**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 G2-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 G2-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 G1-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 G1-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 G1-phase arrest, but G2 arrest. To clarify the antitumor action of PKC activator gnidimacrin and the mechanism of G2 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.

**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 g nidimacrin 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 (4x10^3 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 g nidimacrin at 37°C, in an atmosphere of 5% CO2 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 Na3VO4, 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 CaCl2, 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 MgCl2, 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 MgCl2, 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 1350
Gnidimacrin is due to dephosphorylation of the Rb protein through inhibition of cdk2 activity. 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 G2 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. 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.
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 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 quiescent
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-carcinogens 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 G1-phase by cdc25A suppression. Whereas, in HLE/PKC βII cells without Rb expression, gnidimacrin inhibited cell growth through cell cycle arrest to the G2-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 G1 arrest in response to p21(WAF1/Cip1) expression correlates with the presence of functional Rb and G2 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.

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References


