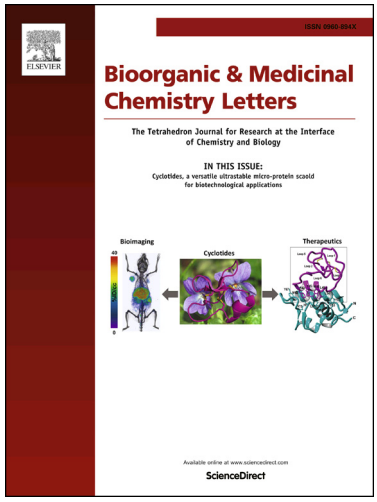

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PII: S0960-894X(18)30279-8
DOI: https://doi.org/10.1016/j.bmcl.2018.03.074
Reference: BMCL 25728

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 13 December 2017
Revised Date: 23 March 2018
Accepted Date: 27 March 2018

Please cite this article as: Tokala, R., Bale, S., Pavan Janrao, I., Vennela, A., Kumar, N.P., Ram Senwar, K., Godugu, C., Shankaraiah, N., Synthesis of 1,2,4-triazole-linked urea/thiourea conjugates as cytotoxic and apoptosis inducing agents, *Bioorganic & Medicinal Chemistry Letters* (2018), doi: https://doi.org/10.1016/j.bmcl.2018.03.074

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Synthesis of 1,2,4-triazole-linked urea/thiourea conjugates as cytotoxic and apoptosis inducing agents

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
1,2,4-Triazoles
Urea
Thiourea
Anticancer
Apoptosis
Flow-cytometry

ABSTRACT

A new series of 1,2,4-triazole-linked urea and thiourea conjugates have been synthesized and evaluated for their *in vitro* cytotoxicity against selected human cancer cell lines namely, breast (MCF-7, MDA-MB-231), lung (A549) prostate (DU145) and one mouse melanoma (B16-F10) cell line and compared with reference drug. The compound **5t** showed significant cytotoxicity on MCF-7 breast cancer cell line with a IC_{50} value of 7.22 ± 0.47 µM among all the tested compounds. Notably, induction of apoptosis by compound **5t** on MCF-7 cells was evaluated using different staining techniques such as acridine orange/ethidium bromide (AO/EB), annexin V-FITC/PI, and DAPI. Further, clonogenic assay indicates the inhibition of colony formation on MCF-7 cells by compound **5t**. Moreover, the flow-cytometric analysis also revealed that compound **5t** caused the arrest of cells at G0/G1 phase of cell cycle. In addition, the compounds when tested on normal human cells (L-132) were found to be safer with low cytotoxicity profile.

Cancer is a major global health problem and one of the leading causes of death when compared to other diseases. The progression of cancer in a person is due to changes in DNA (mutations)[1] or a compromised immune system. Besides, the preventive measures (vaccination) and avoiding behavioural risk factors (smoking etc.,) the occurrence of the disease is still accounted statistically.[2] Evading apoptosis stands as the major hallmark of cancer cellular pathways. The tolerance, along with resistance and subsequent effect of existing anticancer agents, demands the search for new chemical entities (NCEs) as chemotherapeutic agents.

The chemistry involving triazoles has a significant role due to their medicinal and industrial properties as drugs and intermediates respectively in various areas.[3,4] The aspects of stability to metabolic degradation, association with drug targets,[5] the formation of hydrogen-bonds makes triazole as pharmacophore in many biologically active molecules.[6,7] These are well-known compounds for diverse biological activities like anticancer,[8,9] antimicrobial,[10] antifungal,[11] antitubercular,[12] anthelmintic, analgesic,[13] anti-inflammatory,[13,14] and anticonvulsant.[15,16] Also, 1,2,4-triazoles are evident for possessing central nervous system (CNS) acting drug candidates (stimulants, anxiolytics)[8] and effective on enzymes like cholinesterase inhibition,[17] etc.

Examples of drugs with different medicinal importance containing 1,2,4-triazole include[8] (a) In breast cancer treatment-letrozole (**Figure 1**) and anastrazole, (b) CNS acting agents like etizolam, alprazolam, triazolam, (c) Antimicrobial agents like ribavirin, (d) Antimycotics like propiconazole, fluconazole, triadimefon, hexaconazole, myclobutanil, (e) In the treatment of migraine headaches-rizatriptan. Further, 1,2,4-triazoles were reported to have industrial applications as polymers, lubricants, dyes and analytical reagents for heavy metal quantification.[18] Moreover, the antiproliferative activity of the triazoles is expressed in both individual, fused systems and highly effective compounds with better selectivity can be obtained by the chemical modulation of the triazole ring.

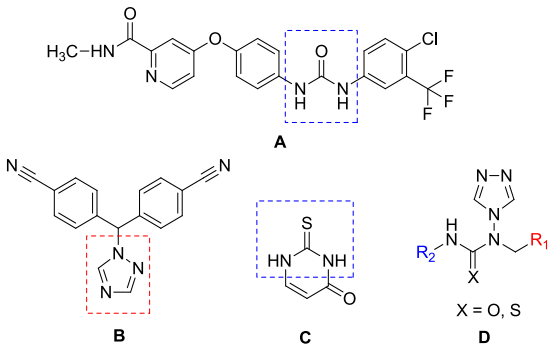

Figure 1. The structures of sorafenib (**A**), letrozole (**B**), thiouracil (**C**), and designed new derivatives (**D**).

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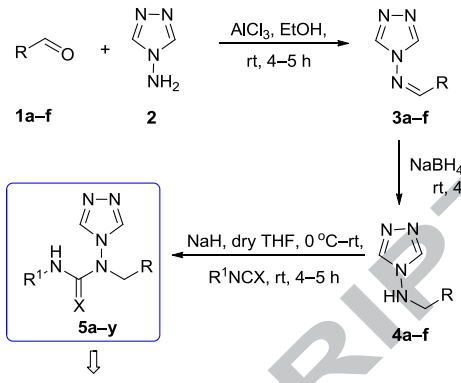
Small molecule kinase inhibitors including urea derivatives (thioureas e.g., thiouracil (**Figure 1**) benzoyl ureas)[19] are drawing substantial interest due to their inhibitory activity against various kinases.[20] Inhibition of protein and receptor tyrosine kinases results in inhibition of tumor generation and proliferation.[21] Sorafenib (**Figure 1**) represents urea containing kinase inhibitors with profound anticancer activity in specific to primary kidney and in advanced liver cancer.[22]

A broad spectrum of anticancer activity was observed in all the cancer cell lines tested when thiourea congeners are synthesized in conjunction with podophyllotoxin.[23] In continuation our earlier reports on 1,2,3-triazole[24,25,26,27] as significant anticancer agents, we pondered to explore antitumor activity of these newly synthesized 1,2,4-triazole and urea/thiourea congeners with various substitutions and explored for their *in vitro* cytotoxicity profile against a panel of selected human cancer cell lines.

The synthesis of designed triazolo-urea/thiourea conjugates was outlined in **Scheme 1**. Schiff bases **3a**–**f** were synthesized in good yields by using commercially available aldehydes **1a**–**f** and 4-amino-4*H*-1,2,4-triazole in presence of aluminium trichloride in ethanol. Thus obtained Schiff bases were reduced to their corresponding amines **4a**–**f** employing sodium borohydride in ethanol. Further, the reduced intermediates were condensed with various isocyanates and isothiocyanates in tetrahydrofuran and sodium hydride as the base to furnish triazolo-urea/thiourea **5a**–**y** congeners in quantitative yields respectively. The reactions were monitored by thin layer chromatography (TLC) and all the products were purified by column chromatography. The newly synthesized compounds were characterized by HRMS, ^{1}H, and ^{13}C NMR spectroscopy. The HRMS (ESI) of all the compounds showed a $[M + H]^+$ peak equivalent to their molecular formulae. The presence of broad singlet at a range of 6.5 to 7.0 ppm accounts for NH of final compound confirms unambiguously. The triazole protons which are deshielded appear as a singlet at a range of 9.3 to 8.5 ppm. The 2 protons of methylene appeared at 4.8 ppm. Characteristic methoxy protons appeared as singlet at a range of 3.7 ppm which accounts for 6 protons indicating the presence of 2 methoxy groups and other aromatic protons lie in range of 6.7 to 8.9 ppm. In ^{13}C NMR spectrum of **5a**, the amide carbonyl carbon appeared at δ 154.1 ppm and the aromatic methoxy-substituted carbons resonated around δ 149.1 ppm. The equivalent carbons of the triazole appear at δ 144.2 ppm. The sp hybridized carbon of the nitrile group resonates at 119.1 ppm. The methoxy carbons appeared at 55.8 ppm whereas the signal attributed to the methylene carbon appeared at 54.8 ppm. The aromatic carbons resonate around δ 111.7-144.2 ppm. A similar pattern was observed in the ^{1}H NMR and ^{13}C NMR of all the other derivatives **5b**–**y**. The HRMS (ESI) of these compounds showed a $[M + H]^+$ peak equivalent to their respective molecular formula.

The newly synthesized triazolo-urea and thiourea conjugates **5a**–**y** were subjected to *in vitro* cytotoxicity studies on MCF-7 and MDA-MB-231 (breast), A549 (non small cell-lung), DU 145 (Prostate), B16-F10 (mouse melanoma), cancer cell lines by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. The IC_{50} (μM) values (concentration required to inhibit 50% of cancer cells growth) of tested compounds **5a**–**y** and reference standard (5-fluorouracil) has been listed in **Table 1**. Results from the **Table 1** indicated that some of the synthesized compounds exhibited potential cytotoxicity against B16-F10 and MCF-7 cancer cell lines and were found to be active in the range of 4.51± 0.05 to 7.22 ± 0.47 μM. From the close examination of IC_{50} values, it is observed that **5g**, **5k**, **5l**, **5p**, **5r**, **5s**, **5t**, and **5y** were active at less than 50 μM on all the tested cancer cell lines.



5a: R = 3,4-dimethoxy phenyl, R^1 = 4-cyanophenyl, X = O
5b: R = 3,4,5-trimethoxy phenyl, R^1 = 4-cyanophenyl, X = O
5c: R = 3,4-dimethoxy phenyl, R^1 = 4-chlorophenyl, X = O
5d: R = 3,4-dimethoxy phenyl, R^1 = 4-chloro-3-(trifluoromethyl)phenyl, X = O
5e: R = 3-methylthiophen-2-yl, R^1 = 3-cyanophenyl, X = O
5f: R = 3-methylthiophen-2-yl, R^1 = 3-chlorophenyl, X = O
5g: R = 3-methylthiophen-2-yl, R^1 = 4-chloro-3-(trifluoromethyl)phenyl, X = O
5h: R = pyridin-2-yl, R^1 = phenyl, X = O
5i: R = pyridin-2-yl, R^1 = benzyl, X = O
5j: R = pyridin-2-yl, R^1 = cyclohexyl, X = O
5k: R = pyridin-2-yl R^1 = 4-methylcyclohexyl, X = O
5l: R = pyridin-2-yl, R^1 = 4-methylphenyl, X = O
5m: R = 3-methylthiophen-2-yl, R^1 = phenyl, X = O
5n: R = 4-cyanophenyl, R^1 = phenyl, X = O
5o: R = 3,4-dimethoxy phenyl, R^1 = 4-ethyl phenyl, X = S
5p: R = 3,4,5-trimethoxy phenyl, R^1 = 4-ethyl phenyl, X = S
5q: R = 3,4-dimethoxy phenyl, R^1 = 4-methyl phenyl, X = S
5r: R = 3,4-dimethoxy phenyl, R^1 = (3,5-Bis(trifluoromethyl)phenyl, X = S
5s: R = 3,4,5-trimethoxy phenyl, R^1 = (3,5-Bis(trifluoromethyl)phenyl), X = S
5t: R = 4-cyano phenyl, R^1 = (3,5-Bis(trifluoromethyl)phenyl), X = S
5u: R = 2,5-dimethoxy phenyl, R^1 = cyclohexyl, X = S
5v: R = 2,5-dimethoxy phenyl, R^1 =nonyl, X = S
5w: R = 2,5-dimethoxy phenyl, R^1 = 4-methylphenyl, X = S
5x: R = 3,4,5-trimethoxy phenyl, R^1 = 4-methylphenyl, X = S
5y: R = 3,4-dimethoxy phenyl, R^1 = 3-chloro-4-fluorophenyl, X = S

Scheme 1. Synthesis of 1,2,4-triazole-linked urea and thiourea conjugates (**5a**–**y**).

From the cytotoxicity results, it was evident that compound **5t** was exhibiting significant cytotoxic activity in all the cell lines screened compared to other compounds. **5s** is showing selective toxicity towards B16-F10 at a concentration <10 μM. From the IC_{50} values, it is evident that the thiourea congeners **5o**-**5y** are potent when compared to urea derivatives **5a**-**5n**. In urea congeners, heterocyclic aldehydes were found to be potent than simple aldehydes and halogen containing derivatives **5f, 5g** showed better activity than alkyl derivatives **5k, 5l**. Though activity of **5t** is potent in B16-F10 cell line compared to MCF-7 cell line and molecular level studies were performed on MCF-7 cell line due to human derived cancer nature.

DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent dye used to detect the nuclear damage, it binds strongly to A-T rich regions in DNA.[28] Dose dependent nuclear changes were remarkably observed with the compound **5t** compared to control. As concentration of the treatment is increased (4 μM, 8 μM), normal oblong shaped cells got shrunk and obtained round shape, whereas at higher concentration of 12 μM, horse shoe shaped (pyknotic) nucleus along with DNA fragmentation that appears as bright color was observed, a typical hallmark of apoptosis was observed as shown in **Figure 2**. DAPI staining demonstrated that compound **5t** is inducing significant nuclear morphological changes.

Table 1. *In vitro* cytotoxic activity (IC_{50} in μM)[a] of triazole-linked urea/thiourea conjugates **5a**–**y**

Compound	MCF-7[b]	A-549[c]	MDA-MB 231[d]	DU-145[e]	B16-F10[f]
5a	>50	>50	>50	24.12±1.03	>50
5b	>50	20.69±0.10	>50	20.59±1.00	21.03±1.56
5c	>50	44.21±0.41	>50	20.41±0.36	42.16±5.34
5d	>50	29.27±5.81	>50	>50	>50
5e	>50	25.96±3.56	>50	31.00±0.66	28.85±1.61
5f	31.16±0.25	21.42±2.32	40.27±0.51	20.41±0.36	>50
5g	30.06±0.38	26.24±0.52	29.60±0.66	24.13±0.88	22.07±3.24
5h	>50	24.85±4.56	>50	20.15±3.19	23.65±5.23
5i	>50	21.98±4.21	>50	21.23±0.59	31.00±0.09
5j	46.94±2.35	>50	44.06±4.07	>50	35.80±1.46
5k	34.02±0.70	43.66±1.25	33.17±1.21	20.14±1.81	39.67±0.27
5l	38.89±0.53	21.90±1.18	39.54±0.92	21.52±1.50	35.19±0.66
5m	>50	22.10±1.42	>50	>50	31.08±0.01
5n	>50	>50	>50	>50	34.72±3.20
5o	>50	28.41±0.11	>50	>50	22.90±0.60
5p	24.80±0.02	17.19±0.29	24.77±0.04	21.37±1.22	24.71±1.39
5q	>50	22.22±1.82	>50	26.29±0.50	29.88±8.05
5r	15.47 ± 1.13	16.18 ± 0.76	25.28±0.35	26.39±1.74	8.54 ± 1.94
5s	23.79±0.93	21.02±2.83	24.92±6.28	23.14±0.68	5.62 ± 0.32
5t	7.22 ± 0.47	9.39 ± 1.61	9.28 ± 0.12	11.75 ± 1.29	4.51 ± 0.05
5u	>50	21.52±0.69	>50	29.81±2.53	39.48±3.48
5v	>50	>50	>50	>50	29.38±2.51
5w	>50	17.38±0.37	>50	20.91±0.25	20.71±2.32
5x	>50	22.78±1.62	>50	20.83±0.11	46.78±6.70
5y	30.07±1.76	21.02±0.69	27.91±3.05	23.32±0.35	20.67±3.36
5-FU[g]	16.7±1.5	39.86	35.91±4.82	39.17±1.25	-

[a]50% Inhibitory concentration after 48 h of compound treatment. [b,d] Breast cancer cells, [c]Lung cancer cells, [e]Prostate cancer cells, [f]Mouse melanoma cells, [g]5-Fluorouracil: Reference standard.

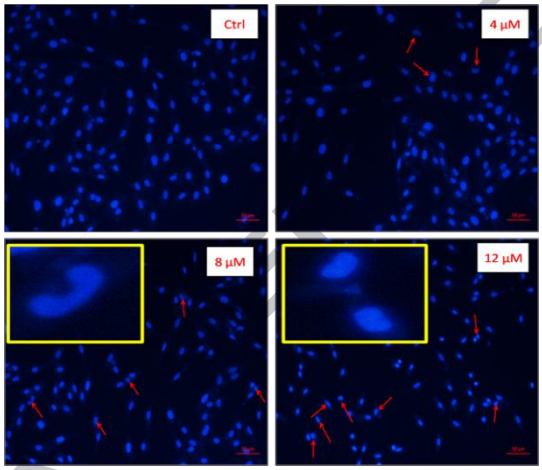


Figure 2. Morphological nuclear changes by **5t** in MCF-7 cells on 48 h exposure as indicated by DAPI staining. Control cells indicating normal oblong shape, 4 μM, 8 μM, and 12 μM, treated cells indicating rounding, shrinkage, pyknotic nucleus of the cells, respectively. Arrows indicate the altered nuclear morphological (horse shoe nucleus and fragmentation) changes in a dose dependant manner.

Acridine Orange and Ethidium Bromide (AO/EB) fluorescent staining assay was carried out for differentiation of live, apoptotic and necrotic cells.[29] This staining solution is a mixture of AO and EB, in which AO is used for live cell identification and EB for identification of dead cells. The AO permeates into the intact cell membrane and thus stains live cells to appear green whereas, EB stained dead cells to appear red when visualized by fluorescent microscopy due to disruption of membrane integrity. It is observed from the results of AO/EB, that control cells showed normal cell morphological features while compound **5t** at the concentration of 4 μM induced morphological changes like membrane blebbing. At the concentration of 8 μM, DNA fragmentation and chromatin condensation are also observed along with membrane blebbing, resulting in loss of membrane permeability. Further, at a concentration of 12 μM, the number of green cells was reduced and orange cells were increased indicating cells underwent late apoptotic stage. These results explore that the compound may induce dose dependent apoptosis as evident by observed hall marks of apoptosis as shown in **Figure 3**.

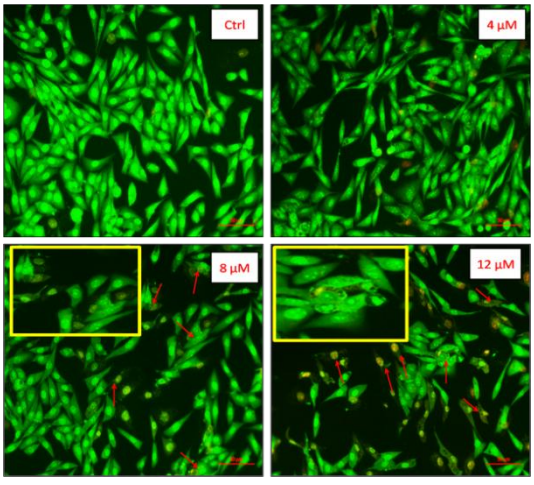


Figure 3. Apoptosis induction by **5t** in MCF-7cells using AO/EB staining. Control cells exhibiting normal green structure. Treatment with 4 μM, 8 μM, and 12 μM. Treated cells indicating rounding, shrinkage, pyknotic nucleus of the cells, respectively. Arrows indicate the altered cell morphological (membrane blebbing and apoptotic bodies) changes at different concentrations of **5t**.

Cell cycle analysis by quantification of DNA content is a reliable method to investigate at which phase cell cycle has been arrested, wherein Propidium Iodide dye is used which binds in proportion to the amount of DNA present in the cell.[31] Results of cell cycle analysis using **5t** against MCF-7 cell lines by flow cytometry showed a transient G0/G1 arrest of cells peaking at 24 h. The results revealed that **5t** caused an increase in % of G0/G1 arrest (61.18% and 69.97% at 4 and 8 µM respectively) than control cells (53.01%).

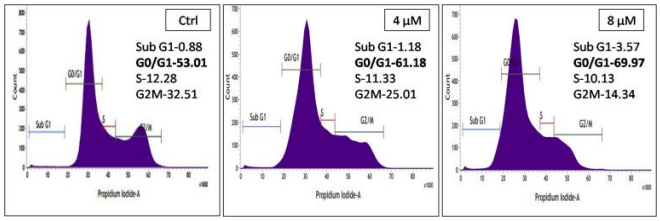


Figure 4. Effect of **5t** on cell cycle progression of MCF-7 using flow cytometry. Cells treated with 4 and 8 µM concentrations of **5t** exhibiting concentration dependent arrest of G0/G1 (61.18% and 69.97%) respectively compared to control cells.

The clonogenic cell reproduction assay determines the ability of a cell to proliferate indefinitely, thereby retaining its proliferative ability to form large colonies.[30] This assay also serves to determine long term viability of cells and growth arrest potential of the compound. **Figure 5** clearly shows inhibition of colony formation by compound **5t** in concentration- dependent manner. At the highest concentration (12 µM) maximum inhibition of colony was observed in comparison to control (**Figure 6**). Total colony count was made possible using molecular imaging system Vilber Fusion Fx software and represented as a total number of colonies verses concentration. The colony count was found to be significantly decreased in 5t treated cells concentration dependently compared to untreated control cells.

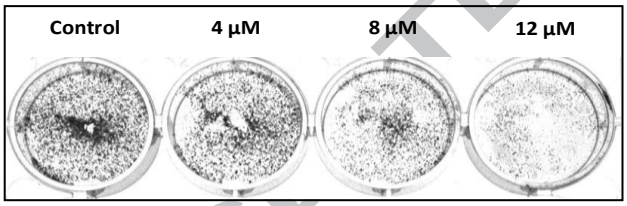


Figure 5. Effect of **5t** on clonogenic assay. Control cells having multiple colonies, while **5t** at concentrations of 4, 8 and 12 µM showing inhibition of number of colonies respectively when compared to control.

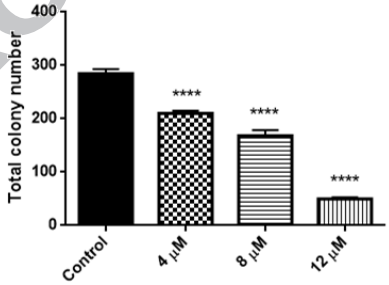


Figure 6. Inhibition of colony formation of **5t** on MCF-7 cell line. Significant decrease in cell reproduction is observed with increase in concentration. All the values are expressed as Mean ± SEM of three experiments in which each treatment was performed in triplicate wells. ****p<0.0001 versus control cells.

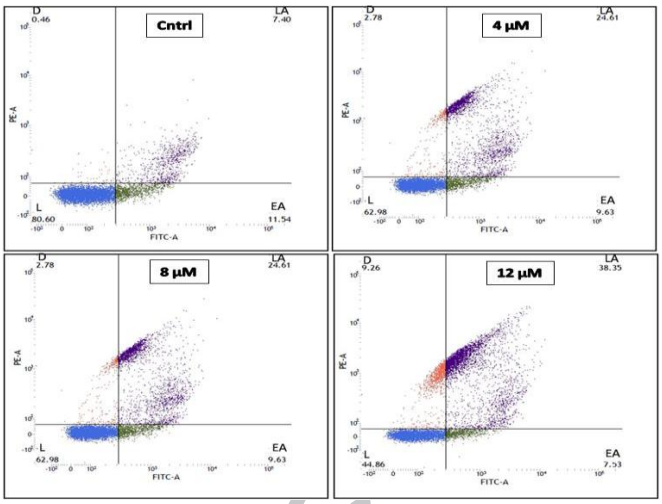


Figure 7. Estimation of apoptotic population by Annexin V and Propidium Iodide (PI) staining by compound **5t** on MCF-7 cells after 48 h using BD C6 Accuri flowcytometer. 10,000 cells from each sample were analysed using flow cytometry. The percentage of cells positive for Annexin V-FITC and/or Propidium Iodide is reported inside the quadrants. Cells in the lower left quadrant (L: AV^-/PI^-): live cells; lower right quadrant (EA: AV+/PI-): early apoptotic cells; upper right quadrant (LA: AV+/PI+): late apoptotic cells and upper left quadrant (D: AV-/PI+): necrotic cells.

To confirm the induction of apoptosis, Annexin V binding studies by flow cytometer was performed. To detect early apoptosis and late apoptosis or necrosis, FITC conjugated human phospholipid phosphatidyl serine binding protein, Annexin V, conjugated with *FITC* is used to stain cells in combination with Propidium Iodide (PI). As phosphatidyl serine externalization is the hallmark of apoptotic cells, Annexin V binds to the membrane and gives fluorescence. Thus, Annexin V stained cells represent cells with intact membranes and externalized phosphatidyl serine. Cells that stained positive for Annexin V/PI represents the cells in the late apoptotic stage that have lost membrane integrity. MCF-7 cells on treatment with 4, 8 and 12 µM for 48 h have shown that increase in the concentration of **5t** resulted in increased Annexin V–FITC binding population when compared to vehicle-treated control cells. It indicates the compound **5t** induces apoptosis in MCF-7 cells in a concentration dependent manner. The percentage of total apoptotic cells (late apoptotic cells) was increased to 38.35% after treatment with 12 µM concentration of compound **5t** for 48 h, in comparison to the control (7.4%) cells **(Figure 7**).

In cancer disease condition, specificity of the compounds towards cancer cell lines, while unaffecting the normal cell functioning gains prime significance. Therefore, performing cell viability assay in the normal cell line of any anticancer agent demonstrates its specific activity towards cancer cell line, and thus can be used further until it doesn't affect normal cell physiology. The L-132 lung epithelial cell was used as a normal cell line to investigate the effect of compound **5t** and found a two-fold difference of cytotoxic activity in L-132 cells compared to MCF-7 cell line. IC_{50} value of **5t** in normal cell line was found to be 15.18 ± 0.84 µM, whereas, on MCF-7 cell line, the IC_{50} value was 7.22 ± 0.47 µM indicating the cancer specificity of compound **5t** while the same concentration is not significantly toxic to normal cell line (**Figure 8**).

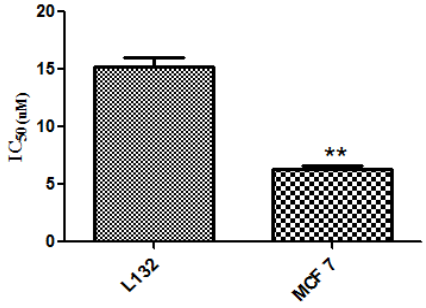


Figure 8. Cytospecificity of compound **5t** towards breast cancer cell line. The cytotoxic effect of compound **5t** was examined on both cancer (MCF-7) and normal cell line (L-132) using MTT assay. The values were expressed as mean ± SEM (n=3) **p<0.01 of normal cell line versus cancer cell line MCF-7.

In conclusion, a new series of 1,2,4-triazole-urea/thiourea conjugates have been synthesized and evaluated for their *in vitro* cytotoxicity against different human cancer cell lines including melanoma mouse cancer cell lines. From the preliminary screening, 1,2,4-triazole-thiourea congeners were found to be potential on MCF-7 cancer cell line. Interestingly, compound **5t** displayed a broad spectrum of activity against all tested cancer cell lines. Specificity in cytotoxicity towards cancer cell lines was studied by performing cell viability assay on healthy lung epithelial cells (L-132) and two-fold lesser cytotoxicity was observed in comparison to MCF-7 cell line. Additionally, the results of detailed studies like AO/EB staining, DAPI nuclear staining, Annexin V/Propidium Iodide assay suggested that the compound **5t** induces apoptosis in a dose-dependent manner. Colony formation in MCF-7 was effectively inhibited by compound **5t**. Moreover, the compound **5t** caused cell cycle arrest at G0/G1 phase by disruption of cytoskeleton in MCF-7 cancer cells. Overall, the current studies demonstrate that the triazole-linked thiourea hybrids have the potential to be developed as lead and their further structural modifications may generate promising new anticancer agents in breast cancer therapy.

Acknowledgments

The authors are thankful to DoP, Ministry of Chemicals & Fertilizers, Govt. of India, for the award of NIPER fellowship. Dr. N. Shankaraiah gratefully acknowledges SERB, DST, Govt. of India for research grant (YSS-2015-001709).

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Graphical Abstract

Synthesis of 1,2,4-triazole-linked urea/thiourea conjugates as cytotoxic and apoptosis inducing agents

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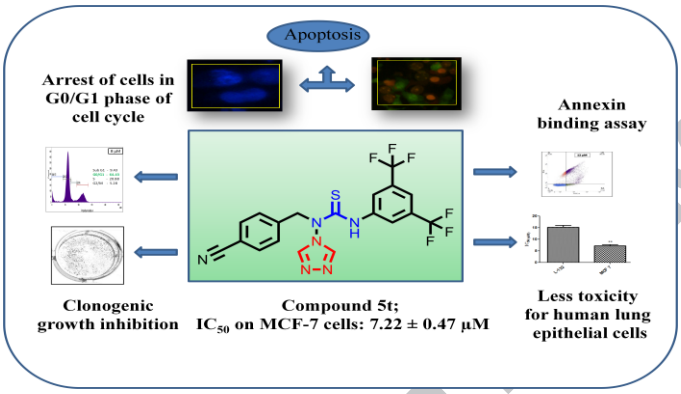

Research Highlights

- A new series of 1,2,4-triazole-urea/thiourea conjugates were synthesized.
- Compound **5t** induced apoptosis and cell cycle arrest in G0/G1 phase in MCF-7 cell line.
- Compound **5t** inhibited colony formation in MCF-7 cell line.
- Compound **5t** was almost 2 times more selective on MCF-7 cells compared to L-132 cells.