In earlier work (3, 4, 6, 8) the extended Hilidebrand solubility approach employed a polynomial regression routine for calculating quantities such as W and $(\log \alpha_2)/A$, and this statistical method has proved successful in most instances. It is demonstrated in the current study that direct polynomial regression sometimes may produce an unsatisfactory fit of solubility data. A nonlinear regression program, NONLIN (17), has been shown to improve the fit when ordinary polynomial regression fails.

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Antitumor Agents LVI: The Protein Synthesis Inhibition by Genkwadaphnin and Yuanhuacine of P-388 Lymphocytic Leukemia Cells

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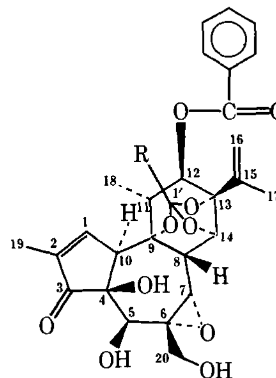
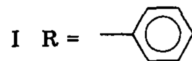
Received August 26, 1981, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.* Accepted for publication January 26, 1982.

Abstract ☐ Two natural products isolated from the plant *Daphne genkwa* have been shown to possess antileukemic activity in mice. Genkwadaphnin and yuanhuacine were observed to inhibit DNA and protein synthesis in P-388 leukemic cells. A detailed study of the effects of these two diterpene esters on protein synthesis of leukemic cells was undertaken. The major effects of genkwadaphnin and yuanhuacine on protein synthesis were blockage of the elongation process and interference with the peptidyl transferase reaction. The latter reaction was suppressed at concentrations of the diterpene esters which were commensurate with concentrations that inhibited whole cell *in vitro* protein synthesis in P-388 cells.

Keyphrases ☐ Antitumor agents—inhibition of DNA and protein synthesis by genkwadaphnin and yuanhuacine in P-388 lymphocytic leukemia cells, daphnane deterpene esters ☐ Genkwadaphnin—antitumor agents, inhibition of DNA and protein synthesis in P-388 lymphocytic leukemia cells, daphnane diterpene esters ☐ Yuanhuacine—antitumor agents, inhibition of DNA and protein synthesis in P-388 lymphocytic leukemia cells, daphnane diterpene esters



I R = 

II R = $-CH{=}CH-CH{=}CH-(CH_2)_4-CH_3$

Daphnane diterpene esters which possess an isopropylene side chain at C_{13} have previously been reported to have antileukemic activity (1). Genkwadaphnin and yuanhuacine (I and II) are two such esters which have been isolated from *Daphne genkwa* and chemically characterized (2). Genkwadaphnin (I) at 0.8 mg/kg/day was shown to produce a T/C% value of 173, whereas yuanhuacine (II) afforded a value of 151% against P-388 lymphocytic leukemia growth (2). These T/C% values were comparable to 5-fluorouracil at 12.5 mg/kg/day in the P-388 screen. Therefore, it was concluded that daphnane diterpene esters may have potential as antineoplastic therapeutic agents and that their modes of action on cellular metabolism were of interest, particularly since these agents resemble, structurally, phorbol esters which are tumor pro-

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moting agents that stimulate nucleic acid and protein synthesis.

Previous studies demonstrated that I and II inhibited DNA synthesis after 60 min of incubation, with ID_{50} values of 5.62 and 7.11 μM in P-388 lymphocytic leukemia cells (2). A higher concentration of daphnane diterpene esters was required to observe protein synthesis inhibition of P-388 cells ($ID_{50} \cong$ 14.8 and 18.5 μM) (2). Similar observations in P-388 cells were made when the agents were administered *in vivo*, in that DNA synthesis was inhibited initially, followed by inhibition of protein synthesis at a later time or at a higher dose (2). Since a cursory review of the literature indicates that effects of daphnane diterpene esters on cellular metabolism have not been studied, the purpose of the present investigation is to establish the mechanism of action of I and II as protein synthesis inhibitors.

EXPERIMENTAL

The studies on the effects of daphnane diterpene esters on protein synthesis were conducted on P-388 lymphocytic leukemia cells harvested on day 10 (3) and maintained according to the NIH Protocol (3). P-388 lymphocytic leukemia lysates from DBA/2 male mice (~25 g) were prepared by a previous method (4). The following were isolated from P-388 lysates by literature techniques: ribosomes[1] (5), pH 5 enzyme (4), and uncharged transfer ribonucleic acid (tRNA) (6). The P-388 lymphocytic leukemia cell initiation factors for protein synthesis were prepared as described previously (7). Standard protein synthesis inhibitors were used as a comparison in the assay at concentrations which were known to cause maximum inhibition. Whole cell *in vitro* incorporation of [^{3}H]leucine (56.6 Ci/mmole) into protein was determined with drug concentrations from 0–50 μM in 1 ml of minimum essential medium[2] for 90 min at 37°. Acid insoluble protein was collected on nitrocellulose filters[3] by vacuum suction (8). [^{3}H]Methionyl-transfer ribonucleic acid (met-tRNA) was prepared from P-388 cell tRNA according to a previous method (9). The effects of the diterpene esters on endogenous protein synthesis of P-388 lysates were performed in a reaction mixture (9) (0.5 ml) containing 10 mM tris(hydroxymethyl)aminomethane, pH 7.6, 76 mM KCl, 1 mM adenosine triphosphate, 0.2 mM guanosine triphosphate, 15 mM creatine phosphate, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM of each of the 19 essential amino acids, 0.9 mg/ml of creatine phosphokinase, and 20 μCi of [^{3}H]leucine (56.6 Ci/mmole). The reaction mixture was incubated at 30°. After 90 sec of incubation, test drugs or the standards, pyrocatechol violet or emetine, were added with a final concentration of 1, 10, and 100 μM. At specific minute intervals, 50-μl aliquots were removed from the reaction tubes and spotted on filter papers[4] which were treated for 10 min in boiling 5% trichloroacetic acid, followed by 10 min in cold 5% trichloroacetic acid and washed with cold 5% trichloroacetic acid, ether–ethanol (1:1), and ether. The filter papers were dried and counted in scintillation fluid.

The effects of daphnane diterpene esters, pyrocatechol violet, cycloheximide, and emetine on the ribosome profile (8) of P-388 cell lysates were assayed using the reaction medium described above (500 μl). The control tubes, as well as tubes containing standards or drugs affording a 100 μM final concentration, were incubated for 4 min at 37°. The reactions were terminated in ice and gradient buffer (1 ml of tris(hydroxymethyl)aminomethane, pH 7.6, 10 mM KCl, and 1.5 mM $MgCl_2 \cdot 6H_2O$) was added. The mixtures were layered over 36 ml of 10–25% linear sucrose gradient (8), prepared in gradient buffer, and centrifuged for 165 min at 25,000 rpm in a swinging bucket rotor[5] at 4°. The absorbance profiles at 260 nm were determined using a flow cell (light path 0.2 cm) attached to a spectrophotometer[6]. The incorporation of [^{3}H]leucine into polypeptide chains was measured for each fraction.

The reaction medium for the polyuridine-directed polyphenylalanine synthesis (10) contained 50 mM tris(hydroxymethyl)aminomethane, pH 7.6, 12.5 mM magnesium acetate, 80 mM KCl, 5 mM creatine phosphate, 0.05 mg/ml of creatine phosphokinase, 0.36 mg/ml of polyuridine[7] (A_{280}/A_{260} = 0.34), 0.5 μCi of [^{14}C]phenylalanine (536 mCi/mmole), 75 μg of uncharged P-388 cell tRNA, 70 μg of P-388 pH 5 enzyme preparation, and 0.9 A_{260} of P-388 cell ribosomes. Test compounds were present in 5, 10, and 50 μmole concentrations. The tubes were incubated for 20 min at 30° after which a 35-μl aliquot was spotted on filter paper[2] and processed as indicated previously.

The reaction medium (200 μl) used to measure the formation of the 80 S initiation complex and the methionyl puromycin reaction (11) contained 15 mM tris(hydroxymethyl)aminomethane, pH 7.6, 80 mM KCl, 1 mM adenosine triphosphate, 0.5 mM guanosine triphosphate, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 3 mM magnesium acetate, 0.1 mM (ethylenedinitrilo)tetraacetic acid, 1 mM dithiothreitol, 0.1 mM of each of the 19 essential amino acids, 3 mg of P-388 cell lysates, 100 μg/ml chlortetracycline[8], 3 × 10^5 cpm of [^{3}H]-met-tRNA, and 20 μg/ml of polyadenosine–uridine–guanosine and 0–50 μM of diterpene esters.

The incubation was carried out at 23°, and after 2 min aliquots were withdrawn to analyze for 80 S complex formation. Puromycin (10 μg/ml) was then added to the reaction medium. The incubation was continued for another 6 min and aliquots were withdrawn to analyze for reaction of the 80 S complex with puromycin. All aliquots (50 μl) were diluted to 250 μl with buffer (20 mM tris(hydroxymethyl)aminomethane, pH 7.6, 80 mM KCl, 3 mM magnesium acetate, 1 mM dithiothreitol, and 0.1 mM (ethylenedinitrilo)tetraacetic acid), layered on 11.8 ml of a 15–30% linear sucrose gradient and centrifuged for 3 hr × 36,000 rpm in a swinging bucket rotor[9]. Fractions (0.4 ml) were collected and protein was precipitated with 10% trichloroacetic acid on filter papers and counted[10].

The reaction mixtures (75 μl) for the ternary complex formation (12) contained 21.4 mM tris(hydroxymethyl)aminomethane, pH 8.0, 80 mM KCl, 0.26 mM guanosine triphosphate, 2.14 mM dithiothreitol, 10 μg of bovine serum albumin, 5 pmoles of P-388 cell [^{3}H]met-tRNA (1 × 10^4 cpm), 100 A_{260}/ml of crude P-388 cell initiation factors, and 25 or 100 μM of drug or standard. The incubation was conducted for 5 min at 37° and terminated by the addition of 3 ml of cold buffer (21.4 mM tris(hydroxymethyl)aminomethane, pH 8.0, 80 mM KCl, 2.14 mM dithiothreitol). The samples were filtered through 0.45-μm nitrocellulose filters, washed twice in buffer, and counted.

The reaction mixture (75 μl) for the 80 S initiation complex (12) formation contained, in addition to the components necessary for the ternary complex formation reaction, 1.9 mM magnesium acetate, 5 A_{260}/ml of polyadenosine-uridine-guanosine[5] and 100 A_{260}/ml of 80 S P-388 cell ribosomes. Incubation was 10 min at 37° which was then cooled to 4°, the samples were diluted with cold buffer (21.4 mM tris(hydroxymethyl)-aminomethane, pH 8.0, 80 mM KCl, 5 mM magnesium acetate, and 2.14 mM dithiothreitol), and filtered as indicated for the ternary complex formation experiment.

RESULTS AND DISCUSSION

Both I and II significantly suppressed *in vitro* whole cell P-388 lymphocytic leukemia protein synthesis (Fig. 1). Compound I afforded an $ID_{50} \simeq$ 6.7 μM and compound II an $ID_{50} \simeq$ 8.9 μM after 90 min of incubation. The studies on the effects of various concentrations of the diterpene esters on endogenous protein synthesis of P-388 lysates (Fig. 2) demonstrated that at low concentration (1 μM) there was a lag of 2–4 min before the inhibition of protein synthesis was observed. At higher concentrations of drug (10–100 μM), cessation of protein synthesis occurred immediately, suggesting that the diterpene esters mimicked the action of an elongation inhibitor such as emetine, more so than an initiation inhibitor such as pyrocatechol violet.

A comparison of the effects of I and II with known inhibitors of protein synthesis on the ribosome profile and [^{3}H]leucine incorporation into polypeptides can be seen in Fig. 3. During the incubation of the P-388 lymphocytic leukemia cell lysates, [^{3}H]leucine was incorporated into the nascent peptide bound to the polysomes (Fig. 3a). However, when I or II was incubated with the P-388 lysates for 4 min at 100 μM, the radioactive peptides were released from the ribosomes. Clearly, all of the agents suppress polypeptide synthesis observed as reduced leucine incorpora-

[1] Ribosomes dissociated from mRNA, runoff ribosomes, or polysomes.
[2] Eagle growth medium × 1 plus 10% fetal calf serum, penicillin, and streptomycin.
[3] Millipore Corp.
[4] Whatman 3.
[5] Beckman SW 27.
[6] Gilford.
[7] Miles Laboratory Inc.
[8] Sigma Chemical Co.
[9] Beckman SW 40.
[10] Fisher Scintiverse in a Packard Scintillation Counter.

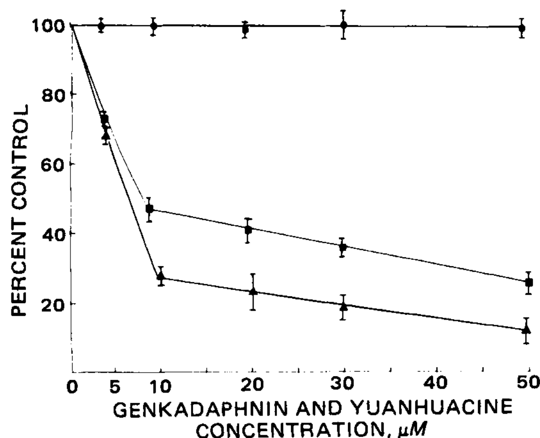

Figure 1—In Vitro *effect of genkwadaphnin and yuanhuacine on whole cells of P-388 lymphocytic leukemia during 90-min incubation with [³H]leucine incorporation* (n = 5). *Key: (●) control; (▲) genkwadaphnin; (■) yuanhuacine.*

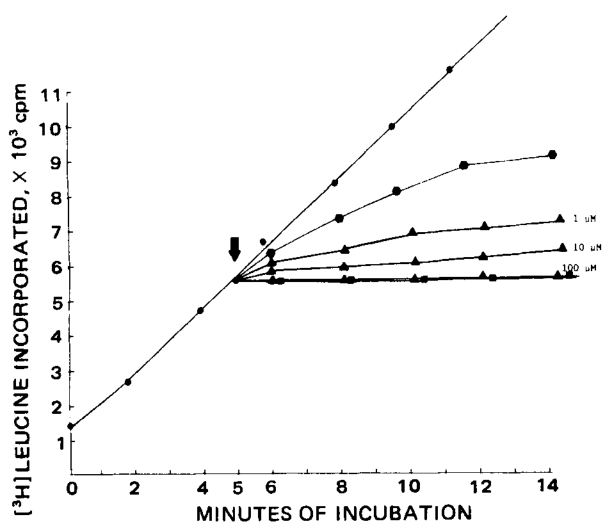

Figure 2a—*Effect of genkwadaphnin on the protein synthesis of P-388 lymphocytic leukemia homogenates using endogenous mRNA* (n = 5). *Key: (●) control; (▲) genkwadaphnin; (■) emetine; (●) pyrocatechol violet; (➧) addition of drug.*

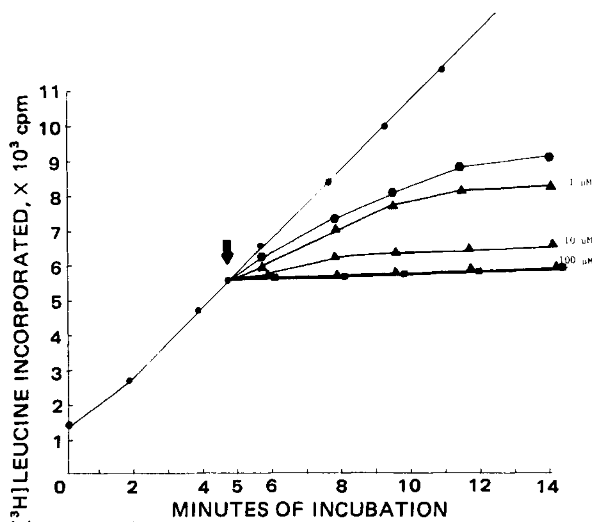

Figure 2b—*Effect of yuanhaucine on the protein synthesis of P-388 lymphocytic leukemia homogenates using endogenous mRNA* (n = 5). *Key: (●) control; (▲) yuanhuacine; (■) emetine; (●) pyrocatechol violet; (➧) addition of drug.*

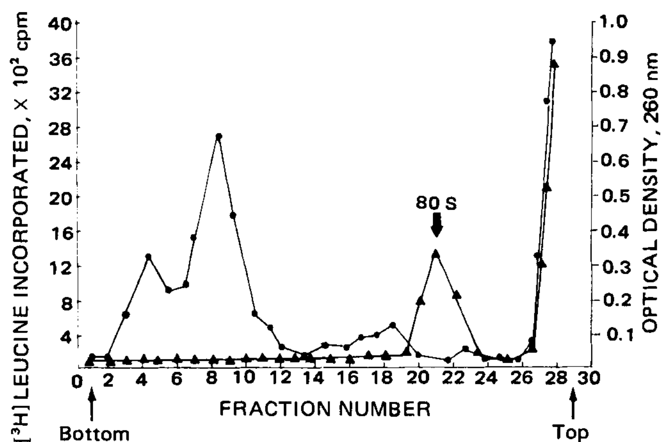

Figure 3a—*Control: Fate of nascent protein on the protein synthesis in P-388 lymphocytic leukemia lysate* (n = 5). *Key: (●) leucine incorporated into polypeptide; (▲) 80 S initiation profile.*

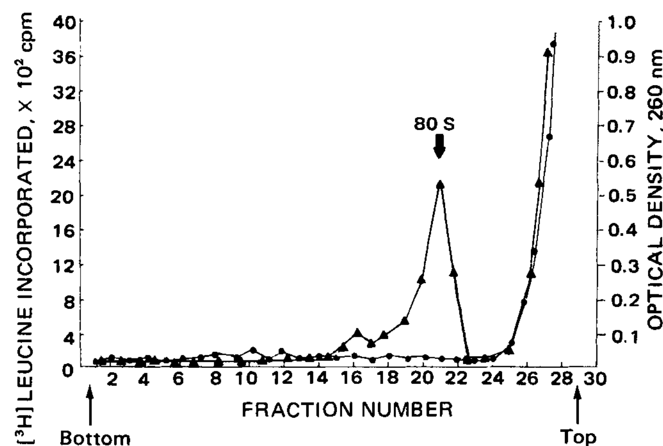

Figure 3b—*Addition of pyrocatechol violet 100* μM. *Effect of pyrocatechol violet on polyribosome structure and release of nascent peptides in P-388 lymphocytic leukemia lysate* (n = 5). *Key: (●) leucine incorporated; (▲) 80 S profile.*

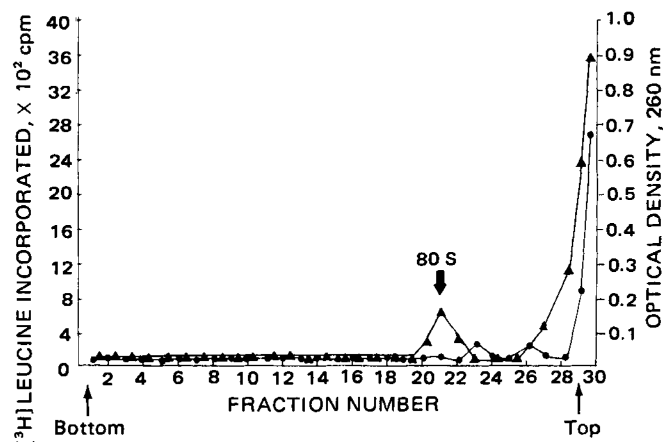

Figure 3c—*Addition of emetine 100* μM. *Effect of emetine on polyribosome structure and release of nascent peptide in P-388 lymphocytic leukemia lysate* (n = 5). *Key: (●) leucine incorporated; (▲) 80 S profile.*

tion. The standard, pyrocatechol violet (Fig. 3b), allows completion or release of the nascent polypeptide chain and the accumulation of the 80 S ribosomal peak, whereas emetine (Fig. 3c), I (Fig. 3d), and II (Fig. 3e), do not allow accumulation of the 80 S ribosomal peak which is reduced from the control. These data suggest that the daphnane diterpene esters

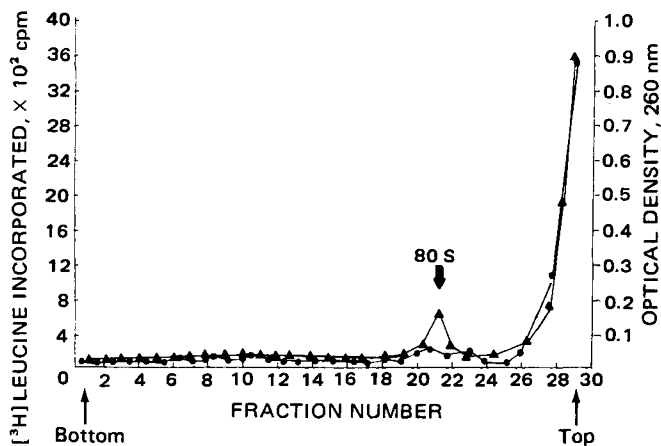

Figure 3d—*Addition of genkwadaphnin 100 μM. Effect of genkwadaphnin on polyribosome structure and release of nascent peptides in P-388 lymphocytic leukemia lysate (n = 5). Key: (●) leucine incorporated; (▲) 80 S profile.*

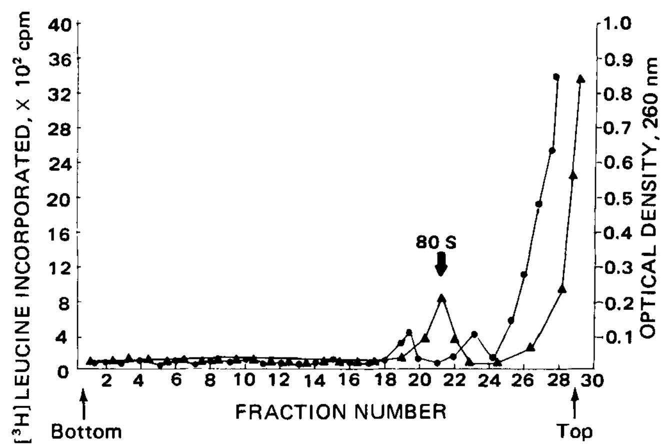

Figure 3e—*Addition of yuanhuacine 100 μM. Effect of yuanhuacine on polyribosome structure and release of nascent peptides in P-388 lymphocytic leukemia lysate (n = 5). Key: (●) leucine incorporated; (▲) 80 S profile.*

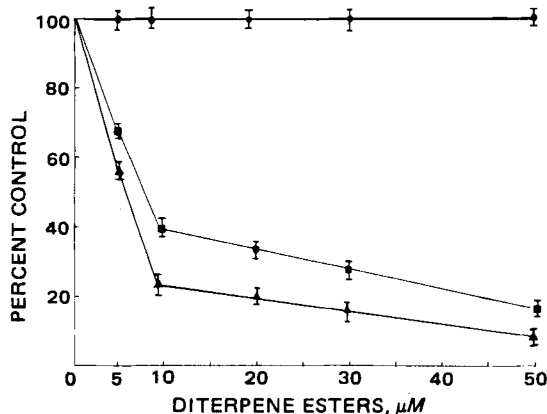

Figure 4—*Effect of genkwadaphnin and yuanhuacine on polyuridine directed poly [³H]phenylalanine synthesis in P-388 run-off ribosomes (n = 5). Key: (●) control; (▲) genkwadaphnin; (■) yuanhuacine.*

are elongation inhibitors of protein synthesis of P-388 lymphocytic leukemia cells at the concentrations employed.

To completely eliminate the possibility that the daphnane diterpene esters were initiation inhibitors, ternary complex[11] formation and the 80 S initiation complex[12] studies were performed (Table I) on P-388 lysates, which showed that at 25 and 100 μM concentrations, the diterpene esters had essentially no effect on the initiation steps of polypeptide chain synthesis. Figure 4 illustrates the effects of the diterpene esters on polyuridine directed polyphenylalanine synthesis of purified ribosomes isolated from 10-day P-388 cells. Polyuridine directed polyphenylalanine synthesis does not require the normal initiation and termination reactions of protein synthesis, and thus, agents that block this reaction are considered exclusively elongation inhibitors. At 50 μM, I suppressed polyuridine directed polyphenylalanine synthesis 92%, while II suppressed it 88%. The degree of inhibition of polyuridine synthesis by the diterpene esters was of sufficient magnitude to account for the degree of inhibition alone observed by these esters on whole cell protein synthesis.

In an effort to further evaluate the effects of diterpene esters on the elongation step of peptide synthesis, the formation of the 80 S initiation complex[12] and peptide bond formation were examined by treating the P-388 cell lysates with the elongation inhibitor chlortetracycline which specifically inhibits the binding of aminoacyl-tRNA to the ribosome A site but does not inhibit the peptidyl transferase reaction of elongation. Thus, when [³H]met-tRNA was added to the system, most of the radioactivity was found associated with the 80 S initiation complex. Addition of polyadenosine-uridine-guanosine to the chlortetracycline-treated lysate allows the formation of the 80 S initiation complex (Fig. 5a) which then reacts with puromycin. The puromycin induces the release of

Table I—Effects of Diterpene Esters on Ternary and 80 S Complex Formation of P-388 Cells

	Concentration, μM	Complex Formation, pmoles	Percent of Control
Ternary Complex Formation (N = 6)			
Control	—	2.12 ± 0.08	100
+ Emetine	100	2.06 ± 0.06	97
+ Pyrocatechol violet	100	0.25 ± 0.03	11
+ Aurintricarboxylic acid	50	0.17 ±0 .02	8
+ Genkwadaphnin	25	1.91 ± 0.05	90
	100	1.89 ± 0.06	
+ Yuanhuacine	25	1.95 ± 0.07	92
	100	1.93 ± 0.05	
80 S Initiation Complex Formation (N = 6)			
Control	—	1.26 ± 0.06	100
+ Emetine	100	1.03 ± 0.07	82
+ Pyrocatechol violet	100	0.20 ± 0.02	15
+ Aurintricarboxylic acid	50	0.13 ± 0.03	10
+ Genkwadaphnin	25	1.13 ± 0.05	90
	100	1.14 ± 0.08	
+ Yuanhuacine	25	1.16 ± 0.07	92
	100	1.16 ± 0.06	

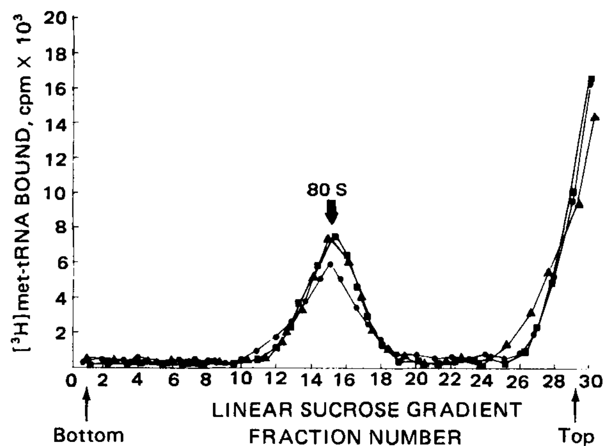

Figure 5a—*Control treated with chlortetracycline. The formation of the 80 S initiation complex of P-388 lymphocytic leukemia lysate (n = 5). Key: (●) control; (▲) genkwadaphnin; (■) yuanhuacine.*

[11] eIF-guanosine triphosphate-[³H]met-tRNA.

[12] 80 S Adenosine-uridine-guanosine-eIF-guanosine triphosphate-[³H]met-tRNA.

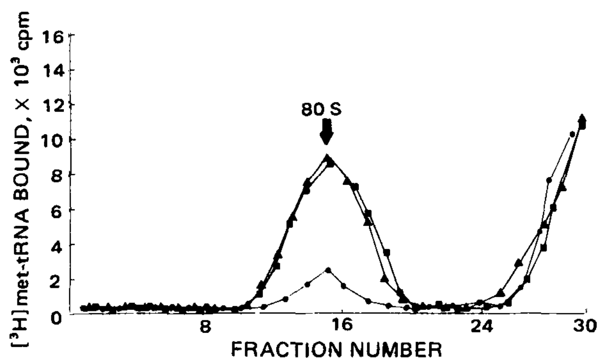

Figure 5b—*Addition of puromycin. Effect of genkwadaphnin and yuanhuacine on the methionyl puromycin reaction of P-388 lymphocytic leukemia lysate (n = 5). Key: (●) control plus puromycin; (▲) genkwadaphnin; (■) yuanhuacine plus puromycin.*

[^{3}H]methionine from the 80 S complex (Fig. 5b). The diterpene esters did not interfere with the formation of a stable 80 S initiation complex but rather inhibited the puromycin release of labeled methionine from the polysome.

These data indicate that I and II similarly block peptide bond formation during elongation peptide chain synthesis. The concentration of drug to block peptide transferase activity was consistent with concentrations required to inhibit whole cell protein synthesis *in vitro*.

The daphnane diterpene esters did not have any significant effects on the individual steps leading to the formation of a stable 80 S initiation complex. The daphnane diterpene esters significantly inhibited both the polyuridine-directed polyphenylalanine synthesis and the formation of the first peptide bond between puromycin and the met-tRNA bound to the 80 S initiation complex. These data strongly indicate that the diterpene esters are potent inhibitors of the peptidyl transferase reaction of the elongation process of protein synthesis of P-388 lymphocytic leukemia cells.

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Bayesian Individualization of Pharmacokinetics: Simple Implementation and Comparison with Non-Bayesian Methods

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Abstract □ One may attempt to individualize drug dosage by estimating an individual's pharmacokinetic parameters. Information useful for this purpose consists of certain population pharmacokinetic parameters (notably those describing the typical relationship between dosage and drug concentrations) and also measured drug concentrations from the individual of concern. Both types of information should be used. A (Bayesian) method that does so has been described in the pharmacokinetic literature. In this report an implementation of the Bayesian method that is readily adapted to a microcomputer is presented. Using simulated data it is compared with two other methods proposed by others, for estimating individual theophylline clearances. Both previously suggested methods are shown to be less precise than the Bayesian method: their typical error magnitudes are 20–70% larger.

Keyphrases □ Bayesian method—individualization of pharmacokinetics, simple implementation and comparison with non-Bayesian methods, theophylline □ Pharmacokinetics—Bayesian individualization, simple implementation and comparison with non-Bayesian methods, theophylline □ Theophylline—Bayesian individualization of pharmacokinetics, simple implementation and comparison with non-Bayesian methods

A great deal of attention has been given to the problem of estimating the pharmacokinetic parameters of individual patients in order to optimize dosage choices. Initially, most attention has been directed at obtaining estimates of individual parameters from the population information relating kinetics to certain patient features (sex, age, renal function, *etc*). More recently, considerable attention has been directed at the estimation of parameters using measured drug levels (1–4).

One particular method, the Bayesian method (3), is intuitively appealing. It involves a continuously changing view of the patient. Before any drug levels are measured,

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