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Anti-breast cancer and antiangiogenic potential of substituted thiazolo[2,3-b] quinazoline derivatives: synthesis, *in vitro* and *in vivo* analysis

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Abstract: Herein, a series of novel substituted thiazolo[2,3-b]quinazoline derivatives has been synthesized. The capability of the synthesized compounds **3a-i** to hinder the viability of human breast cancer cell line (MCF-7) was assessed. The compounds were evaluated as possible inhibitors of angiogenesis by using *in vivo* chorioallantoic membrane (CAM) model. Amongst the compounds **3a-i** screened, **3d** and **3f** exhibited excellent cytotoxicity with IC_{50} values 6.0±0.03 µM & 5.0±0.36 µM respectively. Compounds were further tested to evaluate potential to inhibit the pro-angiogenic cytokines associated with tumor development. Both the compounds were found to be potent antiangiogenic agents against VEGF, TNFα, IL6, TGFb, and EGF. The outcome of the present study reveals that, compound **3d** and **3f** showed the promising inhibitory activity on the viability of MCF-7 cells. In the *in vivo* CAM model, treatment with all the compounds resulted in the significant decrease in blood vessels density. The findings of the study suggest that, compounds **3d** & **3f** may act as potential anti-breast cancer and antiangiogenic agents.

Keywords: Breast cancer, angiogenesis, chorioallantoic membrane, angiogenic cytokines.

Introduction

Breast cancer is the most frequent cancer worldwide and a second foremost cause of cancer related death among females in the world [1, 2]. Unfortunately, in spite of enhanced diagnostic approaches and development of effective treatment, breast cancer continues to be the major reason of cancer associated deaths amongst the women worldwide. The trouble alters between countries and regions exhibiting divergence in incidence, mortality and survival rates [3].

Although there are several therapeutic approaches including chemotherapy to treat cancer, high systemic toxicity and drug resistance limit the successful results in the

majority cases [4]. The toxicity of presently existing anticancer drugs and the incompetence of chemotherapeutic treatments, have restricted the improvement of clinical drug combinations and efficient chemotherapeutic protocols [5]. Multidisciplinary scientific investigations are building best efforts to battle this disease and finding novel anticancer agents. As a result of that, up till now there is a clinical experience with several novel classes of targeted and non-targeted therapies [6]. The wide occurrence of the heterocycles in bioactive natural products made them important synthetic targets. Thiazole is a class of heterocyclic compounds of enormous importance in biological chemistry. They exist in numerous condensed fused systems that lead to possess a wide range of activities [7, 8]. Moreover, fused pyrimidines have drained the consideration of medicinal chemists as chemotherapeutic agents, where several members of this class have earned valuable places in chemotherapy as effective agents. Various literature reports displayed numerous fused pyrimidine ring systems and their chemotherapeutic activities as anticancer [9], antibacterial [10], antifungal [11], and antiviral agents [12]. Also, substituted thiazolo pyrimidine ring systems were reported to possess antitumor activities [13]. Literature survey has pointed out the inherited antitumor potency in compounds containing α, β-unsaturated ketone [14-17], fused pyridine [16-18], condensed pyrimidine and fused quinazoline moieties [19-26]. Quinazoline represent the pharmaceutically important heterocyclic scaffold in the field of medicinal chemistry research, owing to their diverse range of pharmacological activities including anticancer potential [27]. Numerous quinazoline derivatives are reported to be structural synthons of several physiological importance and pharmaceutical applications [28]. The derivatives are most commonly used in the drug design since they have been reported to exhibit various biological activities such as anti-inflammatory, anticancer, antifungal, antimicrobial etc. [29, 30]. The reported significance of such synthons generated the attraction to exploit this bioactive structure in the designing and the synthesis of new thiazolo quinazoline analogues as anti-breast cancer agent. Although there are few reports present on benzo thiazolo quinazoline derivatives which demonstrate anticancer activities [31-36], here in we report the synthesis of thiazolo[2,3-b] quinazoline derivatives by green method and their screening for further activities such as antiangiogenic potential by using different *in vitr*o and *in vivo* assays.

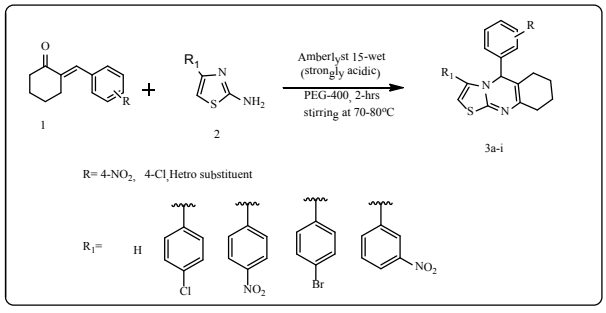
Results and Discussion

Chemistry

The α, β-unsaturated ketones were prepared by reacting cyclohexanone with various benzaldehydes analogues and hetero aldehydes in PEG-400 in the presence of catalytic amount of bleaching earth clay, pH (12.5). The target compounds tetrahydro-5H-thiazolo [2,3-b] quinazoline were prepared by reacting α, β-unsaturated ketones with substituted-2-amino thiazole in the presence of catalytic amount of Amberlyst 15–wet (strongly acidic). Applications of this catalyst allow mild and highly selective transformations and synthesis in a facile and environmentally friendly manner. The catalyst can be regenerated and recycled [37] in polyethylene glycol 400 (PEG-400), benzothiazolo-[2,3-*b*]-quinazolinone derivatives were also prepared earlier by using Amberlyst 15 and PEG-400 as catalytic and efficient systems [38, 39]. Here in this report we prepared tetrahydro-5H-thiazolo [2,3-b] quinazoline derivatives by using substituted 2 amino thiazole with α, β-unsaturated ketones as shown in **scheme 1** and were evaluated for further various biological screening.

There is a condensation reaction take place between α, β-unsaturated ketones and

substituted-2-amino thiazole followed by Michel addition take place to obtain the desired product. The structures of all the synthesized molecules were confirmed by spectral (IR, ^{1}H NMR, and Mass) data. The IR spectrum of compound (**3d**) showed the absence of characteristic peak of NH_2 and C=O group and presence of C=N peak at 1590 cm^{-1} and the peak at 2841 cm^{-1} and 3104 cm^{-1} observed for symmetric and asymmetric stretching of CH_2 group present and C-Cl stretching is at 854 cm^{-1}. In the ^{1}H NMR spectra observed the multiplet of 8H at δ1.75-181 the presence of CH_2 proton, δ 2.16 singlet of 1H,CH of quinazoline ring, δ 8.30 singlet of 1H, thiazole and all other protons are in the respective aromatic region. The peak appear at 158.3 is for fused carbon atom surrounded with nitrogen atom. CH_2 carbon atoms appear in range of 33.7-22.1 δ ppm. Rest of carbon atom they were appeared with their respective region. The mass spectrum of the compound showed M+ ion peak at m/z 423.92 for the molecular formula $C_{22}H_{18}N_2Cl_2SO_2$. The typical synthetic procedure and characterization data of compounds (**3a-i**) are presented in the experimental section. The physico chemical data of substituted tetrahydro-5H-thiazolo[2,3-b] quinazoline derivatives is as shown in **Table 1.**



Scheme 1: Synthetic protocol of tetrahydro-5H-thiazolo[2,3-b]quinazoline derivatives.

Table 1 Physico-chemical data of substituted tetrahydro-5H-thiazolo[2,3-b]quinazoline derivatives.

Entry	Yield(%)[a]	Temp (°C)	M. p.(°C)
3a	90	70	227-230
3b	85	70	232-235
3c	80	70	248-250
3d	80	70	255-257
3e	84	70	248-251
3f	80	70	253-255
3g	87	70	252-254
3h	80	70	273-275
3i	85	70	262-264

Biology

In-vitro cytotoxic activity by SRB assay

This is a colorimetric assay and SRB is bright pink anionic protein staining dye which binds to the basic amino acid of the cellular protein and estimate the cell number indirectly [40]. The results are summarized in **Table 2** and expressed as the IC_{50} values Adriamycin was used as a positive control. The results of the present study revealed that, most of the tested compounds exhibited cytotoxicity against the MCF-7 cells when compared with the reference drug Adriamycin. Most remarkably compounds **3d** and **3f** demonstrated promising cytotoxic activity against breast cancer cell line with IC_{50} – 6.0±0.03 μM & 5.0±0.36 μM respectively. While the compounds **3e, 3g, 3i** showed the significant activity (IC_{50} 10 μM). The other compounds 3a (30±0.02 μM), **3b** (20±0.18 μM), **3c** (40±0.80 μM), and **3h** (20±0.22 μM) showed the moderate activity. SAR study reveals that the presence of halogen substituent's at para position increases the activity as compared to other substituent present in the compound. In the present investigation, it was observed that the presence of **4-Cl and 4-Br** substituent on the phenyl nucleus of **3d** and **3f** enhances these types of activity.

Table 2 *In-vitro* cytotoxic activity of compounds **3a-i** against breast cancer cell line MCF-7. Adriamycin = Positive control. Results are expressed as the mean values from three independent experiments ± Standard deviation (SD).

Compound code	IC_{50} value (µM)
3a	30± 0.12
3b	20± 0.18
3c	40± 0.80
3d	**6.0± 0.13**
3e	10± 0.12
3f	**5.0± 0.36**
3g	10± 0.13
3h	20± 0.22
3i	10± 0.31
Adriamycin	0.5± 0.21

Bold values indicate the compounds with promising activity.

In-vivo chorioallantoic membrane (CAM) model

Angiogenesis refers to the formation of new blood vessels from the pre-existing ones. Moreover in tumor growth and tumor metastasis an uncontrolled angiogenic condition often provokes the disease [41]. The growth of tumors is dependent on their capability to induce angiogenesis as the blood vessels are required to supply the oxygen and nutrients to the growing tumor and it fails to grow beyond the 1-2 mm^3 size without the vascular support [42]. Stabilization of tumor vasculature by anti-angiogenic drugs is a promising model for improving the efficiency of cytotoxic chemotherapy. The chorioallantoic membrane (CAM) is an extraembryonic membrane, which serves as gas exchange surface and the process is regulated by the dense capillary network. Since its extensive vascularization and easy accessibility, it has been employed to study morphofunctional features of the angiogenesis progression *in vivo* and to examine the potency of pro- and anti-angiogenic molecules [43]. The profile of antiangiogenic activities of all synthesized compounds (**3a-i**) using the CAM model is summarized in **Table 3**. The IC_{50} concentrations of test compounds were used to evaluate the efficiency of test compounds as anti-angiogenic agents in CAM model. The inhibition of angiogenesis by using synthesized compounds clearly exhibited the potential of compounds as antiangiogenic agents in CAM model. Amongst the tested compounds, **3d** (92.69±0.52%) and **3f** (94.34±0.22%) displayed the most promising antiangiogenic activity. Since these both compounds showed the almost similar efficacy thereby making them better lead molecules intended for further optimization. However, the compounds **3b** (75.32±0.42 %), **3e** (89.90±0.55 %), **3g** (86.98±0.32 %), **3h** (77.23±0.23 %), **3i** (85.99±0.87 %) demonstrated the effective antiangiogenic activity. The remaining compounds **3a** (64.87±0.26 %) and **3c** (56.45±0.74 %) also exhibited the significant activity. The representative photographs of the CAMs indicating the reduced vascularization by treated compounds are shown in **Fig. 1.** The digitized control and CAMs treated with the IC_{50} concentrations of the test samples were further subjected to image analysis software AngioQuant v 1.33 (a MATLAB-based software tool for quantification of angiogenesis) for the analysis of number, length, size, and the junctions of the tubule complexes. The results acquired from analysis specify the progressive reduction in length, size, number and junctions of tubule complexes.

Fig. 1 Representative digitized illustrations of the CAMS exposed to IC_{50} concentrations of test compounds **3a-i**. The images of CAMS were digitized using Olympus make SZ61TR Zoom Trinocular Microscope with CCD attached camera and an image capturing

software Pinnacle v. 6.0.2 (build 152).

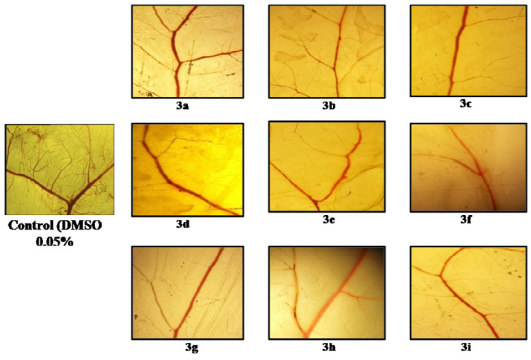


Table 3 Antiangiogenic activities (%) of compounds **3a-i** Results are expressed as the mean values from three independent experiments ± Standard deviation (SD).

Compound code	Anti-angiogenic activity (%)
3a	64.87±0.26
3b	75.32±0.42
3c	56.45±0.74
3d	92.69±0.52
3e	89.90±0.55
3f	94.34±0.22
3g	86.98±0.32
3h	77.23±0.23
3i	85.99±0.87

Bold values used to point out the active compounds.

Assay for inhibition of pro-angiogenic cytokines using human angiogenesis ELISA Strip I Pathological angiogenesis is associated with several diseases comprising cancer angiogenesis [44]. This process is regulated by balance amongst the endogenous angiogenesis activators and inhibitors. Presently there are around 30 activators of angiogenesis along with the equal number of inhibitors are reported [45]. The process of tumor blood vessel development initiation is regulated by vascular endothelial growth factor (VEGF), hypoxia inducible factor (HIF), basic fibroblast growth factor (bFGF), and angiopoietins. VEGF is the most essential angiogenic growth factor however other factors also play a major role in tumor angiogenesis [46]. Targeting angiogenesis has been an essential therapeutic approach for the treatment of cancer. The compounds **3d** and **3f** showing promising cytotoxicity against selected breast cancer cell line were further assessed to evaluate potential for inhibition of selected cytokines like TNFα, IGF1, VEGF, IL6, FGFb, TGFβ, EGF and Leptin using human angiogenesis ELISA Strip I. The results are summarized in **Table 4**. Compound **3d** is found to be potent antiangiogenic agent against VEGF (75.44±0.07%), IL6 (69.76±0.09 %), TGFβ (70.33±0.10 %), EGF (71.43±0.08 %) and the compound **3f** has shown potent TNFα (74.85±0.21 %), VEGF (72.94±0.09 %), TGFβ (73.76±0.12 %), leptin (68.65±0.10 %) inhibition. Whereas both the compounds have showed significant inhibition against remaining pro angiogenic cytokines when compared with reference compound suramin.

Table 4 Effect of compounds **3d** and **3f** on the tumor growth promoting cytokines (growth factors) at 100 µM concentration. Results are expressed as the mean values from three independent experiments ± Standard deviation (SD).

Compound code	Inhibition of tumor growth promoting cytokine (%)							
	TNFα	IGF1	VEGF	IL6	FGFb	TGFb	EGF	Leptin
3d	62.77 ±0.08	65.45 ±0.06	75.44 ±0.07	69.76 ±0.09	51.44 ±0.18	70.33 ±0.10	71.43 ±0.08	58.72 ±0.20
3f	74.85 ±0.21	52.63 ±0.19	72.94 ±0.09	65.78 ±0.04	60.66 ±0.21	73.76 ±0.12	67.74 ±0.05	68.65 ±0.10
Suramin	89.79 ±0.05	88.64 ±0.03	93.83 ±0.04	89.57 ±0.11	90.64 ±0.07	91.81 ±0.04	87.37 ±0.07	85.46 ±0.07

SAR

Compounds of the present investigation belong to substituted tetrahydro-5H-thiazolo[2,3-b] quinazoline derivatives. Furthermore, the cytotoxic activity against breast cancer cells and antiangiogenic activity were massively subject expected due to the substitution pattern present on the aryl ring of substitutes thiazole and α, β-unsaturated ketones and different substituent's at the 4-position of thiazole such as halogen group and substituent's at 4-position of α, β-unsaturated ketones. The presence of **(-NO_2)** group enhances cytotoxic activity as shown in compound **3d** and **3f** respectively. Also **3d** and **3f** shows potent antiangiogenic activities. SAR study reveals that the presence of **4-Cl, 4-Br** and **4-NO_2** substituent on the phenyl nucleus of **3d** and **3f** enhances these types of activity. On the other hand the compounds **3e**, **3g**, **3i** shows the significant cytotoxic activity and **3b, 3e, 3g, 3h** and **3i** shows effective antiangiogenic activity due to the presence of only halogen on any of phenyl nucleus. The other compound such as **3a**, **3b, 3c** shows the moderate cytotoxic activity where as compound such as **3a,** and **3c** shows significant antiangiogenic activity. From the results of SAR study we conclude that presence of halogen and electron withdrawing substituent's enhances the activity.

Experimental

Chemistry

All reagents were obtained from commercial suppliers and used without further purification. Reaction progress was monitored through thin layer chromatography (TLC) on pre-coated Merck alu-foil plate (silica gel 60F-254, 0.25 mm thickness) visualized by iodine vapors. Melting points were determined by open capillary method and are uncorrected. IR spectra were recorded (in KBr pallets) on Schimadzu spectrophotometer or Perkin Elimer Spectrum Version.^{1}H NMR spectra were recorded on Avance/ Bruker 400 MHz spectrophotometer using TMS as an internal standard. All NMR spectra were obtained in DMSO d_6/deuterated chloroform ($CDCl_3$); chemical shifts are reported in parts per million, and coupling constant in hertz (Hz). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet).The mass spectra were recorded on GC–MS Shimdzu (Q2010 PLUS) in the EI mode spectrometer and mass values are reported in m/z.

General method for synthesis of substituted 2-amino thiazole.

A mixture of thiourea (0.01 mol,) and substituted phenacyl bromide (0.005 mol,) was dissolved in 100 ml of EtOH in a round-bottom flask and refluxed for 1- 2 h. After completion of the reaction, as monitored using TLC, the mixture was cooled to room temperature (R.T) and then poured into cold water. The crude Product was recrystallized from absolute EtOH to afford the pure product.

General procedure for the synthesis of (substituted-tetrahydro-5H-thiazolo [2,3-b] quinazoline.

A mixture of substituted α-β unsaturated ketones (5 mmol), and substituted 2-amino thiazole / 2-amino thiazole (5 mmol) were stirred in PEG-400 (20 mL) at 70-80 °C for 2 hours, in the presence catalytic amount of Amberlyst 15–wet (strongly acidic) as a heterogeneous catalyst. After completion of the reaction (TLC), the catalyst is separated by means of simple filtrations. The reaction mixture was diluted with water and extracted with diethyl ether (2×20 mL). The combined organic layers were dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. The crude Product was recrystallized from acetic acid to afford the pure product (**3a-i**).

Product **3a-i** was characterized by means of IR, ^{1}HNMR, ^{13}CNMR, and Mass spectroscopy. The remaining derivatives were also prepared by the same procedure.

5-(4-chlorophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b quinazoline (3a);

Yellow crystals; mp 227-230^{o}C; IR (KBr, cm^{-1}) νmax; 3018 (Ar C-H), 2916 (CH_2), 1625 (C=N), 1520 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.10 (s, 1H,thiazole), 7.90-7.30 (m, 6H, Ar-H), 1.88-1.73. (m, 8H, CH_2), 2.11 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.3, 141.4, 138.0, 133.4, 132.6, 132.3, 128.6, 126.1, 97.9, 62.1, 32.2, 22.9, 22.6. ESI-MS: m/z: 302.82 [M]+ Chemical Formula: $C_{16}H_{15}N_2ClS$.

3,5-bis(4-chlorophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3b);

Yellow crystals; mp 232-235^{o}C; IR (KBr, cm^{-1}) νmax; 3021 (Ar C-H), 2906 (CH_2), 1633 (C=N), 1522 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 7.99 (s, 1H,thiazole), 7.80-7.30 (m, 8H, Ar-H), 1.81-1.71. (m, 8H, CH_2), 1.95 (s, 1H, CH quinazoline ring);^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.2, 144.9, 141.4, 138.0, 133.5, 133.4, 132.3, 128.6, 126.1, 120.2, 108.3, 59.2, 32.1, 22.8, 22.5; ESI-MS: m/z: 413.36 [M]+ Chemical Formula: $C_{22}H_{18}N_2Cl_2S$.

***5-(4-chlorophenyl)-3-(4-nitrophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3c)*;**

Yellow crystals; mp 248-250^{o}C; IR (KBr, cm^{-1}) νmax; 3011 (Ar C-H), 2936 (CH_2), 1645 (C=N), 1622 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.09 (s, 1H,thiazole), 7.90-7.30 (m, 8H, Ar-H), 1.80-1.74. (m, 8H, CH_2), 1.99 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.1, 147.1, 144.9, 141.4, 138.1, 136.8, 128.7, 126.3, 123.7, 107.7, 59.5, 31.9, 22.9, 22.4. ESI-MS: m/z: 423.92 [M]+ Chemical Formula: $C_{22}H_{18}N_2Cl_2SO_2$.

***3-(4-chlorophenyl)-5-(4-nitrophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3d)*;**

Yellow solid; mp 255-257^{o}C; IR (KBr, cm^{-1}) νmax; 3104 (Ar C-H), 2841 (CH_2), 1669 (C=N), 1690 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.32 (s, 1H,thiazole), 8.20-7.01 (m, 8H, Ar-H), 1.81-1.71. (m, 8H, CH_2), 2.16 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.4, 152.5, 151.6, 141.6, 131.2, 130.8, 130.1, 128.6, 127.7, 124.3, 122.0, 119.0, 105.9, 89.7, 44.6, 33.7, 22.5, 22.1; ESI-MS: m/z: 423.92 [M]+ Chemical Formula: $C_{22}H_{18}N_2Cl_2SO_2$.

3,5-bis(4-nitrophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3e);

Yellow crystals; mp 248-251^{o}C; IR (KBr, cm^{-1}) νmax; 3104 (Ar C-H), 2841 (CH_2), 1669 (C=N), 1622 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.12 (s, 1H,thiazole), 8.10-7.01 (m, 8H, Ar-H), 1.80-1.71. (m, 8H, CH_2), 2.10 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 159.1, 149.1, 147.1, 145.9, 144.9, 138.0, 136.9, 133.4, 128.3, 126.7, 123.3, 108.2, 59.5, 32.1, 22.8, 22.6. ESI-MS: m/z: 434.47 [M]+ Chemical Formula: $C_{22}H_{18}N_4SO_4$.

***3-(4-bromophenyl)-5-(4-nitrophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3f)*;**

Yellow solid; mp 253-255^{o}C; IR (KBr, cm^{-1}) νmax; 3081 (Ar C-H), 2894 (CH_2), 1665 (C=N), 1588 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.32 (s, 1H,thiazole), 8.20-7.01 (m, 8H, Ar-H), 1.81-1.75. (m, 8H, CH_2), 2.16 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.4, 149.4, 145.9, 138.1, 133.4, 131.5, 129.7, 128.6, 128.3, 122.3, 108.5, 89.7, 59.5, 32.1, 22.9, 22.4; ESI-MS: m/z: 468.37 [M]+ Chemical Formula: $C_{22}H_{18}N_3BrSO_3$.

5-(5-chloro-3-methyl-1-phenyl-1H-pyrazol-4-

***yl)-3-(4-chlorophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3g)*;**
Yellow crystals; mp 252-254°C; IR (KBr, cm^{-1}) νmax; 3123 (Ar C-H), 2941 (CH_2), 1659 (C=N), 1612 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.12 (s, 1H,thiazole), 8.10-7.01 (m, 9H, Ar-H), 2.11 (s, 3H, CH_3), 1.81-1.71. (m, 8H, CH_2), 2.16 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.3, 147.1, 144.9, 138.0, 137.2, 133.4, 133.3, 129.3, 129.0, 128.7, 126.2, 124.2, 120.2, 119.1,108.3, 50.8, 32.4, 22.9,22.6, 19.3. ESI-MS: m/z: 493.45 [M]+ Chemical Formula: $C_{26}H_{22}N_4Cl_2$.

***5-(5-chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl)-3-(3-nitrophenyl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3h)*;**
Yellow crystals; mp 273-275°C; IR (KBr, cm^{-1}) νmax; 3104 (Ar C-H), 2841 (CH_2), 1669 (C=N), 1622 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.32 (s, 1H,thiazole), 8.20-7.01 (m, 9H, Ar-H), 2.21 (s, 3H, CH_3), 1.81-1.71. (m, 8H, CH_2), 2.16 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.3, 147.8, 144.9, 138.0, 137.2, 133.4, 133.3, 129.5, 129.0, 124.7, 123.2, 120.5, 120.2, 119.1,108.5, 50.7, 32.2, 22.7, 22.2, 20.3. ESI-MS: m/z: 504.00 [M]+ Chemical Formula: $C_{26}H_{22}N_5ClSO_2$.

***3-(4-bromophenyl)-5-(5-chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6,7,8,9-tetrahydro-5H-thiazolo[2,3-b]quinazoline (3i)*;**
Yellow crystals; mp 262-264°C; IR (KBr, cm^{-1}) νmax; 3104 (Ar C-H), 2841 (CH_2), 1669 (C=N), 1622 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, 25 °C) δ ppm; 8.12 (s, 1H,thiazole), 8.18-7.01 (m, 9H, Ar-H), 2.19 (s, 3H, CH_3), 1.81-1.71. (m, 8H, CH_2), 2.17 (s, 1H, CH quinazoline ring); ^{13}CNMR (DMSO-d_6, 75 MHz, 25 °C) δ ppm; 158.4, 144.9, 138.0, 137.2, 133.4, 133.3, 129.3, 129.0, 128.7, 126.2, 124.8, 120.5, 119.1, 108.6, 50.9, 32.5, 22.9, 22.6, 19.7. ESI-MS: m/z: 537.90 [M]+ Chemical Formula: $C_{26}H_{22}N_4ClSBr$.

Biology

Human breast cancer cell line (MCF-7) was procured from National Centre for Cell Science (NCCS: a National Cell Line Facility) Pune (MS), India. Human Angiogenesis I ELISA Strip Kit was purchased from Signosis, Inc (Santa Clara, CA, USA). Other chemicals, solvents, reagents used were of AR grade and were obtained from commercial sources.

In-vitro cytotoxic activity by SRB assay

The synthesized compounds **3a-i** was evaluated for their *in vitro* cytotoxicity in selected human breast cancer cell line (MCF-7). The anticancer activity of the tested compounds was measured *in vitro* using the SRB assay according to [47]. Every test compound was assessed for four concentrations (0.1, 1, 10 and 100 µM) and each was prepared in triplicate wells. After the addition of test compound, plates were further incubated for 48 h and assay was terminated by the adding up cold TCA (trichloro acetic acid). Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm.

In-vivo chorioallantoic membrane (CAM) model

The CAM assay was performed in an identical

manner as depicted by [48]. The fertilized chicken eggs were cleaned for the disinfection. Eggs were kept in a humidified incubator at 37 ºC. Egg development and morphology was analyzed with the help of egg Candler. On the 9th day of incubation, the eggs were opened on the snub side and 1×1 cm window was cut into the eggshell. The concentrations of the test compounds **3a-i**prepared in dimethyl sulfoxide (DMSO, 0.05%, v/v) were applied (20 µl/disc) onto sterile glass discs (10 mm) separately and permitted to dry under laminar air flow conditions. The discs loaded with samples were inverted and applied over the CAM surface embryos. DMSO 0.05%, v/v was utilized as a control. The windows were sealed with tape and the eggs were again incubated in a humidified incubator at 37ºC. After 2 days of incubation, the CAMs were harvested and the vessels meeting the glass disc were counted under an Olympus make SZ 61TR Zoom Trinocular Microscope. The anti-angiogenic effect was expressed using an equation 1-T/C × 100, where T, indicates the no. of blood vessels intersecting the disc treated with samples, while C indicates the no. of blood vessels intersecting the disc in control. The results obtained were expressed in percent values.

Assay for inhibition of pro-angiogenic cytokines using human angiogenesis ELISA Strip I

The evaluation of the inhibition of cytokines promoting tumor growth such as TNFα, IGF1, VEGF, IL6, FGFb, TGFβ, EGF and Leptin was carried out as per the previously described method [49]. Human Angiogenesis I ELISA Strip Kit was used for the experiment. In brief, the MCF-7 cells were treated with test sample (**3d** and **3f**) up to 48 hours. After treatment, cell medium was removed and cells were rinsed once with ice-cold 1X PBS. Further the cells were thawed on ice and 100 µl of 1x cell lysis buffer was added and incubated for 10 minute with gentle shaking. The sample was centrifuged at 3,000 RPM for 5 minute and 90 µl of supernatant was transferred to wells of ELISA plate. Afterward, 100 µl of standard and sample (100 µM) was added in each well and incubated for 1 h at room temperature with gentle shaking. After that, the contents were aspirated from each well followed by washing the well by adding 200µl of 1X assay wash buffer. After the final wash, the residual liquid was evacuated by inverting the plate. 100 µl of diluted biotin-labeled antibody mixture was added to every well and incubated for 1 h at room temperature. The contents were again washed as depicted above. To every well a 100 µl of diluted streptavidin-HRP conjugate was added and incubated for 45 min at room temperature. Again the contents were washed. A 100 µl of substrate was added to each well and again incubated for 25 minutes followed by addition of 50 µl stop solution. The change in the color of the mixture from blue to yellow signifies the incidence of reaction. The optical density of each well was recorded by using Microplate reader at 450 nm within 30 minutes.

Conclusion

In conclusion the present investigation demonstrate the synthesis of new substituted thiazolo[2,3-b]quinazoline derivatives, characterization and evaluation of anti-breast cancer and antiangiogenic activities. Amongst the synthesized derivatives, the compounds **3d** and **3f** were the most promising cytotoxic agents against the breast cancer cells while the remaining derivatives demonstrated the significant cytotoxic activity. In case of antiangiogenic activity using the *in vivo* chorioallantoic membrane (CAM) model the results revealed that, the compounds **3d, 3e, 3f, 3g** and **3i** showed the potent activity. The most promising compounds 3d and 3f were further evaluated for inhibition of selected cytokines and found that **3d** was effective antiangiogenic

agent against VEGF, IL6, TGFβ, and EGF whereas **3f** showed the effective antiangiogenic activity against TNFα, VEGF, TGFβ, and leptin. Therefore, the potency of these two compounds could furnish as promising lead candidates for further study.

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