G_2-Phase Arrest Through *p21(WAF1/Cip1)* Induction and cdc2 Repression by Gnidimacrin in Human Hepatoma HLE Cells

MITSUZI YOSHIDA[1,2], YUKI MATSUI[2], AKIRA IIZUKA[1,2] and YOSHINORI IKARASHI[1,2]

[1]Chemotherapy and [2]Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan

Abstract. *Gnidimacrin (NSC252940) shows significant antiproliferating activity against human tumor cell lines. This compound binds to and directly activates protein kinase C (PKC). Human hepatoma HLE cells, which lose p53 function and retinoblastoma protein (Rb) expression, are resistant to gnidimacrin. However, PKC βII gene-transfected HLE (HLE/PKC βII) cells became sensitive to gnidimacrin, through which cdc2 inhibition and G_2-phase arrest was caused. p21(WAF1/Cip1) induction and cdc2 reduction were observed and this reduction was abolished through the suppression of p21(WAF1/Cip1) induction by the MEK1/2 inhibitor U0126. Translocation of E2F-4 to the nucleus was also observed in the cells but not in parental HLE cells. Consequently gnidimacrin inhibited cell growth through G_2-phase arrest not only by the p21(WAF1/Cip1)-dependent suppression of cdc2 activity, but also by subsequent transcriptional suppression of cdc2 itself. In addition, involvement of E2F-4 in cdc2 suppression through a long-lasting induction of p21(WAF1/Cip1) by gnidimacrin is suggested in HLE/PKC βII cells.*

Daphnane-type diterpene gnidimacrin (NSC252940) strongly inhibits cell growth of human tumor cell lines at 10^{-9}-10^{-10} M (1). In gnidimacrin-sensitive K562 cells, gnidimacrin bound to and directly activated protein kinase C (PKC), and then arrested the cell cycle at the G_1-phase by the inhibition of cdk2 kinase activity (2). Gnidimacrin induced the cyclin-dependent kinase (CDK) inhibitor p21(WAF1/Cip1) and suppressed cdc25A, a phosphatase that activates cdk2 (3). Complete inhibition of cdk2 activity and retinoblastoma protein (Rb) phosphorylation, and maximum G_1-phase arrest were observed during 24 h exposure to gnidimacrin. Northern blot analysis demonstrated cdc25A mRNA was down-regulated in a concentration-dependent manner during 24 h exposure to gnidimacrin (3).

Human hepatoma HLE cells are completely resistant to gnidimacrin. This cell line loses p53 function (4), as do K562 cells (3). To prove a link between PKC activation and biological functions of gnidimacrin, PKC isoform genes were transfected to HLE cells. The PKC βII gene-transfected HLE (HLE/PKC βII) cells became sensitive to gnidimacrin in accordance with the degree of PKC βII expression (5). In this cell line, however, gnidimacrin did not cause G_1-phase arrest, but G_2 arrest. To clarify the antitumor action of PKC activator gnidimacrin and the mechanism of G_2 arrest, different biochemical effects of gnidimacrin on cell cycle mediators were examined using HLE, HLE/PKC βII and K562 cells. As a result, the transcriptional suppression of cdc2 was found through *p21(WAF1/Cip1)* induction and MEK1/2 signal pathway by gnidimacrin in HLE/PKC βII cells. The signal pathway to cdc2 suppression *via* E2F-4 by gnidimacrin was similar to cdc25A suppression in K562 cells.

Correspondence to: Dr. Yoshinori Ikarashi, Section for Studies on Host-immune Response, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Tel: +81 335422511, Fax: +81 335421886, e-mail: yikarash@gan2.res.ncc.go.jp

Key Words: Gnidimacrin, PKC βII, G_2-phase arrest, p21(WAF1/Cip1), cdc2, E2F-4.

Materials and Methods

Chemicals. The isolation and identification of gnidimacrin has been previously outlined (1). Polyclonal anti-PKC βII antibody was obtained from Sigma (St. Louis, MO, USA). Monoclonal antihuman cdc2 (cdk1) and p21(WAF1/Cip1) antibodies and polyclonal antihuman E2F-4, anti-p107 and anti-p130 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antihuman Rb protein antibody was purchased from PharMingen (San Diego, CA, USA). U0126 was purchased from LC Laboratories (Woburn, MA, USA).

Tumor cells. Human K562 leukemia cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Human HLE hepatoma cells were cultured in modified Eagle's medium supplemented with 10% bovine serum. Transfection of HLE cells with PKC expression plasmids encoding both PKC βII and Neomycin resistance gene were carried out as reported previously (5). PKC βII gene-transfected HLE (HLE/PKC βII) cells were selected and cloned in the medium with 500 μg of G-418/mL. Selected clones were maintained in the medium with 100 μg of G-418/mL and used in this study after removal of G-418 from the culture medium.

Antitumor activity in vitro (MTT assay). The effect of gnidimacrin on cell growth was examined with an MTT [3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide] assay, as outlined in a previous report (1). Briefly, K562 (4×10^3 cells/mL), HLE and HLE/PKC βII (10^4 cells/mL) were seeded into a 96-well microplate and cultured for 4 days with various concentrations of gnidimacrin at 37˚C, in an atmosphere of 5% CO_2 in air. After this MTT (2 mg/mL) were added to each well and the plates were incubated for 4 h. The MTT-formazan crystals were dissolved in DMSO and the optical density of each solution was measured at 570 and 630 nm using a Microplate Manager III (BIO-RAD Laboratories, Hercules, CA, USA) analysis program on a Macintosh computer interfaced with a Benchmark Microplate Reader (BIO-RAD Laboratories.

Western blot analysis. Expression of Rb, PKC βII, p21(WAF1/Cip1), cdc2 and E2F-4 proteins were assessed *via* immunoblot assays. K562, HLE and HLE/PKC βII cells were washed and lysed in a lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 1 mM Na_3VO_4, 0.1% SDS, 2 mM EGTA, 80 μg/mL each of leupeptin and aprotinin and 0.6 mM PMSF (phenylmethylsulfonyl fluoride)), at 4˚C for 20 min. In the case of PKC βII expression, the cells were lysed in a cell solubilization buffer (10 mM Tris-HCl, 0.25 M sucrose, 50 μM $CaCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, pH 6.7 and 0.5% Nonidet P-40) for 30 min at room temperature. After centrifugation, the supernatant served as the cell lysate. The cell lysate was also subjected to immunoprecipitation using anti-cdc2 antibody. For preparation of cytosolic and nuclear extract, the cells were solubilized in other lysis buffer (0.6% Nonidet P-40, 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 2 μg/mL aprotinin and 0.01 mM PMSF). Supernatants were used as a cytosolic extract. The pellets were extracted with vigorous agitation at 4˚C in buffer containing 20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol and 2 μg/mL aprotinin. After centrifugation, supernatants were used as nuclear extracts. Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The cell lysate and the immunoprecipitate were applied to 7.5 to 14/16% SDS-PAGE, and then transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The filters were stained with their respective antibodies and secondarily with horseradish peroxidase-conjugated antibody. Antigens were detected with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Flow cytometry. Flow cytometric analysis was performed as previously reported (2). HLE/PKC βII cells in logarithmic growth were cultured with gnidimacrin for 24 h and the cells were washed with PBS(–) and fixed with 70% ethanol after harvesting with 0.05% trypsin and 0.53 mM EDTA. After propidium iodide (PI) staining, the DNA contents of 10^4 cells for each sample were analyzed with the FACSCalibur automated system (BD Biosciences, San Jose, CA, USA).

Cdc2 kinase assay. The histone H1 kinase activity of the human cdc2 protein kinase was measured using anti-cdc2 immunoprecipitate. The cells were washed after gnidimacrin treatment and lysed in lysis buffer. After immunoprecipitation of the cell lysate with the monoclonal mouse anti-human cdc2 antibody, the histone H1 kinase activity of the immunoprecipitate was measured at 25˚C for 30 min in a reaction mixture (40 μL) containing 5 μg of histone H1 (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), 25 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 7.4) and 2.5 μCi of [γ-^{32}P]ATP. The reaction was terminated by adding sample buffer and boiling for 5 min. Fifteen-microliter samples were analyzed with a 12.5% SDS-PAGE, followed by autoradiography.

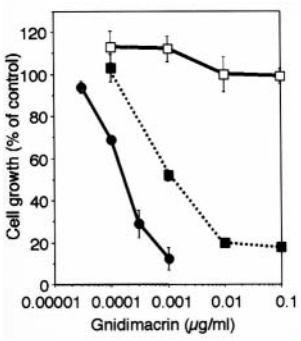

Figure 1. *Concentration-response curves of K562, HLE and HLE/KC βII cells. Points represent average±SD of 3 independent experiments performed in quadruplicate with K562 (●), HLE (□) and HLE/PKC βII (■) cells. Cell growth was measured by an MTT assay, as described in the Materials and Methods.*

Reverse-transcription polymerase chain reaction (RT-PCR) analysis. RT-PCR analysis was carried out by the method described by Furukawa *et al*. (6). Briefly, 1 μg of total RNA from the cells was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) and oligo(dT) primers (Invitrogen Corporation). Subsequent PCR amplification was carried out with cDNA derived from 100 ng of RNA using 1 unit of *Ex Taq*™ (TAKARA BIO Inc., Otsu, Japan) polymerase in a 50-μL reaction mixture containing 200 μM dNTP and 200 nM primer pairs. Each cycle of PCR consisted of 1 min of denaturation at 94˚C, 1 min of annealing at 60˚C, and 2 min of extension at 72˚C. Ten microliters of each reaction mixture were applied to 3% agarose gels in Tris acetate-EDTA buffer and the signals were detected by ethidium bromide staining. The following oligonucleotides were used: 5'-GGGGATTCAGAAATTGATCA-3'(sense) and 5'-TGTCAG AAAGCTACATCTTC-3' (antisense) for the cdc2 and 5'-GAGAGACAGGCATTTGGA-3'(sense) and 5'-TCAGTGGTTTA GGAGGGT-3' for the Rb primers.

Results

Figure 1 shows the concentration-response curves of K562, HLE and HLE/PKC βII cells after continuous gnidimacrin exposure over 4 days. PKC βII gene-transfected cells (HLE/PKC βII) became sensitive to gnidimacrin (5).

Mechanism of the sensitization of HLE/PKC βII cells to gnidimacrin was examined using the parental refractory HLE and constitutionally sensitive K562 cells. It has been clarified that the G_1 arrest of the cell cycle in K562 cells caused by

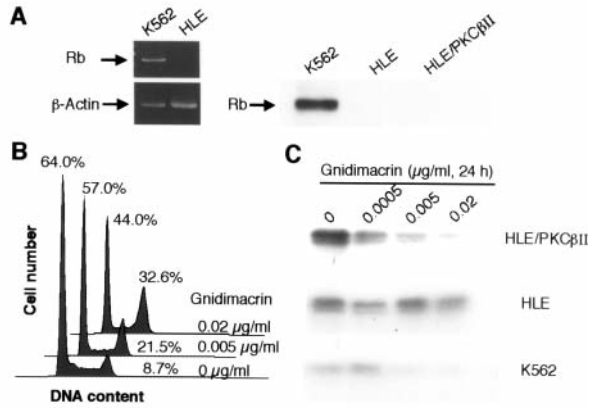

Figure 2. *Rb expression (A) and effects of gnidimacrin on cell cycle (B) and cdc2 activity (C) in HLE, HLE/PKC βII and K562 cells. Rb expressions were examined by RT-PCR (A left)) and Western blotting (A right). Total RNA was isolated from K562 and HLE cells and applied to RT-PCR. RT-PCR analysis was performed as described in Materials and Methods. Twenty five of PCR cycle was performed for PCR analysis of Rb expression. Cell extracts (10 μg protein per lane) were applied to 7.5% SDS-PAGE and then Rb expression was examined by Western blot analysis using anti-Rb monoclonal antibody. Western blot and flow cytometry analyses were performed as described in the Materials and Methods. Cdc2 kinase activity was measured using anti-cdc2 immunoprecipitate and histone H1 as a substrate.*

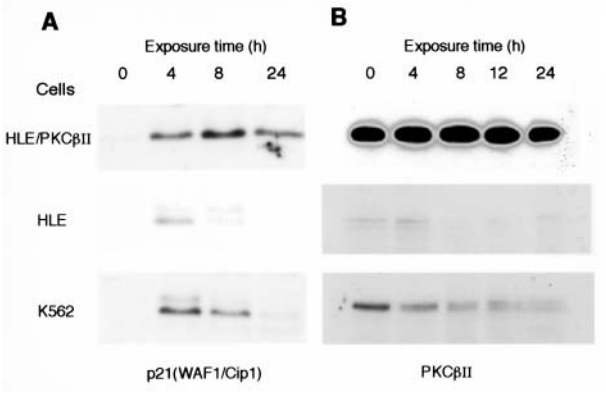

Figure 3. *Induction of p21(WAF1/Cip1) (A) and PKC βII down-regulation (B). HLE/PKC βII, HLE and K562 cells were cultured with gnidimacrin (0.02 μg/mL) for the indicated hours. Cell extracts (10 μg protein per lane) were applied to 14/16% (A) and 7.5% (B) SDS-PAGE. Western blot analysis was performed as described in the Materials and Methods.*

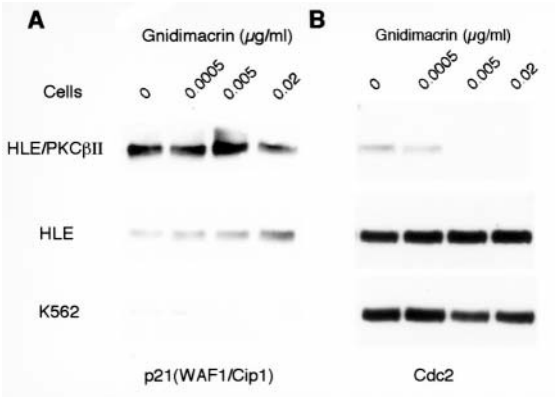

Figure 4. *p21(WAF1/Cip1) associated with cdc2. After the cells were cultured with various concentrations of gnidimacrin for 24 h, cell lysate (1.7 mg protein) was treated with 1.0 μg anti-p21(WAF1/Cip1) antibody for 2 h. Aliquots of the immunoprecipitate were applied to 14/16% SDS-PAGE and stained with anti-p21(WAF1/Cip1) (A) and anti-cdc2 antibodies (B).*

gnidimacrin is due to dephosphorylation of the Rb protein through inhibition of cdk2 activity (3). In HLE and HLE/PKC βII cells, however, expression of Rb protein was not detected (Figure 2A right). Rb mRNA in HLE cells could not be found even by RT-PCR method (Figure 2A left). Gnidimacrin (0.02 μg/mL) caused G_2 phase arrest after continuous exposure over 24 h to gnidimacrin in the HLE/PKC βII cells (Figure 2B). Therefore, the effect of gnidimacrin on cdc2 kinase, which is involved in regulation of mitosis in mammalian cells, was examined using histone H1 as the substrate. As shown in Figure 2C, gnidimacrin inhibited cdc2 histone H1 kinase activity of HLE/PKC βII cells in a dose-dependent manner (0.0005-0.02 μg/mL). This cdc2 inhibition was not observed in refractory HLE cells, while cdc2 activity was scarcely detected in sensitive K562 cells. In addition, the large amount of cdc2 in K562 cells was inactivated by phosphorylation on Tyr15 (data not shown).

In K562 cells, the CDK inhibitor p21(WAF1/Cip1) was induced in association with cdk2 during a 4 h exposure to gnidimacrin (3). Effect of gnidimacrin on p21(WAF1/Cip1) induction and expression of the transfected-gene product PKC βII were examined by incubation time and Western blot analysis (Figure 3A, B). In low PKC βII-expressing HLE cells, the p21(WAF1/Cip1) induction was detectable but weaker than K562 cells. It then diminished in parallel with PKC βII expression as well as K562 cells. In HLE/PKC βII cells overexpressing PKC βII, p21(WAF1/Cip1) was significantly induced at 4 h, reaching a maximum 8 h after treatment and then both p21(WAF1/Cip1) and PKC βII expression were retained over a 24 h exposure. Although there is an approximately 4 h time lag between PKC βII expression and p21(WAF1/Cip1) induction, their expression patterns were similar. These results suggest PKC βII expression was responsible for p21(WAF1/Cip1) induction.

CDK inhibitor p21(WAF1/Cip1) inhibits cdc2 activity as well as cdk2. Whether p21(WAF1/Cip1) induced by gnidimacrin is associated with cdc2 to inhibit the cdc2 activity was examined by immunoprecipitation using anti-cdc2 antibody (Figures 4A, B). In HLE and K562 cells, the same level of cdc2 proteins were detected after treatment

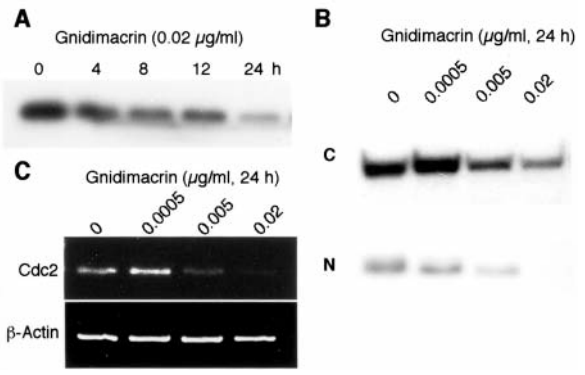

Figure 5. *Time- and concentration-dependent reduction of cdc2 protein in gnidimacrin treated HLE/PKC βII cells. Effects of gnidimacrin on cdc2 expression were examined by Western blot analysis (A, B) and RT-PCR (C). HLE/PKC βII cells were cultured with 0.02 μg/mL gnidimacrin for the indicated times (A) or various concentrations of gnidimacrin for 24 h (B). Whole cell extract (10 μg protein per lane) (A), and cytosolic (25 μg protein) and nuclear (6.5 μg protein) extracts (B) were applied to 14/16% SDS-PAGE. Western blot analysis was performed as described in the Materials and Methods. Total RNA was isolated from HLE/PKC βII cells treated with various concentrations of gnidimacrin for 24 h and applied to RT-PCR. RT-PCR analysis was performed as described in the Materials and Methods. The numbers of PCR cycles were varied at 20 to 25 to demonstrate that the amount of amplified PCR product was directly proportional to the amount of input RNA. The optimal number was found to be 22 cycles for cdc2.*

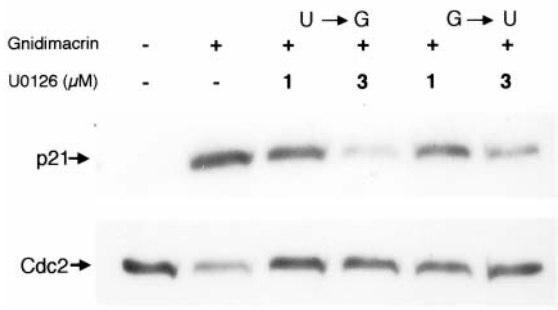

Figure 6. *Effect of U0126 on p21(WAF1/Cip1) induction and cdc2 repression by gnidimacrin. HLE/PKC βII cells were cultured for 18 h with 0.005 μg/mL of gnidimacrin, and 1 or 3 μM MEK inhibitor U0126. U0126 at 1 or 3 μM was added to the culture medium at a 30-min before or after gnidimacrin. Cell extracts (10 μg protein per lane) were applied to 14/16% SDS-PAGE and Western blot analysis was performed as described in Figure 4 and the Materials and Methods.*

with various concentrations of gnidimacrin for 24 h, but p21(WAF1/Cip1) associated with cdc2 was low and hard to detect in these cells, respectively. A large amount of p21(WAF1/Cip1) associated with cdc2 was observed in HLE/PKC βII cells, however, this was not related to the gnidimacrin concentration. Surprisingly, cdc2 protein was low and decreased in a concentration-dependent manner. From these results, it is implied that in HLE/PKC βII cells cdc2 activity was strongly inhibited at a high dose of gnidimacrin because of the increase in the amount of associated p21(WAF1/Cip1) per cdc2 protein.

In HLE/PKC βII cells, cdc2 expression significantly decreased during gnidimacrin treatment (Figure 4B). Therefore, effects of gnidimacrin on the cdc2 protein level in cytosol and nuclear fractions and on cdc2 expression were examined by both Western blot analysis and RT-PCR. Figure 5 shows time-dependent inhibition of cdc2 expression by gnidimacrin (A) and dose-dependent reduction of cdc2 in both cytosol and nuclear extracts (B). These results suggest the reduction of cdc2 by gnidimacrin was not the result of a change of cdc2 distribution in HLE/PKC βII cells. Furthermore, the RT-PCR results showed gnidimacrin inhibited cdc2 mRNA expression in a dose-dependent manner (C).

Promoter of CDK inhibitor p21(WAF1/Cip1) was transcriptionally activated through Ras-MEK [mitogen-activated protein kinase/extracellular signal-regulated kinase(ERK)-kinase]/ERK-Sp1 pathway (7, 8). Therefore, whether p21(WAF1/Cip1) induction could be inhibited by MEK inhibitor U0126 was examined and thereby the cdc2 repression could be recovered. U0126 (3 μM), prior to gnidimacrin treatment caused complete inhibition of p21(WAF1/Cip1) induction and overcame cdc2 suppression (Figure 6). In the case of gnidimacrin treatment prior to the addition of U0126, inhibition of p21(WAF1/Cip1) induction was weak, but cdc2 expression recovered over a 18 h culture. U0126 alone at concentrations up to 10 μM did not show any effect on HLE/PKC βII cells (data not shown). These results suggest transcriptional suppression of cdc2 was down-stream of p21(WAF1/Cip1) induction, which was responsible for cdc2 suppression.

Taylor *et al.* (9) reported overexpression of p21(WAF1/Cip1) represses the cdc2 promoter through the binding of E2F-4 and p130 to the cdc2 promoter. Zhu *et al.* (10) also reported E2F-4 binds to the negative-acting site in the cdc2 promoter. Therefore, the existence and partition of endogenous E2F-4 were examined by Western blot analysis using a preparation of cytosolic and nuclear extracts after gnidimacrin treatment. By gnidimacrin treatment, significant translocation of E2F-4 to nuclear fraction was observed in HLE/PKC βII cells (Figure 7). Moderate translocation of E2F-4 was also found in K562 cells, but any change was not detected in HLE cells. These findings imply E2F-4 might be related to cdc2 repression after gnidimacrin treatment.

Discussion

Gnidimacrin binds to and directly activates PKC in K562 cells (1). PKC is a major receptor for the phorbol ester family (11), and phorbol esters act as mitogens for quiesent

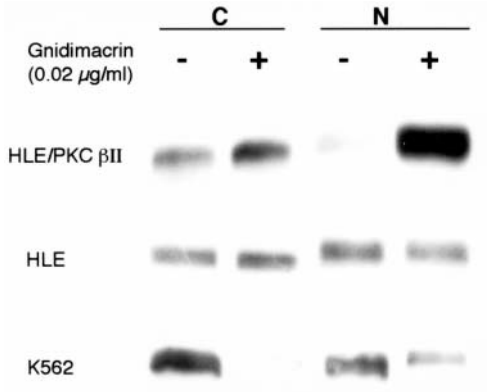

Figure 7. *Effects of gnidimacrin on partition of E2F-4. After HLE/PKC βII, HLE and K562 cells were cultured with 0.02 μg/mL gnidimacrin for 24 h, the amount of E2F4 in cytosol and nuclear extracts was examined by Western blot analysis.*

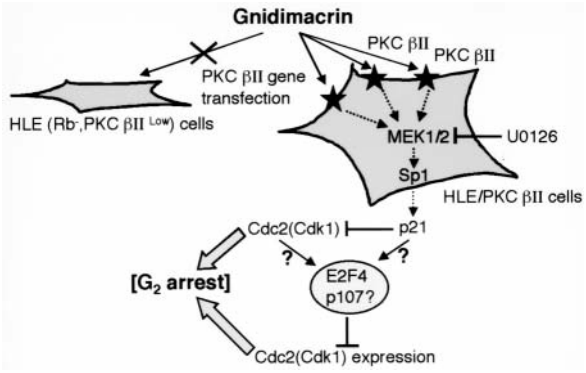

Figure 8. *Proposed scheme for the signaling pathway involved in the antitumor activity of gnidimacrin via PKC activation, p21(WAF1/Cip1) induction and the cell-cycle regulatory system.*

cells (12-14). However, PKC activators including phorbol esters can also inhibit tumor growth (15-21). PKC activator bryostatin I is a potent antileukemic agent (22), while other prostratin and 12-deoxyphorbol 13-phenylacetate function as anti-carcinogenics *in vivo* (23). However, relatively little is known about the biochemical mechanism of growth inhibition by these PKC activators.

While PKC was down-regulated in sensitive K562 cells by pretreatment of bryostatin I, the cells changed to become gnidimacrin-resistant in response to the degree of down-regulation (5). In contrast, PKC βII gene-transfected HLE cells were sensitive in accordance with the degree of PKC βII expression. These findings imply PKC, in particular PKC βII, is necessary for the antitumor effect of gnidimacrin. It has been reported that gnidimacrin exerts antitumor activity through inhibition of cdk2 activity and suppression of cdc25A (3). However, the relationship between PKC activation and this gnidimacrin suppression is unclear.

The CDK inhibitor p21(WAF1/Cip1) was induced not only by p53 (24), but also by PKC activation (19, 20, 25-27). In K562 cells, p21(WAF1/Cip1) was induced after gnidimacrin treatment but this was transient and disappeared after 8 h of gnidimacrin exposure in parallel with the down-regulation of PKC βII. On the other hand, transcriptional activation of p21(WAF1/Cip1) was dependent on Sp1 through MEK/ERK signal pathways (7, 8, 26). Taylor *et al*. (9) reported overexpression of p21(WAF1/Cip1) represses the cdc2 promoter. Therefore, gnidimacrin was likely to repress the cdc2 expression through cdc2 promoter because inhibition of cdc2 expression was abolished by the suppression of p21(WAF1/Cip1) induction using the MEK inhibitor U0126. In addition, Iavarone and Massague (28) reported that in the cdc25A promoter sequence, the E2F-A site and adjacent CHR site exist and correspond to the promoter sequence from cdc2. These reports support our findings of cdc25A suppression by gnidimacrin in K562 cells. HLE/PKC βII cells expressed Rb family p107, however, repressor complexes such as E2F-4-p130 and E2F-4-p107 were not detected by immunoprecipitation in the gnidimacrin treatment HLE/PKC βII cells. Taken together, in sensitive K562 cells expressing Rb, gnidimacrin transiently inhibited cdk2 by the induction of p21(WAF1/Cip1) through PKC activation and arrested the cell cycle to the G_1-phase by cdc25A suppression. Whereas, in HLE/PKC βII cells without Rb expression, gnidimacrin inhibited cell growth through cell cycle arrest to the G_2-phase by p21(WAF1/Cip1)-dependent suppression of cdc2 activity and subsequent transcriptional suppression of cdc2 (Figure 8). Niculescu *et al*. (29) also reported the predominant G_1 arrest in response to p21(WAF1/Cip1) expression correlates with the presence of functional Rb and G_2 arrest is more prominent in Rb-negative cells. These findings correspond to this study results.

This study demonstrates that it is possible to inhibit tumor growth by regulation of cell-cycle mediators including p21(WAF1/Cip1) even though the tumor cells lacked both functional p53 and Rb. Though further work is necessary to clarify the mechanism of repression of cdc2 promoter *via* E2F family or their complexes with Rb family by the p21(WAF1/Cip1) induction, PKC and p21(WAF1/Cip1) might be important and interesting molecular targets for antitumor chemotherapy.

Acknowledgements

This work was supported in part by Grant-in-aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

References

1 Yoshida M, Feng W, Saijo N and Ikekawa T: Antitumor activity of daphnane-type diterpene gnidimacrin isolated from *Stellera chamaejasme* L. Int J Cancer *66*: 268-273, 1996.

2 Yoshida M, Yokokura H, Hidaka H, Ikekawa T and Saijo N: Mechanism of antitumor action of PKC activator, gnidimacrin. Int J Cancer *77*: 243-250, 1998.

3 Yoshida M, Feng W, Nishio K, Takahashi M, Heike Y, Saijo N, Wakasugi H and Ikekawa T: Antitumor action of the PKC activator gnidimacrin through cdk2 inhibition. Int J Cancer *94*: 348-352, 2001.

4 Pu H, Tsuji T, Kondo A, Fushimi K, Ohashi R, Inoue Y, Mimura T, Hamazaki K, Miyazaki M and Namba M: Comparison of cellular characteristics between human hepatoma cell lines with wild-type *p53* and those with mutant-type *p53* gene. Acta Medica Okayama *51*: 313-319, 1997.

5 Yoshida M, Heike Y, Ohno S, Ikekawa T and Wakasugi H: Involvement of PKC βII in anti-proliferating action of a new antitumor compound gnidimacrin. Int. J. Cancer *105*: 601-606, 2003.

6 Furukawa Y, Iwase S, Terui Y, Kikuchi J, Sakai T, Nakamura M, Kitagawa S and Kitagawa M: Transcriptional activation of the cdc2 gene is associated with Fas-induced apoptosis of human hematopoietic cells. JBC *271*: 28469-28477, 1996.

7 Vaque JP, Navascues J, Shiio Y, Laiho M, Ajenjo N, Mauleon I, Matallanas D, Crespo P and Leon J: Myc antagonizes Ras-mediated growth arrest in leukemia cells through the inhibition of the Ras-ERK-$p21^{Cip1}$ pathway. JBC *280*: 1112-1122, 2005.

8 De Siervi A, Marinissen M, Diggs J, Wang X-F, Pages G and Senderowicz A: Transcriptional activation of $p21^{waf1/cip1}$ by alkylphospholipids: role of the mitogen-activated protein kinase pathway in the transactivation of the human $p21^{waf1/cip1}$ promoter by Sp1. Cancer Res *64*: 743-750, 2004.

9 Taylor WR, Schonthal AH, Galante J and Stark GR: p130/E2F4 binds to and represses the cdc2 promoter in response to p53. JBC *276*: 1998-2006, 2001.

10 Zhu W, Giangrande PH and Nevins JR: E2Fs link the control of G_1/S and G_2/M transcription. EMBO J *23*: 4615-4626, 2004.

11 Nishizuka Y: The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature *308*: 693-698, 1984.

12 Dicker P and Rozengurt E: Stimulation of DNA synthesis by tumor promoter and pure mitogenic factors. Nature *276*: 723-726, 1978.

13 Collins MKL and Rozengurt E: Binding of phorbol esters to high-affinity sites on murine fibroblastic cells elicits a mitogenic response. J Cell Physiol *112*: 42-50, 1982.

14 Rozengurt E: Early signals in the mitogenic response. Science *234*: 161-166, 1986.

15 Choi PM, Tchou-Wong KM and Weinstein IB: Overexpression of protein kinase C in HT29 colon cancer cells cause growth inhibition and tumor suppression. Mol Cell Biol *10*: 4650-4657, 1990.

16 Nishio K, Sugimoto Y, Nakagawa K, Niimi S, Fujiwara Y, Bungo M, Kasahara K, Fujiki H and Saijo N: TPA and okadaic acid with tumor promoting activity inhibit the growth of K562, PC9 cells. Br J Cancer *62*: 415-419, 1990.

17 Rogalsky V, Todorov G, Den T and Ohnuma T: Increase in protein kinase C activity is associated with human fibroblast growth inhibition. FEBS Lett *304*: 153-156, 1992.

18 Powell MB, Rosenberg RK, Graham MJ, Birch ML, Yamanishi DT, Buckmeier JA and Meyskens FL Jr: Protein kinase C β expression in melanoma cells and melanocytes: differential expression correlates with biological responses to 12-*O*-tetradecanoylphorbol 13-acetate. J Cancer Res Clin Oncol *119*: 199-206, 1993.

19 Asiedu C, Biggs J, Lilly M and Kraft AS: Inhibition of leukemic cell growth by the protein kinase C activator bryostatin 1 correlates with the dephosphorylation of cyclin-dependent kinase 2. Cancer Res *55*: 3716-3720, 1995.

20 Blagosklonny MV, Prabhu NS and El-Deiry WS: Defects in $p21^{WAF1/CIP1}$, Rb, and c-myc signaling in phorbol ester-resistant cancer cells. Cancer Res *57*: 320-325, 1997.

21 Dale IL, Bradshaw TD, Gesher A and Pettit GR: Comparison of effect of bryostatins and 12-*O*-tetradecanoylphorbol-13-acetate on protein kinase C activity in A549 human lung carcinoma cells. Cancer Res *49*: 3242-3245, 1989.

22 Lilly M, Brown C, Pettit G and Kraft A: Bryostatin 1: a potential anti-leukemic agent for chronic myelomonocytic leukemia. Leukemia *5*: 283-287, 1991.

23 Szallasi Z, Krsmanovic L and Blumberg PM: Nonpromoting 12-deoxyphorbol 13-esters inhibit phorbol 12-myristate 13-acetate-induced tumor promotion in CD-1 mouse skin. Cancer Res *53*: 2507-2512, 1993.

24 EI-Deiry W, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell *75*: 817-825, 1993.

25 Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE and Givol D: Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res *54*: 3391-3395, 1994.

26 Schavinsky-Khrapunsky Y, Huleihel M, Aboud M and Torgeman A: Role of protein kinase C and the sp1-p53 complex in activation of $p21^{WAF-1}$ expression by 12-*O*-tetradecanoylphorbol-13-acetate in human T cells. Oncogene *22*: 5315-5324, 2003.

27 Barboule N, Lafon C, Chadebech P, Vidal S and Valette A: Involvement of p21 in the PKC-induced regulation of the G_2/M cell cycle transition. FEBS Lett *444*: 32-37, 1999.

28 Iavarone A and Massague J: E2F and histone deacetylase mediate transforming growth factor β repression of cdc25A during keratinocyte cell cycle arrest. Mol Cell Biol *19*: 916-922, 1999.

29 Niculescu AB, Chen X, Smeets M, Hengst L, Prives C and Reed SI: Effect of $p21^{Cip1/Waf1}$ at both the G_1/S and the G_2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and preventing endoreduplication. Mol Cell Biol *18*: 629-643, 1998.

Received September 9, 2008
Revised December 23, 2008
Accepted February 9, 2009