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Original article

Synthesis of novel 1,2,4-triazoles, triazolothiadiazines and triazolothiadiazoles as potential anticancer agents

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ABSTRACT

A series of new *N*-substituted-3-mercapto-1,2,4-triazoles (**3a**,**b** and **7a**–**d**), triazolo[1,3,4]thiadiazines (**5a**,**b**) and triazolo[1,3,4]thiadiazoles (**4a**–**d**, **6** and **8a**–**d**) have been synthesized starting from isonicotinic acid hydrazide. The structure of the newly synthesized compounds was confirmed on the basis of their spectral data and elemental analyses. All the compounds were screened for their *in vitro* anticancer activity against 6 human cancer cell lines and normal fibroblasts. Seven of the tested compounds (**3a**,**b**, **4c**, **5a** and **8b**–**d**) exhibited significant cytotoxicity against most cell lines. Among these derivatives compound **4c** exhibited equivalent cytotoxic effect to the standard CHS 828 against gastric cancer cell line (IC_{50} = 25 nM). Normal fibroblast cells (WI38) were affected to a much lesser extent (IC_{50} > 10,000 nM).

1. Introduction

Cancer is a life threatening disease and remains a major health problem around the globe. It is the second most occurring disease after cardiovascular diseases. Thus, the development of potent and effective novel antineoplastic drugs is one of the most intensely persuaded goals of contemporary medicinal chemistry.

The chemistry of 1,2,4-triazoles and their fused heterocyclic derivatives have received considerable attention owing to their synthetic and effective biological importance. For example, a large number of 1,2,4-triazoles have been incorporated into a wide variety of therapeutically interesting drug candidates possessing antimicrobial [1–5], anti-inflammatory [6], analgesic [7] and anticancer activities [8,9]. Literature survey reveals that important chemotherapeutics, such as Vorozole, Letrozole and Anastrozole (Fig. 1) that consist of substituted 1,2,4-triazole ring, are currently being used for the treatment of breast cancer [10]. Among these heterocycles, the mercapto and thione substituted 1,2,4-triazole ring systems have been well studied and so far a variety of biological activities have been reported for a large number of their derivatives [11,12]. In addition to these important biological applications, mercapto 1,2,4-triazoles are also of great utility in preparative organic chemistry as useful intermediates for the preparation of some triazolothiadiazoles and triazolothiadiazines. The amino and mercapto groups are ready made nucleophilic centers for the synthesis of condensed heterocyclic rings [13].

Meanwhile, the triazolothiadiazoles and triazolothiadiazines exhibiting broad spectrum biological profile have matured into indispensable heterocyclic scaffolds [14,15]. New analogs of triazolothiadiazoles and triazolothiadiazines bearing different substituents on the 3 and 6 positions were found to possess significant anticancer activity. Holla et al. [16] reported the synthesis of several structurally modified triazolothiadiazines which were evaluated for their anticancer activity against full panel of 60 cell lines derived from seven cancer types. The screening results showed that the compound bearing a 2-chloroaryloxy methyl group at C-3 and 4-chlorobenzylidine at C-7 positions exhibited the highest activity with GI_{50} < 10 μM against all cell lines. Subsequently, Poojary and co-workers [17] prepared and screened a series of novel triazolothiadiazoles and triazolothiadiazines for their anti-tumor activity. The structure activity relationship indicated that compounds containing chlorine atoms at various positions, especially the triazolothiadiazoles bearing chlorophenyl groups at position 6, are more active as compared to other derivatives. Moreover, Isloor et al. [18] carried out the synthesis of 6-[3-(4-fluorophenyl)-1*H*-pyrazol-4-yl]-3-[(2-naphthyloxy)methyl][1,2,4]triazolo[3,4-*b*][1,3,4]-thiadiazole (FPNT). The anticancer activity along with possible mechanism of action of the triazolothiadiazole in HepG2 cells was

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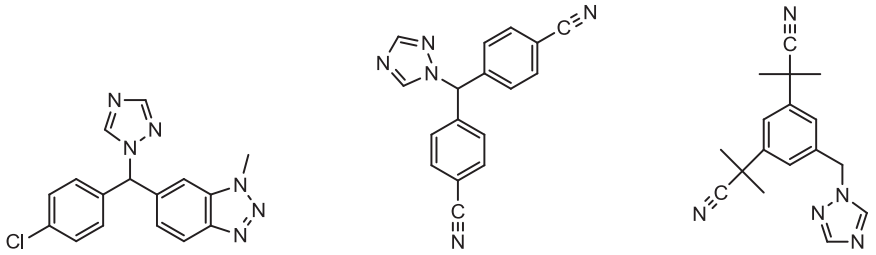


Fig. 1. Chemical structures of breast cancer drugs.

explored using MTT assay, [^{3}H] thymidine assay, flow cytometry and chromatin condensation studies. It was concluded that FPNT exhibited potent cytotoxic activity through induction of growth inhibition followed by apoptosis in HepG2 cells.

Recently, Husain et al. [19] reported the *in vitro* anticancer activity of a library of triazolothiadiazole and triazolothiadiazine derivatives incorporating benzimidazole scaffold. The anticancer screening results revealed that the triazolothiadiazole derivatives carrying a benzimidazole moiety exhibited better anticancer activity than their thiadiazine analogs.

In a valuable attempt to explore the mode of action of triazolothiadiazoles, Ibrahim [20] reported the synthesis of a new series of 3,6-disubstituted triazolo[3,4-*b*]thiadiazole derivatives which were designed to act as tyrosine kinase inhibitors. The synthesized compounds were evaluated for their cytotoxic activity against a panel of 60 human cancer cell lines by the National Cancer Institute (NCI) and some of them demonstrated inhibitory effects on the growth of a wide range of cancer cell lines generally at 10^{-5}–10^{-7} M concentrations. However, the anti-tumor activity of the synthesized compounds could not be interpreted in terms of tyrosine kinase inactivation but more likely as a relatively broad specificity for the ATP-binding domain of other kinases. The biochemical assay of the synthesized compounds as CDK inhibitors was determined and expressed as IC_{50}. Some compounds showed modest activity (IC_{50} < 10 mM).

In our search for new classes of potential anticancer agents, the aforementioned findings promoted us to synthesize a series of *N*-substituted-3-mercapto-[1,2,4]triazoles, 3,6-disubstituted-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles and 3,6-disubstituted-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines. The newly synthesized compounds were evaluated for their *in vitro* anticancer activity.

2. Results and discussion

2.1. Chemistry

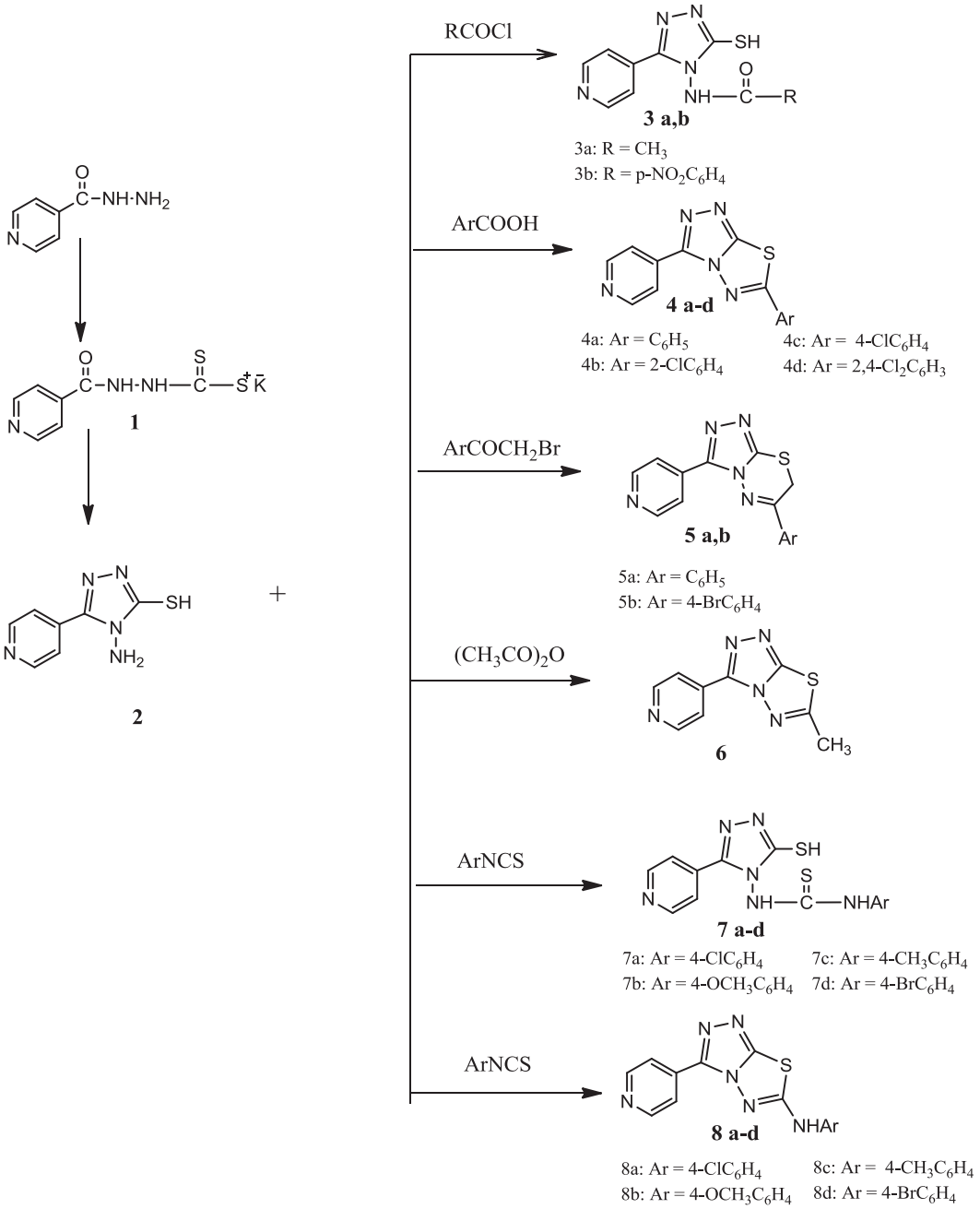
The reaction sequence employed for the synthesis of the title compounds is shown in Scheme 1. The reaction of isonicotinic acid hydrazide with CS_2 in methanolic KOH yielded the corresponding dithiocarbazinate **1** which upon heating under reflux with hydrazine hydrate afforded the starting 3-mercaptotriazole **2**. The N-acylated derivatives **3a**,**b** were obtained by the reaction of **2** with acetyl chloride and p-nitrobenzoyl chloride respectively. On the other hand, condensation of **2** with various aromatic carboxylic acids in $POCl_3$ yielded the corresponding 6-aryltriazolothiadiazoles **4a**–**d**. The triazolothiadiazine derivatives **5a**,**b** were obtained from the reaction of **2** with phenacyl bromides. Moreover, the reaction of **2** with acetic anhydride afforded the 6-methyltriazolothiadiazole **6**. The synthesis of thiourea derivatives **7a**–**d** was achieved via heating under reflux a mixture of **2** with various aryl isothiocyanates in pyridine for 30 min. Meanwhile, carrying the latter reaction for 2–3 h till complete evolution of H_2S (tested by lead acetate paper) afforded the cyclized analogs **8a**–**d**. The disappearance of the singlet signal at δ 5.8 ppm assignable to the 2NH groups of the thiourea moiety present in the ^{1}H NMR spectra of compounds **7a**–**d** confirmed formation of the latter cyclized derivatives.

2.2. In vitro cytotoxicity

The heterocyclic compounds, prepared in this study, were evaluated according to standard protocols for their *in vitro* cytotoxicity against six human cancer cell lines including cells derived from human gastric cancer (NUGC), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), nasopharyngeal carcinoma (HONE1), human breast cancer (MCF) and normal fibroblast cells (WI38). For comparison purposes CHS 828, a pyridyl cyanoguanidine, was used as a standard cytotoxic drug (Fig. 4) [21]. All of IC_{50} values in nanomolar (nM) are listed in (Table 1) and the results are represented graphically in (Figs. 2 and 3). Seven of the tested compounds namely: **3a**, **3b**, **4c**, **5a**, **8b**, **8c** and **8d** showed significant cytotoxic effects with IC_{50} values <800 nM. Normal fibroblast cells (WI38) were affected to a much lesser extent (IC_{50} > 10,000 nM). The N-substituted-mercaptotriazole derivative **3a** showed better cytotoxic activity than **3b** with IC_{50} in the range of 42–53 nM against colon, gastric and liver cancer cell lines. Moreover, compound **5a**, a triazolothiadiazine derivative, possessed potential cytotoxic activity against all the tested cancer cell lines except colon cancer. The triazolothiadiazole derivatives **4c**, **8b**, **8c** and **8d** showed remarkable cytotoxic activity against most cell lines. Among these derivatives compound **4c** exhibited equivalent cytotoxic effect to the standard CHS 828 against gastric cancer cell line (IC_{50} = 25 nM). While some of the compounds were not the most potent, their specific activity against particular cell lines makes that of interest for further development as anticancer drugs.

3. Conclusion

The present research reports the successful synthesis, characterization and anticancer activity of new 1,2,4-triazole derivatives starting from isonicotinic acid hydrazide. Seven of the tested compounds namely: the 3-mercaptotriazoles **3a**, **3b**; triazolothidiazine derivative **5a** and triazolothiadizoles **4c**, **8b**, **8c** and **8d** showed significant cytotoxic effects with IC_{50} values <800 nM. Among these derivatives compound **4c** exhibited equivalent cytotoxicity effect to the standard CHS 828 against gastric cancer cell line (IC_{50} = 25 nM). Normal fibroblast cells (WI38) were affected to a much lesser extent (IC_{50} > 10,000 nM). The obtained results suggest that these compounds may serve as lead chemical entities



Scheme 1. Synthesis of compounds: 3a,b; 4a–d; 5a,b; 6; 7a–d and 8a–d.

for further modification in the search of new classes of potential anticancer agents.

4. Experimental

4.1. Chemistry

All melting points were determined on a Stuart apparatus and the values given are uncorrected. IR spectra (KBr, cm^{-1}) were determined on a Shimadzu IR 435 spectrophotometer (Faculty of Pharmacy, Cairo University, Egypt). ^1H NMR and ^{13}C NMR spectra were recorded on Varian Gemini 300 MHz (Microanalysis Center, Cairo University, Egypt) and Bruker Ascend 400 MHz spectrophotometers (Microanalytical Unit, Faculty of Pharmacy, Cairo University, Egypt) using TMS as internal standard. Chemical shift values are recorded in ppm on δ scale. Mass spectra were recorded on a Hewlett Packard 5988 spectrometer (Microanalysis Center, Cairo University, Egypt). Elemental analyses were carried out at the Microanalysis Center, Cairo University, Egypt; found values were within ±0.35% of the theoretical ones. Progress of the reactions was monitored using thin layer chromatography (TLC) sheets precoated with UV fluorescent silica gel Merck 60F 254 and were visualized using UV lamp.

4.1.1. General method for preparation of 4-amino-5-pyridin-4-yl-4H-[1,2,4]triazole-3-thiol (**2**)

Isonicotinic acid hydrazide (11.37 g, 0.083 mol) was treated with a solution of KOH (6.4 g, 0.125 mol) dissolved in methanol (50 mL)

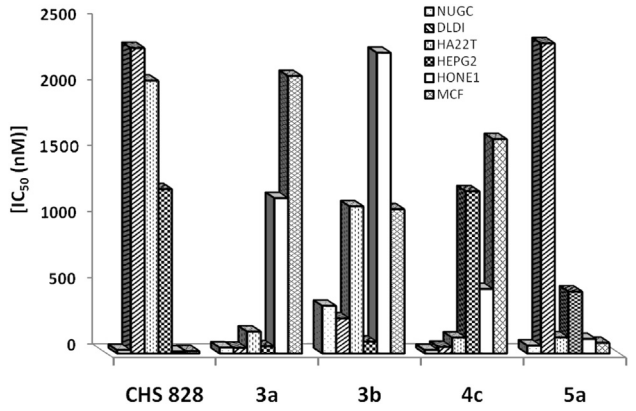


Fig. 2. Cytotoxicity of **3a**, **3b**, **4c**, **5a** and CHS 828 against NUGC, gastric cancer; DLDI, colon cancer; HA22T, liver cancer; HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer.

at 0–5 °C with stirring. Carbon disulphide (9.6 g, 0.125 mol) was added slowly and the reaction mixture was stirred over night at room temperature. The solid product of potassium dithiocarbazinate **1** was filtered, washed with chilled methanol and dried. It was directly used in the next step without further purification.

Hydrazine hydrate (9 g, 0.2 mol) was added to a solution of **1** (22 g, 0.1 mol) in water (20 mL) and the reaction mixture heated under reflux till complete evolution of H_2S. The reaction mixture was poured on ice cold water and acidified with HCl. The yellow precipitate was filtered, washed with cold water, dried and crystallized from ethanol. Yield: 80%; m.p.: 240–242 °C; IR (kBr, cm^{-1}): 3271 (NH_2), 3159 (Ar–H), 2881 (CH aliphatic), 2561 (SH), 1608 (C=N); ^{1}H NMR (DMSO-d_6): δ 5.87 (s, 2H, NH_2, D_2O exchangeable), 8.01 (d, 2H, J = 6.0 Hz, Pyridine H-3 and H-5), 8.75 (d, 2H, J = 6.0 Hz, Pyridine H-2 and H-6), 14.12 (s, 1H, SH, D_2O exchangeable).

*4.1.2. General method for the preparation of compounds **3a**,**b***

A mixture of 4-amino-5-pyridin-4-yl-4*H*-[1,2,4]triazole-3-thiol (**2**) (0.01 mol) and the appropriate acid chloride (0.01 mol) was heated under reflux in DMF in presence of triethylamine (0.5 mL) for 5 h. The reaction mixture was allowed to cool, poured onto ice cold water and acidified by HCl. The obtained white solid was filtered, washed with water, dried and crystallized from ethanol.

*4.1.2.1. N-(3-Mercapto-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-yl)acetamide(**3a**).* Yield: 60%; m.p.: 275–277 °C; IR (kBr, cm^{-1}): 3248 (NH), 2966 (CH aliphatic), 2621 (SH), 1714 (C=O), 1608 (C=N); ^{1}H NMR(DMSO-d_6): δ 2.07 (s, 3H, CH_3), 3.38 (s, 1H, NH, D_2O exchangeable), 7.68 (s, 2H, Pyridine H-3 and H-5), 8.76 (s, 2H, Pyridine H-2 and H-6), 11.53 (s, 1H, SH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ 20.8, 122.1, 133.4, 147.8, 150.6, 168.2, 170.8; MS: m/z (%) 235 (M^+, 41). Anal. Calcd. for $C_9H_9N_5OS$: C, 45.95; H, 3.86; N, 29.77. Found: C, 45.87; H, 3.77; N, 29.61.

*4.1.2.2. N-(3-Mercapto-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-yl)-4-nitrobenzamide(**3b**).* Yield: 68%; m.p.: 235–237 °C; IR (kB, cm^{-1}): 3316 (NH), 3062 (Ar–H), 2551 (SH), 1691 (C=O), 1608 (C=N); ^{1}H NMR (DMSO-d_6): δ 3.32 (s, 1H, NH, D_2O exchangeable), 7.35–7.87 (m, 4H, Ar–H), 8.15–8.61 (m, 4H, Pyridine-H), 13.56 (s, 1H, SH, D_2O exchangeable); MS: m/z (%) 342 (M^+, 21). Anal. Calcd. for $C_{14}H_{10}N_6O_3S$: C, 49.12; H, 2.94; N, 24.55. Found: C, 49.40; H, 2.87; N, 24.42.

*4.1.3. General method for the preparation of compounds **4a**–**d***

An equimolar mixture (0.01 mol) of 4-amino-5-pyridin-4-yl-4*H*-[1,2,4]triazole-3-thiol (**2**) and the appropriate aromatic acid in $POCl_3$ (10 mL) was refluxed for 8–10 h. The reaction mixture was cooled to room temperature and gradually poured onto ice cold water with stirring. The mixture was allowed to stand overnight and the solid separated out was filtered, treated with dilute NaOH solution and washed thoroughly with cold water. The solid obtained was filtered, dried and crystallized from ethanol.

*4.1.3.1. 6-Phenyl-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**4a**).* Yield: 55%; m.p.: 216–218 °C; IR (kBr, cm^{-1}): 3080 (Ar–H), 1633, 1600 (C=N). ^{1}H NMR (DMSO-d_6): δ 7.65–8.12 (m, 5H, Ar–H), 8.46 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.96 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6); MS: m/z (%) 279 (M^+, 100). Anal. Calcd. for $C_{14}H_9N_5S$: C, 60.20; H, 3.25; N, 25.07. Found: C, 60.12; H, 3.07; N, 25.30.

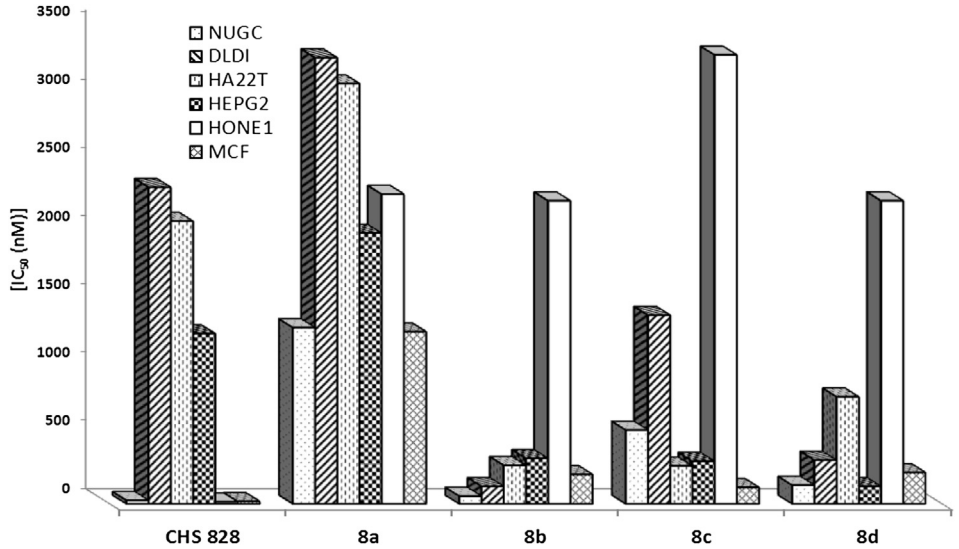


Fig. 3. Cytotoxicity of **8a**, **8b**, **8c**, **8d** and CHS 828 against NUGC, gastric cancer; DLDI, colon cancer; HA22T, liver cancer; HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer.

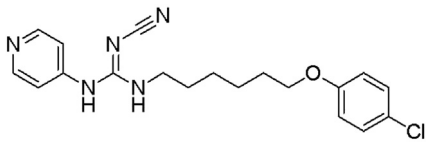


Fig. 4. Chemical structure of CHS 828.

*4.1.3.2. 6-(2-Chlorophenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**4b**).* Yield: 75%; m.p.: 235–237 °C; IR (kBr, cm^{-1}): 3080 (Ar–H), 1602 (C=N). ^{1}H NMR (DMSO-d_6): δ 7.04–7.53 (m, 4H, Ar–H), 8.14 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.81 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6); MS: m/z (%) 313 (M^+, 51). Anal. Calcd for $C_{14}H_8ClN_5S$: C, 53.59; H, 2.57; N, 22.32. Found: C, 53.34; H, 2.76; N, 22.54.

*4.1.3.3. 6-(4-Chlorophenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**4c**).* Yield: 82%; m.p.: 278–280 °C, IR (kBr, cm^{-1}): 3022 (Ar–H), 1604 (C=N). ^{1}H NMR (DMSO-d_6): δ 7.36–8.15 (m, 4H, Ar–H), 8.24 (d, 2H, J = 5.8 Hz, Pyridine H-3 and H-5), 8.84 (d, 2H, J = 5.8 Hz, Pyridine H-2 and H-6); ^{13}C NMR (DMSO-d_6): δ 120.2, 128.8, 129.7, 131.4, 134.0, 134.3, 143.4, 148.6, 151.3, 167.7; MS: m/z (%) 313 (M^+, 53). Anal. Calcd. for $C_{14}H_8ClN_5S$: C, 53.59; H, 2.57; N, 22.32. Found: C, 53.78; H, 2.32; N, 22.60.

*4.1.3.4. 6-(2,4-Dichlorophenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**4d**).* Yield: 84%; m.p.: 215–217 °C; IR (kBr, cm^{-1}): 3039 (Ar–H), 1600 (C=N). ^{1}H NMR (DMSO-d_6): δ 7.73–8.20 (m, 3H, Ar–H), 8.22 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.83 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6); MS: m/z (%) 348 (M^+, 11). Anal. Calcd. for $C_{14}H_7Cl_2N_5S$: C, 48.29; H, 2.03; N, 20.11. Found: C, 48.50; H, 2.18; N, 20.09.

*4.1.4. General method for the preparation of compounds **5a**,**b***

A mixture of 4-amino-5-pyridin-4-yl-4*H*-[1,2,4]triazole-3-thiol (**2**) (0.01 mol) and the appropriate phenacyl bromide (0.012 mol) in absolute ethanol was heated under reflux for 6 h. The reaction mixture was poured onto crushed ice and neutralized with Na_2CO_3. The solid product obtained was filtered, washed with water, dried and crystallized from ethanol.

Table 1
Cytotoxicity of IMT-samples against a variety of cancer cell lines [IC_{50}[a] (nM)].

Compd.	Cytotoxicity (IC_{50} in nM)						
	NUGC	DLDI	HA22T	HEPG2	HONE1	MCF	WI38
3a	44	42	166	53	1177	2102	na
3b	360	266	1114	89	2279	1092	na
4a	1280	3360	1265	1365	2426	1538	na
4b	3220	2665	1260	1328	1169	2177	na
4c	25	49	120	1228	488	1624	na
4d	2157	1188	3068	2265	1170	3224	na
5a	59	2350	122	469	110	80	na
5b	2489	2377	2063	2289	1784	2273	na
6	2188	3285	1723	2735	1078	219	650
7a	2120	2248	2014	2022	1220	3231	2189
7b	1824	2288	2371	1299	2269	1672	na
7c	2766	2152	2379	1230	1289	2170	na
7d	3289	3270	3196	2782	1684	1592	1288
8a	1289	3263	3074	1980	2265	1259	na
8b	56	128	282	333	2217	215	na
8c	540	1380	277	312	3283	120	480
8d	136	320	782	128	2217	229	na
CHS 828	25	2315	2067	1245	15	18	na

NUGC, gastric cancer, DLDI, colon cancer, HA22T, liver cancer, HEPG2, liver cancer HEPG2, liver cancer; HONEI, nasopharyngeal carcinoma; MCF, breast cancer; WI38, normal fibroblast cells.

[a] The sample concentration produces a 50% reduction in cell growth.

*4.1.4.1. 6-Phenyl-3-(pyridin-4-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (**5a**).* Yield: 87%; m.p.: 220–222 °C; IR (kBr, cm^{-1}): 3039 (Ar–H), 1598 (C=N); ^{1}H NMR (DMSO-d_6): δ 4.48 (s, 2H, CH_2), 7.36–8.02 (m, 5H, Ar–H), 8.04 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.79 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6); ^{13}C NMR (DMSO-d_6): δ 156.5, 150.3, 149.6, 143.8, 133.2, 132.9, 132.1, 129.1, 127.7, 121.4, 22.8; MS: m/z (%) 293 (M^+, 85). Anal. Calcd. for $C_{15}H_{11}N_5S$: C, 61.42; H, 3.78; N, 23.87. Found: C, 61.21; H, 3.58; N, 23.92.

*4.1.4.2. 6-(4-Bromophenyl)-3-(pyridin-4-yl)-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (**5b**).* Yield: 84%; m.p.: 283–285 °C; IR (kBr, cm^{-1}): 3078 (Ar–H), 1600 (C=N). ^{1}H NMR (DMSO-d_6): δ 4.52 (s, 2H, CH_2), 7.68–8.06 (m, 4H, Ar–H), 8.39–8.73 (m, 4H, Pyridine-H); MS: m/z (%) 372 (M^+, 42). Anal. Calcd. for $C_{15}H_{10}BrN_5S$: C, 48.40; H, 2.71; N, 18.81. Found: C, 48.13; H, 2.71; N, 18.52.

*4.1.5. General method for the preparation of 6-methyl-3-(pyridin-4-yl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (**6**)*

A mixture of 4-amino-5-pyridin-4-yl-4*H*-[1,2,4]triazole-3-thiol (**2**) (0.01 mol) and acetic anhydride (10 mL) was heated under reflux for 7 h. The reaction mixture was left to cool, poured onto ice cold water. The obtained solid, filtered, washed with water, dried and crystallized from ethanol. Yield: 60%; m.p.: 246–248 °C; IR (kBr, cm^{-1}): 3043 (Ar–H), 2958 (CH aliphatic), 1604 (C=N); ^{1}H NMR (DMSO-d_6): δ 2.48 (s, 3H, CH_3), 8.13 (d, 2H, J = 5.9 Hz, Pyridine H-3 and H-5), 8.80 (d, 2H, J = 5.9 Hz, Pyridine H-2 and H-6); MS: m/z (%) 217 (M^+, 42). Anal. Calcd. for $C_9H_7N_5S$: C, 49.76; H, 3.25; N, 32.24. Found: C, 49.56; H, 3.12; N, 31.95.

*4.1.6. General method for the preparation of compounds **7a**–**d***

An equimolar mixture (0.01 mol) of 4-amino-5-pyridin-4-yl-4*H*-[1,2,4]triazole-3-thiol (**2**) and the appropriate aryl isothiocyanate was heated under reflux in pyridine (10 mL) for 30 min. The reaction mixture was poured onto ice cold water. The obtained solid was filtered, washed with water, dried and crystallized from ethanol.

*4.1.6.1. 1-(4-Chlorophenyl)-3-(3-mercapto-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-yl)thiourea (**7a**).* Yield: 90%; m.p.: 235–238 °C; IR (kBr, cm^{-1}): 3300 (NH), 3138 (Ar–H), 1608 (C=N), 1315 (C=S); ^{1}H NMR (DMSO-d_6): δ 5.86 (s, 2H, NH, D_2O exchangeable), 7.34–7.77 (m, 4H, Ar–H), 8.02 (d, 2H, J = 6.1 Hz, Pyridine H-3 and H-5), 8.75 (d, 2H, J = 6.1 Hz, Pyridine H-2 and H-6), 10.9 (s, 1H, SH, D_2O exchangeable); MS: m/z (%) 362 (M^+, 35). Anal. Calcd. for $C_{14}H_{11}ClN_6S_2$: C, 46.34; H, 3.06; N, 23.16. Found: C, 46.14; H, 3.20; N, 23.40.

*4.1.6.2. 1-(3-Mercapto-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-yl)-3-(4-methoxyphenyl)thiourea (**7b**).* Yield: 92%; m.p.: 200–202 °C; IR (kBr, cm^{-1}): 3215 (NH), 3020 (Ar–H), 1610 (C=N), 1340 (C=S); ^{1}H NMR (DMSO-d_6): δ 3.78 (s, 3H, OCH_3), 5.86 (s, 2H, NH, D_2O exchangeable), 6.88–7.56 (m, 4H, Ar–H), 8.02 (d, 2H, J = 6.1 Hz, Pyridine H-3 and H-5), 8.75 (d, 2H, J = 6.1 Hz, Pyridine H-2 and H-6), 9.42 (s, 1H, SH, D_2O exchangeable); MS: m/z (%) 359 (M+H, 7). Anal. Calcd. for $C_{15}H_{14}N_6OS_2$: C, 50.26; H, 3.94; N, 23.45. Found: C, 49.98; H, 3.70; N, 23.28.

*4.1.6.3. 1-(3-Mercapto-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-yl)-3-(p-tolyl)thiourea (**7c**).* Yield: 88%; m.p.: 210–212 °C; IR (kBr, cm^{-1}): 3233 (NH), 3159 (Ar–H), 1608 (C=N), 1315 (C=S); ^{1}H NMR (DMSO-d_6): δ 1.91 (s, 3H, CH_3), 5.84 (s, 2H, NH, D_2O exchangeable), 6.83–7.79 (m, 4H, Ar–H), 8.02 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.74 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6); MS: m/z (%) 342 (M^+, 14). Anal. Calcd. for $C_{15}H_{14}N_6S_2$: C, 52.61; H, 4.12; N, 24.54. Found: C, 52.43; H, 4.12; N, 24.72.

*4.1.6.4. 1-(4-Bromophenyl)-3-(3-mercapto-5-(pyridin-4-yl)-4H-1,2,4-triazol-4-yl)thiourea (**7d**).* Yield: 94%; m.p: 290–292 °C; IR (kBr, cm^{-1}): 3300 (NH), 3134 (Ar–H), 1610 (C=N), 1313 (C=S); ^1H NMR (DMSO-d_6): δ 5.86 (s, 2H, NH, D_2O exchangeable), 7.29–7.53 (m, 4H, Ar–H), 8.02 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.76 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6), 10.9 (s, 1H, SH, D_2O exchangeable); MS: m/z (%) 407 (M^+, 29). Anal. Calcd. for $C_{14}H_{11}BrN_6S_2$: C, 41.28; H, 2.72; N, 20.63. Found: C, 40.96; H, 2.52; N, 20.71.

*4.1.7. General method for the preparation of compounds **8a–d***

An equimolar mixture (0.01 mol) of 4-amino-5-pyridin-4-yl-4*H*-[1,2,4]triazole-3-thiol (**2**) and the appropriate aryl isothiocyanate was heated under reflux in pyridine (10 mL) for 2–3 h until the complete evolution of H_2S (detected by lead acetate paper). The reaction mixture was poured onto ice cold water. The obtained solid was filtered, washed with water, dried and crystallized from ethanol.

*4.1.7.1. N-(4-Chlorophenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-amine (**8a**).* Yield: 91%; m.p: >300 °C; IR (kBr, cm^{-1}): 3300 (NH), 3100 (Ar–H), 1643, 1612 (C=N); ^1H NMR (DMSO-d_6): δ 7.50–7.65 (m, 4H, Ar–H), 8.13 (d, 2H, J = 5.9 Hz, Pyridine H-3 and H-5), 8.81 (d, 2H, J = 5.9 Hz, Pyridine H-2 and H-6), 10.89 (s, 1H, NH, D_2O exchangeable); MS: m/z (%) 328 (M^+, 17). Anal. Calcd. for $C_{14}H_9ClN_6S$: C, 51.14; H, 2.76; N, 25.56. Found: C, 51.32; H, 2.96; N, 25.40.

*4.1.7.2. N-(4-Methoxyphenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-amine (**8b**).* Yield: 87%; m.p.: 180–182 °C; IR (kBr, cm^{-1}): 3213 (NH), 3020 (Ar–H), 1630, 1610 (C=N); ^1H NMR (DMSO-d_6): δ 3.78 (s, 3H, OCH_3), 6.88–7.55 (m, 4H, Ar–H), 8.14 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.81 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6), 9.42 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ 161.9, 156.5, 155.7, 150.6, 132.1, 126.0, 120.5, 119.1, 114.6, 113.6, 55.3; MS: m/z (%) 325 (M+H, 14). Anal. Calcd. for $C_{15}H_{12}N_6OS$: C, 55.54; H, 3.73; N, 25.91. Found: C, 55.80; H, 3.91; N, 25.71.

*4.1.7.3. N-(4-Methylphenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-amine (**8c**).* Yield: 94%; m.p.: 290–291 °C; IR (kBr, cm^{-1}): 3249 (NH), 2951 (Ar–H), 1608, 1589 (C=N); ^1H NMR (DMSO-d_6): δ 2.27 (s, 3H, CH_3), 7.13–7.39 (m, 4H, Ar–H), 8.04 (d, 2H, J = 5.9 Hz, Pyridine H-3 and H-5), 8.76 (d, 2H, J = 5.9 Hz, Pyridine H-2 and H-6), 10.9 (s, 1H, NH, D_2O exchangeable); MS: m/z (%) 308 (M^+, 62). Anal. Calcd. $C_{15}H_{12}N_6S$: C, 58.43; H, 3.92; N, 27.25. Found: C, 55.34; H, 3.51; N, 27.42.

*4.1.7.4. N-(p-Bromophenyl)-3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-amine (**8d**).* Yield: 89%; m.p: >300 °C; IR (kBr, cm^{-1}): 3300 (NH), 2918 (Ar–H), 1645, 1612 (C=N); ^1H NMR (DMSO-d_6): δ 7.44–7.84 (m, 4H, Ar–H), 8.02 (d, 2H, J = 6 Hz, Pyridine H-3 and H-5), 8.71 (d, 2H, J = 6 Hz, Pyridine H-2 and H-6), 10.95 (s, 1H, NH, D_2O exchangeable); MS: m/z (%) 373 (M^+, 63). Anal. Calcd. for $C_{14}H_9BrN_6S$: C, 45.05; H, 2.43; N, 22.52. Found: C, 45.14; H, 2.39; N, 22.38.

4.2. In vitro cytotoxic assay

4.2.1. Chemicals

Fetal bovine serum (FBS) and L-glutamine, were purchased from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was purchased from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (Saint Louis, USA).

4.2.2. Cell cultures

Cell cultures were obtained from the European Collection of cell Cultures (ECACC, Salisbury, UK) and human gastric cancer (NUGC), human colon cancer (DLD1), human liver cancer (HA22T and HEPG2), human breast cancer (MCF), nasopharyngeal carcinoma (HONE1) and normal fibroblast cells (WI38) were kindly provided by the National Cancer Institute (NCI, Cairo, Egypt). They were grown as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% heat inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 lg/mL), at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were obtained by plating 1.5×10^5 cells/mL for the six human cancer cell lines followed by 24 h of incubation. The effect of the vehicle solvent (DMSO) on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (0.5%) of DMSO used in each assay.

References

[1] A.M. Isloor, B. Kalluraya, P. Shetty, Eur. J. Med. Chem. 44 (2009) 3784–3787.
[2] N. Demirbas, S.A. Karaoglu, A. Demirbas, K. Sanack, Eur. J. Med. Chem. 39 (2004) 793–804.
[3] O. Prakash, D.K. Aneja, K. Hussain, P. Lohan, P. Ranjan, S. Arora, C. Sharma, K.R. Aneja, Eur. J. Med. Chem. 46 (2011) 5065–5073.
[4] S.N. Swamy, Basappa, B.S. Priya, B. Prabhuswamy, B.H. Doreswamy, J.S. Prasad, K.S. Rangappa, Eur. J. Med. Chem. 41 (2006) 531–538.
[5] V.S. Palekar, A.J. Damle, S.R. Shukla, Eur. J. Med. Chem. 44 (2009) 5112–5116.
[6] M.F. Shehy, A. Abu-Hashem, E.M. El-Telbani, Eur. J. Med. Chem. 45 (2010) 1906–1911.
[7] V. Mathew, J. Keshavayya, V.P. Vaidya, D. Giles, Eur. J. Med. Chem. 42 (2007) 823–840.
[8] B.S. Holla, K.N. Poojary, B.S. Rao, M.K. Shivananda, Eur. J. Med. Chem. 37 (2002) 511–517.
[9] V. Padmavathi, G.S. Reddy, A. Padmaja, P. Kondaiah, Ali-Shazia, Eur. J. Med. Chem. 44 (2009) 2106–2112.
[10] M. Clemons, R.E. Coleman, S. Verma, Cancer Treat. Rev. 30 (2004) 324–332.
[11] M. Ashok, B.S. Holla, J. Pharmacol. Toxicol. 2 (3) (2007) 256–263.
[12] B.S. Holla, B. Veerendra, M.K. Shivananda, B. Poojary, Eur. J. Med. Chem. 38 (2003) 759–767.
[13] N. Demirbas, A. Demirbas, S.A. Karaoglu, E. Celik, Arkivoc I (2005) 75–91.
[14] V. Sumangala, B. Poojary, N. Chidananda, T. Arulmoli, S. Shenoy, Eur. J. Med. Chem. 54 (2012) 59–64.
[15] I. Khan, A. Ibrar, N. Abbas, Eur. J. Med. Chem. 63 (2013) 854–868.
[16] B.S. Holla, B.S. Rao, B.K. Sarojini, P.M. Akberali, N.S. Kumari, Eur. J. Med. Chem. 41 (2006) 657–663.
[17] K.S. Bhat, B. Poojary, D.J. Prasad, P. Naik, B.S. Holla, Eur. J. Med. Chem. 44 (2009) 5066–5070.
[18] D. Sunil, A.M. Isloor, P. Shetty, K. Satyamoorthy, A.S.B. Prasad, Arab. J. Chem. 3 (2010) 211–217.
[19] A. Husain, M. Rashid, M. Shaharyar, A.A. Siddiqui, R. Mishra, Eur. J. Med. Chem. 62 (2013) 785–798.
[20] D.A. Ibrahim, Eur. J. Med. Chem. 44 (2009) 2776–2781.
[21] P.J. Hjarnaa, E. Jonsson, S. Latini, S. Dhar, R. Larsson, E. Bramm, T. Skov, L. Binderup, Cancer Res. 59 (22) (1999) 5751–5757.