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ORIGINAL ARTICLE

Head-to-head bisbenzazole derivatives as antiproliferative agents: design, synthesis, in vitro activity, and SAR analysis

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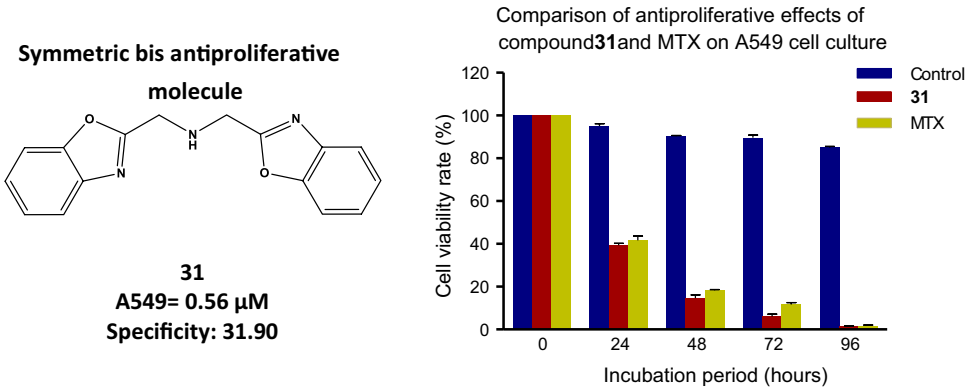
Abstract

In the present work, a series of bisbenzazole derivatives were designed and synthesized as antiproliferative agents. The antiproliferative activity of these compounds was investigated using MTT assay. Bisbenzazole derivatives showed significant antiproliferative activity against all the four tested cancer cell lines. Among the various bisbenzazole derivatives, bisbenzoxazole derivatives exhibited the most promising anticancer activity followed by bisbenzimidazole and bisbenzothiazole derivatives. All the derivatives were found to be less toxic as compared to methotrexate (positive control) in normal human cells, indicating selective and efficient antiproliferative activity of these bisbenzazole derivatives. The structure–activity relationships of heteroaromatic systems and linkers present in bisbenzazole derivatives were analyzed in detail. In silico ADMET prediction revealed that bisbenzazole is a drug-like small molecule with a favorable safety profile. Compound **31** is a potential antiproliferative hit compound that exhibits unique cytotoxic activity distinct from methotrexate.

Graphic abstract

Twenty-one bisbenzoxazole derivatives have been designed synthesized and evaluated to be an antiproliferative activity against four human tumor cell lines.



Keywords Bisbenzimidazole · Bisbenzoxazole · Bisbenzothiazole · Antiproliferative activity · SAR · ADMET

Introduction

Benzazoles are a family of heterocyclic compounds having a chemical skeleton consisting of a benzene ring fused with azole rings. Benz-fused azoles are among the most imperative class of molecules having a common heterocyclic

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scaffold found in several biologically active and medicinally significant compounds.

These compounds are known to exhibit several therapeutic activities, including anticancer, antimicrobial, antiparasitic, antiviral, antihistamine, fungicidal, and antitubercular activity [1–10]. Some of the well-known drugs with benzazole rings that are used in clinical applications are omeprazole, emedastine, candesartan, astemizole, bezitramide, domperidone, lansoprazole, flunoxaprofen, and riluzole.

Bisbenzazoles consist of two benzazole nuclei fused together using a variety of linkers. The wide spectrum of pharmacological activities displayed by bisbenzazole derivatives makes them a highly important scaffold from drug development perspective. There are several reports on the medicinal activities/properties of this class of compounds. Various studies have revealed that bis-derivatives show better antiproliferative activity than the monomeric compounds [11]. Ueki et al. and Sato et al. demonstrated the cytotoxic activity of UK-1 and AJI95618 against B-16, HeLa, and P-338 cancer cell lines [12, 13]. Hoechst 33258 has undergone phase I clinical evaluation as an anticancer agent. It has been proposed that Hoechst 33258 acts via inhibition of topoisomerase and DNA helicase [14, 15]. Rance et al. demonstrated the antiproliferative activity of novel bisbenzothiazole analogs [16]. Among them, bisbenzazole moiety is one of the most important structures in drug discovery. A number of studies have been conducted in past that involved design and synthesis of novel bioactive bisbenzazole derivatives and evaluation of their antiproliferative activity against different human cancer cell lines (Fig. 1) [17–19].

All these studies indicated the therapeutic significance of bisbenzazole derivatives. In the present study, head-to-head bisbenzazole derivatives were designed and synthesized by accumulation of two benzazole units with different aliphatic and heteroaliphatic linkers with the aim to obtain/achieve better antiproliferative agents.

As shown in Fig. 2, the designing of bisbenzazole derivatives with different linkers was broadly divided into two segments. The first segment involves main backbone

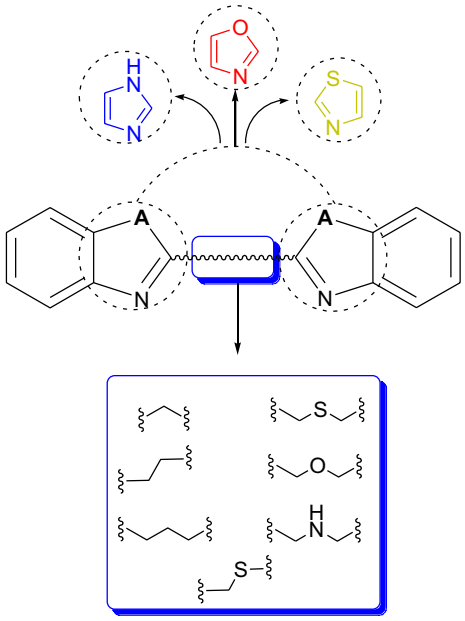
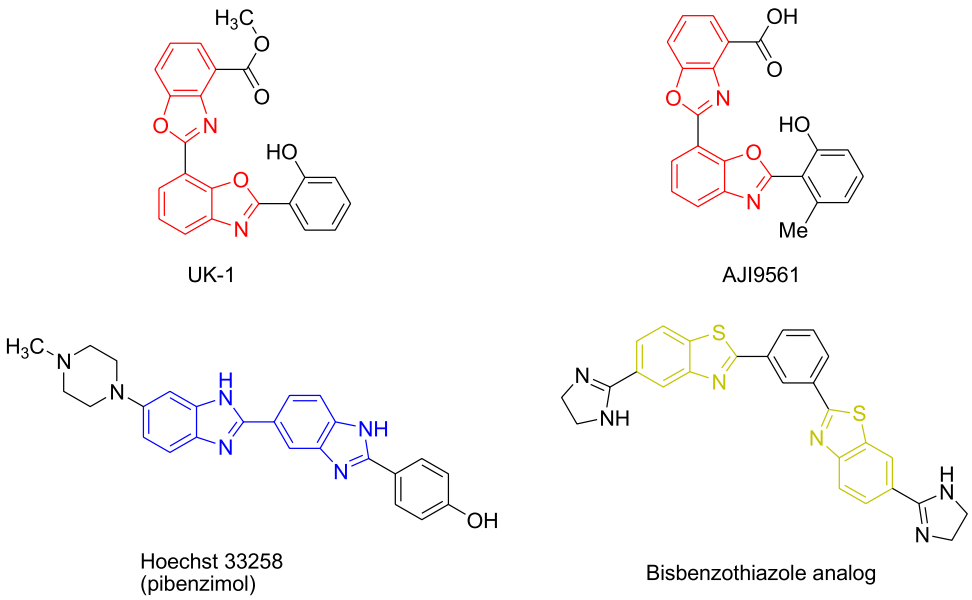
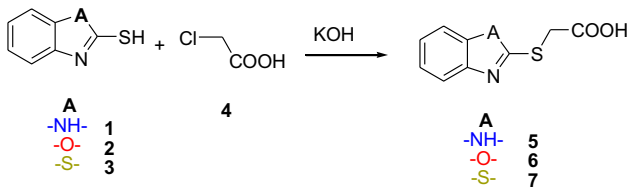


Fig. 2 Molecular design strategy for the synthesis novel bisbenzazole derivatives connected via aliphatic and heteroaliphatic linkers

Fig. 1 Bisbenzazole scaffolds as antiproliferative agents



of the design, a benzazole unit that acts as a source of hydrogen-bond acceptor and donor. This would help to enhance the pharmacophoric properties as they exhibit drug-like properties. The second segment included aliphatic linkages with or without heteroatoms, which were generally used to control the lipophilicity and flexibility of the skeleton, and in silico ADMET prediction was also performed using the compounds.



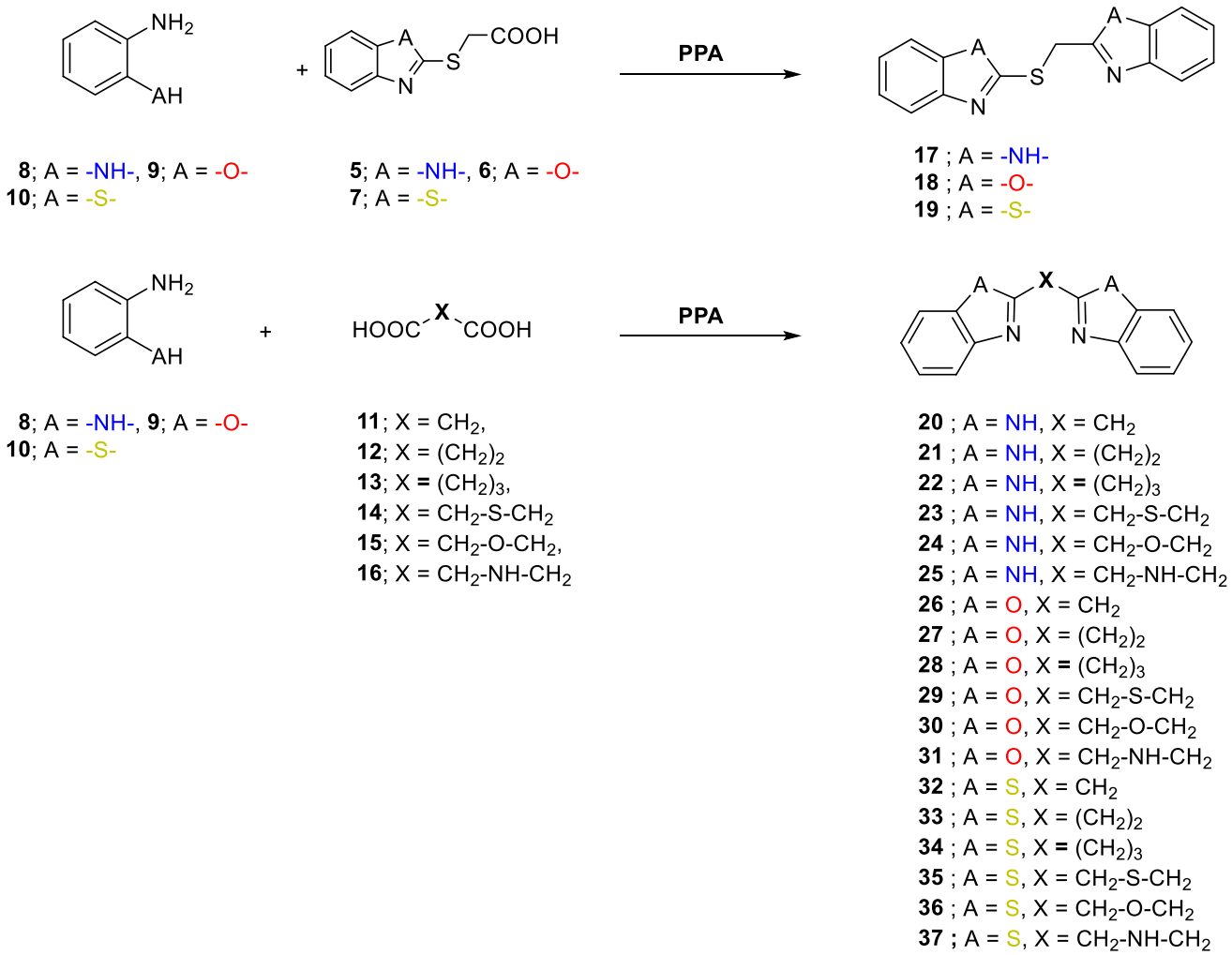
Scheme 1 Synthesis 2-((1*H*-benzazol-2-yl)thio)acetic acid (**5**–**7**) from 2-mercaptobenzazoles (**1**–**3**) and chloroacetic acid (**4**)

Results and discussion

Chemistry

The symmetric and asymmetric bisbenzazole derivatives (**17**–**37**) were synthesized using one-step and multi-step synthetic pathways as illustrated in Schemes 1 and 2. Polyphosphoric acid (PPA) method was used for the synthesis of desired compounds (**20**–**37**) (Scheme 2). For the synthesis of asymmetric compounds (**17**–**19**), 2-((1*H*-benzazol-2-yl)thio)acetic acid derivatives (**5**–**7**) were used as the starting material. These were prepared from 2-mercaptobenzazoles (**1**–**3**) and chloroacetic acid, followed by treatment with potassium hydroxide (Scheme 2).

The structures of all the designed and synthesized bisbenzazole derivatives (**17**–**37**) are shown in Scheme 2. All synthetic analogs (**17**–**37**) were characterized by physical and spectral analysis (FTIR, ^{1}H NMR, ^{13}C NMR) and



Scheme 2 Synthesis of symmetric and asymmetric bisbenzazole derivatives using PPA method (**20**–**37**)

elemental analysis. (For details, please see the Supplementary file.)

The chemical shifts of the ^{1}H NMR signals of bisbenzazole derivatives were investigated, and the signal of two protons of $–CH_2–$ linker group and four protons of $–CH_2–CH_2–$ linker group between benzazole rings is observed as a singlet with chemical shift values 3.35–4.88 ppm, while the signal of six protons of $–CH_2–CH_2–CH_2–$ linker group between benzazole rings is observed as a triplet of four protons of two terminals $–CH_2–$ attached to benzazole rings with chemical shift values 2.88–3.21 ppm and pentet or multiplet of two protons of $–CH_2–$ center of symmetry with chemical shift values 2.26–2.45 ppm. The signal of two protons of $–CH_2–S–$ linker group and four protons of $–CH_2–S–CH_2–$, $–CH_2–NH–CH_2–$, $–CH_2–O–CH_2–$ linker groups between benzazole rings is observed as a singlet with chemical shift values 3.58–5.20 ppm. The signal of aromatic –NH– protons of bisbenzimidazole rings is observed as a singlet with chemical shift values 12.70–13.69 ppm. The signal of aromatic protons of benzazole rings is observed as (s, d, dd, ddd, td, t, and m) with chemical shift values 6.87–8.17 ppm. The chemical shifts of the ^{13}C NMR signals of bisbenzazole derivatives were investigated; the values were 22.6–69.9 ppm for aliphatic carbons and 169.3–109.3 ppm for aromatic carbons.

Antiproliferative Activity

The in vitro antiproliferative activities of all the synthesized compounds (**17**–**37**) were evaluated in four human cancer cell lines, lung cancer (A549), kidney cancer (A498), cervical cancer (HeLa), and liver cancer (HepG2), using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) [20–22]. An antiproliferative drug, methotrexate, was used as the standard for comparison. The results of the MTT assay are summarized in Table 1. The antiproliferative activity of each analog was presented as the concentration of the compound that led to a 50% inhibition (IC_{50}) of cancer cell growth. These compounds were further screened for their cytotoxic nature using Vero cells (normal cell line). This was used to establish the selectivity of these active compounds toward cancerous cells. A potent anticancer compound should be more selective toward cancer cells and less toxic toward the normal cells.

To assess the specificity of the compounds, their toxicity levels were tested against Vero cells. The specificity of the compounds was calculated as their IC_{50} values for normal cells divided by their IC_{50} values for the specific cancer cells. Table 1 shows the specificity of all compounds.

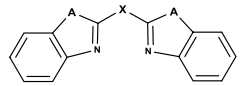
Additionally, antiproliferative activity studies were performed at non-toxic concentrations of the compounds that were determined on the Vero cell line. The most specific compound **31** and MTX were exposed for a period of 0–96 h, and A549 cells were evaluated and subjected to an assessment of their cytotoxicity responses using an MTT assay (Fig. 3). The proliferation of cancer cells treated with a non-toxic and lower-concentration dose of the compound was inhibited in a time-dependent manner. After 24, 48, and 72 h of treatment, compound **31** was more effective, and the number of live cells was lower than was observed with MTX. The antiproliferative activity of the compound increased as the exposure time was extended, and after 96-h treatment, the tested compounds had an activity equal to that of MTX.

As shown in Table 1, all the synthesized compounds showed significant antiproliferative activity against all the tested cancer cell lines. Among the synthesized compounds, bisbenzimidazole compounds **20**, **21**, and **23**; bisbenzoxazole compounds **18, 27**, **30**, and **31**; and bisbenzothiazole compounds **32** and **33** showed promising antiproliferative activity against all the tested cancer cell lines. Among the asymmetric compounds bisbenzoxazole **18** and among the symmetric compounds bisbenzoxazole derivatives **30** and **31,** and bisbenzothiazole **32** were found to be most potent as antiproliferative agents and showed low toxicity and high specificity.

As most of the compounds from this series showed potency against tested human cancer cell lines, they can serve as attractive lead molecules for the discovery of novel antiproliferative agents in future. When the compounds were evaluated according to selectivity, for A549 cells, **18**, **30**, and **31** were found to be most potent antiproliferative agents with IC_{50} values of 0.28, 0.28, and 0.56 µM, respectively. The compounds **18**, **30**, **32**, and **33** showed good antiproliferative activities against both A498 and HeLa cells. For these two cell lines, **18**, **30**, and **33** displayed IC_{50} values of 0.28 µM, 0.56 µM, and 0.52 µM, respectively. Among the various compounds screened, **27** showed better specificity against A498, HeLa, and HepG2 cell lines. For HepG2 cells, **30** and **33** were found to be most potent as indicated by IC_{50} values of 0.28 µM. In addition to this, the compounds **18**, **30**, and **33** displayed potency against all the four cell lines. The compounds **18**, **30**, and **33** showed IC_{50} values of 0.28, 0.28, and 0.68 µM, respectively, in A549; IC_{50} values of 0.28, 0.56, and 0.52 µM, respectively, in A498; IC_{50} values of 0.56, 0.56, and 0.56 µM, respectively, in HeLa; and IC_{50} values of 0.56, 0.28, and 0.28 µM, respectively, in HepG2 cells.

For cytotoxicity test in Vero cells, **31** showed IC_{50} value of 17.90 µM, indicating 32 times more selectivity toward A549 and eight times more selectivity toward A498 and HeLa cancer cell lines. Similarly, **27** displayed IC_{50} value of 18.92 µM against Vero cells. Thus, **27** was found to be 16 times more selective toward A498, HeLa, and HepG2 cells and eight times more selective toward A549 cancer cell line.

Table 1 In vitro antiproliferative activity of synthesized bisbenzazole compounds studied using MTT assay



Compound no.	*A*	*X*	IC_{50} (μM)[a]					Specificity (SI)[d]			
			Vero[b]	A549[c]	A498[c]	HeLa[c]	HepG2[c]	A549	A498	HeLa	HepG2
17	–NH–	$–S–CH_2–$	27.66 ± 2.19	221.38 ± 10.7	221.38 ± 5.36	442.70 ± 5.12	221.38 ± 7.06	0.12	0.12	0.06	0.12
18	–O–	$–S–CH_2–$	1.13 ± 0.84	0.28 ± 0.12	0.28 ± 0.63	0.56 ± 0.79	0.56 ± 0.41	4.00	4.00	2.00	2.00
19	–S–	$–S–CH_2–$	12.44 ± 0.86	48.60 ± 2.24	49.52 ± 2.57	49.35 ± 2.81	49.24 ± 3.56	0.26	0.25	0.25	0.25
20	–NH–	$–CH_2–$	5.030 ± 0.07	1.26 ± 1.55	1.26 ± 0.07	2.52 ± 1.77	2.52 ± 0.78	4.01	4.01	2.00	2.00
21	–NH–	$–CH_2–CH_2–$	0.60 ± 0.25	0.60 ± 0.38	1.19 ± 0.27	0.60 ± 0.29	0.60 ± 0.13	1.00	0.50	1.00	1.00
22	–NH–	$–CH_2–CH_2–CH_2–$	36.19 ± 1.17	18.10 ± 2.22	36.19 ± 2.88	36.18 ± 2.65	36.19 ± 2.43	2.00	1.00	1.00	1.00
23	–NH–	$–CH_2–S–CH_2$	2.12 ± 0.22	2.12 ± 0.45	1.06 ± 0.15	2.12 ± 0.54	2.12 ± 0.01.89	1.00	2.00	1.00	1.00
24	–NH–	$–CH_2–O–CH_2$	17.970 ± 3.41	8.98 ± 1.35	4.49 ± 1.02	8.98 ± 1.56	4.49 ± 0.96	2.00	4.00	2.00	4.00
25	–NH–	$–CH_2–NH–CH_2$	14.10 ± 4.32	55.17 ± 3.52	56.36 ± 5.22	56.35 ± 2.63	56.36 ± 3.31	0.26	0.25	0.25	0.25
26	–O–	$–CH_2–$	9.99 ± 1.28	4.99 ± 0.98	4.99 ± 1.67	4.99 ± 1.48	9.99 ± 2.57	2.00	2.00	2.00	1.00
27	–O–	$–CH_2–CH_2–$	18.92 ± 3.67	2.37 ± 0.85	1.18 ± 0.66	1.18 ± 0.29	1.18 ± 0.45	8.00	16.03	16.03	16.03
28	–O–	$–CH_2–CH_2–CH_2–$	35.93 ± 2.98	35.93 ± 1.88	17.97 ± 2.33	17.96 ± 2.53	35.93 ± 2.49	1.00	2.00	2.00	1.00
29	–O–	$–CH_2–S–CH_2$	33.75 ± 1.02	16.87 ± 1.05	16.87 ± 2.63	16.87 ± 0.36	16.87 ± 2.33	2.00	2.00	2.00	2.00
30	–O–	$–CH_2–O–CH_2$	2.23 ± 0.62	0.28 ± 0.37	0.56 ± 0.25	0.56 ± 0.36	0.28 ± 0.10	8.01	4.01	4.01	8.01
31	–O–	$–CH_2–NH–CH_2$	17.90 ± 0.71	0.56 ± 0.38	2.24 ± 0.84	2.24 ± 0.45	2.24 ± 0.63	31.90	8.00	8.00	8.00
32	–S–	$–CH_2–$	4.42 ± 0.83	1.11 ± 0.52	0.56 ± 0.96	1.11 ± 0.32	1.11 ± 0.06	4.01	8.01	4.01	4.01
33	–S–	$–CH_2–CH_2–$	1.05 ± 0.16	0.68 ± 0.55	0.52 ± 0.39	0.56 ± 0.72	0.28 ± 0.96	2.00	2.00	2.00	4.00
34	–S–	$–CH_2–CH_2–CH_2–$	32.21 ± 2.97	16.11 ± 1.49	16.11 ± 5.33	16.10 ± 1.43	16.11 ± 0.85	2.00	2.00	2.00	2.00
35	–S–	$–CH_2–S–CH_2$	7.61 ± 1.04	7.61 ± 1.56	3.81 ± 0.44	3.81 ± 0.36	7.61 ± 1.79	1.00	2.00	2.00	1.00
36	–S–	$–CH_2–O–CH_2$	4.00 ± 1.73	4.00 ± 1.46	2.00 ± 1.01	4.00 ± 0.75	4.00 ± 1.39	1.00	2.00	1.00	1.00
37	–S–	$–CH_2–NH–CH_2$	32.11 ± 3.55	16.10 ± 1.86	16.10 ± 2.57	16.05 ± 3.55	16.10 ± 2.84	2.00	2.00	2.00	2.00
Methotrexate[e]			0.030 ± 0.41	0.025 ± 0.53	0.016 ± 0.28	0.022 ± 0.36	0.041 ± 0.12	1.20	1.87	1.36	0.73

Abbreviation Vero; African green monkey kidney epithelial cell, A549; human lung adenocarcinoma epithelial cell line, A498; human renal cancer cell line HeLa; human cervical cancer cell line HepG2; human hepatocellular cell line. [a]The reported values represent the mean ± SD for each compound based on three independent experiments, [b]normal kidney epithelial cell, [c]cancer cells, [d]the SI values are calculated as the ratio of the IC_{50} between the Vero's and cancer cells, [e]used as reference

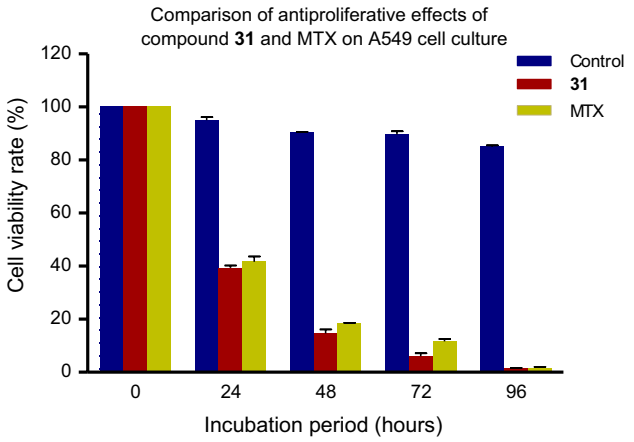


Fig. 3 MTT assay on A549 cell line after 96 h with MTX and compound **31**. The absorbance values were selected as 570 nm for the MTT method. Control cells not containing compounds and MTX were incubated same conditions. Cell viability was calculated as the ratio of absorbance of treated cells with compound or MTX to untreated cells. Given values show the mean standard deviations from three independent experiments carried out in triplicate. *Note*: *p* values: 24 h: control—31; $p < .000$; control—MTX; $p < .000$; control—MTX $p < .05$. 48 h: control—31; $p < .000$; control—MTX; $p < .000$; control—MTX $p < .05$. 72 h: control—31; $p < .000$; control—MTX; $p < .000$; control—MTX $p < .05$. 96 h: control—31; $p < .000$; control—MTX; $p < .000$; control—MTX. NS: nonsignificant

Furthermore, compound **30** with IC_{50} value 2.23 µM against Vero cells showed eight times more selectivity toward A549 and HepG2. The results of this comparative analysis suggested that the most potent compounds from this newly synthesized series were characterized by more selectivity toward cancerous cells and very less toxicity toward the normal cells.

SAR analysis

Most of the compounds from the series displayed moderate to good antiproliferative activity toward cancer cell lines. A careful examination of the data helped to establish a significantly regular SAR. The synthesized compounds were analogs of bisbenzazoles with varying alkyl or heteroalkyl linkers. Accordingly, SAR was established by the comparison of the antiproliferative activities of the different linker and bisbenzazole groups. Among these compounds, bisbenzimidazole derivatives **20, 21**, and **23**; bisbenzoxazole derivatives **18, 26, 27, 30**, and **31**; and bisbenzothiazole derivatives **32** and **33** exhibited the most potent antiproliferative activity. In contrast, compounds **22, 24, 25, 28, 29,** and **34–37** with different linker and bisbenzazole groups showed the lowest antiproliferative activity against all cancer cells. In particular, the 1C linker in bisbenzothiazole **32** and 2C linker in bisbenzimidazole and bisbenzothiazole **21** and **33** were found to be associated with increased potency of the compounds, as compared to the bisbenzoxazoles **26** and **27**, respectively. In addition, the three-atom linker groups ($–CH_2–CH_2–CH_2–$, $–CH_2–O–CH_2–$, and $–CH_2–NH–CH_2–$) in the benzazole derivatives (**22–25, 28, 29,** and **34–37**) did not significantly improve the activities against cancer cell lines, except for compound **31** against A549 and compound **30** in all cancer cell lines. In general, the 3C linkers in all benzazole scaffolds were inactive compounds for antiproliferative activity.

The resulting data are presented in Table 1. It shows that all the active compounds had generally considerable activity against cancer cell lines with IC_{50} values of 0.28–2.12 µM. Compound **30** was the most active agent against A549 and HepG2 cells with an IC_{50} value of 0.28 µM.

Physicochemical and toxicology properties

After completing structural characterization and biological activity studies, important points to know about these compounds were their physicochemical and toxicological properties. (Details are provided in supplementary content of Table S1.) All compounds were analyzed using PREADMET and DATAWARRIOR 4.07.02 software. (Details are provided in supplementary content of Table S2.) With respect to the toxicological parameters, all compounds were evaluated as good, as none showed the potential to be carcinogenic or mutagenic, and they showed a medium-level ability to inhibit the hERG potassium channel and CYP450. For ADME properties, in general, the compounds' inhibitors were well evaluated; only compound **31** presented a weak binding to plasma proteins, low absorption in the brain–blood barrier, and good absorption in the intestinal system. In the case of drug-like parameters, almost the all of the compounds reached values allowing them to be considered as potential oral drugs.

All of the synthesized compounds were found to comply with Lipinski's rule of five. With respect to the toxicological parameters, none of the synthesized compounds had estimated mutagenic, carcinogenic, irritant, or reproduction effects. In the case of drug-like parameters, all compounds can be considered as potential oral drugs because they are well absorbed in the human intestine. Except for compounds **29** and **35,** all compounds inhibit the hERG potassium channel. Only **25** of the compounds does not inhibit CYP450. In addition, compound **25**, unlike most of the others, follows the lead-like rule and is weakly bound to plasma proteins. (Details are provided in supplementary content of Table S2.)

Conclusion

Bisbenzazole analogs (**17**–**37**) were synthesized, characterized, and evaluated for their antiproliferative activities against different cell lines (A549, A498, HeLa, and HepG2). Having a $–CH_2–O–CH_2–$ linker at the 2-position of the benzoxazole moiety enhanced the cytotoxic activity against the designated cell lines at low micromolar concentrations. The structure–activity relationships of heteroaromatic systems and linkers present in bisbenzazole derivatives were analyzed in detail.

Compounds **18**, **21**, **30,** and **33** displayed the highest antiproliferative activity and lower IC_{50} (μM) values against all cancer cell lines, compared to MTX. More remarkably, compound **31** showed approximately 16-fold better cytotoxicity on A549 cells, with approximately 13-fold high specificity, compared to MTX. The results also demonstrated that compound **27** has significant antiproliferative effects on A498, HeLa, and HepG2 cells, in comparison with MTX. Moreover, compound **27** showed more selective but much higher IC_{50} values than other active compounds and MTX; therefore, it was a less active compound. As a result, we can conclude that the linker group consists of two or three atoms and that one of the atoms should be a heteroatom.

The clinical application of most anticancer drugs has been found to be associated with certain side effects or toxicity. These limitations of anticancer drugs are mostly attributed to the poor selectivity of drugs toward cancer cells. The selectivity of drugs is an important factor affecting their utility. In the present study, a pharmacophore hypothesis was developed to analyze SARs between the molecular structures of the synthesized bisbenzazole derivatives and observed biological activity in the A549 cell line. At the same time, the physicochemical properties of the compounds play an important role in reaching the active region of the cell and the interactions with the active region.

In conclusion, our studies have shown that bisbenzazole scaffolds have good antiproliferative effects on all cancer cell lines. However, linkers are also important for changing antiproliferative activities. SAR studies of symmetrical derivatives showed that 1C linker compounds have moderate activity, although 2C linker compounds displayed more improved antiproliferative activity against all tested cancer cell lines. Interestingly, all three-atom linker compounds were inactive. Also, in general, three-atom linker compounds are less active than 2C linker compounds, except for compound **30**, which showed good antiproliferative activity against all cancer cell lines.

For asymmetrical bisbenzoxazole derivative, compound **18** exhibited good antiproliferative activity compared to other benzazoles (**17** and **19**).

Finally, in silico ADMET prediction highlighted that all compounds have desirable drug-like properties, favorable safety profiles, and comply with Lipinski's rule of five.

Experimental

Chemistry

All reagents used were commercially available unless otherwise specified, and all solvents were distilled before use. The precursor (**5**–**7**) was synthesized using the reported method in the literature [23]. Melting points (mp) were determined with Mettler Toledo MP90 melting point device. General reaction visualization was achieved by thin-layer chromatography (TLC) purchased from Merck KGaA (silica gel 60 F254) based on Merck DC plates (aluminum based) by using UV light (254 nm). Chromatographic separations were carried out using silica gel 60 (Merck, 63–200 μm particle size, 60–230 mesh). FTIR spectra were recorded as ATR on a Perkin Elmer Spectrum One FTIR spectrometer. All the nuclear magnetic resonance spectra of the analogs obtained on Bruker spectrometers (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) in ppm (δ) refer to the solvent signal center at δ (7.19 and 76.0) ppm, δ (2.52 and 39.5) ppm, and δ (3.34 and 49.0) ppm for $CDCl_3$, d_6-DMSO, and d_4-CH_3OH, respectively. Chemical shifts (δ) are reported in ppm. Coupling constants (*J*) are reported in Hz. Standard abbreviations indicating multiplicity were used as follows: br s (broad singlet), s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), m (multiplet), and dd (doublet of doublets).

General procedure for preparation of compounds (20–37) 1,2-Phenylenediamine (**8**), 2-aminophenol (**9**), or 2-mercaptoanilin (**10**) (2 eq) and the corresponding dicarboxylic acid derivatives (**11**, **12**, **13**, **14**, **15**, and **16**) (1 eq) are heated for a period of 13–15 h in PPA at 180 °C. The reaction was monitored by thin-layer chromatography (TLC). UV (ultraviolet) light was used in the determination of stains in the works of TLC (Kieselgel 60 F254, ready-to-use aluminum plate coated with 0.2 mm thickness) which was made by using ready-made plates. After cooling, the reaction mixture was poured into ice water and neutralized by mixing with 5 M NaOH till slightly basic pH (8–9) to get the precipitate. The resulting precipitate was filtered off, washed with cold water, and crystallized with a suitable solvent. The resulting crystalline compounds were filtered, and the vacuumed product was dried.

Bis(1H-benzo[d]imidazol-2-yl)methane (20) The above procedure was followed with **8** and **11** to yield **20** as a brown powder solid (36% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.60; **mp** = 119–123 °C; **IR** (KBr, cm^{-1}) Vmax 3058, 2962, 1656, 1314, 756, 672. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [24, 25]. **Anal. calcd.** for $C_{15}H_{12}N_4$: C, 72.56; H, 4.87; N, 22.57. Found: C, 72.44; H, 4.95; N, 22.63.

1,2-Bis(1H-benzo[d]imidazol-2-yl)ethane (21) **[26, 27]** The above procedure was followed with **8** and **12** to yield **21** as a white powder solid (55% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.58; **mp** = 119–123 °C; **IR** (KBr, cm^{-1}) Vmax 3387, 3200, 2719, 2608, 1626, 1574, 814, 764; **^{1}H NMR** (400 MHz, d_6-DMSO) δ 7.84–7.76 (m, 4H, Ar–H), 7.60–7.49 (m, 4H, Ar–H), 3.91 (s, 4H, CH_2); **^{13}C NMR** (100 MHz, d_6-DMSO) δ 151.5, 131.3, 125.3, 113.8, 23.7. **Anal. calcd.** for $C_{16}H_{14}N_4$: C, 73.26; H, 5.38; N, 21.36. Found: C, 73.34; H, 5.29; N, 21.25.

1,3-Bis(1H-benzo[d]imidazol-2-yl)propane (22) The above procedure was followed with **8** and **13** to yield **22** as a brown powder solid (65% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.48; **mp** = 270–275 °C; **IR** (KBr, cm^{-1}) Vmax 3048, 2952, 1547, 1435, 735. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [28, 29]. **Anal. calcd.** for $C_{17}H_{16}N_4$: C, 73.89; H, 5.84; N, 20.27. Found: C, 73.74; H, 5.79; N, 20.25.

Bis((1H-benzo[d]imidazol-2-yl)methyl)sulfane (23) The above procedure was followed with **8** and **14** to yield **23** as a brown powder solid (47% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.71; **mp** = 119–122 °C; **IR** (KBr, cm^{-1}) Vmax 3055, 2781, 1510, 1437, 725. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [30–32]. **Anal. calcd.** for $C_{16}H_{14}N_4S$: C, 65.28; H, 4.79; N, 19.03; S, 10.89. Found: C, 65.14; H, 4.89; N, 19.15; S, 10.95.

2,2′-(Oxybis(methylene))bis(1H-benzo[d]imidazole) (24) The above procedure was followed with **8** and **15** to yield **24** as an orange powder solid (70% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (chloroform/methanol 9:1) = 0.72; **mp** = 122–126 °C; **IR** (KBr, cm^{-1}) Vmax 3056, 2915, 1439, 731. The **^{1}H NMR** spectrum is in agreement with the reported data [33, 34]. **^{13}C NMR** (100 MHz, DMSO-d_6) δ 157.5, 151.3, 136.6, 134.3, 129.6, 127.8, 123.8, 121.6, 116.1, 114.8, 114.6, 62.5. **Anal. calcd.** for $C_{16}H_{14}N_4O$: C, 69.05; H, 5.07; N, 20.13. Found: C, 69.14; H, 4.97; N, 20.25.

Bis((1H-benzo[d]imidazol-2-yl)methyl)amine (25) The above procedure was followed with **1** and **16** to yield **25** as a brown powder solid (47% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.60; **mp** = 170–175 °C; **IR** (KBr, cm^{-1}) Vmax 3469, 3139, 2991, 1506, 1422, 747. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [35–37]. **Anal. calcd.** for $C_{16}H_{15}N_5$: C, 69.29; H, 5.45; N, 25.25. Found: C, 69.32; H, 5.51; N, 25.37.

Bis(benzo[d]oxazol-2-yl)methane (26) The above procedure was followed with **9** and **11** to yield **26** as a brown powder solid (62% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.86; **mp** = 121–123 °C; **IR** (KBr, cm^{-1}) Vmax 3093, 3066, 2956, 1614, 1570, 1239, 833, 742. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [38, 39]. **Anal. calcd.** for $C_{15}H_{10}N_2O_2$: C, 71.99; H, 4.03; N, 11.19. Found: C, 72.12; H, 3.92; N, 11.15.

1,2-Bis(benzo[d]oxazol-2-yl)ethane (27) **[40, 41]** The above procedure was followed with **9** and **12** to yield **27** as an orange powder solid (60% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (chloroform/methanol 95:05) = 0.59; **mp** = 135–138 °C; **IR** (KBr, cm^{-1}) Vmax 3098, 3059, 2926, 1611, 1569, 1242, 831, 752. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.66–7.57 (m, 2H, Ar–H), 7.47–7.38 (m, 2H, Ar–H), 7.26–7.23 (m, 4H, Ar–H), 3.51 (s, 4H, CH_2); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 163.9, 160.0, 140.2, 123.8, 123.2, 118.8, 109.4, 24.5. **Anal. calcd.** for $C_{16}H_{12}N_2O_2$: C, 72.72; H, 4.58; N, 10.60. Found: C, 72.61; H, 4.63; N, 10.49.

1,3-Bis(benzo[d]oxazol-2-yl)propane (28) **[42, 43]** The above procedure was followed with **9** and **13** to yield **28** as a pink powder solid (70% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.63; **mp** = 146–150 °C; **IR** (KBr, cm^{-1}) Vmax 3051, 2997, 1571, 1450, 1240, 756; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.62–7.54 (m, 2H, Ar–H), 7.43–7.36 (m, 2H, Ar–H), 7.26–7.20 (m, 4H, Ar–H), 3.06 (t, J = 7.34 Hz, 4H, $–CH_2$), 2.45 (p, J = 7.34 Hz, 2H, $–CH_2$); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 165.0, 149.9, 140.3, 123.6, 123.2, 109.3, 26.8, 22.6. **Anal. calcd.** for $C_{17}H_{14}N_2O_2$: C, 73.37; H, 5.07; N, 10.07. Found: C, 73.41; H, 4.98; N, 10.21.

Bis(benzo[d]oxazol-2-ylmethyl)sulfane (29) **[44, 45]** The above procedure was followed with **9** and **14** to yield **29** as an orange powder solid (55% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.50; **mp** = 189–193 °C; **IR** (KBr, cm^{-1}) Vmax 3131, 2900, 1548, 1455, 1278, 744; **^{1}H NMR** (400 MHz, d_4-CH_3OH) δ 7.78 (dd, J = 1.44, 8.01 Hz, 2H, Ar–H), 7.00 (td, J = 1.53, 8.06 Hz, 2H, Ar–H), 6.87 (dd, J = 1.24, 8.08 Hz, 2H, Ar–H), 6.87 (td, J = 1.44, 8.03 Hz, 2H, Ar–H), 3.63 (s, 4H, $–CH_2$); **^{13}C NMR** (100 MHz, DMSO-d_6) δ 157.6, 130.9, 128.8,

128.6, 116.6, 35.4. **Anal. calcd.** for $C_{16}H_{12}N_2O_2S$: C, 64.85; H, 4.08; N, 9.45; S, 10.82. Found: C, 64.96; H, 4.03; N, 9.38; S, 10.95.

2,2′-(Oxybis(methylene))bis(benzo[d]oxazole) (30) [46] The above procedure was followed with **9** and **15** to yield **30** as a brown powder solid (35% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.50; **mp** = 191–194 °C; **IR** (KBr, cm^{-1}) Vmax 3048, 2948, 1589, 1452, 1251, 795; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.14 Hz, 2H, Ar–H), 7.83 (d, *J* = 7.94 Hz, 2H, Ar–H), 7.47 (t, *J* = 7.74 Hz, 2H, Ar–H), 7.36 (t, *J* = 7.19 Hz, 2H, Ar–H), 3.74 (s, 4H, $–CH_2$); **^{13}C NMR** (100 MHz, DMSO-d_6) δ 159.7, 151.3, 129.3, 126.3, 125.0, 122.2, 116.6, 63.9. **Anal. calcd.** for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 9.99. Found: C, 68.65; H, 4.43; N, 10.11.

Bis(benzo[d]oxazol-2-ylmethyl)amine (31) The above procedure was followed with **9** and **16** to yield **31** as a red powder solid (30% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (chloroform/methanol 95:05) = 0.25; **mp** = 132–136 °C; **IR** (KBr, cm^{-1}) Vmax 3453, 3058, 2930, 1569, 1459, 1243, 783; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.68 (dd, *J* = 3.35, 5.66 Hz, 2H, NH), 7.52–7.46 (m, 2H, Ar–H), 7.32 (t, *J* = 6.43 Hz, 4H, Ar–H), 3.58 (s, 4H, $–CH_2$); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 149.3, 127.0, 126.7, 123.5, 120.6, 116.7, 37.2. **Anal. calcd.** for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 68.75; H, 4.59; N, 15.17.

Bis(benzo[d]thiazol-2-yl)methane (32) The above procedure was followed with **10** and **11** to yield **32** as a green powder solid (49% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (chloroform) = 0.33; **mp** = 170–174 °C; **IR** (KBr, cm^{-1}) Vmax 3053, 2951, 1592, 1590, 1503, 1062, 854, 729. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [24, 25]. **Anal. calcd.** for $C_{15}H_{10}N_2S_2$: C, 63.80; H, 3.57; N, 9.92; S, 22.71. Found: C, 63.71; H, 3.66; N, 9.81; S, 22.64.

1,2-Bis(benzo[d]thiazol-2-yl)ethane (33) The above procedure was followed with **10** and **12** to yield **33** as a yellow powder solid (55% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.37; **mp** = 137–140 °C; **IR** (KBr, cm^{-1}) Vmax 3055, 2993, 1591, 1510, 1088, 877, 724. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [47, 48]. **Anal. calcd.** for $C_{16}H_{12}N_2S_2$: C, 64.83; H, 4.08; N, 9.45; S, 21.64. Found: C, 64.95; H, 3.96; N, 9.56; S, 21.76.

1,3-Bis(benzo[d]thiazol-2-yl)propane (34) [49, 50] The above procedure was followed with **10** and **13** to yield **34** as a green powder solid (61% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (chloroform) = 0.61; **mp** = 199–202 °C; **IR** (KBr, cm^{-1}) Vmax 3050, 2981, 1515, 1436, 1050, 760; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 8.13 Hz, 2H, Ar–H), 7.77 (d, *J* = 7.99 Hz, 2H, Ar–H), 7.39 (t, *J* = 8.10, 2H, Ar–H), 7.29–7.27 (m, 2H, Ar–H), 3.21 (t, *J* = 7.05, 4H, $–CH_2$), 2.43 (m, 4H, $–CH_2$); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.6, 153.3, 135.2, 126.0, 124.8, 122.7, 121.5, 33.4, 29.0. **Anal. calcd.** for $C_{17}H_{14}N_2S_2$: C, 65.77; H, 4.55; N, 9.02; S, 20.66. Found: C, 65.85; H, 4.66; N, 9.17; S, 20.47.

Bis(benzo[d]thiazol-2-ylmethyl)sulfane (35) [51, 52] The above procedure was followed with **10** and **14** to yield **35** as a brown powder solid (49% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.54; **mp** = 189–193 °C; **IR** (KBr, cm^{-1}) Vmax 3059, 2966, 1513, 1433, 1095, 762; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.12 Hz, 2H, Ar–H), 7.76 (d, *J* = 7.95 Hz, 2H, Ar–H), 7.40–7.27 (m, 4H, Ar–H), 4.17 (s, 4H, $–CH_2$); **^{13}C NMR** (100 MHz, DMSO-d_6) δ 155.9, 135.5, 135.1, 129.0, 128.7, 127.2, 125.8, 115.8, 33.6. **Anal. calcd.** for $C_{16}H_{12}N_2S_3$: C, 58.50; H, 3.68; N, 8.53; S, 29.29. Found: C, 58.63; H, 3.74; N, 8.67; S, 29.12.

2,2′-(Oxybis(methylene))bis(benzo[d]thiazole) (36) The above procedure was followed with **10** and **15** to yield **36** as a cream powder solid (67% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.72; **mp** = 103–106 °C; **IR** (KBr, cm^{-1}) Vmax 3062, 2884, 1528, 143, 1038, 757. The **^{1}H NMR** spectrum is in agreement with the reported data [53, 54]. **^{13}C NMR** (100 MHz, DMSO-d_6) δ 169.0, 152.6, 134.5, 126.3, 125.3, 122.7, 122.4, 69.9. **Anal. calcd.** for $C_{16}H_{12}N_2OS_2$: C, 61.51; H, 3.87; N, 8.97; S, 20.53. Found: C, 61.42; H, 3.73; N, 8.84; S, 20.44.

Bis(benzo[d]thiazol-2-ylmethyl)amine (37) The above procedure was followed with **10** and **16** to yield **37** as a yellow powder solid (40% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (chloroform/methanol 95:05) = 0.27; **mp** = 101–104 °C; **IR** (KBr, cm^{-1}) Vmax 3055, 2995, 1510, 1437, 1012, 724. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data [55, 56]. **Anal. calcd.** for $C_{16}H_{13}N_3S_2$: C, 61.71; H, 4.21; N, 13.49; S, 20.59. Found: C, 61.82; H, 4.19; N, 13.37; S, 20.48.

Synthesis of 2-((benzazol-2-yl)thio)acetic acid (5–7) A mixture of 2-mercaptobenzimidazole (**1**), 2-mercaptobenzoxazole (**2**), 2-mercaptobenzothiazole (**3**) (1 eq), and potassium hydroxide (1.2 eq) in methanol (20 mL) was stirred for 1 h. Then, 2-chloroacetic acid (**4**) (1.1 eq) was added into the mixture and the reaction mixture was refluxed for 8 h. After monitoring the reaction with TLC, the solvent was removed under reduced pressure, washed with cold water, dried, and recrystallized in ethanol to furnish the desired compounds (**5**–**7**).

2-((1H-benzo[d]imidazol-2-yl)thio)acetic acid (5) **[23]** The above procedure was followed with **1** and **4** to yield **5** as a white powder (91% yield). The crystallization solvent is ethanol. $\mathbf{R_f}$ (ethyl acetate/hexanes 2:1) = 0.16; **mp** = 120–123 °C; **^{1}H NMR** (400 MHz, d_6-DMSO) δ 7.46–7.44 (m, 2H, Ar–H), 7.15–7.12 (m, 2H, Ar–H), 4.15 (s, 2H, $–CH_2$).

2-(Benzo[d]oxazol-2-ylthio)acetic acid (6) **[23]** The above procedure was followed with **2** and **4** to yield **6** as a pink crystal (75% yield). The crystallization solvent is ethanol. $\mathbf{R_f}$ (ethyl acetate/hexanes 2:1) = 0.2; **mp** = 110–113 °C; **^{1}H NMR** (400 MHz, d_6-DMSO) δ 7.66–7.63 (m, 2H, Ar–H), 7.36–7.32 (m, 2H, Ar–H), 4.22 (s, 2H, $–CH_2$).

2-(benzo[d]thiazol-2-ylthio)acetic acid (7) **[23]** The above procedure was followed with **3** and **4** to yield **7** as a white powder (83% yield). The crystallization solvent is ethanol. $\mathbf{R_f}$ (ethyl acetate/hexanes 2:1) = 0.16; **mp** = 118–121 °C; **^{1}H NMR** (400 MHz, d_6-DMSO) δ 8.03 (d, J = 7.71 Hz, 1H, Ar–H), 7.87 (d, J = 7.14 Hz, 1H, Ar–H), 7.51–7.41(m, 1H, Ar–H), 7.41–7.36 (m, 1H, Ar–H), 4.12 (s, 2H, $–CH_2$).

General Procedure for preparation of compounds (17–19) A mixture of *o*-phenylenediamine (**8**), 2-aminophenol (**9**), 2-mercaptoaniline (**10**) (1 eq), and 2-((benzazol-2-yl)thio) acetic acid (**5**–**7**) (1 eq) in PPA (5–7 g) was heated for 12 h in an oil bath at 150 °C. The reaction mixture was poured into ice water and neutralized by mixing with 5 M NaOH till slightly basic pH (8–9) to get the precipitate. The resulting precipitate was filtered off, washed with cold water, and recrystallized with ethanol–water.

2-(((1H-benzo[d]imidazol-2-yl)methyl)thio)-1H-benzo[d]imidazole (17) **[57, 58]** The above procedure was followed with **8** and **5** to yield **17** as a white powder (65% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.67; **mp** = 258–262 °C; **IR** (KBr, cm^{-1}) Vmax 3053, 2931, 1504, 1407, 849, 741. The **^{1}H NMR** and **^{13}C NMR** spectra are in agreement with the reported data. **Anal. calcd.** for $C_{15}H_{12}N_4S$: C, 64.26; H, 4.31; N, 19.98; S, 11.44. Found: C, 64.22; H, 4.19; N, 20.07; S, 11.58.

2-((Benzo[d]oxazol-2-ylmethyl)thio)benzo[d]oxazole (18) **[57, 58]** The above procedure was followed with **9** and **6** to yield **18** as a pink powder (56% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.63; **mp** = 146–150 °C; **IR** (KBr, cm^{-1}) Vmax 3093, 2995, 1501, 1445, 1238, 743; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.74–7.69 (m, 1H, Ar–H), 7.66–7.61 (m, 1H, Ar–H), 7.54–7.45 (m, 2H, Ar–H), 7.32 (m,,4H, Ar–H), 4.84 (s, 2H, $–CH_2$); **^{13}C NMR** (100 MHz, DMSO-d_6) δ 156.1, 131.0, 115.9, 115.5, 115.3, 33.0. **Anal. calcd.** for $C_{15}H_{10}N_2O_2S$: C, 63.81; H, 3.57; N, 9.92; S, 11.36. Found: C, 63.92; H, 3.53; N, 10.01; O, S, 11.30.

2-((Benzo[d]thiazol-2-ylmethyl)thio)benzo[d]thiazole (19) **[59]** The above procedure was followed with **10** and **7** to yield **19** as a green powder (55% yield). The crystallization solvent is ethanol–water. $\mathbf{R_f}$ (ethyl acetate/hexanes 1:1) = 0.69; **mp** = 202–206 °C; **IR** (KBr, cm^{-1}) Vmax 3153, 2878, 1656, 1462, 1212, 768; **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.01 (d, J = 8.16 Hz, 1H, Ar–H), 7.93 (d, J = 8.10 Hz, 1H, Ar–H), 7.82–7.75 (m, 2H, Ar–H), 7.50–7.29 (m, 4H, Ar–H), 5.05 (s, 2H, $–CH_2$); **^{13}C NMR** (100 MHz, DMSO-d_6) δ 163.4, 161.0, 155.5, 138.4, 135.9, 130.5, 125.2, 116.0, 115.9, 115.8, 114.0, 113.8, 33.5. **Anal. calcd.** for $C_{15}H_{10}N_2S_3$: C, 57.29; H, 3.21; N, 8.91; S, 30.59. Found: C, 57.18; H, 3.28; N, 8.87; S, 30.44.

Biochemistry

Cell culture Studies

All the synthesized compounds (**17**–**37**) were evaluated for their in vitro antiproliferative activity against four human cancer cell lines by comparing the results with the standard antiproliferative drug, methotrexate (MTX). In vitro antiproliferative screening of titled compounds against human hepatocellular cell line (HepG2), human renal cancer cell line (A498), human lung adenocarcinoma epithelial cell line (A549), and human cervical cancer cell line (HeLa) were performed using MTT assay [22]. The obtained results of in vitro antiproliferative activities are summarized in Table 1. The selectivity of these compounds toward cancerous cells is evaluated against Vero (African green monkey kidney epithelial cell) to determine the non-toxic concentrations of the compounds on relatively healthy cells. All cell lines were obtained from the cell culture collections of Mustafa Kemal University. Cell incubations were done at 37 °C in 5% (v/v) CO_2.

MTT cell viability assay

The cytotoxic activity of the compounds was determined by using MTT assay [20, 21]. The compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted to the appropriate concentrations in Dulbecco's modified Eagle's medium (DMEM). Concentration of DMSO was less than 1% in the culture medium. Subsequently, the cells were seeded at 104 cells/well in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 µg/mL streptomycin in each well of 96-well microculture plates. The cells were cultured at 37 °C for 24, 48, 72, and 96 h in an incubator containing 5% CO_2.

After incubation, cells were treated with test compounds of appropriate concentrations for 24, 48, 72, and 96 h. After incubation period, 10 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and the plates were further incubated for 4 h. Then, the medium of each well was carefully removed and formazan crystals were dissolved in 100 µL of DMSO. Absorbance was determined at 570 nm for each well using a microplate reader (Bioteck) [60].

The cell viability was expressed as percentage of the viable cells in each sample with respect to the control wells. Three independent experiments in triplicates were done for the determination of the growth inhibition of each compound. The IC_{50} values were calculated from concentration–response curves using the SPSS (SPSS Inc., Chicago) software.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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