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3-(5-Nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c]quinazoline induces ROS-mitochondrial mediated death signaling and activation of p38 MAPK in murine L1210 leukemia cells

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Quinazoline derivatives are multitarget agents with a broad spectrum of biological activity. 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c]quinazoline (NTCHMTQ) is a new synthetically prepared derivative, which in our previous studies showed antiproliferative and apoptosis inducing activities towards murine L1210 leukemia cells. The aim of this study was to provide the insight into the molecular mechanism regulating NTCHMTQ-induced apoptosis in L1210 cells. The activity of caspases 3, 8 and 9, generation of reactive oxygen species (ROS), mitochondrial membrane potential changes, release of cytochrome *c*, degradation of PARP and activation of c-Jun N-terminal kinase 1/2 (JNK1/2), p38 MAPK and extracellular-regulated kinase 1/2 (ERK1/2) were investigated. NTCHMTQ induced production of ROS, activation of caspases 3 and 9, cytochrome *c* release, PARP cleavage and activation of p38 MAPK, with no activation of JNK1/2 and ERK1/2. Our resuls clearly demonstrate that NTCHMTQ induces apoptosis of L1210 leukemia cells through ROS-mitochondrial mediated death signaling and activation of p38 MAPK.

Keywords: *quinazoline derivative; apoptosis; caspase; reactive oxygen species; leukemia; p38 MAPK*

Apoptosis is a physiological mode of cell death, which can be selectively triggered by cells in response to different stimuli [1–3]. Despite various agents having ability to induce apoptosis in a dose dependent manner, search for new drugs that induce apoptosis is still highly desirable.

Reactive oxygen species (ROS) are recognized as a mediator of apoptotic cell death, playing pivotal roles in signaling pathways. Mitogen-actived protein kinases (MAPK), a family of serine/threonine kinases, play an important role in ROS-mediated apoptosis. The MAPK superfamily consists of at least three major groups: extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK [4]. Erk1/2 is activated by mitogens and growth factors through a Ras/Raf/MEK signaling cascade. SAPK/JNK1/2 and p38 MAPK are preferentially activated by proinflammatory cytokines and cellular stressors including oxidative stress, UV irradiation, hydrogen peroxide, DNA damage, heat or osmotic shock [5].

Quinazolines represent a class of drugs with a variety of biological activities [6–12]. As documented in the literature, many derivatives act as anticancer active compounds [13, 14] and they act as multitarget agents [15]. A number of quinazoline-based thymidylate synthase inhibitors have broad spectrum of activity against different kinds of human cancer. Some of them (ZD1836, ZD 6474, OSI-774,GW-2016,) are currently in clinical testing[16, 17]. There is also a number of [1,2,4]triazoles having a wide range of pharmacological activities [18].

Based on the above mentioned effects of quinazolines and triazoles, a new series of triazoloquinazolines was prepared by Špirková et al. [19, 20]. These compounds were screened for antibacterial and cytotoxic activities *in vitro* [21–23]. The most potent agent was found to be 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c]quinazoline (NTCHMTQ). This quinazoline was highly cytotoxic and induced apoptosis in L1210 leukemia cells.

The aim of the present work was to provide the insight into the molecular mechanism regulating NTCHMTQ-induced apoptosis in L1210 leukemia cells, and to demonstrate the

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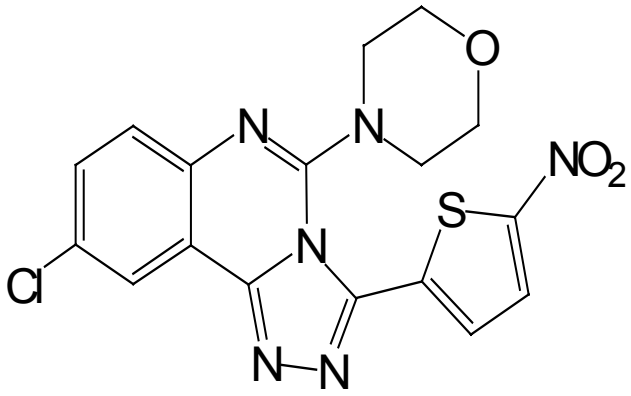
involvement of ROS signaling in mitochondria-mediated apoptosis induced by NTCHMTQ.

Material and methods

Cells. The murine L1210 leukemia cells (ATCC, MD, USA) were cultured in RPMI medium in a 5% CO_2 at 37°C. The medium was enriched with penicillin/streptomycin (100 mg/l) and 10% fetal calf serum (Biocom, Bratislava, Slovakia). Cell growth and viability was determined by trypan blue exclusion test.

Materials. Substituted 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo [4,3-c] quinazoline (Scheme)



was prepared by Spirkova et al. [19, 20]. Chromatographically pure quinazoline was dissolved in 100% dimethyl sulfoxide (final concentration was 0.1 % (v/v) in either control or treated samples). The solutions of tested derivative were prepared freshly before each incubation. RPMI medium, fetal calf serum and antibiotics (penicillin/streptomycin) were obtained from Biocom (Slovakia). Antibodies were obtained from Santa Cruz Biotechnology Inc. (CA, USA). Other chemicals were obtained from Sigma (St. Louis, MI, USA).

Measurement of intracellular reactive oxygen species (ROS). The level of ROS was examined using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were treated with 12 μM NTCHMTQ for 0 – 180 min to detect the change of ROS level. The cells were harvested, washed and resuspended in PBS and 1 μl of DCFH-DA (10mM) was added. The cells were incubated at 37°C for 30 min and analyzed by fluorescent microscopy. L1210 cells treated with 0.1% DMSO were used as negative control.

Caspase assays. L1210 cells were treated with 1.0% DMSO (control vehicle) or with quinazoline for indicated times. Cell lysates were isolated and caspase 3 activity was measured according to the manufacturer´s protocol (CaspACETM Assay System, Promega Corporation, USA). Briefly, 28 μg total protein was added to the reaction mixtures containing colorimetric substrate peptides specific for caspase 3 (Ac-DEVD-pNA) at 37°C for 24, 48 and 72 h. Absorbance was determined using a spectrophotometric microplate reader (Humareader, Wiesbaden, SRN) (wavelength 405 nm). Cellular protein was determined according to Lowry et al. [24].

Caspase 8 and caspase 9 activities were measured according to the manufacturer`s protocol (Caspase-Glo® 8/9 Assay, Promega Corporation, USA). Briefly, 100 μl of Caspase - Glo™ 8 Reagent (for measuring caspase 8 activity, containing specific substrate (Z-LETD-aminoluciferin) and 100 μl of Caspase - Glo™ 9 Reagent (for measuring caspase 9 activity, containing specific substrate (Z-LEHD-aminoluciferin), respectively, were added to the test tube with 100 μl of cell suspension containing 50000 cells, mixed and the luminescent signal was measured immediately for 2h (in 30 min intervals).

Westen blot analyses. The cells were washed twice with PBS, lysed in lysis buffer (25 mM Tris-HCl (pH 7.2), 0.1% SDS, 0.1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mg/ml aprotinin, and 5 mg/ml leupeptin) for 30min on ice, centrifugated and supernatants were collected. Equal amounts of cell lysate proteins were separated on 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and analysed with various antibodies. Anti-cytochrome c, anti-PARP, as well as anti-ERK1/2, anti-p38 MAPK, anti-JNK1/2, anti-phospho-ERK1/2, anti-phospho-p38 MAPK and anti-phospho-JNK1/2 antibodies were used as primary antibodies. Horseradish-peroxidase-conjugated goat anti-rabbit/mouse IgG were used as the secondary antibodies. The proteins of interest were visualized using an enhanced chemiluminescence detection reagents and memrane was exposed to an X-ray film. Protein concentration was determined by Lowry et al. [24].

Measurement of mitochondrial membrane potential ($\Delta\psi_m$). The changes in mitochondrial membrane potential ($\Delta\psi_m$) were determined using 3,3´-dihexyloxacarbocyanine iodide ($DiOC_6$). Cells were treated with 12 μM NTCHMTQ for 24 - 72h to detect the changes of mitochondrial membrane potential. The cells were harvested, washed and resuspended in PBS and 4 μl of $DiOC_6$ (40 μM) was added. The cells were incubated at 37°C for 30 min and analyzed by fluorescence microscopy.

Statistical analysis. Results obtained from caspase 3, 8 and 9 activity measuring are shown as the arithmetic means ± S.D. (standard deviation) of the mean of three separate experiments (each experiment was done with five parallels). The statistical significance of the results was evaluated by Student t-test, with probability values of 0.05 being considered as significant.

Results

To evaluate the reactive oxygen species (ROS) production in NTCHMTQ-treated cells, the intracellular ROS level was monitored using intracellular peroxide-dependent oxidation of DCFH-DA to form fluorescent DCF. DCF fluorescence was detected in cells treated with 12 μM NTCHMTQ for 0-180 min (Fig. 1). Increased level of ROS is connected with reduced mitochondrial membrane potential. In the next experiments, we examined the changes in mitochondrial membrane potential ($\Delta\psi_m$) using fluorescence sensitive probe

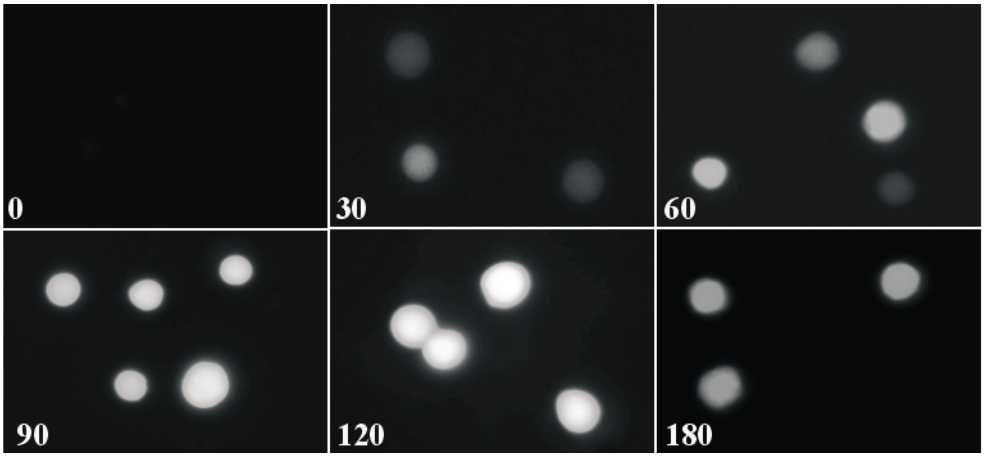


Figure 1. Effect of NTCHMTQ concentration of 12 μM on ROS production analyzed by fluorescence microscopy using DCFH-DA. L1210 cells were treated with quinazoline for 30, 60, 90, 120 and 180 min. 0, negative control (cells treated with 0.1% DMSO). Magnification is 600x.

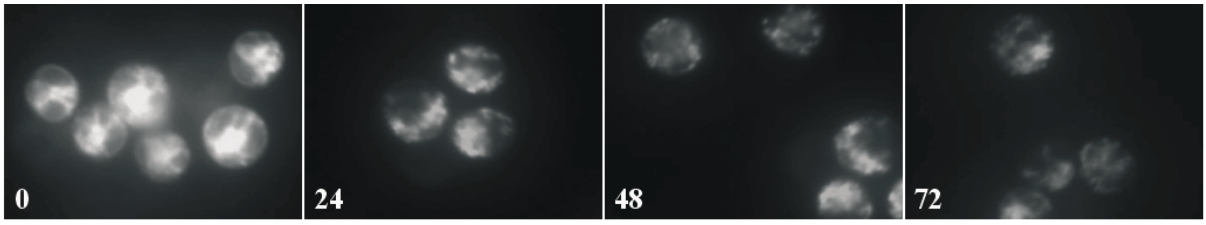


Figure 2. Fluorescence microscopy detection of mitochondrial membrane potential changes in murine L210 leukemia cells treated with NTCHMTQ (12 μM) for 24, 48 and 72 h using DiOC6. 0, negative control (cells treated with 0.1% DMSO). Magnification is 800x.

$DiOC_6$. As shown in Fig. 2, NTCHMTQ induced time-dependent decrease in $\Delta\psi_m$. Western blot analysis confirmed the release of cytochrome *c* in NTCHMTQ -treated cells (Fig. 4).

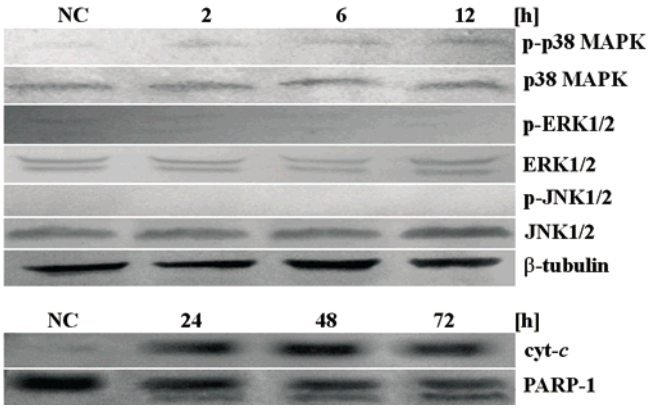


Figure 3. Effect of NTCHMTQ (12 μM) on activity of caspase 8 (A), caspase 9 (B) and caspase 3 (C) in murine L1210 leukemia cells after 24, 48 and 72 h of treatment. NC - control cells treated with 0.1% DMSO, PC - cells treated with 6 μM cisplatine.

To characterize the molecular events involved in NTCHMTQ-induced apoptosis, we examined the NTCHMTQ-induced activation of caspase 3 and caspase 8 and 9, up-regulators of caspase 3/7 activation, by monitoring the increase in spectrophotometric and luminescent signal of treated cells (Fig. 3). In our preliminary experiments [23], NTCHMTQ induced concentration- and time- dependent increase in caspase-3 activity. As presented in Fig. 3, NTCHMTQ treatment activated caspase 9, with slightly increased activity of caspase 8. Induction of apoptosis in L1210 cells by NTCHMTQ was also confirmed by detection of PARP-1 cleavage as a result of caspase 3 activation. 85 kDa fragment of PARP was detected by western blot analysis (Fig. 4).

To assess the changes in MAPK signaling in NTCHMTQ-treated cells, we exposed L1210 cells to NTCHMTQ for various times and the phosphorylation of extracellular-regulated ERK1/2, stress activated p38 MAPK and JNK1/2 kinases was determined by western blot analysis. As shown in Fig. 4, activation of p38 MAPK was evident after 6h and was maximal after 12h, respectively. No significant increase in phosphorylation of ERK1/2 or JNK1/2 was observed.

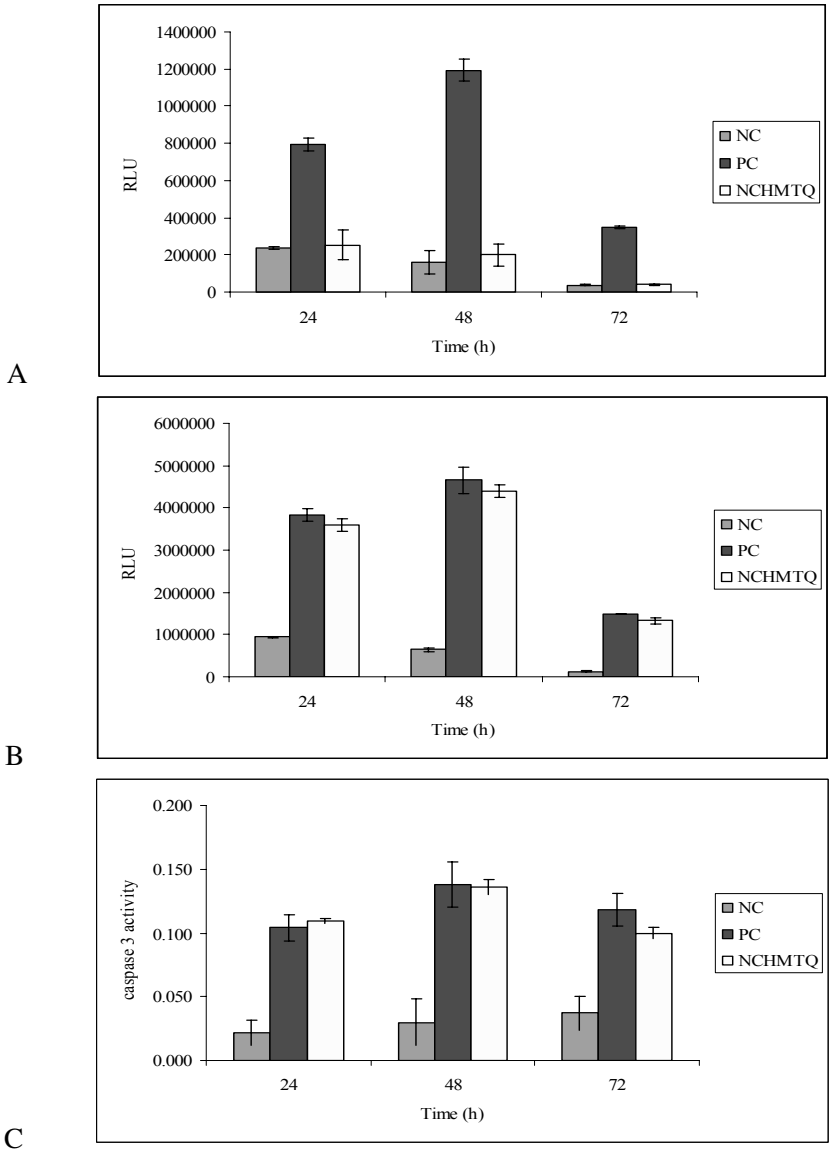


Figure 4. Effect of 12 μM NCHMTQ on phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK1/2, JNK1/2 (for 2, 6 and 12 h) and cyt-c, PARP-1 (for 24, 48 and 72 h) in L1210 cells. NC - negative control cells treated with 0.1% DMSO.

Discussion

Quinazolines are multitarget agents which have broad spectrum of biological activities and some of them are now in clinical testing. 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c]quinazoline (NTCHMTQ) is new synthetically prepared derivative, which in our previous study induced morphological changes and at nanomolar concentrations demonstrated changes of F-actin cytoskeleton. This derivative did not have mutagenic effect and did not behave as typical intercalating agent [15]. In addition, reduction of tumor volume (~11.2 %) in intramuscular transplanted B16 cells was observed on day 16 at doses of 50 mg/kg. Interestingly, NTCHMTQ induced the increase of the sub-G_0 cell fraction, induced apoptotic DNA fragmentation and morphological changes and activated caspase 3 in murine L1210 leukemia cells [21–23]. These results demonstrated that NTCHMTQ is potent inducer of apoptosis in L1210 cells and futher studies on the molecular mechanism regulating the NTCHMTQ-induced apoptosis are necessary.

In the present study, the molecular mechanisms underlying the NTCHMTQ-induced apoptosis of L1210 cells were investigated. It is known, that ROS play important role in the signaling pathways of cell proliferation, survival and apoptosis

[25]. Therefore, in our experiments we looked if reactive oxygen species (ROS) are elevated in NTCHMTQ-treated cells. We found, that NTCHMTQ is increasing the ROS level immediatelly after its application (Fig. 1). L1210 cells treated with 0.1% DMSO (negative control, Fig. 1) did not show ROS production analyzed by fluorescence microscopy. Increased level of ROS has been related to reduced mitochondrial membrane potential [25]. To investigate if elevated ROS caused changes in mitochondrial function, the mitochondrial membrane potential changes were studied. Our finding that in NTCHMTQ-treated cells the mitochondrial membrane potential is decreased (Fig. 2), was futher supported by the observation that this drug is releasing cytochrome *c* from mitochondria (Fig. 4). This indicates that mitochondrial pathway is directly involved in NTCHMTQ-induced apoptosis. Additionally, we demonstrated NTCHMTQ-induced activation of caspase 9 and caspase 3 followed by degredation of PARP-1 (Fig. 3 and 4).

Apoptosis can be initiated via two alternative signal pathways: the extrinsic pathway, which acts through death receptors on cell surfaces, and the intrinsic pathway, which acts through the mitochondria [26–28]. In the intrinsic pathway, the mitochondrial damage caused by apoptotic stimuli triggers the release of proapoptotic proteins including cytochrome *c*. Then cytochrom c caused caspases activation leading to PARP degradation and DNA fragmentation. Our results demonstrated the involvement of the intrinsic pathway in apoptosis of L1210 cells treated with NTCHMTQ. These results are consistent with the reported findings that some quinazolines (gefitinib, erlotinib, 2-phenoxymethyl-3H-quinazolin-4-one) stimulate mitochondrial apoptotic death of cancer cells [29–32]. The family of MAPKs plays central role in the signaling pathways, including extracellular-regulated kinase 1/2 (Erk1/2), stress activated protein kinase/c-Jun N-terminal kinase 1/2 (SAPK/JNK1/2) and p38 MAPK [32, 33]. In our experiments we found that NTCHMTQ treatment resulted in activation of p38 MAPK, with no activation of JNK1/2 and ERK1/2 (Fig. 4). These results imply that NTCHMTQ induces p38 MAPK activation, which seems to be mediated by NTCHMTQ-induced ROS. A connection of p38 MAPK activation with apoptosis in response to ROS was established recently [33, 34].

In summary, we investigated the molecular mechanisms involved in NTCHMTQ-induced apoptosis of L1210 leukemia cells. We showed NTCHMTQ-induced apoptosis involves the activation of caspase 9 pathway that results in caspase 3 activation. We also demonstrated that NTCHMTQ induces ROS generation and loss of mitochondrial membrane potential to cause the release of cytochrome *c* from mitochondria to result in apoptosis. Additionally, NTCHMTQ induces the p38 MAPK activation, mediated by NTCHMTQ-induced ROS generation.

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