# Synthesis and biological activity of newly designed 2-benzo[*b*]thienyl and 2-bithienyl amidino-substituted benzothiazole and benzimidazole derivatives

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**Abstract**

Novel benzo[*b*]thienyl- and 2,2'-bithienyl-derived benzothiazoles (**5a**–**5d**, **7a**–**7d)** and benzimidazoles (**6a**–**6d**, **8a**–**8d**) were designed and synthesized to study their antiproliferative and antitrypanosomal activities *in vitro*. The prepared benzazoles were substituted with different types of amidine groups to study their influence as well as the influence of the type of thiophene backbone on the biological activity.

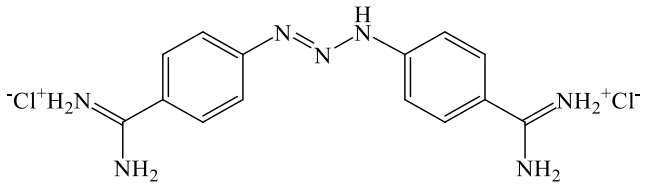
In general, the benzothiazole derivatives were more active than their benzimidazole analogs as both antiproliferative and antitrypanosomal agents. The 2,2'-bithienyl-substituted benzothiazoles **7a** and **7b** showed the most potent antitrypanosomal activity, and the greatest selectivity was observed for the benzimidazole derivatives **6d**, **6a**, and **8b**. The 2,2'-bithiophene derivatives showed most selective antiproliferative activity. Whereas the benzothiazoles (**7a**–**7d**) were selective against H 460, the benzimidazoles (**8a**–**8d**) were selective against HeLa cells. The compounds with unsubstituted amidine group (**7a**, **8a**) showed also strong antiproliferative effect.

The more pronounced antiproliferative activity of the benzothiazole derivatives was attributed to different cytotoxicity mechanisms. Additional cell cycle analysis, fluorescence and confocal microscopy, and DNA binding experiments indicate that the benzimidazoles likely target DNA, whereas the benzothiazoles probably have a different cellular target because they are localized in the cytoplasm and do not interact with DNA.

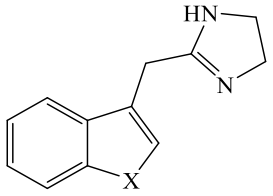
**Key words:** amidines, antiproliferative activity, antitrypanosomal activity, benzimidazoles, benzothiazoles

## 1. Introduction

Amidines, the nitrogen analogues of carboxylic acids, continue to be preferred structural motifs in rational drug design in organic and medicinal chemistry due to their occurrence as structural components of numerous biologically active synthetic or natural compounds.[1,2,3] The amidines form an important class of compounds used both in medicinal treatment (Fig. 1) and as biologically active compounds with a wide range of biological activities.[4,5] Among the most important activities, amidine derivatives have been described as compounds with antitumor,[6,7] antibacterial,[8] antiviral,[9,10]antifungal,[11] antiprotozoal,[12] antitrypanosomal, antioxidant,[13] and anti-inflammatory activities.




*Diminazene* – anti-infective drug




X = O Coumazilne
X = S Metizoline
vasoconstrictors

**Figure 1.** Amidine derivatives used in medical treatment

Since unsubstituted and substituted amidines are useful intermediates for the synthesis of versatile heterocycles due to their reactivity, numerous synthetic methods have been developed for their preparation, the most common being synthesis from amides, nitriles or thioamides.[14] The introduction of an aryl/heteroaryl group on a nitrogen atom of the amidine functionality could attenuate their strong basic properties, allowing the attachment of various lipophilic groups, leading to many new compounds with different biological activities.[15,16] In addition, amidines are also important starting materials for the synthesis of functional materials as well as useful ligands for metal complexes or organocatalysis.[17,18]

Continuing our scientific efforts related to the synthesis of heteroaromatic biologically active derivatives, our research group has recently published several papers on amidino-substituted benzazole derivatives. The targeted compounds were substituted with either an acyclic or cyclic amidine group, placed as a positively charged functional group, at the ends of the heteroaromatic core. The introduction of the amidine group significantly improved the biological activity and solubility. Many of the derivatives that exhibited outstanding biological activity were identified as agents that bind to an electronegatively charged DNA molecule, either as intercalators and/or groove binders.[19,20]

Thus, amidine moiety intercalate in ds-RNA by binding in the minor groove of AT-DNA, interact with ss-RNA, but only 2-imidazolinyl 2-phenylbenzothiazole showed a well-defined orientation and dominant binding mode with poly A and poly G, and amidino-substituted benzimidazole-2-carboxamides bind as groove binders with sequence-selective binding in the A-T rich side.[21] In previous studies, we have demonstrated the strong influence of the type of amidine functionality on the biological activity and binding mode, with the derivatives bearing cyclic amidine groups being the most active compared to other amidine substituents (Fig. 2). Some benzazole derivatives substituted with 2-imidazolinyl group showed $IC_{50}$ values in the submicromolar concentration range with selectivity toward cancer cells as well as very low cytotoxicity against normal fibroblasts.[22]

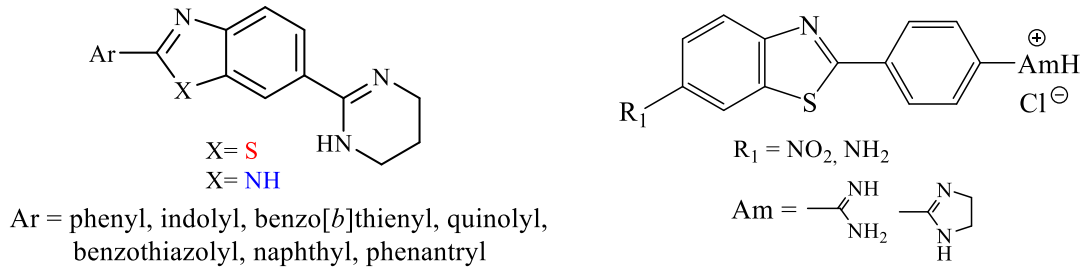


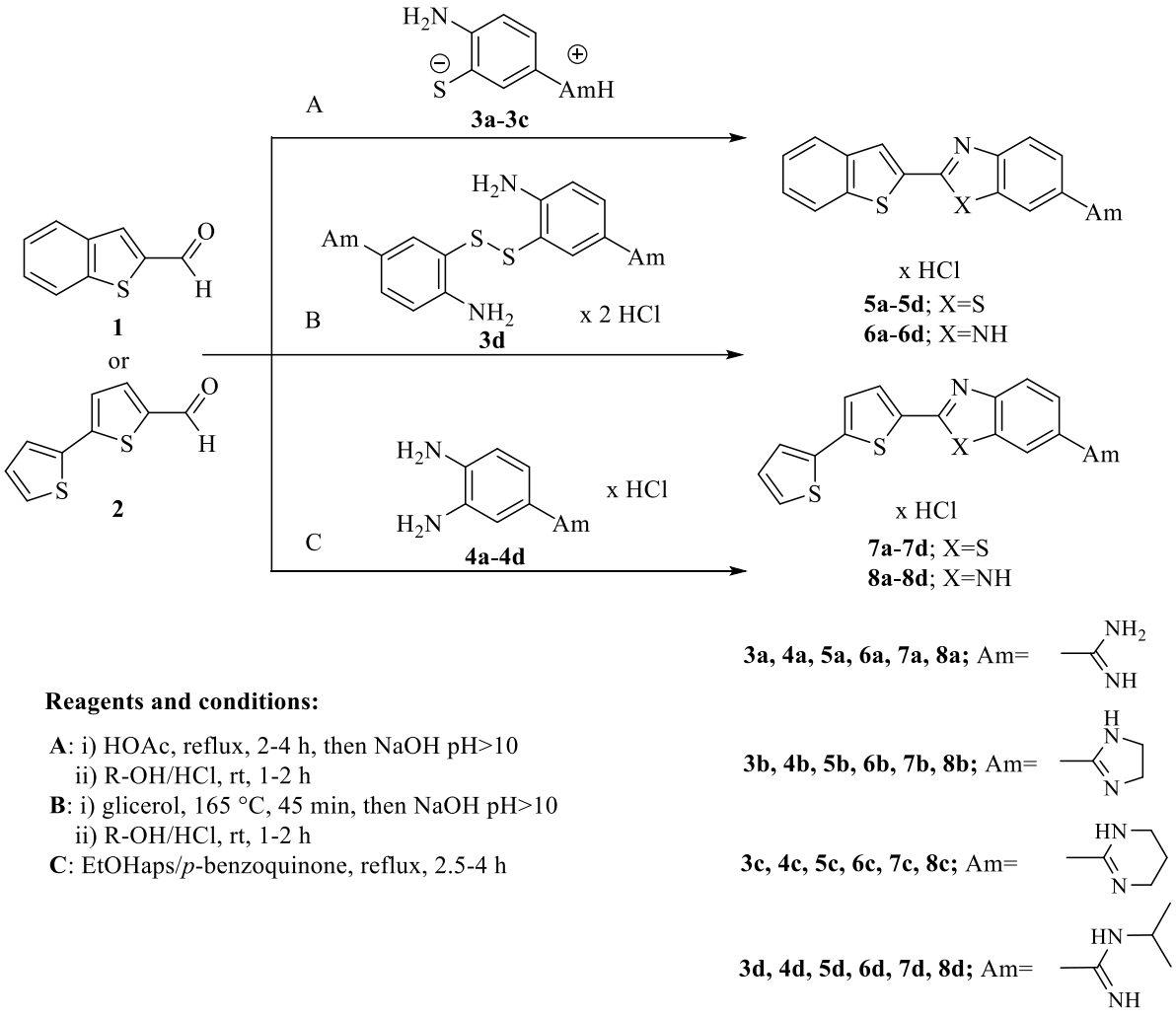

**Figure 2.** Biologically active amidine derivatives synthesized in our research group

Most of the previously prepared amidino-substituted benzazoles were evaluated *in vitro* for their antiproliferative activity on a series of cancer cells, and several promising, selective, and active lead compounds were selected for further optimization. Several types of amidino-substituted benzazoles also showed promising antibacterial and antioxidant activity. Amidino-substituted 2-phenyl-benzothiazole and benzimidazole derivatives with a variable number of hydroxy and methoxy groups attached to the phenyl ring showed promising antioxidant activity.[23,24] In addition, we were also interested in the synthesis of diamidino-substituted derivatives of phenylbenzothiazolyl and dibenzothiazolyl furans and thiophenes,[25] bisbenzothiazolyl-pyridines and pyrazines,[26] phenylenebis-benzothiazoles,[20] amidino-substituted 2-arylbenzothiazole hydrochlorides and mesylates,[27] and benzothienyl-substituted benzothiazoles.[28] Since the benzothiophene and thiophene derivatives are also recognized as highly-privileged substructures and building blocks in medicinal chemistry, and considering the bioactivity and pharmacological effectiveness of benzazoles and amidines, within this work we designed and synthesized novel 2-benzo[*b*]thienyl and 2-bithienly-substituted benzazoles with four types of amidine group. All compounds were evaluated for their antiproliferative and antitrypanosomal activity *in vitro*.

## 2. Result and Discussion

### 2.1. Chemistry

An efficient synthesis of targeted 2-benzothiophene and 2,2'-bithiophene-6-amidino-substituted benzothiazoles **5a**–**5d** and **7a**–**7d**, as well as the corresponding benzimidazole analogues **6a**–**6d** and **8a**–**8d** was carried out by cyclocondensation reaction outlined in Scheme 1.




**Scheme 1.** Synthesis of 2-benzothiophene and 2-bithiophene-6-amidino-substituted benzothiazoles **5a**–**5d** and **7a**–**7d** and benzimidazole **6a**–**6d** and **8a**–**8d**

Recently, we have found two complementary environmentally friendly methods for the condensation of 5-amidino-substituted 2-aminothiophenols with various aryl/heteroaryl carbaldehydes, using acetic acid and glycerol, without the need for a catalyst or an additional oxidation step.[27,28]

We have also found a convenient and green synthetic protocol using glycerol for the introduction of substituents to the C-6 position of the 2-arylbenzothiazole scaffold by the condensation reaction of bis(2-aminophenyl) disulfide derivatives with carbaldehydes.[29] The key precursors 2-amino-5-amidino-substituted benzenethiolates **3a**–**3c** and disulfide **3d** were prepared by the Pinner reaction according to our described procedure,[30,31] while benzo[*b*]thiophene-2-carbaldehyde **1** and 2,2'-bithiophene-5-carbaldehyde **2** were commercially available. Condensation of aldehydes **1** and **2** with amidino-substituted precursors in zwitterionic form **3a**–**3c** in acetic acid followed by a simple acid–base reaction step (Method A) afforded 2-benzothiphene/bithiophene-6-amidino substituted benzothiazole hydrochlorides **5a**–**5c** and **7a**–**7c** in moderate to good overall yields. On the other hand, for the condensation of *N*-isopropilamidino-substituted precursor in disulfide form **5d**, we use a condensation reaction in glycerol (Metod B). The corresponding isolated free bases, were then converted to targeted hydrochlorides in a second reaction step, and 6-*N*-isopropilamidino-substituted benzothiazole derivatives **5d** and **7d** were isolated in moderate overall yields of 47 % and 41 % respectively. Amidino substituted benzo[*b*]thiehyl- and 2,2'-bithiehyl-benzimidazoles **6a**–**6d** and **8a**–**8d** were prepared according to the synthesis shown in Scheme 1. Benzo[b]thiophene-2-carbaldehyde **1** and 2,2'-bithiophene-5-carbaldehyde gave the corresponding 2-benzothiophene/bithiophene-6-amidino-substituted benzimidazole hydrochlorides **6a**–-**6c** and **8a**–**8c** in moderate reaction yields in the cyclocondensation reaction with previously prepared 4-amidino-1,2-phenylenediamines **4a**–**4d** in ethanol using *p*-benzoquinone as oxidant. The amidino-substituted precursors **4a**–**4d** were obtained in the acidic Pinner reaction from the corresponding cyano-substituted precursors according to previously published procedures.The structures of all novel benzothiazole hydrochlorides **5a**–**5d**, **7a**–**7d** and benzimidazole hydrochlorides **6a**–**6d**, **8a**–**8d** were determined by $^{1}$H and $^{13}$C NMR spectroscopy, mass spectrometry and elemental analysis. The chemical shifts in the $^{1}$H and $^{13}$C NMR spectra and the H–H coupling constants were consistent with the proposed structures. The formation of the benzimidazole ring was confirmed by the signal associated with the proton of the NH group on the benzimidazole nuclei. Signals related to the nitrogen protons of the amidine groups were observed as singlets from 9 to 10 ppm, while the signals for the protons of the methyl and methylene groups are placed the aliphatic part of the $^{1}$H NMR spectra. The Pinner reaction was monitored by IR spectroscopy by the disappearance of the signal for cyano group at 2200 cm$^{-1}$. Elemental analyses showed that the hychlorides **5a** and **7a** were isolated as hydrate.

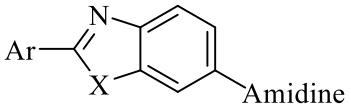
## 2.2. Biology

### 2.2.1. Antiproliferative activity *in vitro*

All prepared amidino-substituted benzothiazoles **5a**–**5d** and **7a**–**7d** and benzimidazoles **6a**–**6d** and **8a**–**8d** were tested *in vitro* for their antiproliferative activity on a series of human cancer cells, including HCT116 and SW620 (colon carcinoma), H460 (lung carcinoma), MCF-7 (breast carcinoma), PC3 (prostate carcinoma) and HeLa (cervical carcinoma), and HEK 293 (human embryonic kidney) cells. The results obtained are shown in Table 1 as $IC_{50}$ values (50% inhibitory concentrations) and compared with etoposide and doxorubicin as standard antiproliferative agents. As can be seen from Table 1, most of the benzazole derivatives tested showed potent and selective antiproliferative activity against the cancer cell lines tested with some compounds exhibiting inhibitory concentrations in the submicromolar range. The compound with the most pronounced activity proved to be 2,2'-bithiophene substituted benzothiazole bearing an unsubstituted amidino group, **7a**, with very potent and selective activity against H 460 cells with an $IC_{50}$ value of 0.02 μM, showing a twofold better inhibitory range compared with the $IC_{50}$ values for other cancer cells tested. All other 2,2'-bithienyl-substituted benzothiazoles **7b**, **7c** and **7d** also showed selective activity against H 460 cells with $IC_{50}$ 0.2 μM. 2,2'-bithienyl substituted benzimidazole derivatives **8a**–**8d** were less active compared to the benzothiazole analogues, with the most active and promising derivative **8a** with unsubstituted amidine showing selective activity against HeLa cells with $IC_{50}$ 0.8 μM. Selective activity against HeLa cells was also observed for **8b**–**8c** with $IC_{50}$ 1-2 μM. Of the benzimidazole analogues **6a**–**6d**, derivative **6b** was the most active but showed no selectivity, whereas compounds **6a**, **6c** and **6d** were generally less active but selective against MCF and HeLa cells.

In summary, the benzothiazole benzothiophenes **5a**–**5d** and the 2,2'-bithiophenes **7a**–**7d** exhibited the most pronounced activity, with the 2,2'-bithiophenyl-substituted benzothiazoles **7a**–**7d** also exhibiting selectivity toward H 460 lung cancer cells. Benzimidazoles **6a**–**6d** and **8a**–**8d** were generally less active but selective toward HeLa cells.

**Table 1.** *In vitro* anti-proliferative activity of amidino-substituted benzazole derivatives

| Cpd |  | | | IC$_{50}$[a] (μM) *Cell lines* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Ar** | **X** | **Amidine** | **HCT116** | **H 460** | **MCF-7** | **PC3** | **HeLa** | **SW620** | **HEK 293** |
| **5a** | benzothiophene | S | unsubstituted | 1.8±0.3 | 1.3±0.8 | 1.6±0.2 | 0.7±0.03 | 0.6±0.03 | 0.8±0.09 | 1.6±0.07 |
| **5b** | benzothiophene | S | 2-imidazolinyl | 1.7±0.1 | 0.9±0.6 | 1.7±0.03 | 1.2±0.08 | 0.7±0.06 | 0.8±0.08 | 1.9±0.4 |
| **5c** | benzothiophene | S | *3,4,5,6-tetrahydro-pyrimidin-1-ium-2-yl* | 1.9±0.6 | 1.4±0.2 | 1.6±0.2 | 6.1±1.5 | 0.7±0.06 | 0.9±0.01 | 2.4±0.98 |
| **5d** | benzothiophene | S | isopropyl | 1.8±0.2 | 0.9±0.6 | 1.3±0.09 | 0.7±0.06 | 0.7±0.02 | 0.8±0.14 | 1.3±0.00 |
| **6a** | benzothiophene | NH | unsubstituted | 11±3.7 | 20±1.7 | 1.3±0.02 | 5.8±1.1 | 0.6±0.05 | 1.3±0.12 | 31±12 |
| **6b** | benzothiophene | NH | 2-imidazolinyl | 3.02±1.8 | 2.6±1.1 | 2.7±0.8 | 2.3±0.40 | 1.3±0.09 | 1.3±0.10 | 2.8±1.7 |
| **6c** | benzothiophene | NH | *3,4,5,6-tetrahydro-pyrimidin-1-ium-2-yl* | 33±0.1 | 26.8±5.4 | 3.5±2.5 | 28.2±0.3 | 5.9±0.7 | 11.9±4.3 | 28±6.3 |
| **6d** | benzothiophene | NH | isopropyl | 30±1.4 | 25±5.2 | 5.2±3.9 | 30.5±3.4 | 2.3±0.03 | 14.2±1.1 | 22±2 |
| **7a** | 2,2'-bithiophene | S | unsubstituted | 1.1±0.6 | 0.02±0.01 | 1±0.6 | 0.8±0.04 | 0.7±0.01 | 0.8±0.09 | 1.7±0.3 |
| **7b** | 2,2'-bithiophene | S | 2-imidazolinyl | 1.5±0.1 | 0.2±0.04 | 1.5±0.3 | 2.0±0.08 | 0.7±0.03 | 0.9±0.01 | 1.5±0.05 |
| **7c** | 2,2'-bithiophene | S | *3,4,5,6-tetrahydro-pyrimidin-1-ium-2-yl* | 1.4±0.0 | 0.2±0.06 | 0.8±0.3 | 1.4±0.26 | 0.6±0.08 | 0.7±0.02 | 0.9±0.6 |
| **7d** | 2,2'-bithiophene | S | isopropyl | 1.6±0.04 | 0.2±0.09 | 1.4±0.07 | 1.2±0.30 | 0.7±0.03 | 0.6±0.14 | 1.2±0.02 |
| **8a** | 2,2'-bithiophene | NH | unsubstituted | 7.2±1.2 | 11.7±0.2 | 1.3±0.05 | 7.5±0.8 | 0.4±0.07 | 2.1±0.29 | 8.8±3 |
| **8b** | 2,2'-bithiophene | NH | 2-imidazolinyl | 4.3±1.6 | 4±0.5 | 2.5±1.08 | 6.6±0.1 | 1.4±0.13 | 2.4±0.8 | 2.9±0.9 |
| **8c** | 2,2'-bithiophene | NH | *3,4,5,6-tetrahydro-pyrimidin-1-ium-2-yl* | 14.9±10 | 5.9±2 | 5.7±0.8 | 17.5±0.7 | 0.8±0.16 | 7.8±1.2 | 2.4±1.5 |
| **8d** | 2,2'-bithiophene | NH | isopropyl | 6.8±3.3 | 4±3.5 | 5.2±3.7 | 6.6±0.1 | 2.1±0.05 | 5.6±0.6 | 6.2±1.4 |
| **doxorubicin** | | | | 0.04±0.01 | 0.01±0.004 | 0.09±0.02 | 0.13±0.02 | 0.003±0.001 | 0.02±0.01 | 0.03±0.003 |
| **etoposide** | | | | 2.2±0.4 | 0.6±0.1 | 9.3±0.9 | 11.6±1.9 | 0.14±0.05 | 0.65±0.02 | 1.6±0.1 |

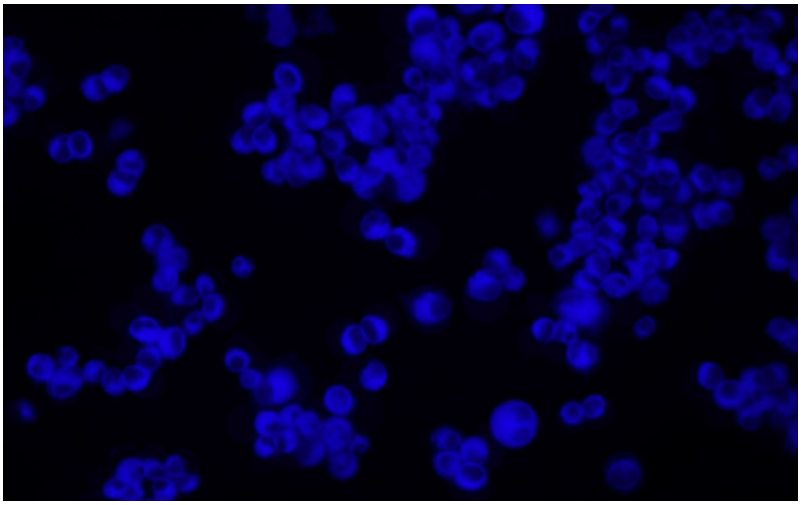
[a]Compound concentration required to inhibit tumor cell proliferation by 50%

### 2.2.2. Additional biological evaluation

#### 2.2.2.1. Fluorescence and confocal microscopy

Fluorescence microscopy on living cells was performed to evaluate the intracellular distribution of compounds. From the fluorescence microscopy images, compounds **5a**, **5b** and **7c** (Figure 3A and Supporting information Figures S1A and B) are localized in the cytoplasm and do not enter the nucleus.

A)



B)

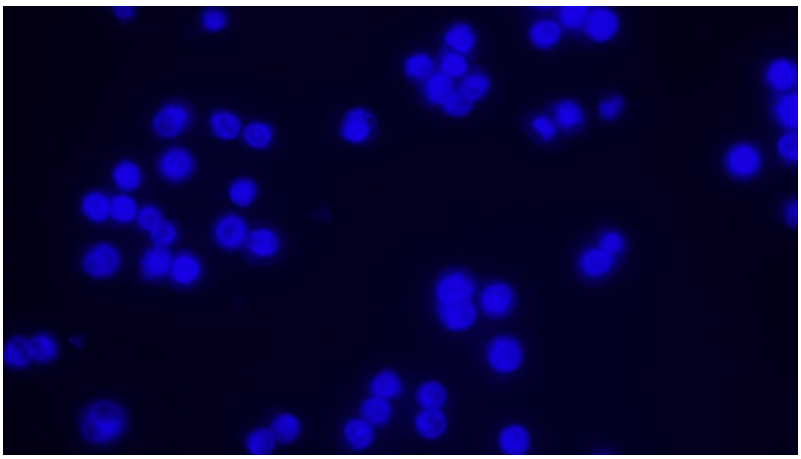


**Figure 3.** Fluorescence micrographs of H460 cells incubated with **7c** (A) and **8b** (B) (5 μM for 20 min) using DAPI filter ($\lambda_{ex}/\lambda_{em}$: 365/465nm) at 40× magnification.

In contrast, fluorescence micrographs indicate that compounds **8b** and **8c** are mainly localized in the nucleus (Figure 4B and Figure S1C).

For other compounds, the fluorescence micrographs were inconclusive, so additional confocal microscopy was performed. The experiments confirmed that compounds **7a** and **7d** did not enter the nucleus (Figure 4A and Supporting information Figure S2A). In contrast, compounds **6a**, **6b** and **8a** (Figure 4B and Supporting information Figure S2B and C) are distributed throughout the cell. The intracellular distribution of the compounds can be correlated primarily with the type of benzazole nucleus, with the benzothiazoles (**5a**–**5d** and **7a**–**7d)** localized in the cytoplasm and the benzimidazoles (**6a**–**6d** and **8a**–**8d**) entering the nucleus and distributed throughout the cell. Moreover, the distribution of the compounds in the cell can be also be related to the antiproliferative activity, since the $IC_{50}$ values for the compounds that enter the nucleus (**6a**–**6d** and **8a**–**8d**) are higher ($IC_{50}$ > 4 μM), while the compounds that are localized in the cytoplasm (**5a**–**5d** and **7a**–**7d)** have $IC_{50}$ values in the submicromolar range.

In summary, the difference in antiproliferative activity and localization suggests a different cytotoxicity mechanism of benzothiazole and benzimidazole derivatives, with the possibility of DNA targeting demonstrated for benzimidazole.

A) B)

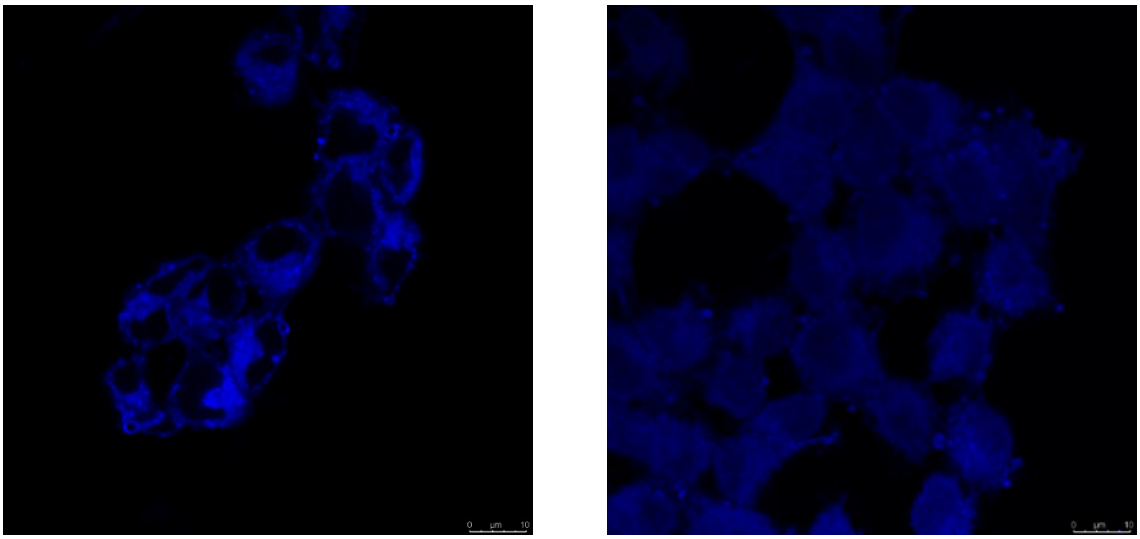


**Figure 4.** Confocal microscopy image of H460 cells incubated with **7a** (1 μM for 1h), $\lambda_{ex}/\lambda_{em}$: 405/450-550 nm (A) and with **8a** (5 μM for 2h), $\lambda_{ex}/\lambda_{em}$: 405/450-550 nm (B).

### 2.2.2.2. Cell cycle analysis

Furthermore, we performed cell cycle analysis to elucidate the mechanism of action of selected compounds with different intracellular distribution. The results show that compound **7a** at $IC_{50}$ concentration (0.02 µM) had no significant effect on the cell cycle after 24- and 48-hour treatment (Figure 5). Interestingly, treatment with the concentration of 0.05 µM resulted in a slight arrest in G1 phase, accompanied by a decrease in the percentage of cells in S phase after 24 hours. However, this effect was not observed after 48 hours of treatment. On the other hand, treatment of cells with **8a** at the $IC_{50}$ concentration (10 µM) resulted in a significant arrest in G2 phase at both time points, accompanied by a drastic reduction in S phase (Figure 6). This effect was also observed when treated with a concentration of 5 µM, although to a slightly lesser extent. Thus, the observed effect of **8a** on the cell cycle was both concentration- and time-dependent. Moreover, such a strong G2 arrest is a characteristic consequence of compounds interacting with DNA. The results obtained can be correlated with the subcellular localization of the compounds, showing that **8a** enters the nucleus and probably interacts with DNA.[32] **7a**, on the other hand, does not enter the nucleus, hence does not interact with DNA, and causes a typical accumulation of cells in G1 phase. These results suggest different targets of these compounds: compound **8a**, which targets DNA, has higher $IC_{50}$ values, as do other benzimidazole derivatives, whereas compound **7a** and other benzothiazoles are more cytotoxic and selective and probably have different cellular targets.

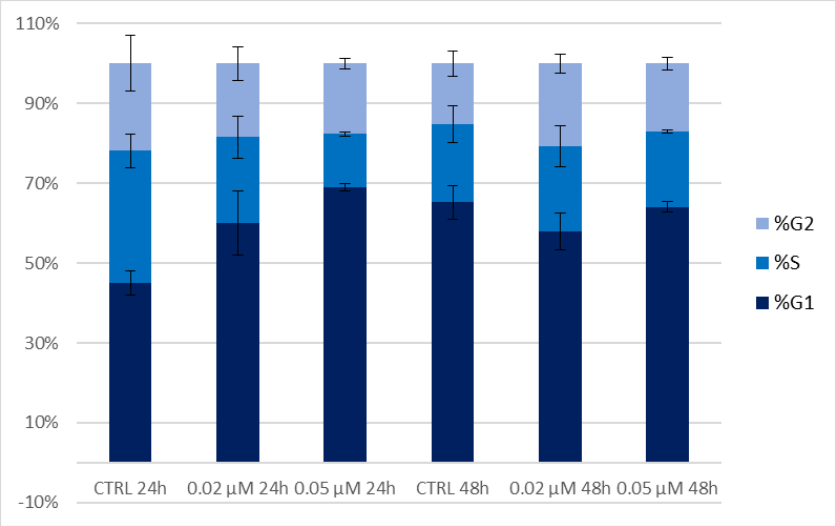



**Figure 5.** The effect of compounds **7a** at 0.02 µM and 0.05 µM concentrations on the cell cycle distribution of H460 cells after 24 h and 48 h treatments. The histograms represent the percentage of cells in the respective cell cycle phase (G1, G2, S), obtained by flow cytometry.

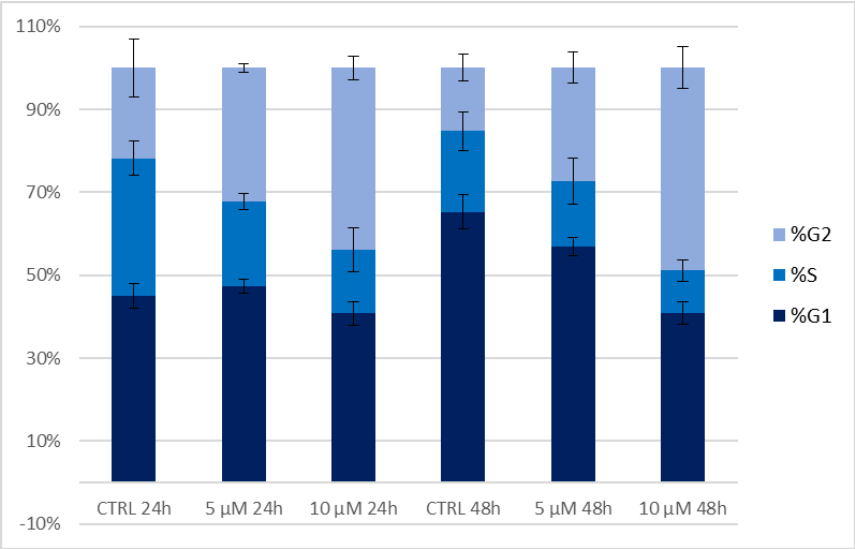



**Figure 6.** The effect of compounds **8a** at 5 µM and 10 µM concentrations on the cell cycle distribution of H460 cells after 24 h and 48 h treatments. The histograms represent the percentage of cells in the respective cell cycle phase (G1, G2, S), obtained by flow cytometry.

### 2.2.2.3. DNA binding study

Because our results suggest that some of the compounds target DNA, we further investigated their interactions with nucleic acids. We selected compounds **6a**, **6d** and **8a** because they exhibit nuclear localization. In addition, we also tested **7a**, which exhibited particularly pronounced activity and selectivity. Prior to the DNA binding studies, we performed spectroscopic characterization of the selected compounds. The tested compounds were dissolved in DMSO ($c$=5 × $10^{-3}$ mol $dm^{-3}$). The absorbance values of the buffered aqueous solutions of the tested compounds were proportional to their concentrations up to $c$=2 × $10^{-5}$ mol $dm^{-3}$, indicating that the compounds do not aggregate by intermolecular stacking in this concentration range. The absorption maxima and corresponding molar extinction coefficients (ε) are shown in Figure 7 and Table S1 (Supporting information Figures S3-S6). Fluorimetric measurements were performed in a region where the emission and excitation spectra did not overlap (Supporting information Figure S7-S11).

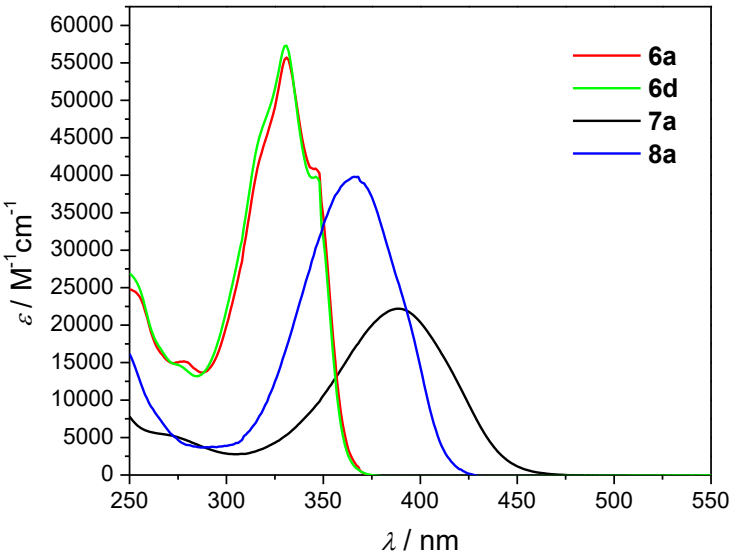



**Figure 7.** UV/Vis spectra of **6a, 6d, 7a** and **8a** at c = 2× $10^{-5}$ mol $dm^{-3}$; pH=7, sodium cacodylate/HCl buffer, $I$ = 0.05 mol $dm^{-3}$.

Compound interactions were studied using calf thymus (ct) DNA (58% AT base pairs, 42% GC base pairs) as a model for classical B-helix.[33] Important parameters can be derived from thermal melting ($T_m$) experiments. The $\Delta T_m$ value is the difference between the $T_m$ value of the free polynucleotide and the complex with a small molecule.[34]

Positive $\Delta T_m$ values are typical of intercalation, for example. However, groove binding can lead to significant (positive $\Delta T_m$ values) or negligible stabilization or destabilization of DNA (negative $\Delta T_m$ values). Compounds with a heterocyclic benzimidazole ring (**8a, 6a, 6d**) showed moderate stabilization effects of ctDNA (Table 2, Figure 8). In contrast, compound **7a** with a benzothiazole ring did not stabilize DNA. Compound **8a** with bithiophene substituent showed the best stabilization of double-stranded (ds-) DNA. Titrations with ctDNA resulted in a decrease in fluorescence of **6a**, **6d**, and **8a** (Table 2, Figure 9, Supporting information Figures S12-S14 in SI). Titration with compound **7a** was not performed because of negligible stabilization effect of ctDNA. The binding constants $K_a$ for ligand-ctDNA complexes were calculated by processing the fluorimetric titration data using the Scatchard equation (Table 2). Compounds **6a** and **8a** with unsubstituted amidine groups showed higher DNA binding affinities than compound **6d** with an isopropyl-substituted amidine group. The obtained results correlate with the $\Delta T_m$ values obtained from the thermal denaturation study.

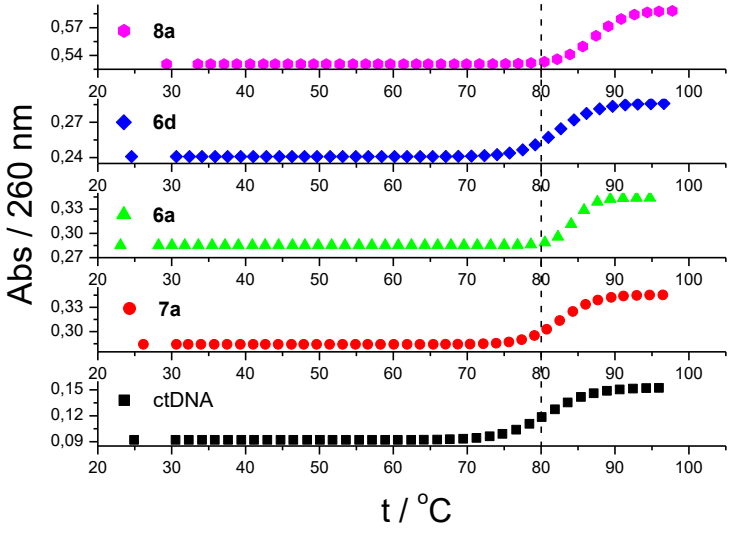



**Figure 8**. a) Melting curve of ctDNA upon addition of ratio, ***r*** ([compound/ [polynucleotide])=0.3 of **6a, 6d, 7a** and **8a** at pH = 7.0 (buffer sodium cacodylate, $I$ = 0.05 mol dm$^{-3}$).

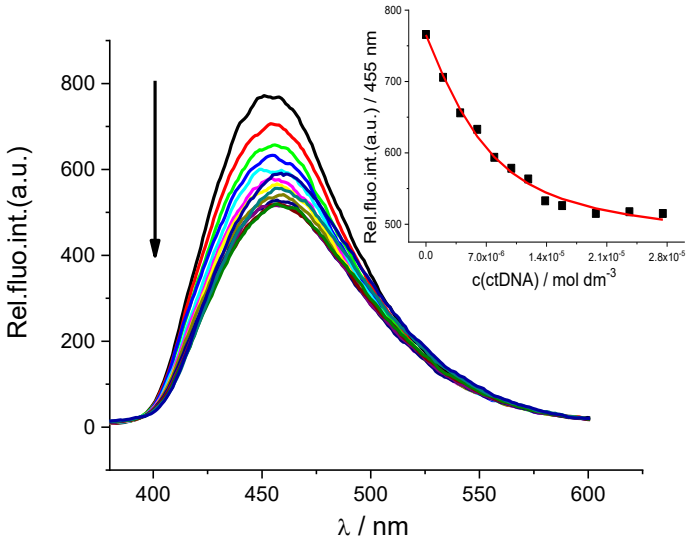



**Figure 9.** Changes in fluorescence spectrum of **8a** ($c$= 2.0 × 10$^{-6}$ mol dm$^{-3}$, $\lambda_{exc}$=340 nm) upon titration with ctDNA ($c$= 2.0 × 10$^{-6}$ – 2.7 × 10$^{-5}$ mol dm$^{-3}$); Inset: Dependence of fluorescence of **8a** at $\lambda_{em}$ = 455 nm on c(ctDNA ); (buffer sodium cacodylate,pH = 7.0, $I$ = 0.05 mol dm$^{-3}$).

**Table 2**. Binding constants ($\log K_a$)[a] for complexes of ligand-ctDNA calculated from the fluorescence titrations and $\Delta T_m$[c] values (°C) of ctDNA upon addition of ratio[d] **r** = 0.3 of **7a, 6a, 6d,** and **8a** at pH 7.0 (sodium cacodylate buffer, $I$ = 0.05 mol dm$^{-3}$).

| compound | $\log K_a$ | $\Delta T_m$ / °C |
|---|---|---|
| **7a** | -[b] | 0.8 |
| **6a** | 6.2 | 2.5 |
| **6d** | 5.3 | 1.8 |
| **8a** | 6.1 | 7.5 |

[a] Processing of titration data using Scatchard equation[35,36] gave values of ratio $n$[bound compound]/[nucleotide phosphate]; for easier comparison all data were recalculated for fixed $n$ = 0.3; correlation coefficients were >0.99 for most of the calculated $K_a$

[b] not determined due to negligible stabilization effect of ctDNA

[c] Error in $\Delta T_m$ : ± 0.5°C;

[d] r = [compound] / [polynucleotide].

CD spectroscopy can be used to monitor the change in nucleic acid conformation upon addition of small molecules. It can also provide information on interaction modes based on the mutual orientation of the small molecule and the chiral axis of the nucleic acid.[37] Achiral small molecules such as **6a**, **6d**, and **8a** can produce an induced CD spectrum (ICD) upon binding to nucleic acids. Useful information about the nature of binding can be obtained in the region where DNA does not absorb (> 300 nm), whereas **6a**, **6d**, and **8a** have UV/vis spectra (Figure 10, Supporting information Figure S15).

Addition of **6a, 6d,** and **8a** caused a decrease in CD intensity of ctDNA at 275 nm. Compounds **6a** and **6d** caused a greater decrease in the CD spectrum of ctDNA than **8a**, likely due to the different aromatic substituent (benzothiophene *versus* bithiophene) on the benzimidazole ring. This significant decrease could be due to a change in DNA conformation or the negative ICD bands of **6a** and **6d** in the 250–300 nm range. However, all tested compounds triggered the appearance of strong positive ICD bands, that correlated with the absorption maxima of the tested compounds (> 300 nm). A positive ICD band whose intensity is stronger than the CD band of DNA strongly suggests binding of the minor groove to ctDNA.[38] These changes are consistent with results obtained for positively charged benzimidazoles, which have been shown to be minor groove binders.**Error! Bookmark not defined.**,[39,40]

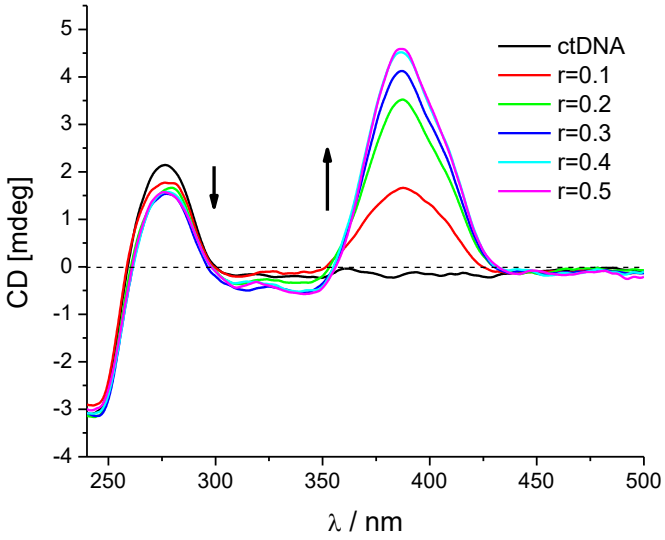



**Figure 10.** CD titration of ctDNA (c = 3.0 × $10^{-5}$ mol $dm^{-3}$) with **8a** at molar ratios at molar ratios **r** = [compound]/[nucleotide phosphate] indicated in the graph legend (pH = 7.0, buffer sodium cacodylate, I = 0.05 mol $dm^{-3}$).

In summary, the nature of the heterocyclic ring and the amidine affect the binding affinity and thermal melting values with ctDNA. Compounds **6a** and **8a** with a heterocyclic benzimidazole ring and unsubstituted amidine groups showed the best binding affinities and stabilization effects for ctDNA.

In addition to the cell cycle analysis and cellular distribution results, the DNA binding experiments confirm that DNA could be a potential target for benzimidazoles **8a**, **6a** and **6d, as the** tested compounds also showed significant DNA binding affinity. We can assume that other benzimidazole derivatives that exhibit comparable antiproliferative activity and localization have similar DNA-binding affinity and mechanism of antiproliferative activity.

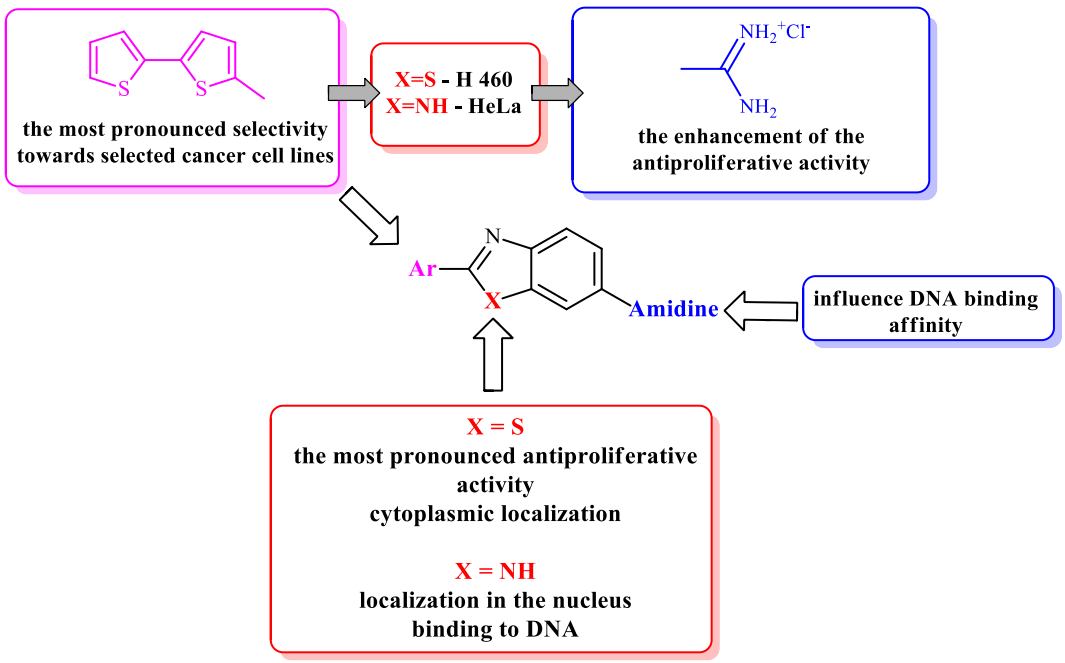
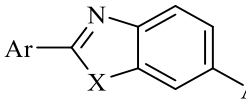



**Figure 11.** Insights into structure-activity relationship of benzazoles on antiproliferative activity and DNA binding affinity

### 2.2.3. Antitrypanosomal activity *in vitro*

All newly synthesized benzo[*b*]thienyl and 2,2'-bithienyl derived benzothiazoles and benzimidazoles were tested *in vitro* for their detailed antitrypanosomal activity against bloodstream-form *T. brucei*. As presented in the Table 3, this evaluation included determination of concentrations that inhibit growth by 50% ($IC_{50}$) and 90% ($IC_{90}$). Nifurtimox was used the standard and reference drug, while cytotoxicity was assessed using the rat myoblast cell line L6. The majority of the amidino substituted benzazoles tested showed potent activity against *T. brucei* having $IC_{50}$ values ranging from 0.26 to 17.8 μM and $IC_{90}$ from 0.34 to 24.0 μM. Most of tested compounds were more active compared to the standard drug. The results presented showed that the most active and promissing compounds were two benzothiazole derivatives substituted with 2,2'-bithiophene skeleton, namely derivative **7a** with an unsubstituted amidino group and derivative **7b** bearing cyclic 2-imidazolinyl group ($IC_{50}$ 0.34 and 0.26 μM; $IC_{90}$ 0.41 and 0.34 μM; respectively). Very strong activity was also observed with the benzo[*b*]thiophene derived benzothiazole with unsubstituted amidino group **5a** ($IC_{50}$ 0.56; $IC_{90}$ 1.13 μM).

**Table 3.** *In vitro* antitrypanosomal activity[a] of amidino-substituted benzazole derivatives against *Trypanosoma brucei*

| Cpd |  | | | ***T. Brucei*** | | **L6 cells** | **S.I.[b]** |
|---|---|---|---|---|---|---|---|
| | **Ar** | **X** | **Amidine** | $IC_{50}$ (μM) | $IC_{90}$ (μM) | $IC_{50}$ (μM) | L6/*Tb* $IC_{50}$ |
| **5a** | benzothiophene | S | unsubstituted | 0.56 ± 0.05 | 1.13 ± 0.08 | 0.96 ± 0.03 | 1.7 |
| **5b** | benzothiophene | S | 2-imidazolinyl | 1.08 ± 0.05 | 1.80 ± 0.46 | 0.89 ± 0.11 | 0.8 |
| **5c** | benzothiophene | S | 3,4,5,6-tetrahydro-pyrimidin-1-ium-2- | 1.86 ± 0.03 | 2.46 ± 0.03 | 1.58 ± 0.18 | 0.8 |
| **5d** | benzothiophene | S | isopropyl | 2.09 ± 0.10 | 3.25 ± 0.21 | 1.88 ± 0.13 | 0.9 |
| **6a** | benzothiophene | NH | unsubstituted | 0.87 ± 0.22 | 17.3 ± 2.4 | 42.8 ± 1.9 | 49 |
| **6b** | benzothiophene | NH | 2-imidazolinyl | 1.57 ± 0.10 | 3.77 ± 0.25 | 11.6 ± 0.6 | 7.4 |
| **6c** | benzothiophene | NH | 3,4,5,6-tetrahydro-pyrimidin-1-ium-2- | 3.74 ± 0.22 | 7.70 ± 0.65 | 143 ± 14 | 38 |
| **6d** | benzothiophene | NH | isopropyl | 1.57 ± 0.25 | 7.67 ± 0.76 | 115 ± 1 | 73 |
| **7a** | 2,2'-bithiophene | S | unsubstituted | 0.34 ± 0.02 | 0.41 ± 0.03 | 1.48 ± 0.30 | 4.4 |
| **7b** | 2,2'-bithiophene | S | 2-imidazolinyl | 0.26 ± 0.05 | 0.34 ± 0.02 | 0.42 ± 0.10 | 1.6 |
| **7c** | 2,2'-bithiophene | S | 3,4,5,6-tetrahydro-pyrimidin-1-ium-2- | 1.10 ± 0.07 | 2.10 ± 0.05 | 1.51 ± 0.07 | 1.4 |
| **7d** | 2,2'-bithiophene | S | isopropyl | 1.14 ± 0.10 | 2.12 ± 0.07 | 0.98 ± 0.07 | 0.9 |
| **8a** | 2,2'-bithiophene | NH | unsubstituted | 12.5 ± 0.7 | 24.0 ± 1.4 | 24.1 ± 6.8 | 1.9 |
| **8b** | 2,2'-bithiophene | NH | 2-imidazolinyl | 1.88 ± 0.05 | 2.48 ± 0.03 | 88.5 ± 9.8 | 47 |
| **8c** | 2,2'-bithiophene | NH | 3,4,5,6-tetrahydro-pyrimidin-1-ium-2- | 9.16 ± 0.25 | 19.8 ± 1.1 | - | - |
| **8d** | 2,2'-bithiophene | NH | isopropyl | 17.8 ± 0.2 | 23.8 ± 0.1 | 33.0 ± 5.0 | 1.9 |
| Nifurtimox[c] | | | | 2.02 ± 0.24 | | | |

[a] *In vitro* activity against bloodstream form *T. brucei* expressed as the concentration that inhibited growth by 50% ($IC_{50}$) and 90% ($IC_{90}$).

[b] Selectivity index, SI = [$IC_{50}$ L6 cells]/[$IC_{50}$ *T. brucei*].

[c] Taken from Wilkinson et al.[41]

*** 6a** was not trypanocidal at any concentration tested – it acted simply as a growth inhibitor

In the case of benzo[b]thienyl-substituted benzazoles, **5a**–**5d** and **6a**–**6d**, the unsubstituted amidine group was shown to have the greatest influence on enhancing antitrypanosomal activity, whereas in the case of 2,2'-thienyl-substituted derivatives, **7a**–**7d** and **8a**–**8d**, the compounds with pentacyclic 2-imidazolinyl amidine group were more active.

Similar to the antiproliferative evaluation, in general, the strongest activity was observed for the benzothiazole derivatives compared to their benzimidazole analogues. In addition, the obtained results showed that benzimidazole derivative **6a** was not trypanocidal at any of the concentrations tested and therefore acted only as a growth inhibitor. Interestingly, some of the compounds were not cytotoxic to the rat myoblast cell line L6 with $IC_{50}$ > 45 μM. Two benzo[*b*]thienyl derived benzimidazole derivatives, substituted with hexacyclic amidine group **6c** and isopropyl amidine group **6d** were not at all cyctotoxic to the rat myoblast cell line L6 ($IC_{50}$ 143 μM, and 115 μM, respectively). The greatest selectivity was observed for three compound tested, benzimidazole derivative **6d** substituted with isopropylamidine group (S.I. 73), benzimidazole derivative **6a** substituted with unsubstituted amidine group (S.I. 49), and benzimidazole derivative **8b** substituted with 2-imidazolinyl group (S.I. 47). The benzothiazole derivatives exhibited significantly lower selectivity compared to the benzimidazole analogues. (Figure 12).

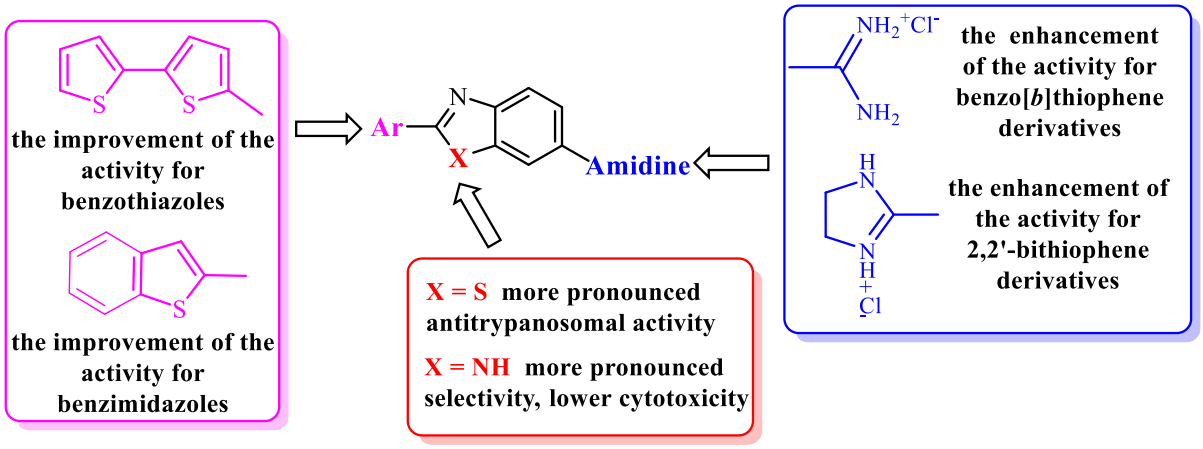



**Figure 12.** Structure-activity relationship regarding the antitrypanosomal activity *in vitro*

## 3. Conclusions

Benzothiazole **5a**–**5d** and **7a**–**7d** and benzimidazole **6a**–**6d** and **8a**–**8d** derivatives substituted with either benzo[*b*]thieny or 2,2'-bithienyl heteroaromatic nuclei at position 2 and with amidino groups on the benzazole backbone were designed and prepared to study their biological activity. Different types of acyclic and cyclic amidino groups were introduced to study their influence on the activity along with the nature of the benzazole skeleton or thiophene-derived nuclei at position 2. All benzazoles were prepared according to optimized synthetic methods previously developed and described in our research group.

New benzothiazoles and benzimidazoles were designed and synthesised to study their antiproliferative and antitrypanosomal activities *in vitro*. In general, the benzothiazole derivatives were more active than their benzimidazole analogues in both activities. The unsubstituted amidine group had the greatest effect on enhancing both biological activities, whereas the benzimidazole analogues with 2-imidazolinyl group showed more pronounced antitrypanosomal activity.

From the results of the antiproliferative evaluation, the majority of the benzazoles tested showed potent and selective antiproliferative activity against the tested cancer cell lines, with some compounds exhibiting inhibitory concentrations in the submicromolar range. The most promising derivative selected as lead compound proved to be 2,2'-bithiophene substituted benzothiazole with unsubstituted amidine group **7a** with potent and selective antiproliferative activity against H 460 in the submicromolar range ($IC_{50}$ 0.02 μM). All other 2,2'-bithienyl substituted benzothiazoles **7b, 7c** and **7d** also showed strong and selective activity against H 460 ($IC_{50}$ 0.2 μM).

In addition, the 2, 2'-bithiophene benzimidazole derivatives **8a**–**8d** also showed significant selectivity against HeLa cells. Interestingly, compounds with unsubstituted amidine group **7a** and **8a** showed significantly stronger activity than compounds with substituted amidine group in selected cell lines, whereas no effect of amidine substitution was not observed in the other cell lines and benzothiophenes (**5a**–**5d**, **6a**–**6d**). On the other hand, the benzothiazoles were generally more active compared with the analogous benzimidazole derivatives. The different antiproliferative activity of the compounds bearing a heterocyclic benzimidazole or benzothiazole ring was explained by different cytotoxicity mechanisms. Additional cell cycle analysis, fluorescence and confocal microscopy, and DNA binding experiments indicate that benzimidazoles likely target DNA because they are localised in the nucleus.

Compounds **8a**, **6a** and **6d** exhibited moderate to high DNA binding affinities, while **8a** also showed strong G2 arrest which is characteristic of compounds that interact with DNA. On the other hand, benzothiazoles are localized in the cytoplasm, the tested **7a** did not cause G2 arrest in the cell cycle and no interactions with DNA were detected, indicating a different cellular target and mechanism of action.

Most of the amidino-substituted benzazoles tested showed potent activity against *T. brucei*. The strongest antitrypanosomal activity was exhibited by the 2,2'-bithienyl-substituted benzothiazoles **7a** and **7b** ($IC_{50}$ 0.34 and 0.26 μM; $IC_{90}$ 0.41 and 0.34 μM; respectively). Very strong activity was also observed for benzo[*b*]thiophene-derived benzothiazole with unsubstituted amidino group **5a** ($IC_{50}$ 0.56; $IC_{90}$ 1.13 μM). Two benzo[*b*]thienyl derived benzimidazole derivatives, **6c** and **6d** were not at all cytotoxic to the rat myoblast cell line L6 at all ($IC_{50}$ 143 μM, and 115 μM, respectively). The benzimidazole derivatives were more selective compared with their benzothiazole analogues. Thus, the greatest selectivity was observed for benzimidazole derivatives **6d** (S.I. 73), **6a** (S.I. 49), and **8b** (S.I. 47).

## 4. Experimental part

### 4.1. Chemistry

#### 4.1.1. General Methods

Melting points were determined by means of Original Kofler Mikroheitztisch apparatus (Reichert, Wien). $^{1}$H NMR and $^{13}$C NMR spectra were recorded with the Bruker Avance DPX-300 or Bruker AV-600 using TMS as an internal standard. Chemical shifts are reported in parts per million (ppm) relative to TMS. LC-MS was performed on the Agilent 6120 Quadrupole coupled to the Agilent 1290 Infinity II UHPLC instrument using electrospray ionization (ESI). Elemental analyses for carbon, hydrogen, and nitrogen were performed on a Perkin-Elmer 2400 elemental analyser. Analyses are indicated as symbols of elements, and the analytical results obtained are within 0.4% of the theoretical value

#### 4.1.2. General methods for preparation of compounds 5a–5d and 7a–7d

**Method A**

To a stirred solution of the corresponding 2-amino-benzenethiolate **3a**-**3c** (0.5 mmol) in glacial acetic acid (5 ml), the corresponding aldehyde **1** or **2** (0.5 mmol) was added and refluxed for 2-4 h. The reaction mixture was poured onto ice and made alkaline (pH 10-12) with 20 % NaOH. The resulting free base was filtered off, washed with water and dried. The crude free base was suspended in ethanol or 2-propanol and an excess of concentrated hydrochloric acid was added and stirred at room temperature for 1-2 h.

**Method B**

To a stirred suspension of disulfide **3d** (0.25 mmol) in glycerol (1.0 g), the corresponding aldehyde **1** or **2** (0.5 mmol) was added and heated at 165 °C for 45 min. The reaction mixture was cooled, diluted with water and made alkaline (pH 10-12) with 20 % NaOH. The resulting free base was filtered, washed with water and dried. The crude free base was suspended in 2-propanol and an excess of concentrated hydrochloric acid was added and stirred at room temperature for 1-2 h.

*6-Amidinium-2-(benzo[b]thiophene-2-yl)benzothiazole chloride* **5a**

According to **Method A,** benzo[*b*]thiophene-2-carbaldehyde (**1**) (81 mg) and amino-5-amidiniumbenzenethiolate (**3a**) (34 mg) were used (reaction time 4 h). Afterwards, the obtained free base was suspended in ethanol (5 mL) followed by the addition of concentred hydrochloric

acid (0.100 ml) and stirring at room temperature for 1h. After cooling overnight, the resulting precipitate was filtered, crystallized from water, and dried at 75 °C. Yield of pure compound **5a** as white solid was 82 mg (45 %); m.p. = 294-298 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ = 9.43 (bs, 4H, -C(N$H_2$)$_2^+$), 8.71 (s, 1H, Ar-H), 8.44 (s, 1H, Ar-H), 8.28 (d, $J$ = 8.3 Hz, 1H, Ar-H), 8.15-7.91 (m, 3H, Ar-H), 7.59-7.46 (m, 2H); $^{13}$C NMR (150 MHz, DMSO-$d_6$) δ = 165.4, 164.9, 155.9, 140.3, 139.2, 135.3, 134.7, 127.8, 126.9, 126.4, 125.2, 125.1, 125.0, 123.2, 122.7 (2C); LC-MS (ESI) m/z: 310.1 [(M-Cl$^-$)$^+$];Analysis calcd for $C_{16}H_{12}ClN_3S_2$ x $H_2O$ (363.88): C, 52.81; H, 3.88; N, 11.55 %; Found: C, 53.01; H, 3.72; N, 11.50 %

*2-(Benzo[b]thiophene-2-yl)-6-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzothiazole chloride* **5b**
According to **Method A,** benzo[*b*]thiophene-2-carbaldehyde (**1**) (81 mg) and 2-amino-5-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)benzenethiolate hydrate (**3b**) (106 mg) were used (reaction time 4 h). Afterwards, the obtained free base was suspended in 2-propanol (10 mL) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 1 h. After cooling overnight, the resulting precipitate was filtered, crystallized from ethanol, and dried at 75 °C. Yield of pure compound **5b** as pale yellow solid was 112 mg (60 %); m.p. > 300 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ = 10.69 (s, 2H, -C(N*H*-)$_2^+$), 8.80 (s, 1H, Ar-H), 8.43 (s, 1H, Ar-H), 8.30 (d, 1H, $J$ = 8.6 Hz, Ar-H), 8.18-7.95 (m, 3H, Ar-H), 7.62-7.41 (m, 2H, Ar-H), 4.03 (s, 4H, C$H_2$C$H_2$);$^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 164.7, 156.4, 140.5, 139.3, 135.32, 135.1, 128.4, 127.2, 126.8, 125.5, 125.4, 123.7, 123.2, 122.9, 119.42 44.6 (2C); LC-MS (ESI) m/z: 336.2 [(M-Cl$^-$)$^+$]; Analysis calcd for $C_{18}H_{14}ClN_3S_2$ (371.91): C, 58.13; H, 3.79; N, 11.30 %; Found: C, 58.18; H, 3.71; N, 11.21 %

*2-(Benzo[b]thiophene-2-yl)-6-(3,4,5,6-tetrahydropyrimidin-1-ium-2-yl)benzothiazole chloride* **5c**
According to **Method A,** benzo[*b*]thiophene-2-carbaldehyde (**1**) (81 mg) and 2-amino-5-(3,4,5,6-tetrahydropyrimidin-1-ium-2-yl)benzenethiolate (**3c**) (104 mg) were used (reaction time 4 h). Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 1 h. After cooling overnight, the resulting precipitate was filtered, crystallized from ethanol, and dried at 75 °C. Yield of pure compound **5c** as pale yellow solid was 108 mg (56 %); m.p. >300 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ = 10.23 (s, 2H, -C(N*H*-)$_2^+$), 8.62 (d, 1H, $J$ = 1.6 Hz, Ar-H), 8.40 (s, 1H, Ar-H), 8.26 (d, 1H, $J$ = 8.6 Hz, Ar-H), 8.11-7.98 (m, 2H, Ar-H), 7.87 (dd, 1H, $J$ = 8.6 Hz, $J$ = 1.8 Hz, Ar-H), 7.55-7.44 (m, 2H, Ar-H), 3.52 (t, 4H, $J$ = 5.5 Hz, C$H_2$CH$_2$C$H_2$), 2.05 – 1.94 (m, 2H, CH$_2$C$H_2$CH$_2$); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 164.8, 159.0, 155.7,

140.4, 139.3, 135.5, 134.9, 128.1, 127.1, 126.3, 125.8, 125.5, 125.3, 123.0, 122.9, 122.8, 39.9 (2C), 17.7; LC-MS (ESI) m/z: 350.2 [(M-$Cl^-$)$^+$]; Analysis calcd for $C_{19}H_{16}ClN_3S_2$ (385.93): C, 59.13; H, 4.18; N, 10.89 %; Found: C, 59.21; H, 4.11; N, 10.87 %;

*2-(Benzo[b]thiophene-2-yl)-6-N-isopropilamidiniumbenzothiazole chloride* **5d**

According to **Method B,** benzo[*b*]thiophene-2-carbaldehyde (**1**) (81 mg) and 3,3'-disulfanediylbis[4-aminobenzo(*N*-isopropylamidinium chloride)] dihydrate (**3d**) (132 mg) were used. Afterwards, the obtained free base was suspended in 2-propanol (5 mL) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 2 h. After cooling overnight, the resulting precipitate was filtered, crystallized from water/acetone mixture, and dried at 75 °C. Yield of pure compound **5d** as white solid was 91 mg (47 %); m.p. = 280-284 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) δ = 9.83 (d, 1H, *J* = 7.2 Hz, -C(N*H*-)N$H_2^+$), 9.67 (bs, 1H, -C(N*H*-)N$H_2^+$), 9.39 (bs, 1H, -C(N*H*-)N$H_2^+$), 8.65 (s, 1H, Ar-H), 8.43 (s, 1H, Ar-H), 8.25 (d, 1H, *J* = 8.3 Hz, Ar-H), 8.10 (d, 1H *J* = 6.9 Hz, Ar-H), 8.03 (d, 1H, *J* = 6.5 Hz, Ar-H), 7.88 (d, 1H, *J* = 8.1 Hz, Ar-H), 7.52 (s, 2H, Ar-H), 4.27-4.12 (m, 1H, -C*H*$(CH_3)_2$), 1.32 (d, 6H, *J* = 5.9 Hz, -CH(C*H*$_3)_2$); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 164.7, 161.4, 155.6, 140.4, 139.3, 135.5, 134.6, 128.0, 127.0, 126.9, 126.4, 125.4, 125.3, 123.6, 122.9, 122.7, 45.3, 21.3 (2C); LC-MS (ESI) m/z: 352.2[(M-$Cl^-$)$^+$]; Analysis calcd for $C_{19}H_{18}ClN_3S_2$ (387.95): C, 58.82; H, 4.68; N, 10.83 %; Found: C, 58.80; H, 4.75; N, 10.98 %

*6-Amidinium-2-(2,2'-bithiophene-5-yl)benzothiazole chloride* **7a**

According to **Method A**, 2,2'-bithiophene-5-carbaldehyde (**2**) (97 mg) and amino-5-amidiniumbenzenethiolate (**3a**) (34 mg) were used (reaction time 4 h). Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 1 h. After cooling overnight, the resulting precipitate was filtered, crystallized from ethanol/diethyl-ether mixture, and dried at 75 °C. Yield of pure compound **7a** as yellow solid was 73 mg (37 %); m.p. = 294-298 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) δ = 9.49 (bs, 2H, -C(N$H_2)_2^+$), 9.26 (bs, 2H, -C(N$H_2)_2^+$), 8.65 (d, 1H, *J* = 1.6 Hz, Ar-H), 8.18 (d, 1H, *J* = 8.6 Hz, Ar-H), 7.97 (d, 1H, *J* = 4.0 Hz, Th-H), 7.92 (dd, 1H, *J* = 8.6 Hz, *J* = 1.8 Hz, Ar-H), 7.68 (dd, 1H, *J* = 5.1 Hz, *J* = 1.0 Hz, Th-H), 7.57 (dd, 1H, *J* = 3.6 Hz, *J* = 1.0 Hz, Th-H), 7.49 (d, 1H, *J* = 4.0 Hz, Th-H), 7.19 (dd, 1H, *J* = 5.0 Hz, *J* = 3.7 Hz, Th-H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 165.4, 164.3, 156.2, 141.9, 135.2, 134.5, 133.6, 132.2, 128.8, 127.5, 126.6, 126.1, 125.3, 124.7, 123.3, 122.3; LC-MS (ESI) m/z: 342.1 [(M-$Cl^-$)$^+$]; Analysis calcd for $C_{16}H_{12}ClN_3S_3$ x $H_2O$ (395.95): C, 48.53; H, 3.56; N, 10.61 %; Found: C, 48.67; H, 3.34; N, 10.58 %

*2-(2,2'-Bithiophene-5-yl)-6-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzothiazole chloride* **7b**

According to **Method A,** 2,2'-bithiophene-5-carbaldehyde (**2**) (97 mg) and 2-amino-5-(4,5-dihydro-1*H*-imidazol-3-ium-2-yl)benzenethiolate hydrate (**3b**) (106 mg) were used (reaction time 3 h). Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 1 h. After cooling overnight, the resulting precipitate was filtered, crystallized from ethanol/diethyl-ether mixture, and dried at 75 °C. Yield of pure compound **7b** as orange solid was 105 mg (52 %); m.p. = 296-300 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ = 10.76 (s, 2H, -C(N*H*-)$_2^+$), 8.78 (s, 1H, Ar-H), 8.19 (d, 1H, *J* = 8.7 Hz, Ar-H), 8.06 (d, 1H, *J* = 8.3 Hz, Ar-H), 7.96 (d, 1H, *J* = 3.9 Hz, Th-H), 7.67 (d, 1H, *J* = 5.0 Hz, Th-H), 7.55 (d, 1H, *J* = 3.4 Hz, Th-H), 7.47 (d, 1H, *J* = 3.9 Hz, Th-H), 7.22-7.12 (m, 1H, Th-H), 4.02 (s, 4H, C*H*$_2$C*H*$_2$); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 164.5, 156.4, 142.0, 135.0, 134.6, 133.4, 132.0, 128.5, 127.2, 126.6, 125.8, 125.1, 123.4, 122.4, 118.5, 44.3 (2C); LC-MS (ESI) m/z: 368.1 [(M-Cl$^-$)$^+$]; Analysis calcd for $C_{18}H_{14}ClN_3S_3$ (403.97): C, 53.52; H, 3.49; N, 10.40 %; Found: C, 53.38; H, 3.61; N, 10.52 %

*2-(2,2'-Bithiophene-5-yl)-6-(3,4,5,6-tetrahydropyrimidin-1-ium-2-yl)benzothiazole chloride* **7c**

According to **Method A,** 2,2'-bithiophene-5-carbaldehyde (**2**) (97 mg) and 2-amino-5-(3,4,5,6-tetrahydropyrimidin-1-ium-2-yl)benzenethiolate (**3c**) (104 mg) were used (reaction time 3 h). Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 1 h. After cooling overnight, the resulting precipitate was filtered, crystallized from water, and dried at 75 °C. Yield of pure compound **7c** as yellow solid was 105 mg (55 %); m.p. > 300 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ = 10.17 (s, 2H, -C(N*H*-)$_2^+$), 8.57 (d, 1H, *J* = 1.4 Hz, Ar-H), 8.18 (d, 1H, *J* = 8.6 Hz, Ar-H), 7.96 (d, 1H, *J* = 4.0 Hz, Th-H), 7.85 (dd, 1H, *J* = 8.6 Hz, *J* = 1.7 Hz, Ar-H), 7.68 (dd, 1H, *J* = 5.0 Hz, *J* = 0.8 Hz, Th-H), 7.57 (d, 1H, *J* = 3.6 Hz, Th-H), 7.49 (d, 1H, *J* = 4.0 Hz, Th-H), 7.18 (dd, 1H, *J* = 5.0 Hz, *J* = 3.7 Hz, Th-H), 3.53 (t, 4H, *J* = 5.5 Hz, C*H*$_2$CH$_2$C*H*$_2$), 2.11-1.91 (m, 2H, CH$_2$C*H*$_2$CH$_2$); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 163.6, 158.5, 155.6, 141.6, 135.0, 134.3, 133.5, 131.6, 128.4, 127.1, 125.9, 125.7, 125.0, 124.9, 122.3, 122.1, 38.6 (2C), 17.55. LC-MS (ESI) m/z: 382.2 [(M-Cl$^-$)$^+$]; Analysis calcd for $C_{19}H_{16}ClN_3S_3$ (418.00): C, 54.59; H, 3.86; N, 10.05 %; Found: C, 54.59; H, 3.86; N, 10.05 %

*2-(2,2'-Bithiophene-5-yl)-6-N-isopropilamidiniumbenzothiazole chloride* **7d**

According to **Method B,** 2,2'-bithiophene-5-carbaldehyde (**2**) (97 mg) and 3,3'-disulfanediylbis[4-aminobenzo(*N*-isopropylamidinium chloride)] dihydrate (**3d**) (132 mg).

Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of concentred hydrochloric acid (0.100 ml) and stirring at room temperature for 2 h. After cooling overnight, the resulting precipitate was filtered, crystallized from ethanol/diethyl-ether mixture, and dried at 75 °C. Yield of pure compound **7d** as orange solid was 86 mg (41 %); m.p. = 255-258 (dec.) °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ = 9.69 (d, 1H, *J* = 7.5 Hz, -C(N*H*-)N$H_2^+$), 9.51 (bs, 1H, -C(N*H*-)N$H_2^+$), 9.14 (bs, 1H, -C(N*H*-)N$H_2^+$), 8.54 (s, 1H, Ar-H), 8.15 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.95 (d, 1H, *J* = 3.7 Hz, Th-H), 7.81 (d, 1H, *J* = 8.7 Hz, Ar-H), 7.66 (d, 1H, *J* = 4.6 Hz, Th-H), 7.55 (d, 1H *J* = 2.5 Hz, Th-H), 7.47 (d, 1H, *J* = 3.5 Hz, Th-H), 7.24-7.10 (m, 1H, Th-H), 4.16-3.96 (m, 1H, -C*H*$(CH_3)_2$), 1.29 (d, 6H, *J* = 6.1 Hz, -CH(C$H_3$)$_2$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ = 163.9, 161.4, 155.7, 141.8, 135.2, 134.3, 133.7, 132.1, 128.7, 127.4, 126.9, 126.0, 125.9, 125.3, 123.4, 122.1, 45.20, 21.24 (2C); LC-MS (ESI) m/z: 384.2 [(M-Cl$^-$)$^+$]; Analysis calcd for $C_{19}H_{18}ClN_3S_3$ (420.01): C, 54.33; H, 4.32; N, 10.00 %; Found: C, 54.41; H, 4.33; N, 9.88 %

### 4.1.3. General method for preparation of compounds 6a–6b (Method C):

Solution of equimolar amounts of aldehydes **1** and **2**, corresponding amidines **4a**–**4d** and *p*-benzoquinone in absolute ethanol was refluxed for 4 hours. After reaction mixture was cooled to the room temperature, the crude product was filtered off and washed with diethyl ether. The crude product was suspended in mixture of ethanol/diethyl ether several times until the powder was analytically pure.

*5(6)-amidino-2-(benzo[b]thiophen-2-yl)benzimidazole hydrochloride* **6a**

Compound **6a** was prepared from benzo[*b*]thiophene-2-carboxaldehyde **1** (0.20 g, 1.23 mmol), 4-amidino-1,2-phenylenediamine hydrochloride **4a** (0.23 g, 1.23 mmol), and *p*-benzoquinone (0.13 g, 1.23 mmol) in absolute ethanol (10 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.24 g (67%) of light blue powder; m.p. >300 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) (δ/ppm): 14.07 (s, 1H, $NH_{benzimidazole}$), 9.37 (s, 2H, $NH_{amidine}$), 9.08 (s, 2H, $NH_{amidine}$), 8.38 (s, 1H, $H_{arom.}$), 8.20 (bs, 1H, $H_{arom.}$), 8.12 – 8.05 (m, 1H, Ar-H), 8.04 – 7.95 (m, 1H, Ar-H), 7.80 (bs, 1H, Ar-H), 7.72 (s, 1H, Ar-H), 7.53 – 7.42 (m, 2H, Ar-H); $^{13}$C NMR (151 MHz, DMSO-$d_6$) (δ/ppm): 166.0, 139.9, 139.4, 132.8, 126.1, 125.1, 124.7, 122.7; LC/MS (ESI) *m/z*: 293.2 [(M-Cl$^-$)$^+$]; Analysis calcd for $C_{16}H_{13}N_4SCl$: C, 58.44; H, 3.98; N, 17.04 %; Found: C, 58.70; H, 4.21; N, 17.33.

*5(6)-(2-Imidazolinyl)-2-(benzo[b]thiophen-2-yl)benzimidazole hydrochloride* **6b**

Compound **6b** was prepared from benzo[*b*]thiophene-2-carboxaldehyde **1** (0.15 g, 0.93 mmol), 4-(2-imidazolinyl)-1,2-phenylenediamine hydrochloride **4b** (0.20 g, 0.93 mmol), and *p*-benzoquinone (0.10 g, 0.93 mmol) in absolute ethanol (10 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.12 g (35%) of grey powder; m.p. >300 °C; $^{1}$H NMR (300 MHz, DMSO-$d_6$) (δ/ppm): 10.73 (s, 2H, $NH_{amidine}$), 8.42 (s, 2H, Ar-H), 8.12 – 8.06 (m, 1H, Ar-H), 8.02 – 7.99 (m, 1H, Ar-H), 7.91 (dd, $J_1$ = 8.6 Hz, $J_2$ = 1.5 Hz, 1H, Ar-H), 7.83 (d, $J$ = 8.5 Hz, 1H, Ar-H), 7.52 – 7.44 (m, 2H, Ar-H), 4.03 (s, 4H, $CH_2$); $^{13}$C NMR (75 MHz, DMSO-$d_6$) (δ/ppm): 165.7, 140.5, 139.9, 133.1, 126.6, 125.7, 125.5, 125.3, 123.2, 116.2, 44.7; LC/MS (ESI) *m/z*: 319.2 [$(M\text{-}Cl^-)^+$]; Analysis calcd for $C_{18}H_{15}N_4SCl$: C, 60.92; H, 4.26; N, 15.79 %; Found: C, 60.66; H, 4.48; N, 15.45.

*2-[2-(benzo[b]thiophen-2-yl)]-5(6)-(1,4,5,6-tetrahydropyrimidin-2-yl)benzimidazole hydrochloride* **6c**

Compound **6c** was prepared from benzo[*b*]thiophene-2-carboxaldehyde **1** (0.05 g, 0.33 mmol), 4-(1,4,5,6-tetrahydropyrimidin-2-yl)-1,2-phenylenediamine hydrochloride **4c** (0.08 g, 0.33 mmol), and *p*-benzoquinone (0.04 g, 0.33 mmol) in absolute ethanol (1.5 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.05 g (41%) of beige powder; m.p. >300 °C; $^{1}$H NMR (600 MHz, DMSO-$d_6$) (δ/ppm): 10.16 (s, 2H, $NH_{amidine}$), 8.23 (s, 2H, Ar-H), 8.00 (s, 1H, Ar-H), 8.00 (d, $J$ = 6.6 Hz, 1H, Ar-H), 7.91 (d, $J$ = 6.8 Hz, 1H, Ar-H), 7.65 (d, $J$ = 8.3 Hz, 1H, Ar-H), 7.47 (d, $J$ = 8.1 Hz, 1H, Ar-H), 7.43 – 7.36 (m, 2H, Ar-H), 3.51 (t, $J$ = 5.3 Hz, 4H, $CH_2$), 1.99 (s, 2H, $CH_2$); $^{13}$C NMR (75 MHz, DMF) (δ/ppm): 160.7, 140.8, 140.6, 126.0, 125.6, 125.0, 123.4, 120.5, 116.3, 39.8, 19.1; LC/MS (ESI) *m/z*: 333.2 [$(M\text{-}Cl^-)^+$]; Analysis calcd for $C_{19}H_{17}ClN_4S$: C, 61.86; H, 4.65; Cl, 9.61; N, 15.19; S, 8.69 %; Found: C, 61.88; H, 4.62; Cl, 9.59; N, 15.21; S, 8.70.

*2-[2-(benzo[b]thiophen-2-yl)]-5(6)-(N-isopropylamidino)benzimidazole hydrochloride* **6d**

Compound **6d** was prepared from benzo[*b*]thiophene-2-carboxaldehyde **1** (0.10 g, 0.62 mmol), 4-(*N*-isopropylamidino)-1,2-phenylenediamine hydrochloride **4d** (0.14 g, 0.62 mmol), and *p*-benzoquinone (0.07 g, 0.62 mmol) in absolute ethanol (3 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.14 g (61%) of green powder; m.p. >300 °C; $^{1}$H NMR (400 MHz, DMSO-$d_6$) (δ/ppm): 14.30 (s, 1H, $NH_{benzimidazole}$), 14.18 (s, 1H, $NH_{benzimidazole}$), 9.55 (s, 2H, $NH_{amidine}$), 9.44 (s, 2H, $NH_{amidine}$), 9.06 (s, 2H, $NH_{amidine}$), 8.42 (s, 2H, Ar-H), 8.08 – 8.01 (m, 6H, Ar-H), 7.79 (bs, 2H, Ar-H), 7.59 – 7.47 (m, 6H, Ar-H), 4.12 (s, 2H, CH), 1.32 (s, 12H, $CH_3$); $^{13}$C NMR (100 MHz, DMSO-$d_6$) (δ/ppm): 162.8, 140.4, 140.0, 133.4, 126.6, 125.7, 125.2, 123.2, 45.5, 21.8; LC/MS (ESI) *m/z*: 335.2 [$(M\text{-}Cl^-)^+$]; Analysis

calcd for $C_{19}H_{17}ClN_4S$: C, 61.69; H, 4.90; Cl, 9.58; N, 15.15; S, 8.67 %; Found: C, 61.67; H, 4.92; Cl, 9.55; N, 15.18; S, 8.68.

*5(6)-amidino-2-[(2,2'-bitiophene)-5-yl]benzimidazole hydrochloride* **8a**

Compound **8a** was prepared from 2,2'-bitiophene-5-carboxaldehyde **2** (0.10 g, 0.51 mmol), 4-amidino-1,2-phenylenediamine hydrochloride **4a** (0.09 g, 0.51 mmol), and *p*-benzoquinone (0.06 g, 0.51 mmol) in absolute ethanol (2.5 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.10 g (56%) of green powder; m.p. >300 °C; $^1$H NMR (400 MHz, DMSO-$d_6$) (δ/ppm): 13.97 (bs, 1H, $NH_{benzimidazole}$), 13.89 (bs, 1H, $NH_{benzimidazole}$), 9.36 (s, 4H, $NH_{amidine}$), 9.07 (s, 4,H, $NH_{amidine}$), 8.16 (s, 2H, Ar-H), 8.01 (s, 2H, Ar-H), 7.83 – 7.63 (m, 4H, Ar-H), 7.63 (s, 1H, Ar-H), 7.62 (s, 1H, Ar-H), 7.48 – 7.46 (m, 4H, Ar-H), 7.17 – 7.15 (m, 2H, Ar-H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) (δ/ppm): 166.5, 140.2, 136.1, 131.5, 129.8, 129.1, 127.2, 125.8, 125.5, 121.8; LC/MS (ESI) *m/z*: 325.2 [$(M\text{-}Cl^-)^+$]; Analysis calcd for $C_{19}H_{17}ClN_4S$: C, 52.95; H, 4.17; Cl, 9.77; N, 15.44; S, 17.67 %; Found: C, 52.97; H, 4.15; Cl, 9.78; N, 15.46; N, 17.66.

*5(6)-(2-imidazolinyl)-2-[(2,2'-bitiophen)-5-yl]benzimidazole hydrochloride* **8b**

Compound **8b** was prepared from 2,2'-bitiophene-5-carboxaldehyde **2** (0.10 g, 0.51 mmol), 4-(2-imidazolinyl)-1,2-phenylenediamine hydrochloride **4b** (0.11 g, 0.51 mmol), and *p*-benzoquinone (0.06 g, 0.51 mmol) in absolute ethanol (2.5 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.06 g (27%) of green powder; m.p. >300 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) (δ/ppm): 10.47 (s, 2H, $NH_{amidine}$), 8.24 (s, 1H, Ar-H), 7.95 (s, 1H, Ar-H), 7.81 (s, 2H, Ar-H), 7.64 (bs, 1H, Ar-H), 7.49 (bs, 2H, Ar-H), 7.17 (bs, 1H, Ar-H), 4.04 (s, 4H, $CH_2$); $^{13}$C NMR (100 MHz, DMSO-$d_6$) (δ/ppm): 165.9, 150.2, 140.7, 136.0, 130.8, 130.1, 129.2, 127.4, 125.9, 125.6, 123.1, 116.1, 44.8 (2C). LC/MS (ESI) *m/z*: 351.1 [$(M\text{-}Cl^-)^+$]; Analysis calcd for $C_{19}H_{17}ClN_4S$: C, 62.26; H, 4.14; Cl, 7.66; N, 12.10; S, 13.85 %; Found: C, 62.23; H, 4.15; Cl, 7.64; N, 12.09; S, 13.87.

*5(6)-(1,4,5,6-tetrahydropyrimidin-2-yl)-2-[(2,2'-bitiophene)-5-yl]benzimidazole hydrochloride* **8c**

Compound **8c** was prepared from 2,2'-bitiophene-5-carboxaldehyde **2** (0.09 g, 0.44 mmol), 4-(1,4,5,6-tetrahydropyrimidin-2-yl)-1,2-phenylenediamine hydrochloride **4c** (0.10 g, 0.44 mmol), and *p*-benzoquinone (0.05 g, 0.44 mmol) in absolute ethanol (2 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.09 g (52%) of green powder; m.p. >300 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) (δ/ppm): 14.12 (s, 1H, $NH_{benzimidazole}$), 13.96 (s,

1H, $NH_{benzimidazole}$), 10.01 (s, 4H, $NH_{amidine}$), 8.05 (bs, 4H, Ar-H), 7.78 (s, 1H, Ar-H), 7.71 (s, 1H, Ar-H), 7.62 (bs, 4H, Ar-H), 7.47 (bs, 4H, Ar-H), 7.16 (s, 2H, Ar-H), 3.35 (s, 8H, $CH_2$), 2.01 (s, 4H, $CH_2$); $^{13}$C NMR (100 MHz, DMSO-d6) (δ/ppm): 160.1, 160.0, 150.2, 149.5, 147.4, 143.7, 140.2, 140.0, 138.7, 136.2, 135.1, 131.6, 130.0, 129.7, 129.1, 127.2, 125.8, 125.5, 122.7, 122.4, 121.7, 119.1, 118.8, 112.2, 111.8, 18.4; LC/MS (ESI) *m/z*: 365.2 [$(M\text{-}Cl^-)^+$]; Analysis calcd for $C_{19}H_{17}ClN_4S$: C, 56.92; H, 4.27; Cl, 8.84; N, 13.97; S, 15.99 %; Found: C, 56.94; H, 4.25; Cl, 8.87; N, 13.94; S, 16.00.

*5(6)-(N-isopropylamidino)-2-[(2,2'-bitiophene)-5-yl]benzimidazole hydrchloride* **8d**

Compound **23** was prepared from 2,2'-bitiophene-5-carboxaldehyde **2** (0.10 g, 0.51 mmol), 4-(*N*-isopropylamidino)-1,2-phenylenediamine hydrochloride **4d** (0.12 g, 0.51 mmol), and *p*-benzoquinone (0.05 g, 0.51 mmol) in absolute ethanol (2.5 ml) after refluxing for 4 hours. The mixture was worked up as it is described to obtain 0.13 g (62%) of light green powder; m.p. >300 °C; $^1$H NMR (300 MHz, DMSO-$d_6$) (δ/ppm): 14.12 (s, 1H, $NH_{benzimidazole}$), 13.99 (s, 1H, $NH_{benzimidazole}$), 9.57 – 9.40 (m, 4H, $NH_{amidine}$), 9.04 (s, 2H, $NH_{amidine}$), 8.09 (s, 1H, Ar-H), 8,04 (bs, 2H, Ar-H), 7.91 (s, 1H, Ar-H), 7.84 – 7.69 (m, 2H, Ar-H), 7.63 – 7.53 (m, 4H, Ar-H), 7.47 (d, 4H, $J$ = 7.0 Hz, Ar-H), 7.16 (s, 2H, Ar-H), 4.11 (d, 2H, $J$ = 4.8 Hz, CH), 1.31 (d, 12H, $J$ = 4.8 Hz, $CH_3$); $^{13}$C NMR (100 MHz, DMSO-$d_6$) (δ/ppm): 162.9, 162.8, 150.2, 149.5, 147.34, 143.5, 140.3, 140.0, 138.7, 136.1, 134.9, 131.6, 131.6, 130.0, 129.7, 129.1, 127.1, 125.7, 125.5, 123.4, 123.1, 123.1, 122.5, 119.5, 118.9, 112.4, 111.9, 45.5, 21.8; LC/MS (ESI) *m/z*: 367.1 [$(M\text{-}Cl^-)^+$]; Analysis calcd for $C_{19}H_{17}ClN_4S$: C, 56.77; H, 4.51; Cl, 8.82; N, 13.94; S, 15.95 %; Found: C, 56.79; H, 4.49; Cl, 8.84; N, 13.92; S, 15.96.

### 4.2. Biology

#### 4.2.1. Antiproliferative activity *in vitro*

Experiments were carried out on 6 human cell lines derived from 6 cancer types according to the previously published experimental procedure. The following cell lines were used: HCT116 and SW620 (colon carcinoma), H460 (lung carcinoma), MCF-7 (breast carcinoma), PC3 (prostate carcinoma) and HeLa (cervical carcinoma), along with HEK 293 (human embryonic kidney) cells. Cells were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM) – HCT116, SW620, H 460, MCF-7, HeLa and HEK 293 cells, or RPMI-1640 medium - PC3 cells, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37˚C. The tested compounds were added in ten-fold dilutions

($10^{-8}$–$10^{-4}$ M) on the next day and incubated for further 72 h. The working dilutions were freshly prepared on the day of testing. After 72 h of incubation the cell growth rate was evaluated using MTT assay. The results obtained are expressed as IC50 value which is the concentration of compound required for 50% growth inhibition. Each test was performed in quadruplicate in at least two individual experiments.

### 4.2.2. Fluorescence and confocal microscopy

**Fluorescence microscopy**

H460 cells were seeded on round microscopic cover slips placed in 24-well-plates (50 000 cells per well) and grown for 24 h in DMEM. Cells were then incubated with the compound (5 or 10 μM) for 20 min. The coverslips were rinsed twice with PBS, placed on the microscopic slides and analyzed immediately. The uptake and intracellular distribution of tested compounds were analyzed and with Olympus BX51 Fluorescence Microscope using DAPI filter ($\lambda_{ex}/\lambda_{em}$: 365/465nm) at 40× and 100× magnification.

**Confocal microscopy**

H460 cells were seeded on round microscopic coverslips placed in 24-well-plates (50 000 cells per well) and grown at 37°C for 24 hours. Cells were then incubated with compound (1, 5 or 10 μM) for 1, 2 or 3 hours. Dishes were rinsed twice with PBS, paraformaldehyde (4%PBS) was added and cells were incubated for 10 minutes. The coverslips were rinsed twice with PBS, placed on the microscopic slides and stored at −20°C. The uptake and intracellular distribution of the tested compounds were analyzed under the Leica TCS SP8 X confocal microscope (Leica Microsystems, Germany).

### 4.2.3 Cell cycle analysis

H460 cells were seeded into six-well plates ($2 \times 10^5$ cells per well). After 24 hours, the tested compounds **7a** (0.02 μM and 0.05 μM) and **8a** (5 μM and 10 μM) were added. After 24 and 48 hours the attached cells were trypsinized, combined with floating cells, washed with PBS, fixed with 70% ethanol, and stored overnight at −20°C. Immediately before analysis, the cells were washed with PBS and stained with 50 μg/mL propidiumiodide with the addition of 0.1 μg/μL RNAse A. The stained cells were then analyzed using BD FACScalibur flow cytometer. The percentage of cells in each cell cycle phase was determined using FlowJo software (TreeStar Inc., USA).

### 4.2.4 DNA binding study

**Spectroscopic characterization**

The UV/vis spectra were recorded on a Varian Cary 100 Bio spectrophotometer (Agilent, Santa Clara, CA, USA), CD spectra on JASCO J815 spectrophotometer (ABL&E Handels GmbH, Wien, Austria) and fluorescence spectra on a Varian Cary Eclipse spectrophotometer (Agilent, Santa Clara, CA, USA) at 25ºC using appropriate 1cm path quartz cuvettes.

**Fluorimetric and CD titration**

Calf thymus DNA, ctDNA was purchased in Sigma-Aldrich (Sigma-Aldrich, St. Louis, MI, USA) and dissolved in Na-cacodylate buffer, $I$=0.05 mol $dm^{-3}$, pH=7. The ctDNA was additionally sonicated and filtered through a 0.45 mm filter.[42] DNA concentration was determined spectroscopically as the concentration of phosphates.[43] Spectrophotometric titrations were performed at pH=7 ($I$=0.05 mol $dm^{-3}$, sodium cacodylate buffer) by adding portions of the polynucleotide solution to the solution of the studied compound for fluorimetric experiments and CD experiments were done by adding portions of the compound stock solution into the solution of a polynucleotide. In the fluorimetric experiments, an excitation wavelength of $\lambda_{exc}$= >300 nm was used to avoid the inner filter effect caused due to increasing absorbance of the polynucleotide. Emission was detected in the range $\lambda_{em}$=350–600 nm. The values for $K_a$ obtained by processing the titration data using the Scatchard equation (Table 1), all have satisfactory correlation coefficients (>0.99). Thermal melting curves for DNA and its complexes with the studied compounds were determined as previously described by tracking the absorbance change at 260 nm as a function of temperature as previously described. The absorbance of the ligands was subtracted from each curve and the absorbance scale normalized. The $T_m$ values are the midpoints of the transition curves, which were determined from the maximum of the first derivative and checked graphically using the tangent method. $\Delta T_m$ values were calculated subtracting $T_m$ of the free nucleic acid from $T_m$ of the complex. Each *ΔTm* value reported here as the average of at least two measurements. The $\Delta T_m$ error is ±0.5ºC.

**4.2.5. Antitrypanosomal activity *in vitro***

**Antitrypanosomal screening**

Bloodstream form T. b. brucei (strain 221) were cultured in modified Iscove's medium and assays were carried out in 96-well microtiter plates (200 ll volumes) to determine the $IC_{50}$ and $IC_{90}$ values of each compound. Parasites growth was initiated at 2.5×104 $ml^{-1}$, compounds were added at a range of concentrations, and the plates were incubated at 37 °C. Resazurin (20

μl at 0.125mg $ml^{-1}$) was added after 48 h, the plates were incubated for a further 16 h, and then read in a Spectramax plate reader, and data analysed using GraphPad Prism. Each drug was tested in triplicate.

**L6 cell proliferation**

For cytotoxicity assays, L6 cells (a rat myoblast line) were seeded into 96-well microtiter plates at 1×104 $ml^{-1}$ in 200 μl of the growth medium, and different compound concentrations were added. The plates were then incubated for 6 days at 37 °C and 20 μl resazurin was added to each well. After a further 8 h incubation, the fluorescence was determined using a Spectramax plate reader, as outlined above.

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**Conflict of interests**

The authors declare no conflict of interest.

**References**

1. Gauter, J. A.; Miocque, M.; Farnoux, C. C. In *The Chemistry of Amidines and Imidates, Vol. 1*; Patai, S., Ed.; John Wiley & Sons; New York, 1975, pp. 283-348.

2. Ashraf A. Aly, Stefan Bräse, Mohsen A. M. Gomaa, Amidines: their synthesis, reactivity, and applications in heterocycle synthesis, ARKIVOC part IV (2018) 85-138

3. R. B. Silverman, *The Organic Chemistry of Drug Design and Drug Action,* Elsevier Academic Press, Amsterdam, **2004**.

4. John V. Greenhill, Ping Lue, Amidines and guanidines in medicinal chemistry, *Progress in medicinal chemistry* 30 (1993) 206-306.

5. Patel, A.; Smith, H.J.; Sturzebecher, J. Introduction to the Principles of Drug Design and Action; Smith, H.J., Ed.; CRC Taylor & Francis: Boca Raton, FL, USA, 2006.

6. Racané, L.; Stojković, R.; Tralić-Kulenović, V.; Cerić, H.; Đaković, M.; Ester, K.; Mišir Krpan, A.; Radić Stojković, M. Interactions with polynucleotides and antitumor activity of amidino and imidazolinyl substituted 2-phenylbenzothiazole mesylates. Eur. J. Med. Chem. 2014, 86, 406–419.

7. Brajša, Karmen; Vujasinović, Ines; Jelić, Dubravko; Trzun, Marija; Zlatar, Ivo; Karminski-Zamola, Grace; Hranjec, Marijana. Antitumor activity of amidino-substituted benzimidazole and benzimidazo[1,2-*a*]quinoline derivatives tested in 2D and 3D cell culture systems. *Journal of enzyme inhibition and medicinal chemistry* **31** (2016) 1139-1145. (IF 4,293; Q1) doi:10.3109/14756366.2015.1101093

8. M. Bhat, S. L. Belagali, Guanidinyl benzothiazole derivatives: Synthesis and structure activity relationship studies of a novel series of potential antimicrobial and antioxidants, Res. Chem. Intermediat 42 (2016) 6195-6208

9. K. Starčević**,** M. Kralj, K. Ester, I. Sabol, M. Grce, K. Pavelić, G. Karminski-Zamola, Synthesis, antiviral and antitumor activity of 2-substituted-5-amidino-benzimidazoles. Bioorg. Med. Chem. 15 (2007) 4419-4426. DOI: 10.1016/j.bmc.2007.04.032

10 Livio Racané, Valentina Rep, Sandra Kraljević Pavelić, Petra Grbčić, Iva Zonjić, Marijana Radić Stojković, Martin C. Taylor, John M. Kelly, Silvana Raić-Malić, Synthesis, antiproliferative and antitrypanosomal activities, and DNA binding of novel 6-amidino-2-arylbenzothiazoles, Journal of Enzyme Inhibition and Medicinal Chemistry, 36:1 (2021) 1952-1967, DOI: 10.1080/14756366.2021.1959572

11. R. K. Arafa, M. A. Ismail, T. Wenzler, R. Brun, A. Paul, W. D. Wilson, A. A. Alakhdar, D. W. Boykin, New antiparasitic flexible triaryl diamidines, their prodrugs and aza analogues: Synthesis, *in vitro* and *in vivo* biological evaluation, and molecular modelling studies, *Eur. J. Med. Chem.* **222** (2021) 113625; doi: 10.1016/j.ejmech.2021.113625

12. Ismail, M.A.; Brun, R.; Easterbrook, J.D.; Tanious, F.A.; Wilson, W.D.; Boykin, D.W. Synthesis and Antiprotozoal Activity of Aza-Analogues of Furamidine. J. Med. Chem. **2003**, 46, 4761–4769.

13. L. Racané, M. Cindrić, N. Perin, P. Roškarić, K.Starčević, T. Mašek, M. Maurić, J. Dogan, G. Karminski-Zamola, Synthesis and Antioxidative Potency of Novel Amidino Substituted Benzimidazole and Benzothiazole Derivatives, Croat. Chem. Acta 90 (2017) 187–195
14. L. Yet, A Survey of Amidine Synthesis, *Albany Molecular Research, Inc. Technical Reports* Volume 4, 03 1-12.
15. Dunn, P. J. In *Comprehensive Organic Functional Group Transformations*; Katritzky, A. R.; Meth- Cohn, O.; Rees, C. W.; Eds.; Pergamon: New York, 1995; Vol. 5, Chapter 5.19.
16. Rescifina, A.; Zagni, C.; Varrica, M.G.; Pistarà, V.; Corsaro, A. Recent advances in small organic molecules as DNA intercalating agents: Synthesis, activity, and modeling. Eur. J. Med. Chem. **2014**, 74, 95–115.
17. Inkyu Lee, Sojin Kim, Seh-na Kim, Yoonsun Jang, Jyongsik Jang, Highly Fluorescent Amidine/Schiff Base Dual-Modified Polyacrylonitrile Nanoparticles for Selective and Sensitive Detection of Copper Ions in Living Cells, ACS Appl. Mater. Interfaces 2014, 6, 17151−17156; doi: dx.doi.org/10.1021/am504824n
18. Tong Tian, Xi Chen, Hong Li, Yao Wang, Lin Guo, Lei Jiang, Amidine-based fluorescent chemosensor with high applicability for detection of CO2: A facile way to “see” CO2, Analyst, 2013,138, 991-994; doi: 10.1039/C2AN36401H
19. Lončar, B.; Perin, N.; Mioč, M.; Boček, I.; Grgić, L.; Kralj, M.; Tomić, S.; Radić Stojković, M.; Hranjec, M. Novel amino substituted tetracyclic imidazo[4,5-b]pyridine derivatives: Design, synthesis, antiproliferative activity and DNA/RNA binding study. Eur. J. Med. Chem. **2021**, 217, 113342
20. L. Racané, S. Kraljević Pavelić, R. Nhili, S. Depauw, C. Paul-Constant, I. Ratkaj, M. H. David-Cordonnier, K. Pavelić, V. Tralić-Kulenović,G. Karminski-Zamola, New anticancer active and selective phenylene-bisbenzothiazoles: Synthesis, antiproliferative evaluation and DNA binding. Eur. J. Med. Chem. 63 (2013) 882-891. DOI: 10.1016/j.ejmech.2013.02.026
21. Cindrić, M.; Jambon, S.; Harej, A.; Depauw, S.; David-Cordonnier, M.H.; Kraljević Pavelić, S.; Karminski-Zamola, G.; Hranjec, M. Novel amidino substituted benzimidazole and benzothiazole benzo[b]thieno-2-carboxamides exert strong antiproliferative and DNA binding properties. Eur. J. Med. Chem. **2017**, 136, 468–479
22. Hranjec, M.; Kralj, M.; Piantanida, I.; Sedić, M.; Šuman, L.; Pavelić, K.; Karminski-Zamola, G. Novel Cyano- and Amidino- Substituted Derivatives of Styryl-2-Benzimidazoles and Benzimidazo[1,2-*a*]quinolines. Synthesis, Photochemical Synthesis, DNA Binding, and Antitumor Evaluation, Part 3. J. Med. Chem. 2007, 50, 5696–5711.

23. Livio Racané, Maja Cindrić, Nataša Perin, Petra Roškarić, Kristina Starčević, Tomislav Mašek, Maja Maurić, Jasna Dogan, Grace Karminski-Zamola, Synthesis and Antioxidative Potency of Novel Amidino Substituted Benzimidazole and Benzothiazole Derivatives, *Croat. Chem. Acta* 2017, *90*(*2*), 187–195; DOI: 10.5562/cca3146

24. Sović, Irena; Cindrić, Maja; Perin, Nataša; Boček, Ida; Novaković, Irena; Damjanović, Ana; Stanojković, Tatjana; Zlatović, Mario; Hranjec, Marijana; Bertoša, Branimir. Biological potential of novel methoxy and hydroxy substituted heteroaromatic amides designed as promising antioxidative agents: Synthesis, 3D-QSAR analysis and biological activity. *Chemical research in toxicology* **32** (2019) 1880-1892. (IF 3,1894; Q2) doi:10.1021/acs.chemrestox.9b00256

25. Racané, L.; Tralić-Kulenović, V.; Kraljević Pavelić, S.; Ratkaj, I.; Peixoto, P.; Nhili, R.; Depauw, S.; Hildebrand, M.P.; David- Cordonnier, M.H.; Pavelić, K.; et al. Novel Diamidino-Substituted Derivatives of Phenyl Benzothiazolyl and Dibenzothiazolyl Furans and Thiophenes: Synthesis, Antiproliferative and DNA Binding Properties. J. Med. Chem. 2010, 53, 2418–2432.

26. L. Racané, S. Kraljević Pavelić, I. Ratkaj, V. Stepanić, K. Pavelić, V. Tralić-Kulenović, G. Karminski-Zamola, Synthesis and antiproliferative evaluation of some new amidino-substituted bis-benzothiazolyl-pyridines and pyrazine. Eur. J. Med. Chem. 55 (2012) 108-116. DOI: doi.org/10.1016/j.ejmech.2012.07.005

27. Racané, Livio, Lucija Ptiček, Mirela Sedić, Petra Grbčić, Sandra Kraljević Pavelić, Branimir Bertoša, Irena Sović, and Grace Karminski-Zamola. 2018. "Eco-Friendly Synthesis, in Vitro Anti-Proliferative Evaluation, and 3D-QSAR Analysis of a Novel Series of Monocationic 2-Aryl/Heteroaryl-Substituted 6-(2-Imidazolinyl)Benzothiazole Mesylates. Molecular Diversity 22 (3): 723–41. https://doi.org/10.1007/s11030-018-9827-2.

28. Racané, Livio, Mirela Sedić, Nataša Ilić, Maja Aleksić, Sandra Kraljević Pavelić, and Grace Karminski-Zamola. 2017, Novel 2-Thienyl- and 2-Benzothienyl-Substituted 6-(2-Imidazolinyl)Benzothiazoles: Synthesis; in Vitro Evaluation of Antitumor Effects and Assessment of Mitochondrial Toxicity, Anti-Cancer Agents in Medicinal Chemistry 17 (1): 57–66. https://doi.org/10.2174/1871520615666160504094753.

29. Racané, Livio, Lucija Ptiček, Glorija Fajdetić, Vesna Tralić-Kulenović, Marko Klobučar, Sandra Kraljević Pavelić, Mihaela Perić, Hana Čipčić Paljetak, Donatella Verbanac, and Kristina Starčević. 2020, Green Synthesis and Biological Evaluation of 6-Substituted-2-(2-Hydroxy/Methoxy Phenyl)Benzothiazole Derivatives as Potential Antioxidant, Antibacterial

and Antitumor Agents, Bioorganic Chemistry 95 (January): 103537. https://doi.org/10.1016/j.bioorg.2019.103537.

30. L. Racanè, V. Tralić-Kulenović, Z. Mihalić, G. Pavlović, G. Karminski-Zamola, *Tetrahedron* 2008, 64, 11594-.

31. Racané, Livio; Cindrić, Maja; Zlatar, Ivo; Kezele, Tatjana; Milić, Astrid; Brajša, Karmen; Hranjec, Marijana. Preclinical in vitro screening of newly synthesised amidino substituted benzimidazoles and benzothiazoles. *Journal of enzyme inhibition and medicinal chemistry* **36** (2021) 163-174. doi:10.1080/14756366.2020.1850711

32. Perin, Nataša; Martin-Kleiner, Irena; Nhili, Raja; Laine, William; David-Cordonnier, Marie-Hélène; Vugrek, Oliver; Karminski-Zamola, Grace; Kralj, Marijeta; Hranjec, Marijana. Biological activity and DNA binding studies of 2-substituted benzimidazo[1,2-*a*]quinolines bearing different amino side chains. *MedChemComm* **4** (2013) 1537-1550. (IF 2,722; Q2)

33. W. Saenger, Principles of nucleic acid structure, Springer-Verlag New York, New York, 1984.

34. J. L. Mergny, L. Lacroix, Analysis of thermal melting curves, Oligonucleotides 13 (6) (2003) 515–537.

35. G. Scatchard, The attractions of proteins for small molecules and ions, Ann. N. Y. Acad. Sci. 51 (4) (1949) 660–672.

36. McGhee JD, von Hippel PH. Theoretical aspects of DNA-protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice. J Mol Biol. 1974;86(2):469-89.

37. N. Berova, K. Nakanishi, R.W. Woody, Circular dichroism Principles and Applications, 2nd Edition, Wiley-VCH, New York, 2000.

38. T. Smidlehner, I. Piantanida, G. Pescitelli, Polarization spectroscopy methods in the determination of interactions of small molecules with nucleic acids - Tutorial, Beilstein J. Org. Chem. 14 (2017) 84-105.

39. M. Eriksson, B. Norden, Linear and circular dichroism of drug-nucleic acid complexes, Method Enzymol. 340 (2001) 68–98.

40. Popov AB, Krstulović L, Koštrun S, Jelić D, Bokulić A, Stojković MR, Zonjić I, Taylor MC, Kelly JM, Bajić M, Raić-Malić S. Design, synthesis, antitrypanosomal activity, DNA/RNA binding and in vitro ADME profiling of novel imidazoline-substituted 2-arylbenzimidazoles. Eur J Med Chem. 2020 Dec 1;207:112802.

41. Wilkinson SR, Taylor MC, Horn D, et al. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. Proc Natl Acad Sci USA 2008;105:5022–7.

42. J. B. Chaires, N. Dattagupta, D.M. Crothers, Studies on Interaction of Anthracycline Antibiotics and Deoxyribonucleic-Acid - Equilibrium Binding-Studies on Interaction of Daunomycin with Deoxyribonucleic-Acid, Biochemistry-Us 21 (17) (1982) 3933–3940.
43. T.V. Chalikian, J. Volker, G.E. Plum, K.J. Breslauer, A more unified picture for the thermodynamics of nucleic acid duplex melting: A characterization by calorimetric and volumetric techniques, P Natl. Acad. Sci. USA 96 (14) (1999) 7853–7858