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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antitumor activity of 4-cyclohexyl/aryl-5-(pyridin-4-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones

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Abstract The reaction of 2-isonicotinoyl-*N*-cyclohexyl/arylhydrazinecarbothioamide (**2a**–**r**) with sodium hydroxide, in each case, a single product was obtained. The structures of the compounds were confirmed on the basis of their elemental analysis and spectral data. The single crystal X-ray analysis confirmed the structure of these products as *N*-4-cyclohexyl/aryl-5-(pyridine-4-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**3a**–**r**). The in vitro antitumor activity of compounds was screened against three cell lines; BEL-7402, HUH-7 and HepG2 human hepatoma using MTT assay. Sorafenib (50 μM) was used as a positive control. The results of the MTT-dye reduction assay indicated that most of the compounds exert potent cytotoxic/antiproliferative effect in a time and dose-dependent manner via induced apoptosis of HepG2 cells. Results also showed that the tested compounds could significantly enhance the activity of caspase-3 which plays a very important role as the central effecter during apoptosis. The effect of different substitutions on the aromatic portion on the activity was found to be in the following order $CH_3 > OCH_3 > I > SO_2NH_2 > OC_2H_5 > C_2H_5 > NO_2 > Cl > CH_3CONH$.

Keywords Isoniazid · 1,2,4-Triazole-3-thione · Cytotoxic activity · MTT assay

Introduction

A number of five membered nitrogen-containing heterocycles have turned out to be potential chemotherapeutic agents. The biological profile of triazoles derivatives is very extensive. Compounds bearing a symmetrical triazole moiety are reported to show a broad spectrum of pharmacological activities such as antibacterial (Sztanke *et al.*, 2006; Prakash *et al.*, 2004), antifungal (Liu *et al.*, 2008; Lebouvier *et al.*, 2007), antimicrobial (Kaplancikli *et al.*, 2008), antimycobacterial (Kucukguzel *et al.*, 2008), antipyretic (Grossi *et al.*, 2002), anticancer (Sztanke *et al.*, 2008; Holla *et al.*, 2002), anticonvulsant (Almasirad *et al.*, 2004), and anti-inflammatory activities (Labanauskas *et al.*, 2004). Also there are known drugs containing 1,2,4-triazole moiety, e.g., alprozolam (tranquilizer), benatraden (diuretic), trapidil (hypotensive), trazodone (antidepressant), anastrozole, letrozole, vorozole (antineoplastic), ribavirin (antiviral) (Mathew *et al.,* 2006), and antimycotic ones such as fluconazole, itraconazole, voriconazole (Haber, 2001). Pyridyl ring, a prominent scaffold present in various bioactive molecules, has played a vital role in the

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development of different medicinal agents (Duan *et al.*, 2011; Luedtke *et al.*, 2010; Packiarajan *et al.*, 2011). Encouraged by these reports, some 1,2,4-triazole derivatives bearing pyridyl moiety were synthesized and screened with the aim to achieve the compounds with better anti-tumor activity.

Chemistry

Experimental

All the solvents were obtained from Merck. The homogeneity of the compounds was checked by TLC performed on Silica gel G coated plates (Merck). Iodine chamber was used for visualization of TLC spots. The FT-IR spectra were recorded in KBr pellets on a (Spectrum BX) Perkin Elmer FT-IR spectrophotometer. Melting points were determined on a Gallenkamp melting point apparatus, and thermometer was uncorrected. NMR Spectra were scanned in DMSO-d_6 on a Bruker NMR spectrophotometer operating at 500 MHz for ^{1}H and 125.76 MHz for ^{13}C at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia. Chemical shifts are expressed in δ-values (ppm) relative to TMS as an internal standard, and D_2O was added to confirm the exchangeable protons. Mass spectra (MS) were measured on triple quadruple mass spectroscopy.

*General procedure for the synthesis of: N-4-cyclohexyl/aryl-2-(pyridin-4-ylcarbonyl) hydrazinecarbothioamide (**2a–r**)*

To a solution of appropriate substituted aniline (0.01 mol) in absolute ethanol (20 ml) were added potassium hydroxide (0.01 mol) and carbon disulphide (0.75 ml), and the mixture was stirred at 0–5 °C for 1 h to form a potassium salt of substituted phenyl dithiocarbamate. To the stirred mixture of phenyl thiocarbamate salt was added isoniazid, INH (0.01 mol) and the stirring was continued at 80 °C for 1 h and on adding crushed ice to obtain *N*-(substituted)-2-isonicotinoylhydrazinocarbothioamides (**2a–r**) (Sriram *et al.*, 2009; Bhat *et al.*, 2014).

*General procedure for the synthesis of 4-cyclohexyl/aryl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3a–r**)*

A solution of thiocarbamate in ethanol was dissolved in 4 N NaOH (2 mL) and refluxed for 2 h. The resulting solution was cooled to room temperature and filtered. The filtrate was adjusted to pH 5–6 with dilute acetic acid and kept aside for 1 h. The crystals produced were washed with water, dried, and recrystallized from EtOH to give the compound (Bayrak *et al.*, 2009).

*4-Phenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3a**)*

Yield: (70 %). m. p.: 270–272 °C. FT-IR (ν, cm^{-1}): 3000 (NH str.), 1600, 1494 (C=N str.), 1284 (C=S str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 7.69–7.40 (m, 7*H*, Ar–H), 8.98–8.75 (m, 2*H*, Ar–H), 14.42 (s, 1*H*, NH, D_2O exch.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.19, 148.47 (triazole ring), 150.09, 134.21, 133.32, 129.83, 129.57, 128.70, 122.01 (Ar–C). MS (EI) m/z = 254.2 [M$^+$]. Anal.: Calcd. for $C_{13}H_{10}N_4S$ (254.3): C (61.40), H (3.96), N (22.03), S (12.61). Found: C (61.30), H (3.95), N (22.04), S (12.58) (Siddiqui and Azam, 2012). The single crystal X-ray of compound **3a** has been obtained (Fig. 1).

*4-Cyclohexyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3b**)*

Yield: (72 %). m. p.: 314–316 °C. FT-IR (ν, cm^{-1}): 3098 (arom. CH), 2941, 2854 (ali. CH), 1558 (C=N), 1284(C=S). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 0.96 (d, J = 12.9 Hz, 1*H* alip.), 1.19 (q, J = 12 Hz, 1*H*, $H_{aliph.}$), 1.57 (d, J = 12.5 Hz, 1*H*, $H_{aliph.}$), 1.72 (d, J = 12.9 Hz, 2*H*, $H_{aliph.}$), 1.76 (d, J = 11.7 Hz, 2*H*, $H_{aliph.}$), 2.13 (br, 2*H*, $H_{aliph.}$), 4.25 (br, 1*H*, CH–N), 7.62 (d, 2*H*, J = 4.6 Hz), 8.8 (d, J = 4.5, 2*H*, Ar–H), 14.08 (s, 1*H*, –NH, D_2O exchang.); MS (EI) m/z = 260.0 [M$^+$]. Anal.: Calcd. for $C_{13}H_{16}N_4S$ (260.3): C (59.97), H (6.19), N (21.52), S (12.32). Found: C (59.87), H (6.2), N (21.56), S (12.33). (Ebrahimi, 2010). The single crystal X-ray of compound (**3b**) has been obtained (Fig. 2).

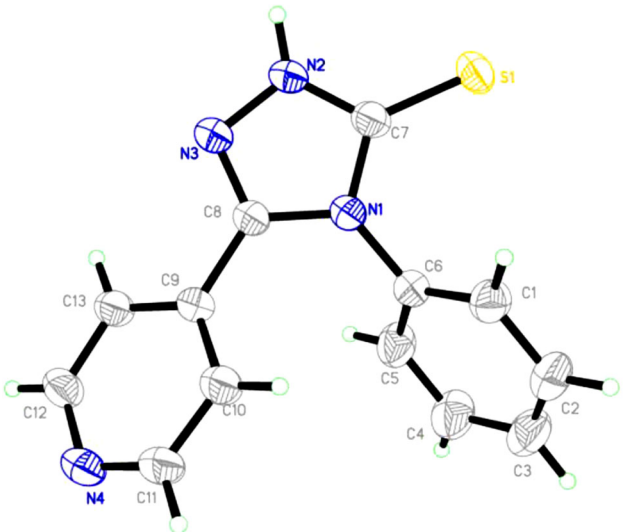


Fig. 1 ORTEP diagram of **3a** at 50 % probability

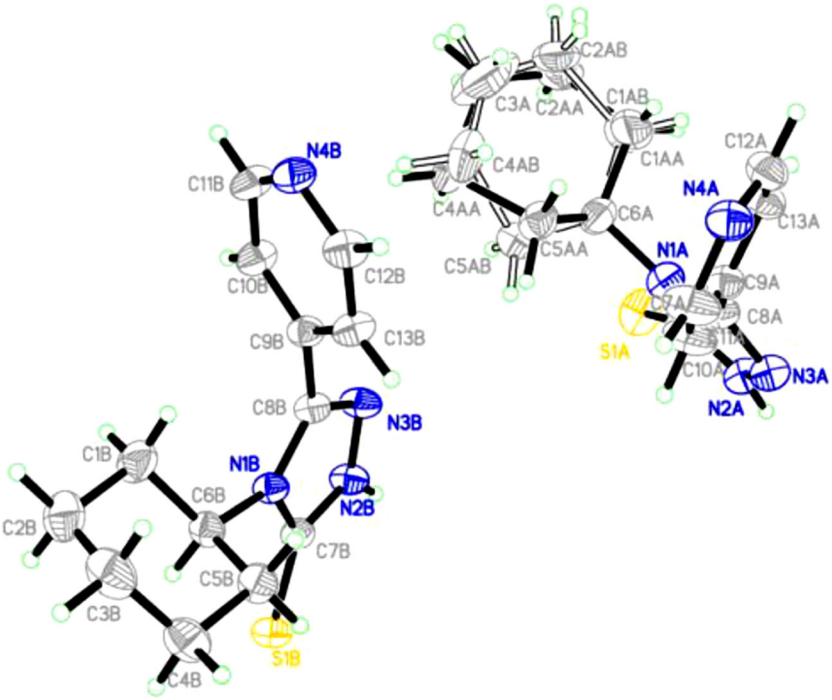


Fig. 2 ORTEP diagram of **3b** at 50 % probability

*4-(4-Chlorophenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3c**)*

Yield: (75 %). m. p.: 310–315 °C. FT-IR (ν, cm^{-1}): 3028 (NH str.), 1606, 1494 (C=N str.), 1288 (C=S str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 7.2–7.5 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.4 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.1, 150.0, 148.0, 135.3, 133.1, 131.1, 130.0, 128.8, 127.6, 122.0. MS (EI) $m/z = 288.5$ [M^+]. Anal.: Calcd. for $C_{13}H_9ClN_4S$ (288.7): C (54.07), H (3.14), N (19.40), S (11.10). Found: C (54.14), H (3.11), N (19.44), S (11.11).

*4-(4-Methoxyphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3d**)*

Yield: (75 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3433 (NH str.), 1653, 1515 (C=N str.), 1290 (C=S str.), 1079 (C–O). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 7.2–7.5 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.4 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.0, 150.0, 148.0, 135.1, 133.0, 131.1, 130.0, 128.1, 127.5, 122.0; MS (EI) $m/z = 284.1$ [M^+]. Anal.: Calcd. for $C_{14}H_{12}N_4OS$ (284.3): C (59.14), H (4.25), N (19.70), S (11.28). Found: C (59.20), H (4.25), N (19.66), S (11.31).

*4-(2,6-Dimethylphenyl)-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3e**)*

Yield: (85 %). m. p.: 310–312 °C. FT-IR (ν, cm^{-1}): 3028 (NH), 1494 (C=N), 1284 (C=S). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.98 (s, 6*H*, CH_3), 7.1–7.4 (m, 5*H*, Ar–H), 8.5 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 168.2, 150.5, 147.4, 135.9, 132.5, 132.2, 130.2, 128.9, 120.0, 18.1, 17.4. MS (EI) $m/z = 282.0$ [M^+]. Anal.: Calcd. for $C_{15}H_{14}N_4S$ (282.3): C (63.80), H (5.00), N (19.84), S (11.36). Found: C (63.71), H (4.98), N (19.86), S (11.38).The single crystal X-ray of compound **3e** has been obtained (Fig. 3).

*4-(3-Ethylphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3f**)*

Yield: (70 %). m. p.: >310 °C. FT-IR (ν, cm^{-1}): 3449 (NH str.), 1606, 1549 (C=N str.), 1280 (C=S str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 3.3 (s, 3*H*, CH_3), 4.1 (2*H*, q, CH_2), 7.24–7.75 (m, 6*H*, Ar–H), 8.5 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 150.02, 145.3, 133.1, 129.3, 129.1, 127.7, 125.7, 121.8, 48.5, 27.7, 15.2. MS (EI) $m/z = 282.0$ [M^+]. Anal.: Calcd. for $C_{15}H_{14}N_4S$ (282.3): C (63.80), H (5.00), N (19.84), S (11.36). Found: C (63.70), H (4.99), N (19.85), S (11.34).

*4-(4-Nitrophenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3g**)*

Yield: (72%). m. p.: 320–322 °C. FT-IR (ν, cm^{-1}): 3450 (NH str.), 1605, 1549 (C=N str.), 1280 (C=S str.); ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 6.7–7.5 (m, 6*H*, Ar–H), 7.8 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 172.0, 155.0, 136.5, 134.0, 125.0,

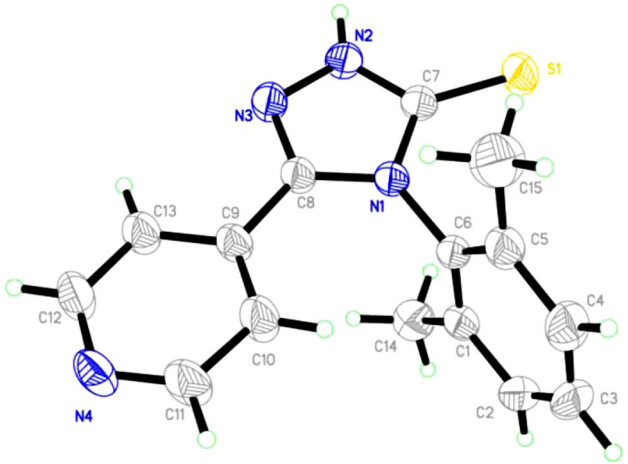


Fig. 3 ORTEP diagram of **3e** at 50 % probability

120.9, 116.0. MS (EI) $m/z = 299.0$ $[M^+]$. Anal.: Calcd. for $C_{13}H_9N_5O_2S$ (299.3): C (52.17), H (3.03), N (23.40), S (10.71). Found: C (52.26), H (3.05), N (23.43), S (10.73).

*4-(4-Sulfapyridinephenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3h**)*

Yield: (60 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3425 (NH str.), 1600, 1586 (C=N str.), 1290 (C=S str.), 1095 (SO_2). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 5.0 (s, 1*H*, SO_2NH, D_2O exchang.), 7.2–7.6 (m, 7*H*, Ar–H), 8.7 (m, 4*H*, Ar–H), 14.4 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.3, 160.4, 157.9, 155.0, 149.5, 110.3, 126.8, 121.0, 110.3. MS (EI) $m/z = 411.20$ $[M]^+$. Anal.: Calcd. for $C_{17}H_{13}N_7O_2S_2$ (411.46): C (49.62), H (3.18), N (23.83), S (15.59). Found: C (49.54), H (3.17), N (23.81), S (15.60).

*4-(4-Methylphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3i**)*

Yield: (75 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3306 (NH str.), 1652, 1558 (C=N str.), 1309 (C=S str.). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 3.3 (s, 3*H*, CH_3), 6.96–7.3 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 9.8 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 179.5, 152.6, 137.2, 133.4, 130.3, 128.9, 123.8, 118.1, 48.5, 20.4. MS (EI) $m/z = 268.2$ $[M]^+$. Anal.: Calcd. for $C_{14}H_{12}N_4S$ (268.3): C (62.66), H (4.51), N (20.88), S (11.95). Found: C (62.56), H (4.50), N (20.92), S (11.93).

*4-(3-Methylphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3j**)*

Yield: (70 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3306 (NH str.), 1652, 1558 (C=N str.), 1309 (C=S str.). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 3.1 (s, 3*H*, CH_3), 7.1–7.4 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 168.8, 149.9, 148.3, 138.9, 134.6, 133.6, 130.0, 128.8, 126.4, 125.5, 123.2, 121.4, 48.5, 21.3. MS (EI) $m/z = 268.1$ $[M]^+$. Anal.: Calcd. for $C_{14}H_{12}N_4S$ (268.3): C (62.66), H (4.51), N (20.88), S (11.95). Found: C (62.57), H (4.49), N (20.90), S (11.91).

*4-(2-Methylphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3k**)*

Yield: (75 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3200 (NH str.), 1607, 1500 (C=N str.), 1319 (C=S str.). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 3.0 (s, 3*H*, CH_3), 7.1–7.4 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 160.4, 155.0, 149.5, 139.2, 136.8, 129.4, 126.1, 124.7, 121.0, 15.8. MS (EI) $m/z = 268.1$ $[M^+]$. Anal.: Calcd. for $C_{14}H_{12}N_4S$ (268.33): C (62.66), H (4.51), N (20.88), S (11.95). Found: C (26.57), H (4.53), N (20.90), S (11.93).

*4-(3-Chorophenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3l**)*

Yield: (70 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3481 (NH str.), 1606, 1507 (C=N str.), 1298 (C=S str.). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 7.2–7.6 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.4 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.0, 150.1, 148.2, 135.3, 133.3, 131.0, 129.9, 128.8, 127.6, 122.0. MS (EI) $m/z = 288.5$ $[M^+]$. Anal.: Calcd. for $C_{13}H_9ClN_4S$ (288.7): C (54.07), H (3.14), N (19.40), S (11.10). Found: C (54.15), H (3.12), N (19.43), S (11.13).

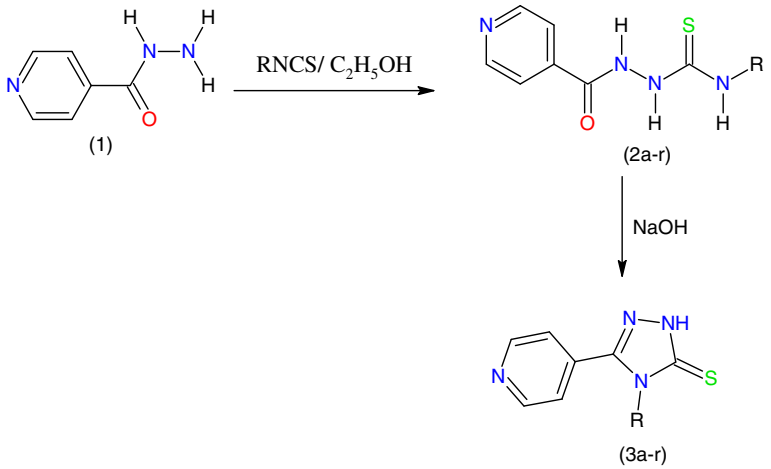
*4-(2-Methoxyphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3m**)*

Yield: (70 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3481 (NH str.), 1606, 1507 (C=N str.), 1298 (C=S str.). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 7.2–7.6 (m, 6*H*, Ar–H), 8.6 (m, 2H, Ar–H), 14.4 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.0, 150.1, 148.2, 135.3, 133.3, 131.0, 129.9, 128.8, 127.6, 122.0. MS (EI) $m/z = 284.2$ $[M^+]$. Anal.: Calcd. for $C_{14}H_{12}N_4OS$ (284.3): C (59.14), H (4.25), N (19.70), S (11.28). Found: C (59.22), H (4.26), N (19.68), S (11.30).

*4-(3-Methoxyphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3n**)*

Yield: (65 %). m. p.: >320 °C. FT-IR (ν, cm^{-1}): 3400 (NH str.), 1600, 1500 (C=N str.), 1200 (C=S str.), 1100 (C–O). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 4.1(s, 3*H*, $-OCH_3$),

Scheme 1 Synthetic protocol of the compounds (**3a**–**r**)



Compound	R
3a	Phenyl
3b	Cyclohexyl
3c	4-Chloro phenyl
3d	4-Methoxy phenyl
3e	2,6-Dimethyl phenyl
3f	3-Ethyl phenyl
3g	4-Nitro phenyl
3h	4-Sulphapyrimidine phenyl
3i	4-Methyl phenyl
3j	3-Methyl phenyl
3k	2-Methyl phenyl
3l	3-Chloro phenyl
3m	2-Methoxy phenyl
3n	3-Methoxy phenyl
3o	4-Acetamido phenyl
3p	4-Ethoxy phenyl
3q	4-Iodo phenyl
3r	2-Nitro phenyl

6.9–7.4 (m, 6*H*, Ar–H), 8.5 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 169.0, 162.3, 159.7, 150.0, 133.1, 130.3, 121.7, 120.5, 115.2, 114.6, 55.4, 48.5, 35.7. MS (EI) $m/z = 284.1$ [M^+]. Anal.: Calcd. for $C_{14}H_{12}N_4OS$ (284.3): C (59.14), H (4.25), N (19.70), S (11.28). Found: C (59.04), H (4.23), N (19.72), S (11.30).

*4-(4-Acetamidophenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3o**)*

Yield: (60 %). m. p.: >320 °C. FT-IR (v, cm^{-1}): 3326 (NH str.), 1680, 1518 (C=N str.), 1335 (C=S str.). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 4.1 (s, 3*H*, –$COCH_3$), 5.5 (s, 1*H*, NH, D_2O exchang.), 6.6–7.2 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 150.0, 149.8, 128.8, 121.7, 113.7, 48.5. MS (EI) $m/z = 311.1$ [M^+]. Anal.: Calcd. for $C_{15}H_{13}N_5OS$ (311.36): C (57.86), H (4.21), N (22.49), S (10.30). Found: C (57.76), H (4.22), N (22.50), S (10.27).

*4-(4-Ethoxyphenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3p**)*

Yield: (60 %). m. p.: 280–282 °C. FT-IR (v, cm^{-1}): 3296 (NH str.), 1636, 1511 (C=N str.), 1246 (C=S str.), 1048 (C–O). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 1.37 (s, 3*H*, CH_3), 4.0 (s, 2*H*, OCH_2), 6.8–7.3 (m, 6*H*, Ar–H), 8.6 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 160.4, 155.0, 149.5, 136.7, 127.1, 125.6, 121.0, 114.7, 64.7, 14.8. MS (EI) $m/z = 298.2$ [M^+]. Anal.: Calcd. for $C_{15}H_{14}N_4OS$ (298.36): C (60.38), H (4.73), N (18.78), S (10.75). Found: C (60.28), H (4.74), N (18.75), S (10.73).

*4-(4-Iodophenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (**3q**)*

Yield: (65 %). m. p.: >320 °C. FT-IR (v, cm^{-1}): 3100 (NH str.), 1600, 1500 (C=N str.), 1200 (C=S str.). ^{1}H NMR

Table 1 IC_{50} values of compounds (**3c–q**)

Compd.	Cell line BEL-7402			Cell line HUH-7			Cell line HepG2		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
3c	2.287 ± 0.0003	1.225 ± 0.0005	0.951 ± 0.0004	0.749 ± 0.0004	0.485 ± 0.0004	0.197 ± 0.0003	0.177 ± 0.0003	0.110 ± 0.0007	0.100 ± 0.0008
3d	2.089 ± 0.0008	1.021 ± 0.0007	0.723 ± 0.0007	0.519 ± 0.0006	0.305 ± 0.0006	0.113 ± 0.0009	0.092 ± 0.0006	0.052 ± 0.0002	0.043 ± 0.0004
3e	2.068 ± 0.0007	1.001 ± 0.0006	0.701 ± 0.0006	0.498 ± 0.0005	0.290 ± 0.0005	0.106 ± 0.0008	0.086 ± 0.0005	0.048 ± 0.0003	0.039 ± 0.0003
3f	2.197 ± 0.0005	1.130 ± 0.0007	0.842 ± 0.0006	0.636 ± 0.0008	0.395 ± 0.0006	0.154 ± 0.0006	0.132 ± 0.0006	0.079 ± 0.0007	0.069 ± 0.0008
3g	2.241 ± 0.0005	1.176 ± 0.0005	0.894 ± 0.0006	0.690 ± 0.0006	0.437 ± 0.0006	0.174 ± 0.0003	0.153 ± 0.0004	0.093 ± 0.0003	0.083 ± 0.0006
3h	2.153 ± 0.0007	1.085 ± 0.0004	0.792 ± 0.0008	0.586 ± 0.0009	0.356 ± 0.0009	0.136 ± 0.0005	0.114 ± 0.0008	0.067 ± 0.0005	0.057 ± 0.0004
3i	2.048 ± 0.0007	0.981 ± 0.0005	0.680 ± 0.0005	0.478 ± 0.0004	0.276 ± 0.0006	0.100 ± 0.0007	0.080 ± 0.0004	0.044 ± 0.0004	0.035 ± 0.0002
3j	2.027 ± 0.0006	0.961 ± 0.0004	0.660 ± 0.0004	0.459 ± 0.0006	0.262 ± 0.0007	0.094 ± 0.0006	0.074 ± 0.0003	0.041 ± 0.0004	0.032 ± 0.0003
3k	2.219 ± 0.0006	1.153 ± 0.0004	0.868 ± 0.0007	0.662 ± 0.0007	0.415 ± 0.0005	0.164 ± 0.0004	0.142 ± 0.0003	0.086 ± 0.0006	0.075 ± 0.0007
3l	2.264 ± 0.0004	1.201 ± 0.0006	0.922 ± 0.0005	0.719 ± 0.0005	0.460 ± 0.0005	0.186 ± 0.0005	0.164 ± 0.0005	0.102 ± 0.0004	0.091 ± 0.0007
3m	2.132 ± 0.0008	1.063 ± 0.0005	0.768 ± 0.0009	0.563 ± 0.0008	0.338 ± 0.0008	0.128 ± 0.0006	0.106 ± 0.0009	0.062 ± 0.0004	0.052 ± 0.0005
3o	2.310 ± 0.0002	1.250 ± 0.0004	0.980 ± 0.0003	0.780 ± 0.0003	0.510 ± 0.0003	0.210 ± 0.0001	0.190 ± 0.0002	0.120 ± 0.0006	0.110 ± 0.0009
3p	2.175 ± 0.0004	1.107 ± 0.0006	0.816 ± 0.0005	0.611 ± 0.0009	0.375 ± 0.0008	0.145 ± 0.0007	0.123 ± 0.0007	0.073 ± 0.0008	0.062 ± 0.0006
3q	2.110 ± 0.0009	1.042 ± 0.0006	0.745 ± 0.0008	0.540 ± 0.0007	0.321 ± 0.0007	0.120 ± 0.0007	0.099 ± 0.0008	0.057 ± 0.0003	0.047 ± 0.0006
[a]C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[b]C	1.220 ± 0.0002	1.012 ± 0.0003	0345 ± 0.0004	0.440 ± 0.0007	0.222 ± 0.0007	0.112 ± 0.0007	0.089 ± 0.0008	0.045 ± 0.0003	0.031 ± 0.0006

Data are the mean ± SD of at least three independent experiments. ** $P < 0.01$ versus the control, the difference was markedly significant

* $P < 0.05$; ** $P < 0.01$. * $P < 0.05$ versus the control, the difference was significant

[a] C = negative control

[b] C = positive control, sorafenib (50 μM)

Table 2 The apoptosis ratio of cells treated after treatment and incubation for 24 h values of compounds (**3c–q**)

Compd.	The apoptosis ratio of cells treated after treatment and incubation for 24 h in HepG2		
	50 μmol/L	100 μmol/L	150 μmol/L
3c	14.23 %	47.95 %	40.54 %
3d	22.07 %	74.39 %	74.54 %
3e	23.18 %	78.11 %	79.75 %
3f	17.29 %	58.29 %	53.14 %
3g	15.69 %	52.87 %	46.42 %
3h	19.07 %	64.26 %	60.84 %
3i	24.33 %	82.02 %	85.34 %
3j	25.55 %	86.12 %	91.31 %
3k	16.47 %	55.51 %	49.67 %
3l	14.94 %	50.35 %	43.385 %
3m	20.02 %	67.485 %	65.10 %
3o	12.33 %	34.49 %	45.16 %
3p	18.16 %	61.20 %	56.86 %
3q	21.02 %	70.85 %	69.66 %
[a]C	0.57 %	1.22 %	3.65 %
[b]C	23.78 %	35.66 %	60.13 %

Data are expressed as mean and evaluated by one-way analysis of variance (ANOVA); results are representative of three replicates (* $P < 0.01$)

[a] C = negative control

[b] C = positive control, sorafenib

Table 3 The Protein Breaks % of cells treated after treatment and incubation for 24 h values of compounds (**3c–q**)

Compd.	The DNA Breaks % of cells treated after treatment and incubation for 24 h values of compounds		
	50 μmol/L	100 μmol/L	150 μmol/L
3c	5.94 %	9.56 %	14.38 %
3d	9.75 %	20.01 %	38.33 %
3e	9.94 %	20.61 %	39.86 %
3f	7.10 %	12.47 %	20.46 %
3g	6.56 %	11.08 %	17.49 %
3h	7.84 %	14.45 %	24.90 %
3i	10.14 %	21.23 %	41.46 %
3j	10.35 %	21.86 %	43.11 %
3k	6.83 %	11.75 %	18.92 %
3l	6.18 %	10.14 %	15.55 %
3m	8.16 %	15.33 %	26.93 %
3o	5.60 %	8.74 %	12.78 %
3p	7.54 %	13.62 %	23.02 %
3q	9.56 %	19.42 %	36.85 %
[a]C	0.20 %	0.44 %	0.75 %
[b]C	6.28 %	20.34 %	45.89 %

Data are expressed as mean and evaluated by one-way analysis of variance (ANOVA)

Results are representative of three replicates (* $P < 0.01$)

[a] C = negative control

[b] C = positive control, sorafenib

(500 MHz, DMSO-d6, δ ppm): 6.8–7.4 (m, 6*H*, Ar–H), 8.0 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 160.4, 155.0, 149.5, 137.9, 136.7, 132.9, 128.1, 121.0, 90.0. MS (EI) $m/z = 380.0$ [M^+]. Anal.: Calcd. for $C_{13}H_9N_4SI$ (380.20): C (41.07), H (2.39), N (14.74), S (8.43). Found: C (41.15), H (2.40), N (14.73), S (8.40).

4-(2-Nitrophenyl-5-(pyridin-4-yl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***3r***

Yield: (70 %). m. p.: >320 °C. FT-IR (v, cm^{-1}): 3100 (NH str.), 1600, 1494 (C=N str.), 1200 (C=S str.). ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 6.7–7.4 (m, 6*H*, Ar–H), 7.8 (m, 2*H*, Ar–H), 14.5 (s, 1*H*, NH, D_2O exchang.). ^{13}C NMR (125.76, DMSO-d_6, δ ppm): 172.2, 155.9, 136.8, 134.7, 125.3, 120.8, 116.3. MS (EI) $m/z = 299.0$ [M^+]. Anal.: Calcd. for $C_{13}H_9N_5O_2S$ (299.3): C (52.17), H (3.03), N (23.40), S (10.71). Found: C (52.27), H (3.04), N (23.44), S (10.70).

In vitro antitumor activity

Cell culture The cells were routinely cultured in RPMI-1640 medium, supplemented with 10 % fetal calf serum. The culture was maintained at 37 °C with a gas mixture of 5 % CO_2/95 % air. The medium was changed every 2 days and the cells were sub cultured every 3 days.

Cell viability assay Cell viability was determined using the MTT assay. Briefly, the cells were collected and resuspended in RPMI-1640 medium at 4×10^4 cells/mL, 100 μL aliquots were added to each well of 96-well flat-bottomed micro titer plates, followed by addition of 100 μL of the complexes one and two. Three replicate wells were used for each data point in the experiments. After incubation for the indicated intervals, 20 μL of MTT (5 mg/mL in PBS) solution was added to each well and plates were then incubated for 4 h at 37 °C. The medium with MTT was removed from the wells. Intracellular formazan crystals were dissolved by adding 150 μL of DMSO to each well, and the plates were shaken for 10 min. The absorbance was read at 490 nm with a microplate reader. Percentage of survival was calculated as a fraction of the negative control (medium only). The half-maximal inhibitory concentration (IC_{50}) was obtained from the dose-response curve with an original 6.0 software.

Flow cytometry analysis

Cells were seeded into 100 mL cell culture bottles at 12×10^6 cells 24 h before treatment. Then cells were treated according to the aforementioned method and incubated for 24 h. After wards, cells were collected, combined into single cell suspension, and centrifuged at 8,000 rpm for 5 min. The supernatant was discarded and the cells were washed three times with cooled PBS and fixed for 24 h with cold alcohol at 4 °C. One milliliter cell suspension (10^6/mL) was washed three times with the cool PBS, treated with RNase for 30 min at 37 °C, stained it with PI for 30 min at 37 °C in a dark environment, and taken for flow cytometry analysis.

Western blotting

The cells were taken in the logarithmic growth phase, treated according to the aforementioned method, and incubated for 24 h. After fragmentation on ice for 20 min, the lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, the protein was collected, quantitated with the BCA method, electrophoresed and isolated by the SDS-PAGE (10 %) using the electrotransfer method, blocked and hybridized on the cellulose nitrate film. The protein expression of cells was detected using the ECL Western blotting method. The densities of protein bands were calculated using the Quantity One software.

Statistical analysis Data were expressed as the mean SD from these independent experiments. Statistic analysis was performed using the SPSS 13.0 for Windows. Comparisons between two groups were performed by unpaired test. Multiple comparisons between more than two groups were performed by one-way analysis of variance (ANOVA). Significance was accepted at value lower than 0.05.

Result and discussion

Chemistry

In the present work, eighteen compounds (**3a–r**) were synthesized. (Scheme 1) illustrates the way used for the preparation of target compounds. The reaction of thiosemicarbazides (**2a–r**) with sodium hydroxide, in each case, a single product was obtained. The FT IR, ^{1}H NMR, ^{13}C NMR, MS, and single crystal X-ray crystallography analysis confirmed the structure of these products as *N*-4-cyclohexyl/aryl-5-(pyridine-4-yl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones (**3a–r**). In IR spectra of all compounds NH were observed at about 3,000–3,481 cm^{-1}. In the ^{1}H NMR spectra of the compounds (**3a–r**) that are taken in DMSO-d_6, D_2O exchangeable NH proton was seen as singlet at 9.8–14.5 ppm. The aromatic protons appear as multiplet at 6.6–8.7 ppm.MS of the compounds showed molecular ion peaks $[M]^+$, in agreement with their molecular formula. In the ^{13}C-NMR spectra of all compounds (**3a–r**), all aromatic and aliphatic carbons were observed at expected regions. All compounds gave satisfactory elemental analysis.

In vitro antitumor studies

The compounds were evaluated for their ability to inhibit the growth of BEL-7402, HUH-7 and HepG2 human hepatoma cell lines using MTT assay. The inhibition was expressed as cell viability relative to control without compound treatments. In the present study, BEL-7402, HUH-7 and HepG2 human hepatoma cells were used which have been recently characterized as a suitable model for in vitro assessment of hepatoma toxicity (Banik *et al.*, 2011; Johnstone *et al.*, 2002). Sorafenib (50 μM) was used as a positive control, which has been used extensively as an efficient anticancer drug (Sonntag *et al.*, 2014). After treated for 24, 48 and 72 h on the selected three cell lines, the cells viability with their the IC_{50} values was calculated (Table 1). The synthesized compounds exhibit antiproliferative effect to human hepatoma cells BEL-7402, HUH-7 and HepG2 in a time and dose-dependent manner with increasing the concentrations of compounds in the following order of activity **3j** > **3i** > **3e** > **3d** > **3q** > **3m** > **3h** > **3p** > **3f** > **3k** > **3g** > **3l** > **3c** > **3o**. The results of the MTT-dye reduction assay unambiguously indicate that the compounds exert potent cytotoxic/antiproliferative effect. Flow cytometric analyses of apoptosis to further examine the effects of the compounds on apoptosis; flow cytometry was used to quantify the apoptotic state (Table 2). After treatment and incubation for 24 h, the apoptosis ratio of cells treated with 50, 100, and 150 μmol/L concentration of the compounds. The results also supported the notion that the tested compounds induced apoptosis of HepG2 cells in a concentration-dependent manner in the following order of activity **3j** > **3i** > **3e** > **3d** > **3q** > **3m** > **3h** > **3p** > **3f** > **3k** > **3g** > **3l** >**3c** > **3o**.After treatment with the compounds (50, 100 and 150 μmol/L, respectively) for 24 h, the caspase-3 zymogen protein bands became thinner. Studies have proved that the unactivated caspase-3 will trigger apoptosis when it is activated and play a very important role as the central effector of apoptosis when cells start apoptosis. Our results showed that the compounds could significantly enhance the activity of caspase-3 (Table 3) in the following order of activity **3j** > **3i** > **3e** > **3d** > **3q** > **3m** > **3h** > **3p** > **3f** > **3k** > **3g** > **3l** > **3c** > **3o**.

Structure activity relationship (SAR)

Careful investigation of the relation between structure and the data of tested activities revealed the following assumption about SAR.

a) The effect of different substitutions on the aromatic portion on the activity was in the following order $CH_3 > OCH_3 > I > SO_2NH_2 > OC_2H_5 > C_2H_5 > NO_2 > Cl > CH_3CONH$.
b) Concerning the alkyl substitutions on aromatic part, the methyl group greatly increases the activity rather than the more bulky ethyl one, and also its position and numbers greatly influence the activity where as the number of alkyl substitutions increases, the activity decreases and its presence in the para position also decreases the activity.
c) Concerning the alkoxy substitutions on aromatic part, the methoxyl group greatly increases the activity rather than the more bulky ethoxyl one, and also its position greatly influence the activity where its presence in the para position increases the activity.
d) Concerning the halide substitutions on aromatic part, the larger the atomic weight and the less inductive effect iodine atom greatly increases the activity than chloro one in the same manner as for alkyl ones, presence of halide in the para position also decreases the activity.

Conclusion

The newly synthesized compounds exhibit antiproliferative effect to human hepatoma cells BEL-7402, HUH-7 and HepG2 via induced apoptosis of HepG2 cells in a concentration-dependent manner in the following order of activity **3j** > **3i** > **3e** > **3d** > **3q** > **3m** > **3h** > **3p** > **3f** > **3k** > **3g** > **3l** > **3c** > **3o**. The exact mechanism of action is enhance the activity of caspase-3.

Supplementary details

Crystallographic Data Center (CCDC) numbers 43737–943738, 943618–943619, and 943735–943736 contains crystallographic data for the structures **3a**, **3b**, and **3e,** respectively. Copies of the data can be obtained, free of charge, on application to CCDC 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk/http://www.ccdc.cam.ac.uk).

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References

Almasirad A, Tabatabai A, Shafiee A (2004) Synthesis and anticonvulsant activity of new 2-substituted-5-[2-(2-fluorophenoxy) phenyl]-1,3,4-oxadiazoles and 1,2,4-triazoles. Bioorg Med Chem Lett 14(11):6057–6059

Banik B, Sasmal PK, Roy S, Majumdar R, Dighe RR, Chakravarty AR (2011) Terpyridine oxovanadium (IV) complexes of phenanthroline bases for cellular imaging and photocytotoxicity in HeLa cells. Eur J Inorg Chem 9:1425–1435

Bayrak H, Demirbas A, Karaoglu SA, Demirbas N (2009) Synthesis of some new 1,2,4-triazoles, their mannich and Schiff bases and evaluation of their antimicrobial activities. Eur J Med Chem 44(3):1057–1066

Bhat MA, Khan AA, Khan S, Al-Omar MA, Parvez MK, Al-Dosari MS, Al-Dhfyan A (2014) Synthesis and anti-candidal activity of *N*-(4-aryl/cyclohexyl)-2-(pyridine-4-yl-carbonyl) hydrazinecarbothioamide. Bioorg Med Chem Lett 24:1299–1302

Duan M, Kazmierski WM, Chong PY, Deanda F, Edelstein M, Ferris R, Peckham J, Wheelan P, Xiong Z, Zhang H, Nishizawa R, Takaoka Y (2011) Discovery of novel pyridyl carboxamides as potent CCR5 antagonists and optimization of their pharmacokinetic profile in rats. Bioorg Med Chem Lett 21(21):6470–6475

Ebrahimi S (2010) Synthesis of some pyridyl cyclohexyl substituted 1,2,4-triazole, 1,3,4-thiadiazole and 1,3,4-oxadiazole derivatives. Eur J Chem 1(4):322–324

Grossi G, Braccio M, Roma G, Ballabeni V, Toglonini M, Calcina F, Barocelli E (2002) 1,5-Benzodiazepines. Part XIII. Substituted 4*H*-[1,2,4]triazolo[4,3-a][1,5]benzodiazepin-5-amines and 4*H*-imidazo[1,2-a][1,5]benzodiazepin-5-amines as analgesic, anti-inflammatory and/or antipyretic agents with low acute toxicity. Eur J Med Chem 37:933–944

Haber J (2001) Present status and perspectives on antimycoties with systematic effects. Cas Lek Cesk 140:596–604

Holla BS, Poojary KN, Rao BS, Shivananda MK (2002) New bis-aminomercaptotriazoles and bis-triazolothiadiazoles as possible anticancer agents. Eur J Med Chem 37(6):511–517

Johnstone RW, Ruefli AA, Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell 108:153–164

Kaplancikli ZA, Turan-Zitouni G, Ozdemir A, Revial G (2008) New triazole and triazolothiadiazine derivatives as possible antimicrobial agents. Eur J Med Chem 43(1):155–159

Kucukguzel I, Tatar E, Kucukguzel SG, Rollas S, De Clercq E (2008) Synthesis of some novel thiourea derivatives obtained from 5-[(4-amino phenoxy) methyl]-4-alkyl/aryl-2,4-dihydro-3*H*-1,2,4-triazole-3-thiones and evaluation as antiviral/anti-HIV and anti-tuberculosis agents. Eur J Med Chem 43(2):381–392

Labanauskas L, Udrenaite E, Brukstus A (2004) Synthesis of 5-(2,3 and 4-methoxyphenyl)-4*H*-1,2,4-triazole-3-thiol derivatives exhibiting anti-inflammatory activity. IL Farmaco 59(4):255–259

Lebouvier N, Pagniez F, Duflas M, Le Pape P, Na YM, Le Bault G, Le Borgne M (2007) Synthesis and antifungal activities of new fluconazole analogues with azaheterocycle moiety. Bioorg Med Chem Lett 17(13):3686–3689

Liu P, Zhu S, Xie P, Xie W, Jiang Y, Zhang D (2008) Synthesis and SAR studies of biaryloxy-substituted triazoles as antifungal agents. Bioorg Med Chem Lett 18:3261–3265

Luedtke GR, Schinzel K, Tan X, Tester RW, Nashashibi I, Xu YJ, Dugar S, Levy DE, Jung J (2010) Amide-based inhibitors of p38alpha MAP kinase. Part 1: discovery of novel *N*-pyridyl amide lead molecules. Bioorg Med Chem Lett 20(8):2556–2559

Mathew V, Keshavayya J, Vaidya VP (2006) Heterocyclic system containing bridgehead nitrogen atom: synthesis and pharmacological activities of some substituted 1,2,4-triazolo [3,4-b]-1,3,4-thiadiazoles. Eur J Med Chem 41(9):1048–1058

Packiarajan M, Coate H, Desai M, Jimenez HN, Reinhard EJ, Jubian VJ, Marzabadi MR, Chandrasena G, Wolinski TC, Walker MW, Andersen K (2011) 5-(2′-Pyridyl)-2-aminothiazoles: alkyl amino sulfonamides and sulfamides as potent NPY (5) antagonists. Bioorg Med Chem Lett 21(21):6500–6504

Prakash OP, Bhardwaj V, Kumar R, Aneja KR (2004) Organoiodine (III) mediated synthesis of 3-aryl/hetryl-5,7-dimethyl-1,2,4-triazolo[4,3-a]pyrimidines as antibacterial agents. Eur J Med Chem 39(12):1073–1077

Siddiqui SM, Azam SA (2012) Synthesis, characterization and antimicrobial activity of some hydrazone and azole derivatives bearing pyridyl moiety as promising heterocyclic scaffold. Eur J Med Chem 49:411–416

Sonntag R, Gassler N, Bangen J-M, Trautwein C, Liedtke C (2014) Pro-apoptotic sorafenib signaling in murine hepatocytes depends on malignancy and is associated with PUMA expression in vitro and in vivo. Cell Death Dis 5:e1030. doi:10.1038/cddis.2013.557

Sriram D, Yogeeswari P, Priya DY (2009) Antimycobacterial activity of novel *N*-(substituted)-2-isoniconinoylhydrazono-carbothioamide endowed with high activity towards isoniazid resistant tuberculosis. Biomed Pharmacother 63:36–39

Sztanke R, Pasternak K, Jozwiak K (2006) Synthesis of imidazoline and imidazo[2,1-c][1,2,4]triazole aryl derivatives containing the methylthio group as possible antibacterial agents. Bioorg Med Chem 14(2):3635–3642

Sztanke K, Tuzimski T, Rzymowska J, Pasternak K, Szerszen MK (2008) Synthesis, determination of the lipophilicity, anticancer and antimicrobial properties of some fused 1,2,4-triazole derivatives. Eur J Med Chem 43:404–419