GROWTH INHIBITION AND APOPTOSIS INDUCED BY 2-PHENOXYMETHYL-3H-QUINAZOLIN-4-ONE IN HL-60 LEUKEMIA CELLS

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Aim: The aim of the study was to investigate anticancer activity of newly synthesized 2-phenoxyethyl-3H-quinazolin-4-one (PMQ).

Materials and Methods: Anticancer activity of PMQ was studied towards human HL-60 leukemia cells. Antiproliferative activity of PMQ was determined by direct counting of cells using trypan blue staining technique. Apoptosis and cell cycle profile changes were analysed using internucleosomal DNA fragmentation assay and flow cytometry. Activation of caspases and changes in glutathione level were monitored using colorimetric or luminiscent methods.

Results: PMQ induced concentration-dependent cytotoxicity in leukemia cells, with IC₅₀ of 10.8 ± 0.9 μM. DNA flow cytometry analysis and DNA ladder formation assay indicated that PMQ actively induced apoptosis of cells accompanied by a block of cells in G₂/M phase and a marked loss of cells in G₁/G₀ and S phases. Additionally, the activities of caspase-3 and caspase-9 were increased significantly and a markedly increased level of oxidized glutathione was observed. Inhibition of glutathione synthesis using buthionine sulfoximine sensitized leukemia cells to PMQ, confirming the involvement of ROS in PMQ-induced apoptosis.

Conclusion: The results of this study clearly demonstrate that PMQ is a promising anticancer drug showing cytostatic and apoptotic effects toward HL-60 leukemia cells mainly through mitochondrial/caspase-9 dependent pathway.

Key Words: quinazoline, leukemia, apoptosis, cell cycle arrest.

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

Quinazolines represent a class of drugs with a variety of biological activities, including antimicrobial, antiinflammatory, diuretic, antiinconvulsant, antiallergic, anticancer, and many others [4–7]. 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, affect DNA topoisomerases and potentiate the efficacy of chemotherapeutics [8–13].

In the work presented, anticancer properties of a newly synthesized 2-phenoxyethyl-3H-quinazolin-4-one (PMQ) were studied. PMQ was chosen from series of fifteen 2-substituted quinazolin-4-ones that shown to inhibit the growth of HeLa cells [14]. The antiproliferative, cell cycle profile modulating and apoptotic properties of PMQ were studied, complemented with experiments elucidating the molecular mechanism of PMQ-induced apoptosis in more detail, such as abilities of PMQ to induce oxidative stress and to activate the caspases -3, -8 and -9.

MATERIALS AND METHODS

Drug. 2-phenoxyethyl-3H-quinazolin-4-one (PMQ) (Fig.1) was synthesized according to Spirkova et al. [14]. The solution of PMQ (20 mM in DMSO) was stored at −20 °C, protected from light. The final concentration of DMSO in the medium was < 0.01% and did not affect cell growth.

Cell line. Human promyelocytic leukemia HL-60 cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in an atmosphere of 5% CO₂ 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 3 x 10⁵ cells/ml. The cells were treated with 1.0–15.0 μM PMQ for 24 h. In experiments with BSO, the cells were exposed to 0.5 mM BSO 24 h prior to PMQ treatment. Cell number and viability were determined by trypan blue staining.

Cell cycle measurement. Untreated and drug-treated cells (0.5 x 10⁵) were harvested, washed twice in phosphate-buffered saline (PBS) and exposed to 0.05%
Triton X-100 in PBS supplemented with RNase A (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 Coulter Epics Altra flow cytometer with the use of software provided by the manufacturer. A minimum of 20,000 cells per sample was analyzed at a flow rate of 200 cells/s.

Electrophoretic analysis of apoptotic DNA fragmentation. Untreated and drug-treated cells (1 × 10⁶) 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 A (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 2 h in 1.5% (w/v) agarose gel complemented with EtBr (1 μg/ml). Separated DNA fragments were visualized using UV transilluminator (254 nm).

Caspase-3 activity assay. Cells were treated with vehicle (DMSO) or 5.0 and 10.0 μM PMQ for 24 h. 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 lystate 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 4 h at 37°C. Absorbance was determined after 8 h using a spectrophotometric microplate reader (Humareader, U.K., 405 nm). Protein concentrations were determined by Bradford method.

Caspase-8 and caspase-9 activities assays. Cells were treated with vehicle (DMSO) or 10.0 μM PMQ for 24 h. Caspase-8 and caspase-9 activities were measured according to the manufacturer’s protocol (CaspGlo™ Assay, Promega Corporation, USA). Briefly, 100 μl of Caspase-Glo™ 8 Reagent (containing Z-LETD-aminoluciferin) and 100 μl of Caspase-Glo™ 9 Reagent (containing Z-LEHD-aminoluciferin), respectively, were added to the test tube with 100 μl of cell suspension containing 50,000 cells and mixed. The luminescence signal was measured after 30 min using a Glomax®/20 Luminometer w/Dual Auto-Injector (Turner BioSystems, USA).

Glutathione assay. To determine total glutathione (GSHt), 10 μl of cell lysate was incubated at 30°C with 80 μl of 0.3 mM NADPH, 125 mM sodium phosphate buffer with 6.3 mM EDTA, pH 7.5, and 10 μl of 6 mM DTNB. After addition of 20 μl of 25 U/ml GSH reductase the change in absorbance at 412 nm was measured using Spekol 221. To measure oxidized glutathione (GSSG), reduced glutathione (GSH) in samples was derivatized by adding 0.2 μl of 20 mM 4-vinylpyridine per 12.5 μl solution and mixed vigorously for 1 min. GSSG was measured in the same manner as GSHt. The concentration of GSH was calculated as the difference between GSHt and GSSG. In parallel experiments, the number of cells was determined in order to express the results as the amount of GSHt (nanomoles) per 10⁶ cells.

Statistical analysis. Data are presented as the means ± S.D. of three independent experiments. The median and the standard deviation were calculated using Excel (Microsoft Office, Version 98). The statistical significance of the results obtained was evaluated by the Student’s t-test, with probability values of 0.05 being considered as significant.

RESULTS

PMQ inhibits growth and induces apoptosis in HL-60 cells. Growth of HL-60 leukemia cells exposed to 1.0–15.0 μM of 2-phenoxy methyl-3H-quainazolin-4-one (PMQ) was monitored within 24 h. As shown in Fig. 2a, PMQ induced concentration-dependent inhibition of cell proliferation with IC₅₀ of 10.8 ± 0.9 μM. Assessment of cytoplasmic membrane integrity revealed that PMQ at tested concentrations did not affect the membrane integrity of treated cells significantly. The growth inhibition induced by PMQ was accompanied by apoptotic cell death and cell cycle profile changes. As presented in Fig. 2b, 5.0 μM PMQ induced significant apoptotic DNA fragmentation. Induction of apoptosis was confirmed by an appearance of sub-Gₒ cell population. Cell cycle analysis revealed that PMQ induced concentration-dependent Gₒ/M cell cycle arrest (53.2 ± 3.2% for cells treated with 15.0 μM PMQ compared to 11.6 ± 2.8% for untreated cells) with a marked loss of cells in G₀/G₁ and S phases (Table 1).
Table 1. Effect of 2-phenoxymethyl-3H-quinazolin-4-one (PMQ) on cell cycle profile of HL-60 leukemia cells after 24 h treatment

<table>
<thead>
<tr>
<th>PMQ</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>1.5 ± 0.9</td>
<td>39.2 ± 1.9</td>
<td>49.2 ± 4.2</td>
</tr>
<tr>
<td>5.0 μM</td>
<td>4.7 ± 1.3</td>
<td>40.9 ± 2.5</td>
<td>37.6 ± 3.1</td>
</tr>
<tr>
<td>10.0 μM</td>
<td>12.6 ± 2.8</td>
<td>29.7 ± 3.6</td>
<td>30.4 ± 2.9</td>
</tr>
<tr>
<td>15.0 μM</td>
<td>24.5 ± 2.9</td>
<td>21.1 ± 2.9</td>
<td>25.7 ± 4.4</td>
</tr>
</tbody>
</table>

Note: Data represent mean values ± S.D. of three independent experiments.

PMQ activates caspases and induces oxidative stress response in HL-60 cells. To address which caspases are activated by PMQ treatment in HL-60 cells were treated with 5.0 μM and 10.0 μM PMQ for 24 h. As shown in Fig. 3a, activity of caspase-3 in cells treated with 5.0 μM PMQ was detected to be 2.8 times higher (P < 0.01), and cells treated with 10.0 μM PMQ had above 5.1 times higher activity (P < 0.01) of the caspase-3 compared to untreated cells. Activity of caspase-9 in cells treated with 10.0 μM PMQ was increased about 1.9 times compared to untreated cells (P < 0.01). Caspase-8 was not activated significantly (Fig. 3b).

Fig. 3. Activation of caspase-3 (a) and caspase-8 and -9 (b) by 2-phenoxymethyl-3H-quinazolin-4-one (PMQ) in HL-60 leukemia cells. Data represent mean values ± S.D. of three independent experiments.

Note: cis-Pt = cisplatin; i = caspase-3 inhibitor; *P < 0.05; **P < 0.01.

Reactive oxygen species (ROS) play an important role in signalling leading to apoptosis. Many chemicals elevate the level of ROS, but only a few are able to change the redox state of cell significantly, with no damage to the cytoplasmic membrane. To examine if PMQ has a potential to change the redox state of cells, levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured. As presented in Table 2, the cells treated with 2.5–10.0 μM PMQ elevated the level of total GSH (GSH + GSSG = GSHt). While the level of GSH in cells treated with 10.0 μM PMQ increased above 2 times (from 5.82 ± 0.53 to 10.97 ± 0.56 nmol/10⁶ cells), the content of GSSG increased nearly 3 times (from 0.24 ± 0.06 to 0.68 ± 0.09 nmol/10⁶ cells). GSSG/GSH ratio changed from 0.041 to 0.062, showing that PMQ induced significant changes in redox state of HL-60 cells (P < 0.01).

Table 2. Effect of 2-phenoxymethyl-3H-quinazolin-4-one (PMQ) on GSSG/GSH ratio in HL-60 leukemia cells after 24 h treatment

<table>
<thead>
<tr>
<th>PMQ</th>
<th>GSHt</th>
<th>GSSG</th>
<th>GSSG/GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>6.6 ± 0.6</td>
<td>0.24 ± 0.06</td>
<td>0.041</td>
</tr>
<tr>
<td>2.5 μM</td>
<td>8.57 ± 0.26</td>
<td>0.39 ± 0.08</td>
<td>0.046*</td>
</tr>
<tr>
<td>5.0 μM</td>
<td>10.00 ± 0.33</td>
<td>0.52 ± 0.11</td>
<td>0.052**</td>
</tr>
<tr>
<td>10.0 μM</td>
<td>10.97 ± 0.56</td>
<td>0.68 ± 0.09</td>
<td>0.062**</td>
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Note: Cellular levels of GSH and GSSG are in nmol per 10⁶ cells. Data represent mean values ± S.D. of three independent experiments. *P < 0.05; **P < 0.01.

Pretreatment of HL-60 cells with buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamyl-cysteine synthetase, was applied to evaluate the involvement of ROS in PMQ-induced apoptosis. Incubation of cells with 0.5 mM BSO for 24 h decreased GSHt content from 6.06 ± 0.62 nmol/10⁶ cells to 0.55 ± 0.12 nmol/10⁶ cells (Fig. 4a). The PMQ did not increase the level of GSHt in cells pretreated with BSO. BSO-pretreated cells were more prone to die by apoptosis when treated with 2.5 μM PMQ, confirming the important role of ROS in apoptotic signalling induced by PMQ (Fig. 4b).
DISCUSSION

Many quinazolines exhibit antiproliferative activity against various tumor cell lines. However, mechanism responsible for their antiproliferative activity varies considerably. It includes induction of cell cycle arrest, direct cytotoxicity through the initiation of apoptosis, effect on membrane integrity, inhibition of antifolate synthase, EGFR tyrosine kinase, dihydrofolate reductase or tyrosine kinase, or effect on activity of DNA topoisomerases [4–13].

In this study, anticancer properties of a newly synthesized 2-phenoxymethyl-3H-quinazolin-4-one (PMQ) were studied. PMQ was chosen as the best candidate from series of fifteen newly synthesized 2-substituted quinazolin-4-ones. These quinazolines have been shown to inhibit growth of HeLa cells [14]. The antiproliferative, cell cycle profile modulating and apoptotic properties of PMQ are presented, complemented with experiments showing that PMQ induced apoptosis through mitochondrial/caspase-9 dependent pathway, and experiments confirming the importance of ROS in signalling leading to PMQ-induced apoptosis.

Antiproliferative activity of PMQ occurred at concentrations as low as 1.0 μM and 5.0 μM of PMQ was required to induce features typically associated with apoptosis. Analysis of HL-60 cells treated with 5.0 μM of PMQ provided clear evidence that PMQ-treated cells were dying by apoptosis as sub-G0 cell fraction (Table 1), apoptotic DNA fragmentation (Fig. 2, b) and active forms of caspase-9 and caspase-3 (Fig. 3) were detected. No significant cytoplasmic membrane damage was observed in conjunction with observed apoptotic features. These changes closely resemble those induced by cisplatin, the clinically used anticancer drug, that was used in our experiments as positive control. Additionally to apoptosis, block of cells in G0/M phase and decrease in percentage of cells in G0/G1, and S phases were observed (Table 1). Experiments leading to elucidation of the molecular mechanism by which PMQ induces cell cycle profile changes are in progress. It is possible that PMQ inhibits some cyclin-dependent kinases or some other regulatory proteins that are directly involved in regulation of cell cycle progression. It has been shown previously that some quinazoline derivatives are potent inhibitors of cyclin-dependent kinases [15–17].

Cell proliferation and survival are influenced by reactive oxygen species. Previous reports showed that some drugs can induce elevation of ROS levels during induction of apoptosis [18, 19]. Here, we showed that in PMQ-induced apoptosis ROS play an important role in signalling leading to apoptotic cell death. Increased levels of GSH and GSSG indicated the presence of significant oxidative stress in cells treated with PMQ for 24 h. Additionally, pretreatment of cells with BSO, the inhibitor of glutathione synthesis, sensitized the cells to PMQ-induced apoptosis. It has been shown previously that depletion of GSH sensitized cancer cells to therapeutic efficacy of some drugs, which anticancer potential depends on ROS production [20, 21].

There is always a temptation to extrapolate the significance of in vitro concentration based cell responses to in vivo response. In vivo experiments using L1210-bearing mice showed that PMQ prolonged the survival rate of leukemia bearing mice significantly, confirming the anticancer activity of PMQ (P < 0.0001). Therapeutic index of PMQ was comparable to therapeutic index of cisplatin (our unpublished results).

In summary, results of this study clearly demonstrate that PMQ is a promising anticancer drug showing cytostatic and apoptotic effects mainly through mitochondrial/caspase-9 dependent pathway.

ACKNOWLEDGEMENTS

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REFERENCES


ИНГИБИРОВАНИЕ РОСТА И АПОПТОЗ, ИНДУЦИРОВАННЫЕ 2-ФЕНОКСИМЕТИЛ-3Н-ХИНАЗОЛИН-4-ОНОМ, В ЛЕЙКОЗНЫХ КЛЕТКАХ ЛИНИИ HL-60

Цель: изучить антипролиферативную активность нового синтезированного 2-феноксиметил-3H-хиназолин-4-она (PMQ).

Материалы и методы: антипролиферативную активность PMQ определяли по отношению к клеткам лейкоза линии HL-60 в тесте с трипановым синим при стандартном подсчете клеток. Апоптоз и клеточный цикл оценивали с помощью проточной цитометрии и анализа фрагментации внутриядерной ДНК. Активацию каспаз и изменения уровня глутатиона определяли колориметрическими или люминесцентными методами.

Результаты: PMQ индуцирует дозозависимую цитотоксичность в клетках линии HL-60 (IC50 при 10,8 ± 0,9 μM). При проведении анализа ДНК с применением проточной цитометрии и определением формирования апоптотической лестницы было показано, что PMQ активно индуцирует апоптоз и блокаду клеточного цикла в G2/M фазе митоза и выраженной потерей клеток в G0/G1 и S фазах. Кроме того, была достоверно повышена активность каспазы-3 и -9 и выраженно увеличен уровень окисленного глутатиона. Применение бутионин сульфоксимина привело к уменьшению синтеза глутатиона и повышению чувствительности клеток HL-60 к PMQ, что подтверждает факт участия РФК в PMQ-индукрованном апоптозе.

Выводы: PMQ проявил себя как потенциальное противоопухолевое средство против клеток лейкоза человека HL-60 с выраженным цитостатическим и проапоптическим действием.

Ключевые слова: хиназолин, лейкоз, апоптоз, блокада клеточного цикла.