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ANTITUMOR ACTION OF THE PKC ACTIVATOR GNIDIMACRIN THROUGH CDK2 INHIBITION

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Daphnane-type diterpene gnidimacrin (NSC252940), isolated from a Chinese plant, exhibited antitumor activity against murine leukemias and solid tumors. At concentrations of 10^{-9} to 10^{-10} M, this agent strongly inhibited the growth of human tumor cell lines. In sensitive human leukemia K562 cells, gnidimacrin is a PKC activator that arrests the cell cycle in the G_1 phase by inhibiting cdk2 activity. A 4 hr exposure of K562 cells to gnidimacrin induced the CDK inhibitor $p21^{WAF1/Cip1}$, but this effect was transient and did not correlate temporally with the onset of G_1 arrest. Expression of cdc25A, a phosphatase that activates cdk2, was reduced during 24-hr exposure to gnidimacrin. Moreover, the suppression corresponded in a concentration- and time-dependent manner to both the inhibition of cdk2 activity and the mobility shift observed when cdk2 was electrophoresed on SDS-PAGE, indicating that the phosphorylation state of cdk2 must change. Cyclin E, the other regulator of cdk2 activity, was not influenced by gnidimacrin. These results suggest that gnidimacrin exerts antitumor activity through suppression of cdc25A and inhibition of cdk2 activity.

Key words: *gnidimacrin; protein kinase C; G_1 arrest; cyclin-dependent kinase-2; $p21^{WAF1/Cip1}$; cdc25A*

In the past, studies we have done involving novel antitumor agents have focused on Chinese plants, especially traditional Chinese medicinal herbs, which are reported to manifest certain antitumor effects and have been used in clinical practice, though their active constituents have not been clarified.

Gnidimacrin (Fig. 1), isolated from the root of the Chinese plant *Stellera chamaejasme* L., showed significant antitumor activity against murine P388 leukemia, Lewis lung carcinoma, B16 melanoma and colon 26 carcinoma (ILS 40–79%, 0.01–0.02 mg/kg). Moreover, it inhibited cell growth in several human leukemias (K562, CCRF-CEM and HL-60), in addition to stomach cancer cells (MKN28 and MKN45) and non-small-cell lung-cancer cells (PC-7 and PC-14) at low concentrations ranging from 0.1 to 1 nM.[1]

A study of the antitumor action of gnidimacrin revealed that it binds to and directly activates, PKC in a dose-dependent manner (3–100 nM) *in vitro;* it also induced a slight and temporal block in the G_2/M phase within 8 hr and then a significant linear increase in the percentage of G_1-phase cells within 12 to 24 hr after the onset of treatment in sensitive K562 cells.[1] To clarify the mechanism by which gnidimacrin induces cell-cycle arrest, its effects on CDKs were examined. Low concentrations (0.6–26 nM) inhibited the cdk2 histone H1 kinase activity of K562 cells.[2]

The antitumor action of gnidimacrin might be associated with its ability to regulate the cell cycle through inhibition of cdk2 activity. In the present study, we clarified the mechanism by which gnidimacrin exerts antitumor activity *via* cell-cycle regulation factors.

MATERIAL AND METHODS

Chemicals

The isolation and identification of daphnane-type diterpene gnidimacrin (NSC252940) has been outlined previously.[1] Polyclonal antihuman cdk2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA). Antihuman Rb protein MAb was purchased from Pharmingen (San Diego, CA). Anti-cyclin E, anti-$p21^{WAF1/Cip1}$ and anti-cdc25A antibodies, along with their respective antigenic peptides, were purchased from Santa Cruz Biotechnology.

Tumor cells and antitumor activity in vitro *(MTT assay)*

Human K562 leukemia and KATOIII stomach cancer cells were grown in RPMI-1640 medium supplemented with 10% FBS. HLE hepatoma cells were grown in modified Eagle medium supplemented with 10% bovine serum. The effect of gnidimacrin on cell growth was examined with an MTT assay, as outlined in a previous report.[1] Briefly, K562 (4×10^3 cells/ml), KATOIII (10^4 cells/ml) and HLE (10^4 cells/ml) cells were seeded in a 96-well microplate in a volume of 180 μl. Twenty microliters of various concentrations of gnidimacrin were added to each well and cells were cultured for 4 days at 37°C in an atmosphere of 5% CO_2 in air. Fifty microliters of MTT (2 mg/ml), dissolved in Ca^{2+}/Mg^{2+}-free Dulbecco's [PBS(–)], were added to each well and the plates incubated for 4 hr. MTT formazan crystals were dissolved in DMSO and absorbance was measured at 562 and 630 nm in a multiwell plate reader (EL-340; BioMetallics, Princeton, NJ).

cdk2 assay

The histone H1 kinase activity of the anti-cdk2 immunoprecipitate was measured as detailed previously.[2] Briefly, cells were washed and lysed in 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] at 4°C for 20 min. After immunoprecipitation of the cell lysate with polyclonal rabbit antihuman cdk2 antibody, the histone H1 kinase activity of the immunoprecipitate was measured at 25°C for 10 min in a reaction mixture (40 μl) containing 0.4 mg/ml histone H1 (Boehringer-Mannheim, Mannheim, Germany), 60 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 7.4) and 2 μCi of [γ-^{32}P]ATP. The reaction was terminated by adding sample buffer and boiling for 5 min. Samples (7 μl) were analyzed by 12.5% SDS-PAGE, followed by autoradiography. The intensity of the spot corresponding to histone H1 was measured with a Fuji (Tokyo, Japan) BAS 2000 Bio-Image Analyzer.

Abbreviations: CAK, cdk-activating kinase; cdk2, cyclin-dependent kinase-2; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; HRP, horseradish peroxidase; IC_{50}, concentration required to inhibit cell growth to 50% of control; ILS, increase in life span; MAb, monoclonal antibody; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide; PKC, protein kinase C; PMA, phorbol myristate acetate; Rb, retinoblastoma.

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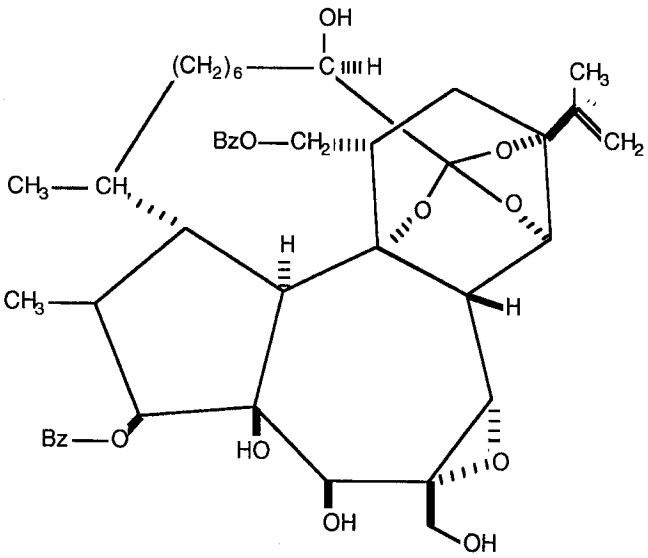

FIGURE 1 – Chemical structure of gnidimacrin. Bz, benzoyl.

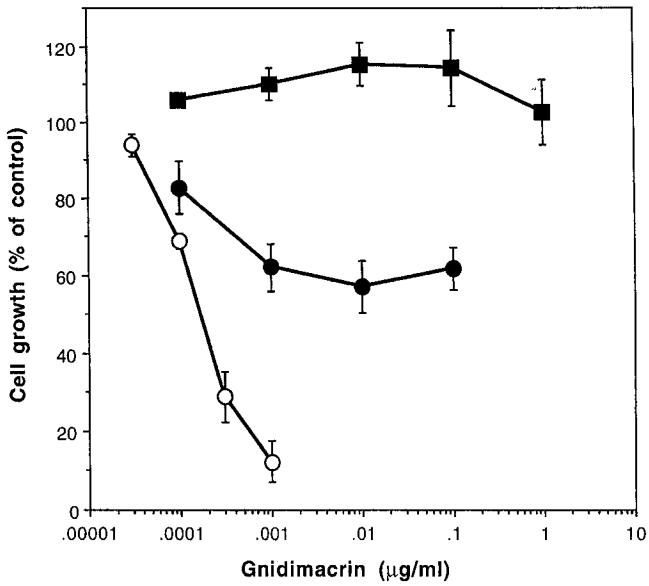

FIGURE 2 – Concentration-response curves of gnidimacrin-sensitive K562 and refractory KATOIII and HLE cells. Points represent averages ± SD of 3 to 5 independent experiments performed in quadruplicate with K562 (open circles), KATOIII (solid circles) and HLE (solid squares) cells. Cells were seeded in a volume of 200 μl/well and cultured for 4 days with various concentrations of gnidimacrin. Cell growth was measured with an MTT assay as described in Material and Methods.

Western blot analysis

The phosphorylation states of cdk2 and Rb protein, as well as the expression of $p21^{WAF1/Cip1}$, cyclin E and cdc25A, were assessed *via* immunoblot assays employing their specific antibodies. K562 (5×10^5 cells/ml) and KATOIII (5×10^5 cells/ml) cells were cultured with gnidimacrin at the indicated concentrations. Following incubation, treated cells were washed and lysed in lysis buffer or RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 50 μg/ml aprotinin and 1 mM sodium orthovanadate) at 4°C for 20 min. After centrifugation, the supernatant served as the cell lysate. The cell lysate was also subjected to immunoprecipitation using specific antibodies against cell-cycle regulation factors. The cell lysate and the immunoprecipitate were applied to 7.5% to 15% SDS-PAGE and then transferred electrophoretically to PVDF membranes (Millipore, Bedford, MA). Filters were stained with their respective antibodies and then secondarily with HRP-conjugated antibodies. Antigens were detected by enhanced chemiluminescence (Amersham, Aylesbury, UK).

Northern blot analysis

Total RNA from gnidimacrin-treated K562 cells was isolated by the method of Chomczynski and Sacchi.[3] Total RNA (10 μg) was loaded in each lane, electrophoresed on a formaldehyde-agarose gel (1%), transferred to a Hybond-N^+ transfer membrane (Amersham) and hybridized with each probe. The probe for cdc25A corresponded to a fragment (nucleotides 1878–2025 of human cdc25A) that had been labeled with ^{32}P to high specificity by PCR.[4] The probe for glucose-3-phosphate dehydrogenase (Clontech, Palo Alto, CA) was labeled with ^{32}P using a random-primer labeling system (Amersham).

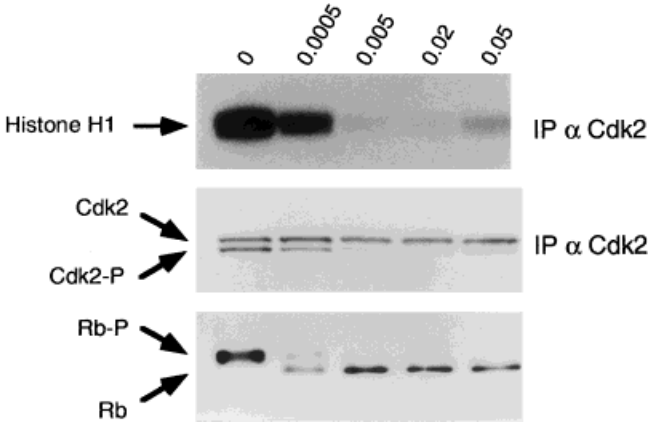

FIGURE 3 – Effects of gnidimacrin on cell-cycle regulatory factors. K562 cells were cultured with various concentrations of gnidimacrin for 24 hr. Histone H1 kinase activity of the anti-cdk2 immunoprecipitate (IP) was measured as described in Material and Methods. Cell lysate (2.5 mg protein) was treated with 0.3 μg anti-cdk2 antibody for 2 hr. Aliquots of IP and cell lysate (10 μg protein/lane) were applied to 15% and 7.5% SDS-PAGE and stained with anti-cdk2 and anti-Rb protein antibodies, respectively.

RESULTS

Effect of gnidimacrin on cell growth and cell-cycle regulation factors

Daphnane-type diterpene gnidimacrin (Fig. 1) exhibits antitumor activity against many human tumor cell lines.[1] However, there are intrinsically refractory cell lines, such as the human hepatoma cell line HLE. In addition, the human stomach-cancer cell line KATOIII exhibited some resistance to gnidimacrin (Fig. 2). Maximal G_1 arrest of the cell cycle was observed 24 hr after addition of gnidimacrin. Therefore, the cdk2 activity of K562 cells was examined 24 hr after gnidimacrin treatment. Figure 3 shows that gnidimacrin inhibited the cdk2 histone H1 kinase activity of K562 cells in a dose-dependent manner (0.0005–0.05 μg/ml). In refractory HLE cells, this cdk2 inhibition was not observed.[2] Thus, to clarify the mechanism governing cdk2 inhibition by gnidimacrin, the phosphorylation state of cdk2 was examined *via* immunoblot analysis using anti-cdk2 antibody after SDS-PAGE. Judging from the electrophoretic mobility of cdk2 during SDS-PAGE, the active form of cdk2,[5] in which Thr^{160} is phosphorylated,[6] decreased significantly in the presence of 0.005 and 0.02 μg gnidimacrin/ml (Fig. 3). The dose-dependent reduction in active cdk2 noted during immunoblot analysis paralleled the observed inhibition of cdk2

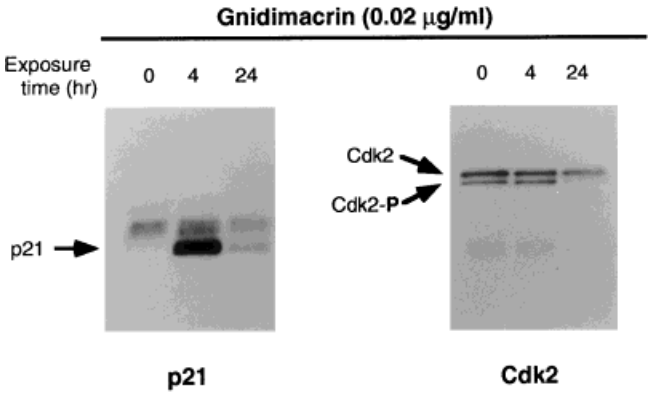

FIGURE 4 – Induction of $p21^{WAF1/Cip1}$ and disappearance of the phosphorylated form of cdk2 after gnidimacrin treatment. K562 cells were cultured with 0.02 μg gnidimacrin/ml for 0, 4 and 24 hr. Anti-cdk2 immunoprecipitate was applied to 15% SDS-PAGE and stained with anti-cdk2 and anti-$p21^{WAF1/Cip1}$ antibodies after being transferred to a PVDF membrane.

histone H1 kinase activity. Notably, exposure to 0.05 μg/ml gnidimacrin for 24 hr caused complete downregulation of PKC, as described previously,[2] and a slight reappearance of the active form and activity of cdk2 (Fig. 3). Our results indicate that a decrease in the active form of cdk2 is responsible for the inhibition in cdk2 activity created by gnidimacrin.

Since the Rb protein is phosphorylated by activated CDKs, including cdk2,[7] inhibition of cdk2 activity by gnidimacrin appears to cause conversion of the Rb protein to its inactive, dephosphorylated form (Fig. 3), resulting in G_1 phase arrest. These results suggest that the G_1 arrest of the cell cycle caused by gnidimacrin is due to dephosphorylation of the Rb protein through inhibition of cdk2 activity.

Induction of the cdk inhibitor $p21^{WAF1/Cip1}$ by gnidimacrin

In K562 cells, which do not express p53 protein,[8] the CDK inhibitor $p21^{WAF1/Cip1}$ was strongly induced in association with cdk2 during a 4 hr exposure to 0.02 μg gnidimacrin/ml (Fig. 4). However, its induction was transient and the phosphorylation state of cdk2 remained the same as it was before treatment, though cdk2 completely converted to its inactive form when exposed to gnidimacrin for 24 hr.

Effect of gnidimacrin on cdk2-regulatory factors

During the transition from G_1 to S, cdk2 is activated by cdc25A, which specifically dephosphorylates Thr^{14} and Tyr^{15} on cdk2. Thus, the effects of various concentrations of gnidimacrin on cdk2 activation were examined (Fig. 5). In a 24-hr exposure to gnidimacrin causing maximal G_1 arrest, cdk2-associated $p21^{WAF1/Cip1}$ was not detected at the indicated concentrations (Fig. 5*f*), though $p21^{WAF1/Cip1}$ was induced 4 hr after gnidimacrin treatment at concentrations exceeding 0.005 μg/ml (Figs. 4, 5*e*). A reduction in cdk2-associated cyclin E was observed during 24 hr exposures to gnidimacrin at concentrations exceeding 0.005 μg/ml. However, cyclin E expression was not affected by gnidimacrin (Fig. 5*a*). Surprisingly, in K562 cells exposed for 24 hr, suppression of cdc25A was observed in a concentration-dependent manner (Fig. 5*b*), though it was not detected in resistant KATOIII cells (Fig. 5*c*). In addition, inhibition of cdc25 expression was detected 12 hr after cell exposure to gnidimacrin and during 24 hr exposures to gnidimacrin at 0.005 and 0.02 μg/ml, stronger cdc25A suppression was observed (Fig. 6). The time-dependence and dose-dependence of this were consistent with the rate at which the active form of cdk2 disappeared upon administration of gnidimacrin. Northern blot analysis (Fig. 7) revealed that *cdc25A* mRNA was also downregulated in a concentration-dependent manner during 24 hr exposures

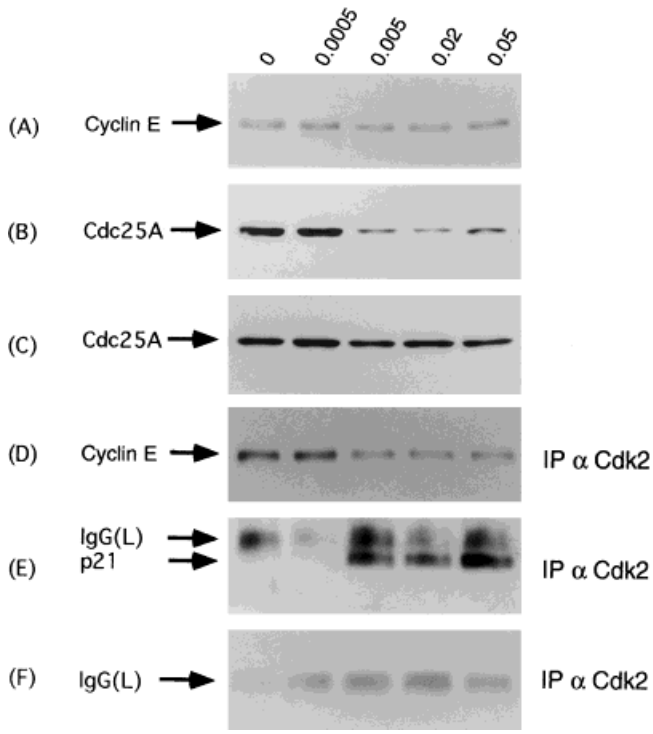

FIGURE 5 – Effects of gnidimacrin on cdk2-regulatory factors. K562 *(a,b,d–f)* and KATOIII *(c)* cells were cultured with gnidimacrin at the indicated concentrations. Four *(e)* and 24 *(a–d,f)* hr after gnidimacrin treatment, the cell lysate digested by lysis buffer and the anti-cdk2 immunoprecipitate (IP) were subjected to 10% *(a,d)* and 15% *(e,f)* SDS-PAGE and stained with anti-cyclin E and anti-$p21^{WAF1/Cip1}$ antibodies, respectively, after being transferred to a PVDF membrane. cdc25A *(b,c)* expression was examined *via* immunoblot analysis (7.5% SDS-PAGE) of the cell lysate (5 μg protein/lane) in RIPA buffer.

to gnidimacrin, in accordance with Western blot results. These results suggest that suppression of cdk2 activity is responsible for the antitumor action that gnidimacrin exerts *via* inhibition of cdc25A expression.

DISCUSSION

The antitumor action of a naturally occurring product, gnidimacrin, which can be isolated from the Chinese plant *S. chamaejasme* L., might be associated with cell-cycle regulation through suppression of cdk2 activity. A similar concentration-dependent pattern of cdk2 inhibition was found to correspond to the G_1 phase arrest induced by gnidimacrin in K562 cells.[2] The other CDK that regulates G_1 phase, cdk4, was not influenced by gnidimacrin treatment (data not shown). The cdk2 kinase is required for G_1/S transition in mammalian cells.[9,10] cdk2 inhibitors appear to have potential as antitumor agents, as reported by Chen *et al.*[11] In the present study, the mechanism of the antitumor activity of gnidimacrin *via* cell-cycle regulation factors, including cdk2, was examined to develop new antitumor agents targeting these factors. Sherr and Roberts[12] reported that the activity of CDKs, including cdk2, can be negatively regulated by the binding of various CDK inhibitors or tyrosine phosphorylation. In K562 cells, the CDK inhibitor $p21^{WAF1/Cip1}$ was strongly induced in association with cdk2 during 4 hr exposure to gnidimacrin. $p21^{WAF1/Cip1}$ is a potential mediator of p53 tumor suppression[13] and p53-mediated G_1 arrest in human cancer cells.[14] However, almost all gnidimacrin-sensitive cell lines tested, including a p53-deficient human leukemic K562 cell line, have been p53-negative. KATOIII cells[15] were found to have a complete deletion and HLE cells[16] to have a

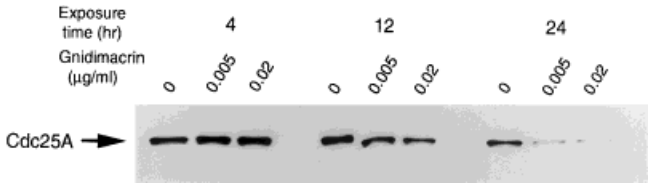

FIGURE 6 – Time- and dose-dependent repression of cdc25A by gnidimacrin. K562 cells were cultured with gnidimacrin at the indicated concentrations, then lysed in RIPA buffer 0, 4, 12 and 24 hr after gnidimacrin treatment. Cell lysate (2 μg protein/lane) was applied to 7.5% SDS-PAGE and stained with anti-cdc25A antibodies after being transferred to a PVDF membrane.

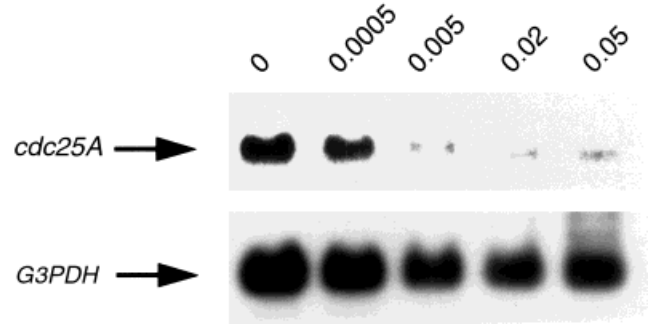

FIGURE 7 – Repression of *cdc25A* mRNA expression. Northern blot analysis was carried out as described in Material and Methods.

mutation in the *p53* gene. Thus, $p21^{WAF1/Cip1}$ induction is not likely to be responsible for the onset of G_1 block as a result of cdk2 inhibition by gnidimacrin, though it can be induced through a p53-independent mechanism.[17] This is also supported by our finding that the inhibition of cdk2 activity and the onset of G_1 phase arrest lagged 8 hr behind the transient induction of $p21^{WAF1/Cip1}$ by gnidimacrin. This finding is consistent with previous results involving another PKC activator, PMA.[6,18] When pretreated with the protein synthesis inhibitor cycloheximide to inhibit $p21^{WAF1/Cip1}$ induction, the cdk2 phosphorylation pattern did not change during 24 hr exposure to gnidimacrin compared to a case in which the cells were not pretreated, though complete inhibition of $p21^{WAF1/Cip1}$ induction was observed (data not shown). In addition, many human cancer cell lines are PMA-resistant, despite $p21^{WAF1/Cip1}$ induction.[19] Wang *et al.*[20] also reported that $p21^{WAF1/Cip1}$ expression in vitamin D_3-treated HL60 cells was transient and did not temporally correlate with the onset of G_1 block.

Gu *et al.*[5] reported that cdk2 activation is accompanied by an increase in its electrophoretic mobility on SDS-PAGE and that this increase is due to phosphorylation of Thr^{160} on cdk2. This increase is not observed upon phosphorylation of Tyr^{15} or Thr^{14}. Thus, the concentration- and time- dependent inhibition of cdk2 must correlate with a reduction in the active phosphorylated Thr^{160} form of cdk2. Our results are consistent with the finding that growth inhibition can be induced by both the PKC activator bryostatin 1 and PMA, as reported by Asiedu *et al.*[6]

Poon *et al.*[21] reported that CDKs are inactivated by a combination of $p21^{WAF1/Cip1}$ and Thr^{14}/Tyr^{15} phosphorylation following UV-induced DNA damage. Our data demonstrate that the inhibition of cdk2 by gnidimacrin correlates with a loss in Thr^{160} phosphorylation. Coppock *et al.*[22] reported that regulation of cdk2 activity is mediated by the availability of cyclins E and A by the CAK enzyme, which phosphorylates cdk2 on Thr^{160}, or by cdc25A, which regulates the phosphorylation of Tyr^{15}. Therefore, these factors that may play a role in suppressing cdk2 kinase activation by gnidimacrin were examined. Expression of cyclin E

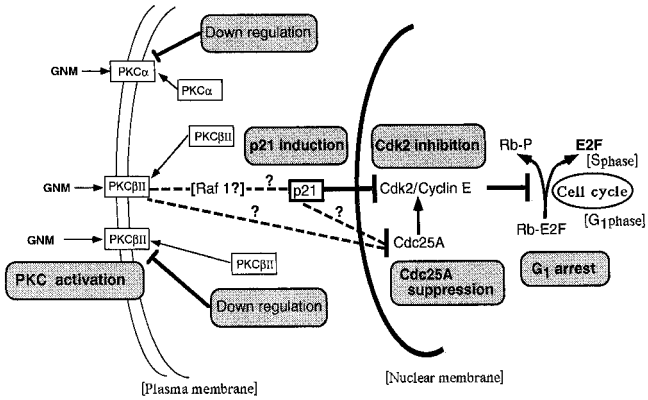

FIGURE 8 – Proposed scheme for the signaling pathway involved in the antitumor activity of gnidimacrin *via* PKC and the cell-cycle regulatory system. GNM, gnidimacrin.

was not affected by gnidimacrin, though the amount of cdk2-associated cyclin E decreased upon its administration. The decrease in cdk2-associated cyclin E at gnidimacrin levels over 0.005 μg/ml paralleled the decrease in active cdk2 shown in Figure 3. This suggests that cdk2 inhibition by gnidimacrin is not due to the binding of a CDK inhibitor to cdk2 but rather to a reduction in the amount of active cdk2 and that gnidimacrin causes this reduction in the active cyclin E–cdk2 complex. Suppression of cdc25A was found when K562 cells were treated with gnidimacrin. Moreover, this dose-dependent suppression was similar to the suppression observed in cdk2 activity. Also, its time dependence paralleled the time required for cdk2 inhibition. cdc25A is a phosphatase that activates cdk2 and a potential human oncogene.[23] In addition, cdc25A is essential for G_1/S transition because microinjection of anti-cdc25A-specific antibody into rat cells blocked cell-cycle progression from G_1 into S phase.[24] Saha *et al.*[25] reported that $p21^{WAF1/Cip1}$ inhibits the association between cdc25A, cyclin and cdk2. However, our results suggest that repression of cdc25A and induction of $p21^{WAF1/Cip1}$ were independent of cdk2 inhibition by gnidimacrin. This is likely the case because cdc25A repression was observed after $p21^{WAF1/Cip1}$ induction by gnidimacrin in our study and because cdc25A repression by TGF-β has been reported in cells lacking the CDK inhibitor p15.[4] The relationship between cdc25A repression and Thr^{160} dephosphorylation in cdk2 is not clear, but it is possible that Thr^{160} phosphorylation in cdk2 occurs simultaneously with, or just after, the dephosphorylation of Thr^{14}/Tyr^{15} in cdk2 during cdk2 activation. It does not exclude the possibility that gnidimacrin itself inhibits cdk2 phosphorylation at Thr^{160} by CAK, as reported for phorbol ester–treated fibroblasts.[26] Moreno *et al.*[27] reported that the *cdc25* gene product acts as an important rate-limiting activator in regulating the onset of the cell cycle. Therefore, cdc25A repression appears to be responsible for G_1 phase arrest through cdk2 inhibition by gnidimacrin. Gnidimacrin is a PKC activator. To prove a link between PKC of tumor cells and these biologic functions caused by gnidimacrin, we are now examining the biologic effects of gnidimacrin using PKC-downregulated cells and PKC isoform gene–transfected cells.

We have shown that the mechanism governing the antitumor action of gnidimacrin operates through cell-cycle regulation factors (Fig. 8). Many factors affect cdc25, such as raf1,[28] c-myc[29] and the signal transducers and activators of transcription-3.[30] The effects of gnidimacrin on these factors need to be determined, to clarify the mechanism of cdc25A suppression by gnidimacrin. For this purpose, studies involving gnidimacrin will be invaluable in delineating the signal-transduction pathway induced by gnidimacrin. Gnidimacrin will also be of use in the clarification of the mechanism by which $p21^{WAF1/Cip1}$ induction and cdc25A repression lead to G_1 arrest in the cell cycle, resulting in inhibition of tumor growth.

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