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G1 Arrest of the Cell Cycle by Gomisin N, a Dibenzocyclooctadiene Lignan, Isolated from *Schizandra chinensis* Baill in Human Leukemia U937 Cells

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We investigated the anti-cancer effects of two dibenzocyclooctadiene lignans, gomisin A and gomisin N, isolated from *Schizandra chinensis* Baill, in human promyelocytic U937 cells. Gomisin N, but not gomisin A, inhibited cell growth in a concentration-dependent manner, which was associated with the induction of G1 arrest of the cell cycle. G1 arrest induced by gomisin N was correlated with down-regulation of cyclin E, cyclin-dependent kinase (Cdk) 2 and Cdk4, and a concomitant up-regulation of Cdk inhibitors such as p16 (INK4A) and p21 (WAF1/CIP1). Furthermore, gomisin N inhibited phosphorylation of retinoblastoma protein (pRB) and p130, and expression of transcription factor E2Fs. The results indicated that growth inhibition by gomisin N is related to cell cycle arrest at G1 in U937 cells and these findings suggest that gomisin N may be a useful chemotherapeutic agent.

Key words : Gomisin N, U937, cell cycle, G1 arrest

Introduction

The progression of eukaryotic cells through the cell cycle is orchestrated by sequential activation and inactivation of the cyclin-dependent kinases (Cdks), which is associated with their respective cyclin subunits [5,18,21]. Early G1 phase is regulated by complexes of D-type cyclins and Cdk4/6, and the G1/S transition is controlled by a complex of cyclin E and Cdk2 [18]. On the other hand, Cdk inhibitors play a key role in negative regulation of cell cycle progression by binding to cyclin/Cdk complexes [18,21]. Furthermore, retinoblastoma protein (pRB) family proteins such as pRB and p130 are also important for cell cycle progression during the G1 to S phase transition [5,18]. Accumulating data have shown that many chemopreventive and/or chemotherapeutic agents can cause cell cycle arrest, which is the preferred method of managing cancer. Therefore, the induction of cell cycle arrest is an important mechanism in the anti-cancer properties of many drugs [6,11].

Schizandra chinensis Baill, a member of the Magnoliaceae family, has been used as a useful material in the regulation of various pathological conditions over the last several decades in Korea, China, and Japan [9,16]. The seeds and fruits of *S. chinensis* are enriched in lignans belong to the dibenzocyclooctadiene type, and more than 40 lignans have been isolated from this plant [1,16,19]. Some lignans have previously been reported to induce cell cycle arrest and apoptosis in human cancer cell lines including leukemia cells [4,8,14,17]. However, the underlying molecular mechanisms for their putative therapeutic effects are not clear. As part of an ongoing study to isolate chemopreventive or therapeutic compounds from *S. chinensis*, we isolated several lignans from the fruit of this plant and compared their anti-cancer properties. In this study, we compared the anti-proliferative activities of two dibenzocyclooctadiene lignans, gomisin N and gomisin A, as potential anticancer agents. To this end, we examined whether these lignans affected cell cycle progression in human leukemia U937 cells.

Materials and Methods

Cell culture and cell viability study

Human leukemia U937 cells were obtained from the American Type Culture Collection (Rockville, MD) and cul-

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tured in RPMI1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 2 mM gutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified environment with 5% CO_2 at 37°C. Gomisin A and gomisin N isolated from dried fruits of *S. chinensis* were prepared as described previously (Fig. 1A,) [2], dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 100 mM concentration, and stored in aliquots at -20°C. Cell viability was assessed using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide] assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. For morphological study, the cells were treated with gomisin A and gomisin N for 48 hr and directly photographed with an inverted microscope (Carl Zeiss, Germany).

DNA flow cytometric analysis

After treatment with gomisin A and gomisin N for 48 h, cells were harvested, washed twice with ice-cold PBS, fixed with 75% ethanol at 4°C for 30 min, and stained using a DNA staining kit (CycleTEST PLUS Kit, Becton Dickinson, San Jose, CA) with propidium iodide (PI). Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber, Becton Dickinson) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence.

Protein extraction and Western blot analysis

Treated cells with gomisin A and gomisin N were collected with ice-cold PBS, and immediately lysed with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris–Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$) containing protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). After normalization, total proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electro-blotting. The membranes were blocked with 5% skim milk, probed with the desired antibodies for 1 hr, incubated with the diluted enzyme-linked secondary antibodies and visualized by

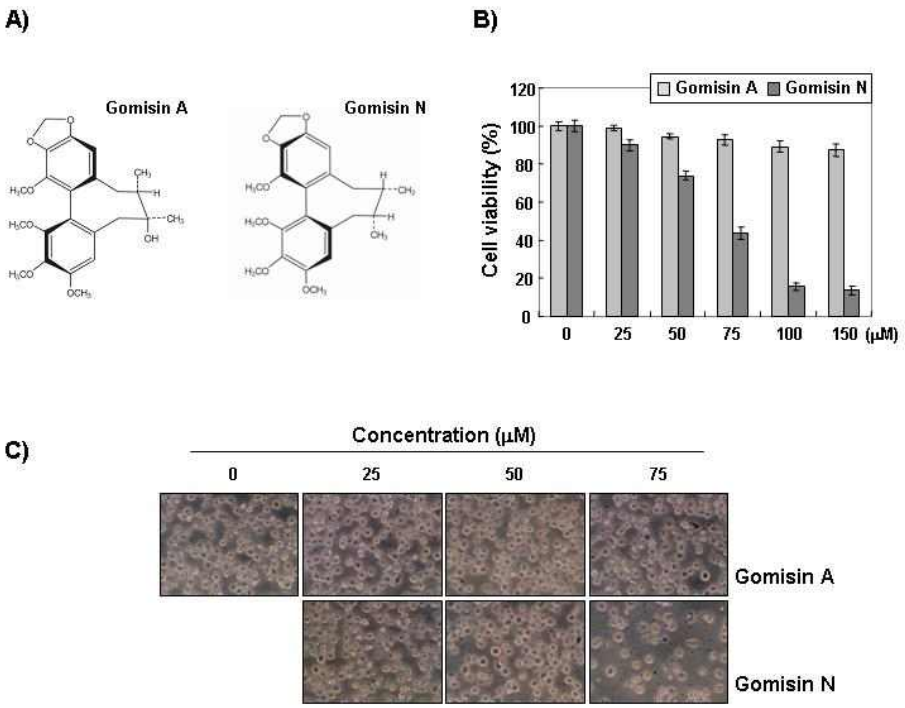


Fig. 1. Growth inhibition of U937 cells by gomisin N treatment. (A) Chemical structures of gomisin A and gomisin N. (B) U937 cells were plated at a concentration of $1x10^5$ cells per 60-mm plate and then incubated for 24 hr, after which they were treated with various concentrations of gomisin A and gomisin N for 48 h. Cell viability was determined by MTT assay. Results are expressed as percentage of control±SD and represent the average of three separate experiments. (C) After treating the cells with gomisin A and gomisin N for 48 hr, they were observed using an inverted microscope (original magnification 200×).

enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp., Arlington Heights, IL). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Cell Signaling Technology, Inc. (Boston, MA). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp.

Statistical analysis

All data are presented as the mean±SD. The results shown in each of the figures in this article are representative of at least two or three independent experiments.

Results

Growth inhibition by gomisin N

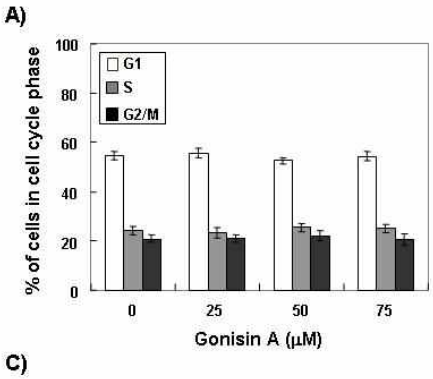
In order to investigate whether gomisin A and gomisin N inhibit cell growth, U937 cells were treated with various concentrations of gomisin A and gomisin N for 48 hr and the MTT assay was used to assess cell viability. As shown in Fig. 1B, treatment with gomisin N decreased the viability of U937 cells in a concentration-dependent manner, whereas gomisin A did not inhibit the growth of U937 cells under the same conditions. Under the inverted microscope, gomisin N-treated cells, but not gomisin A, exhibited granulated morphology, and eventually degraded after treatment with 75 mM gomisin N (Fig. 1C).

Induction G1 arrest by gomisin N

To determine whether gomisin N treatment of cells resulted in the alteration of cell cycle progression, the cell cycle patterns of the U937 cells were examined. As shown in Fig. 2, the growth inhibiting action of gomisin N was the consequence of a G1 phase arrest. At a concentration of 75 μM, 69.26% of cells were in G1 phase and far fewer cells were in S and G2/M phases (16.41% and 14.33%, respectively) compared with the control. At the same concentration of gomisin A, there was no significant increase in the proportion of U937 cells in G1 phase. These results suggest that the growth inhibitory effect of gomisin N, but not gomisin A, in U937 cells is the result of G1 arrest.

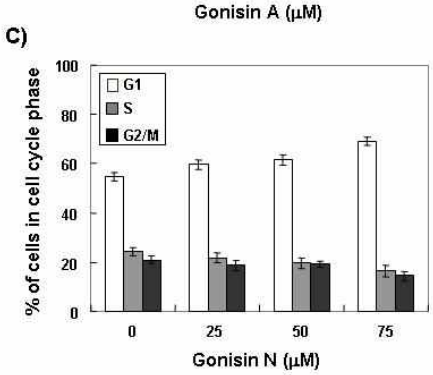
Effects of gomisin N on the expression of cell cycle-regulated proteins

To more clearly characterize the G1 phase arrest induced by gomisin N, we examined changes in the expression of



B)

Gomisin A (μM)	Number of cells (%)		
	G1	S	G2/M
0	54.80	24.29	20.91
25	55.71	23.20	21.09
50	52.55	25.36	22.09
75	54.36	24.96	20.68



D)

Gomisin N (μM)	Number of cells (%)		
	G1	S	G2/M
0	54.80	24.29	20.91
25	59.64	21.71	18.65
50	61.58	19.43	19.00
75	69.26	16.41	14.33

Fig. 2. Induction of G1 arrest of the cell cycle by gomisin N treatment in U937 cells. (A and C) Cells grown under the same conditions as Fig. 1 were collected, fixed, and stained with PI for flow cytometry analysis. (B and D) The percentages of cells in each cell cycle phase are presented. The data represent the average of two independent experiments.

proteins that have been known to control G1 cell cycle phase transition in U937 cells. In comparison to control cells, cells treated with gomisin N exhibited a dose-dependent decrease in the levels of cyclin E, Cdk2 and Cdk4 (Fig. 3), which is consistent with the role of these proteins in the regulation of the G1 to S phase transition. Since the pRB family gene products such as pRB and p130 are also important checkpoint proteins in the G1 phase of the cell cycle, we next determined the kinetics between phosphorylation of pRB and p130 and the transcription factors, E2F-1 and E2F-4. Total levels of E2F-1 and E2F-4 were down-regulated in gomisin N-treated cells, and pRB and p130 expression decreased remarkably and changed from the hyperphosphorylated form to the hypophosphorylated form after gomisin N treatment. This effect of gomisin N was dose-dependent (Fig. 3).

Furthermore, incubation of U937 cells with gomisin N resulted in a concentration-dependent increase in expression of the Cdk inhibitors such as p16 (INK4A) and p21 (WAF1/CIP1), but not p27 (KIP1), whereas gomisin A did not significantly affect expression levels of Cdk inhibitors (Fig. 4). Because the tumor suppressor p53 gene is deleted in U937 cells [3], it is most likely that the induction of p21 (WAF1/CIP1) by gomisin N is mediated in a p53-independent fashion. These data suggest that gomisin N induces G1 arrest *via* the modulation of cell cycle-regulating proteins.

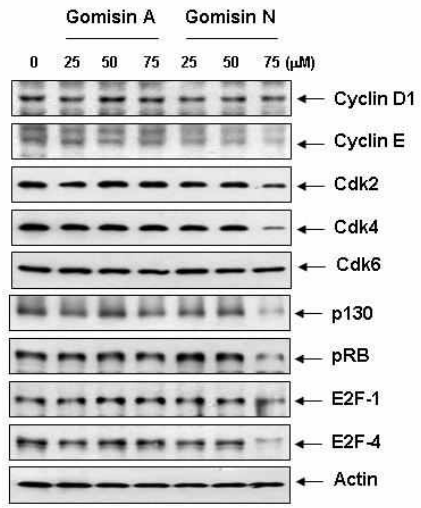


Fig. 3. Effect of gomisin A and gomisin N on the expression of cyclins, Cdks and pRB family proteins in U937 cells. U937 cells were treated with the indicated concentrations of gomisin A and gomisin N for 48 hr. The cells were lysed and proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Next, the membranes were probed with the indicated antibodies and the proteins were visualized using an ECL detection system. Actin was used as an internal control.

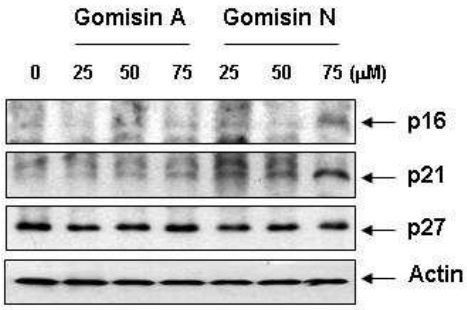


Fig. 4. Induction of Cdk inhibitors p16 (INK4A) and p21 (WAF1/CIP1) expression by gomisin N in U937 cells. U937 cells were treated with the indicated concentrations of gomisin A and gomisin N for 48 hr. The cells were lysed and proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Next, the membranes were probed with the anti-p16 (INK4A), anti-p21 (WAF1/CIP1) and anti-p27 (KIP1) antibodies and the proteins were visualized using an ECL detection system. Actin was used as an internal control.

Discussion

Many previous studies indicated that lignans isolated from *S. chinensis* Baill have revealed anti-cancer properties, anti-hepatocarcinogenesis, anti-oxidant and anti-inflammatory activities [2,10,17,13,22]. In particular, some lignans restored cytotoxic activities of anticancer agents in multidrug-resistant human cancer cells, and enhanced cytotoxic and proapoptotic effects of anti-cancer drugs [7,15,20]. In addition, other lignans including gomisin N, schisantherin C and G and propinquanin E were selectively cytotoxic in human cancer cells [2,12,23], suggesting the anticancer and cancer chemopreventive potential of lignans. However, the mechanisms underlying antiproliferative effects of lignans in human cancer cells have not been thoroughly studied.

In the present study, we found that gomisin N, but not gomisin A, strongly suppresses the proliferation (Fig. 1) and causes G1 phase arrest in human leukemia U937 cells (Fig. 2), which appeared to account for its anti-proliferative activity. Progression from G1 to S phase of the cell cycle is regulated by D-type cyclins and cyclin E, and their cognate kinases, Cdk2, 4 and 6, which act by phosphorylating and

inactivating pRB family proteins such as pRB and p130 prior to the restriction point at which cells commit to DNA synthesis, and by cyclin E-Cdk2, which acts later in G1 phase [18,21]. Therefore, any factor affecting the activity of these kinases could abrogate the normal inactivation of pRB and/or p130 and cause an accumulation of cells in G1 phase. Under normal conditions, pRB and p130 proteins bind to the members of the E2F family of transcription factors. However, growth factors induce phosphorylation and dissociation of the pRB and/or p130 from E2F, which triggers G1 cell cycle progression [18,21]. Therefore, the complex between cyclin D or cyclin E and Cdks is an obvious candidate for control of pRB and p130 phosphorylation. If decreased levels of either protein or the association between respective binding partners were observed, a concomitant decrease in the degree of pRB phosphorylation would be expected [18,21]. The data generated in this study demonstrate that in U937 cells, gomisin N, but not gomisin A, blocks phosphorylation of pRB and p130 proteins and decreases levels of E2F-1 and E2F-4 (Fig. 3). Furthermore, gomisin N inhibits Cdk2 and Cdk4 expression in association with enhanced expression of the Cdk inhibitor p16 (INK4A) and p21 (WAF1/CIP1) (Fig. 3 and 4). Additionally, our data suggest that gomisin N-induced p21 (WAF1/CIP1) upregulation involves a p53-independent pathway because U937 cells lack functional p53 [3].

In conclusion, these results provide the first evidence that gomisin N induces abnormal cell cycle transitions at G1 phase. Although further studies are needed, we believe that gomisin N is a promising candidate for cancer chemoprevention and/or chemotherapy.

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References

1. Cho, J. Y., A. R. Kim, E. S. Yoo, K. U. Baik, and M. H. Park. 1999. Immunomodulatory effect of arctigenin, a lignan compound, on tumour necrosis factor-a and nitric oxide production, and lymphocyte proliferation. *J. Pharm. Pharmacol.* **51,** 1267-1273.
2. Choi, Y. W., S. Takamatsu, S. I. Khan, P. V. Srinivas, D. Ferreira, J. Zhao, and I. A. Khan. 2006. Schisandrene, a dibenzocyclooctadiene lignan from *Schisandra chinensis*: structure-antioxidant activity relationships of dibenzocyclooctadiene lignans. *J. Nat. Prod.* **69,** 356-359.
3. Danova, M., M. Giordano, G. Mazzini, and A. Riccardi. 1990. Expression of p53 protein during the cell cycle measured by flow cytometry in human leukemia. *Leuk. Res.* **14,** 417-422.
4. Hausott, B., H. Greger, and B. Marian. 2003. Naturally occurring lignans efficiently induce apoptosis in colorectal tumor cells. *J. Cancer Res. Clin. Oncol.* **129,** 569-576.
5. Kastan, M. B. and J. Bartek. 2004. Cell-cycle checkpoints and cancer. *Nature* **432,** 316-323.
6. Khan, N., V. M. Adhami, and H. Mukhtar. 2008. Apoptosis by dietary agents for prevention and treatment of cancer. *Biochem. Pharmacol.* **76,** 1333-1339.
7. Li, L., Q. Lu, Y. Shen, and X. Hu. 2006. Schisandrin B enhances doxorubicin-induced apoptosis of cancer cells but not normal cells. *Biochem. Pharmacol.* **71,** 584-595.
8. Lin, S., M. Fujii, and D. X. Hou. 2008. Molecular mechanism of apoptosis induced by schizandrae-derived lignans in human leukemia HL-60 cells. *Food Chem. Toxicol.* **46,** 590-597.
9. Liu, G. T. 1988. Biochemical and pharmacologic effects of wuweizi (*Schizandra chinensis* (Turcz.) Baill.) and its chemical contents on animal livers. *Sheng Li Ke Xue Jin Zhan* **19,** 197-203.
10. Lu, H. and G. Liu. 1992. Anti-oxidant activity of dibenzocyclooctene lignans isolated from *Schisandraceae*. *Planta Med.* **58,** 311-313.
11. Meiler, J. and M. Schuler. 2006. Therapeutic targeting of apoptotic pathways in cancer. *Curr. Drug Targets* **7,** 1361-1369.
12. Min, H. Y., E. J. Park, J. Y. Hong, Y. J. Kang, S. J. Kim, H. J. Chung, E. R. Woo, T. M. Hung, U. J. Youn, Y. S., Kim, S. S. Kang, K. Bae, and S. K. Lee. 2008. Antiproliferative effects of dibenzocyclooctadiene lignans isolated from *Schisandra chinensis* in human cancer cells. *Bioorg. Med. Chem. Lett.* **18,** 523-526.
13. Ohtaki, Y., T. Hida, K. Hiramatsu, M. Kanitani, T. Ohshima, M. Nomura, H. Wakita, M. Aburada, and K. I. Miyamoto. 1996. Deoxycholic acid as an endogenous risk factor for hepatocarcinogenesis and effects of gomisin A, a lignan component of *Schizandra fruits*. *Anticancer Res.* **16,** 751-755.
14. Pan, M. H. and C. T. Ho. 2008. Chemopreventive effects of natural dietary compounds on cancer development. *Chem. Soc. Rev.* **37,** 2558-2574.
15. Pan, Q., Q. Lu, K. Zhang, and X. Hu. 2006. Dibenzocyclooctadiene lingnans: a class of novel inhibitors of P-glycoprotein. *Cancer. Chemother. Pharmacol.* **58,** 99-106.
16. Panossian, A. and G. Wikman. 2008. Pharmacology of *Schisandra chinensis* Bail.: an overview of Russian research and uses in medicine. *J. Ethnopharmacol.* **118,** 183-212.
17. Park, C., Y. W. Choi, S. K. Hyun, H. J. Kwon, H. J. Hwang, G. Y. Kim, B. T. Choi, B. W. Kim, I. W. Choi, S. K. Moon, W. J. Kim, and Y. H. Choi. 2009. Induction of G1 arrest and apoptosis by schisandrin C isolated from *Schizandra chinensis* Baill in human leukemia U937 cells. *Int. J. Mol. Med.* **24,**

495-502.
18. Sherr, C. J. and J. M. Roberts. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9,** 1149-1163.
19. Slanina, J., E. Táborská, and L. Lojková. 1997. Lignans in the seeds and fruits of *Schisandra chinensis* cultured in Europe. *Planta Med.* **63,** 277-280.
20. Wan, C. K., G. Y. Zhu, X. L. Shen, A. Chattopadhyay, S. Dey, and W. F. Fong. 2006. Gomisin A alters substrate interaction and reverses P-glycoprotein-mediated multidrug resistance in HepG2-DR cells. *Biochem. Pharmacol.* **72,** 824-837.
21. Weinberg, R. A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81,** 323-330.
22. Wu, M. D., R. L. Huang, L. M. Y. Kuo, C. C. Hung, C. W. Ong, and Y. H. Kuo, 2003. The anti-HBsAg (human type B hepatitis, surface antigen) and anti-HBeAg (human type B hepatitis, e antigen) C18 dibenzocyclooctadiene lignans from *Kadsura matsudai* and *Schizandra arisanensis*. *Chem. Pharm. Bull.* **51,** 1233-1236.
23. Xu, L., F. Huang, S. Chen, S. Chen, and P. Xiao. 2006. A new triterpene and dibenzocyclooctadiene lignans from *Schisandra propinqua* (WALL.) BAILL. *Chem. Pharm. Bull. (Tokyo)* **54,** 542-545.

초록 : 오미자에서 분리된 dibenzocyclooctadiene lignan의 일종인 gomisin N에 의한 인체혈구암세포의 세포주기 G1 arrest 유발

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본 연구에서는 오미자에서 분리된 dibenzocyclooctadiene lignan의 일종인 gomisin A와 gomisin N에 대한 항암효능을 U937 인체혈구암세포를 대상으로 조사하였다. Gomisin N은 처리농도의 증가에 따라 U937 세포의 증식을 억제하였으나 동일조건에서 gomisin A는 세포독성 효과를 나타내지 못하였으며, gomisin N에 의한 U937 세포의 증식억제는 세포주기 G1 arrest 유발에 의한 것이었다. Gomisin N에 의한 G1 arrest는 cyclin E, cyclin-dependent kinase (Cdk) 2 및 Cdk4의 발현 억제와 Cdk inhibitor인 p16 (INK4A) 및 p21 (WAF1/CIP1)의 발현 증가와 연관성이 있었으나, gomisin A는 세포주기 인자들의 발현에 유의적인 변화를 유발하지 않았다. 또한 gomisin N은 retinoblastoma 단백질군인 pRB 및 p130의 인산화를 억제하였으며, 전사조절인자인 E2F 단백질들의 발현을 억제하였다. 본 연구의 결과에서 gomisin N에 의한 U937 세포의 증식억제는 세포주기 조절인자들의 발현 변화를 통한 G1 arrest 유발에 의한 것임을 알 수 있었으며, gomisin N은 암예방 및 항암활성 후보물질로서의 개발 가능성이 있음을 알 수 있었다.