New [1,2,4]triazolo[4,3-c]quinazoline enhances cisplatin- and temozolomide-induced growth inhibition and apoptosis in HL-60 cells

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The purpose of this study was to investigate the therapeutic potential of a newly synthesized [1,2,4]triazolo[4,3-c]quinazoline (NTCHMTQ) alone and in combination with two anticancer drugs (cisplatin and temozolomide) against HL-60 leukemia cell line. The IC_{50} value of NTCHMTQ toward HL-60 cells was 19.7 μM. No apoptosis and cell cycle changes were observed in cells treated with 5 μM NTCHMTQ alone. Combination of non-toxic concentrations of NTCHMTQ (1 – 5 μM) with cisplatin or temozolomide sensitized HL-60 cells to these two drugs and significantly enhanced their efficacies, that is illustrated by combination indexes, sub-G_0 cell fraction, apoptotic DNA fragmentation and caspase-3 activity. The results suggest that combined therapy of non-toxic concentrations of NTCHMTQ with chemotherapeutics may provide synergistic regimen for treatment of leukemia. However, further *in vitro* and *in vivo* experimental drug-cell and drug-drug studies are warranted.

Key words: chemotherapy, modulator, quinazoline, apoptosis, cisplatin, temozolomide

Quinazolines represent a class of drugs with a variety of biological activities, including antimicrobial, antiinflammatory, diuretic, anticonvulsant, antiallergic, antihypertensive, anticancer, antiparkinsonian and many others [1-4]. It has been shown that they act as antifolate synthase inhibitors, EGFR tyrosine kinase inhibitors, inhibitors of dihydrofolate reductase and tyrosine kinase. Some quinazolines interact with cytoskeleton, induce apoptosis and affect DNA topoisomerases [5-8].

In the work presented, a newly synthesized 3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c] quinazoline (NTCHMTQ) was studied. Previously it has been shown that this drug has antiproliferative effects on HeLa and B16 cells [9]. This work presents the antiproliferative/cytotoxic, cell cycle and apoptosis inducing effects of newly synthesized quinazoline, together with its effects on therapeutic efficacies of two currently used chemotherapeutics (cisplatin and temozolomide) in human promyelocytic leukemia HL-60 cell line.

Materials and methods

Drugs. Quinazoline, (3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c]quinazoline, NTCHMTQ, Fig.1), was synthesized according to Spirkova et al. [10]. Cisplatin and temozolomide were obtained from Sigma (St. Louis, MO, USA). The solution of NTCHMTQ (25 mM in DMSO) was stored at -20°C, protected from light. The final concentration of DMSO in the medium was <0.02% and did not affect cell growth.

Cell line. Human promyelocytic leukemia HL-60 cells were kindly provided by Dr. P. Ujhazy (Roswell Park Cancer Institute, Buffalo, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% FCS (PAN Biotech GmbH, Germany), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in an atmosphere of 5% CO_2 in humidified air at 37°C.

Drug treatment. Exponentially growing cells were harvested by centrifugation and resuspended in fresh medium to

Table 1. The cell cycle profile data of HL-60 cells treated with cisplatin, quinazoline derivative (NTCHMTQ) or their combination for 24 h.

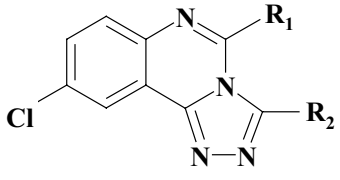
	sub-G_0	G_0/G_1	S	G_2/M
control	2.0 ± 0.6	40.3 ± 2.1	48.1 ± 3.7	11.6 ± 2.5
5 μM NTCHMTQ	1.7 ± 0.4	42.3 ± 3.5	45.5 ± 4.8	12.2 ± 3.1
4 μM cisplatin	2.2 ± 1.3	18.7 ± 4.9**	72.9 ± 6.2**	8.4 ± 2.8*
4 μM cisplatin + 5 μM NTCHMTQ	8.5 ± 3.1*	16.8 ± 2.7**	71.8 ± 3.3**	11.4 ± 3.9
6 μM cisplatin	2.6 ± 0.8	18.6 ± 4.3**	68.5 ± 2.4**	12.9 ± 2.1
6 μM cisplatin + 5 μM NTCHMTQ	19.3 ± 4.2**	15.2 ± 2.3**	79.4 ± 1.7**	5.4 ± 1.3**

Note: Data represent mean values ± s.d. from three independent experiments
*$P<0.05$ and **$P<0.01$ represent significant differences compared with control value

achieve culture density of $3x10^5$ cells/ml. The cells were treated with 1-30 μM NTCHMTQ, 2-6 μM cisplatin, 1-10 μM temozolomide, or with their combinations for 24 h (cisplatin) and 48 h (temozolomide), respectively. Cell viability was determined by trypan blue staining (0.4% Trypan blue stain solution).

Analysis of drug combination. The cell viability data were analyzed using CalcuSyn Version 2.0 program to determine the IC_{50} of each drug alone. The CI-isobologram by Chou and Talalay was used to analyze the drug combinations [11]. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were applied to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate). CI>1, CI=1, and CI<1 indicate antagonism, additive effect, or synergism, respectively.

Cell cycle measurement. The control and drug-treated cells ($0.5x10^6$) were harvested, washed twice in phosphate-buffered saline (PBS) and exposed to 0.05% Triton X-100 in PBS supplemented with RNAse (50 μg/ml) for 25 min at 37°C. Afterwards, DNA was stained by propidium iodide (50 μg/ml) for 15 min at 4°C. Samples were analyzed by FACStar (Becton-Dickinson, Mountain View, CA, USA) with the use of software provided by the manufacturer. A minimum of 10.000 cells per sample was analyzed at a flow rate of 200 cells/s.



R_1 - Morpholine
R_2 - 5-nitro-2-thienyl

Figure 1. Chemical structure of (3-(5-nitro-2-thienyl)-9-chloro-5-morpholin-4-yl[1,2,4]triazolo[4,3-c]quinazoline (NTCHMTQ).

Electrophoretic analysis of DNA fragmentation. The control and drug-treated cells ($1x10^6$) were harvested, washed in PBS and lysed with 100 μl of solution (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100) supplemented with proteinase K (1 mg/ml). Samples were then incubated at 37°C for 1 h and heated at 70°C for 10 min. Following lysis, RNAse (200 μg/ml) was added and repeated incubation at 37°C for 1 h followed. The samples were subjected to electrophoresis at 40 V for 3 h in 1.5% (W/V) agarose gel complemented with ethidium bromide (1 μg/ml). Separated DNA fragments were visualized using UV transilluminator (254 nm).

Caspase-3 activity assay. The cells were treated with vehicle (DMSO) or drugs for 24 or 48 h, respectively. Cell lysates were prepared and caspase-3 activity was measured according to the manufacturer's protocol (CaspACE™ Assay System, Promega Corporation, USA). Briefly, an equal amount of cell lysate proteins (adjusted to 10 μl with lysate buffer) was added to the reaction mixtures containing colorimetric substrate peptide specific for caspase-3

Table 2. Cytotoxicity and combination indexes (CIs) of cisplatin and quinazoline derivative (NTCHMTQ) after 24 h treatment of HL-60 cells.

Drugs	Fraction affected ± s.d.	CIs
2 μM cisplatin	0.238 ± 0.026	–
4 μM cisplatin	0.345 ± 0.042	–
6 μM cisplatin	0.603 ± 0.097	–
5 μM NTCHMTQ	0.000 ± 0.000	–
10 μM NTCHMTQ	0.126 ± 0.017	–
15 μM NTCHMTQ	0.368 ± 0.034	–
2 μM cisplatin + 5 μM NTCHMTQ	0.342 ± 0.083	0.966
4 μM cisplatin + 5 μM NTCHMTQ	0.563 ± 0.108	0.951
6 μM cisplatin + 5 μM NTCHMTQ	0.720 ± 0.124	0.866

Note: CI values are obtained from the mean values ± s.d. of three independent experiments
IC_{50} (cisplatin/24h) = 5.1 ± 1.2 μM
IC_{50} (NTCHMTQ/24h) = 19.7 ± 2.3 μM
CI > 1.0 antagonism
CI = 1.0 additive effect
CI < 1.0 synergism

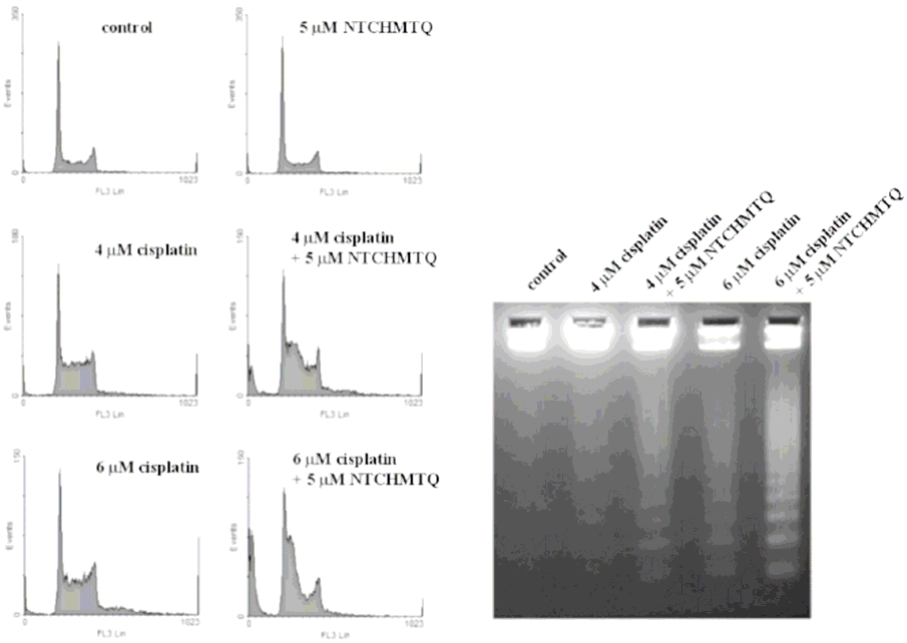


Figure 2. Effect of quinazoline derivative (NTCHMTQ), cisplatin or their combinations on cell cycle profile and apoptotic DNA fragmentation of HL-60 cells after 24 h treatment. The figure is representative of three independent experiments.

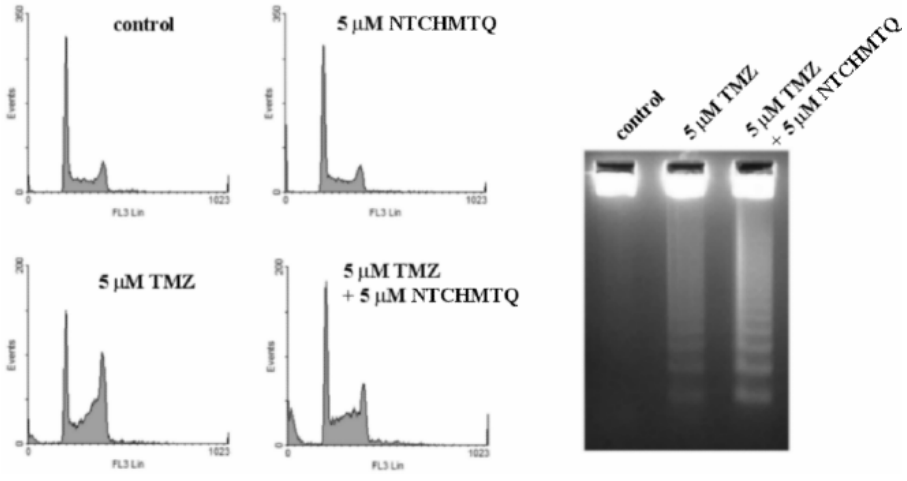


Figure 3. Effect of quinazoline derivative (NTCHMTQ), temozolomide (TMZ) or their combination on cell cycle profile and apoptotic DNA fragmentation of HL-60 cells after 48 h treatment. The figure is representative of three independent experiments.

(Ac-DEVD-pNA). The plate was incubated in the dark for 4 h at 37°C. Absorbance was determined using a spectrophotometric microplate reader (Humareader U.K.) (wavelength 405 nm). Protein concentrations were determined by Bradford method.

Statistical analysis. Student's *t*-test was used in statistical analysis between treated and control cells. $P<0.05$ and $P<0.01$ were considered as statistically significant.

Results and discussion

Many current cancer treatments approaches, including certain classes of chemotherapeutic agents, induce cytotoxicity via damaging the DNA. However, many cancers are resistant to these therapies thus, searching the agents that enhance the sensitivity of cancer cells to chemotherapy is highly desirable.

In this work, we studied the antiproliferative, cell cycle and apoptosis inducing activities of newly synthesized quinazoline derivative (see Fig. 1), as well as its ability to sensitize the HL-60 cells to cisplatin (DNA alkylating agent) and temozolomide (DNA methylating agent). These two drugs were chosen as chemotherapeutic agents that induce single strand DNA breaks [12]. It has been shown that some quinazoline derivatives are potent enhancers of therapeutic activities of these drugs [13, 14].

We found that NTCHMTQ has antiproliferative effect toward HL-60 cells. Value of IC_{50} was 19.7 ± 2.3 μM after 24 h treatment. Previously it has been shown that NTCHMTQ has antiproliferative activity [9]. In our study, 5 μM NTCHMTQ did not induce apoptosis of HL-60 cells even when used at concentrations inhibiting the cell growth to more than 90%. As it is presented, we did not detect any significant changes in cell cycle profile (see Table 1 and Table 3) and did not observe induction of apoptosis in HL-60 cells (data not shown).

Despite of low potential of NTCHMTQ to change the cell cycle profile and induce apoptosis, in the next experiments we evaluated a combined treatment of this drug with cisplatin and temozolomide. Recently it has been shown that some quinazoline derivatives can act as selective inhibitors of DNA repair pathways, and can act as very potent modulators of cell sensitivity to chemotherapeutics [15, 16].

Additionally, lack of p53 (HL-60 cells) or mismatch repair function are commonly associated with tumor cell resistance to antineoplastic agents [13]. The combined therapy of NTCHMTQ with cisplatin and temozolomide led to significant sensitization of HL-60 cells to these two chemotherapeutics. As it is shown, the combinations of non-toxic concentration of NTCHMTQ (5 μM) with cisplatin induced an increase in percentage of cells in sub-G_0 cell fraction from 2.2% and 2.6% for 4 μM and 6 μM cisplatin, respectively, to 8.5% and 19.3% for combination of NTCHMTQ and 4 μM or 6 μM cisplatin, respectively (see Table 1) and enhanced the apoptotic DNA fragmentation of HL-60 cells (see Fig. 2). The same results were observed for combination of NTCHMTQ with temozolomide after 48 h treatment (see Table 3 and Fig. 3). For both combinations (NTCHMTQ and cisplatin or NTCHMTQ and temozolomide), the activity of caspase-3 was significantly enhanced in comparison to cells treated with chemotherapeutics only (see Fig. 4). As it is presented in Table 2 and Table 4, the combination indexes (CIs) indicate that combined treatment led to synergism in antiproliferative activities of the drugs used. Differences in incubation time for drug combinations were due to various molecular mechanisms of their chemotherapeutic action and as well as due to differences in processing of DNA damage induced by these two drugs. In addition, it is worth to mention that the combination of cisplatin or temozolomide with a concentration of NTCHMTQ that showed antiproliferative effects led to rapid cell death after 8 h of treatment of HL-60 cells (data not shown). To check the effects of NTCHMTQ pre- and post-treatments on therapeutic efficacies of the chemotherapeutics used, HL-60 cells were treated with quinazoline 12 h before cisplatin or temozolomide applications, or 12 h after cisplatin or temozolomide applications. We did not observe significant differences compared to simultaneous treatment protocol (data not shown).

Taken together, results of this study provide strong evidence that combined treatment of non-toxic concentration of the newly synthesized derivative of quinazoline may provide synergistic regimen for treatment of leukemia. However, further *in vitro* and *in vivo* experiments leading to elucidation of molecular mechanisms of HL-60 cell sensitization by the tested quinazoline are warranted.

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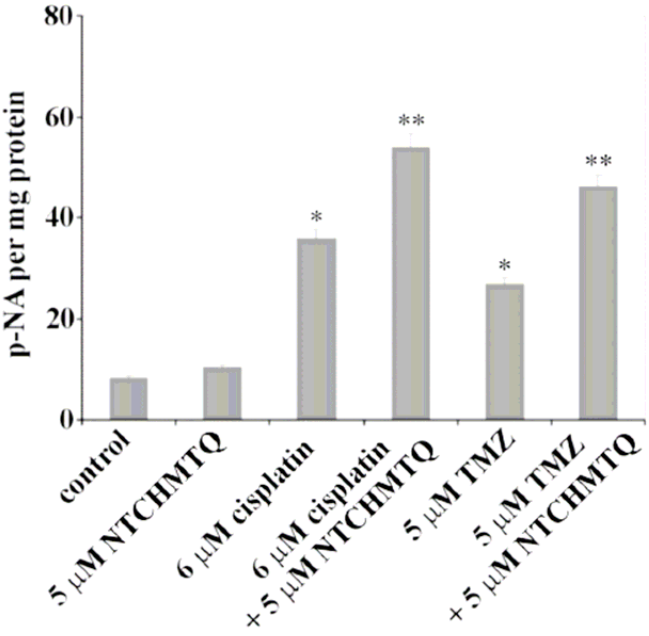

Figure 4. Caspase-3 activity of drug-treated HL-60 cells. The rate of DEVD-*p*NA cleavage was measured at 405 nm. The data represent the mean values ± s.d. of three independent experiments. *Note:* $^{*}P<0.05$ and $^{**}P<0.01$ represent significant differences compared with control value.

Table 3: The cell cycle profile data of HL-60 cells treated with temozolomide (TMZ), quinazoline derivative (NTCHMTQ) or their combination for 48 h.

	sub-G_0	G_0/G_1	S	G_2/M
control	2.3 ± 1.1	39.6 ± 1.9	47.3 ± 4.5	13.1 ± 2.7
5 μM NTCHMTQ	3.1 ± 1.4	38.1 ± 2.8	46.7 ± 3.6	15.3 ± 3.9
5 μM TMZ	6.3 ± 0.9*	22.7 ± 4.7**	54.3 ± 5.4*	23.1 ± 2.4*
5 μM TMZ + 5 μM NTCHMTQ	18.4 ± 2.4**	28.9 ± 3.3*	55.2 ± 3.8*	16.0 ± 2.8

Note: Data represent mean values ± s.d. from three independent experiments
$^{*}P<0.05$ and $^{**}P<0.01$ represent significant differences compared with control value

Table 4. Cytotoxicity and combination indexes (CIs) of temozolomide (TMZ) and quinazoline derivative (NTCHMTQ) after 48 h treatment of HL-60 cells.

Drugs	Fraction affected ± s.d.	CIs	
5 μM TMZ		0.370 ± 0.062	–
7.5 μM TMZ		0.482 ± 0.098	–
10 μM TMZ		0.667 ± 0.121	–
5 μM NTCHMTQ		0.000 ± 0.000	–
10 μM NTCHMTQ		0.527 ± 0.085	–
15 μM NTCHMTQ		0.944 ± 0.102	–
5 μM TMZ + 5 μM NTCHMTQ		0.640 ± 0.078	0.932
7.5 μM TMZ + 5 μM NTCHMTQ		0.789 ± 0.141	0.897

Note: CI values are obtained from the mean values ± s.d. of three independent experiments
IC_{50} (TMZ/48h) = 7.1 ± 0.8 μM
IC_{50} (NTCHMTQ/48h) = 11.2 ± 2.5 μM
CI > 1.0 antagonism
CI = 1.0 additive effect
CI < 1.0 synergism

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