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# ANTIPROLIFERATIVE ACTIVITY AND APOPTOSIS INDUCED BY 6-BROMO-2-(MORPHOLIN-1-YL)-4-ANILINOQUINAZOLINE ON CELLS OF LEUKEMIA LINES

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**Quinazolines are known to be multitarget agents with broad spectrum of biological activity. *Aim:* To investigate anticancer activity of newly prepared 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) towards L1210, HL-60 and U-937 leukemia cells. *Materials and Methods:* Growth inhibition of BMAQ-treated cells was determined by cell counting using trypan blue staining technique. Apoptosis and cell cycle profile changes were analysed using internucleosomal DNA fragmentation assay, fluorescence microscopy and flow cytometry. Activity of caspase-3 was determined using colorimetric method. *Results:* Cell proliferation assay showed that BMAQ caused significant decrease of cell number in a dose-dependent manner. BMAQ induced cell death by apoptosis, based on results from DNA fragmentation, fluorescence microscopy and caspase-3 assays. *Conclusion:* Presented results clearly demonstrate that BMAQ is a promising anticancer agent with significant antiproliferative and apoptotic activities towards leukemia cells *in vitro*.**

***Key Words:*** **quinazoline, apoptosis, leukemia.**

Quinazoline derivatives are known to possess a broad spectrum of biological activities and are used in pharmaceutical industry, in medicine and in agriculture because of their antimicrobial, antiinflammatory, diuretic, anticonvulsant, antiallergic, and other properties [1, 2]. As documented in the literature, many derivatives of quinazoline act as anticancer drugs [3, 4]. They act as multitarget agents possessing inhibitory activities against thymidylate synthase, dihydrofolate reductase, tyrosine kinase, and cyclic GMP phosphodiesterase [5–7]. Some quinazolines interact with cytoskeleton, induce apoptosis and inhibit DNA topoisomerase [8–10].

Based on the above-mentioned effects of quinazolines, a new series of substituted 4-anilinoquinazolines was prepared by Stankovsky *et al.* [11, 12]. These compounds were screened for antibacterial, mutagenic and cytotoxic activities *in vitro*. 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) was found be the most potent derivative. This drug inhibited the growth of HeLa and B-16 cells and induced changes in actin of HepG2 cells [13, 14].

The aim of this study was to evaluate the anticancer activity of BMAQ towards selected leukemia cells, i. e. murine L1210, human promyelocytic HL-60 and human promonocytic U-937 leukemia cells. Additionally, the effects of BMAQ on cell cycle and its potential to induce apoptosis were studied.

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*Abbreviations used:* BMAQ – 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline; DMSO – dimethyl sulfoxide; EDTA – ethylenediaminetetraacetic acid; EGFR – epidermal growth factor receptor; EtBr – ethidium bromide; FCS – fetal calf serum; PBS – phosphate-buffered saline; PI – propidium iodide; RNAse – ribonuclease A; RT – room temperature.

## MATERIALS AND METHODS

***Drug.*** 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) (Fig. 1) was synthesized according to Stankovsky et al. [11, 12]. The solution of BMAQ (10 mM in 100% DMSO) was stored at –20 °C, protected from light. The final concentration of DMSO in the medium was < 0.5% and did not affect cell growth.

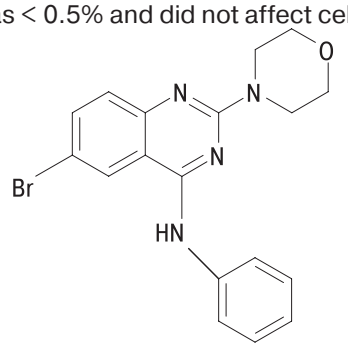


**Fig. 1.** Chemical structure of 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ)

***Cell line.*** Murine L1210 leukemia cells, human promyelocytic HL-60 leukemia cells and human promonocytic U-937 leukemia cells (ATCC, Rockville, MD, USA) were grown in RPMI-1640 medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in an atmoshere 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 achieve culture density of 8 x $10^4$/ml for L1210, 3 x $10^5$/ml for HL-60 and 2 x $10^5$/ml for U-937 cells. The cells were treated with 0.26–104.0 μM BMAQ for 24, 48 and 72 h. Cell number and viability were determined by trypan blue staining.

***Cell cycle measurement.*** Untreated and drug-treated cells (0.5 x $10^6$) were harvested, washed twice in 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 PI (50 μg/ml) for 15 min at 4 °C. Samples were analyzed by a Beckman-Coulter FC 500 flow cytometer (Beckman Coulter Inc, Fullerton, California, USA) with the use of DNA Cell Cycle Analysis Software distributed by Phoenix Flow Systems — MultiCycle AV for Windows. A minimum of 10000 cells per sample were analyzed at a flow rate of 200 cells/s.

***Analysis of apoptotic DNA fragmentation.*** Untreated and drug-treated cells ($1 \times 10^6$) 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 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 1.3% (w/v) agarose gel complemented with EtBr (1μg/ml). Separated DNA fragments were visualized using UV transilluminator.

***Fluorescence microscopy.*** Untreated and drug-treated cells were resuspended in 1 ml of fresh medium and 40 μl of Hoechst 33 258 (1 μg/ml) and 15 μl of PI (5 μg/ml) were added. Cell suspension was incubated for 30 min at RT. Cells were centrifuged, resuspended in 40 μl of fresh medium and monitored by fluorescence microscopy (Zeiss Jenalumar, Jena, Germany).

***Caspase-3 activity assay.*** Cells were treated with vehicle (DMSO) or 104 μM BMAQ for 24 h. Cell lysates were prepared and caspase-3 activity was measured according to the manufacturer`s protocol (CaspACE™ Assay System Colorimetric, 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 (Ac-DEVD-pNA). The plate was incubated in the dark for 24 h at 37 °C. Absorbance at 405 nm was determined using microplate reader (Humareader, Wiesbaden, SRN). Protein concentration was determined by Lowry method [15].

## RESULTS

***BMAQ inhibits growth and induces cell cycle profile changes.*** Proliferation of L1210, HL-60 and U-937 leukemia cells exposed to 0.26–104 μM of 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) was monitored within 24, 48 and 72 h. As shown in Table 1, BMAQ induced concentration- and time-dependent inhibition of cell proliferation. After 24 h of treatment, the highest tested concentration of BMAQ (104 μM) completely inhibited cell division and after 72 h, part of cell population degenerated. Assessment of cytoplasmic membrane integrity by trypan blue staining revealed that BMAQ did not affect the membrane integrity of leukemia cells significantly (data not shown).

As antiproliferative activity of anticancer drugs is connected with cell cycle arrest, in the next experiments we checked the cell cycle profile of BMAQ-treated leukemia cells. As presented in Table 2, BMAQ induced $G_2$/M cell cycle arrest of L1210 ($P < 0.05$) and HL-60 cells. U-937 cells were found to be arrested in $G_0/G_1$ phase ($P < 0.05$).

***BMAQ induces apoptosis of leukemia cells.*** To evaluate the type of cell death induced by BMAQ, we analyzed the cells treated with BMAQ for markers related to programmed cell death. As shown in Fig. 2, 104 μM BMAQ induced significant apoptosis represented by typical patern of internucleosomal DNA fragmentation. The double staining of treated cells by Hoechst and PI revealed the apoptotic bodies formation in treated leukemia cells (Fig. 3). Additionally, the activation of caspase-3 was confirmed by colorimetric assay ($P < 0.01$) (Fig. 4).

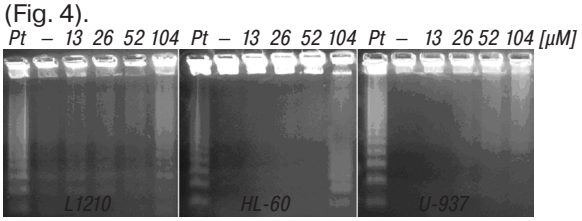


**Fig. 2.** Electrophoretic analysis of internucleosomal DNA fragmentation of A: L1210, B: HL-60 and C: U-937 leukemia cells treated with 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) for 24 h. Pt = 6.0 μM cisplatin. Figure is representative of three independent experiments

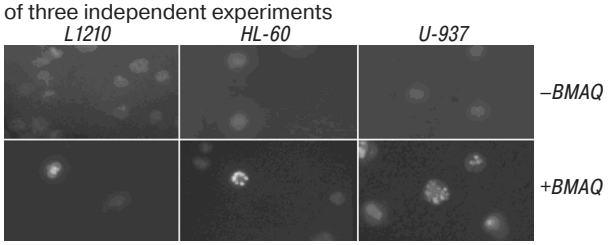


**Fig. 3.** Detection of apoptotic body formation in L1210, HL-60 and U-937 leukemia cells treated with 104.0 μM of 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) for 24 h by fluorescent microscopy (magnification = 600 X)

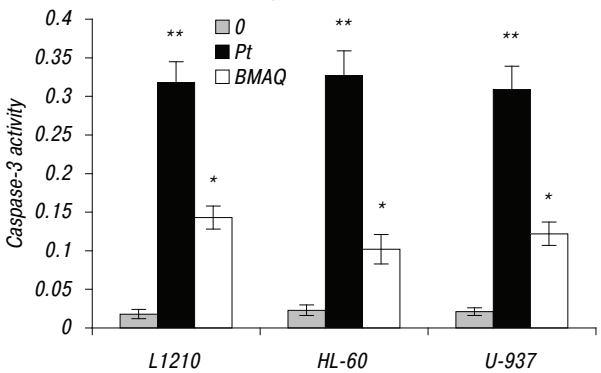


**Fig. 4.** Activity of caspase-3 after 24 h treatment of leukemia cells with the 104.0 μM of 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ). Pt = 6 μM cisplatine. Data represent mean values ± s. d. of three independent experiments
*$P < 0.01$, **$P < 0.001$.

## DISCUSSION

Recently we have synthetized a series of substituted 4-anilinoquinazolines. Some of these compounds showed biological activities towards bacteria and cancer cells [13, 14]. Although 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) did not possess antibacterial activities, this derivative manifested significant antiproliferative activity towards HeLa, B-16 and HepG2 cells [14].

In this study, we monitored the anticancer activity of BMAQ towards murine L1210, human promyelocytic HL-60 and human promonocytic U-937 leukemia cells. We found that BMAQ inhibits proliferation of all tested leuke-

mia cells in time- and concentration- dependent manner (see Table 1). This finding is consistent with our previous observation that BMAQ can significantly reduced the growth of HeLa, B-16 and HepG2 cells [14]. The fact that antiproliferative activities of anticancer drugs is connected with cell cycle changes prompted us to analyze the cell cycle profile of BMAQ-treated leukemia cells. We found that cell treated with the drug are arrested in $G_2$/M phase (L1210 and HL-60 cells) or in $G_0/G_1$ phase (U-937 cells) (see Table 2). Although we do not know reason for different effects of BMAQ on cell cycle profile of leukemia cells, it is likely that the differences in cell cycle perturbation are due to differences in responce of particular leukemia cells to this drug. The experiments elucidating the effects of BMAQ on cell cycle regulatory proteins (e. g. cyclins and cyclin-dependent kinases) are currently in progress.

**Table 1.** The values of $IC_{50}$ and $IC_{100}$ of 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) towards L1210, HL-60 and U-937 cells after 24, 48 and 72 h treatment

| | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ | $IC_{100}$ | $IC_{50}$ | $IC_{100}$ | $IC_{50}$ | $IC_{100}$ |
| L1210 | 35.8 ± 1.4 | > 104.0 | 13.3 ± 0.5 | 104.0 ± 1.6 | 10.9 ± 0.7 | 104.0 ± 1.2 |
| HL-60 | 47.1 ± 2.8 | > 104.0 | 15.3 ± 0.9 | > 104.0 | 13.8 ± 0.8 | 104.0 ± 1.8 |
| U-937 | 37.7 ± 1.9 | > 104.0 | 13.7 ± 0.8 | 104.0 ± 1.7 | 12.0 ± 0.8 | 104.0 ± 1.9 |

*Note.* Data represent mean values ± s. d. (μM) of three independent experiments.

**Table 2.** Cell cycle analysis of L1210, HL-60 and U-937 leukemia cells treated with 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline (BMAQ) for 24 h

| | BMAQ [μM] | $G_0/G_1$ | S | $G_2$/M |
|---|---|---|---|---|
| L1210 | 0 | 44.08 ± 4.26 | 50.40 ± 4.84 | 5.53 ± 0.47 |
| | 13.0 | 38.37 ± 3.75 | 52.29 ± 4.97 | 9.34 ± 0.76 |
| | 26.0 | 38.71 ± 3.74 | 51.63 ± 4.87 | 9.65 ± 0.83 |
| | 52.0 | 35.39 ± 3.47* | 54.10 ± 5.17 | 10.51 ± 0.97* |
| HL-60 | 0 | 49.86 ± 3.47 | 38.83 ± 3.51 | 11.31 ± 0.95 |
| | 13.0 | 46.08 ± 3.98 | 39.64 ± 3.02 | 14.28 ± 0.98 |
| | 26.0 | 47.58 ± 4.00 | 39.29 ± 3.24 | 13.13 ± 1.00 |
| | 52.0 | 46.38 ± 4.10 | 39.37 ± 2.95 | 14.25 ± 1.23 |
| U-937 | 0 | 43.19 ± 4.21 | 41.71 ± 3.86 | 15.10 ± 1.16 |
| | 13.0 | 52.90 ± 4.68* | 41.44 ± 3.97 | 5.66 ± 0.41* |
| | 26.0 | 55.78 ± 4.95* | 40.13 ± 3.78 | 4.10 ± 0.26* |
| | 52.0 | 57.47 ± 5.01* | 35.73 ± 2.98 | 6.81 ± 0.51* |

*Note.* Data represent mean values ± s.d. of three independent experiments. *$P < 0.05$.

Numerous studies have demonstrated that apoptosis may be involved in cell death induced by different chemotherapeutic agents. Apoptosis can be executed through two basic signalling pathways: one is mediated by death receptors on the cell surface and the other is mediated by mitochondria [16, 17]. Accumulating evidence reveals that the efficacy of antitumor agents is related to the intrinsic propensity of the tumor cells to respond to particular agents by apoptosis. Therefore, in the next experiments we analysed BMAQ-treated leukemia cells for apoptotic DNA fragmentation, apoptotic bodies formation and activation of caspase-3. We found that all leukemia cells treated with BMAQ are presenting typical apoptotic markers, as internucleosomal DNA fragmentation (see Fig. 2), apoptotic bodies formation (cells had undamaged cytoplasmic membrane) (see Fig. 3) and activation of caspase-3 (see Fig. 4). Our findings are consistent with previous studies demonstrating the ability of some quinazoline derivatives to induce changes in cell cycle profile and to induce apoptosis [3–7, 14].

In summary, we can conclude that BMAQ possesses significant antiproliferative activity and is potent inducer of programmed cell death in leukemia cells.

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# АНТИПРОЛИФЕРАТИВНОЕ И ПРОАПОПТОТИЧЕСКОЕ ДЕЙСТВИЕ 6-БРОМО-2-(МОРФОЛИН-1-ИЛ)-4-АНИЛИНОИНАЗОЛИНА НА ЛЕЙКОЗНЫЕ КЛЕТКИ

**Квиназолины известны как химиопрепараты широкого спектра действия. *Цель:* на моделях лейкозных клеток линий L1210, HL-60 и U-937 изучить противоопухолевую активность нового препарата 6-бромо-2-(морфолин-1-ил)-4-аналиноиназолина (BMAQ). *Методы:* ингибирование роста клеток под действием BMAQ изучали путем подсчета количества клеток, окрашенных трипановым синим. Апоптоз и изменения профиля клеточного цикла исследовали с помощью флуоресцентной микроскопии, электрофореза ДНК и проточной цитометрии. Активность каспазы-3 определяли колориметрическим методом. *Результаты:* показано, что BMAQ вызывает значительное дозозависимое уменьшение количества лейкозных клеток. При этом клетки, обработанные BMAQ, погибают путем апоптоза, что подтверждается образованием апоптотических телец, межнуклеосомной фрагментацией ДНК и активацией каспазы-3. *Выводы:* представленные результаты свидетельствуют о том, что BMAQ обладает антипролиферативной и проапоптотической активностью в отношении лейкозных клеток *in vitro*.**

***Ключевые слова:*** **иназолин, апоптоз, лейкоз.**