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SYNTHESIS, MOLECULAR MODELING STUDIES AND BIOLOGICAL EVALUATION OF NOVEL PYRAZOLE DERIVATIVES AS ANTITUMOR AND EGFR INHIBITORS

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Abstract

Abstract

In this study, a new series of 4-(5-methylisoxazol-3-ylamino)thiazole derivatives incorporated with different heterocyclic moieties was synthesized and screened for their *in vitro* antitumor activity against human HCT-116, HePG-2 and MCF-7 cell lines. Among them, compound **3** revealed the most potent cytotoxic activity against HePG-2 cell line with IC_{50} = 20.2 µg ml^{-1} and moderate activity with MCF-7 cell line (IC_{50} = 60.9 µg ml^{-1}) in comparison with doxorubicin (IC_{50} = 21.6 and 26.3 µg ml^{-1}) respectively. Furthermore, compound **5b** showed the highest antitumor activity with IC_{50} 16.2 µg ml^{-1}. The most promising compound **3** was subjected to further kinase assay study against a panel of twelve protein kinases and exhibited good EGFR inhibitory activity (95%) and moderate PI3K (p110b/p85a) and p38α inhibitory activities (89, 85%) respectively. Molecular modeling simulation was done to explore the binding mode of the ligand **3** within active sites EGFR, PI3K (p110b/p85a) and p38α kinases.

Keywords: 4-(5-methylisoxazol-3-ylamino)thiazole, antitumor activity, kinase assay, Molecular modeling simulation.

Introduction:

Cancer is a serious disease in which characterized by uncontrolled growth of abnormal cells. Cancer may affect all ages of people even fetuses. Almost cancers are caused by abnormalities in the genetic material of the cells [(1, 2)]. Several efforts were exerted to develop new drugs with high toxicity toward cancer cells and with low toxicity against normal cells [(3)]. Protein kinases were proved to have an important role in anticancer drug development [(4)]. The epidermal growth factor receptor (EGFR) is one of the most vital kinases that play a fundamental role in signal

transduction pathways [5]. The EGFR activity correlates with many human tumors, including breast, lung, head, neck, bladder, prostate, and kidney cancers [6]. It has been also noted the overexpression of EGFR in ovarian and breast cancer [6]. Therefore, inhibition of EGFR tyrosine kinase activity is of potential interest for the development of novel anticancer agents [7].

Thiazole derivatives are involved in many natural and synthetic products with several pharmacological activities, such as anticancer, anti-inflammatory, antifungal, antibacterial, antiviral activities. Among them, 2-aminothiazole derivatives have an anticancer activity through the inhibition of the kinases [8]. In addition, pyrazoles are reported as cytotoxic, antiviral, antimicrobial and anti-inflammatory agents [9]. Above observation directed us to synthesize novel thiazole compounds incorporating different heterocycles hopping to possess great antitumor activity.

Materials and Methods

1. Chemistry

NMR spectra were recorded on a General Electric QE 300 instrument and chemical shifts were given with respect to TMS. IR spectra were recorded on a Perkin-Elmer 1420 spectrometer and a Broad FTS7 (KBr). Mass spectra were obtained on a Jeol JMS D-300 spectrometer operating at 70 eV.

Microanalysis was conducted using an Elemental analyzer 1106. Melting points were determined on a Reichet Hot Stage and uncorrected.

N-4-(5-methylisoxazol-3-yl)thiazole-2,4-diamine (3)

A mixture of **2** [10] (0.01 mol), thiourea (0.01 mol) and absolute ethanol (30 ml) was refluxed for 4 h. The reaction mixture was cooled, and then poured onto ice/cold water acidified with hydrochloric acid. The formed precipitate was filtered and washed with water to give **3**. Yield: 64%, Crystallized from benzene, m.p. ≈226-228 °C. IR (KBr) ν, cm^{-1}: 3423, 3385, 3124 (NH_2, NH), 1610 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.38 (s, 3H, CH_3 of isoxazole), 6.60 (s, 1H, CH of thiazole), 6.70 (s, 1H, CH of isoxazole), 7.47 (s, 2H, NH_2, D_2O exchangeable), 9.93 (s, 1H, NH, D_2O exchangeable). MS m/z: M^+.196 (3.76%). Anal. Calcd. for $C_7H_8N_4OS$ (196.23): C, 42.85; H, 4.11; N, 28.55%; found: C, 42.50; H, 3.82; N, 28.32%.

N-(2-hydrazinylthiazol-4-yl)-5-methylisoxazol-3-amine (4)

A mixture of **2** [10] (0.01 mol), thiosemicarbazide (0.01 mol) and absolute ethanol (30 ml) was refluxed for 4 h. The reaction mixture was cooled, and then poured onto ice/cold water acidified with hydrochloric acid. The formed precipitate was filtered and washed with water to give **4**. Yield: 75%, Crystallized from acetic acid, m.p. ≈ 258-260

°C. IR (KBr) ν, cm^{-1}: 3419, 3318, 3210, 3181 (NH_2, 2NH), 1614 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.35 (s, 3H, CH_3 of isoxazole), 5.75 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 7.47 (s, 2H, NH_2, D_2O exchangeable), 8.50, 9.93 (2s, 2H, 2NH, D_2O exchangeable). MS m/z: M^+.211 (15.95%). Anal. Calcd. for $C_7H_9N_5OS$ (211.24): C, 39.80; H, 4.29; N, 33.15%; found: C, 39.50; H, 3.95; N, 32.98%.

***N*-(2-(2-((substituted)(4-methylpiperazin-1-yl)methyl)hydrazinyl)thiazol-4-yl)-5-methylisoxazol-3-amine (5a, b)**

A mixture of compound **4** (0.01 mol), the appropriate aldehyde, namely: vanillin and/ or 4-fluoro benzaldehyde in the presence of methylpiperazine (0.01 mol) was refluxed for 8 h. After cooling, the formed precipitate was filtered to give **5a, b**.

2-Methoxy-4-((2-(4-((5-methylisoxazol-3-yl)amino) thiazol-2-yl) hydrazinyl) (4-methyl piperazin-1-yl) methyl) phenol (5a)

Yield: 60%, Crystallized from absolute ethanol, m.p. ≈ 248-250 °C. IR (KBr) ν, cm^{-1}: 3520 (OH), 3422, 3314, 3181 (3NH), 1613 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.25 (s, 3H, N-CH_3), 2.36 (s, 3H, CH_3 of isoxazole), 2.73 (t, 8H, 4CH_2 of piperazine), 3.75 (s, 3H, OCH_3), 5.10 (s, 1H, CH), 5.50 (s, 1H, OH, D_2O exchangeable), 5.80 (s, 1H, CH of thiazole), 6.50 (s, 1H, CH of isoxazole), 7.10-7.80 (m, 3H, Ar), 8.50, 8.90, 9.93 (3s, 3H, 3NH, D_2O exchangeable). MS m/z: M^+.445 (1.47%). Anal. Calcd. for $C_{20}H_{27}N_7O_3S$ (445.54): C, 53.92; H, 6.11; N, 22.01%; found: C, 53.50; H, 5.95; N, 21.89%.

***N*-(2-(2-((4-fluorophenyl)(4-methylpiperazin-1-yl)methyl)hydrazinyl)thiazol-4-yl)-5-methylisoxazol-3-amine (5b)**

Yield: 74%, Crystallized from benzene, m.p. ≈ 268-270 °C. IR (KBr) ν, cm^{-1}: 3430, 3319, 3281 (3NH), 1622 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.26 (s, 3H, N-CH_3), 2.34 (s, 3H, CH_3 of isoxazole), 2.76 (t, 8H, 4CH_2 of piperazine), 5.30 (s, 1H, CH), 5.90 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 7.30-7.90 (m, 4H, Ar), 8.20, 8.70, 9.60 (3s, 3H, 3NH, D_2O exchangeable). MS m/z: M^+.417 (1%). Anal. Calcd. for $C_{19}H_{24}FN_7OS$ (417.50): C, 54.66; H, 5.79; N, 23.48%; found: C, 54.46; H, 5.65; N, 23.22%.

***N*-(2-(3,5-dimethyl-1*H*-pyrazol-1-yl)thiazol-4-yl)-5-methylisoxazol-3-amine (6)**

A mixture of compound **4** (0.001 mol) and acetylacetone (0.001 mol) in glacial acetic acid (15 mL) was heated under reflux for 10 h. The reaction mixture was cooled and the precipitated solid was collected by filtration, washed with water and dried to give **6**. Yield: 69%, Crystallized from toluene, m.p. > 300 °C. IR (KBr) ν, cm^{-1}: 3225 (NH), 1615 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.30 (2s, 6H, 2CH_3 of pyrazole), 2.80 (s, 3H, CH_3 of isoxazole), 5.90

(s, 1H, CH of pyrazole), 6.50 (s, 1H, CH of isoxazole), 6.80 (s, 1H, CH of thiazole), 8.20 (s, 1H, NH, D_2O exchangeable). MS m/z: M^+.275 (12%). Anal. Calcd. for $C_{12}H_{13}N_5OS$ (275.33): C, 52.35; H, 4.76; N, 25.44%; found: C, 52.15; H, 4.52; N, 25.25%.

3-Methyl-1-(4-((5-methylisoxazol-3-yl) amino)thiazol-2-yl)-1*H*-pyrazol-5(4*H*)-one (7)

A mixture of compound **4** (0.001 mol), ethyl acetoacetate (0.001 mol) and anhydrous potassium carbonate (0.0015 mol) in absolute ethanol (15 mL) was heated under reflux for 10 h. The reaction mixture was cooled and the precipitated solid was collected by filtration, washed with water and dried to give **7**.

Yield: 70%, Crystallized from benzene, m.p. ≈ 283-285 °C. IR (KBr) ν, cm^{-1}: 3311 (NH), 1717 (C=O), 1606 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.10 (s, 3H, CH_3 of pyrazole), 2.30 (s, 2H, CH_2 of pyrazole), 2.40 (s, 3H, CH_3 of isoxazole), 5.40 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 8.40 (s, 1H, NH, D_2O exchangeable). MS m/z: M^+.277 (1.19%). Anal. Calcd. for $C_{11}H_{11}N_5O_2S$ (277.30): C, 47.64; H, 4.00; N, 25.26%; found: C, 47.52; H, 3.92; N, 25.01%.

***N*-(substituted)-2-(4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl) substituted hydrazine (8a-c)**

A mixture of compound **4 (**0.01 mol) and the appropriate isothiocyanate and/or isocyanate, namely: 2-chloro phenyl isothiocyanate, 3-chloro phenyl isocyanate, ethyl isothiocyanate (0.01 mol) in dry benzene (20 ml) was refluxed for 6 h. The formed precipitate was filtered and dried to give **8a-c**.

***N*-(2-chlorophenyl)-2-(4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)hydrazinecarbothioamide (8a)**

Yield: 75%, Crystallized from benzene, m.p. ≈ 253-255 °C. IR (KBr) ν, cm^{-1}: 3414, 3251, 3242, 3160 (4NH), 1630 (C=N), 1330 (C=S). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.40 (s, 3H, CH_3 of isoxazole), 5.60 (s, 1H, CH of thiazole), 6.30 (s, 1H, CH of isoxazole), 7.10-8.10 (m, 4H, Ar), 8.40, 8.90, 9.10, 9.40 (4s, 4H, 4NH, D_2O exchangeable). MS m/z: M^+.380 (1.52%), M^{+2}.382 (0.62%). Anal. Calcd. for $C_{14}H_{13}ClN_6OS_2$ (380.88): C, 44.15; H, 3.44; N, 22.06%; found: C, 44.02; H, 3.22; N, 21.95%.

***N*-(3-chlorophenyl)-2-(4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)hydrazinecarboxamide (8b)**

Yield: 80%, Crystallized from DMF, m.p. ≈ 239-241 °C. IR (KBr) ν, cm^{-1}: 3433, 3290, 3255, 3179 (4NH),1719 (C=O), 1632 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.20 (s, 3H, CH_3 of isoxazole), 5.40 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 7.30-8.40 (m, 4H, Ar), 8.50, 8.70, 9.20, 9.50 (4s, 4H, 4NH, D_2O exchangeable). MS m/z: M^+.364 (12%), M^{+2}.366 (6%). Anal. Calcd. for $C_{14}H_{13}ClN_6O_2S$ (364.81): C, 46.09; H, 3.59; N, 23.04%; found: C, 45.91; H, 3.32; N, 22.92%.

_N_-ethyl-2-(4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)hydrazinecarbothioamide (8c)

Yield: 70%, Crystallized from absolute ethanol, m.p. ≈ 262-264 °C. IR (KBr) ν, cm^{-1}: 3410, 3270, 3230, 3108 (4NH), 1632 (C=N), 1340 (C=S). ^{1}H NMR spectrum (d_6-DMSO, δ ppm): 1.28 (t, 3H, CH_3 of ethyl), 2.30 (s, 3H, CH_3 of isoxazole), 4.50 (q, 2H, CH_2 of ethyl), 5.60 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 8.10, 8.30, 9.40, 9.70 (4s, 4H, 4NH, D_2O exchangeable). MS m/z: M^+.298 (3.63%), Anal. Calcd. for $C_{10}H_{14}N_6OS_2$ (298.39): C, 40.25; H, 4.73; N, 28.16%; found: C, 40.03; H, 4.51; N, 27.96%.

(*Z*)-3-(3-chlorophenyl)-2-(2-(4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)hydrazono)oxazolidin-4-one (9)

A mixture of compound **8b** (0.002 mol), monochloroacetic acid (0.003 mol) and a few drops of pyridine was fused for 6 h. The reaction mixture was cooled, and then poured onto ice/cold water. The formed precipitate was filtered and washed with water to give **9**. Yield: 75%, Crystallized from benzene, m.p. ≈ 126-128 °C. IR (KBr) ν, cm^{-1}: 3405, 3219 (2NH), 1710 (C=O), 1603 (C=N). ^{1}H NMR spectrum (d_6-DMSO, δ ppm): 2.72 (s, 3H, CH_3 of isoxazole), 4.35 (s, 2H, CH_2 of oxazolidine), 5.40 (s, 1H, CH of thiazole), 6.50 (s, 1H, CH of isoxazole), 7.20-8.10 (m, 4H, Ar), 8.50, 9.00 (2s, 2H, 2NH, D_2O exchangeable). MS m/z: M^+.404 (4.32%), M^{+2}.406 (2.12%). Anal. Calcd. for $C_{16}H_{13}ClN_6O_3S$ (404.83): C, 47.47; H, 3.24; N, 20.76%; found: C, 47.25; H, 3.10; N, 19.92%.

1-(3-Chlorophenyl)-3-((4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)amino)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (10)

A mixture of compound **8b** (0.002 mol) and malonic acid (0.002 mol) in absolute ethanol (20 mL) was refluxed for 16h. The precipitated solid formed upon cooling and filtered to give **10**.

Yield: 65%, Crystallized from DMF, m.p. > 300 °C. IR (KBr) ν, cm^{-1}: 3432, 3301 (2NH), 1720, 1710, 1698 (3C=O), 1610 (C=N). ^{1}H NMR spectrum (d_6-DMSO, δ ppm): 2.35 (s, 3H, CH_3 of isoxazole), 3.23 (s, 2H, CH_2 of pyrimidine), 5.30 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 7.30-8.00 (m, 4H, Ar), 8.40, 8.90 (2s, 2H, 2NH, D_2O exchangeable). MS m/z: M^+.432 (1.41%), M^{+2}.434 (0.70%). Anal. Calcd. for $C_{17}H_{13}ClN_6O_4S$ (432.84): C, 47.17; H, 3.03; N, 19.42%; found: C, 46.97; H, 2.95; N, 19.01%.

_N_-(2-(2-((substituted)methylene)hydrazinyl)thiazol-4-yl)-5-methylisoxazol-3-amine (11a,b)

A mixture of compound **4** (0.01 mol) and the appropriate aldehydes, namely: Vanillin and/ or Indole-3-carboxaldehyde (0.01 mol) in absolute ethanol (20 mL) in the presence of a few drops of acetic acid was refluxed for 12 h. The reaction mixture was cooled and the formed precipitate was filtered off to give **11a, b**.

2-Methoxy-4-((2-(4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)hydrazono)methyl)phenol (11a)

Yield: 76%, Crystallized from benzene, m.p. ≈ 265-267 °C. IR (KBr) ν, cm^{-1}: 3436 (OH), 3213, 2988 (2NH), 1608 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.50 (s, 3H, CH_3 of isoxazole), 3.48 (s, 3H, OCH_3), 5.60 (s, 1H, OH, D_2O exchangeable), 5.50 (s, 1H, CH of thiazole), 6.30 (s, 1H, CH of isoxazole), 7.10-8.00 (m, 3H, Ar), 8.28 (s,1H, CH=N), 9.02, 11.8 (2s, 2H, 2NH, D_2O exchangeable). MS m/z: M^+.345 (7.75%). Anal. Calcd. for $C_{15}H_{15}N_5O_3S$ (345.38): C, 52.16; H, 4.38; N, 20.28%; found: C, 52.01; H, 4.12; N, 20.03%.

***N*-(2-(2-((*1H*-indol-3-yl)methylene)hydrazinyl)thiazol-4-yl)-5-methylisoxazol-3-amine (11b)**

Yield: 80%, Crystallized from DMF, m.p. ≈ 227-229 °C. IR (KBr) ν, cm^{-1}: 3420, 3231, 2910 (3NH), 1615 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.40 (s, 3H, CH_3 of isoxazole), 5.60 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 7.10-8.50 (m, 5H, Ar), 8.90 (s, 1H, CH=N), 9.12, 9.50, 10.10 (3s, 3H, 3NH, D_2O exchangeable). MS m/z: M^+.338 (15.31%). Anal. Calcd. for $C_{16}H_{14}N_6OS$ (338.39): C, 56.79; H, 4.17; N, 24.84%; found: C, 56.62; H, 3.97; N, 24.74%.

2-(Substituted)-3-((4-((5-methylisoxazol-3-yl) amino) thiazol-2-yl) amino)-2*H*-benzo[e][1,3]thiazin-4(3*H*)-one (12a,b)

A solution of compounds **11a, b** (0.01 mol) and thiosalicylic acid (0.02 mol) in dioxane (20 mL) was stirred at room temperature for 48 hrs. The solvent was evaporated under vacuum and the residue was washed with 4 N Na_2CO_3 solutions then with water. The separated solid was filtered off, washed with water and dried under vacuum at room temperature to give **12a, b**.

2-(4-Hydroxy-3-methoxyphenyl)-3-((4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)amino)-2*H*-benzo[*e*][1,3]thiazin-4(3*H*)-one (12a)

Yield: 75%, Crystallized from benzene, m.p. ≈ 255-257 °C. IR (KBr) ν, cm^{-1}: 3423 (OH), 3051, 2981 (2NH), 1698 (C=O), 1602 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.50 (s, 3H, CH_3 of isoxazole), 3.77 (s, 3H, OCH_3), 5.40 (s, 1H, CH of thiazole), 6.90 (s, 1H, CH of isoxazole), 7.20-8.10 (m, 7H, Ar), 8.80 (s, 1H, CH of thiazin), 9.80, 11.70, 11.80 (3s, 3H, 2NH, OH, D_2O exchangeable). MS m/z: M^+.481 (5.08%). Anal. Calcd. for $C_{22}H_{19}N_5O_4S_2$ (481.55): C, 54.87; H, 3.98; N, 14.54%; found: C, 54.79; H, 3.84; N, 14.33%.

2-(1*H*-indol-3-yl)-3-((4-((5-methylisoxazol-3-yl)amino)thiazol-2-yl)amino)-2*H*-benzo[*e*][1,3]thiazin-4(3*H*)-one (12b)

Yield: 70%, Crystallized from DMF, m.p.≈ 242-246 °C. IR (KBr) ν, cm^{-1}: 3423, 3310, 3065 (3NH), 1683 (C=O), 1602 (C=N). ^1H NMR spectrum (d_6-DMSO, δ ppm): 2.30 (s, 3H, CH_3 of isoxazole), 5.50 (s, 1H, CH of thiazole),

5.90 (s, 1H, CH of thiazin), 6.60 (s, 1H, CH of isoxazole), 7.10-8.50 (m, 9H, Ar), 8.80, 9.80, 10.50 (3s, 3H, 3NH, D_2O exchangeable). MS m/z: M^+.474 (1.53%). Anal. Calcd. for $C_{23}H_{18}N_6O_2S_2$ (474.56): C, 58.21; H, 3.82; N, 17.71%; found: C, 58.01; H, 3.75; N, 17.63%.

Chemicals: 3-Amino-5-methyl isoxazole **(1)** from (**New Lab Company**), 15th of May City, Cairo, Egypt.

2. Pharmacology

Some of the newly synthesized target compounds are subjected to two assays of biological evaluation; cytotoxicity assays and biochemical assays (kinase inhibitor activity).

2-1 . Cytotoxic effect on human cell line (HCT-116, HePG-2 and MCF-7)

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan [(11)].

Procedure:

All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for HCT-116, HePG-2 and MCF-7. The media are supplemented with 1% antibiotic antimycotic mixture (10,000 U ml^{-1} Potassium Penicillin, 10,000 µg ml^{-1} Streptomycin Sulfate and 25 µg ml^{-1} Amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37 °C under 5% CO_2. Cells were batch cultured for 10 days, then seeded at concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well Microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg ml^{-1}). After 48 h of incubation, the medium was aspirated, 40 µl MTT salt (2.5 µg ml^{-1}) was added to each well and incubated for a further 4 h at 37 °C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 µl of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37 °C. A positive control which composed of 100 µg ml^{-1} was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions [(12, 13)]. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration in the cells was less

than 0.2%. The percentage of change in viability was calculated according to the formula: (Reading of extract/Reading of negative control)-1) x 100. A probit analysis was carried for IC_{50} determination using SPSS 11 program. Cytotoxic activity test (*In vitro* bioassay on human tumor cell lines) was conducted and determined by the Bioassay-Cell Culture Laboratory, National Research Centre, El-Tahrir St., Dokki, Cairo 12622, Egypt.

2-2. Kinase screening at 100 µM

The *in vitro* enzyme inhibition determination for compound **3** was carried out in KINEXUS Corporation, Vancouver, British Columbia, Canada. Kinexus has developed an open-access, on-line resource called DrugKiNET, www.drugkinet.ca. The evaluation performed profiling of the compound **3** against a range of twelve protein kinases (AKT1, AKT2, BRAF (V600E), CDK2/CyclinA1, CHK1, EGFR, VEGFR-2, p38α, PDGFRβ, PI3K (p110a/p85a and p110b/p85a) and c-RAF at 1 concentration (100 µM) in single measurements using the radiometric or ADP-Glo assay method. Kinexus evaluated the profile of compound **3** against different kinases by employing the standardized assay methodology. The intra-assay variability was determined to be less than 10%. Inhibition of target activity by the compound gives negative (-) values while activation of target activity gives positive (+) value. Kinexus considers only values of > 25% change in activity compared to control to be significant.

Materials: Quality control and reagents:

The PKs employed in the compound profiling process was cloned, expressed and purified using proprietary methods. Quality control testing is routinely performed to ensure compliance to acceptable standards. ^{33}P-ATP was purchased from PerkinElmer. The various lipid substrates were purchased from Echelon. All other materials were of standard laboratory grade. The compound **3** was supplied by Kinexus as a powder and stock solution was made in DMSO. The stock solution was then diluted to form an assay stock solution and this was used to profile against the protein kinases (AKT1, AKT2, BRAF (V600E), CDK2/CyclinA1, CHK1, EGFR, VEGFR-2, p38α, PDGFRβ and c-RAF) and lipid protein kinases PI3K (p110a/p85a and p110b/p85a).

The assay condition for the PK targets was optimized to yield acceptable enzymatic activity. In addition, the assays were optimized to give high signal-to-noise ratio.

Protein Kinase (PK) Assays:

A radioisotope assay format was used for profiling evaluation of PK targets and all assays were carried out in a designated radioactive working area. PK assays (in singlicate) were performed at ambient temperature for 30 min in a final volume of 25 µl according to the following assay reaction recipe: **Component 1:** 5 µl of diluted active PK target

(~10-50 nM final concentration in the assay). **Component 2:** 5 µl of stock solution of substrate (1-5 µg of peptide substrate). **Component 3:** 5 µl of kinase assay buffer. **Component 4:** 5 µl of compound (50 µM) or 10% DMSO. **Component 5:** 5 µl of ^{33}P-ATP (250 µM stock solution, 0.8 µCi).

The assay was initiated by the addition of ^{33}P-ATP and the reaction mixture incubated at ambient temperature for 30 min. After the incubation period, the assay was terminated by spotting 10 µl of the reaction mixture onto Multiscreen phosphocellulose P81 plate. The Multiscreen phosphocellulose P81 plate was washed 3 times for approximately 15 min each in a 1% phosphoric acid solution. The radioactivity on the P81 plate was counted in the presence of scintillation fluid in a Trilux scintillation counter. Blank control was set up that included all the assay components except the addition of the appropriate substrate (replaced with equal volume of assay dilution buffer). The corrected activity for PK target was determined by removing the blank control value.

Lipid Protein Kinase Assays: ADP-Glo assay format was used for profiling evaluation of lipid protein kinase targets using the ADP-Glo™ assay kit from Promega. This kit measures the generation of ADP by the lipid protein kinase which leads to an increase in luminescence signal. The lipid protein kinase assays were performed at 30 ^{o}C for 30 min in a final volume of 25 µl according to the following assay reaction recipe: **Component 1:** 5 µl of diluted active lipid protein kinase. **Component 2:** 5 µl of 125 µM stock solution of lipid substrate. **Component 3:** 5 µl of kinase assay buffer. **Component 4:** 5 µl of compound or 10 % DMSO. **Component 5:** 5 µl of 250 µM ATP stock solution.

The assay was started by incubating the reaction mixture in a 96-well plate at ambient temperature for 30 min. Then, the assay was terminated by the addition of 25 µl of ADP-Glo™ Reagent (Promega). The 96-well plate was shaken and then incubated for 40 min at ambient temperature. Kinase Detection Reagent (50 µl) was added, the 96-wellplate shaken and then incubated for further 30 min at ambient temperature. The 96-well reaction plate was read using the ADP-Glo™ Luminescence Protocol on a GloMax plate reader (Promega; Cat# E7031).

Blank control was set up that included all the assay components except the addition of the lipid substrate (replace with equal volume of kinase assay buffer). The corrected activity for lipid protein kinase targets were determined by removing the blank control value.

3-Molecular modeling studies

All the molecular modeling calculations and docking simulation studies were performed using Molecular Operating Environment (MOE®) [(14)] 2008.10. All the interaction energies and different calculations were automatically calculated.

3-1. Optimization of Target compound 3.

The target compound **3** was constructed as a 3D model using the builder interface of the MOE program. After checking its structure and the formal charges on atoms by 2D depiction, the following steps were carried out: the target compound **3** was subjected to a conformational search. All conformers were subjected to energy minimization, all the minimizations were performed with MOE until a RMSD gradient of 0.01 Kcal mol^{-1} and RMS distance of 0.1 Å with MMFF94X force-field, and the partial charges were automatically calculated. The obtained database was then saved as MDB file to be used in the docking calculations.

3-2. Optimization of active sites of the enzymes [EGFR, PI3K (p110b/p85a) and p38α].

The three dimensional X-ray structures of EGFR (PDB code: 1M17), PI3K (p110b/p85a) (PDB code: 4BFR) and p38α (PDB code: 3FMK) were obtained from the Protein Data Bank through the internet [(15-17)]. The enzymes were prepared for docking studies by removing the ligand molecules from the active sites. Hydrogen atoms were added to the system with their standard geometry.

Atoms connection and type were checked for any errors with automatic correction. Selection of the receptor and its atoms potential were fixed. MOE Alpha Site Finder was used for the active sites search in the enzymes structures using all default items. Dummy atoms were created from the obtained alpha spheres. Re-docking of co-crystalline ligands to the receptors active sites to insure the docking method was efficient and the active pocket was saved as MOE file to be used for docking simulation of compound **3**.

3-3. Docking of the target molecule 3 to the active sites of receptors.

Docking of the conformation database of compound **3** was done using MOE-Dock software. The following methodology was generally applied via loading of the enzymes active site files, and the dock tool was initiated. The program specifications were adjusted to: - Dummy atoms as the docking sites.

- Triangle matcher as the placement methodology to be used.
- London dG as scoring methodology to be used and was adjusted to its default values.

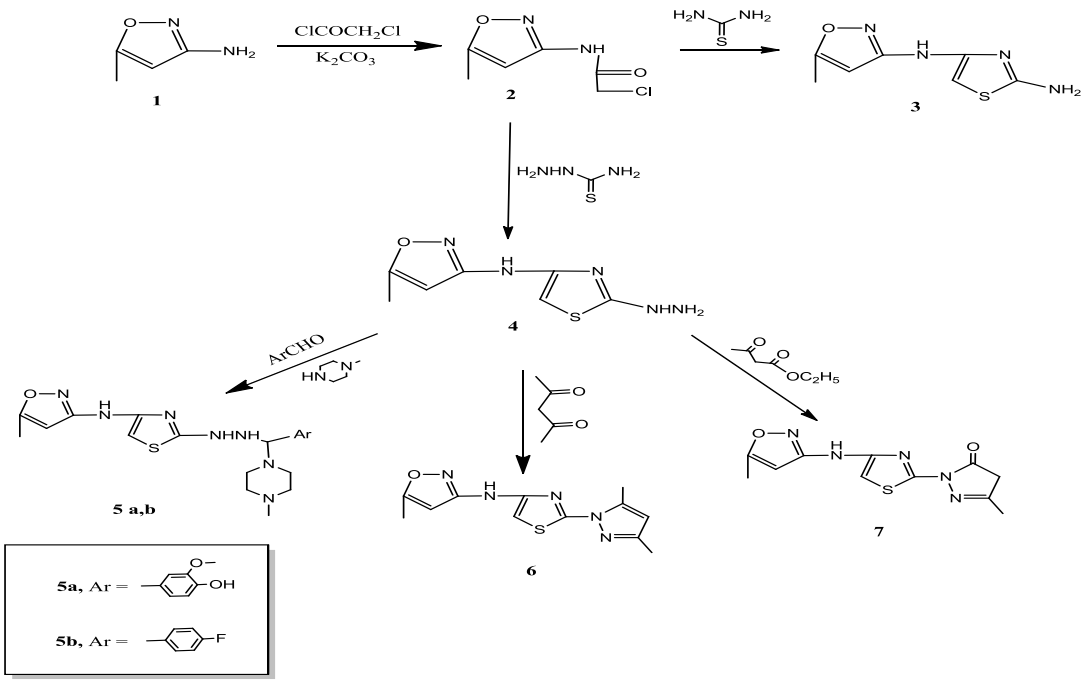
The MDB file of the ligand **3** to be docked was loaded and dock's calculations were run automatically. The obtained poses were studied, and the poses showed best ligand-enzyme interactions were selected and stored for energy calculations.

The 2D interaction and stereo view for compound **3** inside the active sites of the selected enzymes were obtained and saved as both MOE and photo files.

Results and Discussion

1- Chemistry

Compounds **3** and **4** were prepared by cyclization of **2** with thiourea and thio semicarbazide, respectively **(Scheme 1)**.



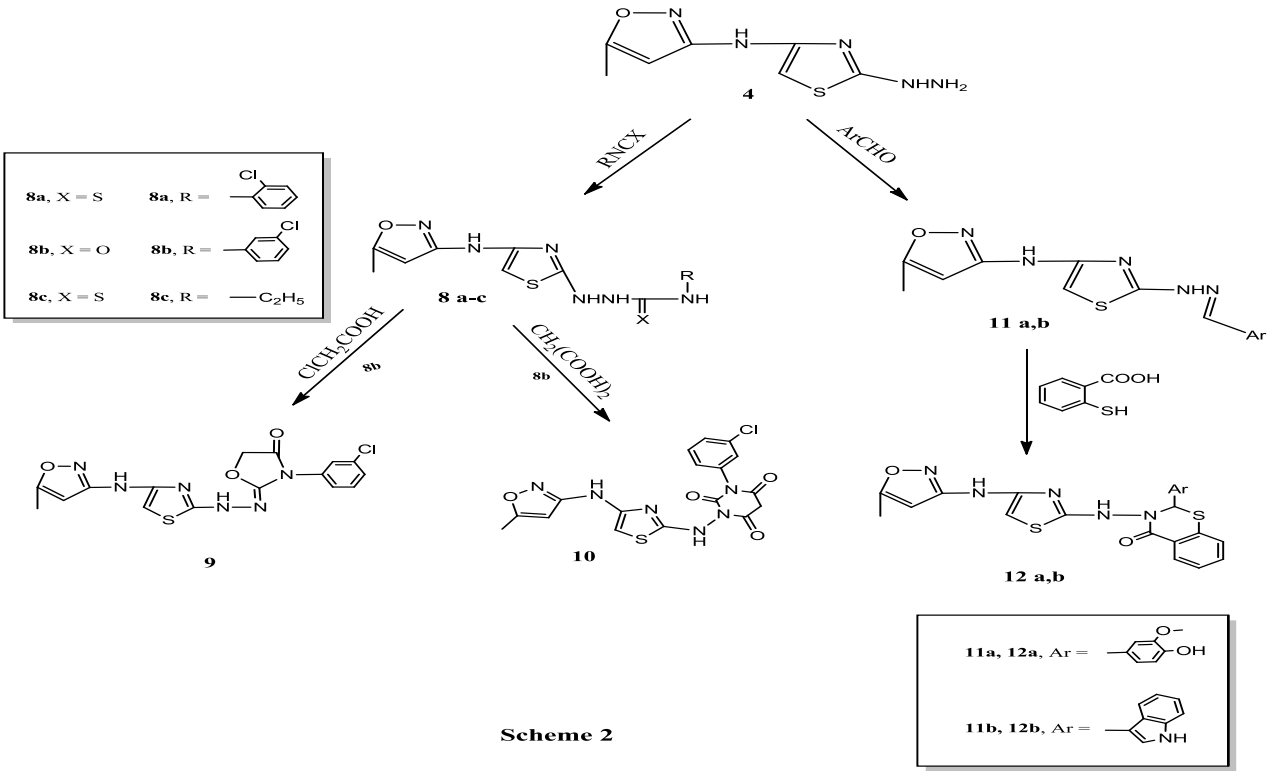
Scheme 1

^{1}H NMR (DMSO-d_6) spectra of **3** revealed signals at 2.38 (s, 3H, CH_3 of isoxazole), 6.60 (s, 1H, CH of thiazole), 6.70 (s, 1H, CH of isoxazole), 7.47 (s, 2H, NH_2, D_2O exchangeable), 9.93 (s, 1H, NH, D_2O exchangeable). IR spectra of **4** exhibited bands at 3419, 3318, 3210, 3181 (NH_2, 2NH), 1614 (C=N). Compound **4** when reacted with the appropriate aldehydes namely: vanillin and/ or 4-fluoro benzaldehyde in the presence of methylpiperazine, gave **5a, b (Scheme 1).** ^{1}H NMR (DMSO-d_6) spectra of compound **5a** revealed signals at 2.25 (s, 3H, N-CH_3), 2.36 (s, 3H, CH_3 of isoxazole), 2.73 (t, 8H, 4CH_2 of piperazine), 3.75 (s, 3H, OCH_3), 5.10 (s, 1H, CH), 5.50 (s, 1H, OH, D_2O exchangeable), 5.80 (s, 1H, CH of thiazole), 6.50 (s, 1H, CH of isoxazole), 7.10-7.80 (m, 3H, Ar), 8.50, 8.90, 9.93 (3s, 3H, 3NH, D_2O exchangeable). IR spectra of compound **5b** exhibited bands at 3430, 3319, 3281 (3NH), 1622 (C=N). Compound **4** also reacted with acetylacetone and ethyl acetoacetate to give **6** and **7**, respectively**. (Scheme1).** ^{1}H NMR (DMSO -d_6) spectra of **6** revealed signals at 2.30 (2s, 6H, 2CH_3 of pyrazole), 2.80 (s, 3H, CH_3 of isoxazole), 5.90 (s, 1H, CH of pyrazole), 6.50 (s, 1H, CH of isoxazole), 6.80 (s, 1H, CH of thiazole), 8.20 (s, 1H, NH, D_2O exchangeable). IR spectra of **7** exhibited bands at 3311 (NH), 1717 (C=O), 1606 (C=N).

8a-c were prepared by the reaction of **4** with the appropriate isothiocyanate and/or isocyanate, namely: 2-chloro phenyl isothiocyanate, 3-chloro phenyl isocyanate, ethyl isothiocyanate, compound **8b** reacted with monochloroacetic acid and malonic acid gave **9** and **10** respectively **(Scheme 2)**. ^{1}H NMR (DMSO-d_6) spectra of **8a** revealed signals at

2.40 (s, 3H, CH_3 of isoxazole), 5.60 (s, 1H, CH of thiazole), 6.30 (s, 1H, CH of isoxazole), 7.10-8.10 (m, 4H, Ar), 8.40, 8.90, 9.10, 9.40 (4s, 4H, 4NH, D_2O exchangeable). IR spectra of **8b** exhibited bands at 3433, 3290, 3255, 3179 (4NH), 1719 (C=O), 1632 (C=N). ^{1}H NMR (DMSO-d_6) spectra of **8c** revealed signals at 1.28 (t, 3H, CH_3 of ethyl), 2.30 (s, 3H, CH_3 of isoxazole), 4.50 (q, 2H, CH_2 of ethyl), 5.60 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 8.10, 8.30, 9.40, 9.70 (4s, 4H, 4NH, D_2O exchangeable). IR spectra of **9** exhibited bands at 3405, 3219 (2NH), 1710 (C=O), 1603 (C=N). ^{1}H NMR (DMSO-d_6) spectra of **10** revealed signals at 2.35 (s, 3H, CH_3 of isoxazole), 3.23 (s, 2H, CH_2 of pyrimidine), 5.30 (s, 1H, CH of thiazole), 6.40 (s, 1H, CH of isoxazole), 7.30-8.00 (m, 4H, Ar), 8.40, 8.90 (2s, 2H, 2NH, D_2O exchangeable).

11a, b can be formed via the reaction of **4** with the appropriate aldehydes, namely: vanillin and/ or Indole-3-carboxaldehyde which, when reacted with thiosalicylic acid gave **12a, b (Scheme 2).** ^{1}H NMR (DMSO-d_6) spectra of **11a** revealed signals at 2.50 (s, 3H, CH_3 of isoxazole), 3.48 (s, 3H, OCH_3), 5.60 (s, 1H, OH, D_2O exchangeable), 5.50 (s, 1H, CH of thiazole), 6.30 (s, 1H, CH of isoxazole), 7.10-8.00 (m, 3H, Ar), 8.28 (s, 1H, CH=N), 9.02, 11.8 (2s, 2H, 2NH, D_2O exchangeable). IR spectra of **11b** exhibited bands at 3420, 3231, 2910 (3NH), 1615 (C=N). ^{1}H NMR (DMSO-d_6) spectra of compound **12a** revealed signals at 2.50 (s, 3H, CH_3 of isoxazole), 3.77 (s, 3H, OCH_3), 5.40 (s, 1H, CH of thiazole), 6.90 (s, 1H, CH of isoxazole), 7.20-8.10 (m, 7H, Ar), 8.80 (s, 1H, CH of thiazin), 9.80, 11.70, 11.80 (3s, 3H, 2NH, OH, D_2O exchangeable). IR spectra of **12b** exhibited bands at 3423, 3310, 3065 (3NH), 1683 (C=O), 1602 (C=N).



Scheme 2

2- Pharmacology

2.1. In-vitro cytotoxic Screening against HCT-116, HePG-2 and MCF-7 cell lines

The cytotoxic potencies of compounds **(3, 4, 5b, 6, 7, 8b, 9, 10, 11b, 12b)** were investigated against human colon carcinoma (HCT-116), hepatocellular carcinoma (HePG-2) and Caucasian breast adenocarcinoma (MCF-7) cell lines using MTT assay [(11-13)]. Doxorubicin, which is one of the most effective anticancer agents, was used as a reference drug. Tumor cells were incubated either alone (negative control) or with different concentrations of the test compounds (100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µM). By analyzing the data in tables **(1, 2)**, we found that compound **3** showed potent antitumor activity against HePG-2 cell line with IC_{50} = 20.2 µg ml^{-1}, higher than the reference drug doxorubicin with IC_{50} of 21.6 µg ml^{-1} but weak activity towards HCT-116 cell line (IC_{50} = 82.6 µg ml^{-1}). Regarding to MCF7 cell line, compound **5b** revealed the highest cytotoxic activity with IC_{50} = 16.2 µg ml^{-1}, while compound **3** exhibited moderate activity (IC_{50} = 60.9 µg ml^{-1}) in comparison with doxorubicin (IC_{50} = 26.3 µg ml^{-1}). Finally, it could be concluded that the anticancer activity was mainly attributed to compound bearing thiazole moiety directly attached to free NH_2 group. Substitution of NH_2 group or insertion of it in cyclized system decrease or abolish the cytotoxic activity.

Table 1: Cytotoxic activity of the newly synthesized compounds against human carcinoma cell lines at 100 µg ml^{-1}.

Compound [a]	Growth inhibition (%)		
	HCT-116	**HePG-2**	**MCF-7**
3	61.5	100	88
4	0	18.2	6.3
5b	46.2	51.9	100
6	0	2.5	13.8
7	0	0	9.8
8b	0	16.8	29.8
9	0	6.2	13.5
10	0	13.5	0
11b	0	39.8	22.5
12b	0	5.6	18.5
DMSO	1	1	3
Negative control[b]	0	0	0
Doxorubicin[a]	100	100	100

[a]Concentration of test compounds and positive control (doxorubicin) were 100 µg ml^{-1}

[b]Untreated cells in DMSO and its final concentration in the cells was less than 0.2 %.

Table 2: IC_{50} of the highly cytotoxic active compounds against human cancer cell lines.

Compounds	IC_{50} (µg ml^{-1})		
	HCT-116	**HePG-2**	**MCF-7**
3	82.6	20.2	60.9
5b	-	-	16.2
Doxorubicin	37.8	21.6	26.3

IC_{50}: Compound concentration required to inhibit the cell viability by 50 %.

2.2. Biochemical Assay (kinase inhibitor activity):

Depending on the results of anticancer screening, the most potent compound **3** was selected for *in vitro* inhibition assessment against a panel of twelve protein kinases (AKT1, AKT2, BRAF (V600E), CDK2/CyclinA1, CHK1, EGFR, VEGFR-2, p38α, PDGFRβ, PI3K (p110a/p85a and p110b/p85a) and c-RAF) at 100 µM using the radiometric or ADP-Glo assay method. Three of the selected kinases (EGFR, PI3K (p110b/p85a) and p38α) were strongly inhibited by the compound by more than 85% with the highest inhibition noted with EGFR at 95%. Moderate inhibitions were observed with six of kinases (AKT2, CDK2/Cylin A1, PDGFRβ, VEGFR-2, BRAF(V600E), and CHK1) ranging from 26% up to 64%. Both PI3K (p110a/p85a) and AKT1 showed little inhibition at 2% to 4%. There was slight activation of the c-RAF kinase with an increase in counts of 16% compared to the control (table **3**).

Table 3: % Inhibition of kinases in the presence of compound 3 at 100 µM using the radiometric or ADP-Glo (*) assay method.

Kinase	Compound 3
	% Inhibition
AKT1	-2
AKT2	-64
BRAF (V600E)	-37
CDK2/Cyclin A1	-57
CHK1	-26
EGFR	-95
KDR (VEGFR)	-42
p38α	-85
PDGFRβ	-56
PI3K (p110a/p85a)	-4
PI3K* (p110b/p85a)	-89
c-RAF*	16

3. Molecular modeling studies

In order to better understand the interaction between the target compound **3** and the kinases (EGFR, PI3K (p110b/p85a) and p38α), molecular docking studies were performed using Molecular Operating Environment (MOE®) [14] 2008.10.

Fig.1 demonstrates the compound **3** docking into the binding site of EGFR kinase (PDB: 1M17) [15]. In this binding model, the free hydrogens of NH_2 linked to thiazole ring formed three H-bonds with **Ala719, Leu764** and **Thr766** as H-donors (distance: 1.7, 2, 2.5 A^o respectively). Furthermore, the gatekeeper residue **Thr766** formed H-bond acceptor with the nitrogen of thiazole moiety (distance: 2.3 A^o). There was a strong hydrogen bond appeared between the nitrogen of isoxazole and the side chain of **Thr830** as H-acceptor (distance: 2.5 A^o).

The binding model of compound **3** into the ATP-binding cavity of P13Kβ kinase (PDB: 4BFR)[16] is depicted in **Fig.2**. This binding model revealed that compound **3** formed three strong hydrogen bonds with receptor. Two H-bonds appeared as H-donors between hydrogens of NH_2 group and the side chains of **Asp807** and **Tyr833** (distance: 1.5, 2.4 A^o respectively), and the third H-bond was linking **Tyr833** as H-bond acceptor with nitrogen of thiazole (distance: 2.7 A^o). Interaction of compound **3** with the binding site of P38α kinase (PDB: 3FMK)[17] is shown in **Fig.3**. The P38α-binding domain demonstrates that the ligand **3** made three favorable hydrogen bonding with the protein. The back bones of **Ala51** and **Leu104** were involved in two H-bond donors with NH_2 group (distance: 2.2, 2.1 A^o respectively). Additionally, H-bond acceptor established between nitrogen of isoxazole and side chain of **Lys 53** (distance: 2.6 A^o).

The above docking analysis was consistent with kinase assay data and thus further confirmed that compound **3** is a potent inhibitor of EGFR, PI3K (p110b/p85a) and p38α kinases and it could be a potential antitumor agent.

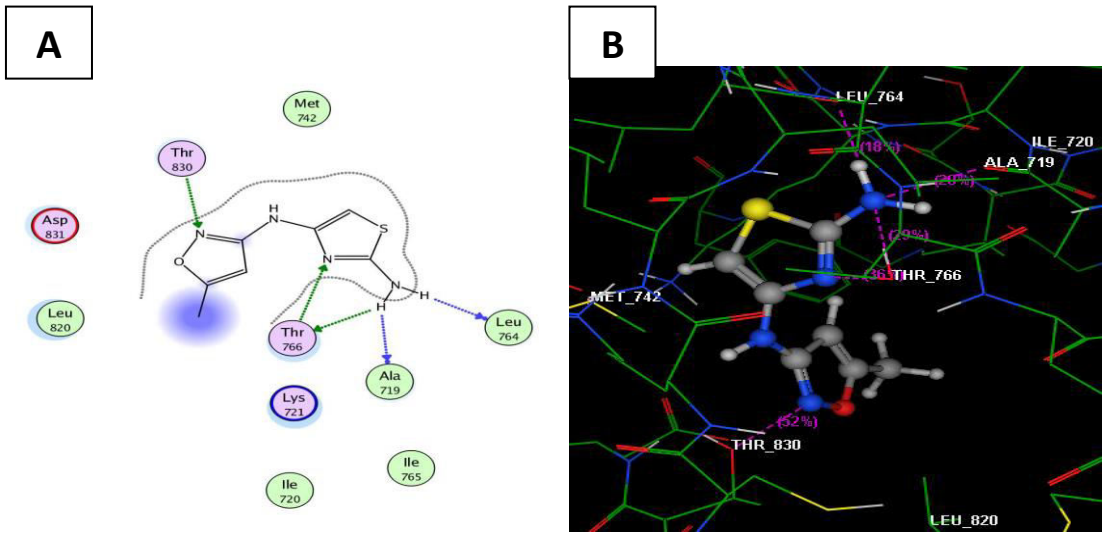


Fig. 1: The proposed binding mode of compound **3** docked in the active site of EGFR; A and B showing 2D and 3D ligand-receptor interactions (hydrogen bonds are illustrated as arrows, C atoms are colored gray, N blue and O red).

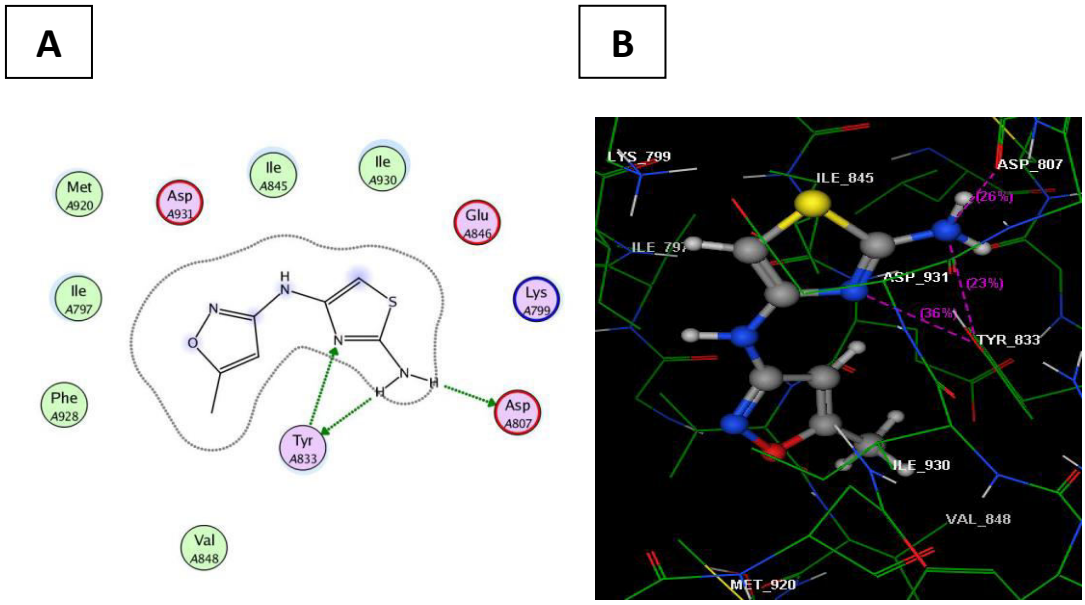


Fig. 2: The proposed binding mode of compound **3** docked in the active site of P13Kβ; A and B showing 2D and 3D ligand-receptor interactions (hydrogen bonds are illustrated as arrows, C atoms are colored gray, N blue and O red).

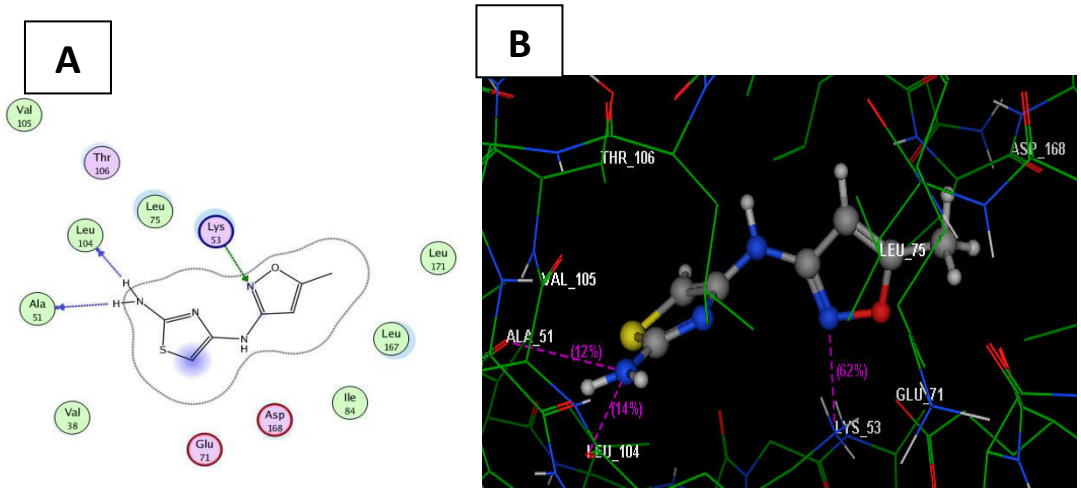


Fig. 3: The proposed binding mode of compound **3** docked in the active site of P38α; A and B showing 2D and 3D ligand-receptor interactions (hydrogen bonds are illustrated as arrows, C atoms are colored gray, N blue and O red).

Conclusion:

A series of 4-(5-methylisoxazol-3-ylamino)thiazole derivatives was synthesized and evaluated for their antitumor activities against the human cancer cell lines HCT-116, HePG-2 and MCF-7. The most potent compound **3** was screened for its inhibitory activity against twelve types of kinases. The results showed that compound **3** could be considered as multi-target EGFR, PI3K (p110b/p85a) and p38α kinases inhibitors. The docking study was achieved to predict the binding modes, affinities and orientations of compound **3** at the active sites of these enzymes.

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Conflict of interests: No conflict of interest is there to declare.

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