical structure. At variance to **5a**, **5b**, **5g** and **5h**, the largest acyclic (**5c** and **5d**) and all cyclic *para* amidine (**5e** and **5f**) derivatives yielded bisignate ICD bands > 300 nm, characterised by an isoelliptic point (only one type of dye-DNA complex) and negative/positive band distribution taken from shorter wavelengths. Such bisignate ICD bands could be attributed to the dye-dimer formation,[35] most likely within the ct-DNA minor groove.

The similarity of bisignate ICD bands > 300 nm observed for **5d–f**/ct-DNA complexes and **5a–e**, **5g** and **5h**/ds-RNA complexes suggests a similar type of dimeric-dye form. Obviously, the fine interplay between the amidine substituent size and the DNA/RNA groove steric and non-covalent interaction properties controls the dye-dimer formation and consequently the characteristic ICD profile.

Ethidium Bromide Displacement Assay

Displacement of EB pre-bound to ds-DNA by successive additions of the studied compounds provides an indirect method of measuring the binding affinity of compounds by qualitative comparison of binding affinities within a series of compounds with similar structure.[40,41] The extent of quenching of the fluorescence of EB bound to DNA would reflect the extent of DNA binding of the studied molecule, thus allowing a rough estimation of the affinity of the studied molecule toward ds-DNA.[42]

The obtained IC_{50} values (Figure 4) suggest that affinity of **5a**, **5c**, **5e**, **5g** and **5h** toward ct-DNA is comparable to the affinity of EB, while in the case of compounds **5b**, **5d** and **5f**, IC_{50} of the fluorescence of ethidium bromide-ct-DNA complexes was not reached. Table 4 presents the DNA association constants (K_{app}) of compounds **5a**, **5c**, **5e**, **5g** and **5h** in comparison with ethidium bromide.

Antiproliferative Capacity of Compounds 5a–h

New compounds **5a–h** may be viewed as amide analogues of compounds **I** and **II** showed in Figure 1. After evaluating the influence of expansion of the central part, by introduction of an amide bond (in the structure of compounds **I**) or replacement of the rigid part (benzimidazole in compounds **II**) with the flexible one (benzamide), on DNA binding mode by UV-Vis, fluorescence, CD and UV-melting methods, cytotoxic effects on normal and tumour cells were checked.

A number of recent studies conducted on the tumour cell lines of different origin showed that some derivatives of benzimidazole, diarylamidine, and bisbenzimidazoles are cytotoxic against tumour cells derived from solid organ tumours,[22,23,43,44] as well as from haematological tumours.[45] The newly synthesized compounds were tested for their antiproliferative effects on normal cells (MDCKI) and seven human tumour cell lines of different histological origin (HT-29, AGS, MIAPaCa2, CaCo2, HEp2, HeLa and NCI H358). The results are presented in Table 5 as IC_{50} values, the concentration achieving 50 % of cell growth inhibition. Doxorubicin was used as a control. Obtained data show that investigated compounds differentially influenced tumour cell growth, depending on the cell line as well as on the dose applied. Compounds **5a**, **5g** and **5h** in concentration of 10^{-4} mol dm^{-3} showed strong inhibitory potential on normal cells and all tumour cell lines (IC_{50} values ranging from $14–37 \times 10^{-6}$ mol dm^{-3} for **5a**, from $15–68 \times 10^{-6}$ mol dm^{-3} for **5g**, and $16–76 \times 10^{-6}$ mol dm^{-3} for **5h**). An exception was the weak inhibitory

Table 5. Sensitivity of human tumour and normal cells to investigated compounds, expressed as IC_{50}/μmol dm^{-3} [(a)]

Compound	Normal cell line MDCKI	Solid tumour cell lines						
		HT29	AGS	MIAPaCa2	CaCo2	HEp2	HeLa	NCI H358
5a	**14±5.1**	**37±26.9**	125±33.1	**22±3.1**	**29±9.8**	**22±4.3**	**17±1.7**	**27±1.7**
5b	161±6.5	172±7.8	149±32.2	154±39.9	157±38.6	123±22.0	170±13.9	147±5.2
5c	181±5.05	154±17.5	**50±7.0**	102±12.0	155±14.4	156±8.9	125±4.9	98±4.8
5d	163±12.2	114±11.9	147±1.2	123±0.6	107±22.3	99±23.6	129±4.1	138±9.3
5e	97±0.1	139±19.05	163±2.9	195±0.1	162±5.7	155±1.6	184±9.0	136±7.10
5f	99±13.8	86±24.6	114±29.7	87±35.1	120±8.6	106±18.6	93±4.9	73±13.3
5g	48±42.5	**35±10.1**	61±47.2	**15±1.1**	68±14.9	66±36.9	55±22.8	**38±12.0**
5h	58±10.0	76±25.0	16±2.95	**30±0.6**	75±23.0	**28±0.6**	**34±3.25**	**29±0.5**
Doxorubicin	0.3±0.1	0.6±0.32	0.2±0.04	0.4±0.05	0.7±0.09	0.4±0.06	4±0.28	0.8±0.13

[(a)] IC_{50} - drug concentration that inhibited cell growth by 50 %. Data represents mean IC_{50} / μmol dm^{-3} values ± standard deviation (SD) of three independent experiments. Exponentially growing cells were treated with substances during 72 hrs period. Cytotoxicity was analysed using MTT survival assay.

effect of compound **5a** on the AGS cell line, which could be explained by high multidrug resistance phenotype of AGS cells.[46,47] Compounds **5b, 5c, 5d** and **5f** showed very weak activity on the proliferative capacity of the majority of tested cells lines. Although a rapid cellular uptake and nuclear accumulation of benzimidazole-based amidines in different cancer cells have been recently evidenced by fluorescence microscopy,[48] the possibility cannot be excluded that the observed difference in cytotoxicity of new compounds was a consequence of different cellular uptake. For that reason, the entry of compounds and intracellular distribution will be investigated.

CONCLUSION

Interactions of amidine derivatives of EDOT such as those in Figure 1 with nucleic acids are of interest for two primary reasons: they have shown very significant anticancer activity that appears to be related to their ability to complex with DNA and they provide unique probes of the nucleic acid sequence depending molecular recognition.

A series of eight diamidine derivatives of EDOT, namely, 2,5-bis[(amidinophenyl)carboxamide]-3,4-ethylenedioxythiophene **5a–h** with terminal amidine group in *para-* or *meta-* position (Scheme 1), was synthesized. Both, the position and nature of substituents were changed to determine their influence on the DNA binding mode and anticancer properties.

Based on the position of terminal amidine groups two set of structural isomers with amidine substituents in *para-para* (**5a–f**) and *meta-meta* (**5g** and **5h**) positions were studied. DNA binding studies revealed that the spectra of investigated compounds supported formation of at least two different types of complexes with ct-DNA as a result of deviation from isosbestic points. Due to several binding modes of compounds **5a–h**, it was not possible to calculate the binding constants. Thermal melting experiments have shown that all investigated compounds **5a–h** interact with ct-DNA to stabilize the duplex structure. ΔT_m measurement at r = 0.1 revealed that alkylation of parent compound **5a** resulted in an overall increase of ΔT_m values, while substitution of the amidine moiety with cyclic ones, imidazoline or 1,4,5,6-tetrahydropyrimidine did not affect the values of ΔT_m.

ICD spectra of investigated compounds **5a–h** with ct-DNA support binding of studied compounds within the DNA minor groove either as single molecules (small substituents on amidines, only one ICD band) or for compounds with larger amidine substituents as dimeric forms (bisignate ICD bands). The bisignate ICD bands observed upon addition of derivatives **5a–h** to poly A–poly U, could be attributed to dimerization of compounds within the RNA major groove. Obviously, the fine interplay between the amidine substituent size and different steric and non-covalent interaction properties of the ds-DNA minor groove and ds-RNA major groove, respectively, control the dye-dimer formation and consequently characteristic ICD profile. Ethidium bromide displacement studies have shown that compounds **5a**, **5c**, **5e**, **5g** and **5h** efficiently compete with ethidium bromide in binding to ct-DNA (K_{app} at IC_{50} Table 4), at variance to **5b**, **5d** and **5f** which needed much higher concentrations to displace EB from DNA. Obtained IC_{50} did not correlate with thermal melting experiments, whereby the compounds **5d** and **5f** showing the highest stabilisation effects on ct-DNA, were the most inefficient in displacing ethidium bromide from DNA. This discrepancy could be attributed to the different binding modes of studied compounds (DNA minor groove) and ethidium bromide (intercalation).

The *in vitro* anticancer properties of the new compounds depend on the tested compound, the cell line, as

well as on the dose applied. Since it cannot be excluded that the observed fractional antiproliferative potential of compounds was a consequence of a somewhat difficult access into cells, the entry of compounds and their intracellular distribution will be investigated.

Supplementary Materials. – Supporting informations to the paper are enclosed to the electronic version of the article. These data can be found on the website of *Croatica Chemica Acta* (http://public.carnet.hr/ccacaa).

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