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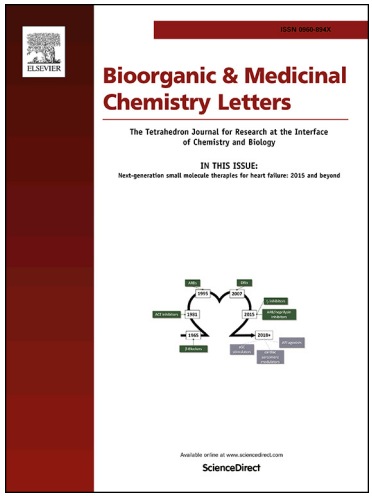

Synthesis, Characterization and Cytotoxicity Studies of 1, 2, 3-triazoles and 1, 2, 4-triazolo [1, 5-a] pyrimidines in Human Breast Cancer Cells

Maryam Gilandoust, Kachigere B. Harsha, Chakrabhavi Dhananjaya Mohan, Ainiah Rushdiana Raquib, Shobith Rangappa, Vijay Pandey, Peter E. Lobie, Basappa, Kanchugarakoppal S. Rangappa

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Synthesis, Characterization and Cytotoxicity Studies of 1, 2, 3-triazoles and 1, 2, 4-triazolo [1, 5-a] pyrimidines in Human Breast Cancer Cells

Maryam Gilandoust,[a,#] Kachigere B. Harsha,[a,#] Chakrabhavi Dhananjaya Mohan,[b,#] Ainiah Rushdiana Raquib,[c] Shobith Rangappa,[d] Vijay Pandey,[c] Peter E. Lobie,[c] Basappa,[e] Kanchugarakoppal S Rangappa[a,*]

[a]*Department of studies in Chemistry, University of Mysore, Manasagangotri, Mysuru-570006, India.*

[b]*Department of studies in Molecular Biology, University of Mysore, Manasagangotri, Mysuru-570006, India.*

[c]*Cancer Science Institute of Singapore, National University of Singapore, Singapore-117599.*

[d]*Adichunchangiri Institute for Molecular Medicine (AIMM), BG Nagara, Nagamangala Taluk, Mandya district-571448, India.*

[e]*Department of studies in Organic Chemistry, University of Mysore, Manasagangotri, Mysuru-570006, India.*

[#]*These authors contributed equally to this work.*

**Corresponding author*

Abbreviation:
EFT: 1-(2'-ethoxy-4'-fluoro-[1,1'-biphenyl]-4-yl)-4-phenyl-1H-1,2,3-triazole

ABSTRACT

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) is essential for physiological functions of tissues and neovasculature. VEGFR signaling is associated with the progression of pathological angiogenesis in various types of malignancies, making it an attractive therapeutic target in cancer treatment. In the present work, we report the synthesis of 1,4-disubstituted 1,2,3-triazoles and 1,2,4-triazolo[1, 5-a]pyrimidine derivatives *via* copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction and screened for their anticancer activity against MCF7 cells. We identified 1-(2'-ethoxy-4'-fluoro-[1,1'-biphenyl]-4-yl)-4-phenyl-1H-1,2,3-triazole (EFT) as lead cytotoxic agent against MCF7 cell lines with an IC_{50} value of 1.69 µM. Further evaluation revealed that EFT induces cytotoxicity on Ishikawa, MDA-MB-231 and BT474 cells with IC_{50} values of 1.97, 4.81 and 4.08 µM

respectively. However, EFT did not induce cytotoxicity in normal lung epithelial (BEAS-2B) cells. Previous reports suggested that 1,2,3-triazoles are the inhibitors of VEGFR1 and therefore, we evaluated the effect of EFT on the expression of VEGFR1. The results demonstrated that EFT downregulates the expression of VEGFR1 in MCF7 cells. In summary, we identified a potent cytotoxic agent that imparts its antiproliferative activity by targeting VEGFR1 in breast cancer cells. The novel compound could serve as a lead structure in developing VEGFR1 inhibitors.

Keywords: 1,2,3-triazole; 1,2,4-triazole; Angiogenesis; VEGFR1; Anticancer agent.

Angiogenesis is the process of formation of a new blood vessel from the pre-existing vasculature and vascular endothelial cells. Angiogenesis is essential for nutrient supply, disposal of metabolic wastes, growth, and development [1]. Angiogenesis is a key player in several pathological conditions including cancer, wound healing, inflammation, rheumatoid arthritis, ischemic cardiovascular diseases and age-related macular degeneration [2]. However, aberrant angiogenesis is one of the fundamental processes responsible for the transformation of the dormant tumor to malignancy [3]. Initially, Judah Folkman postulated that angiogenesis is essential for tumor proliferation and tumor releases proangiogenic factors called tumor angiogenesis factors (TAF) which are essential for neovasculature [4]. Further studies on angiogenesis revealed that vascular endothelial growth factor (VEGF) is the pivotal proangiogenic mitogen that stimulates the growth of new blood vessels by interacting with vascular endothelial growth factor receptors (VEGFR) [5]. VEGFR belongs to receptor tyrosine kinase superfamily with three major subtypes namely, VEGFR1, VEGFR2, and VEGFR3. The binding of VEGF to VEGFR results in the receptor dimerization and activation of an intracellular signaling cascade that leads to the expression of genes responsible for cell survival, proliferation, migration, angiogenesis, and vascular permeability

[6]. Even though VEGFR2 has relatively major role than VEGFR1 in VEGF-mediated signaling, it is noteworthy that VEGFR1 mediates the survival of endothelial cells and contributes to the progression of cancer [7, 8]. VEGF has been reported to act as an intracrine survival factor in breast cancer cells through its binding to VEGFR1 and the same study also revealed that knockdown of VEGFR2 had no effect on the survival of tested cancer cells [9]. In another study, Dales et al. investigated the prognostic value of VEGFRs and reported that expression of VEGFR1 is positively correlated with the high risk of metastasis and relapse [10]. They concluded that VEGFR1 may further be considered as a potential tool to evaluate the aggressiveness of breast cancer [10]. Taken together, these reports support the intimate relationship between VEGFR1 and cancer progression and encourage the development of VEGFR1 inhibitors as anticancer therapeutics.

Triazole-based heterocycles were identified as biologically important pharmacophores and were reported to possess good anticancer potential targeting in multiple types of malignancies [11]. Several studies have suggested that triazoles target VEGFR to induce their anticancer activity. Specifically, (1,2,3-triazol-4-yl)benzenamines were identified as potent inhibitors of VEGFR1 and VEGFR2, and the activity was comparable to Vatalanib in both homogenous time-resolved fluorescence (HTRF) enzymatic and cellular assays [12]. In another study, 1,2,4-triazol-3-yl-anilines were described as potent ATP-competitive inhibitors of VEGFR1 and 2 [13]. In continuation of our efforts in exploring the anticancer potential of various heterocycles [14-20], we prepared the series of novel 1,2,3- and 1,2,4-triazoles and investigated the effect of newly synthesized compounds on breast cancer (MCF7) cells. The lead compounds 1-(2'-ethoxy-4'-fluoro-[1,1'-biphenyl]-4-yl)-4-phenyl-1H-1,2,3-triazole (EFT, 3a) and 1-(4-(benzo [b]thiophen-2-yl)phenyl)-4-phenyl-1H-1,2,3-triazole (3j) were chosen to investigate the possible VEGFR inhibitory activity in breast cancer cells.

Preparation of 1,4 disubstituted 1,2,3 triazoles 3(a-j): The synthesis of the title compounds was carried out as illustrated in Scheme 1 (Figure 1A). In the present work, 1-(4-bromophenyl)-4-phenyl-1,2,3-triazole (**1**) was synthesized *via* copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) between 4-bromophenylazide and phenylacetylene in the presence of sodium ascorbate and $CuSO_4.5H_2O$ in tert-butanol and water mixture as the solvent system. In the next step, 1-(4-bromophenyl)-4-phenyl-1,2,3-triazole was used for Suzuki-Miyaura cross-coupling reactions. We treated 1-(4-bromophenyl)-4-phenyl-1,2,3-triazole with various aryl /heteroaryl boronic acids (**2**) in presence of K_2CO_3 and Pd(II) catalyst under a nitrogen atmosphere to yield a novel series of 1,4 disubstituted 1,2,3 triazoles **3(a-j)** (Scheme 1) with a yield of 70-92% as summarised in **table 1**. The structure of all the synthesized compounds **3(a-j)** were confirmed by nuclear magnetic resonance (NMR) and high-resolution mass spectral (HRMS) analysis. All the final compounds **3(a-j)** showed a characteristic singlet around 8.2 δ corresponding to a proton on the triazole ring. Spectral analysis data is provided as supplementary information.

Figure 1

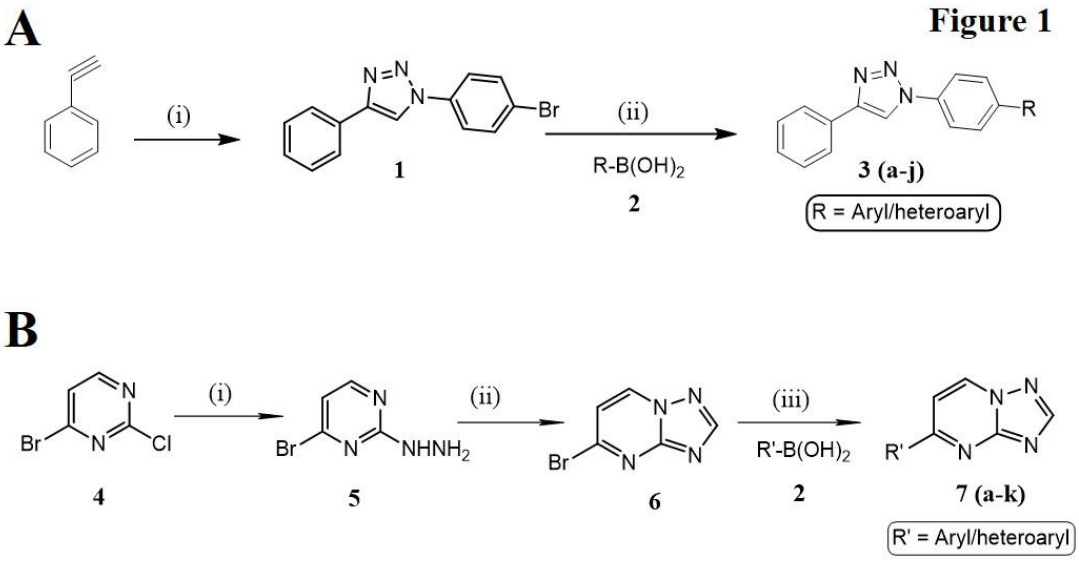
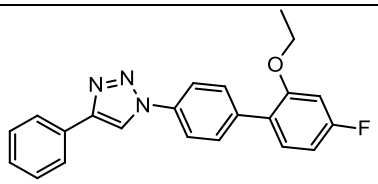
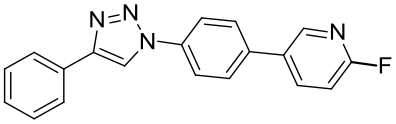
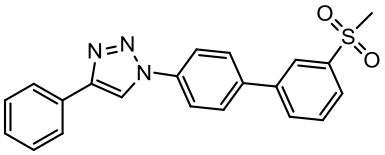
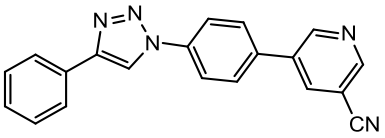
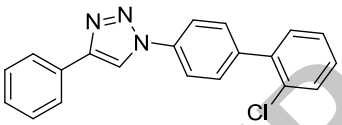
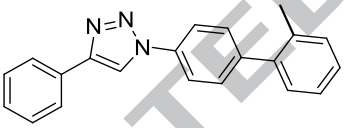
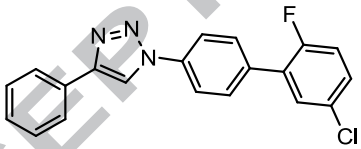
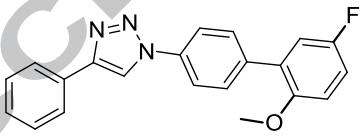
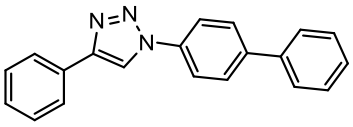
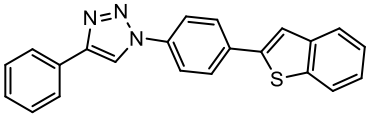


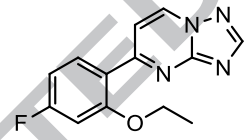
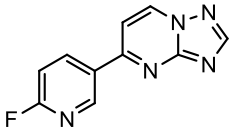
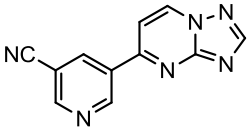
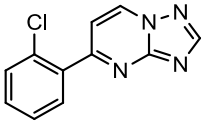
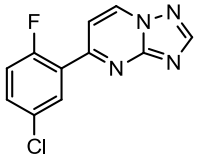
Table 1: Structure of 1,4-disubstituted 1,2,3-triazole derivatives **3**(**a-j**) and cytotoxic activity against breast cancer cells.

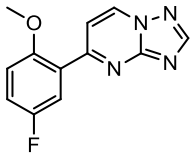
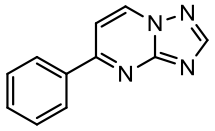
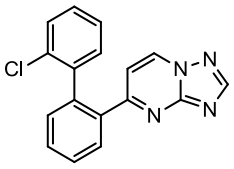
Entry	Compound	Yield (%)	IC_{50} (µM))			
			MCF7	BT474	MDA-MB-231	Ishikawa
3a		92	1.69	4.08	4.81	1.97
3b		82	NT	NT	NT	NT
3c		70	<50	NT	NT	NT
3d		73	<50	NT	NT	NT
3e		90	<50	NT	NT	NT
3f		92	<50	NT	NT	NT
3g		70	<50	NT	NT	NT
3h		90	<50	NT	NT	NT
3i		80	<50	NT	NT	NT
3j		85	14.4	<50	NT	NT

NT: Not Tested

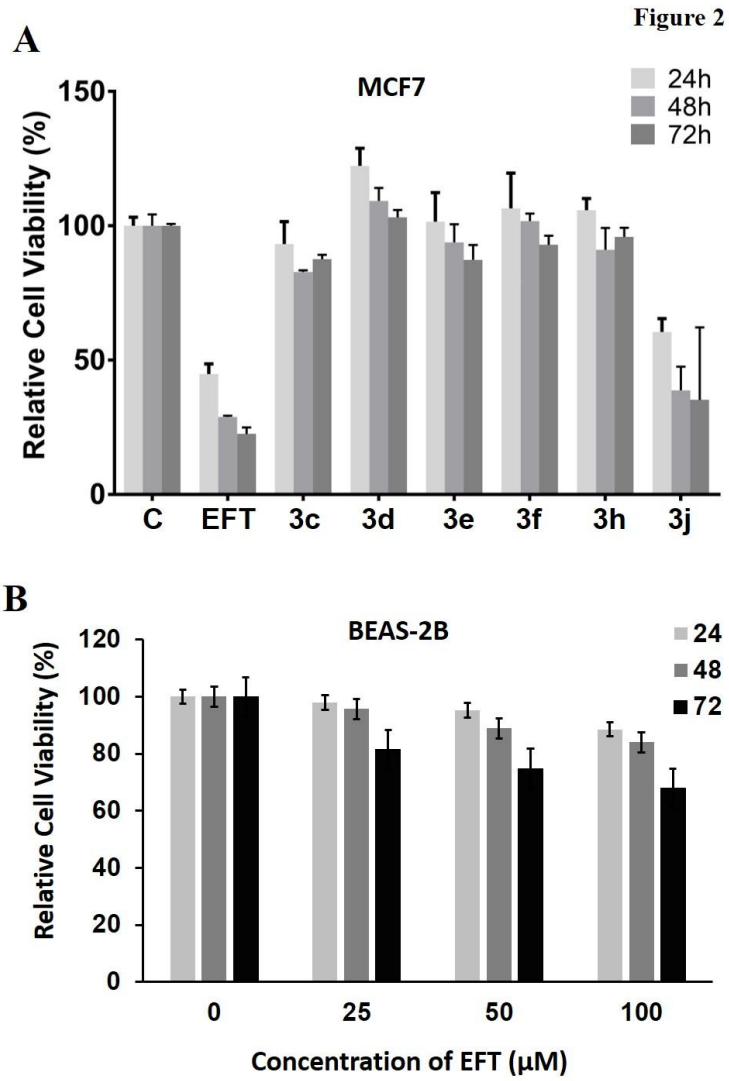
Preparation of [1, 2, 4] triazolo [1, 5-a] pyrimidine derivatives 7(a-k) [21]: The synthesis of the title compounds was carried out as illustrated in Scheme 2 (Figure 1B). Initially, starting from the transition metal free coupling reaction between 2-chloro-5-bromopyrimidine (**4**) with hydrazine hydrate to produce the corresponding hydrazine (**5**) which was condensed with formaldehyde to afford hydrazones. Further, hydrazone intermediate was *in situ* cyclized in the presence of $IPh(OAc)_2$ to yield 5-bromo-[1,2,4]-triazolo[1,5-a]pyrimidine (**6**). Similarly, scaffold (**6**) was crossed-coupled with various aryl/heteroaryl boronic acids under inert (nitrogen) atmosphere in the presence of K_2CO_3 and palladium(II) catalyst to obtain novel series of [1,2,4]triazolo[1,5-a]pyrimidines **7**(**a-k**) in 65-90% yield as shown in **table 2.**

Table 2: Structure of [1,2,4]-triazolo [1,5-a] pyrimidine derivatives **7**(**a-k**) and cytotoxic activity against breast cancer cells.

Entry	Compound	Yield (%)	IC_{50} (µM)	
			MCF7	MDA-MB-231
7a		90	93.8	50.3
7b		73	>100	>100
7d		70	NT	>100
7e		85	>100	>100
7g		80	NT	>100

7h		90	>100	>100
7i		88	3.91	>100
7k		65	>100	>100
NT: Not Tested				

1,2,3-triazoles elicit growth inhibitory effect on breast cancer cells: Initially, we investigated the growth inhibitory effect of 1,2,3-triazoles against MCF7 cells and 1,2,4-triazolo-pyrimidines against MCF7 and MDA-MB-231 cells using the AlamarBlue assay [22, 23]. The detailed methodology is provided in the supplementary information. Among the tested compounds, 1-(2'-ethoxy-4'-fluoro-[1,1'-biphenyl]-4-yl)-4-phenyl-1H-1,2,3-triazole (EFT, 3a) and 1-(4-(benzo[b]thiophen-2-yl)phenyl)-4-phenyl-1H-1,2,3-triazole (3j) were identified as lead anticancer compounds with an IC_{50} values of 1.69 and 14.4 µM for MCF7 cells respectively. Further, we evaluated the effect of EFT on Ishikawa, MDA-MB-231 and BT474 cells and IC_{50} values were found to be 1.97, 4.81 and 4.08 µM respectively. In addition, we also evaluated the effect of new compounds on the viability of MCF7 cells at different time points (24, 48 and 72 h) at 25 µM. The results revealed that EFT significantly decreases the cell viability of MCF7 in a time-dependent manner (Figure 2A). However, 1,2,4-triazolo-pyrimidines did not induce significant growth inhibition in the tested cells at 25 µM for 72 h. Further, we tested the effect of EFT on normal lung epithelial (BEAS-2B) cells at 100 µM for 72 h using MTT assay as described earlier [24, 25]. The lead compound, EFT, did not induce significant cytotoxicity against the tested non-diseased cells (Figure 2B).



EFT reduces the expression of VEGFR1 in breast cancer cells: Kiselyov and co-workers described triazole derivatives as potent and ATP competitive inhibitors of VEGFR1 [12]. Therefore, we evaluated the effect of EFT and 3j on the expression of VEGFR1 in MCF7 cells by immunoblotting as described previously [26, 27]. The treatment with EFT significantly downregulated the expression of VEGFR1 in a dose-dependent manner (Figure 3A and B). We also observed a marginal reduction in the expression of VEGFR1 on treatment with compound 3j. VEGFR signaling activates a cascade of downstream pathways including P38-MAPK, JNK, ERK, Akt/PKB and NF-κB [28]. Among them, Akt signaling leads to an increased production of the hypoxia-inducible factor α (HIF1α and HIF2α) transcription factors [29] and VEGFR1 (FLT-1) was reported as one of the target genes of

HIF-1 [30]. Moreover, in another similar study, Li et al. reported a new small molecule that inhibited VEGFR2 tyrosine kinase activity and also reduced the expression of VEGFR2 [31]. Therefore, we speculate that EFT downregulates the expression of VEGFR1 *via* modulating the activity of Akt and HIF-1 and further investigation is essential to decipher the possible mechanism. No significant change was observed in the levels of β-Actin.

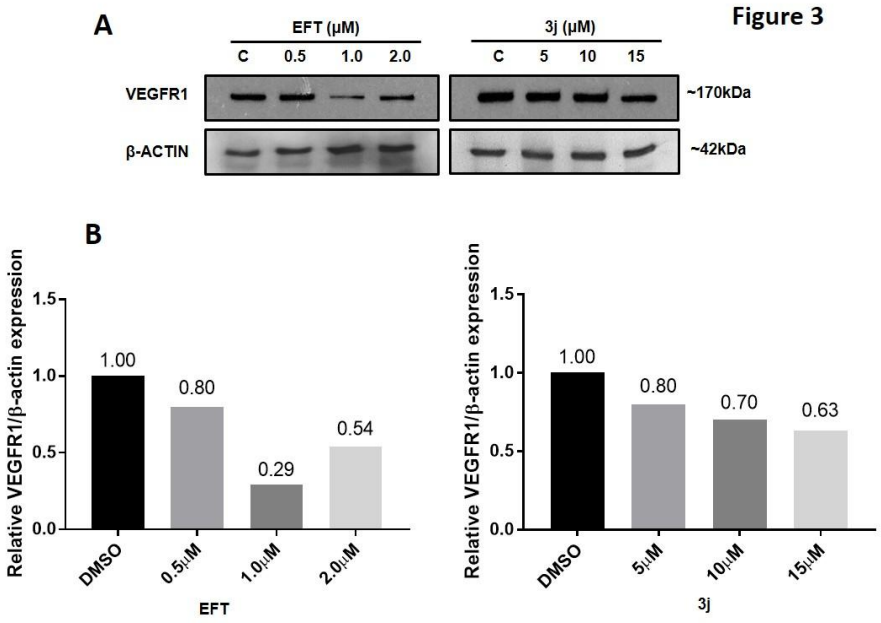


Figure 3

In conclusion, VEGFR family proteins have been emerged as potential drug targets to treat various malignancies and several attempts have been made in implementing them as therapeutic targets. Many antibodies and small-molecule based drugs targeting VEGFR family proteins are approved for the treatment of several cancers. Sunitinib, sorafenib, and pazopanib are the multikinase inhibitors that inhibit VEGFR1/2/3, PDGFR, c-Kit and approved for the treatment of renal cell cancer and for gastrointestinal stromal tumors; renal cell cancer and hepatocellular carcinoma; and advanced renal cell cancer respectively [32]. Several VEGFR inhibitors are in the different phases of clinical trials. These reports emphasize the role of VEGFR family proteins as potential drug targets in the treatment of cancers. 1,2,3- and 1,2,4-triazoles were identified as pharmacologically important scaffolds because of their medicinal properties. Several research groups have developed various

methodologies for the preparation of triazoles and their conjugation with a broad range of heterocyclic derivatives. Multiple reports have been demonstrated the action of triazole derivatives on various cellular targets. In this report, we prepared series of 1,2,3- and 1,2,4-triazole derivatives and identified EFT as a lead antiproliferative agent against breast cancer cells. Further evaluation demonstrated that EFT decreases the expression of VEGFR in breast cancer cells and these results are in agreement with the available literature. We hypothesize that the reduction in VEGFR1 levels is due to the modulation of Akt and HIF-1 signaling that needs to be verified in future studies. This is a report presenting triazoles as VEGFR1 inhibitors and the further comprehensive investigation is essential to dissect the detailed molecular mechanism of lead compound and its off-targets.

Acknowledgments

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Conflict of interest

None declared

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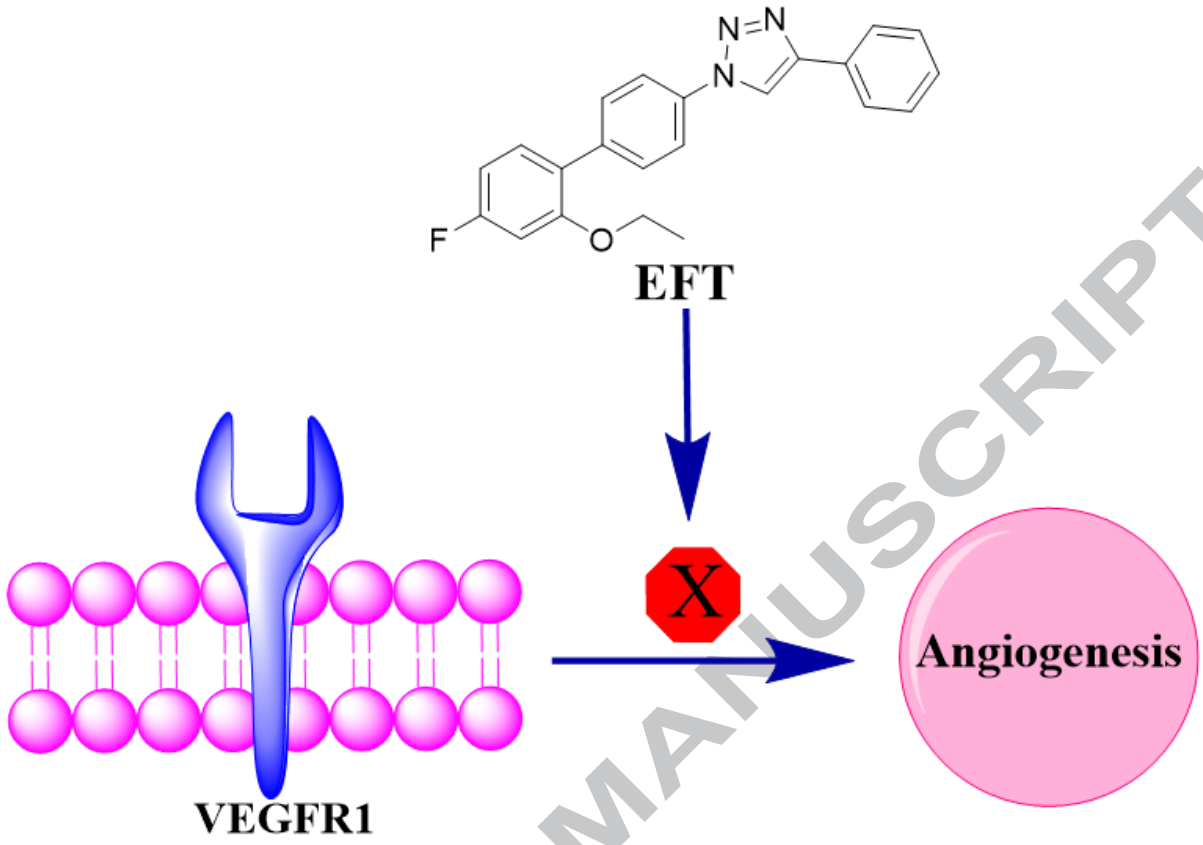
Figure legends

Figure 1: A. Scheme 1. Synthesis of 1,4 disubstituted 1,2,3 triazole derivatives. Reagents and condition: i) 4-Bromophenylazide, (+)-Sodium L-ascorbate, $CuSO_4$. $5H_2O$, H_2O:t-BuOH, RT, 18 h; ii) R′-B(OH)2, Dikis, EtOH: H_2O: Dioxan (1:1:5), 120° C, 40 min. B. Scheme 2. Synthesis of [1, 2, 4]triazolo[1,5-a]pyrimidine derivatives. Reagents and condition: i) $NH_2NH_2 \cdot H_2O$, EtOH, Δ, 1.5 h; ii) CH_2O, EtOH, RT, 2 h; and $IPh(OAc)_2$, CH_2Cl2, RT, 15 h; iii) R′-B(OH)2, Dikis, EtOH: H_2O:Dioxan (1:1:5), 120° C, 40 min.

Figure 2: 5000 MCF7 cells were seeded per well (96-well plate) and 25μM of respective compounds were added after 24h. Cell viability assay was performed using AlamarBlue reagent with 4h incubation after 24, 48, and 72h. B. 5000 BEAS-2B cells were seeded per

well (96-well plate) and MTT assay was performed to determine the cell viability of BEAS-2B cells upon treatment with 25, 50 and 100µM of EFT for 24, 48 and 72h. EFT did not induce a significant cytotoxic effect on BEAS-2B cells. Readings were taken in triplicates and normalized against DMSO control.

Figure 3: A. 300,000 MCF7 (in RPMI+10%FBS) cells were seeded per 6cm dish. Cells were allowed to grow for 24h before being subjected to compound treatment (in RPMI+2%FBS). Cells were collected after 48h and harvested for Western Blot. 40µg of protein was loaded per lane. Primary antibodies used were against VEGFR1 (ab32152) and β-actin (sc-47778). B. Quantification and graphical representation of VEGFR1 expression after the treatment with EFT and 3j in breast cancer cells. A gradual decrease in the expression of VEGFR1 was observed in a dose-dependent manner.



1,2,3-Triazoles as inhibitors of VEGFR1

Highlights

- VEGFR signalling is associated with the progression of various malignancies.
- 1,2,3-triazoles and 1,2,4-triazolo-pyrimidines were synthesized *via* CuAAC reaction.
- 1,2,3-triazole derivative (EFT) was identified the lead cytotoxic agent.
- EFT reduced the expression of VEGFR1 in MCF7 cells.