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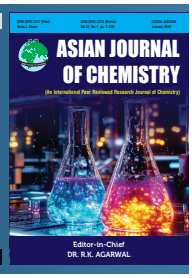

Synthesis, Anticancer and EGFR Inhibitory Activity of Novel [1,2,4]Triazolo[3,4-*b*][1,3,4]thiadiazine-isoxazoles

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A novel approach was utilized for the synthesis of new [1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazine containing isoxazoles (**6a-o**) from 3-ethynyl-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine-8,8-dioxide (**4**) in combination with freshly prepared substituted nitrile oxides (**5**) using Cu(I)-catalyzed [3+2] cycloaddition method. The *in vitro* cancer activity of the synthesized compounds was carried out against two lung cancer cell lines, A-549 and H1299. Among the compounds tested, **6h**, **6k** and **6l** exhibited significantly stronger anticancer activity compared to standard drug erlotinib, with IC_{50} values ranging from 0.62 ± 0.20 to 4.40 ± 0.14 µM. The cytotoxicity of the more potent compounds was also tested against HEK-293 normal cell line and showed no toxic activity. Compound **6k** appears to have higher inhibitory activity against EGFR (IC_{50} = 0.41 ± 0.03 µM) compared to the standard drug erlotinib (IC_{50} = 0.42 ± 0.03 µM), while compounds **6h** and **6l** demonstrated prominent activity. Further potent molecule believed to be a future drug for lung cancer.

Keywords: Anticancer activity, EGFR, Isoxazole, [1,3,4]Thiadiazine, [1,2,4]Triazole.

INTRODUCTION

Cancer, a collection of disease marked by unregulated cellular proliferation with the ability to metastasize to various regions of the body, ranks as the second primary cause of mortality globally, following cardiovascular conditions [1,2]. Lung cancer exhibits significant morbidity and stands as the primary cause of cancer-related fatalities, accounting for approximately 20% of all cancer mortalities [3-6]. The advancement of innovative anticancer agents has led to significant progress in lung cancer treatment in recent years. Mortality and morbidity rates are still significant, nevertheless, with the total 5-year survival rate at about 15% [7,8]. Based on the information provided, it appears that a number of research have concentrated on EGFR as a means of regulating cancer cell proliferation [9].

A number of cellular processes that influence the growth and evolution of tumors are regulated by EGFR. These functions encompass growth, differentiation, inhibition of apoptosis, spread (through its influence on cell movement, invasiveness and absence of adhesion dependence) and the formation of new blood vessels. Moreover, it is essential in regulating these various cellular functions [10]. The unusual expression of EGFR-TK has been observed in various human cancers, such as those of liver, breast and colon. In several studies, the presence of EGFR was shown to correlate with unfavorable outcomes and resistance to standard cancer treatments.Therefore, the inhibitors of EGFR-TK activity that compete with its cognate ligands have the potential to become a significant class of medicines that are effective in the treatment of cancer [11,12].

Triazolothiadiazine, a hybrid nucleus resulting from the amalgamation of two pharmacologically active components, triazole and thiadiazine, is a significant structure owing to its extensive applications as synthetic intermediates and potential pharmaceuticals [13,14], especially in anticancer activity [15-17]. Recent reports disclosed novel [1,2,4]triazolo[3,4-*b*][1,3,4]-thiadiazine-1,2,3-triazoles as effective EGFR-targeting thera-

peutics against breast cancer, with compound **C** demonstrating significant anticancer and EGFR activity. In a similar vein, a series of phenyl-linked 1,2,3-triazole[1,2,4]triazolo[3,4-*b*]-[1,3,4]thiadiazine hybrids by a click reaction, with compound **D** demonstrating significant anticancer activity against the tested cancer cell lines and robust EGFR inhibitory effect [18]. On the other hand, the isoxazole moiety is a significant heterocycle that consists of five members and contains one oxygen atom along with nitrogen. Isoxazole-based heterocycles are among the most abundant of the biologically active chemicals [19,20]. It has been demonstrated that a number of isoxazole derivatives have the ability to exercise their anticancer activity by targeting a variety of proteins, including EGFR [21]. As an instance, the isoxazole derivative **E** showed remarkable anticancer activity [22], whilst derivative **F** was demonstrated to excellent possess antitumor and EGFR-TK inhibitory properties (Fig. 1) [23]. The molecular hybridization (MH) technique is an effective method for the rational design of novel ligands or prototypes of active compounds [24-27]. The design of novel hybrid structures that keep the pre-selected properties of the original templates can be accomplished by linking or fusing together the pharmacophoric sub-units that are present in the molecular structure of two or more recognized bioactive molecules [28].

Considering the significant roles of [1,2,4]triazolo[3,4-*b*]-[1,3,4]thiadiazine and isoxazole in the advancement of various anticancer pharmaceuticals (Fig. 1). The ongoing study involved in the development and biological evaluation of fused 1,2,3-triazole-based compounds [29-31]. The utilization of molecular hybridization strategies [32] to obtain potent biologically active entities, considering the significant roles of 1,2,4-triazolo isoxazoles, we wish to synthesize several novel [1,2,4]triazolo-[3,4-*b*][1,3,4]thiadiazine-linked isoxazoles by molecular hybridization strategies. The efficacy of the newly synthesized compounds in combating cancer was also assessed against A-549 and H1299 cell lines. The promising compounds were further assessed for their inhibitory effects against EGFR kinase.

EXPERIMENTAL

All the commercially available chemicals were utilized without further purification. The purity of compounds was anal-

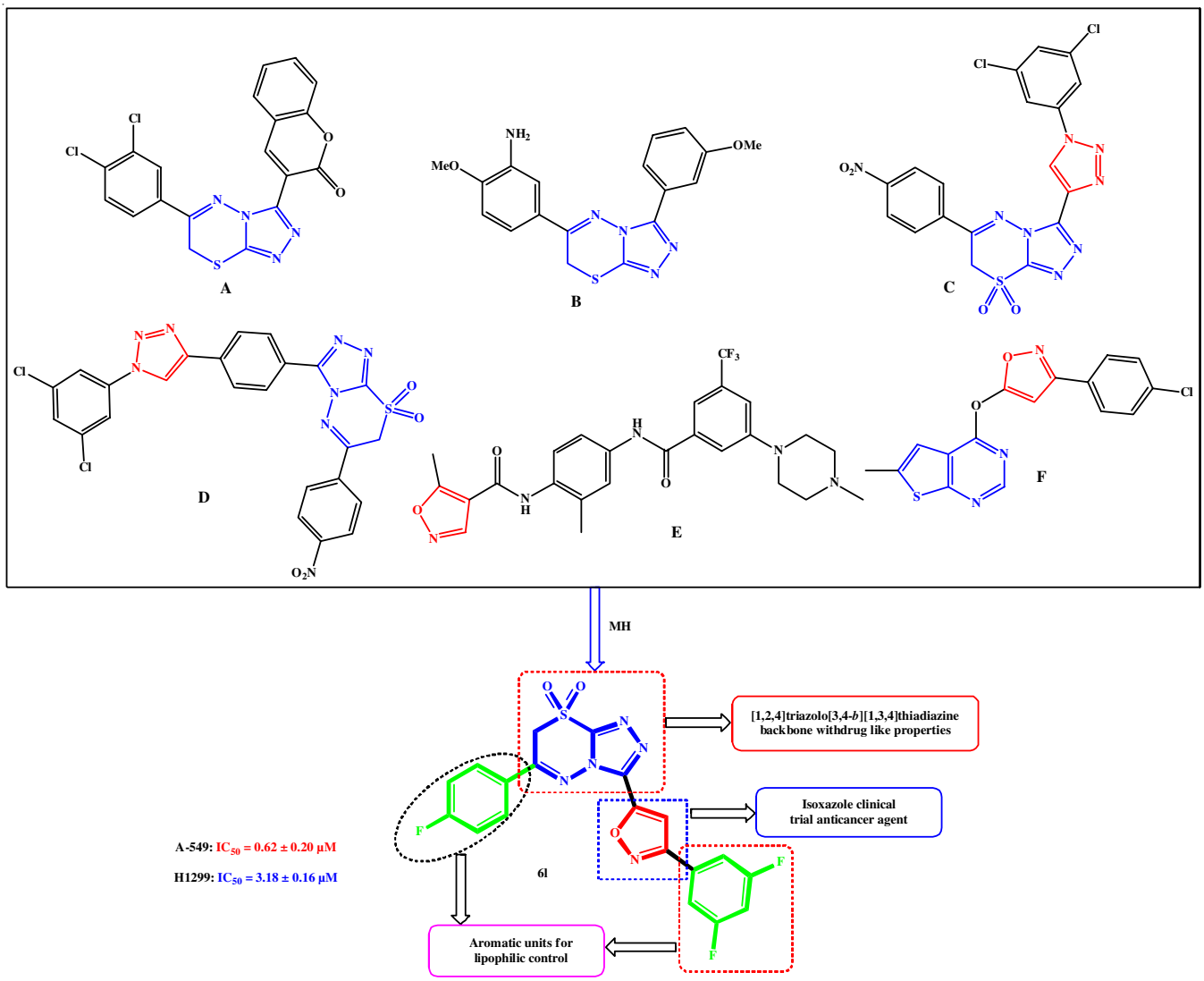


Fig. 1. Rational design of new [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine-isoxazole hybrids *via* pharmacophore hybridization approach

yzed on Merck $60F_{254}$ silica gel TLC plates. Melting points were recorded on a hot stage melting point apparatus in Ernst Leitz-Wetzlar, Germany and were uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded using the Mercuryplus spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C), and the chemical shifts were referenced to TMS. The ESI (electrospray ionization) mass spectra at an ionizing voltage of 70 eV were obtained with the help of a Shimadzu QP5050A quadrupole-based mass spectrometer.

Synthesis of 3-ethynyl-6-(4-fluorophenyl)-7*H*-[1,2,4]-triazolo[3,4-*b*][1,3,4]thiadiazine (3): A mixture of **2** (0.02 mol) and 2-bromo-1-(4-fluorophenyl)ethan-1-one (0.02 mol) in anhydrous ethanol (40 mL) was refluxed for 5 h. The solvent was removed under reduced pressure, diethyl ether (30 mL) was added and the reaction mixture was left at 0 ºC overnight. The precipitated solid was filtered off, dried and recrystallized with ethanol to give **5** (72 %) as a pale red solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.19 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.94 (d, *J* = 8.0 Hz, 2H, Ar-H), 3.48 (s, 2H, -CH_2), 2.09 (s, 1H, CH); ESI-MS (*m/z*): calcd. for $C_{12}H_7FN_4S$: $[M]^+$ *m/z:* 258, found: 259 $[M+H]^+$.

3-Ethynyl-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*]-[1,3,4]thiadiazine 8,8-dioxide (4): To 3-ethynyl-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (**3**) (0.01 mol) in DCM (30 mL), *m*-CPBA (0.04 mol) was added slowly at 0 ºC and the reaction mixture was stirred at room temperature for 12 h. The progress of the reaction was analyzed by TLC and the reaction mixture was then quenched with a saturated aqueous solution of sodium bicarbonate (40 mL) and extracted with DCM (2 × 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and finally concentrated under reduced pressure to afford compound **6** (65%) as a dirty white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.20 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.96 (d, *J* = 8.0 Hz, 2H, Ar-H), 4.21 (s, 2H, -CH_2), 2.11 (s, 1H, CH); ESI-MS (*m/z*): calcd. for $C_{12}H_7FN_4O_2S$: $[M]^+$ *m/z:* 290, found: 291 $[M+H]^+$.

General procedure for synthesis of aryl nitrile oxides (5a-o): Various aryl aldehydes (2 mmol) was added to hydroxylamine hydrochloride (2 mmol) in 8 mL of 1:1 *t*-BuOH:H_2O. To this was added NaOH solution and after being stirred for 30 min at ambient temperature. TLC analysis indicated that oxime formation was complete. Chloramine-T trihydrate (2 mmol) was added in small portions over 5 min then formed nitrile oxides without isolation used for next step [33].

General procedure for the synthesis of 6-(4-fluorophenyl)-3-(3-(aryl)isoxazol-5-yl)-7*H*-[1,2,4]triazolo[3,4-*b*]-[1,3,4]thiadiazine 8,8-dioxide (6a-o): To a stirred solution of alkyne (**6**, 0.001 mol) and freshly prepared nitrile oxide (**5a-o**, 0.0012) in THF (20 mL) was added CuI (10 mol%) and the reaction mixture was stirred at room temperature for 8-10 h. After completion of the reaction, the reaction mixture was diluted with water (20 mL) and the product was extracted with ethyl acetate (2 × 20 mL). The combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. After filtration, the solvent was evaporated under vacuum and the crude product obtained was purified by column chromatography (hexane/ethyl acetate gradient) to afford the compounds **6a-o** (**Scheme-I**).

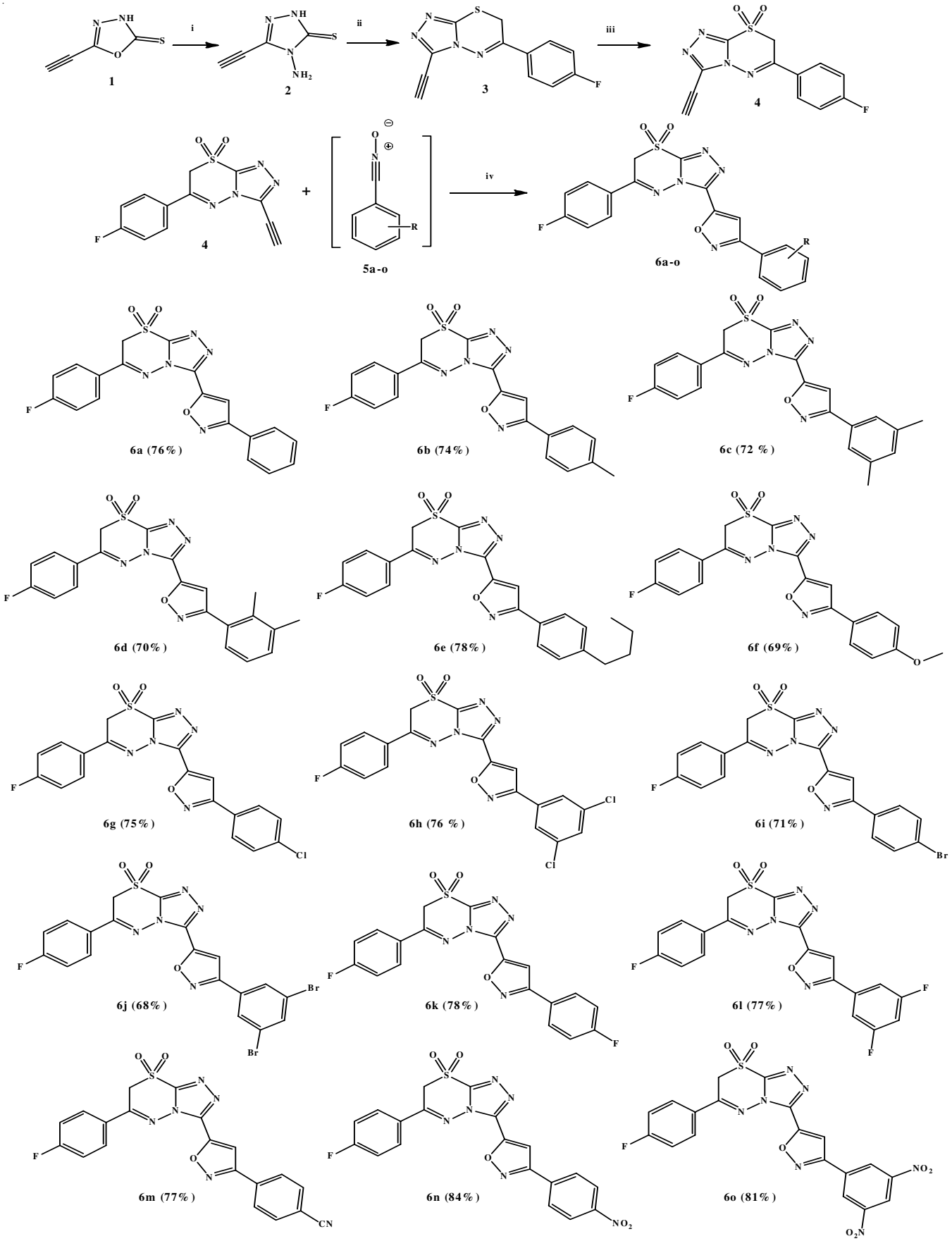
6-(4-Fluorophenyl)-3-(3-phenylisoxazol-5-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine-8,8-dioxide (6a): Pale red solid, m.p.: 122-124 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.20 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.93 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.65 (d, *J* = 8.0 Hz, 2H), 7.25-7.21 (m, 3H), 6.71 (s, 1H) 5.11 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.29, 164.29, 163.25, 162.19, 161.25, 146.58, 143.17, 132.69, 130.48, 129.87(2C), 129.01, 128.10(2C), 127.86, 127.06(2C), 116.61, 116.15, 100.78, 44.89; Anal. calcd. (found) % for $C_{19}H_{12}FN_5O_3S$: C, 55.74 (55.72); H, 2.95 (2.93); N, 17.11 (17.14). ESI-MS (*m/z*): calcd. for $C_{19}H_{12}FN_5O_3S$: $[M]^+$: 409, found: 410 $[M+H]^+$.

6-(4-Fluorophenyl)-3-(3-(*p*-tolyl)isoxazol-5-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6b): Pale red solid, m.p.: 127-129 ºC, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.19 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.94 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.18 (d, *J* = 8.0 Hz, 2H), 6.72 (s, 1H) 5.12 (s, 2H, S-CH_2), 2.32 (s, 3H, -CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.49, 164.50, 163.20, 162.10, 161.43, 146.45, 143.59, 139.52, 132.15, 131.41, 129.67(2C), 129.02(2C), 127.86(2C), 116.83, 116.25, 100.42, 45.26, 22.37; Anal. calcd. (found) % for $C_{20}H_{14}FN_5O_3S$: C, 56.73 (56.70); H, 3.33 (3.31); N, 16.54 (16.56). ESI-MS (*m/z*): calcd. for $C_{20}H_{14}FN_5O_3S$: $[M]^+$: 423, found: 424 $[M+H]^+$.

3-(3-(3,5-Dimethylphenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazine 8,8-dioxide (6c): Red solid, m.p.: 132-134 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.17 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.92 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.51 (s, 2H), 7.14 (s, 1H), 6.75 (s, 1H), 5.13 (s, 2H, S-CH_2), 2.33 (s, 6H, 2Ar-CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.15, 164.87, 163.56, 162.33, 161.39, 146.65, 143.16, 137.69(2C), 132.84, 132.10, 130.47, 130.04, 129.34(2C), 126.99, 126.16(2C), 116.83, 116.17, 109.53, 44.61, 20.13; Anal. calcd. (found) % for $C_{21}H_{16}FN_5O_3S$: C, 57.66; H, 3.69; N, 16.01. Found: C, 57.64; H, 3.66; N, 16.03. ESI-MS (*m/z*): calcd. for $C_{21}H_{16}FN_5O_3S$: $[M]^+$: 437, found: 438 $[M+H]^+$.

3-(3-(2,3-Dimethylphenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6d): Red solid, m.p.: 129-131 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.21 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.96 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.58-7.53 (m, 3H), 6.76 (s, 1H) 5.12 (s, 2H, S-CH_2), 2.26 (s, 3H, -CH_3), 1.97 (s, 3H, -CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.45, 164.59, 163.54, 162.39, 161.49, 146.68, 143.84, 137.66, 136.44, 133.22, 131.65, 129.81 (2C), 126.61, 125.61, 116.80, 116.19, 109.83, 44.61, 19.80, 16.17; Anal. calcd. (found) % for $C_{21}H_{16}FN_5O_3S$: C, 57.64 (57.66); H, 3.66 (3.69); N, 16.04 (16.01). ESI-MS (*m/z*): calcd. for $C_{21}H_{16}FN_5O_3S$: $[M]^+$: 437, found: 438 $[M+H]^+$.

3-(3-(4-Butylphenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4] thiadiazine 8,8-dioxide (6e): Dull white solid, m.p.: 121-123 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.18 (d, *J* = 8.0 Hz, 2H), 7.97 (d, *J* = 8.0 Hz, 2H), 7.62 (d, *J* = 8.0 Hz, 2H), 7.21 (d, *J* = 8.0 Hz, 2H), 6.76 (s, 1H) 5.11 (s, 2H, S-CH_2), 2.64 (t, *J* = 8.0 Hz, 2H, -CH_2), 1.69-1.62 (m, 2H, -CH_2), 1.33-1.28 (m, 2H, -CH_2), 0.98 (t, *J* = 4.0 Hz, 3H, -CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.26,



Scheme-I: (iii) NH_2-NH_2, EtOH, reflux, **6h**; (iv) 2-bromo-1-(4-fluorophenyl)ethan-1-one, EtOH, reflux, 5 h and ether 0 ºC, 12 h; (v) *m*-CPBA, DCM, 0 ºC, **6h**; (iv) CuI, THF, rt, 10-12 h

164.49, 163.29, 162.31, 161.07, 146.57, 143.57, 142.89, 132.84, 132.01, 129.81(2C), 128.71(2C), 126.51(2C), 116.82, 116.23, 100.76, 44.19, 36.36, 32.13, 21.19, 14.80; Anal. calcd. (found) % for $C_{23}H_{20}FN_5O_3S$: C, 59.35 (59.33); H, 4.33 (4.31); N, 15.05 (15.08). ESI-MS (*m/z*): calcd. for $C_{23}H_{20}FN_5O_3S$: $[M]^+$: 465, found: 466 $[M+H]^+$.

6-(4-Fluorophenyl)-3-(3-(4-methoxyphenyl)isoxazol-5-yl)-7*H*-[1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazine 8,8-dioxide (6f): White solid, m.p.: 135-137 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.19 (d, *J* = 8.0 Hz, 2H), 7.95 (d, *J* = 8.0 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.00 (d, *J* = 8.0 Hz, 2H), 6.73 (s, 1H) 5.11 (s, 2H, S-CH_2), 3.84(s, 3H, -OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.56, 164.40, 163.34, 162.20, 161.38, 159.51, 146.61, 143.39, 132.56, 129.57(2C), 129.02 (2C), 121.38, 116.89, 116.34, 114.32(2C), 109.04, 56.50, 44.62; Anal. calcd. (found) % for $C_{20}H_{14}FN_5O_4S$: C, 54.67 (54.65); H, 3.21 (3.18); N, 15.94 (15.96). ESI-MS (*m/z*): calcd. for $C_{20}H_{14}FN_5O_4S$: $[M]^+$: 439, found: 440 $[M+H]^+$.

3-(3-(4-Chlorophenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazine 8,8-dioxide (6g): Pale yellow solid, m.p.: 126-128 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.19 (d, *J* = 8.0 Hz, 2H), 7.95 (d, *J* = 8.0 Hz, 2H), 7.75 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 6.77 (s, 1H) 5.13 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.58, 164.69, 163.41, 162.14, 161.24, 146.54, 143.50, 135.31, 132.62, 129.86(2C), 128.71, 128.21 (2C), 127.89(2C), 116.86, 116.19, 109.25, 44.63; Anal. calcd. (found) % for $C_{19}H_{11}ClFN_5O_3S$: C, 51.42 (51.40); H, 2.50 (2.47); N, 15.78 (15.80). ESI-MS (*m/z*): calcd. for $C_{19}H_{11}ClFN_5O_3S$: $[M]^+$: 443, found: 444 $[M+H]^+$.

3-(3-(3,5-Dichlorophenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6h): Pale yellow solid, m.p.: 126-128 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.20 (d, *J* = 8.0 Hz, 2H), 7.95 (d, *J* = 8.0 Hz, 2H), 7.68 (s, 2H), 7.24 (s, 1H), 6.76 (s, 1H) 5.13 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.53, 164.76, 163.52, 162.17, 161.24, 146.55, 143.17, 133.62 (2C), 132.47, 131.93, 130.94, 129.84(2C), 125.57(2C), 116.76, 116.16, 109.49, 44.19; Anal. calcd. (found) % for $C_{19}H_{10}Cl_2FN_5O_3S$: C, 47.71 (47.68); H, 2.11 (2.09); N, 14.64 (14.66). ESI-MS (*m/z*): calcd. for $C_{19}H_{10}Cl_2FN_5O_3S$: $[M]^+$: 477, found: 478 $[M+H]^+$.

3-(3-(3,5-Dibromophenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazine 8,8-dioxide (6i): White solid, m.p.: 159-161 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.20 (d, *J* = 8.0 Hz, 2H), 7.97 (d, *J* = 8.0 Hz, 2H), 7.53 (s, 2H), 7.19 (s, 1H), 6.74 (s, 1H) 5.13 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.36, 164.74, 163.88, 162.77, 161.70, 146.55, 143.43, 135.61, 132.16, 130.69, 129.43(2C), 128.94(2C), 122.21(2C), 116.68, 116.16, 109.40, 44.57; Anal. calcd. (found) % for $C_{19}H_{10}Br_2FN_5O_3S$: C, 40.24 (40.21); H, 1.78 (1.76); N, 12.35 (12.38). ESI-MS (*m/z*): calcd. for $C_{19}H_{10}Br_2FN_5O_3S$: $[M]^+$: 565, found: 568 $[M+3H]^+$.

3-(3-(4-Bromophenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6j): White solid, m.p.: 142-144 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.18 (d, *J* = 8.0 Hz, 2H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.63-7.58 (m, 4H), 6.76 (s, 1H) 5.13 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.34, 164.33, 163.19, 162.34, 161.02, 146.39, 143.74, 132.03, 130.39(2C), 129.13(2C), 127.92, 126.49(2C), 125.66, 116.78, 116.16, 109.51, 44.20; Anal. calcd. (found) % for $C_{19}H_{11}BrFN_5O_3S$: C, 46.74 (46.72); H, 2.27 (2.25); N, 14.34 (14.36). ESI-MS (*m/z*): calcd. for $C_{19}H_{11}BrFN_5O_3S$: $[M]^+$: 487, found: 488 $[M+H]^+$.

6-(4-Fluorophenyl)-3-(3-(4-fluorophenyl)isoxazol-5-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6k): Red solid, m.p.: 130-132 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.29 (d, *J* = 8.0 Hz, 2H), 8.19 (d, *J* = 8.0 Hz, 2H), 7.98 (d, *J* = 8.0 Hz, 2H), 7.92 (d, *J* = 8.0 Hz, 2H), 6.78 (s, 1H) 5.14 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.54, 164.86, 163.75, 162.81, 161.69, 160.66, 158.99, 146.61, 143.84, 142.14, 132.56, 130.43(2C), 129.17(2C), 128.27, 127.95, 125.54, 118.65, 118.18, 116.75, 116.13, 109.34, 44.59; Anal. calcd. (found) % for $C_{19}H_{11}F_2N_5O_3S$: C, 53.40 (53.38); H, 2.59 (2.56); N, 16.39 (16.42). ESI-MS (*m/z*): calcd. for $C_{19}H_{11}F_2N_5O_3S$: $[M]^+$: 427, found: 428 $[M+H]^+$.

3-(3-(3,5-Difluorophenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazine 8,8-dioxide (6l): Red solid, m.p.: 146-148 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.21 (d, *J* = 8.0 Hz, 2H), 8.11(s, 2H), 7.96 (d, *J* = 8.0 Hz, 2H), 7.88 (s, 1H), 6.76 (s, 1H) 5.14 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.78, 166.02, 164.94, 164.26, 163.84, 162.33, 161.13, 160.20, 146.74, 143.83, 134.73, 132.37, 129.74(2C), 118.66, 118.11, 116.78, 116.16, 114.13, 113.29, 112.80, 112.21, 109.25, 106.35, 105.69, 100.76, 44.60; Anal. calcd. (found) % for $C_{19}H_{10}F_3N_5O_3S$: C, 51.24 (51.21); H, 2.26 (2.24); N, 15.72 (15.75). ESI-MS (*m/z*): calcd. for $C_{19}H_{10}F_3N_5O_3S$: $[M]^+$: 445, found: 446 $[M+H]^+$.

4-(5-(6-(4-Fluorophenyl)-8,8-dioxido-7*H*-[1,2,4]triazolo-[3,4-*b*][1,3,4]thiadiazin-3-yl)isoxazol-3-yl)-benzonitrile (6m): White solid, m.p.: 152-154 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.18 (d, *J* = 8.0 Hz, 2H), 7.94 (d, *J* = 8.0 Hz, 2H), 7.80 (d, *J* = 8.0 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 6.77 (s, 1H) 5.13 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.43, 164.42, 163.42, 162.40, 161.59, 146.64, 143.23, 135.32, 132.27(2C), 131.63, 129.88(2C), 128.24 (2C), 119.16, 116.91, 116.25, 114.33, 109.42, 44.87; Anal. calcd. (found) % for $C_{20}H_{11}FN_6O_3S$: C, 55.30 (55.27); H, 2.55; (2.52); N, 19.35 (19.38). ESI-MS (*m/z*): calcd. for $C_{20}H_{11}FN_6O_3S$: $[M]^+$: 434, found: 435 $[M+H]^+$.

6-(4-Fluorophenyl)-3-(3-(4-nitrophenyl)isoxazol-5-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6n): Yellow solid, m.p.: 154-156 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.33 (d, *J* = 8.0 Hz, 2H), 8.20 (d, *J* = 8.0 Hz, 2H), 8.04 (d, *J* = 8.0 Hz, 2H), 7.95 (d, *J* = 8.0 Hz, 2H), 6.78 (s, 1H) 5.14 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.42, 164.32, 163.31, 162.22, 161.44, 149.29, 146.34, 143.47, 135.46, 132.92, 129.61(2C), 127.94(2C), 125.74(2C), 116.89, 116.37, 109.61, 44.22; Anal. calcd. (found) % for $C_{19}H_{11}FN_6O_5S$: C, 50.22 (50.19); H, 2.44 (2.41); N, 18.50 (18.53). ESI-MS (*m/z*): calcd. for $C_{19}H_{11}FN_6O_5S$: $[M]^+$: 454, found: 455 $[M+H]^+$.

3-(3-(3,5-Dinitrophenyl)isoxazol-5-yl)-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (6o): Yellow solid, m.p.: 162-164 ºC, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: δ 8.31 (s, 1H), 8.20 (d, *J* = 8.0 Hz,

2H), 8.11 (s, 2H), 7.98 (d, *J* = 8.0 Hz, 2H), 6.79 (s, 1H) 5.15 (s, 2H, S-CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 166.66, 164.43, 163.49, 162.41, 161.55, 147.05(2C), 146.13, 143.40, 132.85, 132.14(2C), 130.36, 129.19(2C), 122.27, 116.78, 116.15, 109.50, 44.56; Anal. calcd. (found) % for $C_{19}H_{10}FN_7O_7S$: C, 45.70 (45.68); H, 2.02 (2.00); N, 19.63 (19.66). ESI-MS (*m/z*): calcd. for $C_{19}H_{10}FN_7O_7S$: $[M]^+$: 499, found: 500 $[M+H]^+$.

RESULTS AND DISCUSSION

The synthesis of the title compounds as outlined in **Scheme-I**. Initially, 5-ethynyl-1,3,4-oxadiazole-2(3*H*)-thione (**1**), upon reaction with hydrazine hydrate, yields corresponding 4-amino-5-ethynyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione (**2**) [34]. Intramolecular cyclization compound **2** with 2-bromo-1-(4-fluorophenyl)ethan-1-one resulted to form 3-ethynyl-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (**3**). Oxidation of sulfur in compound **3** by using *m*-CPBA in the presence of DCM to form a key intermediate 3-ethynyl-6-(4-fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 8,8-dioxide (**4**). The 1,3-dipolar cycloaddition reaction of compound **4** with different nitrile oxides (**5a-o**) at room temperature afforded [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine containing isoxazoles (**6a-o**) in good to excellent yields.

***In vitro* cytotoxic activity:** Newly synthesized isoxazoles (**6a-o**) were then investigated for their *in vitro* cytotoxicity against human cancer cell lines A-549 and H1299. Erlotinib was used as positive control [22,23]. From the obtained cytotoxicity results (Table-1), it is obvious that the compounds showed varied levels of cytotoxic activity against tested cancer cell lines ranging from strong, good, moderate and weak. In specific, compounds **6h**, **6k** and **6l** showed superior cytotoxic results against tested cell lines than the erlotinib with IC_{50} values ranging from 0.62 ± 0.20 μM to 4.40 ± 0.14 μM, respectively. In addition, compounds **6g**, **6i** and **6j** showed good cytotoxicity against both tested cell lines with IC_{50} values ranging from 10.48 ± 0.17 μM to 16.86 ± 0.17 μM. Similarly, compounds **6m**, **6n** and **6o** have shown good activity against A-549 cell line with IC_{50} values 11.64 ± 0.41 μM, 15.88 ± 0.26 μM and 14.90 ± 0.12 μM. Besides, the compounds exhibited moderate to poor activity against tested cell lines with IC_{50} values 16.63 ± 0.23 μM to 50.21 ± 0.26 μM.

TABLE-1
In vitro CYTOTOXICITY OF [1,2,4]TRIAZOLO[3,4-*b*][1,3,4]THIADIAZINE CONTAINING ISOXAZOLES WITH IC_{50} (μM)

Compd.	R	IC_{50} (μM)[a]		
		A-549	H1299	HEK-293
6a	H	43.04 ± 0.08	50.21 ± 0.26	NT
6b	4-Me	42.80 ± 0.48	44.95 ± 0.22	NT
6c	3,5-diMe	40.26 ± 0.09	36.52 ± 0.33	NT
6d	2,3-diMe	43.29 ± 0.30	21.2 ± 0.17	NT
6e	4-C_4H_9	34.32 ± 0.09	36.07 ± 0.09	NT
6f	4-OMe	30.57 ± 0.19	40.65 ± 0.08	NT
6g	4-Cl	14.01 ± 0.59	11.99 ± 0.04	12.89 ± 0.69
6h	3,5-diCl	4.20 ± 0.34	2.99 ± 0.62	10.44 ± 0.38
6i	3,5-diBr	14.02 ± 0.24	10.48 ± 0.17	13.67 ± 0.51
6j	4-Br	16.86 ± 0.17	15.22 ± 0.17	14.73 ± 0.64
6k	4-F	2.57 ± 0.36	4.40 ± 0.14	10.91 ± 0.38
6l	3,5-diF	0.62 ± 0.20	3.18 ± 0.16	9.37 ± 0.27
6m	4-CN	11.64 ± 0.41	21.03 ± 0.18	NT
6n	4-NO_2	15.88 ± 0.26	16.65 ± 0.43	NT
6o	3,5-diNO_2	14.90 ± 0.12	16.63 ± 0.23	NT
Std.	Erlotinib	4.30 ± 0.44	6.09 ± 0.11	12.22 ± 0.19

[a]Values are expressed as mean ± SD. NT = Not tested.

The relationship between the surviving fraction and drug concentration was illustrated to derive the survival curves of A-549 and H1299 in Fig. 2. It is crucial to elucidate the pharmacophore characteristics of freshly synthesized compounds, as the cytotoxic effects of these compounds might be attributed to the substituents present on the phenyl group attached to isoxazoles. Among all synthesized compounds compound **6h** and **6l** have shown more potent activity due to more electronegative fluoro groups containing phenyl on triazolo ring. Similarly, the compound which containing 3,5-dichlorophenyl group on the isoxazole ring also shown more potent activity as compared standard drug. Otherwise, the electron-withdrawing groups such as bromo, cyano and nitro groups on isoxazole ring also shown good activity as compared to the electron-donating methyl, methoxy groups on isoxazoles.

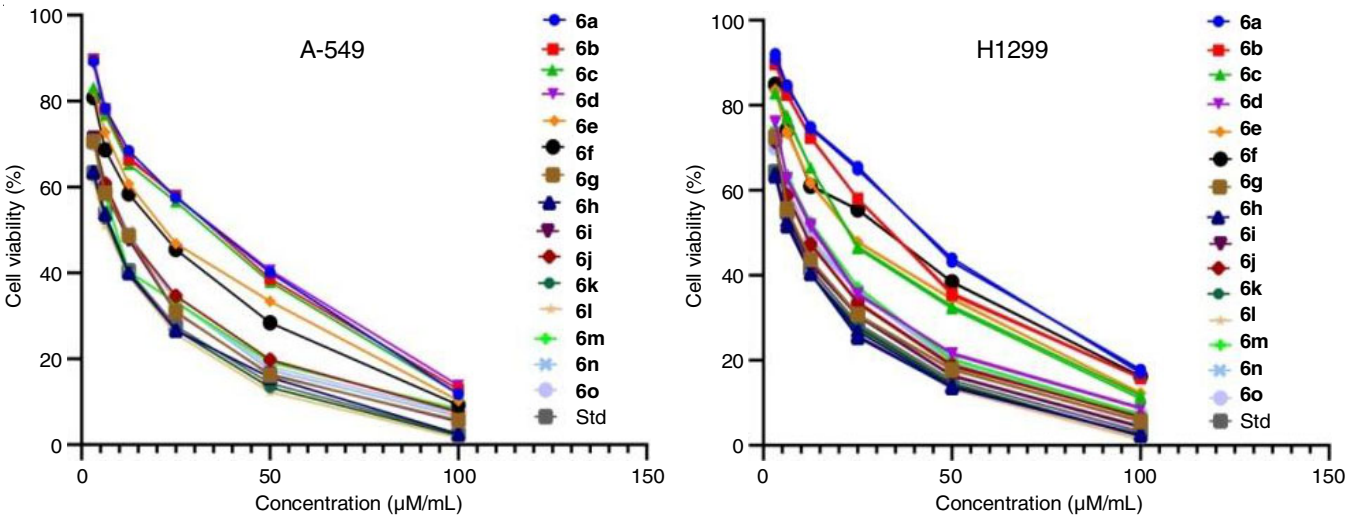


Fig. 2. Survival curves of A-549 and H1299 for isoxazole (**6a-o**)

Tyrosine kinase EGFR inhibitory activity: Three active compounds (**6h**, **6k** and **6l**) were evaluated for their inhibitory effect on the tyrosine kinase EGFR by an enzymatic assay, based on their cytotoxic activity [24]. Table-2 indicates that all the evaluated drugs exhibit powerful to excellent efficacy in comparison to the standard erlotinib. Among the three drugs, compound **6k** had more efficacy than the positive control, erlotinib ($IC_{50} = 0.42 \pm 0.03$ µM), in inhibiting EGFR tyrosine kinase, with an IC_{50} value of 0.41 ± 0.03 µM for the other compounds examined (Fig. 3).

TABLE-2
EGFR INHIBITORY ACTIVITY OF POTENT COMPOUNDS

Compound	EGFR (µM)
6h	0.88 ± 0.07
6k	0.41 ± 0.03
6l	0.78 ± 0.07
Erlotinib	0.42 ± 0.03

Molecular docking: Compounds **6h**, **6k** and **6l** have shown greater *in vitro* anticancer activity, when docked for binding interactions with the EGFR. According to Table-3, all the compounds have shown highest binding energy as compared to the standard erlotinib. Among all the compound **6h** which contains 3,5-dichlorophenyl on isoxazole ring has exhibited the highest binding energy (-10.51 kcal/mol) and formed three hydrogen bonds with LYS721, ASP831, MET769 (Fig. 4). Similarly compound **6l** which containing 3,5-difluorophenyl on isoxazole ring has exhibited the second highest binding energy (-9.67 kcal/mol) and formed five hydrogen bonds with PHE699, GLY700, THR766, LYS721(2). Compound **6k** having 4-fluorophenyl on isoxazole ring has exhibited the third highest binding energy (-9.64 kcal/mol) and formed two hydrogen bond with MET769, THR766 (Fig. 5). These results are comparable to standard erlotinib with binding energy (-8.25 kcal/mol) with two hydrogen bonds MET769, CYS773. It is important to mention that compiunds **6h**, **6k** and standard erlotinib having similar hydrogen bond interactions with MET-769 amino acid (Fig. 6).

TABLE-3
BINDING ENERGY AND HYDROGEN BINDING INTERACTIONS OF COMPOUNDS WITH EGFR (PDB ID: 4HJO)

Compd.	Binding energy (kcal/mol)	No. of hydrogen bonds	Residues involved in the hydrogen bonding
6h	-10.51	3	LYS721, ASP831, MET769
6k	-9.64	2	MET769, THR766
6l	-9.67	5	PHE699, GLY700, THR766, LYS721(2)
Erlotinib	-8.25	2	MET769, CYS773

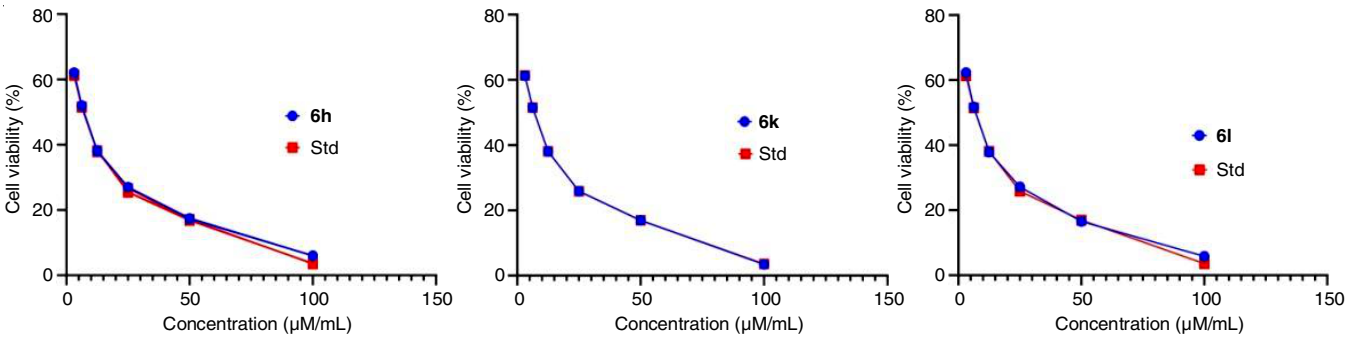


Fig. 3. Survival curves of compounds **6h**, **6k** and **6l** against EGFR protein kinases

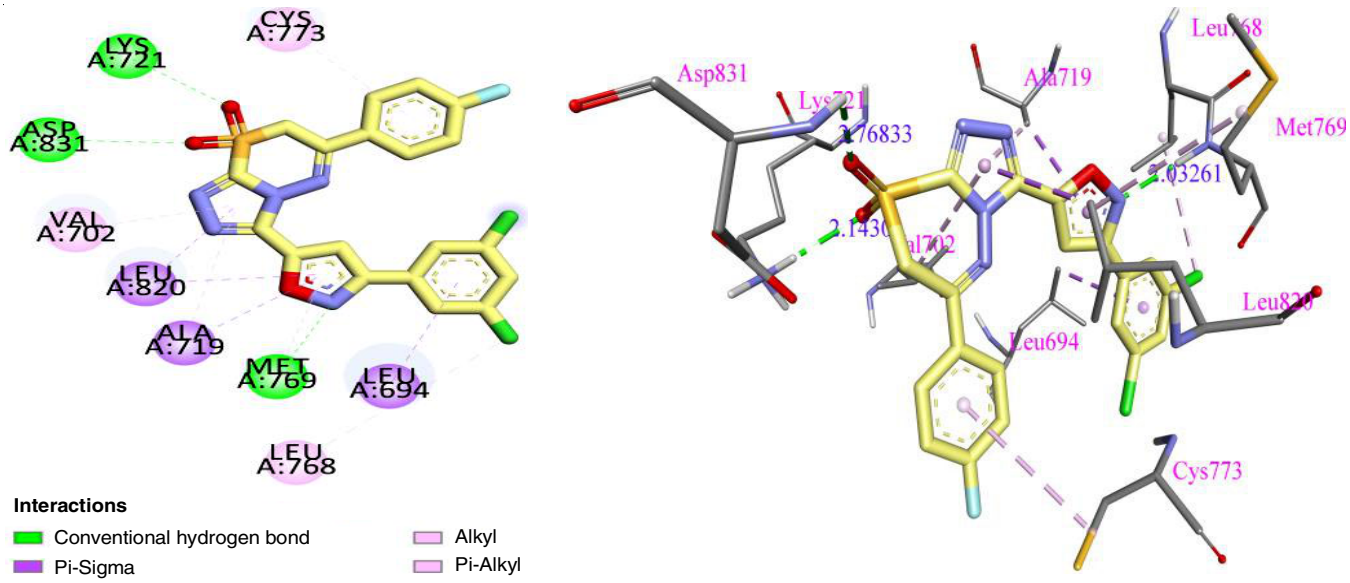


Fig. 4. 2D and 3D Surface interaction of the compound **6h** with EGFR

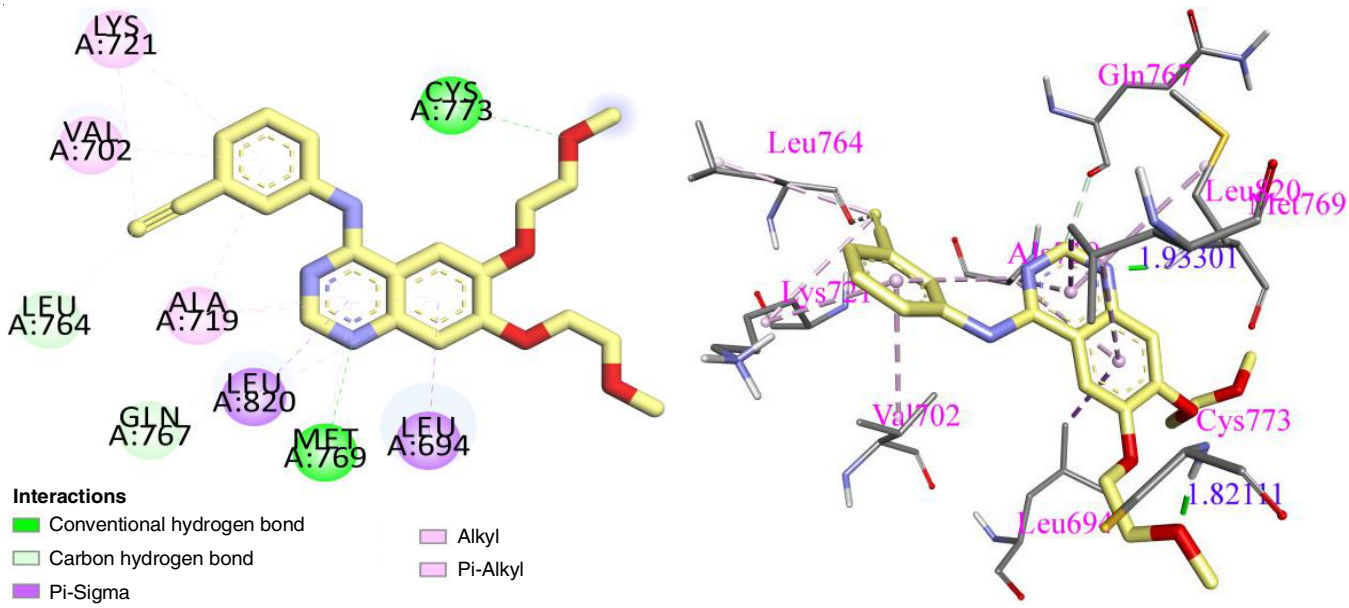


Fig. 5. 2D and 3D Surface interaction of the erlotinib with EGFR

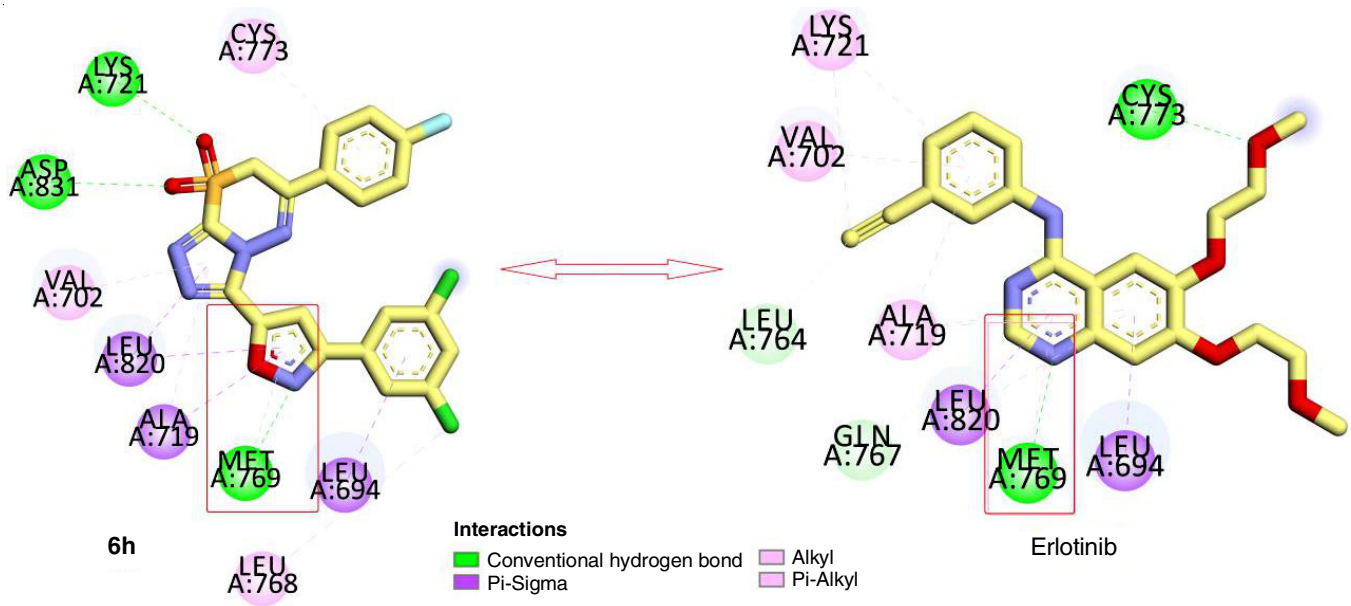


Fig. 6. MET769 H-bond interactions of target compound **6h** and erlotinib

Conclusion

In conclusion, some novel isoxazole derivatives of [1,2,4]-triazolo[3,4-*b*][1,3,4]thiadiazine (**6a-o**) were synthesized using Cu(I) catalyzed [3+2] cycloaddition reaction of alkyne **4** with different nitrile oxides (**5**). All the synthesized derivatives were further evaluated for their *in vitro* anticancer activity against A-459 and H1299. Among them, compounds **6h**, **6k** and **6l** exhibited superior activity compared to the remaining compounds and showed the highest activity as compared to standard erlotinib. The highest activity was observed for compound **6l**, which has IC_{50} values of 0.62 ± 0.20 µM and 3.18 ± 0.16 µM against both lung cancer cells. The *in vitro* EGFR results of more potent compounds **6h**, **6k** and **6l** revealed that the compounds **6k** (IC_{50} = 0.41 ± 0.03 µM) was more effective than the conventional medicine Erlotinib (IC_{50} = 0.42 ± 0.03 µM) and remaining compounds **6h** (IC_{50} = 0.88 ± 0.07 µM) and **6l** (IC_{50} = 0.78 ± 0.07 µM) have shown good activity. Remarkably, the *in silico* studies like molecular docking of more potent compounds **6h**, **6k** and **6l** were also found to be promise with the corresponding *in vitro* activity IC_{50} data.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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