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Original article

Luotonin-A based quinazolinones cause apoptosis and senescence *via* HDAC inhibition and activation of tumor suppressor proteins in HeLa cells

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ABSTRACT

A series of novel quinazolinone hybrids were synthesized by employing click chemistry and evaluated for anti-proliferative activities against MCF-7, HeLa and K562 cell lines. Among these cell lines, HeLa cells were found to respond effectively to these quinazolinone hybrids with IC_{50} values ranging from 5.94 to 16.45 μM. Some of the hybrids (**4q**, **4r**, **4e**, **4k**, **4t**, **4w**) with promising anti-cancer activity were further investigated for their effects on the cell cycle distribution. FACS analysis revealed the G1 cell cycle arrest nature of these hybrids. Further to assess the senescence inducing ability of these compounds, a senescence associated β-gal assay was performed. The senescence inducing nature of these compounds was supported by the effect of hybrid (**4q**) on p16 promoter activity, the marker for senescence. Moreover, cells treated with most effective compound (**4q**) show up-regulation of p53, p21 and down-regulation of HDAC-1, HDAC-2, HDAC-5 and EZH2 mRNA levels. Docking results suggest that, the triazole nitrogen showed Zn^{+2} mediated interactions with the histidine residue of HDACs.

1. Introduction

Cancer is one of the most common diseases worldwide and is the second leading cause of deaths in the world. Drug discovery has played an important role in the development of newer and safer anti-cancer agents that have a broader spectrum of cytotoxicity to tumor cells [1]. Histone deacetylases (HDACs) are important class of enzymes involved in many significant biological functions. They have been linked to a variety of cancers. Altered expression and mutations of genes that encode HDACs have been linked to tumor development as these two factors induce the aberrant transcription of key genes which regulate important cellular functions such as cell proliferation, cell-cycle regulation and apoptosis. HDAC inhibitors have been shown to inhibit cell proliferation by inducing apoptosis and senescence. Thus, HDACs are among the most promising therapeutic targets for cancer treatment [2–4].

Histone deacetylases are functionally a class of enzymes that remove acetyl groups from an ε-N-acetyllysine aminoacid on histone, allowing the histones to wrap the DNA more tightly. HDACs are classified into class I, class II, class III (SIRT proteins) and class IV (HDAC-11). Class I HDAC proteins includes HDAC-1, 2, 3 and 8 whereas class II includes HDAC-4, 5, 6, 7, 9 and both these classes are associated with Zn^{+2} as co-factor [3]. Recent studies have indicated that Histone methyl transferases [HMTs] work along with HDACs and cause epigenetic deregulation that eventually leads to cancer phenotype [5]. Members of HMTs include Enhancer of Zeste Homolog 2 (EZH2), a key component of polycomb repressive complex that catalyzes H3 lysine 27 trimethylation and cause transcriptional gene silencing [6,7]. Recent studies have indicated that EZH2 is a cell regulatory protein that governs senescence and was found to interact with HDAC-1/2 proteins in cancer cells and alter the chromatin structure [8]. Among HDAC inhibitors, SAHA was found to inhibit gall bladder carcinoma [9] and LBH 589 [10–12] was known to inhibit both HDACs and EZH2 in acute leukemia cells. Several HDAC inhibitors (HDACi) are currently under

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clinical trials on either monotherapy or combination therapy for cancer treatment [13,14]. HDACi induce different phenotypes in various transformed cells, including growth arrest, activation of the extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, reactive oxygen species (ROS)-induced cell death, mitotic cell death and senescence clearly indicating inhibition of HDACs as a novel promising strategy in human cancer therapy [15,16].

The p53 tumor suppressor is well known to regulate genes that mediate cell cycle arrest, apoptosis, senescence, DNA repair [17]. During tumorigenesis, multiple cooperating genetic events result in inactivation of p53 tumor suppressor pathway, a major regulator of senescence and tumorigenesis [18]. Cellular senescence could be triggered by activation of pathways that are dependent on p53-p21 and p16-pRB with over expression of p16 markedly retarding the growth of HCT-116 cells and evoking the senescent phenotype in cancer cells [19]. Studies have also demonstrated that the HDAC inhibitors such as sodium butyrate and Trichostatin-A are known to induce senescence even in the absence of DNA damage in normal human fibroblast cells that cause tumor cell death [20,21]. Apart from p53, HDAC, the transcriptional repressor EZH2 that belongs to polycomb group is also found to play a critical regulatory role in senescence [22].

The quinazolinone moiety contained in natural products (Luotonin, Rutaecarpine, Tryptanthrin, Chloroqualone, Alloqualone, etc) represents medicinally and pharmaceutically important class of compounds [23,24] because of their diverse range of biological activities such as anti-cancer, diuretic, anti-inflammatory, anti-convulsant and anti-hypertensive [25,26]. In recent years, quinazolinone embedded numerous natural products have been identified [27]. Several other quinazolinone derivatives have been identified with anticancer, mPTP modulators, EGFR and VEGFR-2 inhibitors [28–30]. The cytotoxic alkaloid Luotonin-A (1) and its derivatives infused with quinazolinone moiety are clinically proved as anti-cancer agents (Fig. 1) [31–33].

Luotonin A is a pyrroloquinazolinoquinoline alkaloid extracted from Chinese herbal medicinal plant *Peganum nigellastrum* that functions as human topoisomerase-I poison. Luotonin-A stabilizes the human DNA topoisomerase I-DNA covalent binary complex and mediates topoisomerase-I dependent cytotoxicity in intact cells [34]. Studies [35] have demonstrated that luotonin-A binds to dsDNA through the minor groove and stabilize the topoisomerase I-DNA covalent binary complex and induce DNA cleavage. In view of great potential of Luotonin A, it has been subjected to manipulations (or modifications) for the generation of novel hybrids that have a better potential to cause cytotoxicity as well as target the key proteins that dictates the cell fate such as HDACs and EZH2 in cancer cells.

Current chemotherapy regimens are comprised mostly of single-target drugs which are often plagued by toxic side effects and resistance development. The attractive targets, histone deacetylase (HDAC) and topoisomerase I (Topo I), are cellular modulators that can broadly arrest cancer proliferation through a range of downstream effects. Presently, Researchers are concentrating on agents that can modulate multiple targets that may have superior advantage and fewer side effects over the use of single-target agents. Studies [36,37] have indicated an unanticipated function of Topo I in regulating senescence. In addition, HDAC inhibitors also were found to induce the senescence of corneal myofibroblasts as evidenced by increased staining of beta-galactosidase and upregulated expression of p16 (ink4a) [38].

Studies on pyrroloquinoline nucleus have indicated the potential cytotoxic effects of these chemical compounds on tumor cells [39,40]. As a part of our on-going programme to discover and develop tumor growth inhibitors as well as apoptotic inducers, we have identified several classes of novel potential anti-cancer molecules [41–44]. Here we synthesized novel quinazolinone hybrids of Luotonin A, by employing click chemistry, to elicit combined anti-tumor efficacy/cytotoxicity against different cancer cell lines *in vitro*. The cytotoxicity of these compounds were evaluated on MCF-7 (Human breast cancer), HeLa (human cervical cancer), K562 (human myelogenous leukemia [CML]) cell lines as well as normal HEK (human embryonic kidney cells).

The computational techniques play a vital role in lead molecule identification and its optimization. Both structure based (homology modeling, docking) and ligand based (pharmacophore, QSAR) drug design approaches have been used for the design of better ligands in order to enhance potency, selectivity, pharmacokinetic parameters [45–48]. Hence, in the current study, we have applied both ligand based and structure based techniques on a series of quinazolinone compounds in both EZH2 and HDAC proteins. The crystal structures of HDAC5 and HDAC6 are unknown and hence we have made homology models using human HDAC2 as a template. The site map analysis has been performed on EZH2 structure for the identification of its catalytic domain. The docking studies have been performed to understand the key active site residues of most potent quinazolinone series of compounds in all HDAC, EZH2 proteins and explored all probable binding modes. Ligand based pharmacophore model (AAAHRR) has been built using dataset compounds and

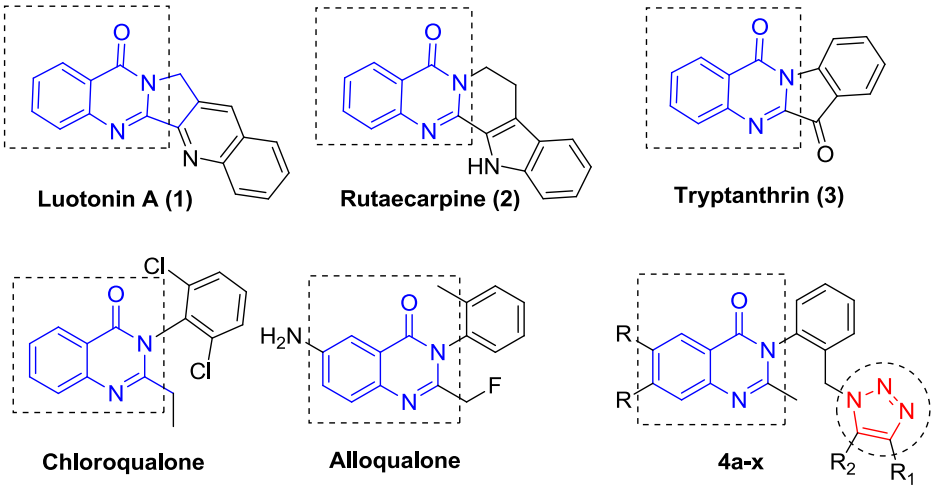

Fig. 1. Quinazolinone scaffold containing natural products and its hybrids (**4a**–**x**).