Spiroquinazolinone-induced cytotoxicity and apoptosis in K562 human leukemia cells: alteration in expression levels of Bcl-2 and Bax

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ABSTRACT — Spiroquinazolinone compounds have been considered as a new series of potent apoptosis-inducing agents. In this study, anti-proliferative and apoptotic effects of the derivatives from the spiroquinazolinone family were investigated in the human chronic myeloid leukemia K562 cells. The K562 cells were treated with various concentrations of the spiroquinazolinone (10-300 μM) for 3 days and cell viability was determined by MTT growth inhibition assay. 4t-QTC was more active among these compounds with IC50 of 50 ± 3.6 μM and was selected for further studies. Apoptosis, as the mechanism of cell death was investigated morphologically by acridine orange/ethidium bromide (AO/EtBr) double staining, cell surface expression assay of phosphatidyl serine by Annexin V/PI technique, as well as the formation of DNA ladder. The K562 cells underwent apoptosis upon a single dose (at IC50 value) of the 4t-QTC compound, and over-expressed caspase-3 expression by more than 1.7-fold, following a 72 hr treatment. Furthermore, RT-PCR and Western blot analysis revealed that treatment of the K562 cells with 4t-QTC down-regulates and up-regulates the expression of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), respectively. Based on the present data, it seems that these compounds from the spiroquinazolinone family are good candidates for further evaluation as an effective chemotherapeutic family acting through induction of apoptosis in chronic myeloid leukemia.

Key words: Apoptosis, Bax, Bcl-2, Caspase-3, Chronic Myeloid leukemia, Spiroquinazolinone

INTRODUCTION

Chronic myelogenous leukemia (CML) is a lethal malignancy characterized by uncontrolled growth, resistance to apoptosis, and the Philadelphia chromosome. This abnormality, the Philadelphia chromosome, is the result of a reciprocal translocation between chromosomes 9 and 22 that leads to the formation of a fusion BCR–ABL gene on chromosome 22 (de Lima et al., 2010; Moosavi et al., 2007). K562 is one of the cell lines that has been established to investigate the advanced phase of CML (Moosavi et al., 2007). Apoptosis is a tightly controlled mode of cell death that eliminates harmful cells upon treatment with anti-cancer drugs. It is morphologically characterized by chromatin condensation, cellular shrinkage, plasma membrane blebbing and nuclear fragmentation (Cheung et al., 2012; Pulido and Parrish, 2003). During apoptosis, a group of cysteine proteases, caspases, which contain a nucleophilic cysteine residue are activated and specifically cleave their substrate proteins after aspartic acid (Strasser et al., 2000). Two main molecular pathways that cause apoptosis are the death receptor (extrinsic) and mitochondrial (intrinsic) pathways. In the death receptor pathway, apoptosis is induced by interaction between ligands such as TNF and FAS with their respective death receptors that cause formation of a death-inducing signaling complex (DISC). These interactions ultimately lead to activation of caspase-8 by induced proximity mechanism. The active caspase-8 can activate other procaspases, such as caspase-3, -6, and -7 (Favalaro et al., 2012; Rastogi and Sinha, 2010). The intrinsic pathway on the other hand is activated in response to a number of stressing conditions including DNA damage and oxidative stress that induces the release of mitochondrial proteins, such as cytochrome c (cyt c) from the intermembrane space to the cytosol. Cyt c with some other molecules such as Apaf-1...
and ATP (or dATP) forms a multimeric complex that promotes caspase-9 activation. The activated caspase-9 then cleaves and activates the effector caspases such as -3, -6 and -7 (Rastogi and Sinha, 2010; Putcha et al., 2002). In the intrinsic apoptosis pathway, the Bcl-2 family of proteins are the central regulators (Adams and Cory, 2007). These related proteins share at least one of four homologous regions termed Bcl homology (BH) domains (BH1 to BH4) which control the ability of the Bcl-2 proteins to bind to each other to form homodimers and heterodimers. BH domains contribute at multiple levels to the function of these proteins in cell death and survival (Adams and Cory, 2007; Tzifi et al., 2011). About 30 members of this family have been currently identified. Based on the functional studies and the conservation of BH domains, these proteins are divided into three subgroups, pro-survival Bcl-2 like subgroup such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, A-1 subgroup which suppress cell death, and pro-apoptotic Bax-like subgroup such as Bak, Bak, Bok and Bik which promote cell death (Tzifi et al., 2011; Billen et al., 2008). The Bcl-2 protein, as one of the Bcl-2 family, binds to the outer surface of mitochondria and sometimes endoplasmic reticulum, and, separating pro-apoptotic members such as Bax from mitochondria, prevents apoptotic cell death (Plati et al., 2011). Increased levels of Bcl-2 expression in cancer cells prevent apoptosis and lead to tumor progression. In contrast, increase in expression of Bax induces cell death and eliminates tumor cells (Tzifi et al., 2011). Reduced expression of Bax and increased expression of Bcl-2 in many drug-resistant tumor cells has been reported (Sakamoto and Kyprianou, 2010). The fate of every cell is ultimately determined by cross-talking of the pro- or anti-apoptotic signaling pathways. These pro- or anti-apoptotic proteins are highlighted not only in the clinical treatments of malignancy, but also in cancer chemoprevention. It is generally believed that chemotherapeutic drugs cause the elimination of cancer cells by induction of apoptosis (Mahdavi et al., 2011). The main reasons of failure in cancer chemotherapy in patients with CML are drug toxicity and occurrence of drug resistance. Chemotherapy is still an effective way to reduce cancer cells, especially in patients with CML in blast crisis (Shi et al., 2010). Heterocyclic compounds such as spiroquinazolinone derivatives have a great importance in a wide spectrum of biological activity such as anti-bacterial, anti-inflammatory, anti-convulsant, anti-malarial, anti-allergic, anti-HIV and anti-parkinsonian (Abdel Gawad et al., 2010; Vijayakumar et al., 2013). These compounds are derivatives of benzopyrimidine with different mechanisms of action. The application of spiroquinazolinone derivatives has been reported in several types of cancer such as lung cancer, pancreatic cancer, thyroid cancer and chronic myeloid leukemia. These compounds are also used for treatment of high blood pressure, prevention of folic acid synthesis through inhibition of the enzyme dihydrofolate reductase and inhibition of activity of EGFR over-expressed in the several forms of cancer. Interaction with cytoskeleton, DNA topoisomerase and induction of apoptosis are other effects of the spiroquinazolinone compounds (Cubedo et al., 2006; Mohamed et al., 2012; Krishnan et al., 2011;OVádeková et al., 2005; Selvam et al., 2011). The spiroquinazolinone compounds can be proposed as effective agents for more investigation in the drug developments. In this study, we investigated twelve new derivatives of three group spiroquinazolinone including spiroquinazolinone benzamide, spiroquinazolinone benzene sulfonamide and spiroquinazolinone thiophene-2-carboxamide, and explored their cytotoxic effects to induce apoptosis in K562 cell line. Furthermore, we demonstrated that a stronger cytotoxic compound (4t-QTC from the spiroquinazolinone thiophene-2-carboxamide group) functions through down-regulation of Bcl-2 and activation of caspase-3.

MATERIALS AND METHODS

Materials

The cell culture medium (RPMI 1640) and penicillin-streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). The culture plates were obtained from Nunc (Kamstrupvej, Denmark). Ethidium bromide and proteinase K were purchased from Sigma Chemical Company (Darmstadt, Germany). All antibodies, including anti-Bcl-2 (ALX-210-701-C100), anti-caspase-3 (ALX-210-806-C100) and anti-β actin (ADI-905-733-100) were purchased from Alexis Biochemicals (San Diego, CA, USA). Propidium iodide (PI), dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) and other reagents were from Sigma-Aldrich (St. Louis, MO, USA). Annexin-V FITC Apoptosis kit, Cell Extraction Buffer and ECL kit were purchased from Roché (Mannheim, Germany). The K562 cell line was obtained from Pasteur Institute of Iran (Tehran, Iran).

General procedure for the preparation of the spiroquinazolinone compounds

The spiroquinazolinone compounds, including spiroquinazolinone benzamide (4-CPQB, 4-CHQB, 3m-CHQB, 4e-CHQB and 4t-CHQB), spiroquinazolinone benzene sulphonamide (4-CPQ, 4-CHQ and 4t-CHQ) and spiroquinazolinone thiophene-2-carboxamide (4-QTC, 5-QTC and 6-QTC) were synthesized following the general procedure.
4e-QTC and 4t-QTC), were prepared according to the previously described method (Tajbakhsh, 2014).

Cell culture
The human K562 cell line was cultured in RPMI 1640 medium supplemented with FBS (10%, v/v), streptomycin (100 μg/mL) and penicillin (100 U/mL) in CO2 humidified atmosphere at 37°C (Zhou et al., 2012).

MTT Assay
Cell proliferation was determined using the MTT assay. K562 cells (2 × 10^4 cells/well) were incubated in 96-well plates with various concentrations of the spiroquinazolinone compounds for 24, 48 and 72 hr in a final volume of 200 μL. After treatment, 20 μL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 hr at 37°C. The purple-blue MTT formazan precipitate was dissolved in 200 μL of DMSO and the absorbance values at 570 nm were determined on a multi-well plate reader (Quant Bio-tek Instruments, Winooski, VT, USA) (Subhashini et al., 2004).

DNA fragmentation assay
DNA fragmentation was measured by extracting genomic DNA from a constant number of cells. K562 cells treated with a single dose (at IC50 value) of the 4t-QTC for 48 and 72 hr were collected and then washed twice with cold PBS. Afterwards, the samples were resuspended in 100 μL of lytic solution (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) and incubated with 10 μL of 10 mg/mL RNase A for 1 hr at 37°C followed by 10 μL of 20 mg/mL proteinase K for 2 hr at 50°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), washed with ethanol and resuspended in TE buffer. Aliquot of each sample was then subjected to electrophoresis in a 2% agarose gel containing ethidium bromide (Mahdavi et al., 2011).

Morphological evaluation of the apoptotic cells
Apoptosis was studied morphologically using the fluorescent dyes, acridine orange and ethidium bromide (AO/EtBr), which intercalate with DNA. The K562 cells (2 × 10^4 cells/well) were seeded in 96-well plates for 24 hr prior to treatment and treated with 4t-QTC for 24, 48 and 72 hr. An Annexin-V FITC and propidium iodide (PI) double staining method was used according to the manufacturer’s protocol. Briefly, after double washing of treated and untreated cells with PBS, 1 × 10^6 cells were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) and then 10 μL of Annexin-V FITC and 10 μL PI were added. The mixture was incubated for 15 min in the dark at room temperature and then measured by flow cytometry (Partec PAS) (Uchakina et al., 2013).

Reverse transcribed-polymerase chain reaction (RT-PCR) of Bax and Bcl-2 genes
After drug treatment, total RNAs were extracted from treated and untreated cells using RNX-plus reagent as described by the manufacturer (Cinagen, Tehran, Iran). One μg of total RNA was reverse transcribed into cDNA using a Revert Aid™ M-MuLV (Fermentas, Burlington, Canada) with each dNTP, 1 μg oligo dT, 20 U RNasin (Promega, Madison, WI, USA) and 1 × RT buffer and 200 U. Specific primers were used for the:

- **GAPDH**: Forward, 5′-CAAGGTCATCCATGACAACTTTG-3′; Reverse, 5′-GTCCACCACCCTGTTGCTGTAG-3′;
- **Bax**: Forward Primer, 5′-TGCCAGCAAACTGGTGCTCA-3′; Reverse Primer, 5′-GCACTCCCGCCACAAAGATG-3′;
- **Bcl-2**: Forward Primer, 5′-CGCATCAGGAAGGCTAGAGT-3′; Reverse Primer, 5′-AGCTTCCAGACATTCGGAGA-3′.
The reaction for Bax, Bcl-2 and GAPDH was run for 30, 30 and 35 cycles, respectively. The PCR products were electrophoresed on a 2% agarose gel and visualized under UV light by ethidium bromide staining (Finaurini et al., 2012; Kalle et al., 2010).

Western blot experiments

The K562 cells treated with the indicated dose (at IC50 values) of the 4t-QTC and harvested after 24, 48 and 72 hr were lysed by cell extraction buffer containing 1% Triton X-100, 0.1% SDS, 10 mM Tris (pH 7.4), 100 mM NaCl, 10% glycerol and 0.5% deoxycholate for 30 min on ice, with vortexing at 10 min intervals, and then, the extract was transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 10 min at 4°C. The protein concentration of each sample was determined using Lowry’s method (Lowry et al., 1951). Equal quantities of protein (40-50 μg) were subjected to SDS-polyacryl-amide gel electrophoresis (PAGE) and then transferred to nitrocellulose (and or PVDF) membranes. Transfer of proteins was assessed by ponceau-red staining. Membranes were blocked by incubation in non-fat dry milk containing 0.1% Tween-20 for 1 hr at room temperature. Following overnight incubation with primary antibodies, HRP conjugated secondary antibodies were added and incubated for 1 hr. The proteins were detected using an enhanced chemiluminescence (ECL) detection system (Thermo Scientific, Mannheim, Germany). Intensities of the bands were quantified using the NIH ImageJ software (Mahdavi et al., 2011).

Statistical Analysis

Data were derived from three independent experiments and presented as means ± S.D. Significant differences between the groups were determined using the one way analysis of variance (ANOVA) software. Differences were considered significant at p < 0.05.

RESULTS

Spiroquinazolinone derivatives decreased viability of K562 cells

The spiroquinazolinone compounds were tested by the traditional cell viability assay to confirm that the active compounds can inhibit tumor cell survival. To determine the effect of the investigated spiroquinazolinones on viability of the K562 cells, 2 × 10^4 cells/mL were treated with various concentrations (10-300 μM) of the compounds for 24, 48 and 72 hr. Cell viability was observed following the treatment of different doses of the compounds for 24, 48 and 72 hr. Cell viability was observed following the treatment of different doses of the compounds for 24, 48 and 72 hr. Cell viability was observed following the treatment of different doses of the compounds for 24, 48 and 72 hr. The IC50 values of the compounds are summarized in Table 1. Among these compounds, 4t-QTC was found to be highly active with IC50 value of 50 ± 3.6 μM in K562 cells (Fig. 1). As shown in Fig. 1, cell viability was reduced following treatment of the cells with the 4t-QTC in a time-dependent manner.

![Fig. 1](image-url) Effect of the 4t-QTC on cell viability of K562 cells. The cells were subjected to various concentrations (10-120 μM) of the 4t-QTC for 24, 48 and 72 hr. Cell viability was assessed by MTT test and presented as percentage of the corresponding controls. The results are the means of three independent experiments ± S.D. (*p < 0.05, **p < 0.01 vs. control).
**Table 1.** The IC50 values of investigated spiroquinazolinone compounds after 48 hr of exposure. For experimental details, see material and method sections. Each value represents the means of three independent experiments ± S.D. (P < 0.05).

| Basic structure                  | Substituent | Compound name | IC50  |
|----------------------------------|-------------|---------------|-------|
| (Spiroquinazoline benzamide)     | n = 1       | R = H         | 4-CPQB | 180 ± 2.8 |
|                                  | n = 2       | R = H         | 4-CHQB | 200 ± 2.4 |
|                                  | n = 2       | R = 4-Me      | 3m-CHQB | 180 ± 2.4 |
|                                  | n = 2       | R = 4-Et      | 4e-CHQB | 140 ± 2.9 |
|                                  | n = 2       | R = 4-tBu     | 4t-CHQB | 120 ± 2.4 |
| (Spiroquinazoline benzenesulphonamide) | n = 1       | R = H         | 4-CPQ  | 250 ± 2.4 |
|                                  | n = 2       | R = H         | 4-CHQ  | 250 ± 3.0 |
|                                  | n = 2       | R = 4-tBu     | 4t-CHQ | 180 ± 2.9 |
| (Spiroquinazoline thiophene-2 carboxamide) | R = H       |               | 4-QTC  | 180 ± 1.7 |
|                                  | R = 4-Et    |               | 4e-QTC | 100 ± 4.6 |
|                                  | R = 4-tBu   |               | 4t-QTC | 50 ± 3.6  |

This compound (4t-QTC) was selected for further studies to investigate the mechanism of cell death.

**Induction of apoptosis**

To induce apoptosis, K562 cells were cultured in the presence of indicated concentrations (IC50 value) of the 4t-QTC. The cellular morphology of the K562 cells treat-
ed with the 4t-QTC was observed with a phase contrast microscope (Zeiss). While the control cells have a round morphology, the treated cells exhibit an increased condensation over time and their adhesion to the culture plate was reduced (Fig. 2A). Chromatin condensation was also detected using the fluorescent DNA binding dye (AO/EtBr). As shown in Fig. 2A, the viable cells are uniformly green, whereas the apoptotic cells exhibit bright green dots in their nuclei (early apoptosis) indicating nuclear fragmentation. The late apoptotic cells appear in bright orange color with condensed and fragmented DNA.

**Flow cytometric assessment of apoptosis and DNA fragmentation**

We used a flow cytometric method to confirm cell apoptosis. Distribution of the cells in different phases of the cell cycle was studied by flow cytometry. We found that the number of cells in the sub-G1 phase was significantly increased after 72 hr treatment with 4t-QTC. The cell cycle distribution of control K562 cells were among sub-G1, G0/G1, S and G2/M phase by almost 3.6%, 54%, 23% and 19.4%, respectively. After treatment of the K562 cells for 72 hr, the cell cycle distribution was 48.4%, 42%, 6.2% and 3.4% at sub-G1, G0/G1, S and G2/M phase, respectively (Fig. 3A). Therefore, this finding suggested that the 4t-QTC induces sub-G1 arrest and apoptosis in the K562 cells. Further confirmation of apoptosis came from the formation of DNA ladders upon treatment of the cells with the 4t-QTC (Fig. 2B). To further confirm the type of cell death, we used a flow cytometric method for the detection of cell surface localization of phosphatidylserine (PS) using Annexin V/PI staining. This method has been widely used to discriminate between normal cells (Annexin V−/PI−), early apoptotic cells (Annexin V+/PI−), late apoptotic cells (Annexin V+/PI+) and necrotic cells (AnnexinV−/PI+). After treatment of the cells with the indicated concentration (IC50 values) of the 4t-QTC for 24-72 hr, the apoptosis cell death was detected by flow cytometry. Consistent with the previous results on morphological changes of the cells, DNA ladder and cell cycle, apoptosis was mostly observed time dependently after 24-72 hr of treatment with 4t-QTC. Shifts from early

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**Fig. 2.** Morphological changes of the K562 cells treated with the 4t-QTC. (A) After 72 hr treatment with the 4t-QTC, the cells were harvested and were stained with AO/EtBr and then were studied using light microscopy and fluorescent microscopy. × 200 magnification. (B) DNA fragmentation was detected after 48 and 72 hr treatment of the K562 cells with the 4t-QTC.
Induction of apoptosis by Spiroquinazolinone Family

Fig. 3. Effect of 4t-QTC on cell cycle and apoptosis of K562 cells. The cells were treated with the indicated concentrations (at IC50 value, 50 μM) of 4t-QTC and harvested after 24, 48 and 72 hr. (A) Cell cycle analysis of the K562 cells treated with the 4t-QTC. The cells were stained with propidium iodide (PI) and then the percentage of cells was calculated in each phase using flow cytometry. (B) Apoptosis was also studied by Annexin-V/PI double staining assay. Flow cytometric analysis showed a shift from bottom-right quadrant panel (early apoptosis) to top-right quadrant panel (late apoptosis or necrosis).
Expression of apoptosis-related genes and proteins upon 4t-QTC treatment

To determine whether the apoptotic effects of the 4t-QTC leads to increased expression of caspases, we examined the ability of the 4t-QTC to activate caspase-3 in the K562 cells. Caspase-3 is an “effector or executioner” caspase in the last and irreversible phase of the apoptotic caspase-dependent pathway (Fan et al., 2005). Caspase-3 cleavage was investigated in the K562 cells treated with 4t-QTC by Western blot analysis, using a caspase-3 antibody after 24-72 hr (Fig. 4B). As shown in Fig. 4B, treatment of the K562 cells with 4t-QTC led to an up-regulation and activation of caspase-3 in this cell line. Caspase-3 activity was found to be elevated in a time-dependent manner compared to the untreated cells (Fig. 4B). Subsequently, to determine the effect of the 4t-QTC on expression of intrinsic apoptosis-related genes and proteins in the K562 cells, we evaluated the expression levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) of the Bcl-2 family before and after treatment with 4t-QTC. Thus, following the treatment of the cells with the 4t-QTC at respective IC50 value (50 μM), RT-PCR and Western blot analysis was performed (Fig. 4A and B). As shown in Fig. 4B, low levels of Bcl-2 expression in K562 cells were observed after 48 and 72 hr of treatment, although this reduction did not appear significantly in K562 after 24 hr. Simultaneously with the down-regulation of Bcl-2, expression of Bax was increased after treatment with 4t-QTC (Fig. 4A). High levels of Bax expression in K562 cells were observed in the time-dependent manner of treatment. Exposure of K562 cells to 4t-QTC for 48-72 hr led to a remarkable increase in Bax expression.
Fig. 4. Expression of apoptosis-related genes and proteins upon 4t-QTC treatment. (A) RT-PCR analysis of Bcl-2, Bax and GAPDH was performed after cell treatment with the indicated concentrations (IC50 value) and incubation times (48 and 72 hr) of the 4t-QTC. (B) Western blotting was performed to determine levels of Bcl-2, cleaved and pro-caspase-3 proteins after treatment with IC50 value of the drug for 24, 48 and 72 hr. The Bcl-2 and Bax gene (C) and Bcl-2, cleaved and pro caspase-3 protein (D) levels in control and treated cells were quantified by ImageJ software and normalized to GAPDH and β-actin band intensity for genes and proteins, respectively. Data are from three independent experiments (*p < 0.05, **p < 0.01 vs. control).
increase in number of the cells in the sub-G1 phase representing of apoptotic event (Fig. 3A). Caspases play an important role in the initiation and execution pathways of apoptosis and they can be activated through both extrinsic and intrinsic signaling pathway. Caspase-3 is an important executioner caspase and proteolytic cleavage is necessary for its activation. For further evaluation, we analyzed protein levels of procaspase-3 and cleavage of caspase-3 by Western blot analysis. Consistently, treatment of K562 cells with 4t-QTC (at IC50 values) resulted in a time-dependent increase in activation of caspase-3 as compared to the respective control cells (untreated cells, Fig. 4B). This increase was 1.7-fold following a 72 hr treatment.

In order to further study the activation of the mitochondrial pathway, the Bcl-2 family proteins which play a key role in the regulation of this pathway were examined. Many studies have demonstrated that over-expression of anti-apoptotic Bcl-2 family proteins give resistance to apoptosis and decreases efficiency of therapeutics (Jiang et al., 2007; Jia et al., 2009).

The members of the Bcl-2 family are the central regulators of the mitochondrial cell-intrinsic apoptosis (Adams and Cory, 1998; Adams and Cory, 2007). The Bcl-2 protein as one of the Bcl-2 family proteins regulates cytochrome c release from the mitochondria and prevents apoptotic cell death (Plati et al., 2011). Down-regulation of Bax and up-regulation of Bcl-2 in many drug-resistant tumor cells has been reported (Sakamoto and Kyprianou, 2010). In Chronic Myeloid Leukemia (CML) cells, over-expression of Bcl-2 protein and association with Bcr/Abl cause accelerated transfer rate of chronic phase to blastic phase (Tzifi et al., 2011) and leads to tumor progression. In contrast, increase in expression of Bax, induce cell death and eliminate the tumor cells (Tzifi et al., 2011).

In this study, changes in the protein expression of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) of the Bcl-2 family, under the influence of 4t-QTC was evaluated. The K562 Cells treated with certain concentration (IC50 values) of 4t-QTC, exhibited widespread and strong induction of apoptosis after 72 hr. Interestingly, levels of Bcl-2 protein expression in K562 cells clearly decreased time-dependently after 48 and 72 hr while not significantly changed after 24 hr (Fig. 4A). The expression of Bax was also changed in the cell line after treatment with the 4t-QTC in a time-dependent manner. In K562 cells, Bax level considerably increased following 48 and 72 hr of treatment (Fig. 4A). Mechanism(s) by which spiroquinazolinones modulate the Bcl-2 and Bax protein levels remains unknown. These compounds might interfere with the expression of Bcl-2 proteins or more likely activate degradation pathways of this protein.

In cancer cells, the Bcl-2 protein, by interacting with Bax-Bak complex prevents them from forming a pore in the mitochondrial membrane and the release of cytochrome c and hence inhibits activation of caspase-9 and -3 in its downstream molecules (Teijido and Dejean, 2010; Pavlović et al., 2007). It has been suggested that a high ratio of Bax to Bcl-2 can lead to collapse of mitochondrial membrane potential, resulting in the release of cytochrome c and consequently causes cell apoptosis (Ismail et al., 2005; Boersma et al., 1997). Our data also confirm that the reduction in Bcl-2 protein expression was removed its inhibitory effect on Bax and leads to over expression of Bax and finally activation of caspase-3. Therefore, altered ratio of pro-apoptotic and anti-apoptotic Bcl-2 family members might be an important key question to understand the sensitizing effect of the 4t-QTC in K562 cells.

In conclusion, here we reported on derivatives from the spiroquinazolinone family with high apoptotic activity in the apoptosis-resistant leukemic K562 cells. 4t-QTC was more active in comparison with the other compounds. We attribute the anti-proliferative effect of this compound to several mechanisms including inhibition of cell growth, induction of apoptosis, and expression of apoptosis-related genes and proteins. Induction of apoptosis was achieved by over expression of Bax, activation of caspase-3 and down-regulation of Bcl-2. Our results suggest that the new spiroquinazolinone derivatives are exciting candidates for further evaluation and can be proposed as effective agents for chemotherapy.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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