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

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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 (Fig. 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.

Small molecule kinase inhibitors including urea derivatives (thioureas e.g., thiouracil (Fig. 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 (Fig. 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[24–27] as significant anticancer agents, we pondered to explore antitumor activity of these newly synthesized

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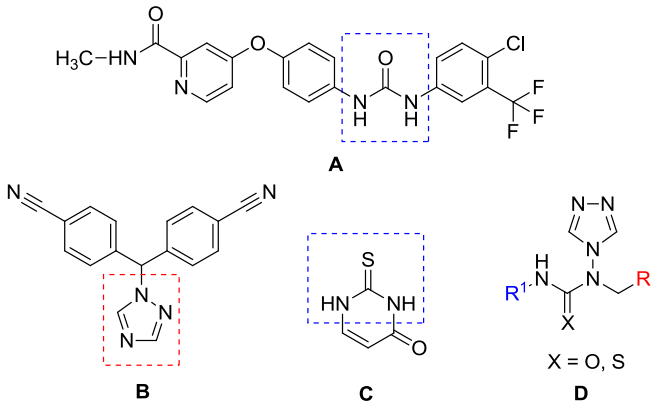
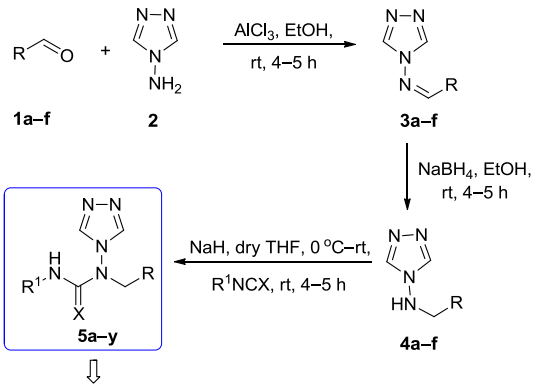


Fig. 1. The structures of sorafenib (**A**), letrozole (**B**), thiouracil (**C**), and designed new derivatives (**D**).

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



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**).

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 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.

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 Fig. 2. DAPI staining demonstrated that compound **5t** is inducing significant nuclear morphological changes.

Acridine Orange and Ethidium Bromide (AO/EB) fluorescent staining assay was carried out for differentiation of live, apoptotic