Synthesis, antiproliferative and antitrypanosomal activities, and DNA binding of novel 6-amidino-2-arylbenzothiazoles

Livio Racané, Valentina Repb, Sandra Kraljević Pavelićc, Petra Grbčićd, Iva Zonićd, Marijana Radić Stojkovicd, Martin C. Taylore, John M. Kellyd and Silvana Raić-Malicd

aFaculty of Textile Technology, Department of Applied Chemistry, University of Zagreb, Zagreb, Croatia; bFaculty of Chemical Engineering and Technology, Department of Organic Chemistry, University of Zagreb, Zagreb, Croatia; cFaculty of Health Studies, University of Rijeka, Rijeka, Croatia; dDivision of Organic Chemistry and Biochemistry, Rudjer Bošković Institute, Zagreb, Croatia; eDepartment of Infection Biology, London School of Hygiene and Tropical Medicine, London, UK

ABSTRACT
A series of 6-amidinobenzothiazoles, linked via phenoxyimethylen or directly to the 1,2,3-triazole ring with a p-substituted phenyl or benzyl moiety, were synthesised and evaluated in vitro against four human tumour cell lines and the protozoan parasite Trypanosoma brucei. The influence of the type of amidino substituent and phenoxyimethylene linker on antiproliferative and antitrypanosomal activities was observed, showing that the imidazine moiety had a major impact on both activities. Benzothiazole imidazoline 14a, which was directly connected to N-1-phenyl-1,2,3-triazole, had the most potent growth-inhibitory effect (IC50 = 0.25 μM) on colorectal adenocarcinoma (SW620), while benzothiazole imidazoline 11b, containing a phenoxyimethylene linker, exhibited the best antitrypanosomal potency (IC50 = 0.12 μM). DNA binding assays showed a non-covalent interaction of 6-amidinobenzothiazole ligands, indicating both minor groove binding and intercalation modes of DNA interaction. Our findings encourage further development of novel structurally related 6-amidino-2-arylbenzothiazoles to obtain more selective anticancer and anti-HAT agents.

GRAPHICAL ABSTRACT

Introduction
The benzothiazoles are constituents of bioactive heterocyclic compounds that exhibit a wide spectrum of biological activities. Electron deficient, bivalent sulphur atoms in sulphur-containing heterocycles were found to participate in attractive nonbonding sulphur aromatic and sulphur halogen interactions that proved to enhance drug – target binding affinity. Functionalization of the benzothiazole scaffold at the C-2 and C-6 positions has been a key determinant for their enhanced biological activity, mainly antiproliferative and antiparasitic. Thus, the antiproliferative activity of amidino- and amino-substituted 2-phenylbenzothiazole derivatives strongly depended on the position of the substituent in the benzothiazole skeleton, as well as on the type of amidino unit. Anticancer effects of 2-arylbenzothiazoles involved metabolic activation by cytochrome P450 to electrophilic reactive species, which generated DNA adducts in sensitive tumour cells. Polyhydroxylated 2-phenylbenzothiazoles were developed as surrogates for the naturally occurring bioactive flavonoid and isoflavone. Among C-2-arylbenzothiazoles, amino-substituted derivatives possessed excellent cytotoxicity in nanomolar concentrations against several breast cancer cell lines. While the methylated analogue of C-2-arylbenzothiazole demonstrated superior in vitro efficacy, albeit, with metabolic instability, the fluorinated analogue exhibited enhanced stability with limited bioavailability. The prodrug concept led to the development of the phortress compound, which had potent antitumor activity against human mammary tumour xenografts and is in clinical trials for the treatment of solid tumours. 2-Piperazinyl benzothiazole derivatives were found to strongly inhibit the growth of hepatocellular, breast, and colorectal cancer cells. Even though a number of structurally related benzothiazoles have been reported to exert antitumor effect, their mechanism is not fully evaluated, as a preliminary lead optimisation and clinical development. Some benzothiazole-based anticancer agents were found to target tyrosine kinase, topoisomerase, microtubule, cytochrome P450, heat shock protein 90 (Hsp90), epidermal growth factor receptor (EGFR) and apoptosis by activation of reactive oxygen species (ROS). Amido benzothiazoles that exhibited strong antiproliferative activity also exerted good DNA binding affinity having both helix groove binding and DNA intercalation properties. Some benzothiazole sulphonamides were found to have a crucial role in the inhibition of the metalloenzyme carbonic anhydrase (CA IX and XII) that is overexpressed in hypoxic tumours.

Human African trypanosomiasis (HAT), or sleeping sickness, is a neglected tropical disease (NTD) caused by Trypanosoma brucei, a protozoan parasite transmitted to humans through the bite of a blood-sucking tsetse fly. The drugs currently used to treat HAT are not effective against all stages and subspecies of the parasite, so further clinical investigation is needed to develop new.
antitrypanosomal drugs. Some 2-benzylsulfanyl- and 2-arylbenzothiazole derivatives have been found to exhibit good trypanocidal activity at low concentrations.\(^{35,36}\) Optimisation of anti-parasite activity, physicochemical parameters and ADME properties afforded the fluoro-substituted benzothiazole, with a 2-cyclopropylbenzoxazole amide at position 2, which displayed promising in vivo efficacy.\(^{37}\)

In continuation of our recent work on the development of aromatic benzimidazole amidines as antitypansomal\(^{38-40}\) and cytotoxic\(^{41}\) agents, we have now synthesised new chemical entities by the fusion of benzothiazole through a phenoxymethylene unit, or directly to 1,2,3-triazole ring with a \(p\)-substituted phenyl or benzyl subunit (Figure 1).

In this context, the influence of linkers and the type of amidino substituents of the benzothiazole derivatives 10a–10d, 11a–11d, 12a–12d, 13a–13d, 14a–14d, 15a–15d on their antiproliferative and antiprotozoal activity has been explored. Compounds with potent antiproliferative and antitypansomal activities were selected for further investigation of their DNA binding affinities by UV-Vis and CD spectroscopy, as well as thermal denaturation experiments.

**Materials and methods**

**General**

Melting points were determined by means of Original Kofler Mikroheiztisch 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. UPLC-MS spectra were recorded with Agilent 1290 Infinity II/6120 Quadruple LC/MS spectrometers using electrospray ionisation (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.

**Experimental procedures for the synthesis of compounds**

Synthesis of main precursors for the synthesis of targeted benzothiazoles, namely 4-(prop-2-yn-1-yloxy)benzaldehyde (2)\(^{41}\), 4-[(1-phenyl-1H-1,2,3-triazol-4-yl)methoxy]benzaldehyde (3a)\(^{41}\), 4-[(1-(4-chlorophenyl)-1H-1,2,3-triazol-4-yl)methoxy]benzaldehyde (3b)\(^{41}\), 4-[(1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl)methoxy]benzaldehyde (3c)\(^{38}\), 4-[(1-benzyl-1H-1,2,3-triazol-4-yl)methoxy]benzaldehyde (3d)\(^{41}\), (1-phenyl-1H-1,2,3-triazol-4-yl)methanol (5a)\(^{42}\), [1-(4-chlorophenyl)-1H-1,2,3-triazol-4-yl]methanol (5b)\(^{41}\), [1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl]methanol (5c)\(^{42}\), (1-benzyl-1H-1,2,3-triazol-4-yl)methanol (5d)\(^{41}\), 1-phenyl-1H-1,2,3-triazole-4-carbaldehyde (6a)\(^{44}\), 1-(4-chlorophenyl)-1H-1,2,3-triazole-4-carbaldehyde (6b)\(^{41}\), 1-(4-methoxyphenyl)-1H-1,2,3-triazole-4-carbaldehyde (6c)\(^{42}\), 1-benzyl-1H-1,2,3-triazole-4-carbaldehyde (6d)\(^{43}\), 2-amino-5-amidinobenzenethiolate (7)\(^{45}\), 2-amino-5-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzenethiolate hydrate (8)\(^{45}\) and 2-amino-5-(3,4,5,6-tetrahydropyrimidin-1-ium-2-yl)benzenethiolate (9)\(^{45}\) was carried out according to the previously published experimental procedures.

**General method for preparation of compounds 10a–10d, 11a–11d, 12a–12d, 13a–13d, and 14a–14d**

To a stirred solution of a corresponding 5-amidino-substituted-2-amino-benzenethiolate (7–9) (0.25 or 0.5 mmol) in glacial acetic acid (5 ml), a corresponding carbaldehyde 3a–3d or 6a–6d (0.25 or 0.5 mmol) was added under a nitrogen atmosphere and heated to reflux for 2–4 h. The reaction mixture was poured onto ice and made alkaline with 20% NaOH. The resulting free base was filtered, washed with water, and dried. The crude free base was converted into a methanesulfonate salt as described below.

**6-Amidinium-2-[(1-phenyl-1H-1,2,3-triazol-4-yl)methoxy]benzothiazole methanesulfonate (10a)**

According to the above-mentioned general method, 4-[(1-phenyl-1H-1,2,3-triazol-4-yl)methoxy]benzaldehyde 3a (70 mg, 0.25 mmol)
and 2-amino-5-aminidinobenzenethiolate 7 (42 mg, 0.25 mmol) were used and refluxed for 4 h, giving 78 mg (0.183 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (5 ml) followed by the addition of methanesulfonic acid (13 μl, 0.2 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off, crystallised from ethanol and dried at 75°C. Yield of pure compound 10a as colourless solid was 60 mg (46.2%), mp = 272–275°C. 1H NMR (300 MHz, DMSO-d6) δ = 9.35 (s, 2H, -C(NH2)2), 8.99 (s, 2H, -C(NH2)2), 8.95 (s, 1H, Ar-H), 8.63 (s, 1H, Ar-H), 8.21 (d, 1H, J = 8.5 Hz, Ar-H), 8.14 (d, 2H, J = 8.9 Hz, Ar-H), 7.81 (d, 2H, J = 8.8 Hz, Ar-H), 7.15 (d, 2H, J = 8.9 Hz, Ar-H), 5.38 (s, 2H, -OCH3), 3.85 (s, 3H, -OCH3), 2.34 (s, 3H, CH3SO3). 13C NMR (151 MHz, DMSO-d6) δ = 171.2 (s), 165.3 (s), 161.1 (s), 159.3 (s), 156.7 (s), 143.1 (s), 134.5 (s), 129.9 (s), 129.4 (d, 2C), 126.3 (d), 125.3 (s), 124.6 (s), 123.2 (d), 123.1 (d), 122.5 (d, 2C), 122.0 (d, 2C), 115.7 (d, 2C), 61.4 (t) 55.6 (q), 39.7 (q). LC-MS (ESI) m/z: 457.2 (M + H+) calcld. for free base M = 456.14. Analysis calcld. for C23H20NO8S2 × H2O (570.64): C, 52.62; H, 4.59; N, 14.73%; Found C, 52.71; H, 4.62; N, 14.68%.

6-Aminidin-2-[(1-(benzyl-1H)2-3,2-triazol-4-yl)methoxy]benzothiazole methanesulfonate (10d)

According to the above-mentioned general method, 4-[(1-benzyl-1H)2-3,2-triazol-4-yl)methoxy]benzaldehyde 3d (148 mg, 0.5 mmol) and 2-amino-5-aminidinobenzenethiolate 7 (84 mg, 0.5 mmol) were used and refluxed for 2 h, giving 149 mg (0.277 mmol) of crude free base. Afterwards, the obtained free base was suspended in 2-propanol (10 ml) followed by the addition of methanesulfonic acid (20 μl, 0.31 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off, crystallised from ethanol and dried at 75°C. Yield of pure compound 10d as pale yellow solid was 76 mg (28.4%), mp = 253–257°C. 1H NMR (300 MHz, DMSO-d6) δ = 9.40 (s, 2H, -C(NH2)2), 9.02 (s, 2H, -C(NH2)2), 8.62 (d, 1H, J = 1.3 Hz, Ar-H), 8.34 (s, 1H, Ar-H), 8.22 (d, 1H, J = 8.6 Hz, Ar-H), 8.11 (d, 2H, J = 8.7 Hz, Ar-H), 7.89 (dd, 1H, J = 1.6 Hz, J = 8.7 Hz, Ar-H), 7.46–7.30 (m, 5H, Ar-H), 7.26 (d, 2H, J = 8.8 Hz, Ar-H), 5.63 (s, 2H, -CH2-), 5.28 (s, 2H, -OCH3), 2.32 (s, 3H, CH3SO3). 13C NMR (75 MHz, DMSO-d6) δ = 171.2 (s), 165.3 (s), 161.2 (s), 156.7 (s), 142.5 (s), 136.0 (s), 134.5 (s), 129.4 (d, 2C), 128.8 (d, 2C), 128.2 (d), 1280 (d, 2C), 126.3 (d), 124.9 (d, 12C), 124.6 (d), 123.2 (d), 122.5 (d, 2C), 115.7 (d, 2C), 61.4 (t) 52.9 (t). LC-MS (ESI) m/z: 441.2 (M + H+) calcld. for free base M = 440.14. Analysis calcld. for C23H22N6O8S2 (536.63): C, 55.95; H, 4.51; N, 15.66%; Found C, 56.08; H, 4.36; N, 15.57%.

6-(4-Dihydro-1H-imidazol-3-ium-2-yl)-2-[(1-phenyl-1H-2,3,2-triazol-4-yl)methoxy]benzothiazole methanesulfonate (11a)

According to the above-mentioned general method, 4-[(1-phenyl-1H)2-3,2-triazol-4-yl)methoxy]benzaldehyde 3a (70 mg, 0.25 mmol) and 2-amino-5-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzenethiolate hydrate 8 (53 mg, 0.25 mmol) were used and refluxed for 2 h, giving 88 mg (0.195 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (5 ml) followed by the addition of methanesulfonic acid (15 μl, 0.23 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75°C. Yield of pure compound 11a as colourless solid was 74 mg (54%), mp = 270–274°C. 1H NMR (600 MHz, DMSO-d6) δ = 10.56 (s, 2H, -C(NH2)2), 9.01 (s, 1H, Ar-H), 8.73 (s, 1H, Ar-H), 8.33–8.85 (m, 6H, Ar-H), 7.69–7.25 (m, 5H, Ar-H), 5.40 (s, 2H, -OCH3), 4.06 (s, 4H, -CH2-CH3), 2.33 (s, 3H, CH3SO3). 13C NMR (75 MHz, DMSO-d6) δ = 171.8 (s), 164.8 (s), 161.2 (s), 157.1 (s), 143.3 (s), 136.5 (s), 134.8 (s), 129.9 (d, 2C), 129.5 (d, 2C), 128.8 (d, 12C), 126.4 (d, 12C), 123.4 (d), 123.1 (d), 122.9 (d), 120.2 (d, 2C), 118.4 (s, 115.7 (d, 2C), 61.3 (t). LC-MS (ESI) m/z: 455.3 (M + H+) calcld. for free base M = 454.12. Analysis calcld. for C23H18N6O8S2 (548.64): C, 56.92; H, 4.41; N, 15.32%; Found C, 56.78; H, 4.37; N, 15.37%.
According to the above-mentioned general method, 4-[(1-chlorophenyl)-1H-1,2,3-triazol-4-yl]methoxy)benzothiazole methanesulfonate (11d) was synthesized by reacting 79 mg (0.25 mmol) and 2-amino-5-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzenethiolate 9 (52 mg, 0.25 mmol) were used and refluxed for 3 h, giving 75 mg (0.160 mmol) of crude free base. Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of methanesulfonic acid (12 μl, 0.18 mmol) and stirring at room temperature for 2h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 11d as colourless solid was 67 mg (45.0%), mp = 220–224 °C. 1H NMR (300 MHz, DMSO-d6) δ = 9.93 (s, 2H, -(CH=N)2–), 8.95 (s, 1H, Ar-H), 8.52 (s, 1H, Ar-H), 8.22 (d, 1H, J = 8.7 Hz, Ar-H), 8.13 (d, 2H, J = 8.3 Hz, Ar-H), 7.92 (d, 2H, J = 7.7 Hz, Ar-H), 7.82 (d, 1H, J = 8.5 Hz, Ar-H), 7.69–7.48 (m, 3H, Ar-H), 7.32 (d, 2H, J = 8.5 Hz, Ar-H), 5.40 (s, 2H, -(CH2=CH2)2), 3.55 (m, 4H, -(CH2=CH2)2), 2.30 (s, 3H, CH3SO3–), 2.04 (m, 4H, -(CH2=CH2)2–). 13C NMR (151 MHz, DMSO-d6) δ = 170.7 (s), 161.0 (s), 159.0 (s), 156.2 (s), 143.2 (s), 136.4 (s), 134.4 (s), 129.7 (d, 2C), 129.2 (d, 2C), 128.6 (d), 125.6 (d, 125.2), 124.8 (s), 122.8 (d), 122.5 (d), 122.2 (d), 120.0 (d, 2C), 115.6 (d, 2C), 61.3 (t), 39.6 (q), 38.8 (t, 2C), 17.6 (t). LC-MS (ESI) m/z: 467.3 (M+H+) calcd. for free base M = 466.16. Analysis calcd. for C27H26N6O4S2 × H2O (580.68): C, 55.85; H, 4.86; N, 14.47%; Found C, 56.09; H, 4.69; N, 14.58.

6-(3,4,5,6-Tetrahydroprymidin-1-ium-2-yl)-2-[(1-phenyl-1H-1,2,3-triazol-4-yl)methoxy]benzothiazole methanesulfonate (12a)
Mixture was cooled overnight, diethyl-ether was added and the reaction was refluxed for 2 h, giving 101 mg (0.203 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (5 ml) followed by the addition of methanesulfonic acid (15 µl, 0.23 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, diethyl-ether was added and the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 12c as colourless solid was 60 mg (39.2%), mp = 259–264 °C. 1H NMR (300 MHz, DMSO-d6) δ = 9.96 (s, 2H, -CH(NH3)+), 8.84 (s, 1H, Ar-H), 8.52 (d, 1H, J = 1.5 Hz, Ar-H), 8.21 (d, 1H, J = 8.6 Hz, Ar-H), 8.13 (d, 2H, J = 8.8 Hz, Ar-H), 7.84–7.80 (m, 3H), 7.31 (d, 2H, J = 8.8 Hz, Ar-H), 7.15 (d, 2H, J = 9.0 Hz, Ar-H), 5.38 (s, 2H, -OCH2), 3.85 (s, 3H, -OCH3), 3.55 (t, 4H, J = 5.6 Hz, -CH2CH2CH2-), 2.31 (s, 3H, CH3SO2). 13C NMR (151 MHz, DMSO-d6) δ = 170.8 (s), 161.0 (s), 159.3 (s), 159.1 (s), 156.2 (s), 134.5 (s), 129.8 (s), 129.2 (d, 2C), 126.5 (d), 125.2 (s), 124.9 (s), 122.8 (s), 122.5 (d, 2H), 122.3 (d, 2C), 116.5 (d, 2C), 114.8 (d, 2C), 61.3 (t), 55.4 (q), 39.6 (q), 38.8 (t, 2C), 17.6 (t). LC-MS (ESI) m/z: 497.3 (M + H+) calc. for base M = 496.17. Analysis calc. for C28H29N2O2S2 · H2O (610.70): C, 55.07%; H, 4.95%; N, 13.76%; Found C, 54.91; H, 4.99; N, 13.87%.

According to the above-mentioned general method, 4-[1-(4-methoxyphenyl)-1H-1,2,3-triazole-4-ylmethoxy]benzaldehyde 3c (148 mg, 0.5 mmol) and 2-amino-5-(3,4,5,6-tetrahydroprimidin-1-ium-2-yl)benzenethiolate 9 (104 mg, 0.5 mmol) were used and refluxed for 2 h, giving 188 mg (0.391 mmol) of crude free base. Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of methanesulfonic acid (28 µl, 0.43 mmol) and stirring at room temperature for 2 h. To the reaction mixture diethyl-ether was added, the resulting precipitate was filtered off, crystallised from 2-propanol and dried at 75 °C. Yield of pure compound 12d as colourless solid was 47 mg (15.8%), mp = 197–199 °C. 1H NMR (300 MHz, DMSO-d6) δ = 10.01 (s, 2H, -CH(NH3)+), 8.52 (d, 1H, J = 1.3 Hz, Ar-H), 8.34 (s, 1H, Ar-H), 8.22 (d, 1H, J = 8.5 Hz, Ar-H), 8.11 (d, 2H, J = 8.7 Hz, Ar-H), 7.80 (dd, 1H, J = 1.5 Hz, J = 8.6 Hz, Ar-H), 7.42–7.32 (m, 5H, Ar-H), 7.26 (d, 2H, J = 8.8 Hz, Ar-H), 5.63 (s, 2H, -CH2-), 5.28 (s, 2H, -OCH2-), 3.53 (t, 4H, J = 5.4 Hz, -CH2CH2CH2-), 2.29 (s, 3H, CH3SO2), 2.01 (m, 2H, -CH2CH2CH2-). 13C NMR (75 MHz, DMSO-d6) δ = 165.1 (s), 162.2 (s), 155.9 (s), 142.2 (s), 134.7 (s), 134.0 (s), 133.6 (s), 129.5 (d, 2C), 126.0 (d), 125.2 (s), 123.0 (d), 122.5 (d), 122.1 (d, 2C), 39.4 (q). LC-MS (ESI) m/z: 355.1 (M + H+) calc. for base M = 354.05. Analysis calc. for C18H16N6O3S2 (450.92): C, 45.28; H, 3.35; N, 18.64%; Found C, 45.28; H, 3.21; N, 18.71%.

According to the above-mentioned general method, 1-(4-chlorophenyl)-1H-1,2,3-triazole-4-carboxaldehyde 6b (104 mg, 0.5 mmol) and 2-amino-5-aminimidobenzenethiolate 7 (84 mg, 0.5 mmol) were used and refluxed for 3 h, giving 151 mg (0.394 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (10 ml) followed by the addition of methanesulfonic acid (29 µl, 0.45 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 13b as colourless solid was 143 mg (63.6%), mp > 300 °C. 1H NMR (300 MHz, DMSO-d6) δ = 9.70 (s, 1H, Ar-H), 9.18 (s, 4H, -CH(NH3)+), 8.70 (s, 1H, Ar-H), 8.25 (d, 1H, J = 8.3 Hz, Ar-H), 8.08 (d, 2H, J = 8.0 Hz, Ar-H), 7.95 (d, 1H, J = 7.8 Hz, Ar-H), 7.73 (d, 2H, J = 7.6 Hz, Ar-H), 3.43 (s, 3H, CH3SO2). 13C NMR (75 MHz, DMSO-d6) δ = 165.1 (s), 162.2 (s), 155.9 (s), 142.2 (s), 134.7 (s), 134.0 (s), 133.6 (s), 129.5 (d, 2C), 126.0 (d), 125.2 (s), 123.0 (d), 122.5 (d), 122.1 (d, 2C), 39.4 (q). LC-MS (ESI) m/z: 355.1 (M + H+) calc. for base M = 354.05. Analysis calc. for C17H16N6O3S2 (409.92): C, 45.28; H, 3.35; N, 18.64%; Found C, 45.28; H, 3.21; N, 18.71%.

According to the above-mentioned general method, 1-(4-methoxyphenyl)-1H-1,2,3-triazole-4-carboxaldehyde 6c (102 mg, 0.5 mmol) and 2-amino-5-aminimidobenzenethiolate 7 (84 mg, 0.5 mmol) were used and refluxed for 2 h, giving 130 mg (0.371 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (10 ml) followed by the addition of methanesulfonic acid (26 µl, 0.40 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 13c as colourless solid was 114 mg (50.0%), mp > 300 °C. 1H NMR (300 MHz, DMSO-d6) δ = 9.65 (s, 1H, Ar-H), 9.45 (s, 2H, -CH(NH3)+), 9.11 (s, 2H, -CH(NH3)+), 8.71 (s, 1H, Ar-H), 8.27 (d, 1H, J = 8.1 Hz, Ar-H), 8.08–7.87 (m, 3H, Ar-H), 7.20 (d, 2H, J = 7.1 Hz, Ar-H).
6-Amidinium-2-(1-benzyl-1H,1,2,3-triazole-4-yl)benzothiazole methanesulfonate (13d)

According to the above-mentioned general method, 1-benzyl-1H,1,2,3-triazole-4-carbaldehyde 6d (94 mg, 0.5 mmol) and 2-amino-5-amidiniumbenzenethiolate 7a (84 mg, 0.5 mmol) were used and refluxed for 3 h, giving 78 mg (0.233 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (5 ml) followed by the addition of methanesulfonic acid (16 μl, 0.25 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 13d as colourless solid was 147 mg (66.5%), mp 334.10. Analysis calcd. for C18H18N6O4S2: C, 47.46; H, 4.20; N, 18.87%; Found C, 47.62; H, 4.12; N, 18.35%.

6-(4,5-Dihydro-1H-imidazol-3-ium-2-yl)-2-(1-phenyl-1H-1,2,3-triazole-4-yl)benzothiazole methanesulfonate (14a)

According to the above-mentioned general method, 1-phenyl-1H,1,2,3-triazole-4-carbaldehyde 6a (87 mg, 0.5 mmol) and 2-amino-5-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzenethiolate hydrate 8 (106 mg, 0.5 mmol) were used and refluxed for 3 h, giving 137 mg (0.396 mmol) of crude free base. Afterwards, the obtained free base was suspended in ethanol (10 ml) followed by the addition of methanesulfonic acid (29 μl, 0.44 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 14a as colourless solid was 147 mg (66.5%), mp 283–287 °C. 1H NMR (300 MHz, DMSO-d6) δ = 10.56 (s, 2H, C(NH)2+), 9.70 (s, 1H, Ar-H), 8.81 (s, 1H, Ar-H), 8.31 (d, 1H, J = 8.6 Hz, Ar-H), 8.13–8.04 (m, 3H, Ar-H), 7.69–7.56 (m, 3H, Ar-H), 4.08 (s, 4H, -CH2NH2+), 2.33 (s, 3H, CH3SO3). 13C NMR (151 MHz, DMSO-d6) δ = 164.7 (s), 162.9 (s), 156.4 (s), 141.9 (s), 135.8 (s), 134.3 (s), 129.5 (d, 2C), 129.0 (d), 126.1 (d), 123.4 (d), 122.8 (d), 122.4 (d), 120.3 (d, 2C), 118.8 (s), 44.3 (t, 2C). LC-MS (ESI) m/z: 347.2 (M + H+) calc. for free base M = 346.10. Analysis calcld. for C19H18N6O4S2: C, 51.57; H, 4.10; N, 18.99%. Found C, 51.67; H, 4.09; N, 18.87%.

6-(4,5-Dihydro-1H-imidazol-3-ium-2-yl)-2-(1-benzyl-1H,1,2,3-triazole-4-yl)benzothiazole methanesulfonate (14d)

According to the above-mentioned general method, 1-benzyl-1H,1,2,3-triazole-4-carbaldehyde 6d (94 mg, 0.5 mmol) and 2-amino-5-(4,5-dihydro-1H-imidazol-3-ium-2-yl)benzenethiolate hydrate 8 (106 mg, 0.5 mmol) were used and refluxed for 3 h, giving 75 mg (0.233 mmol) of crude free base. Afterwards, the obtained free base was suspended in 2-propanol (5 ml) followed by the addition of methanesulfonic acid (16 μl, 0.25 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75 °C. Yield of pure compound 14d as colourless solid was 75 mg (32.9%), mp 236–240 °C. 1H NMR (300 MHz, DMSO-d6) δ = 10.52 (s, 2H, C(NH)2+), 9.09 (s, 1H, Ar-H), 8.76 (s, 1H, Ar-H), 8.27 (d, 1H, J = 8.3 Hz, Ar-H), 8.03 (d, 1H, J = 8.8 Hz, Ar-H), 7.47–7.35 (m, 5H, Ar-H), 5.75 (s, 2H, -CH2NH2+), 4.07 (s, 4H, -CH2NH2+), 3.23 (s, 3H, CH3SO3). 13C NMR (151 MHz, DMSO-d6) δ = 164.8 (s), 163.6 (s), 156.6 (s), 141.5 (s), 135.3 (s), 134.3 (s), 128.7 (d, 2C), 128.3 (d), 128.0 (d, 2C), 126.4 (d), 124.8 (d), 123.7 (d), 123.1 (d), 118.9 (s), 53.4 (t, 4H, 2C), 39.7 (q). LC-MS (ESI) m/z: 361.2 (M + H+) calc. for free base.
6-(3,4,5,6-Tetrahydroprymidin-1-ium-2-yl)-2-(1-phenyl-1H-1,2,3-triazole-4-yl)benzothiazole methanesulfonate (15a)

According to the above-mentioned general method, 1-phenyl-1H-1,2,3-triazole-4-carbaldehyde 6a (87 mg, 0.5 mmol) and 2-amino-5-(3,4,5,6-tetrahydroprymidin-1-ium-2-yl)benzenethiolate 9 (106 mg, 0.5 mmol) were used and refluxed for 2 h, giving 145 mg (0.371 mmol) of crude free base. Afterwards, the obtained free base was suspended in 2-propanol (10 ml) followed by the addition of methanesulfonic acid (26 µl, 0.40 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75°C. Yield of pure compound 15a as colourless solid was 117 mg (47.6%), mp = 269–274°C. ′1H NMR (300 MHz, DMSO-d6) δ = 9.94 (s, 2H, C(NH)2), 9.62 (s, 1H, Ar-H), 8.60 (d, 1H, J = 1.5 Hz, Ar-H), 8.27 (d, 1H, J = 8.5 Hz, Ar-H), 8.04 (d, 2H, J = 7.9 Hz, Ar-H), 7.88 (dd, 1H, J = 1.9 Hz, J = 8.6 Hz, Ar-H), 7.69–7.76 (m, 3H, Ar-H), 3.57 (t, 4H, J = 5.7 Hz, -CH2CH2CH2-), 2.32 (s, 3H, CH3SO3), 2.06 (m, 2H, -CH2CH2-). ′13C NMR (151 MHz, DMSO-d6) δ = 162.3 (s), 159.1 (s), 155.8 (s), 142.3 (s), 136.0 (s), 134.1 (s), 129.8 (d, 2C), 129.3 (d), 125.9 (d), 125.5 (s), 122.8 (d), 122.7 (d), 122.5 (d), 120.5 (d, 2C), 39.6 (q), 38.8 (t, 2C), 17.6 (t). LC-MS (ESI) m/z: 361.2 (M+H)+ calcd. for free base M = 360.12. Analysis calcd. for C20H19ClN6O3S2 × 1.25H2O: C, 49.54; H, 4.85; N, 16.51%; Found C, 49.41; H, 4.83; N, 17.63%.

6-(3,4,5,6-Tetrahydroprymidin-1-ium-2-yl)-2-(1-benzyl-1H-1,2,3-triazole-4-yl)benzothiazole methanesulfonate (15d)

According to the above-mentioned general method, 1-benzyl-1H-1,2,3-triazole-4-carbaldehyde 6d (94 mg, 0.5 mmol) and 2-amino-5-(3,4,5,6-tetrahydroprymidin-1-ium-2-yl)benzenethiolate 9 (106 mg, 0.5 mmol) were used and refluxed for 2 h, giving 106 mg (0.283 mmol) of crude free base. Afterwards, the obtained free base was suspended in 2-propanol (10 ml) followed by the addition of methanesulfonic acid (14 µl, 0.21 mmol) and stirring at room temperature for 2 h. The reaction mixture was cooled overnight, the resulting precipitate was filtered off and dried at 75°C. Yield of pure compound 14d as colourless solid was 74 mg (32.9%), mp = 170–174°C. ′1H NMR (300 MHz, DMSO-d6) δ = 9.94 (s, 2H, C(NH)2), 9.01 (s, 1H, Ar-H), 8.56 (s, 1H, Ar-H), 8.21 (d, 1H, J = 8.4 Hz, Ar-H), 7.85 (d, 1H, J = 7.9 Hz, Ar-H), 7.47–7.34 (m, 5H, Ar-H), 5.75 (s, 2H, -CH2), 3.56 (m, 4H, -CH2CH2CH2-), 2.32 (s, 3H, CH3SO3), 2.05 (m, 2H, -CH2CH2-). ′13C NMR (151 MHz, DMSO-d6) δ = 162.7 (s), 159.1 (s), 155.8 (s), 141.6 (s), 135.3 (s), 134.0 (s), 128.7 (d, 2C), 128.3 (d), 128.0 (d, 2C), 125.8 (d), 125.5 (s), 124.6 (d), 122.8 (d), 122.7 (d), 53.4 (t), 38.9 (t, 2C), 17.6 (t). LC-MS (ESI) m/z: 375.2 (M+H)+ calcd. for free base M = 374.13. Analysis calcd. for C21H22N6O4S2 × 1.5H2O (497.59): C, 50.69; H, 5.06; N, 16.89%; Found C, 50.91; H, 5.14; N, 16.86%.

Biological evaluations

Cell culturing

Human cell lines SW620 (colorectal adenocarcinoma, metastatic), MCF-7 (human breast adenocarcinoma), CFPAC-1 (pancreatic adenocarcinoma), HeLa (cervical carcinoma), and HFF-1 (human foreskin fibroblasts) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in a humidified atmosphere at 37°C with 5% CO2. As a growth medium, Dulbecco’s modified Eagle medium (DMEM) was used with the addition of foetal bovine serum (10%), L-glutamine (2 mM), and antibiotics: streptomycin (100 µg/ml) and penicillin (100 U/ml).

Proliferation assay

Cells were seeded onto 96-well microtiter plates at a seeding density of 3000 cells/well for carcinoma cell lines, and 5000 cells/well for normal human fibroblasts. The next day, cells were treated with test agents at five different concentrations (0.01–100 µM) and further incubated for 72 h. DMSO (solvent) was tested for potential cytotoxic effect but it did not exceed 0.1%.
Fluorouracil (5-FU, 0.384 M fluorouracilum, Pliva 500 mg/10 ml) dissolved in physiological solution was used as a positive control. Following 72 h incubation, the MTT assay was performed and measured absorbances were transformed into a percentage of cell growth as described previously. Results were obtained from three independent experiments. IC50 values were calculated using linear regression analysis (FORECAST option taking into account the concentration range of two experimental points above and below IC50).

Antitrypanosomal screening and cytotoxicity assays

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 μl volumes) to determine the IC50 and IC90 values of each compound. Parasites growth was initiated at 2.5 x 10^4 ml^-1, compounds were added at a range of concentrations, and the plates were incubated at 37°C. Resazurin (20 μl at 0.125 mg 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 x 10^4 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.

DNA binding study

Compounds 11a, 11b, 12b, 14a, 14c and 15b were dissolved (c = 5 x 10^-3 M for 12b, 14a, 14c, 15b, c = 4 x 10^-3 M for 11a, 11b) in water, while compounds 10b and 14b were dissolved (c = 5 x 10^-4 M) in DMSO. These solutions were used for measurements in an aqueous buffer (pH = 7, sodium cacodylate buffer, I = 0.05 mol dm^-3). Polynucleotides were purchased as noted: poly (dAdT)_2 and calf thymus ctDNA (Sigma-Aldrich). Polynucleotides were dissolved in Na-cacodylate buffer, I = 0.05 mol dm^-3, pH = 7. The calf thymus ctDNA was additionally sonicated and filtered through a 0.45 mm filter. Polynucleotide concentration was determined spectrophotometrically as the concentration of phosphates.

UV/vis measurements

The UV/Vis spectra were recorded on a Varian Cary 100 Bio spectrophotometer using 1 cm path quartz cuvettes. Calibration experiments were performed at 25°C and pH = 7 (I = 0.05 mol dm^-3, sodium cacodylate buffer). Absorption maxima and corresponding molar extinction coefficients (ε) of benzothiazole derivatives are given in Table S1 (Supplementary material). Thermal melting curves for DNA and their complexes with studied compounds were determined as previously described by following the absorbance change at 260 nm as a function of temperature. The absorbance of the ligands was subtracted from every curve and the absorbance scale was normalised. Tm values are the midpoints of the transition curves determined from the maximum of the first derivative and checked graphically by the tangent method. The ΔTm values were calculated by subtracting Tm of the free nucleic acid from Tm of the complex. Every ΔTm value reported here was the average of at least two measurements. The error in ΔTm is ±0.5°C.

Fluorimetric measurements

Fluorescence spectra were recorded on a Varian Cary Eclipse spectrophotometer at 25°C using appropriate 1 cm path quartz cuvettes. Fluorimetric experiments were performed at pH = 7 (I = 0.05 mol dm^-3, sodium cacodylate buffer) by adding portions of polynucleotide solution into the solution of the studied compound. In fluorimetric experiments, an excitation wavelength of λ_ex ≥ 300 nm was used to avoid the inner filter effect caused due to increasing absorbance of the polynucleotide. Emissions were determined in the range λ_em = 350–650 nm. Values for Kf were obtained by processing titration data using the Scatchard equation. All had satisfactory correlation coefficients (>0.99).

CD measurements

CD spectra were recorded on a JASCO J815 spectrophotometer in 1 cm path quartz cuvettes. CD parameters: range = 500–220 nm, data pitch = 2, standard sensitivity, scanning speed = 200 nm/min, accumulation = 3–5. Titrations were performed at 25°C and pH = 7 (I = 0.05 mol dm^-3, sodium cacodylate buffer). CD experiments were done by adding portions of the compound stock solution into the polynucleotide solution.

Results and discussion

Chemistry

The synthesis of novel 1,2,3-triazolyl linked 6-amidino substituted benzothiazole derivatives 10a–10d, 11a–11d, 12a–12d, 13a–13d, 14a–14d, 15a–15d were synthesised according to the procedure shown in Scheme 1. 4-(1,2,3-Triazol-1-yl)benzaldehyde derivatives (3a–3d) were prepared by propargylation of 4-hydroxy benzaldehyde to give 4-O-propargylated benzaldehyde (2). Intermediate 2 was then used as a dipolarophile in copper(l)-catalysed Huisgen 1,3-dipolar cycloaddition with unsubstituted, p-chloro- and p-methoxy-substituted phenyl azides to afford 3a–3d. With the aim of assessing the influence of the phenoxyimethylene linker between the benzothiazole and 1,2,3-triazole moieties on the anti-proliferative and antitrypanosomal activities, the 1,2,3-triazole ring was introduced directly (13a–13d, 14a–14d, and 15a–15d) to the benzothiazole ring as displayed in Scheme 1. 1-Aryl-substituted 1,2,3-triazolyl aliphatic alcohols (5a–5d) were prepared in excellent yields by using the ultrasound-assisted reaction of propargyl alcohol and corresponding aromatic azides with Cu(OAc)2. 1-Aryl-substituted 1,2,3-triazole-carbaldehyde precursors (6a–6d) were subsequently obtained by the Swern oxidation.

Amidino-substituted 2-aminothiophenones (7–9) were prepared from 6-cyanobenzothiazole by the Pinner method, as previously reported. To study the influence of the type of the hydrophilic amidino substituents, non-substituted amidino, imidazolino and pyrimidino moieties were introduced at C6 of benzothiazole scaffold. Condensation of various amidino-substituted 2-aminothiophenones (7–9) with 4-(1,2,3-triazol-1-yl)benzaldehyde precursors (3a–3d) and 1,2,3-triazole-4-carbaldehyde precursors (6a–6d) was carried out in acetic acid, followed by a simple acid-base reaction step to afford the targeted amidino-substituted benzothiazole...
Table 1. The growth-inhibition effects * in vitro of compounds 10a–10d, 11a–11d, 12a–12d, 13a–13d, 14a–14d, 15a–15d on human tumour cell lines and normal fibroblasts.

| Compd. | R₁ | X | R₂ | IC₅₀ (µM) |
|--------|----|---|----|----------|
|        |    |   |    | SW620    | CFPAC-1  | MCF-7   | HeLa    | HFF-1/WI-38 |
| 10a    |     | PhOCH₂– | NH₂ | 2.89 ± 0.03 | 3.34 ± 0.19 | 3.39 ± 1.17 | 2.89 ± 0.11 | 0.17 ± 0.04 |
| 10b    |     | PhOCH₂– | NH₂ | 3.05 ± 0.82 | 1.76 ± 0.17 | 0.87 ± 0.18 | 1.24 ± 0.60 | 0.17 ± 0.13 |
| 10c    |     | PhOCH₂– | NH₂ | 3.86 ± 0.29 | 3.45 ± 0.29 | 3.66 ± 1.49 | 2.87 ± 1.24 | 0.31 ± 0.08 |
| 10d    |     | PhOCH₂– | NH₂ | 1.18 ± 0.18 | 5.15 ± 0.23 | 3.02 ± 0.98 | 1.77 ± 0.45 | 0.76 ± 0.18 |
| 11a    |     | PhOCH₂– | NH₂ | 0.46 ± 0.03 | 0.45 ± 0.01 | 0.49 ± 0.01 | 0.57 ± 0.19 | 0.26 ± 0.02 |
| 11b    |     | PhOCH₂– | NH₂ | 2.84 ± 0.19 | 3.09 ± 0.19 | 3.12 ± 0.36 | 2.61 ± 0.42 | 0.17 ± 0.09 |
| 11c    |     | PhOCH₂– | NH₂ | 0.50 ± 0.16 | 2.06 ± 0.70 | 0.86 ± 0.97 | 2.53 ± 0.01 | 0.24 ± 0.13 |
| 11d    |     | PhOCH₂– | NH₂ | 1.81 ± 0.55 | 1.21 ± 0.74 | 2.21 ± 1.17 | 1.40 ± 0.75 | 0.18 ± 0.02 |
| 12a    |     | PhOCH₂– | NH₂ | 4.17 ± 0.28 | 3.98 ± 0.29 | 3.98 ± 0.04 | 2.95 ± 0.38 | 0.44 ± 0.03 |
| 12b    |     | PhOCH₂– | NH₂ | 0.66 ± 0.21 | 3.55 ± 0.81 | 3.91 ± 1.19 | 2.81 ± 0.58 | 0.24 ± 0.09 |
| 12c    |     | PhOCH₂– | NH₂ | 5.22 ± 1.24 | 3.31 ± 0.60 | 3.46 ± 0.45 | 2.64 ± 0.46 | 0.46 ± 0.27 |
| 12d    |     | PhOCH₂– | NH₂ | 7.94 ± 2.58 | 23.42 ± 0.29 | 6.01 ± 0.53 | 8.60 ± 0.85 | 1.74 ± 0.29 |
| 13a    |     | NH₂ |   | 24.22 ± 4.31 | 26.58 ± 4.69 | 25.58 ± 3.15 | 7.77 ± 0.05 | 2.07 ± 0.13 |
| 13b    |     | NH₂ |   | 3.55 ± 0.85 | 3.17 ± 0.11 | 3.32 ± 0.01 | 2.74 ± 0.21 | 0.85 ± 0.05 |
| 13c    |     | NH₂ |   | 5.39 ± 0.41 | 5.23 ± 0.87 | 4.50 ± 0.43 | 3.64 ± 1.44 | 2.99 ± 1.17 |
| 13d    |     | NH₂ |   | 9.48 ± 0.29 | 25.93 ± 3.71 | 22.56 ± 0.91 | 14.90 ± 1.54 | 4.04 ± 1.35 |
| 14a    |     |     | NH₂ | 0.25 ± 0.16 | 0.45 ± 0.19 | 0.52 ± 0.26 | 0.67 ± 0.06 | 0.12 ± 0.01 |

(continued)
Benzothiazoles:

- **6a–10d, 11a–11d, 12a–12d, 13a–13d, 14a–14d, 15a–15d**.

The structures of novel 6-amidino-substituted benzothiazoles mesylates were determined by using $^1$H and $^{13}$C NMR spectroscopy, mass spectrometry, and elemental analysis (Materials and methods section). The chemical shifts in the $^1$H and $^{13}$C NMR spectra and the H–H coupling constants were consistent with the proposed structures (Supplementary material).

**Biological evaluations**

**Evaluation of antiproliferative activity**

Antiproliferative evaluations of all synthesised compounds were performed in vitro on human tumour cell lines, SW620 (colorectal adenocarcinoma, metastatic), MCF-7 (human breast adenocarcinoma), CFPAC-1 (pancreatic adenocarcinoma), HeLa (cervical carcinoma), and non-tumour HFF-1 (human foreskin fibroblasts) cells. 5-Fluorouracil (5-FU) was used as a reference drug. The results are presented in Table 1.

| Compd. | R$_1$ | X | R$_2$ | IC$_{50}$ (μM) |
|--------|-------|---|-------|---------------|
| 14b    |       |   |       | SW620 | CFPAC-1 | MCF-7 | HeLa | HFF-1/WI-38 |
| 14c    |       |   |       | 0.35 ± 0.13 | 2.42 ± 0.06 | 1.77 ± 0.18 | 0.48 ± 0.01 | 0.36 ± 0.18 |
| 14d    |       |   |       | 0.36 ± 0.09 | 0.48 ± 0.03 | 0.54 ± 0.01 | 0.38 ± 0.10 | 0.18 ± 0.01 |
| 15a    |       |   |       | 4.15 ± 1.58 | 37.57 ± 4.58 | 8.31 ± 0.12 | 7.62 ± 0.84 | 0.53 ± 0.10 |
| 15b    |       |   |       | 0.85 ± 0.61 | 8.71 ± 3.74 | 1.76 ± 1.17 | 0.82 ± 0.01 | 0.11 ± 0.01 |
| 15c    |       |   |       | 4.07 ± 1.06 | 4.74 ± 0.14 | 4.28 ± 0.06 | 4.30 ± 0.41 | 1.33 ± 0.17 |
| 15d    |       |   |       | 31.86 ± 8.38 | 49.89 ± 12.5 | 40.58 ± 10.6 | 30.99 ± 4.50 | 18.93 ± 0.19 |
| 5-FU$^b$ |       |   |       | 6.4 ± 0.22 | 6.45 ± 0.66 | 0.09 ± 0.01 | 47.52 ± 1.93 | 0.94$^c$ |

$^a$50% inhibitory concentration or compound concentration required to inhibit tumour cell proliferation by 50%; $^b$control substance 5-fluorouracil; $^c$WI-38 fibroblast cell line.

It can be observed that the tested compounds exhibited strong to moderate inhibitory activity towards tumour cell lines. However, compounds with pronounced antiproliferative effects affected the proliferation of the non-tumour HFF-1 cell line. The type of amidino moiety at the benzothiazole influenced the antiproliferative activities (Figure 2). For example, among the 6-amidino benzothiazoles, imidazolino-substituted derivatives 11a–11d and 14a–14d showed the best inhibitory effects, particularly on colorectal adenocarcinoma (SW620) and cervical carcinoma (HeLa) cells. This correlates with our earlier findings for the benzimidazole series$^{11}$. Unsubstituted amidino-benzothiazole 13a–13d exhibited up to 10-fold lower activities relative to their imidazolines congeners 14a–14d. The cytostatic effects decreased in the following order: imidazoline > pyrimidine > non-substituted amide.

The effect of the aromatic substituent at N-1 of 1,2,3-triazole ring on antiproliferative activities varied depending on the type of 6-amidino moiety. In most cases, the $p$-chlorophenyl aromatic unit contributed to the enhanced antiproliferative effects of 10b, 12b, 13b, and 15b, except in the case of imidazolino-benzothiazoles. In benzothiazole imidazolines, the unsubstituted phenyl ring in 11a and 14a had the highest influence on inhibitory activity. The 2-imidazolino-substituted derivative 14a exhibited the highest activity on SW620 (IC$_{50}$ = 0.25 μM) and CFPAC-1 (IC$_{50}$ = 0.45 μM) cell lines, while 14c, which contains a $p$-methoxyphenyl ring, was the most potent against HeLa cells (IC$_{50}$ = 0.38 μM). The benzothiazole imidazoline 11a was the most active representative of this class (IC$_{50}$ = 0.49 μM) against MCF-7 cells. Comparison of the effect of the aryl substituents at the N-1 position of the 1,2,3-triazole ring on activity revealed that, among 2-imidazolino-substituted derivatives, phenyl and $p$-methoxyphenyl substituents caused better potencies than $p$-chlorophenol and benzyl substituents.

The introduction of a phenoxymethylene linker improved the activity of the resulting 10a–10d and 12a–12d up to 8-fold, relative to the corresponding benzothiazoles directly connected to 1,2,3-triazole in 13a–13d and 15a–15d. In contrast, with imidazolino-benzothiazoles, direct fusion of benzothiazole to 1,2,3-triazole in 14a–14d enhanced antiproliferative activity compared to 11a–11d, which contained a phenoxymethylene spacer.

**Evaluation of antitrypanosomal activity**

Preliminary screening of 2-arylbenzimidazole amidines 6a–6c, 7a–7c, 8, 14a–18a, 14b–18b, and 14c–18c against bloodstream-
form *T. brucei* in vitro was performed at a range of concentrations to select compounds with the highest inhibition for further evaluation (Table S1, Supplementary material). Thus, compounds, 10a–10c, 11a–11d, 12b, 12c, 13a, 13b, 14a–14d, and 15b, were submitted for more detailed evaluation and the concentrations that inhibited growth by 50% (IC₅₀) and 90% (IC₉₀) were determined (Table 2). Nifurtimox was included as a reference drug. Cytotoxicity was assessed using the rat myoblast cell line L6.

All tested compounds showed potent activity against *T. brucei* with IC₅₀ values ranging from 0.09 to 3.68 μM and IC₉₀s ranging from 0.12 to 8.66 μM, more potent than the front-line drug nifurtimox. We investigated the influence of unsubstituted and cyclic amidino moiety, and aromatic substituent attached to the N-1 of the 1,2,3-triazole ring on antitrypanosomal activity (Figure 2). Similar to antiproliferative evaluations, imidazolino-substituted benzothiazoles 11a–11c and 14a–14c exhibited the best anti-trypanosomal activity, with the p-chlorophenyl analogue 11b being the most promising compound (IC₅₀ = 0.09 μM, IC₉₀ = 0.12 μM). With the exception of compounds 11d and 14d, which contain the N-1-benzyl-1,2,3-triazolyl moiety, benzothiazole imidazolines 11a–11c and 14a–14c showed potency in submicromolar concentrations, with IC₅₀ in the range of 0.12–0.53 μM. However, these compounds were cytotoxic to rat myoblast cell line L6 (IC₅₀ > 0.2 μM). Some selectivity (SI = 1.8–6.9) was observed for benzothiazole pyrimidines and non-substituted amidines.

Replacement of the phenoxymethylene linker in 10a–10d, 11a–11d, and 12a–12d with a direct fusion of benzothiazole to 1,2,3-triazole in 13a–13d, 14a–14d, and 15a–15d reduced antitrypanosomal activity against bloodstream-form *T. brucei*. Furthermore, the introduction of an electron-withdrawing p-chloro substituent in 10b–15b improved the inhibitory effect. The relationship between the type of aromatic substituents and their activities revealed that antitrypanosomal effects decreased in the following order: p-CIPh > p-OCH₃Ph > Ph > Bn.

**DNA binding study**

As a carrier of genetic information and a great influence on vital processes in the cell, DNA is the main target of a large number of drugs with anticancer and antiprotozoal activity. Among them, anthracyclines that target DNA topoisomerase II cause highly lethal DNA breaks in proliferating cancer cells, while some drugs, such as quinacrine, with a long history of clinical use in the treatment of malaria, modulate cellular pathways that lead to cellular toxicity and death.
With the aim of investigating possible mechanisms of antiproliferative and antitrypanosomal action, we examined the DNA interactions of compounds that exhibited the most potent submicromolar activities, \(10b, 11a, 11b, 12b, 14a–14c, \) and \(15b\). The study was performed with a double-stranded (ds) poly-nucleotide, calf-thymus (ctDNA) which represents a classical B-helix consisting of 58% AT base pairs (42% GC base pairs). Titration with ctDNA yielded fluorescence quenching of the studied compounds (Figure 3, Table 3, Figures S19–S26, Supporting material). It can be observed that the addition of ctDNA caused a small redshift (\(\Delta = 1–5\) nm) of emission maxima. The binding constants, \(Ks\) obtained by processing of fluorimetric titration data with the Scatchard equation \(^{52}\) are summarised in Table 3. Interestingly, the 6-amidinobenzothiazoles \(10b, 11a, 11b, \) and \(12b\), which have a phenoxymethylene linker, showed higher binding affinities towards ds-DNA than compounds \(14a–14c\) and \(15b\), which lack the linker. Non-covalent interaction of small molecules with DNA can affect the thermal stability of the double helix, which results in either an increase or decrease of the melting temperature \(Tm\). \(^{42}\) The intercalative mode of binding usually results in an increased \(Tm\) value, while groove binding molecules can stabilise or destabilise the double-stranded structure, resulting in increased or decreased \(Tm\) values, respectively. The majority of studied compounds showed a small stabilisation effect of ctDNA (58% AT). Moreover, all 6-amidinobenzothiazoles stabilised DNA consisted of only AT sequences. The best stabilisation effects of AT-DNA were observed for \(11a, 14c, \) and \(15b\) (Table 4).

The formation of complexes between small molecules and DNA can be monitored using circular dichroism (CD) spectroscopy. A mutual orientation of achiral small molecule and polynucleotide chiral axis, which results in an induced CD (ICD) signal, could give us additional information about modes of interaction. \(^{59,60}\) Hence, the most reliable insight into modes of interaction between small molecules and DNA can be retrieved at wavelength area, \(\lambda > 300\) nm, where ligands possess UV/Vis spectra, while DNA does not.

The addition of studied compounds to ctDNA resulted in a decreased intensity of the CD band of ds-polynucleotide, ctDNA (Figure 4, Figures S31 and S32). Furthermore, compounds \(12b\) and \(15b\) exhibited positive induced CD spectra (ICD) with ctDNA around 300 nm and 345 nm, respectively, supporting a minor groove binding mode to ctDNA. \(^{39,40,61,62}\) Similar changes in CD spectra were noticed for classical minor groove binders like DAPI or Hoechst 33258. \(^{44}\)

The addition of compound \(11a\) to a ctDNA solution resulted in the appearance of a bisignate signal with maxima at 317 and 360 nm. Such change suggests the binding of \(11a\) in the form of a dimer within the minor groove (Figure 4). \(^{52}\)

In the titration of ctDNA with \(10b\) and \(11b\), a small negative ICD signal appeared, while \(14a–14c\) caused the small positive ICD signal upon binding to ctDNA (Figure 4, Figures S31 and S32). Such changes, whether small negative, or positive, point to an intercalative way of binding. This is additionally supported by thermal stabilisation of ctDNA and/or AT-DNA (Table 4), as well as binding constants of \(\sim 1\) \(\mu\)M (Table 3), which were observed with the majority of studied benzothiazoles. The positive sign of the ICD band observed with \(14a–14c\) suggests that the long axis of the benzothiazole moiety is approximately perpendicular to the long axis of the basepair pocket, but still in the plane with the adjacent base pairs. The negative sign of the ICD band found for \(10b\) and \(11b\) indicates that the transition moment of the ligand is oriented “parallel” to the long axis of adjacent base pairs. \(^{42,63}\)

At higher ratios, \(r > 0.3\), a big decrease of intensity of the CD bands and strong negative ICD spectra observed with \(14a–14c\) can be attributed to non-specific aggregation of non-intercalated molecules along the DNA backbone, possibly within major grooves. \(^{43}\)

**Conclusions**

The 6-amidinobenzothiazoles \(10a–10d, 11a–11d, 12a–12d, \) \(13a–13d, 14a–14d, \) and \(15a–15d\), containing distributed highly hydrophilic cationic moieties and hydrophobic aromatic components were designed and synthesised with the aim of performing antiproliferative and antiprotozoal evaluations. The antiproliferative assessment showed that imidazoline moieties improved the growth-inhibitory effects of \(11a–11d\) and \(14a–14d\) and that benzothiazole directly connected to the 1,2,3-triazole in \(14a–14d\) additionally increased inhibitory activity. Thus, benzothiazole imidazolines \(11a, 14a, \) and \(14c\) showed the most pronounced inhibitory effects on all the tumour cell lines studied \(11a\): \(IC_{50} = 0.49\) \(\mu\)M, MCF-7; \(14a\): \(IC_{50} = 0.25\) \(\mu\)M, SW620, \(IC_{50} = 0.45\) \(\mu\)M, CFPAC-1; \(14c\): \(IC_{50} = 0.38\) \(\mu\)M, HeLa). However, 6-amidinobenzothiazoles also affected the proliferation of the non-tumour HFF-1 cell line.

Similarly, the antitrypanosomal evaluations showed that benzothiazole imidazolines \(11a–11c\) and \(14a–14c\) exhibited the best potency, with values that paralleled antiproliferative activity.
| Compd. | R₁       | X          | R₂       | T. brucei | L6 cells | SI<sup>c</sup> |
|-------|----------|------------|----------|-----------|----------|---------------|
|       |          |            |          | IC₅₀ (µM) | IC₉₀ (µM) | IC₅₀ (µM) |
| 10a   | PhOCH₂   | NH₂        |          | 1.17 ± 0.10 | 1.45 ± 0.11 | <2.0    |
| 10b   | PhOCH₂   | NH₂        |          | 0.54 ± 0.13 | 0.81 ± 0.04 | 1.46 ± 0.45 | 2.7 |
| 10c   | PhOCH₂   | NH₂        |          | 1.00 ± 0.07 | 1.26 ± 0.02 | <2.0    |
| 11a   | PhOCH₂   | NH₂        |          | 0.23 ± 0.02 | 0.28 ± 0.03 | <2.0    |
| 11b   | PhOCH₂   | NH₂        |          | 0.09 ± 0.02 | 0.12 ± 0.03 | <2.0    |
| 11c   | PhOCH₂   | NH₂        |          | 0.19 ± 0.04 | 0.23 ± 0.05 | <2.0    |
| 11d   | PhOCH₂   | NH₂        |          | 1.09 ± 0.02 | 1.30 ± 0.02 | <2.0    |
| 12b   | PhOCH₂   | NH₂        |          | 0.91 ± 0.08 | 1.19 ± 0.02 | 2.32 ± 0.18 | 2.5 |
| 12c   | PhOCH₂   | NH₂        |          | 0.90 ± 0.05 | 1.18 ± 0.02 | 2.92 ± 0.26 | 3.2 |
| 13a   | PhOCH₂   | NH₂        |          | 2.02 ± 0.10 | 2.52 ± 0.34 | 7.97 ± 0.96 | 3.9 |
| 13b   | PhOCH₂   | NH₂        |          | 0.67 ± 0.07 | 1.02 ± 0.02 | 2.69 ± 0.42 | 4.0 |
| 14a   | PhOCH₂   | NH₂        |          | 0.40 ± 0.02 | 0.53 ± 0.03 | <2.0    |
| 14b   | PhOCH₂   | NH₂        |          | 0.32 ± 0.06 | 0.48 ± 0.02 | <2.0    |
| 14c   | PhOCH₂   | NH₂        |          | 0.31 ± 0.02 | 0.49 ± 0.01 | <2.0    |
| 14d   | PhOCH₂   | NH₂        |          | 3.68 ± 0.22 | 8.66 ± 0.20 | 6.64 ± 0.53 | 1.8 |
| 15b   | PhOCH₂   | NH₂        |          | 1.12 ± 0.23 | 3.84 ± 0.65 | 7.69 ± 0.16 | 6.9 |

Nifurtimox<sup>b</sup> | 2.0 ± 0.24<sup>b</sup> |

<sup>a</sup>In vitro activity against bloodstream form *T. brucei* expressed as the concentration that inhibited growth by 50% (IC₅₀) and 90% (IC₉₀). Data are the mean of triplicate experiments ± SEM. <sup>b</sup>Taken from Wilkinson et al.<sup>54</sup>. <sup>c</sup>Selectivity index, SI = [IC₅₀ L6 cells] / [IC₅₀ T. brucei].
1,2,3-triazole displayed the highest antitrypanosomal potency (IC50 = 304 nm) upon titration with ctDNA (c = 2 x 10^-6 to 7.8 x 10^-5 M). Inset: dependence of 14a absorbance at λ = 385 nm on c(ctDNA), at pH = 7, sodium cacodylate buffer, l = 0.05 mol dm^-3.

Table 3. Binding constants (logKs) calculated from the fluorescence titrations of benzothiazole compounds with ds-DNA at pH = 7.0 (buffer sodium cacodylate, l = 0.05 mol dm^-3).

| Compd. | LogKs | I/I0 |
|--------|-------|------|
| 10b    | 6.6   | 0.12 |
| 11a    | 6.8   | 0.18 |
| 11b    | 6.6   | 0.21 |
| 12b    | 7.0   | 0.31 |
| 14a    | 5.6   | 0.54 |
| 14b    | 5.0   | 0.45 |
| 14c    | 5.4   | 0.35 |

*Accuracy of n ± 10 – 30%, consequently logKs values vary in the same order of magnitude; †Processing of titration data by means of Scatchard Equation gave values of ratio n[bound compound]/[polynucleotide] = 0.8–0.2, for easier comparison all log Ks values were recalculated for fixed n = 0.6; correlation coefficients were >0.99 for most of calculated Ks. ‡Starting fluorescence intensity of compound/polynucleotide complex calculated by Scatchard equation; §due to the several types of binding of compound 14c with ctDNA, it was not possible to calculate the polynucleotide – compound complex stability constant.

Table 4. The ΔTm values (°C) of ds-DNA upon addition of ratio p = 0.3 of 6-amidinobenzothiazoles at pH 7.0 (sodium cacodylate buffer, l = 0.05 mol dm^-3).

| Compd. | ΔTm |
|--------|-----|
| 10b    | 2.7 |
| 11a    | 2.1 |
| 11b    | 1.2 |
| 14a    | 0.7 |
| 14b    | 2.2 |
| 14c    | 0   |
| 15b    | 0   |

*Difference between Tm value of free polynucleotide and complex with small molecule; error in ΔTm = ±0.5°C; ‡r = [compound]/[polynucleotide]; ‡Changes of 12b with increase of temperature were significant, thus ΔTm values could not be determined.

In contrast to antitumor results, a direct fusion of benzothiazole to 1,2,3-triazole decreased antitrypanosomal activity. 6-Imidazolinobenzothiazole containing 3-chlorophenyl at N-1 of 1,2,3-triazole displayed the highest antitrypanosomal potency (IC50 = 0.09 μM, IC90 = 0.12 μM). UV-Vis and CD spectroscopy, as well as thermal denaturation assays, indicated the binding affinities of 6-amidinobenzothiazoles towards ctDNA. Strong positive ICD bands supported minor groove binding, as the dominant binding mode of 11a, 12b and 15b, while small negative and positive ICD signals identified intercalation, as the predominant binding mode of 10b and 11b and 14b.

Figure 3. Changes in fluorescence spectrum of 14a (c = 1 x 10^-6 M): λexc = 304 nm) upon titration with ctDNA (c = 2 x 10^-6 to 7.8 x 10^-5 M). Inset: dependence of 14a absorbance at λ = 385 nm on c(ctDNA), at pH = 7, sodium cacodylate buffer, l = 0.05 mol dm^-3.

Figure 4. CD titration of ctDNA (c = 3.0 x 10^-3 M) with 11a, 15b at molar ratio, r = [compound]/[polynucleotide] = 0.5 and 11b (molar ratio, r = 0.1) (pH = 7.0, buffer sodium cacodylate, l = 0.05 mol dm^-3).

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

The present work was financially supported by the Croatian Science Foundation (projects No. IP-2018-01-4682 and No. IP-2018-01-4694).

**References**

1. Irfan A, Batool F, Zahra Naqvi SA, et al. Benzothiazole derivatives as anticancer agents. J Enzyme Inhib Med Chem 2020; 35:265–79.
2. Pathak N, Rathi E, Kumar N, et al. A review on anticancer potentials of benzothiazole derivatives. Mini Rev Med Chem 2020;20:12–23.
3. Kamal A, Syed MAH, Mohammed SM. Therapeutic potential of benzothiazoles: a patent review (2010–2014). Expert Opin Ther Pat 2015;25:335–49.
4. Ahmed K, Yellamelli Valli Venkata S, Mohammed NAK, et al. Recent advances on structural modifications of benzothiazoles and their conjugate systems as potential chemotherapeutics. Expert Opin Investig Drugs 2012;21:619–35.
5. Singh M, Singh SK. Benzothiazoles: how relevant in cancer drug design strategy? Anti-Cancer Agent Med Chem 2014;14:127–46.
6. Beno BR, Yeun KS, Bartberger MD, et al. A survey of the role of noncovalent sulfur interactions in drug design. J Med Chem 2015;58:4383–438.
7. Nicaise Djuidje E, Sciabica S,uzzi R, et al. Design, synthesis, and evaluation of benzothiazole derivatives as multifunctional agents. Bioorg Med Chem 2020;101:103960–75.

8. Linciano P, Pozzi C, dello Iacono L, et al. Enhancement of benzothiazoles as Pteridine reductase-1 inhibitors for the treatment of trypanosomatid infections. J Med Chem 2019;62:3989–4012.

9. Navarrete-Vázquez G, Chávez-Silva F, Colin-Lozano B, et al. Synthesis of nitro(benzothiazole) acetaldehydes and in vitro antiprotozoal effect against amitochondriate parasites Giardia intestinalis and Trichomonas vaginalis. Bioorg Med Chem 2015;23:2204–10.

10. Pudhom K, Kasai K, Terauchi H, et al. Synthesis of three classes of rhodacyanine dyes and evaluation of their in vitro and in vivo antimalarial activity. Bioorg Med Chem 2006;14:8550–63.

11. Patrick DA, Gillespie JR, McQueen J, Hulverson MA, et al. Urea derivatives of 2-aryl-benzothiazol-5-amines: a new class of potential drugs for human African trypanosomiasis. J Med Chem 2017;60:957–71.

12. Racané L, Tralić-Kulenović V, Kraljević Pavelić S, et al. Novel diamidino-substituted derivatives of phenyl benzothiazolyl and dibenzothiazolyl furans and thiophenes: synthesis, anti-proliferative and DNA binding properties. J Med Chem 2010;53:2418–32.

13. Racané LK, Pavelić S, Nhili R, et al. New anticancer active and selective phenylene-bis-benzothiazoles: synthesis, antiproliferative evaluation and DNA binding. Eur J Med Chem 2013;63:882–91.

14. Racané L, Stojković R, Tralić-Kulenović V, et al. Interactions with polynucleotides and antitumor activity of amidino and imidazolyl substituted 2-phenylbenzothiazole mesylates. Eur J Med Chem 2014;86:406–19.

15. Racané L, Sedić M, Ilić N, et al. Novel 2-thienyl- and 2-benzothienyl-substituted 6-(2-imidazolyl)benzothiazoles: synthesis; in vitro evaluation of antitumor effects and assessment of mitochondrial toxicity. Anti-Cancer Agents Med Chem 2017;17:57–66.

16. Racané L, Kralj M, Suman L, et al. Novel amidino substituted 2-phenylbenzothiazoles: synthesis, antitumor evaluation in vitro and acute toxicity testing in vivo. Bioorg Med Chem 2010;18:10384–44.

17. Brantley E, Trapani V, Alley MC, et al. Fluorinated 2-(4-amino-3-methylphenyl)benzothiazoles induce CYP1A1 expression, become metabolized, and bind to macromolecules in sensitive human cancer cells. Drug Metab Dispos 2004;32:1392–401.

18. Stevens MFG, McCall CJ, Lelievald P, et al. Structural studies on bioactive compounds. 23. Synthesis of polyhydroxylated 2-phenylbenzothiazoles and a comparison of their cytotoxicities and pharmacological properties with genistein and quercetin. J Med Chem 1994;37:1689–95.

19. Shi DF, Bradshaw TD, Wrigley S, et al. Antitumor benzothiazoles. 3. Synthesis of 2-(4-amino phenyl)benzothiazoles and evaluation of their activities against breast cancer cell lines in vitro and in vivo. J Med Chem 1996;39:3375–84.

20. Bradshaw TD, Stevens MFG, Westell AD. The discovery of the potent and selective antitumour agent 2-(4-amino-3-methylphenyl)benzothiazole (DF 203) and related compounds. Curr Med Chem 2001;8:203–10.

21. Fichtner I, Monks A, Hose C, et al. The experimental antitumor agents Phortress and doxorubicin are equipotent against human-derived breast carcinoma xenograft models. Breast Cancer Res Treat 2004;87:97–107.

22. Gurdal EE, Durmaz I, Cetin-Atalay R, Yarim M. Cytotoxic activities of some benzothiazole-piperazine derivatives. J Enzym Inhib Med Chem 2015;30:649–54.

23. Bhuvá HA, Kini SG. Synthesis, anticancer activity and docking of some substituted benzothiazoles as tyrosine kinase inhibitors. J Mol Graph Model 2010;29:32–7.

24. Choi SJ, Park HJ, Lee SK, et al. Solid phase combinatorial synthesis of benzothiazoles and evaluation of topoisomerase II inhibitory activity. Bioorg Med Chem 2006;14:1229–35.

25. Lion CJ, Matthews CS, Wells G, et al. Antitumour properties of fluorinated benzothiazole substituted hydroxycyclohexa-2,5-dienones (‘quinols’). Bioorg Med Chem Lett 2006;16:5005–8.

26. Zhang L, Fan J, Vu K, et al. 7-substituted benzothiazolo-thio and pyridinothiazolo-purines as potent heat shock protein 90 inhibitors. J Med Chem 2006;49:5352–62.

27. Singh M, Singh SK, Thakur B, et al. Design and synthesis of novel Schiff base-benzothiazole hybrids as potential epidermal growth factor receptor (EGFR) inhibitors. Anticancer Agents Med Chem 2016;16:722–39.

28. Sharma PC, Sinharma A, Sharma A, et al. Medicinal significance of benzothiazole scaffold: an insight view. J Enzym Inhib Med Chem 2013;28:240–66.

29. Osmaniye D, Levent S, Karaduman AB, et al. Synthesis of new benzothiazole acylhydrazones as anticancer agents. Molecules 1975;24:1639–41.

30. Sović I, Jambon S, Kraljević Pavelić S, et al. Synthesis, antitumor activity and DNA binding features of benzothiazolyl and benzimidazolyl substituted isoindolines. Bioorg Med Chem 2018;26:1950–60.

31. Supuran CT. Carbonic anhydrase inhibitors as emerging agents for the treatment and imaging of hypoxic tumors. Expert Opin Investig Drugs 2018;27:963–70.

32. Ibrahim DA, Lasheen DS, Zaky MY, et al. Design and synthesis of benzothiazole-6-sulfamides acting as highly potent inhibitors of carbonic anhydrase isoforms I, II, IX and XII. Bioorg Med Chem 2015;23:4989–99.

33. De Koning HP. The drugs of sleeping sickness: their mechanisms of action and resistance, and a brief history. Trop Med Infect Dis 2020;5:14–37.

34. Bhattacharya A, Corbel A, Do Monte-Neto RL, Fernandes-Prada C. Of drugs and trypanosomatids: new tools and knowledge to reduce bottlenecks in drug discovery. Genes 1975;24:1639–746.

35. Avila-Sorrosa A, Tapia-Alvarado JD, Nogueda-Torres B, et al. Facile synthesis of a series of non-symmetric thioethers including a benzothiazole moiety and their use as efficient in vitro anti-Trypanosoma cruzi agents. Molecules 2019;24:3077–85.

36. Cuevas-Hernández RL, Girard RMBM, Martínez-Cerón S, et al. A fluorinated phenylbenzothiazole arrests the Trypanosoma cruzi cell cycle and diminishes the infection of mammalian host cells. Antimicrob Agents Chemother 1975;24:e01742–641.

37. Fleau C, Padilla A, Miguel-Siles J, et al. Chagas disease drug discovery: multiparametric lead optimization against Trypanosoma cruzi in Acylaminobenzothiazole series. J Med Chem 2019;62:10362–75.

38. Bistrić A, Krstulović L, Stolić I, et al. Synthesis, anti-bacterial and anti-protozoal activities of amidinobenzimidazole derivatives and their interactions with DNA and RNA. J Enzym Inhib Med Chem 2018;33:1323–34.
39. Bistrović Popov A, Stolić I, Krstulović L, et al. Novel symmetric bis-benzimidazoles: synthesis, DNA/RNA binding and antitrypanosomal activity. Eur J Med Chem 2019;173:63–75.

40. Bistrović Popov A, Krstulović L, Kostrun S, et al. Design, synthesis, antitrypanosomal activity, DNA/RNA binding and in vitro ADME profiling of novel imidazole-substituted 2-arylbenzimidazoles. Eur J Med Chem 2020;207:112802–21.

41. Bistrović A, Krstulović L, Harej A, et al. Design, synthesis and biological evaluation of novel benzimidazole amidines as potent multi-target inhibitors for the treatment of non-small cell lung cancer. Eur J Med Chem 2018;143:1616–34.

42. Boechat N, Ferreira VF, Ferreira SB, et al. Novel 1,2,3-triazole derivatives for use against Mycobacterium tuberculosis H37Rv (ATCC 27294) strain. J Med Chem 2011;54:5988–99.

43. Bistrović L, Ferreira GR, Spyvoo M. Preparation of aminopyrimidines as heparan sulfate biosynthesis inhibitors for the treatment of diseases. Patent WO 2015-US54761, USA; 2015.

44. Eriksson M, Nordén B. Linear and circular dichroism of drug-nucleic acid complexes. Methods Enzymol 2001;340:68–98.

45. Racané L, Tralić-Kulenović V, Mihalić Ž, et al. Synthesis of new amidino-substituted 2-aminothiophenoles: mild basic ring opening of benzothiazole. Tetrahedron 2008;64:11594–602.

46. Racané L, Cindrič M, Zlatar I, et al. Preclinical in vitro screening of newly synthesised amidino substituted benzimidazoles and benzothiazoles. J Enzyme Inhib Med Chem 2021;36:163–74.

47. Gazivoda T, Račič-Malić S, Krštafor V, et al. Synthesis, cystostatic and anti-HIV evaluations of the new unsaturated acyclic C-5 pyrimidine nucleoside analogues. Bioorg Med Chem 2008;16:5624–34.

48. Taylor MC, Lewis MD, Fortes AF, et al. The Trypanosoma cruzi vitamin C dependent peroxidase confers protection against oxidative stress but is not a determinant of virulence. PLoS Negl Trop Dis 2015;9:e0003707–23.

49. Chaires JB, Datta Gupta N, Crothers DM. Studies on interaction of anthracycline antibiotics and deoxyribonucleic acid: equilibrium binding studies on interaction of daunomycin with deoxyribonucleic acid. Biochemistry 1982;21:3933–40.

50. Bresloff JL, Crothers DM. Equilibrium studies of ethidium-polynucleotide interactions. Biochemistry 1981;20:3547–53.

51. Chalikian TV, Völker J, Plum GE, Breslauer KJ. A more unified picture for the thermodynamics of nucleic acid duplex melting: a characterization by calorimetric and volumetric techniques. Proc Natl Acad Sci USA 1999;96:7853–8.

52. Scatbard G. The attractions of proteins for small molecules and ions. Ann NY Acad Sci 1949;51:660–72.

53. Glover NR, MacDonald GC, Entwistle J, et al. Tumor specific antibody. Patent WO2005121341, USA; 2005.

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

55. Gurova K. New hopes from old drugs: revisiting DNA-binding small molecules as anticancer agents. Future Oncol 2009;5:1685–704.

56. Dardonneville C, Nue Martinez JJ. Bis(2-aminoimidazolines) and Bisguanidines: synthetic approaches, antiparasitic activity and DNA binding properties. Curr Med Chem 2017;24:3606–32.

57. Millan CR, Acosta-Reyes FJ, Lagartera L, et al. Functional and structural analysis of AT-specific minor groove binders that disrupt DNA-protein interactions and cause disintegration of the Trypanosoma brucei kinetoplast. Nucleic Acids Res 2017;45:8378–91.

58. Margery JL, Lacroix L. Analysis of thermal melting curves. Oligonucleotides 2003;13:515–37.

59. Rodger A, Nordén B, Circular dichroism and linear dichroism. New York (NY): Oxford University Press; 1997.

60. Berova N, Nakanishi K, Wooy RW, eds. Circular dichroism: principles and applications. 2nd ed. New York (NY): Wiley-VCH; 2000.

61. Tidwell RR, Boykin DW. Dicationic DNA minor groove binders as antimicrobial agents. In: Demeunynck M, Bailly C, Wilson WD, eds. Small molecule DNA RNA binders from synthesis to molecules with nucleic acids - tutorial. Beilstein J Org Chem 2017;10:614–23.

62. Smidler K, Piantanida I, Pescitelli G. Polarization spectroscopy methods in the determination of interactions of small molecules with nucleic acids - tutorial. Beilstein J Org Chem 2018;14:84–105.

63. Radić Stojković M, Plantanida I. Tuning urea-phenanthridinium conjugates for DNA/RNA and basepair recognition. Tetrahedron 2008;64:7807–14.