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# The Effects of Genkwadaphnin and Gnidilatidin on the Growth of P–388, L–1210 Leukemia and KB Carcinoma Cells *in Vitro*

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**Abstract**—*Daphnane diterpene esters have previously been shown to have antineoplastic activity* in vivo *against the growth of* P–388 *lymphocytic leukemia cells. These studies demonstrate cytotoxic activity of genkwadaphnin and gnidilatidin against* P–388 *lymphocytic leukemia,* L–1210 *lymphoid leukemia and human KB carcinoma cell growth* in vitro. *At the* $ED_{50}$ *values in the respective tumor lines DNA synthesis was preferentially suppressed in all three cell lines. RNA synthesis was essentially unaffected by the agents. Protein synthesis inhibition by the two agents demonstrated selectivity, e.g. in* P–388 *cells significant inhibition, in* L–1210 *cells marginal inhibition and in KB cells no inhibition was observed at these concentrations. Multiple sites in DNA synthesis were found to be inhibited by the daphnane diterpene esters. Two to three times the* $ED_{50}$ *concentration in the respective tumor lines was required to observe suppression of DNA synthesis. Purine* de novo *synthesis appeared to be the major site of inhibition, with inosine monophosphate dehydrogenase and phosphoribosyl pyrophosphate amido transferase activities being inhibited in all three tissue lines. Dihydrofolate reductase activity was inhibited, significant only in the* P–388 *and KB cells. The magnitude of the enzyme suppression by the agents varied with the tumor line. However, the degree of enzyme suppression was of sufficient magnitude to account for the observed purine and DNA synthesis inhibition by the daphnane diterpene esters.*

## INTRODUCTION

THE FLOWERS of *Daphne genkwa* (Thymelaeaceae) are known in Chinese folklore as herbal remedies and have recently been reported to be useful in cancer against tumor growth [1]. From this plant have been isolated novel daphnane diterpene esters. Two of these esters, genkwadaphnin and gnidilatidin [2], structurally resemble phorbol esters and have many structural features in common with the phorbol esters; however, there are some minor structural differences. The daphnane esters contain an ortho-ester moiety and an epoxy group which are not present in phorbol esters. The phorbol esters possess an intact cyclopropane ring whereas daphnane esters do not. Apparently, these structural differences denote major alterations in the biological activity of the two types of esters since phorbol esters are not known to be antineoplastic agents, whereas the daphnane diterpene esters have been reported to be active against P–388 leukemic growth [2].

Genkwadaphnin and gnidilatidin, two daphnane diterpene esters, have been shown to be active against the growth of P–388 lymphocytic leukemia cells in $BDF_1$ mice *in vivo*. Genkwadaphnin produced an optimum *T/C*% value of 173 and gnidilatidin a *T/C*% of 151 at 0.8 mg/kg/day i.p. in $DBA_2$ mice [3]. Previous studies after *in vivo* administration at 6, 12, and 72 hr have shown that daphnane diterpene esters inhibit significantly multiple sites in nucleic acid *de novo* synthesis including nuclear DNA polymerase, IMP dehydrogenase, PRPP amido transferase and dihydrofolate reductase activities of P–388 cells. In an attempt to determine which biochemical event correlated most directly with cell death of the tumor cell, we elected to examine these same biochemical parameters *in vitro* at multiples of the $ED_{50}$ values of the drugs and we expanded the study to include a human KB carcinoma of the mouth and the murine L–1210 lymphoid leukemia to ascertain if the same mode of action was valid in other tumor lines for these daphnane diterpene esters.

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## MATERIALS AND METHODS

*Isolation of drugs*

Genkwadaphnin and gnidilatidin were isolated

from the air-dried flowers of *Daphne genkwa* as previously described by Hall *et al.* [3].

*Cytotoxic assays.* The mouse P–388 lymphocytic leukemia, L–1210 lymphoid leukemia cells and human KB (epidermoid carcinoma of the mouth) cells were maintained in minimum essential medium (MEM) with antibiotics and 10% fetal calf serum. Drugs were suspended in 0.05% Tween 80–water by homogenization and sterilized by filtration. The $ED_{50}$ values for genkwadaphnin and gnidilatidin were determined over the range of 1–50 μM final concentration of drug. The $ED_{50}$ values (concentration of drug required to cause 50% inhibition of cell growth) in all cell lines were calculated on day 3 according to NCI's standard protocol [4]. KB cells were treated with 0.25% trypsin to digest and suspend the KB cells. Cell number was determined per ml with a hemocytometer by counting viable cells using the 0.04% trypan blue exclusion technique [5]. In other P–388 tissue culture cells, the drugs were washed out after 5 min. Cells were harvested by centrifugation (600 ***g***), placed in fresh MEM + 10% FCS and antibiotics and allowed to grow for 3 days.

*DNA, RNA and protein synthesis*

The *in vitro* incorporation studies of precursors into DNA, RNA and protein in KB, L–1210 or P–388 cells was determined for 60 min by the method of Liao *et al.* [6]. *In vitro* incorporation studies were conducted with $10^6$ tumor cells, 1 μCi thymidine (methyl–$^3$H, 84 Ci/mmol), uridine (6–$^3$H, 22.4 Ci/mmol) or leucine (4, 5–$^3$H(N), 56.5 Ci/mmol), drugs present at 0.5, 1, 1.5, 2 and 4 times their respective $ED_{50}$ values and MEM + 10% fetal calf serum for a total volume of 1 ml. After incubation at 37°C for 60 min the reactions were inactivated with acid and the acid-insoluble precipitate collected on filter discs. DNA was collected on glass fiber GF/F discs by vacuum suction and washed with 10% perchloric acid containing 1% pyrophosphate. RNA and protein were collected on nitrocellulose Millipore filters washed with 10% trichloroacetic acid. The discs were placed in scintillation fluid and counted. The control value for thymidine incorporation was 47317 dpm/$10^6$ P–388 cells; uridine incorporation for the control was 42931 dpm/$10^6$ P–388 cells; and leucine incorporation for the control was 9353 dpm/$10^6$ P–388 cells. The control value for thymidine incorporation was 31891 dpm/$10^6$ KB cells; uridine incorporation was 43340 dpm/$10^6$ KB cells; and leucine incorporation was 18380 dpm/$10^6$ KB cells. The control values from L–1210 cell DNA synthesis was 13516 dpm/$10^6$ cells; for RNA synthesis 20781 dpm/$10^6$ cells; and for protein synthesis 12256 dpm/$10^6$ cells.

*Enzymatic studies*

Daphnane diterpene esters were present at 0.5, 1, 1.5 and 2 times the $ED_{50}$ value for inhibition of cell growth in the P–388 cells, 0.5, 1, 2 and 4 times the $ED_{50}$ values for L–1210 cells and 1, 2, 3 and 4 times the $ED_{50}$ values for the KB cells. The following enzymatic activities were determined on P–388, KB and L–1210 cells. Nuclear DNA polymerase activity was determined in isolated nuclei of P–388 cells [7]. DNA α-polymerase activity was determined on a cytoplasmic preparation of the enzyme isolated from P–388 cells by the method of Eichler *et al.* [8], using activated DNA digested with pancreatic DNase I. Nuclear DNA and DNA (α-)polymerase activities were determined using dTTP (methyl$^3$H, 82.4 Ci/mmol), dCTP, dATP and dGTP for 60 min at 37°C. Acid-insoluble DNA was collected on glass fiber discs and counted. The control value for P–388 cell DNA nuclear polymerase activity was 24, 568 dpm/mg of nuclear protein. The control value for KB cells' DNA nuclear polymerase activity was 1022 dpm/mg of nuclear protein. The control value for L–1210 nuclear polymerase activity was 4035 dpm/mg of nuclear protein. The control value for DNA polymerase (α) activity of P–388 cells was 47,644 dpm/mg of protein. Formate incorporation into purine was determined in the three cell lines by the method of Spassova *et al.* [9]. Formate incorporation into purines for 40 min at 37°C was determined using 0.5 μCi [$^{14}$C]formic acid (52 mCi/mmol). Purines were separated by silica gel TLC eluted with *N*-butanol:acetic acid: water (4:1:5). Using the standards, guanine and adenine, the appropriate spots were scraped and counted. The control value for P–388 cells was 23, 682 dpm/mg of protein. The control value for KB cells was 24, 970 dpm/mg of protein. The control value for L–1210 cells was 15,286 dpm/mg of protein. Phosphoribosyl pyrophosphate (PRPP) amido transferase activity was determined by the spectrophotometric method of Wyngaarden and Ashton [10]. PRPP amido transferase activity was determined at 340 nm utilizing a 600 ***g*** × 10 min supernatant for 20 min. The net optical density change/hr/μg protein for the P–388 control was 0.806, for the KB control 0.942 and for the L–1210 0.936. Inosinic acid dehydrogenase activity was determined by the method of Becker and Löhr [11]. Inosinic acid dehydrogenase activity for 30 min at 37°C was determined using [8–$^{14}$C]inosine–5′–monophosphate (61 mCi/mmol). XMP was separated from IMP by TLC on PEI plastic plates eluted with 0.5 M $(NH_4)_2SO_4$. Using standards, the XMP spot was scraped and counted. The P–388 control value was 40,059 dpm/mg of protein, the KB control value was 37,935 and the L–1210 control value was 22,980 dpm/mg of protein. Dihydrofolate reduc-

tase activity was determined by the method of Ho *et al.* [12]. Dihydrofolate reductase activity was determined at 340 nm for 30 min using a supernatant (600 ***g*** × 10 min). The control value for reoxidation of NADH was 0.761 optical units change/hr/mg of protein for P–388 cells, 0.682 for KB cells and 0.868 for L–1210 cells.

*Metaphase cell blockage*

P–388 cells ($2 \times 10^6$) were treated first with hydroxyurea and then with colcemid (0.025 μg/ml) for 7 hr, which arrests the cells during the mitotic stage. The cells were washed in cold MEM and 10% fetal calf serum and cultured in fresh water medium. Drugs were added at 50 μM concentration at 0, 0.5 and 1 hr after removal of colcemid from the medium [13]. Adriamycin (2 μg/ml) was used as a standard in the assay.

*DNA strand scission studies*

Previously labeled [$^3$H]thymidine P–388 DNA was incubated with daphnane diterpene ester at twice the $ED_{50}$ value to determine single-strand scission using alkaline sucrose gradients as described by Suzuki *et al.* [13], Pera *et al.* [14] and Woynarowski *et al.* [15]. P–388 cells ($5 \times 10^6$) were incubated for 24 hr at 37°C in MEM + 10% fetal calf serum and 10 μCi [$^3$H]thymidine (methyl-$^3$H, 84.0 Ci/mmol). Cells were centrifuged at 600 ***g*** × 10 min in phosphate-buffered saline (PBS), washed and resuspended in 1 ml of PBS. Genkwadaphnin (11.18 μM) and gnidilatidin (12.82 μM) were added and incubated at 37°C for 2 hr. Cells were centrifuged and washed with PBS. Lysis buffer (0.5 ml—0.5 M NaOH, 0.02 M EDTA, 0.01% Triton X–100) was layered onto a 5–20% alkaline-sucrose gradient (4.0 ml of 0.3 M NaOH, 0.7 M NaCl, 0.01 M EDTA) and 0.2 ml cell preparation was added. After incubating for 30 min at room temperature, the gradient was centrifuged at 140,000 ***g*** and 20°C for 60 min (Beckman rotor SW 60). Fractions were collected (0.2 ml) from the top of the gradient, neutralized with 0.2 ml of 0.3 N HCl and counted.

## RESULTS

*Cytotoxic assays*

The daphnane diterpene esters effectively inhibited the growth of P–388 lymphocytic leukemia, L–1210 lymphoid leukemia and KB human epidermoid carcinoma tissue culture cells. The $ED_{50}$ value for genkwadaphnin for the P–388 cytotoxic assay was 5.39 μM while gnidilatidin afforded a value of 6.42 μM. The $ED_{50}$ in the L–1210 cells for genkwadaphnin was 2.8 μM, whereas gnidilatidin afforded a value of 10.1 μM. KB tissue cells appeared to be more sensitive to the agents with genkwadaphnin, resulting in an $ED_{50}$ value of 1.15 μM and a gnidilatidin value of 1.95 μM. Examination of the P–388 cell growth after the removal of drugs after 5 min demonstrated that the cells did not recover from the inhibition by the drugs, but cell growth was suppressed approximately at the same level, i.e. affording identical $ED_{50}$ values as previously observed when the drug was present for the entire 3-day period. Thus the effects of the agents were essentially immediate and non-reversible.

*Metabolic studies*

Genkwadaphnin produced an $ID_{50}$ of 6.9 μM and gnidilatidin 8.4 μM for the inhibition of DNA synthesis in P–388 cells (Figs 1 and 2). For protein synthesis inhibition an $ID_{50}$ value of 12.4 μM was obtained for genkwadaphnin and 19.1 μM for gnidilatidin. RNA synthesis of P–388 cells was inhibited by approximately 15% for genkwadaphnin at 5–16 μM, whereas gnidilatidin had essentially no effect on RNA synthesis in P–388 cells. Nuclear DNA polymerase activity was inhibited 30% by genkwadaphnin and 13% by gnidilatidin at three times the $ED_{50}$ values for antineoplastic activity. DNA polymerase (α) activity was reduced 25% by genkwadaphnin and 15% by gnidilatidin at 100 μM. Purine synthesis of P–388 cells was markedly inhibited by the daphnane diterpene esters, with genkwadaphnin affording an $ID_{50}$ value of 16.17 μM and gnidilatidin 23.2 μM concentration. The $ID_{50}$ value for PRPP amido transferase activity was 20.5 μM for genkwadaphnin and > 26.5 μM for gnidilatidin. For IMP dehydrogenase activity the $ID_{50}$ value for genkwadaphnin was 18.8 μM and for gnidilatidin 19.5 μM, whereas for dihydrofolate reductase activity the $ID_{50}$ value for genkwadaphnin was 3.79 μM and for gnidilatidin 5.6 μM.

In the KB cells the $ID_{50}$ value for DNA synthesis inhibition for genkwadaphnin was 3.06 μM and for

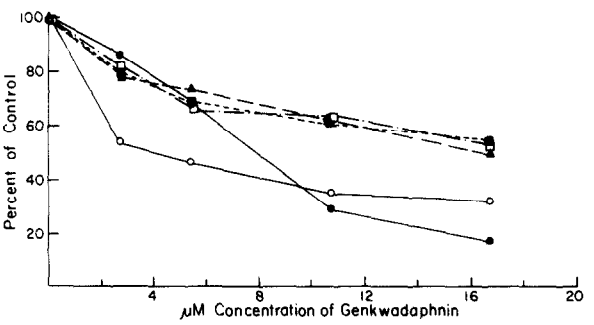


*Fig. 1. The effects of genkwadaphnin on P-388 lymphocytic leukemia cell metabolism. ●–●, DNA synthesis; ▲–▲, purine synthesis; ■– – –■, PRPP amido transferase activity; □– – –□, IMP dehydrogenase activity; and ○–○, dihydrofolate reductase activity.* n = 6; *standard deviation was less than 7%.*

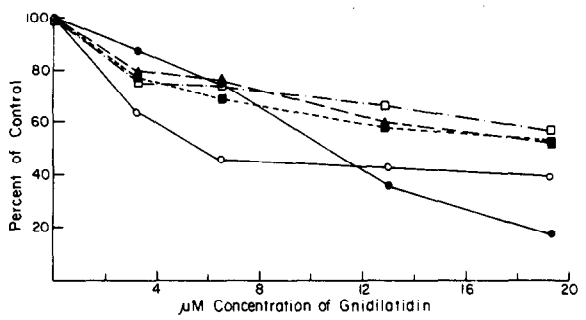


*Fig. 2. The effects of gnidilatidin on P-388 lymphocytic leukemia cell metabolism. ●–●, DNA synthesis; ▲– –▲, purine synthesis; ■– – –■, PRPP amido transferase activity; □–□, IMP dehydrogenase activity; and ○–○, dihydrofolate reductase activity.* n = 6; *standard deviation was less than* 7%.

gnidilatidin 6.02 μM (Figs 3 and 4). Protein synthesis in KB cells was not affected by the daphnane diterpene esters at the concentrations employed. RNA synthesis in KB cells was not inhibited significantly, e.g. at four times the $ED_{50}$ value genkwadaphnin caused 32% inhibition and gnidilatidin 26%. Nuclear DNA polymerase activity was suppressed 18% by genkwadaphnin and 26% by gnidilatidin at four times the $ED_{50}$ value for antineoplastic activity. Purine synthesis of KB cells was inhibited significantly, with an $ID_{50}$ value of 2.72 μM for genkwadaphnin and 4.04 μM for gnidilatidin. PRPP amido transferase activity was inhibited to a lesser degree in the KB cells, with $ID_{50}$ values > 10 μM for genkwadaphnin. IMP dehydrogenase activity afforded an $ID_{50}$ of 4 μM, and for dihydrofolate reductase activity the $ID_{50}$ for genkwadaphnin was 7.1 μM. For gnidilatidin, IMP dehydrogenase activity was suppressed 33% at 7.8 μM and for dihydrofolate reductase activity an $ID_{50}$ of 7.8 μM was afforded.

In the L-1210 cells, significant inhibition of DNA synthesis was observed with genkwadaphnin (Figs 5 and 6). The $ID_{50}$ value was ~4.9 μM for suppression of DNA synthesis by genkwadaphnin.

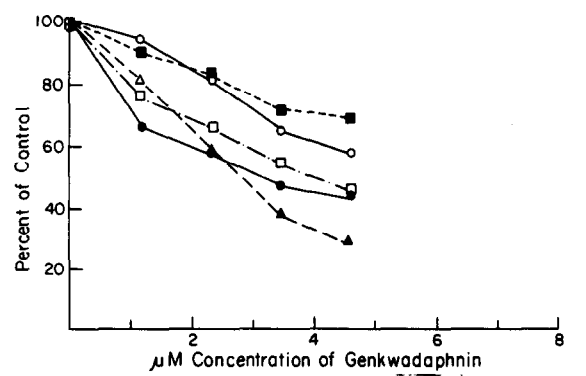


*Fig. 3. The effects of genkwadaphnin on KB epidermoid carcinoma cell metabolism. ●–●, DNA synthesis; ▲– –▲, purine synthesis; ■– – –■, PRPP amido transferase activity; □–□, IMP dehydrogenase activity; and ○–○, dihydrofolate reductase activity.* n = 6; *standard deviation was less than* 7%.

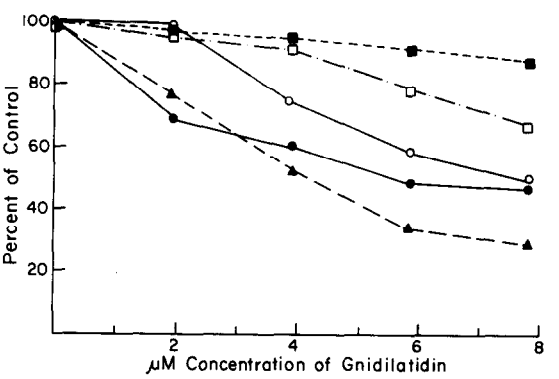


*Fig. 4. The effects of gnidilatidin on KB epidermoid carcinoma cell metabolism. ●–●, DNA synthesis; ▲– –▲, purine synthesis; ■– – –■, PRPP amido transferase activity; □–□, IMP dehydrogenase activity; and ○–○, dihydrofolate reductase activity.* n = 6; *standard deviation was less than* 7%.

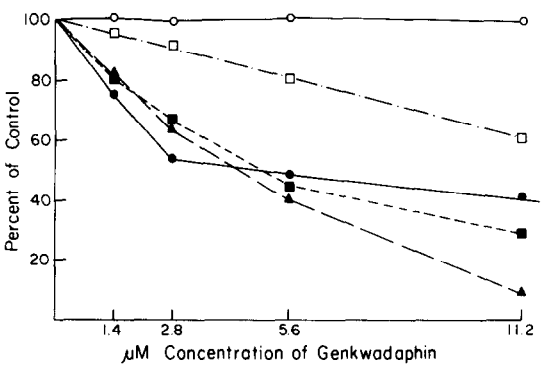


*Fig. 5. The effects of genkwadaphnin on L-1210 lymphoid leukemia cell metabolism. ●–●, DNA synthesis; ▲– –▲, purine synthesis; ■– – –■, PRPP amido transferase activity; □–□, IMP dehydrogenase activity; and ○–○, dihydrofolate reductase activity.* n = 6; *standard deviation was less than* 7%.

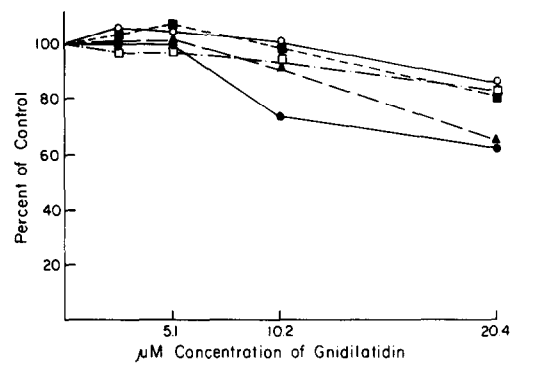


*Fig. 6. The effect of gnidilatidin on L-1210 lymphoid leukemia cell metabolism. ●–●, DNA synthesis; ▲–▲, purine synthesis; ■–■, PRPP amido transferase activity; □–□, IMP dehydrogenase activity; and ○–○, dihydrofolate reductase activity.* n = 6; *standard deviation was less than* 7%.

RNA synthesis was essentially unaffected (8–9%) by the daphnane diterpene esters and protein synthesis was suppressed only 26–29% by the drugs at the highest concentration employed. Formate incorporation into purines was suppressed

significantly by genkwadaphnin, affording an $ID_{50}$ value of 4.1 μM. The enzyme PRPP amido transferase appeared to be the key site of drug inhibition, resulting in an $ID_{50}$ of ~4.8 μM for genkwadaphnin. IMP dehydrogenase activity was inhibited ~41% at the maximum drug concentration of 11.2 μM. Dihydrofolate reductase and nuclear DNA polymerase activities were not inhibited significantly by genkwadaphnin, with inhibitions of 3 and 10% respectively at the highest concentrations employed. Gnidilatidin was not an effective agent against the growth of L-1210 cells. Only 38% inhibition of DNA synthesis, 35% inhibition of purine synthesis, 19% suppression of PRPP amido transferase activity, 16% reduction of IMP dehydrogenase activity and 14% inhibition of dihydrofolate reductase activity by gnidilatidin at 20.4 μM concentration was observed.

*Metaphase cell blockage and DNA strand scission studies*

After synchronized P-388 cells were treated with colcemid, no cell growth inhibition was observed with the daphnane diterpene esters at 50 μM concentration (Fig. 7). Since this concentration far exceeds the $ED_{50}$ values in P-388 cells, it can be assumed that these agents do not block the cell cycle in the metaphase stage. The DNA strand scission studies using labeled P-388 cells showed that at twice the $ED_{50}$ value of the daphnane diterpene esters there was no breakage of the DNA strands by the drugs (Fig. 8).

All data in Figs 1–6 are expressed as a percentage of control. The standard deviations did not exceed 7% for any of the assays and six aliquots of cells were used for each assay value. $ED_{50}$ (50% of the cell growth) and $ID_{50}$ values (50% inhibition of activity) were determined using semi-log plots. The significant level used for these studies was $P < 0.001$ calculated by the Student's $t$ test.

## DISCUSSION

Daphnane diterpene esters are diterpene esters which resemble tigliane esters structurally but with the cyclopropane ring D of the tigliane esters opened in the daphnane esters to afford an isopropylene side chain at C-13 [16]. Although genkwadaphnin and gnidilatidin share many similar structural features with the tigliane esters, they also possess an ortho-ester and an epoxy ring. These latter structural changes may be critical to the type of biological activity afforded by the ester. Tigliane esters, e.g. croton oil and TPA, are co-carcinogens and promote cellular proliferation by stimulating DNA synthesis, increasing tRNA, rRNA and mRNA synthesis, followed by increasing protein synthesis and decreasing cAMP levels in several cell lines [16, 17]. It has been suggested that croton oil inhibits the DNA repair

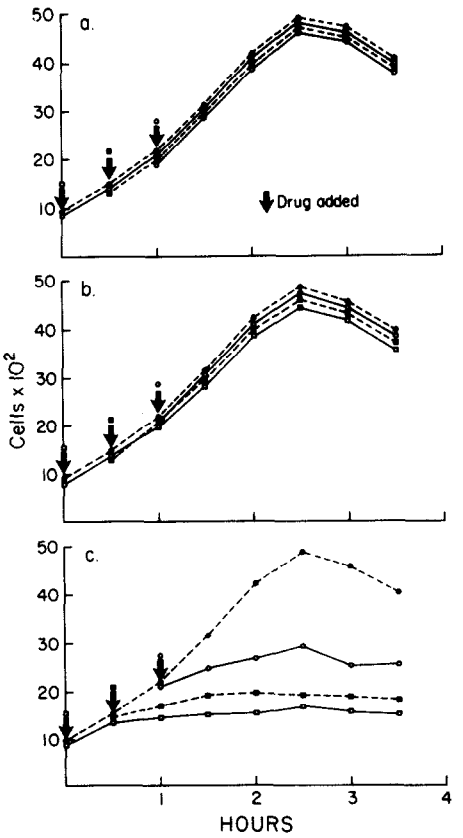


*Fig. 7. Effects of genkwadaphnin and gnidilatidin on the division of synchronized P-388 lymphocytic leukemia cells treated with colcemid. P-388 cells* ($2 \times 10^5$) *were incubated with* 0.025 μg/ml *of colcemid for* 7 hr. *The cells were then washed with cold MEM* + 10% *FCS and suspended in fresh warm medium. Drugs* (50 μM) *were added to tissue culture cells at* 0, 0.5 *and* 1 hr *after the removal of colcemid. The cell number was determined with the use of a hemocytometer over the next* 3.5 hr. *Adriamycin* (2 μg/ml) *was used as a standard. (a) Genkwadaphnin; (b) gnidilatidin; (c) adriamycin* 2 μg/ml.

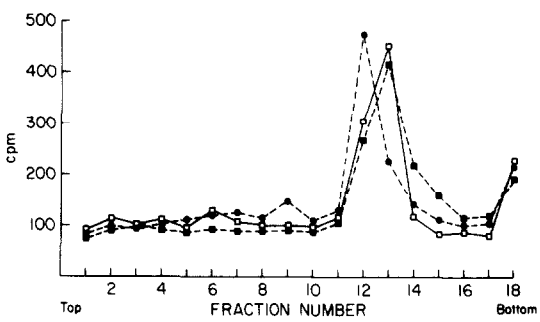


*Fig. 8. Effect of genkwadaphnin and gnidilatidin on DNA strand scission of P-388 lymphocytic leukemia cells. P-388 cells* ($5 \times 10^6$) *were incubated for* 24 hr *at* 37°C *in MEM* + 10% *fetal calf serum and* 10 μCi [$^3$H] *thymidine* (*methyl-*$^3$H, 84.0 Ci/mmol). *Cells were centrifuged at* 600 *g* × 10 min *in PBS, washed and resuspended in* 1 ml *of PBS. Genkwadaphnin* (11.18 μM) *and gnidiliatidin* (12.82 μM) *were added and incubated at* 37°C *for* 2 hr. *Cells were centrifuged and washed with PBS. Lysis buffer* (0.5 ml—0.5 M NaOH, 0.02 M *EDTA*, 0.01% *Triton X-100*) *was layered onto a* 5–20% *alkaline-sucrose gradient* (4.0 ml—0.3 M NaOH, 0.7 M NaCl, 0.01 M *EDTA*) *and* 0.2 ml *cell preparation was added. After incubating for* 30 min *at room temperature, the gradient was centrifuged at* 35,000 rpm *and* 20°C *for* 60 min (*Beckman rotor SW60*). *Fractions were collected* (0.2 ml) *from the top of the gradient, neutralized with* 0.2 ml *of* 0.3 N HCl *and counted.* ●–●, *Control;* ▲–▲, *genkwadaphin, and* ■–■, *gnidilatidin.*

mechanism of cells. Unlike the tigliane diterpene esters, the daphnane diterpene esters possess P-388 antileukemic activity, e.g. 12-hydroxydaphnetoxin, gnidin, gniditrin, gnidicin, gnidimacrin and mezerein. Kupchan and coworkers [18–21] have suggested that C–20 and C–12 ester groups are necessary for antineoplastic activity; however, with genkwadaphnin and gnidilatidin, the C–20 ester group is not required for antileukemic activity against *in vivo* P-388 growth, nor does it appear to be required for cytotoxic activity in P-388, KB or L–1210 tissue culture cells. Both compounds have a C–12 ester group, thus this requirement would be consistent with literature observations. However, it should be noted that these are not the only functional moieties present in these daphnane diterpene esters and other structural components may contribute to the antineoplastic/cytotoxic activity of this group of compounds.

Genkwadaphnin and gnidilatidin proved to be cytotoxic against the growth of murine P-388 lymphocytic leukemia, L–1210 lymphoid leukemia and human KB carcinoma cells. The NCI protocol has denoted 4 μg/ml or less as the concentration required for significant cytotoxicity activity *in vitro* [4]. All of the $ED_{50}$ values for the three tumor lines fall within this value with the exception of gnidilatidin in the L–1210 screen at 10.2 μM (6.6 μg/ml). The $ED_{50}$ values for the two daphnane diterpene esters in the P-388 or KB cell lines were very similar but the values were quite different in the L–1210 screen. DNA synthesis was preferentially inhibited in the three tumor cells. Values of twice the $ED_{50}$ concentration required for cell death were necessary to observe 50% inhibition of DNA synthesis in P-388 and L–1210 cells, and three times the $ED_{50}$ concentration required for cell death were necessary in the KB cells to observe 50% inhibition of DNA synthesis. RNA synthesis inhibition did not appear to be a factor in causing cell death by the daphnane diterpene esters in any of the three types of cells. The suppression of RNA synthesis by the daphnane diterpene esters lagged behind the effects of the drugs on DNA synthesis, probably due to the fact that even though the agents suppressed purine synthesis, which is required in both DNA and RNA *de novo* pathways, most eukaryotic cells have a higher pool level of ribonucleotides rather than deoxyribonucleotides. Suppression of purine synthesis by any agent would be reflected in the deoxyribonucleotide pool quicker than in the ribonucleotide pool; thus DNA synthesis would be inhibited initially, followed at a later time by RNA synthesis suppression. Protein synthesis was significantly inhibited only in the P-388 cells. The inhibition of protein synthesis in the P-388 cells no doubt contributed to the $ED_{50}$ value obtained for the cytotoxic assay in P-388 cells, since these values were lower for both drugs than the $ID_{50}$ values obtained for the individual enzymes of the purine pathways which were inhibited by these agents. A detailed study on the mechanism of protein synthesis inhibition by daphnane diterpene esters has shown that they are inhibitors of the elongation process of protein synthesis by interference with the peptidyl transferase reaction in P-388 cells [3, 22].

Since the inhibition of DNA synthesis by the daphnane diterpene esters appeared to be the key factor, it was decided to examine regulatory enzymes known to be affected by these agents to determine which event was the most important in affording DNA synthesis inhibition. Multiples of the $ED_{50}$ concentrations for the respective tumor lines were employed. The inhibition of purine synthesis was effectively reduced in all three tumor lines. At the $ED_{50}$ value for cytotoxic activity in the respective cell lines the inhibition of DNA synthesis and the inhibition of purine synthesis were of a similar magnitude of reduction, suggesting that the two events were directly related. In P-388 cells the inhibition of the DNA synthesis became greater at higher multiples of the $ED_{50}$ values, probably due to the additive effect of the inhibition of dihydrofolate reductase activity and reduction of protein synthesis by the daphnane diterpene esters. The $ID_{50}$ values obtained for the agents in P-388 cells for the inhibition of dihydrofolate reductase activity were more similar to the $ED_{50}$ concentrations in the P-388 than the inhibition of regulatory enzymes involved in the purine pathway, i.e. suppression of dihydrofolate reductase in P-388 correlated most directly with cell death ($ED_{50}$ value). In the KB cells the purine synthesis inhibition correlated with the inhibition of DNA synthesis by genkwadaphnin. The inhibition of IMP dehydrogenase activity in KB cells appeared to be the major enzyme responsible for purine synthesis reduction. In the L–1210 lymphoid leukemia cells the inhibition of PRPP amido transferase activity by genkwadaphnin correlated more directly with reduction of purine synthesis and thus DNA synthesis. This was evident at the $ED_{50}$ value in L–1210 cells. The inhibition of IMP dehydrogenase lagged behind the other metabolic effects caused by the drug; however, the inhibition of IMP dehydrogenase by genkwadaphnin was probably synergistic with the inhibition of PRPP amido transferase at the higher concentration of drug in the L–1210 cells. These observations suggest that there are differences in sensitivities of the enzymes from various tumor lines to the daphnane diterpene esters, resulting in different magnitudes of enzyme reduction. This may be due to genetic differences in the polypeptides of the enzymes or due to isoenzymes

being produced in the carcinogenic process of the tissue. An alternative hypothesis which might explain the differences in effects in various tumor lines is that it may not be due to the parent drug but rather to possible different metabolites which are generated by the individual cancer cells. These metabolites may vary with the species as well as the histological type of tissue, resulting in different chemical species of the drug which bind to the enzyme with different affinities, creating different magnitudes of inhibition of the catalytic action of the enzyme.

Previous u.v. studies with genkwadaphnin and gnidilatidin have shown that there was no binding of the drugs to DNA or the base, guanine [3]. The present study shows that when the drugs were incubated at twice the $ED_{50}$ concentration in P–388 tissue culture cells for 24 hr, there was no evidence of DNA strand scission. Thus the DNA macromolecule cell does not appear to be a target of the drugs. Colcemid is an agent which blocks the cell cycle in the $G_2$ phase and allows the cell to synchronize. After removal of colcemid, growth is initiated after 30 min in P–388 cells. The addition of genkwadaphnin or gnidilatidin did not block cell growth after the 30 min as did adriamycin, once the colcemid was removed from the cultured cells. This datum indicates that the daphnane diterpene esters do not affect events in the latter stages of the cell cycle. In other studies the inhibition of cell growth of P–388 cells only required 5 min of drug incubation to cause permanent effects which were non-reversible when the drug was removed from the medium. The cytotoxic activity of the agents was determined by the technique of dye exclusion by the cell. This parameter appears to be affected by the daphnane diterpene ester within 5 min of drug incubation at a relative low concentration of the drug. On the other hand, DNA synthesis or purine synthesis inhibition requires 2–3 times the $ED_{50}$ value of the drug for a 60-min incubation to observe 50% reduction. These two observations suggest that the kinetics of cell death by the daphnane diterpene esters is not related directly to the inhibition of DNA synthesis. The ability of the agents to cause cell death may be related more to changes in the permeability of the membrane of the cancer cells whereas the ability to block proliferation is related more to suppression of DNA synthesis of the cell.

In conclusion, the daphnane diterpene esters appear to block cancer cell growth by suppressing DNA synthesis at multiple sites in the purine pathway, i.e. IMP dehydrogenase, PRPP amido transferase and dihydrofolate reductase. The magnitude of the suppression of the activity of the individual enzyme varies with the type of cancer cell involved.

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