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Synthesis and Biological Evaluation of Some New Substituted-Pyrazole Attached to 4-(Benzothiazol-2-Yl) Moiety as Anti-angiogenic Agents.

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ABSTRACT

Some substituted-pyrazole derivatives attached to 4-(benzothiazol-2-yl) moiety were prepared starting from o-aminothiophenol **1** by reacting with different electrophilic and nucleophilic reagents. compound **10d** exhibited the higher cytotoxic activity against breast cancer cell line MCF-7 (IC_{50} = 0.01µmol/ml), also higher inhibition of human VEGF in MCF-7 cancer cell line with percentages of inhibition (99%) in comparison to the positive drug, Tamoxifen (IC_{50} = 0.02µmol/ml, % inhibition = 98%) as compared with control untreated cells.
Keywords: 4-(Benzothiazol-2-yl), substituted-pyrazole, MCF7, VEGF, VEGFR2.

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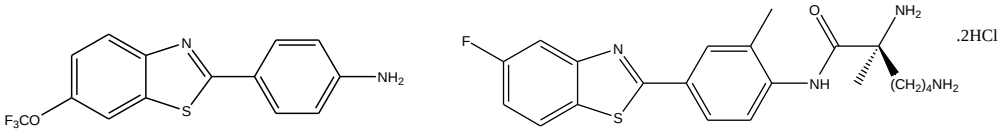
INTRODUCTION

Angiogenesis, new blood vessels formation, plays a central role in cancer cell survival, tumor growth which is considered as a precondition of metastasis for all solid tumors [1-3]. This process consists of the formation of new capillaries through splitting from pre-existing blood vessels. There are several growth factors involved in tumor angiogenesis which include vascular endothelial growth factor (VEGF) [4], basic fibroblast growth factor [5], epidermal growth factor (EGF) [6], platelet-derived growth factor (PDGF) [7] and angiopoietin Ref. [8]. Indeed, VEGF is the most important signaling protein among the other growth factors that plays a vital role in stimulation of angiogenesis [9]. The VEGF family consists of six members of proteins (VEGF-A, B, C, D, E and placenta growth factor). These proteins can bind to their VEGF receptors (VEGFR1, VEGFR2 and VEGFR3). These receptors are belonging to receptor tyrosine kinases (RTK). VEGFR2 is considered as important receptor mediating of all the cellular responses to VEGF [10]. Binding of VEGF to its family of receptors (VEGFR), is the key mediator that promotes the proliferation and survival of endothelial cells and consequently cancer progression [11]. Therefore, looking for an effective anti-VEGF/VEGFR drug became the main interest for many research groups aiming to discover a new cancer therapy *via* angiogenesis inhibition.

Benzothiazole is a core subunit in various marine or terrestrial natural compounds which have useful biological activities [12-17], such as neuroprotective for e.g., **Riluzole** [16] as illustrated in figure **1** and **Pramipexole** [17] (known commercially as Mirapex), a potent inhibitor for muscarinic receptor [18], and also for HIV-protease and reverse transcriptase [19] and many others. Molecules based on benzothiazole derivatives have attracted a great deal of interest due to their antitumor [20-25], antimicrobial [15], antipyretic, analgesic, anti-inflammatory [26], lipid-lowering properties [27] and hypoglycemic activity [28]. The fluorinated analogue, 2-(4-aminophenyl)-5-fluorobenzothiazole is a novel agent with potent and selective anti-tumor properties which in the form of its L-lysylamide prodrug, **Phortress**, as illustrated in figure **1** is in early phase clinical studies [29].



Riluzole **Phortress**

Figure 1

In the same direction and in continuing effort to find more potent and selective anticancer compounds, herein, we designed and synthesized a series of benzothiazole compounds. Their biological activities against tumor cell-line MCF-7 that may act through VEGF inhibition were evaluated.

Chemistry

2-(4-Cyanophenyl)benzothiazole **2** was prepared from reacting o-aminothiophenol **1** with 4-cyanobenzaldehyde in absolute ethanol according to reported procedure [30]. Acid oxidation of carbonitrile group by stirring with 70% sulfuric acid gave 4-(benzothiazol-2-yl) benzoic acid **3** followed by esterification and reacting with hydrazine hydrate to form the corresponding 4-(benzothiazol-2-yl)benzohydrazide **5**. 5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid ethyl ester (**6**) was prepared by refluxing compound **5** with ethyl (ethoxy methylene) cyanoacetate in ethanol (***Scheme 1***).

Reacting **6** with sulfanilamide or sulfathiazole in presence of sodium hydroxide (aqueous solution) and ethanol, the sulfa derivatives **7a,b** were obtained. Also, reacting **6** with different amines, compounds **8a-d** were obtained as illustrated in ***Scheme 2***.

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid **9** was prepared by treating **6** with sodium hydroxide solution. Products **10a-d** were prepared from coupling compound **9** with the appropriate amino acid ethyl ester (D-alanine, L-methionine, L-glutaminic or D-tyrosine ethyl ester hydrochloride, respectively) in presence of coupling reagent benzotriazol-1-yloxytris (dimethylamino) phosphonimhexafluorophosphate (BOP) and diisopropyl ethylamine as represented in ***Scheme 3***.

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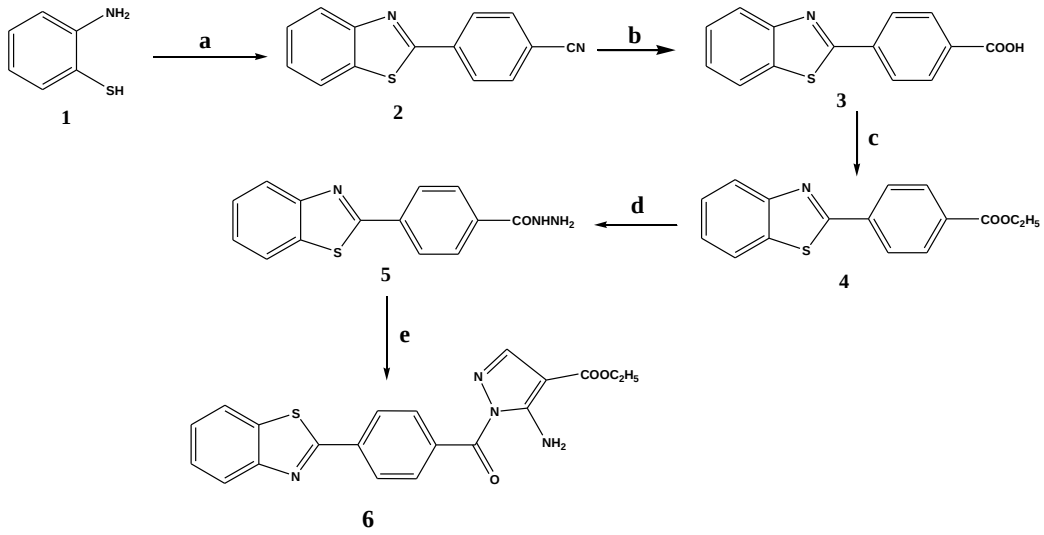
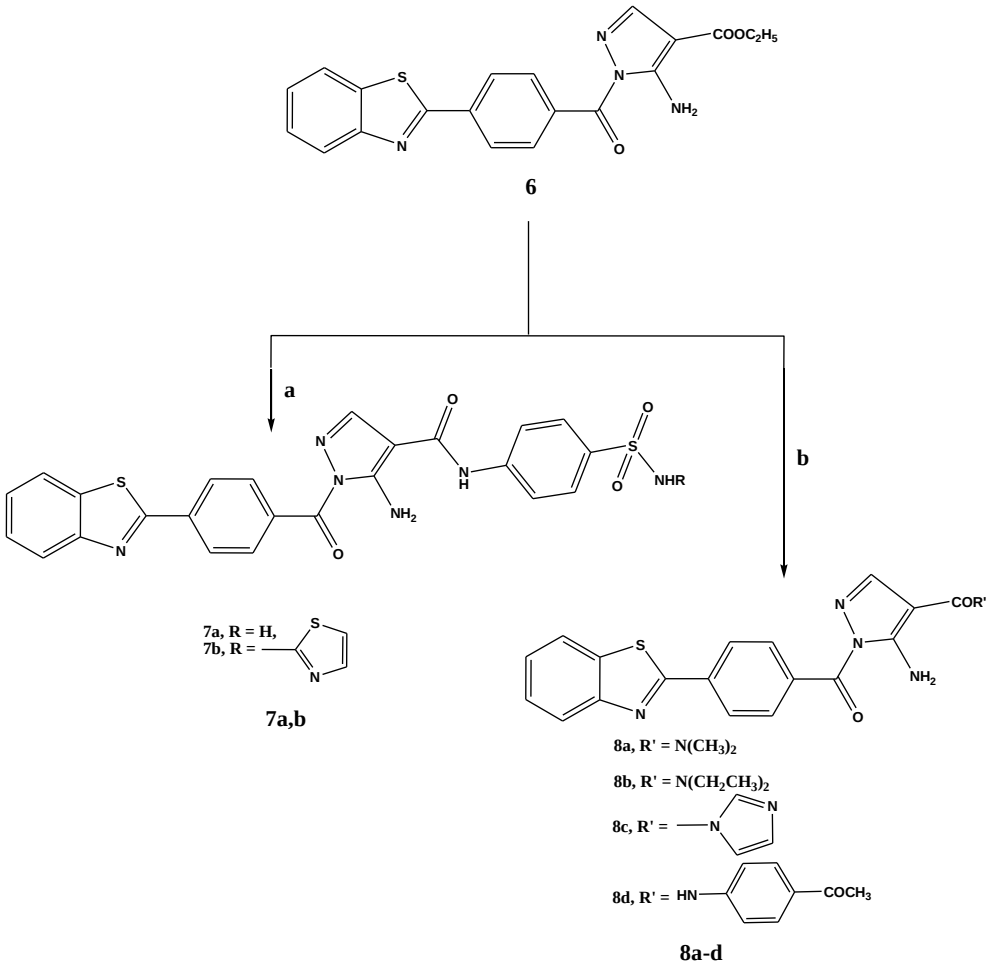


Scheme 1: Reagents: **(a)** 4-Cyanobenzaldehyde, EtOH; **(b)** 70% H_2SO_4; **(c)** EtOH, H_2SO_4; **(d)** $NH_2NH_2.H_2O$, EtOH; **(e)** ethyl(ethoxymethylene) cyanoacetate, EtOH



Scheme 2: Reagents: (a) sulfanilamide or sulfathiazole, NaOH, EtOH; (b) dimethylamine, diethylamine, 1H-imidazole or 4-aminoacetophenone, EtOH.

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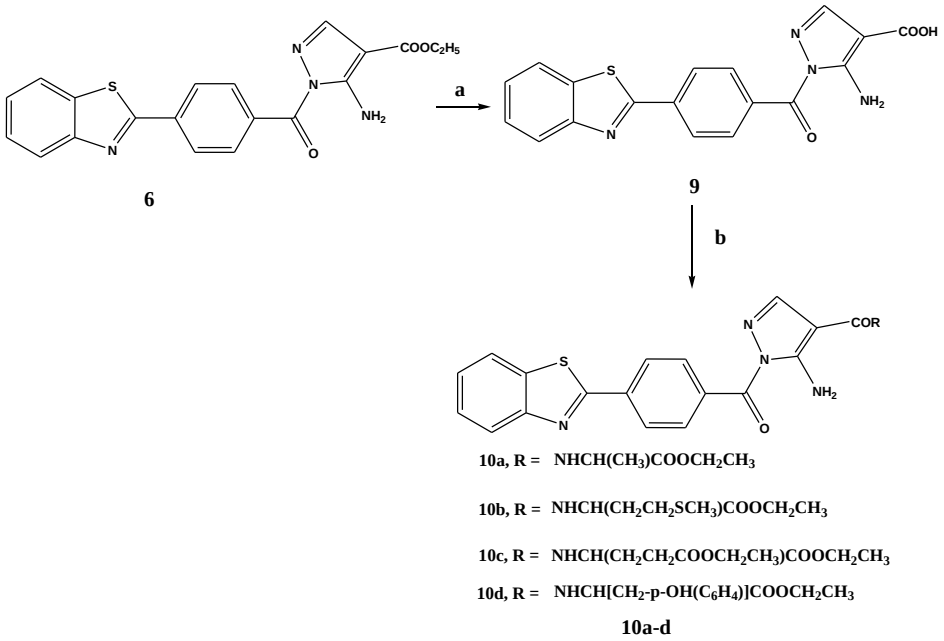

6

10a-d

Scheme 3: Reagents: (a) NaOH; (b) D-alanine, L-methionine, L-glutaminic or D-tyrosine ethyl ester hydrochloride, methylene chloride, BOP, diisopropyl ethylamine

RESULTS AND DISCUSSION

Biological evaluation

Cytotoxicity against human breast cancer cell line MCF-7

Cytotoxicity of the synthesized compounds was tested using Skehan et al method [31] in human breast cancer cell line MCF-7. The Cytotoxicity results were compared to that of standard drug, Tamoxifen. While compounds **7a** and **8c** exhibited no anticancer effect against MCF-7 cell line, compound **10d** is more potent than tamoxifen (IC_{50} = 0.01 µmol/ml) with IC_{50} = 0.02µmol/ml. The rest of the compounds were of moderate to weak activity against MCF-7 cell line as shown in (Table 1).

Table 1: Effect of the synthesized compounds on the human breast cancer cell line MCF-7 and the VEGF level (pg/ml) in breast cancer cell line MCF-7.

Compound	IC_{50} (µmol/ml)	VEGF (pg/ml)
6	0.13	5160.90±550.00 (1.70%)
7a	NA	5500.00±576.00 (-4.76%)
7b	0.03	2071.00±230.80 (60.58%)
8a	0.13	5161.90±550.00 (1.70%)
8b	0.12	5040.90±550.00 (1.98%)
8c	NA	-
8d	0.14	5200.70±545.22 (0.94%)
9	0.03	2070.00±230.80 (60.57%)
10a	0.11	4800.00±230.80 (3.57%)
10b	0.04	296.56±20.00 (93.25%)
10c	0.06	685.76±20.00 (88.25%)
10d	0.01	106.76±20.00 (99.05%)
DMSO	-	5250.00
Tamoxifen	0.02	110.75 (98%)

Data were expressed as M±SE of four independent experiments.
Values between brackets indicated percentage changes as compared with control cancer cells.
NA is no activity

In vitro VEGF inhibition in human breast cancer cell line MCF-7

This biological *in vitro* study was done using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of human VEGF in human breast cancer cell line MCF-7 samples as compared to the inhibition for the untreated cells. The screening results (Table 1) showed that compound **10d** were found to be more potent and selectively similar to the positive drug, Tamoxifen (98%) against human VEGF with percentage of inhibition values 99% as compared with control untreated cells. These results were consistent with cell cytotoxicity activity against MCF-7 cell line where this compound exhibited excellent activity with IC_{50} ranging from 0.01µmol/ml.

CONCLUSION

Our main goal throughout this manuscript was the synthesis of new substituted-pyrazole derivatives attached to 4-(benzothiazol-2-yl) moiety as antiangiogenic agents working *via* inhibiting VEGF-VEGFR2 complex formation, thus suppressing proliferation and survival of endothelial cells and consequently preventing cancer progression.

The bioactivity of the compounds **6-10d** showed that 5 compounds (**7b**, **9**, **10b**, **10c** and **10d**) have shown promising cytotoxic activity against breast cancer cell line MCF-7 with (IC_{50} = 0.01-0.06 µmol/ml) and potential inhibition of human VEGF in MCF-7 cancer cell line with percentages of inhibition (60-99%) in comparison to the positive drug, Tamoxifen (IC_{50} = 0.02µmol/ml, % inhibition = 98%) as compared with control untreated cells.

Experimental

Chemistry

Melting points (˚C) were taken in open capillary tubes using silicon oil on Gallen Kamp apparatus. ^{1}H-NMR Spectra were measured in DMSO-d_6 on JEOL-270 MHz Spectrometer with TMS as an internal standard. Mass Spectra were obtained with a Schimadzu GCS-QP1000EX Spectrometer at 70 eV. The IR Spectra were recorded with a Philips Infra cord Spectrophotometer Model PU 9712 in KBr discs. Elemental analysis was performed at the Micro analytical Laboratory of the National Research Center. The antitumor activity of the synthesized compounds was carried out at the National Research Centre, Giza, Egypt.

2-(4-Cyanophenyl)benzothiazole (2)

4-Cyanobenzaldehye (1.05g, 0.21 mol) and o-aminothiophenol **1** (1 ml, 0.21 mol) were dissolved in ethanol. This mixture was refluxed for 5h. and cooled to room temperature. Then, water was added slowly to the mixture with stirring. The suspension was maintained at -5ºC overnight. The product was washed repeatedly with ethanol-water (1:1) mixture and then recrystallized from acetone. Yield = 1.2g (64%), m.p. = 150-2˚C. Analysis for $C_{14}H_8N_2S$ (236.2): Calcd.: C, 69.0; H, 4.5; N, 12.4; S, 14.2; Fd.: C, 69.2; H, 4.6; N, 12.5; S, 14.3.

4-(Benzothiazol-2-yl)benzoic acid (3)

A mixture of **2** (2g, 0.01 mol) and 30 ml 70% sulfuric acid was stirred in 100 ml three-necked flask at 140ºC for 5h, then suspended in 150 ml water and the resulting precipitate was filtered off. Recrystallization from diluted ethanol afforded white crystals. Yield = 0.8g (74%), m.p. = 250-3˚C. Analysis for $C_{14}H_9NO_2S$ (255.3): Calcd.: C, 65.9; H, 3.6; N, 5.5; S, 12.6; Fd.: C, 66.0; H, 3.6; N, 5.6; S, 12.8. MS: m/z (%): 256 ($M^{+\cdot}$, 19); 255 (M^+, 100).

Ethyl 4-(benzothiazol-2-yl)benzoate (4)

To a solution of compound **3** (2g; 0.073 mol) in absolute ethanol, few drops of concentrated sulfuric acid were added and the mixture was refluxed for 4h. The crude product was filtered, air-dried and crystallized from ethanol. Yield = 2.1g (94%), m.p. = 102-6˚C. Analysis for $C_{16}H_{13}NO_2S$ (283.3): Calcd.: C, 67.8; H, 4.6; N, 4.9;

S, 11.3; Fd.: C, 68.0; H, 4.6; N, 5.0; S, 11.5. MS: m/z (%): 284 ($M^{+\cdot}$, 25); 283 (M^+, 5). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.32 (t, 3H, C<u>H</u>$_3$); 4.32 (q, 2H, C<u>H</u>$_2$CH$_3$); 7.48-8.22 (m, 8H, Ar-H).

4-(Benzothiazol-2-yl)benzohydrazide (5)

To a solution of ester compound **4** (1g; 0.033 mol) in ethanol, hydrazine hydrate (98%; 2 ml) was added and heated for 5h on a water-bath. The reaction mixture was cooled. The crude product was filtered, washed with water and dried. It was crystallized from ethanol. Yield = 0.8g (84%), m.p. = 234-8˚C. Analysis for $C_{14}H_{11}N_3OS$ (269.3): Calcd.: C, 62.4; H, 4.1; N, 15.6; S, 11.9; Fd.: C, 62.5; H, 4.2; N, 15.7; S, 12.0. IR (cm^{-1}): 3453 (NH), 3314 (NH$_2$), 1684 (CO). MS: m/z (%): 270 ($M^{+\cdot}$, 6); 269 (M^+, 16). ^{1}H-NMR: δ, ppm (DMSO-d_6); 7.50-8.24 (m, 8H, Ar-H); 9.96 (s, 2H, N<u>H</u>$_2$); 10.45 (s, 1H, N<u>H</u>).

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid ethyl ester (6)

To a solution of hydrazide **5** (1g, 0.01 mol) in 30 ml ethanol, ethyl(ethoxy methylene)cyanoacetate (1.62g, 0.01 mol) was added. The reaction mixture was refluxed for 8h. The reaction mixture was cooled to room temperature and the solvent was removed under reduced pressure. The solid product was recrystallized from ethanol.

Yield = 0.9g (62%); m.p. = above 300˚C. Analysis for $C_{20}H_{16}N_4O_3S$ (392.4): Calcd.: C, 61.2; H, 4.1; N, 14.3; S, 8.2; Fd.: C, 61.3; H, 4.5; N, 14.6; S, 8.4. IR (cm^{-1}): 3224 (NH$_2$), 1697 (CO), 1662 (CO). MS: m/z (%): 392 (M^+, 57). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.19 (t, 3H, C<u>H</u>$_3$); 3.30 (q, 2H, C<u>H</u>$_2$); 4.19 (s, 2H, N<u>H</u>$_2$); 7.48 (s, 1H, C<u>H</u>); 7.50-8.25 (m, 8H, Ar-H).

General procedure for the preparation of 5-amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid (4-sulfamoylphenyl) / [4-(thiazol-2-yl-sulfamoyl)-phenyl] amide (7a,b)

Compound **6** (1g, 0.0034 mol) was dissolved in ethanol (20 ml) and 10% aqueous sodium hydroxide solution, the sulfa drug (sulfanilamide or sulfathiazole) (0.0034 mol) was added dropwisely and the reaction mixture was stirred for 4h. at room temperature. The mixture was then poured onto ice/cold water and neutralized with dilute hydrochloric acid. The crude product was filtered and crystallized from petroleum ether.

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid (4-sulfamoyl phenyl)-amide (7a)

Yield = 1.2g (91%), m.p. = 265-9˚C. Analysis for $C_{24}H_{18}N_6O_4S_2$ (518.5): Calcd.: C, 55.6; H, 3.5; N, 16.2; S, 12.4; Fd.: C, 55.7; H, 3.7; N, 16.4; S, 12.5. IR (cm^{-1}): 3373 (NH), 3224 (NH$_2$), 1687 (CO), 1662 (CO), 1375 (SO$_2$). MS: m/z (%): 519 ($M^{+\cdot}$, 18); 518 (M^+, 21). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.23 (s, 2H, N<u>H</u>$_2$, exchangeable with D$_2$O); 4.19 (s, 2H, N<u>H</u>$_2$, exchangeable with D$_2$O); 7.48-8.28 (m, 12H, Ar-H); 7.61 (s, 1H, C<u>H</u>); 10.60 (s, 1H, N<u>H</u>, exchangeable with D$_2$O).

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid [4-(thiazol-2-yl-sulfamoyl)-phenyl]-amide (7b)

Yield = 1.3g (85%), m.p. = above 300˚C. Analysis for $C_{27}H_{19}N_7O_4S_3$ (601.7): Calcd.: C, 53.9; H, 3.2; N, 16.3; S, 15.9; Fd.: C, 53.8; H, 3.5; N, 16.1; S, 15.9. IR (cm^{-1}): 3373 (NH), 3224 (NH$_2$), 1687 (CO), 1662 (CO), 1375 (SO$_2$). MS: m/z (%): 602 ($M^{+\cdot}$, 32); 601 (M^+, 91). ^{1}H-NMR: δ, ppm (DMSO-d_6); 2.46 (s, 1H, N<u>H</u>); 4.19 (s, 2H, N<u>H</u>$_2$); 7.42-8.29 (m, 12H, Ar-H); 7.61 (s, 1H, C<u>H</u>); 8.01 (d, 1H, C<u>H</u>); 8.09 (d, 1H, C<u>H</u>); 10.40 (s, 1H, N<u>H</u>).

General procedure for the preparation of 5-amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid substituted-amide (8a-d)

A mixture of compound **6** (1g, 0.002 mol), the respective amine (dimethylamine, diethylamine, 1H-imidazole or 4-aminoacetophenone) (0.002 mol) and 20 ml of ethanol were heated under reflux for 10h. The reaction mixture was then cooled and the precipitate was filtered off, dried and recrystallized from methanol.

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid dimethylamide (8a)

Yield = 0.9g (90%), m.p. = 83-6˚C. Analysis for $C_{20}H_{17}N_5O_2S$ (391.5): Calcd.: C, 61.4; H, 4.4; N, 17.9; S, 8.2; Fd.: C, 61.6; H, 4.2; N, 17.9; S, 8.3. IR (cm^{-1}): 3224 (NH_2), 1685 (CO), 1663 (CO). MS: m/z (%): 392 ($M^{+\cdot}$, 13); 391 (M^+, 35). ^{1}H-NMR: δ, ppm (DMSO-d_6); 2.46 (s, 6H, 2C$\underline{H}_3$); 4.19 (s, 2H, N$\underline{H}_2$); 7.47-8.33 (m, 8H, Ar-H); 7.61 (s, 1H, C$\underline{H}$).

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid diethylamide (8b)

Yield = 0.9g (84%), m.p. = 93-6˚C. Analysis for $C_{22}H_{21}N_5O_2S$ (419.5): Calcd.: C, 62.9; H, 5.1; N, 16.7; S, 7.6; Fd.: C, 63.1; H, 5.2; N, 16.6; S, 7.6. IR (cm^{-1}): 3224 (NH_2), 1685 (CO), 1663 (CO). MS: m/z (%): 420 ($M^{+\cdot}$, 12); 419 (M^+, 25). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.19 (t, 6H, 2C$\underline{H}_3$); 3.30 (q, 4H, 2C$\underline{H}_2$); 4.19 (s, 2H, N$\underline{H}_2$, exchangeable with D_2O); 7.47-8.33 (m, 8H, Ar-H); 7.61 (s, 1H, C$\underline{H}$).

5-Amino-4-(imidazole-1-carbonyl)-pyrazol-1-yl]-(4-benzothiazol-2-yl-phenyl)-methanone (8c)

Yield = 0.8g (76%), m.p. = 103-6˚C. Analysis for $C_{21}H_{14}N_6O_2S$ (414.4): Calcd.: C, 60.8; H, 3.4; N, 20.3; S, 7.7; Fd.: C, 60.9; H, 3.6; N, 20.1; S, 7.7. IR (cm^{-1}): 3224 (NH_2), 1685 (CO), 1663 (CO). MS: m/z (%): 415 ($M^{+\cdot}$, 22); 414 (M^+, 45). ^{1}H-NMR: δ, ppm (DMSO-d_6); 4.19 (s, 2H, N$\underline{H}_2$, exchangeable with D_2O); 7.10 (d, 2H, 2C$\underline{H}$); 7.47-8.33 (m, 8H, Ar-H); 7.61 (s, 1H, C$\underline{H}$); 7.71 (s, 1H, C$\underline{H}$).

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid (4-acetyl phenyl)-amide (8d)

Yield = 1g (82%), m.p. = 85-9˚C. Analysis for $C_{26}H_{19}N_5O_3S$ (481.5): Calcd.: C, 64.9; H, 3.9; N, 14.5; S, 6.7; Fd.: C, 64.8; H, 3.9; N, 14.6; S, 6.8. IR (cm^{-1}): 3392 (NH), 3222 (NH_2), 1706 (CO), 1689 (CO), 1667 (CO). MS: m/z (%): 482 ($M^{+\cdot}$, 22); 481 (M^+, 35). ^{1}H-NMR: δ, ppm (DMSO-d_6); 2.58 (s, 3H, C$\underline{H}_3$); 4.19 (s, 2H, N$\underline{H}_2$, exchangeable with D_2O); 6.57 (s, 1H, N$\underline{H}$, exchangeable with D_2O); 7.47-8.33 (m, 12H, Ar-H); 7.61 (s, 1H, C$\underline{H}$).

5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carboxylic acid (9)

Compound **6** (1g, 0.0034 mol) was dissolved in ethanol (20 ml) and 10% aqueous sodium hydroxide solution and the reaction mixture was refluxed for 4h. The mixture was then poured onto ice/cold water and neutralized with dilute hydrochloric acid. The crude product was filtered and crystallized from petroleum ether. Yield = 0.8g (86%), m.p. = 255-9˚C. Analysis for $C_{18}H_{12}N_4O_3S$ (364.4): Calcd.: C, 59.3; H, 3.3; N, 15.4; S, 8.8; Fd.: C, 59.5; H, 3.2; N, 15.3; S, 8.9. IR (cm^{-1}): 3392 (NH_2), 3222 (OH), 1680 (CO), 1664 (CO). MS: m/z (%): 465 ($M^{+\cdot}$, 22); 464 (M^+, 35). ^{1}H-NMR: δ, ppm (DMSO-d_6); 4.19 (s, 2H, N$\underline{H}_2$, exchangeable with D_2O); 7.47-8.33 (m, 8H, Ar-H); 7.61 (s, 1H, C$\underline{H}$); 10.75 (s, 1H, O$\underline{H}$, exchangeable with D_2O).

General procedure for the preparation of 2-{[5-amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carbonyl]-amino} substituted-ethyl ester (10a-d)

A stirring mixture of the respective amino acid ethyl ester (D-alanine, L-methionine L-glutaminic or D-tyrosine ethyl ester hydrochloride) (0.038 mol) and compound **9** (1g, 0.038 mol) in anhydrous methylene chloride (30ml) was cooled to 0˚C. Diisopropylethylamine (1.86g, 0.14 mol) was then slowly added to the mixture, followed by the addition of the coupling reagent benzotriazol-1-yloxytris(dimethylamino) phosphonimhexafluorophosphate reagent (BOP) (1.91g, 0.042 mol) dissolved in 5 ml of anhydrous methylene chloride. The reaction was stirred for 12h. at 20˚C. Ethyl acetate (50 ml) was added to the reaction mixture and the organic layer was successively washed with a 1N hydrochloric acid solution (2x35 ml), a 20% sodium carbonate solution (2x30 ml) and brine. The organic layer was dried over magnesium sulfate and concentrated in vacuum. The crude residue was purified by chromatography on a silica gel column using ethyl acetate/petroleum ether as eluent (1:1).

2-{[5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carbonyl]-amino}-propionic acid ethyl ester (10a)

Yield = 1.1g (87%); m.p. = 214-7˚C. Analysis for $C_{23}H_{21}N_5O_4S$ (463.5): Calcd.: C, 59.6; H, 4.6; N, 15.1; S, 6.9; Fd.: C, 59.6; H, 4.7; N, 15.2; S, 6.9. IR (cm^{-1}): 3430 (NH), 3222 (NH_2), 1706 (CO), 1689 (CO), 1667 (CO). MS: m/z (%): 464 ($M^{+\cdot}$, 12); 463 (M^+, 35). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.17 (t, 3H, C$\underline{H}_3$); 2.48 (d, 3H, C$\underline{H}_3$); 4.15 (q, 2H, C$\underline{H}_2$CH$_3$); 4.19 (s, 2H, N$\underline{H}_2$, exchangeable with D_2O); 4.97 (q, 1H, C$\underline{H}$); 7.49-8.29 (m, 8H, Ar-H); 7.61 (s, 1H, C$\underline{H}$);

9.30 (s, 1H, NH, exchangeable with D_2O). ^{13}C-NMR δ, ppm (DMSO-d_6): showed the presence of 23 signals correspond to the 23 different carbon groups, signals appeared at δ 13.9 (CH_3CH$_2$), 30.5 (CH_3CH), 60.9 (CHCH$_3$), 61.4 (CH_2CH$_3$), 122.4-130.7 (Ar-8CH), 133.2 (C-C), 134.7 (C=C), 137.0 (CS), 153.3 (C-N), 164.5 (C=C), 165.7 (C=N), 167.5 (2CO) and 171.6 (COO).

2-{[5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carbonyl]-amino}-4-methyl sulfanyl-butyric acid ethyl ester (10b)

Yield = 1.2g (84%); m.p. = 205-9˚C. Analysis for $C_{25}H_{25}N_5O_4S_2$ (523.6): Calcd.: C, 57.3; H, 4.8; N, 13.4; S, 12.3; Fd.: C, 57.3; H, 4.9; N, 13.3; S, 12.4. IR (cm^{-1}): 3430 (NH), 3222 (NH$_2$), 1706 (CO), 1689 (CO), 1667 (CO). MS: m/z (%): 524 ($M^{+\cdot}$, 12); 523 (M^+, 35). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.32 (t, 3H, CH_3); 2.09 (s, 3H, CH_3); 2.27 (q, 2H, CH_2); 2.45 (t, 2H, CH_2S); 4.12 (q, 2H, CH_2CH$_3$); 4.19 (s, 2H, NH$_2$); 4.43 (t, 1H, CH); 7.48-8.26 (m, 8H, Ar-H); 7.61 (s, 1H, CH); 9.30 (s, 1H, NH). ^{13}C-NMR δ, ppm (DMSO-d_6): showed the presence of 25 signals correspond to the 25 different carbon groups, signals appeared at δ 14.1 (CH_3CH$_2$), 19.5 (CH_3S), 38.6 (CH_2CH), 40.2 (CH_2CH$_2$), 58.0 (CHCH$_2$), 61.0 (CH_2CH$_3$), 122.4-132.8 (Ar-8CH), 134.6 (C=C), 136.5 (CS), 153.4 (C-N), 164.9 (C=C), 165.8 (C=N), 166.0-166.5 (2CO) and 171.6 (COO).

2-{[5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carbonyl]-amino}-pentanedioic acid diethyl ester (10c)

Yield = 1.2g (80%); m.p. = 210-3˚C. Analysis for $C_{27}H_{27}N_5O_6S$ (549.6): Calcd.: C, 59.0; H, 4.9; N, 12.7; S, 5.8; Fd.: C, 58.8; H, 4.9; N, 12.8; S, 5.9. IR (cm^{-1}): 3430 (NH), 3222 (NH$_2$), 1706 (CO), 1692 (CO), 1689 (CO), 1667 (CO). MS: m/z (%): 550 ($M^{+\cdot}$, 22); 549 (M^+, 32). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.32 (m, 6H, 2CH_3); 2.25 (t, 2H, CH_2); 2.29 (q, 2H, CH_2); 4.12 (m, 4H, 2CH_2CH$_3$); 4.19 (s, 2H, NH$_2$); 4.42 (t, 1H, CH); 7.48-8.26 (m, 8H, Ar-H); 7.61 (s, 1H, CH); 9.30 (s, 1H, NH).

2-{[5-Amino-1-(4-benzothiazol-2-yl-benzoyl)-1H-pyrazole-4-carbonyl]-amino}-3-(4-hydroxy-phenyl)-propionic acid ethyl ester (10d)

Yield = 1.3g (86%); m.p. = 187-9˚C. Analysis for $C_{29}H_{25}N_5O_5S$ (555.6): Calcd.: C, 62.7; H, 4.5; N, 12.6; S, 5.8; Fd.: C, 62.7; H, 4.5; N, 12.8; S, 5.7. IR (cm^{-1}): 3430 (NH), 3322 (NH$_2$), 3212 (OH), 1706 (CO), 1689 (CO), 1667 (CO). MS: m/z (%): 556 ($M^{+\cdot}$, 22); 555 (M^+, 32). ^{1}H-NMR: δ, ppm (DMSO-d_6); 1.30 (t, 3H, CH_3); 3.48 (d, 2H, CH_2); 4.15 (q, 2H, CH_2CH$_3$); 4.19 (s, 2H, NH$_2$); 4.81 (t, 1H, CH); 5.19 (s, 1H, OH); 6.68-8.23 (m, 12H, Ar-H); 7.61 (s, 1H, CH); 8.37 (s, 1H, NH).

Bioactivity materials and methods

Cytotoxicity against human breast cancer cell line MCF-7

Fetal bovine serum (FBS) and L-glutamine, were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), Tamoxifen, penicillin, streptomycin and Sulfo-Rhodamine-B stain (SRB) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA). Human Vascular Endothelial Growth Factor (VEGF) ELISA kit was purchase from Glory Science Co., Ltd (Del Rio, TX 78840, USA).

Anticancer activity screening for the tested compounds utilizing human breast MCF-7 cancer cell line obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 ^{o}C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm^2 flask in 5 ml of complete culture medium.

In vitro VEGF inhibition in human breast cancer cell line MCF-7

The antiproliferative activity was measured *in vitro* using the Sulfo-Rhodamine-B stain (SRB) assay according to the previous reported standard procedure [31]. Cells were inoculated in 96-well microtiter plate

(10^4 cells/ well) for 24 h before treatment with the tested compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the compounds under test (0-100 µg/ml) were added to the cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h. at 37°C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1.

After that, the cells in culture medium were treated with 20 µl of 1/10 of IC_{50} values of the compounds and the standard reference drug, tamoxifen, then incubated for 24 h at 37 ºC, in a humidified 5% CO_2 atmosphere. The cells were harvested and homogenates were prepared in saline using a tight pestle homogenizer until complete cell disruption, the lysate was used in studying the effect of tested compounds on the level of human vascular endothelial growth factor (VEGF) using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit to assay the level of human VEGF in samples. Which depend on the principle that, add VEGF to monoclonal antibody enzyme well which is pre-coated with human VEGF monoclonal antibody, incubation; then, add VEGF antibodies labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme. Then add Chromogen solution A, B, the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of color and the concentration of the human TRK of sample were positively correlated and the optical density was determined at 450 nm. The level of human VEGF in samples was calculated (pg/ml) as triplicate determinations from the standard curve.

Statistical analysis

The results are reported as Mean ± Standard error (S.E.) for at least four times experiments. Statistical differences were analyzed according to one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

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