Full Paper

Synthesis and Cytotoxicity of Bis-1,3,4-oxadiazoles and Bis-pyrazoles Derived from 1,4-Bis[5-thio-4-substituted-1,2,4-triazol-3-Yl]-butane and Their DNA Binding Studies

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A new series of 1,4-bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-substituted-1,2,4-triazol-3-yl]-butane **7-12** and 1,4-bis[5-(1-oxo-1-(3,5 dimethyl pyrazol-1-yl)-methyl)-thio-4-substitued-1,2,4-triazol-3-yl]-butane **13-18** were prepared from 1,4-bis(5[hydrazinocarbonylmethylthio]-4-substituted-1,2,4-triazol-3-yl) butane based derivativess were synthesized **1-6**. All the synthesized compounds were characterized by IR, NMR and Mass spectral studies. The synthesized compounds **7-18** were screened for *in-vitro* cytotoxicity potential using the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay against a panel of three human cancer cell lines: Lung carcinoma A-549, colon carcinoma HT-29 and breast cancer MDA MB-231. DNA binding studies were conducted for three potent molecules by absorption titration method.

Keywords: Bis-1,2,4-triazole / Bis-1,3,4-oxadiazole / Bis-pyrazole / Cytotoxic activity / MTT assay

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Introduction

Dimeric analogues of various heterocyclic compounds are drawing much attention in the recent past. This is largely due to the wide spectrum of activity exhibited by these molecules. Many dimeric compounds designed as bis-DNA intercalators have been evaluated as anticancer agents. Dimers of more lipophilic compounds have shown potent and broad spectrum activity against human solid tumor cell lines both in culture and as xenografts in nude mice. Some of the bis-intercalators were found to possess high selective toxicity against human colon carcinoma [1, 2]. The broad spectrum antifungal agent fluconazole is a bis-triazole derivative. The bis heterocyclic molecules have also shown to exhibit several diverse pharmacological activity such as antimicrobial [3], anti-protozoal [4], anti-inflammatory [5], anti-HIV [6], and cytotoxicity [7–12].

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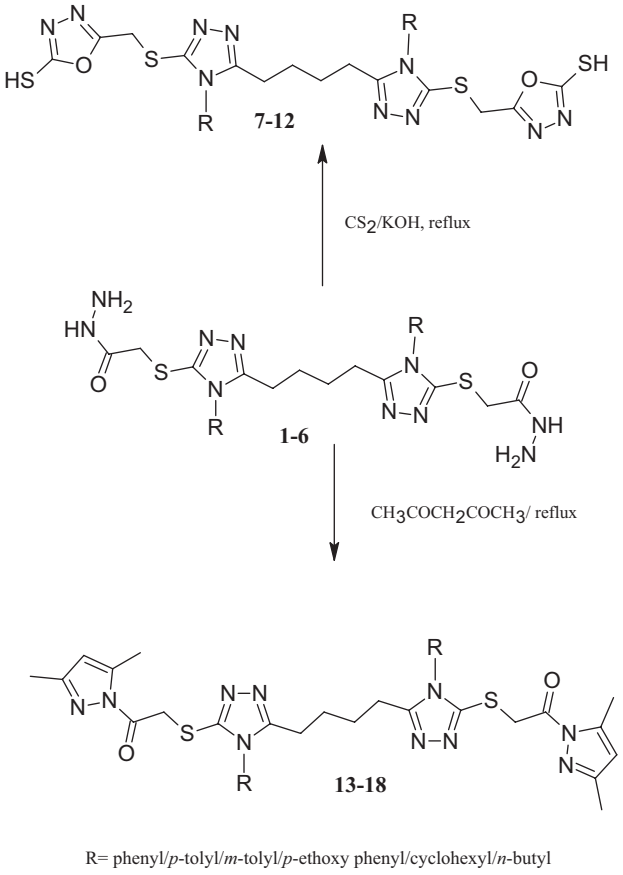
Many of the bis-1, 2, 4-triazoles [13–16], bis-oxadiazoles [17], and bis-pyrazoles [18, 19] have also been reported to possess wide spectrum of biological activity. Keeping these observations in mind and in continuation of our work on the synthesis of bis-heterocyclic compounds [20]; we report herein the synthesis and *in-vitro* cytotoxic activity of certain novel oxadiazole and pyrazole incorporated bis-1,2,4-triazoles separated by a butyl side chain. Then the most potent three molecules were subjected to DNA intercalation studies by UV-absorption method.

Results and discussion

Chemistry

Synthesis of the intermediate and target compounds was accomplished according to the steps depicted in Scheme 1. The carbohydrazides **1–6** which served as key intermediates were prepared starting from adipoyldihydrazide according to the previously reported reaction conditions [21]. Briefly, the adipoyldihydrazide was treated with six different substituted isothiocyantes namely phenyl, *p*-tolyl, *m*-tolyl, *p*-ethoxyphenyl, cyclohexyl and *n*-butyl under reflux condition



Scheme 1. Synthesis of the intermediate and target compounds.

to obtain corresponding thiosemicarbazides in quantitative yield, which in turn were further converted to respective bis-(5-mercapto or 4-substituted)-1,2,4 triazoles by heating under strong alkaline conditions. Stirring these triazoles with ethyl bromoacetate in dry acetone under reflux temperature yielded corresponding thioesters in moderate to good yield. Heating these thioesters with excess of hydrazine hydrate in absolute ethanol afforded acid hydrazides **1–6** in good yield. The spectral data and physical characteristics of **1–6** is presented else where. The bis-1,3,4-oxadiazole-5-thiones **7–12** were synthesized from intermediate acid hydrazides by refluxing with excess of carbon disulfide and potassium hydroxide. Similarly the acid hydrazides on heating with excess of acetyl acetone yielded 1,4-bis[5-(1-oxo-1-(3,5 dimethyl pyrazol-1-yl)-methyl)-thio-4-substitued-1,2,4-triazol-3-yl]-butane **13–18**. The list of compounds synthesized is given in Table 1 along with corresponding melting points and lipophilicity values. The structures of all compounds were elucidated on the basis of elemental analysis, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data.

The IR spectrum of the compound **7**, which was prepared by refluxing the carbohydrazide **1** with excess of carbon disulfide and potassium hydroxide showed absence of strong absorptions due to C=O stretching and N–H stretching indicating the cyclization of hydrazide group. The ^{1}H-NMR spectrum of the compound showed a broad hump corresponding to two protons between 5.5–5.9 ppm which was assigned to the mercapto group of the 1,3,4-oxadiazole. Further the absence CONH and NH_2 signals corresponding to the carbohydrazide moiety of the starting material **1** confirms the formation of oxadiazole. The molecular ion peak at *m/z* 637 (M + H) also is in agreement with the proposed structure. The bis-1,3,4-oxadiazoles **8-12** were similarly characterized.

Table 1. Physical and lipophilicity data of synthesized compounds.

Compound	R	Yield	MP (°C)[a]	clog *P*[b]
7	Phenyl	55	203	3.53
8	*p*-Tolyl	62	212	4.52
9	*m*-Tolyl	60	225	4.52
10	*p*-Ethoxy phenyl	45	245	4.59
11	Cyclohexyl	50	207	3.42
12	*n*-butyl	45	198	2.53
13	Phenyl	60	232	5.98
14	*p*-Tolyl	65	247	6.97
15	m-Tolyl	62	263	6.97
16	*p*-Ethoxy phenyl	60	271	7.04
17	Cyclohexyl	55	217	5.87
18	*n*-butyl	60	220	4.98

[a] Average of three trials[b] Determined by using software Bioloom (BioByte corp, USA)

In the ^{1}H-NMR spectrum of the compound **13** the aromatic protons resonated as multiplet between 7.3–7.6 ppm. The proton of C_4 of pyrazole appeared as a singlet 4.6 ppm. The singlet signal at 3.8 ppm was assigned to -SCH_2 protons. The two methyl protons of the pyrazole resonated as singlet at 2.8 and 2.6 ppm respectively. The alkyl protons of the butyl chain resonated at 2.3 and 1.2 ppm. The ^{13}C-NMR spectrum shows a signal due to carbonyl group at 195.23 ppm. The signals at 153.25 and 148.65 ppm were assigned to the carbon atoms of the triazole moiety (C_3 and C_5). The carbons of pyrazole ring appeared at 145.23, 142.75, and 112.64 ppm. The six aromatic carbon atoms of the phenyl ring were assigned to the signal at 131.42, 129.75, 128.82, and 127.39 ppm. The thiomethylene group resonated at 34.92, 17.43, and 15.83 ppm, respectively. The signals at 32.33 and 25.12 ppm were attributed to the methylene carbon atoms of the butyl chain. In conclusion, ^{1}H-NMR and ^{13}C-NMR spectral data were consistent with the proposed structures. The mass spectra of all the triazole derivatives were analyzed under ESI conditions. Molecular ions were observed in the form of M + H. Most of the compounds yield abundant molecular ions in the form of M + H peaks.

Biological activity

Lipophilicity

The lipophilic character plays a major role in the cytotoxic effect of the compounds since the efficiency of the cytotoxicity of the drug depends on the accumulation of the compound into the cell. The partition coefficient ($\log_{10}P$) of the compounds which is a measure of lipophilicity was calculated using the software Bioloom (version 1) from Biobyte Corp. (Claremont, CA). The lipophilicity data of all the compounds expressed in $\log_{10}P$, are given in Table 1. The $\log_{10}P$ values range from 2.53 to 7.04. The compounds having phenyl and substituted phenyl groups have higher $\log_{10}P$ values. The compounds **7–12** which contain tautomeric 5-mercapto-1,3,4-oxadiazole substitution were found to have lower lipophilicity (2.53–4.52) than the compounds substituted with dimethyl pyrazole **13–18** which showed higher $\log_{10}P$ values (4.98–7.04). Presence of a methyl group or ethoxy group on the phenyl ring markedly increased the $\log_{10}P$ values, while the replacement of aryl ring with cyclohexyl moiety slightly decreased the $\log_{10}P$ values. The triazoles **8**, **9**, **14** and **15** are the positional isomers which exhibit identical $\log_{10}P$ values. It is clear from $\log_{10}P$ data given in Table 1 that the triazoles have shown lipophilicity in the following order, **16** > **15** > **14** > **13** > **17** > **18** > **10** > **9** > **8** > **7** > **11** > **12**. The analysis of the relationship between $\log_{10}P$ values and the efficiency of the compounds cytotoxicity in cancer cells showed a poor correlation. The bis-triazole dimethyl pyrazole derivative **16** with ethoxy phenyl substitution having a $\log_{10}P$ value (7.04) showed significant cytotoxicity while the bis-triazole 5-mercapto-1,3,4-oxadiazole derivative **12** having n-butyl substitution with lower $\log_{10}P$ value was not effective against cancer cell lines. It is also speculated that the bis-triazole nucleus with substitution may also exhibit higher affinity for membranes or be more readily taken up into cells than that with hydrogen atom present. Therefore, we can conclude that the degree of lipophilicity of each drug would seem to be important, but it is not the sole determinant of potency for the anticancer activity of the triazoles.

Cytotoxicity studies

The cytotoxicity of all the compounds was evaluated by *in-vitro* method against the following human cancer cell lines: A-549 lung carcinoma, HT-29 colon adenocarcinoma and MDA-MB-231 breast carcinoma. The standard MTT assay was used to determine IC_{50} values *i.e.* the drug concentration that causes 50% cell-growth inhibition after 72 h of continued exposure to the test compounds and the mean of the results obtained from triplicate assays are shown in Table 2 [22–24]. The IC_{50} data reported in Table 2 indicate that the bis-triazoles substituted with 1,3,4-oxadiazole moiety **7–12** did not show significant anticancer activity where as the bis-triazoles substituted with pyrazole moiety **14**, **15**, and **16** exhibited higher cytotoxic activity against all the tested cell lines as indicated by lower IC_{50} values. The presence of electron releasing substituent such as methyl and ethoxy group seems to be contributing factor for higher activity. Apparently the high lipophilic nature of these compounds also contributes for their higher cytotoxic activity. The ethoxy substituted triazole **16** exhibited higher cytotoxicity than other molecules. Among three cell lines tested A-549 lung carcinoma cell line was found to be more susceptible against the compounds.

Table 2. *In vitro* anticancer activity of synthesized compounds.

Compound	IC_{50} (μM)[a]		
	A-549	HT-29	MDA-MB-231
7	49.91 ± 2.11	45.81 ± 2.53	71.63 ± 3.90
8	61.23 ± 2.98	48.18 ± 5.75	66.17 ± 1.74
9	55.25 ± 1.84	65.31 ± 4.72	70.34 ± 2.44
10	63.06 ± 3.63	69.82 ± 2.15	42.17 ± 1.13
11	69.94 ± 1.98	65.31 ± 1.48	24.31 ± 2.03
12	52.30 ± 3.34	31.18 ± 2.49	52.19 ± 3.51
13	35.25 ± 5.92	38.25 ± 2.66	40.17 ± 1.48
14	5.09 ± 1.68	9.24 ± 2.43	12.83 ± 3.68
15	9.23 ± 3.36	12.90 ± 3.16	15.10 ± 2.61
16	4.73 ± 2.98	6.90 ± 1.24	6.34 ± 2.91
17	63.37 ± 5.66	51.87 ± 1.10	70.20 ± 4.16
18	58.86 ± 4.57	61.50 ± 2.78	59.82 ± 3.83

[a] The drug concentration that causes 50% cell growth inhibition. A-549: Lung carcinoma; HT-29: Colon adenocarcinoma; MDA-MB-231: Breast carcinoma.SEM: average of three experiments.

DNA binding studies

The DNA-binding properties of the three potent compounds were evaluated based on their affinity or intercalation with CT-DNA measured with absorption titration method [25]. The DNA-binding properties of the compounds were studied by monitoring the changes in the UV-VIS absorption spectra of the triazole derivatives upon addition of CT-DNA. In the range from 330 to 350 all the three derivatives exhibited strong absorption peaks with maxima near 340–345 nm. Progressive addition of DNA led to strong hypochromism in the absorption intensities in all the compounds studied (Fig. 1). The percentage hypochromism were found to be 50.2, 55.3 and 54.1. The half-reciprocal plots for binding of triazoles with CT DNA were presented (Fig. 2). The compounds exhibited the similar absorption spectra pertaining to the chromophore. The selection of ionic strength (150 mM NaCl) in the absorption titration experiment was mainly based on the avoidance of DNA deposition in all drug solution (20 μm). The DNA-binding constants and related properties of derivatives were calculated after intercalation

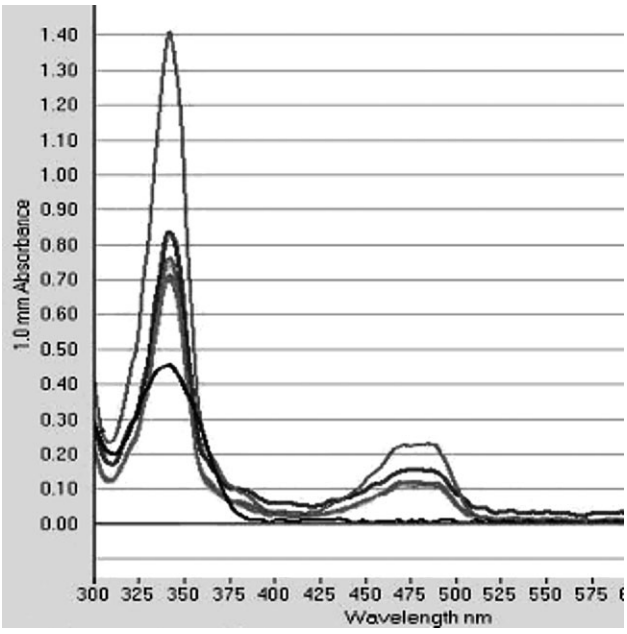


Figure 1. Absorption titration of compound 14 at 25 μM in 30 mM sodium phosphate buffer (pH 6.5) with 150 mM NaCl at increasing CT DNA concentration.

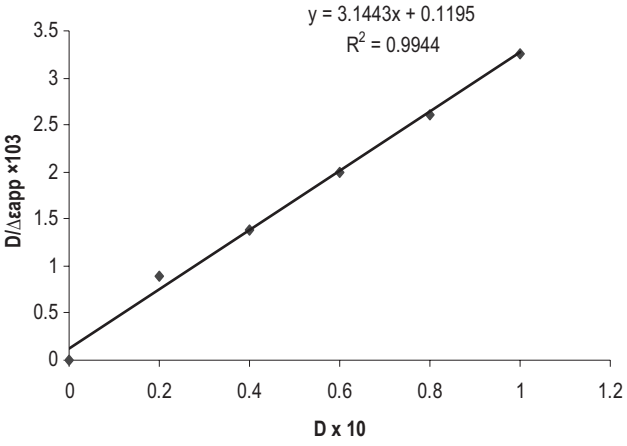


Figure 2. Half-reciprocal plot for binding of compound **14** with CT DNA.

with CT-DNA. One compound **14** containing *p*-tolyl among the derivatives studied showed strong DNA-binding affinities like hypochromicity and isobastic points.

Conclusion

Twelve new bis 1,2,4-triazoles substituted with oxadiazole and pyrazole moiety were synthesized starting from 1,4-bis-(5[hydrazinocarbonylmethylthio]-4-(substituted)-1,2,4-triazol-3-yl)-butane derivatives and characterized using IR, NMR, and LCMS data. The yields of triazoles were between 45-65%. The *in-vitro* cytotoxicity potential of the compounds was tested against the panel of three human cancer cell lines: A-549 lung carcinoma, HT-29 colon adenocarcinoma and MDA-MB-231 breast carcinoma by using standard MTT assay method. The bis-triazoles **14**, **15**, and **16** possessing the pyrazole moiety with substituents like *p*-tolyl, *m*-tolyl and *p*-ethoxy phenyl respectively were found to be more active than other compounds. Further studies are underway to understand the possible role of DNA affinity in cytotoxic activity of these compounds. DNA binding studies indicate that compound **14** has strong binding affinity among the derivatives studied.

Experimental

Materials and methods

The melting points were determined in open glass capillaries and are uncorrected. The follow-up of the reactions and checking the purity of the synthesized compounds was made by thin layer chromatography (TLC) on silica gel precoated aluminium sheets (Type $60GF_{254}$; Merck, Germany) and the spots were detected by exposure to UV lamp at λ_{254} for a few seconds. Elemental analysis was performed and found values are ±0.4% of theoretical values unless otherwise noted. IR spectra were recorded on Shimadzu FT-IR 8400-S spectrophotometer by KBr pellet technique. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on AMX-400 NMR spectrophotometer at 400 MHz using DMSO-d_6 as the solvent and tetra methyl silane (TMS) as internal standard. The chemical shifts are expressed in δ ppm. The splitting patterns were designated as follows; b: broad; s: singlet; d: doublet; m: multiplet. LCMS were recorded by using Shimadzu LCMS-2010A instrument by ESI.

General procedure for the preparation of 1,4-bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(substituted)-1,2,4-triazol-3-yl]-butane ***(7–12)***

A suspension of 1, 4-bis-(5[hydrazinocarbonylmethylthio]- 4-(substituted)-1,2,4-triazol-3-yl)-butane derivatives **1–6** (0.01 mol) in 15 mL of absolute ethanol was added to a mixture of carbon disulfide (2.28 mL, 0.03 mol) and potassium hydroxide (1.68 g, 0.03 mol) in 15 mL of absolute ethanol with continuous stirring during 10minutes. The resultant reaction mixture was heated under reflux till the evolution of hydrogen sulfide ceased. After the completion of reaction, the excess of the solvent was removed under reduced pressure and the residue was dissolved in 30 mL cold water. The resulting solution was acidified with acetic acid. The white solid that separated was filtered and crystallized from ethyl acetate. The physical data of the synthesized compounds is presented in Table 1.

1,4-Bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(phenyl)-1,2,4-triazol-3-yl]-butane ***(7)***

Yield: 55%, M.p. 203–205°C, IR (ν cm^{-1}, KBr): 3045, 2965, 1225, ^{1}H-NMR (DMSO-d_6, δ ppm): 7.2–7.6 (m, Ar–H, 10H), 5.5–5.9 (b, 2H,SH), 4.0 (s, 4H, SCH_2), 2.45 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 1.4 (t, 4H, C_2 and C_3 methylene protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 158.3 (C=S), 155.42 (C_2 of

oxadiazole), 153.20 (C_3 of triazole), 148.56 (C_5 of triazole), 131.46 (C_1 of phenyl ring), 129.85 (C_2 and C_6 of phenyl ring), 128.92 (C_3 and C_5 of phenyl ring), 127.35 (C_4 of phenyl ring), 34.92 (SCH_2), 32.31 (C_2 and C_3 of butyl chain), 25.18 (C_1 and C_4 of butyl chain); LCMS *m/z*: 637 (M + H, 40), 609 (25), 495 (45), 409 (100). Anal. calcd. for $C_{26}H_{24}N_{10}O_2S_4$: C, 49.04; H, 3.80; N, 22.00. Found: C, 49.14; H, 3.92; N, 22.06.

1,4-Bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(p-tolyl)-1,2,4-triazol-3-yl]-butane ***(8)***

Yield: 62%, M.p. 212–215°C, IR (ν cm^{-1}, KBr): 3035, 2972, 1232; ^{1}H-NMR (DMSO-d_6, δ ppm): 7.2–7.4 (m, Ar-H, 8H), 5.5–6.0 (b, 2H,SH), 3.9 (s, 4H, SCH_2), 2.5 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 2.2 (s, 6H, *p*-tolyl CH_3), 1.3 (t, 4H, C_2 and C_3 methylene protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 158.22 (C=S), 155.56 (C_2 of oxadiazole), 153.24 (C_3 of triazole), 148.55 (C_5 of triazole), 137.35 (C_4 of phenyl ring), 131.66 (C_1 of phenyl ring), 129.85 (C_2 and C_6 of phenyl ring), 128.92 (C_3 and C_5 of phenyl ring), 34.59 (SCH_2), 32.35 (C_2 and C_3 of butyl chain), 25.20 (C_1 and C_4 of butyl chain), 20.32 (*p*-tolyl CH_3); LCMS *m/z*: 665 $(M + H)^+$. Anal. calcd. for $C_{28}H_{28}N_{10}O_2S_4$: C, 50.58; H, 4.24; N, 21.07. Found: C, 50.40; H, 4.21; N, 21.16.

1,4-Bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(m-tolyl)-1,2,4-triazol-3-yl]-butane ***(9)***

Yield: 60%, M.p. 221–225°C IR (ν cm^{-1}, KBr): 3043, 2972, 1224; ^{1}H-NMR (DMSO-d_6, δ ppm): 7.1–7.3 (m, Ar-H, 8H), 5.6–6.0 (b, 2H, SH), 4.0 (s, 4H, SCH_2), 2.54 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 2.25 (s, 6H, *p*-tolyl CH_3), 1.34 (t, 4H, C_2 and C_3 methylene protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 158.25 (C=S), 155.65 (C_2 of oxadiazole), 153.31 (C_3 of triazole), 148.45 (C_5 of triazole), 137.38 (C_4 of phenyl ring), 131.62 (C_1 of phenyl ring), 129.85 (C_2 and C_6 of phenyl ring), 128.92 (C_3 and C_5 of phenyl ring), 34.79 (SCH_2), 32.35 (C_2 and C_3 of butyl chain), 25.20 (C_1 and C_4 of butyl chain), 20.45 (*p*-tolyl CH_3); LCMS *m/z*: 665 $(M + H)^+$. Anal. calcd. for $C_{28}H_{28}N_{10}O_2S_4$: C, 50.58; H, 4.24; N, 21.07. Found: C, 50.51; H, 4.28; N, 21.06.

1,4-Bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(p-ethoxyphenyl)-1,2,4-triazol-3-yl]-butane ***(10)***

Yield: 45%, M.p. 245–248°C IR (ν cm^{-1}, KBr): 3048, 2970, 1233; ^{1}H-NMR (DMSO-d_6, δ ppm): 7.1–7.6 (m, Ar-H, 8H), 5.5–6.0 (b, 2H, SH), 4.3 (q, 4H, OCH_2), 4.0 (s, 4H, SCH_2), 2.55 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 1.32 (t, 4H, C_2 and C_3 methylene protons of butyl chain), 1.13 (t, 6H, CH_3 of ethoxy group); ^{13}C-NMR (DMSO-d_6, δ ppm): 158.52 (C=S), 156.05 (C_2 of oxadiazole), 153.30 (C_3 of triazole), 148.45 (C_5 of triazole), 137.40 (C_4 of phenyl ring), 131.65 (C_1 of phenyl ring), 129.86 (C_2 and C_6 of phenyl ring), 128.90 (C_3 and C_5 of phenyl ring), 62.37 (OCH_2), 34.90 (SCH_2), 32.33 (C_2 and C_3 of butyl chain), 25.20 (C_1 and C_4 of butyl chain), 15.11 (methyl of ethoxy group); LCMS *m/z*: 725 $(M + H)^+$. Anal. calcd. for $C_{30}H_{32}N_{10}O_4S_4$: C, 49.71; H, 4.45; N, 19.32. Found: C, 49.52; H, 4.34; N, 19.51.

1,4-Bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(cyclohexyl)-1,2,4-triazol-3-yl]-butane ***(11)***

Yield: 50%, M.p. 205–207°C IR (ν cm^{-1}, KBr): 2984, 1239; ^{1}H-NMR (DMSO-d_6, δ ppm): 5.5–6.0 (b, 2H, SH), 4.0 (s, 4H, SCH_2), 3.69 (m, 2H, C_1 protons of cyclohexyl), 2.44 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 2.05 (m, 8H, C_2 and C_6 protons of cyclohexyl), 1.75 (m, 8H, C_3 and C_5 protons of cyclohexyl), 1.64 (m, 4H, C_4 protons of cyclohexyl), 1.32 (t, 4H, C_2 and C_3 methylene protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 157.52 (C=S), 155.05 (C_2 of oxadiazole), 153.39 (C_3 of triazole), 148.65 (C_5 of triazole), 40.25 (C_1 of cyclohexyl), 34.50 (SCH_2), 34.05 (C_2 and C_6 of cyclohexyl), 32.38 (C_2 and C_3 of butyl chain), 28.45 (C_4 of cyclohexyl), 25.20 (C_1 and C_4 of butyl chain), 22.11 (C_3 and C_5 of cyclohexyl); LCMS *m/z*: 649 $(M + H, 35)^+$, 535 (60), 421 (100). Anal. calcd. for $C_{26}H_{36}N_{10}O_2S_4$: C, 48.12; H, 5.59; N, 21.59. Found: C, 48.32; H, 5.48; N, 21.51.

1,4-Bis[5-(5-mercapto-1,3,4-oxadiazol-2-yl-methyl)-thio-4-(n-butyl)-1,2,4-triazol-3-yl]-butane ***(12)***

Yield: 45%, M.p. 198–202°C IR (ν cm^{-1}, KBr): 2975, 1237; ^{1}H-NMR (DMSO-d_6, δ ppm): 5.45–5.80 (b, 2H, SH), 3.90 (s, 4H, SCH_2), 3.73 (t, 4H, C_1 protons of *n*-butyl), 2.35 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 1.75 (m, 4H, C_2 protons of *n*-butyl), 1.47 (t, 4H, C_2 and C_3 methylene protons of butyl chain), 1.30 (m, 4H, C_3 protons of *n*-butyl), 0.91 (t, 6H, C_4 protons of *n*-butyl); ^{13}C-NMR (DMSO-d_6, δ ppm): 157.42 (C=S), 155.15 (C_2 of oxadiazole), 153.42 (C_3 of triazole), 148.55 (C_5 of triazole), 36.21 (C_1 of butyl), 33.88 (S-CH_2), 32.90 (C_2 of butyl), 32.10 (C_2 and C_3 of butyl chain), 25.2 (C_1 and C_4 of butyl chain), 20.12 (C_3 of butyl), 12.32 (C_4 of butyl); LCMS *m/z*: 597 $(M + H, 15)^+$, Anal. calcd. for $C_{22}H_{32}N_{10}O_2S_4$: C, 44.27; H, 5.40; N, 23.47. Found: C, 44.32; H, 5.48; N, 23.51.

General procedure for the preparation of 1,4-bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4-substituted-1,2,4-triazol-3-yl]-butane ***(13–18)***

The intermediate hydrazide derivative 1,4-bis-(5[hydrazinocarbonylmethylthio]-4-(substituted)-1,2,4-triazol-3-yl)butane derivatives **1–6** (0.01 mol) was suspended in 10 mL acetyl acetone in presence of a drop of conc. HCl and refluxed for 18–22 h (reaction monitored by TLC). The resulting solution was concentrated and poured in to crushed ice to get the solid which was further washed with petroleum ether to obtain pyrazoles derivative. Recrystallization was done by using mixture of DMF.

1,4-Bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4-(phenyl)-1,2,4-triazol-3-yl]-butane ***(13)***

Yield: 60%, M.p. 230–234°C, IR (ν cm^{-1}, KBr): 3045, 2965, 1652, 1225, ^{1}H-NMR (DMSO-d_6, δ ppm): 7.3–7.6 (m, 10H, Ar-H), 4.6 (s, 2H, C_4 of pyrazole), 3.8 (s, 4H, SCH_2), 2.8 (s, 6H, CH_3), 2.6 (s, 6H, CH_3), 2.3 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 1.2 (t, 4H, C_2 and C_3 methylene protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 195.23 (C=O), 153.25 (C_3 of triazole), 148.65 (C_5 of triazole), 145.23 (C_3 of pyrazole), 142.75 (C_5 of pyrazole), 131.42 (C_1 of phenyl ring), 129.75 (C_2 and C_6 of phenyl ring), 128.82 (C_3 and C_5 of phenyl ring), 127.39 (C_4 of phenyl ring), 112.64 (C_4 of pyrazole), 34.92 (SCH_2), 32.33 (C_2 and C_3 of butyl chain), 25.12 (C_1 and C_4 of butyl chain), 17.43 (CH_3), 15.83 (CH_3); LCMS *m/z*: 682 (M + H, 20). Anal. calcd. for $C_{34}H_{36}N_{10}O_2S_2$: C, 59.98; H, 5.33; N, 20.57. Found: C, 59.64; H, 5.12; N, 20.46.

1,4-Bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4-(p-tolyl)-1,2,4-triazol-3-yl]-butane ***(14)***

Yield: 65%, M.p. 245–248°C, IR (ν cm^{-1}, KBr): 3048, 2960, 1662, 1225, ^{1}H-NMR (DMSO-d_6, δ ppm): 7.2–7.5 (m, 8H, Ar-H), 4.5 (s, 2H, C_4 of pyrazole), 3.82 (s, 4H, SCH_2), 2.7 (s, 6H, CH_3), 2.5 (s, 6H, CH_3), 2.35 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 2.2 (s, 6H, *p*-tolyl CH_3), 1.3 (t, 4H, C_2 and C_3 methylene

protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 197.30 (C=O), 153.32 (C_3 of triazole), 148.69 (C_5 of triazole), 145.35 (C_3 of pyrazole), 142.70 (C_5 of pyrazole), 137.32 (C_4 of phenyl ring), 131.60 (C_1 of phenyl ring), 129.75 (C_2 and C_6 of phenyl ring), 128.72 (C_3 and C_5 of phenyl ring), 112.61 (C_4 of pyrazole), 34.88 (SCH_2), 32.23 (C_2 and C_3 of butyl chain), 25.32 (C_1 and C_4 of butyl chain), 20.70 (*p*-tolyl CH_3), 17.43 (CH_3), 15.83 (CH_3); LCMS *m*/*z*: 709 (M + H, 20). Anal. calcd. for $C_{36}H_{40}N_{10}O_2S_2$: C, 60.99; H, 5.69; N, 19.76. Found: C, 60.84; H, 5.72; N, 20.06.

1,4-Bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4-(m-tolyl)-1,2,4-triazol-3-yl]-butane **(15)**

Yield: 62%, M.p. 250–254°C, IR (ν cm^{-1},KBr): 3114, 2950, 1668, 1229, ^{1}H-NMR (DMSO-d_6, δ ppm): 7.0–7.5 (m, 8H, Ar-H), 4.5 (s, 2H, C_4 of pyrazole), 3.85 (s, 4H, SCH_2), 2.6 (s, 6H, CH_3), 2.5 (s, 6H, CH_3), 2.38 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 2.1 (s, 6H, *m*-tolyl CH_3), 1.3 (t, 4H, C_2 and C_3 methylene protons of butyl chain); ^{13}C-NMR (DMSO-d_6, δ ppm): 196.58 (C=O), 153.50 (C_3 of triazole), 148.67 (C_5 of triazole), 145.35 (C_3 of pyrazole), 142.74 (C_5 of pyrazole), 137.33 (C_4 of phenyl ring), 131.60 (C_1 of phenyl ring), 129.79 (C_2 and C_6 of phenyl ring), 128.62 (C_3 and C_5 of phenyl ring), 112.69 (C_4 of pyrazole), 34.98 (SCH_2), 32.27 (C_2 and C_3 of butyl chain), 25.36 (C_1 and C_4 of butyl chain), 20.73 (*m*-tolyl CH_3), 17.40 (CH_3), 15.87 (CH_3); LCMS *m*/*z*: 709 (M + H, 20). Anal. calcd. for $C_{36}H_{40}N_{10}O_2S_2$: C, 60.99; H, 5.69; N, 19.76. Found: C, 60.94; H, 5.62; N, 19.85.

1,4-Bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4-(p-ethoxyphenyl)-1,2,4-triazol-3-yl]-butane **(16)**

Yield: 60%, M.p. 270–273°C, IR (ν cm^{-1}, KBr): 3058, 2970, 1674, 1233; ^{1}H-NMR (DMSO-d_6, δ ppm): 7.1–7.4 (m, Ar-H, 8H), 4.6 (s, 2H, C_4 of pyrazole), 4.1 (q, 4H, OCH_2), 3.75 (s, 4H, SCH_2), 2.7 (s, 6H, CH_3), 2.5 (s, 6H, CH_3), 2.3 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 1.4 (t, 4H, C_2 and C_3 methylene protons of butyl chain), 1.1 (t, 6H, CH_3 of ethoxy group); ^{13}C-NMR (DMSO-d_6, δ ppm): 196.50 (C=O), 152.50 (C_3 of triazole), 148.63 (C_5 of triazole), 145.33 (C_3 of pyrazole), 142.75 (C_5 of pyrazole), 137.43 (C_4 of phenyl ring), 131.49 (C_1 of phenyl ring), 129.65 (C_2 and C_6 of phenyl ring), 128.62 (C_3 and C_5 of phenyl ring), 112.69 (C_4 of pyrazole), 62.37 (OCH_2), 34.96 (SCH_2), 32.22 (C_2 and C_3 of butyl chain), 25.36 (C_1 and C_4 of butyl chain), 17.40 (CH_3), 15.87 (CH_3) 15.10 (methyl of ethoxy group); LCMS *m*/*z*: 769 (M + H, 40). Anal. calcd. for $C_{38}H_{44}N_{10}O_4S_2$: C, 59.35; H, 5.77; N, 18.22. Found: C, 59.54; H, 5.72; N, 18.36.

1,4-Bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4- (cyclohexyl)-1,2,4-triazol-3-yl]-butane **(17)**

Yield: 55%, M.p. 215–217°C, IR (ν cm^{-1}, KBr): 2984, 1675, 1233; ^{1}H-NMR (DMSO-d_6, δ ppm): 4.5 (s, 2H, C_4 of pyrazole), 3.8 (s, 4H, SCH_2), 3.66 (m, 2H, C_1 protons of cyclohexyl), 2.6 (s, 6H, CH_3), 2.5 (s, 6H, CH_3), 2.4 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 2.10 (m, 8H, C_2 and C_6 protons of cyclohexyl), 1.77 (m, 8H, C_3 and C_5 protons of cyclohexyl), 1.60 (m, 4H, C_4 protons of cyclohexyl), 1.34 (t, 4H, C_2 and C_3 methylene protons of butyl chain); LCMS *m*/*z*: 693 (M + H, 15)$^+$. Anal. calcd. for $C_{34}H_{48}N_{10}O_2S_2$: C, 58.93; H, 6.98; N, 20.21. Found: C, 58.82; H, 6.85; N, 20.32.

1,4-Bis[5-(1-oxo-1-(3,5-dimethyl pyrazol-1-yl)-methyl)-thio-4- (n-butyl)-1,2,4-triazol-3-yl]-butane **(18)**

Yield: 55%, M.p. 219–222°C, IR (ν cm^{-1}, KBr): 2985, 1680, 1238; ^{1}H-NMR (DMSO-d_6, δ ppm): 4.4 (s, 2H, C_4 of pyrazole), 3.90 (s, 4H, SCH_2), 3.75 (t, 4H, C_1 protons of *n*-butyl), 2.7 (s, 6H, CH_3), 2.5 (s, 6H, CH_3), 2.36 (t, 4H, C_1 and C_4 methylene protons of butyl chain), 1.75 (m, 4H, C_2 protons of *n*-butyl), 1.44 (t, 4H, C_2 and C_3 methylene protons of butyl chain), 1.30 (m, 4H, C_3 protons of *n*-butyl), 0.91 (t, 6H, C_4 protons of *n*-butyl); LCMS *m*/*z*: 641(M + H, 20)$^+$, Anal. calcd. for $C_{30}H_{44}N_{10}O_2S_2$: C, 56.22; H, 6.92; N, 21.86. Found: C, 56.32; H, 6.88; N, 21.71.

In-vitro cytotoxicity activity

The cytotoxicity of the compounds was evaluated *in vitro* against the following human cancer cell lines: A-549 lung carcinoma, HT-29 colon adenocarcinoma and MDA-MB-231 breast carcinoma. The cell lines were procured from National Centre for Cell Sciences, Pune, India, and were cultured in DMEM medium supplemented with 10% FBS, 1% L-glutamine and 50 μg/mL gentamicin sulphate in a CO_2 incubator in a humidified atmosphere of 5% CO_2 and 95% air. The *in-vitro* cytotoxicity was determined using a standard MTT assay [22–24]. Briefly, the exponentially growing cells were plated in 96- well plates (10^4 cells/well in 100 μL of medium) and incubated for 24 h for attachment. The test compounds were prepared prior to the study by dissolving in 0.1% DMSO and diluted with medium. The cells were then exposed to different concentration of test compounds (10, 20, 50 and 100 μM) in a volume of 100 μL/well. The cells in the growth control wells received only the same volume of medium containing 0.1% DMSO. After 72 h of exposure, the medium was removed and the cell cultures were incubated with 100 μL of MTT reagent (0.1%) for 4 h at 37°C. the pink colored formazan was dissolved in 100 μL of DMSO and absorbance of each well was read in an ELISA micro plate reader at 570 nm. The experiment was performed in triplicate and the percentage cytoxicity was calculated using the following formula.

$$\%\,\text{Cytotoxicity} = (\text{Control abs} - \text{Test abs}) \times 100 / \text{Control abs}.$$

The drug concentration that causes 50% cell growth inhibition after 72 h of continuous exposure to the test compounds (IC_{50}) was determined by plotting the graph of concentration of the drug against the percent cytotoxicity and performing the regression analysis. The IC_{50} values of the test compounds are shown in Table 2.

DNA binding assay by absorption titration

The spectrometric titration was conducted by Nano Drop ND-1000 UV Spectrophotometer at room temperature (~30°C). The CT DNA (Sigma, St. Louis, MO) was dissolved in double distilled de-ionized water with 50 mM NaCl, and dialyzed against a buffer solution for 2 days. Its concentration was determined by absorption spectrometry at 340 nm using a molar extinction coefficient 6600 M^{-1} cm^{-1}. The ratio A325/A355 > 1.80 was used as an indication of a protein-free DNA. Absorption titration was performed at a fixed concentration of drugs (25 μM) in a sodium phosphate buffer (20 mM sodium phosphate, 150 mM NaCl, pH 6.5). Small aliquots of concentrated CT DNA (5 mM) were added into the solution at final concentrations from 0 to 100 μM, and stirred for 5 min before measurement. The parameters, λ_{max}, hypochromicity, isobastic point and binding constant (Table 3) were found from the absorption spectra. The intrinsic binding constant (K_i) for a given complex with DNA was obtained from a plot of $D/\Delta\varepsilon_{app}$ *versus* D according to the following equation, $D/\Delta\varepsilon_{app} = D/\Delta\varepsilon + 1/\Delta\varepsilon \times K$, where D = concentration of DNA in

Table 3. Binding constant (K_i) and photometric properties of triazoles in contact with CT-DNA

Compound	K_i ($\times 10 \times M^{-1}$)	λ_{max} (nm)	Hypochromicity (%)	Isobastic point
14	3.92	341	57.2	391
15	3.13	338	51.3	327
16	3.52	336	53.1	345

base molarities, $\Delta\varepsilon_{app} = |\varepsilon_a - \varepsilon_f|$ and $\Delta\varepsilon = |\varepsilon_b - \varepsilon_f|$, where ε_a and ε_f are respective extinction coefficient of the complex in the presence and absence of DNA. The apparent extinction coefficient ε_a is obtained by calculating A_{obs}/ [bis compounds]. The data were fitted to the equation with a slope equal to $1/\Delta\varepsilon$ and Y-intercept equal to $1/(\Delta\varepsilon \times K)$.The intrinsic binding constant (K_i) is determined from the slope of Y-intercept.

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