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Indirect Inactivation of Rabbit Reticulocyte Initiation Factor eIF-2 by Helenalin and Bis(helenalinyl) Malonate†

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ABSTRACT: Helenalin and bis(helenalinyl) malonate, sesquiterpene lactones that react primarily with exposed sulfhydryl groups, were shown to be equally effective inhibitors of endogenous protein synthesis in rabbit reticulocyte lysates. By use of partially fractionated systems, it was possible to show that helenalin preferentially inhibited the conversion of the ternary initiation complex to the 48S preinitiation complex. Previous experiments have shown that this preferential inhibition is due to selective inactivation of eIF-3 [Williams, W. L., Chaney, S. G., Willingham, W., Considine, R. T., Hall, I. H., & Lee, K.-H. (1983) *Biochim. Biophys. Acta 740*, 152–162]. Bis(helenalinyl) malonate was much less active as an inhibitor of 48S complex formation than helenalin and clearly did not possess sufficient activity in that assay to explain its effectiveness as a protein synthesis inhibitor in whole lysates. Kinetic studies also showed a clear difference between the mechanism of action of these two drugs. Bis(helenalinyl) malonate inactivated protein synthesis in reticulocyte lysates only after a lag of 10 min, and the inhibition of protein synthesis could be completely reversed by the addition of 5 mM cAMP. Helenalin showed more complex kinetics. While full inhibition only occurred after a lag of 10–15 min, a partial inhibition was observed from very early times. cAMP at 5 mM was only partially able to reverse inhibition by helenalin. Phosphorylation studies showed that both helenalin and bis(helenalinyl) malonate were equally effective at activating eIF-2α kinase and indirectly causing phosphorylation of eIF-2. Furthermore, both drugs were able to activate eIF-2α kinase at low enough concentrations to account for their effectiveness as protein synthesis inhibitors in whole lysates. These data were interpreted to indicate that the activation of eIF-2α kinase was the primary mode of action of bis(helenalinyl) malonate as a protein synthesis inhibitor. Helenalin probably acts preferentially at the level of eIF-2α kinase activation in vivo. However, at sufficiently high concentrations it also directly inactivates eIF-3.

The biologically active sesquiterpene lactones usually contain an α-methylene γ-lactone, an α,β-unsaturated cyclopentenone ring, or an α-epoxycyclopentenone ring system (Lee et al., 1971; Hall et al., 1978; Kupchan et al., 1971). These are all electrophilic reactive centers that are capable of alkylating sulfhydryl compounds by a rapid Michael-type addition. While they are, in theory, capable of reacting with any nucleophile, several lines of evidence suggest that the sesquiterpene lactones exert their cytotoxic effects primarily through inactivation of essential sulfhydryl groups. Several studies with model biological nucleophiles have shown that thiols are the only nucleophiles alkylated to any significant extent by sesquiterpene lactones (Kupchan et al., 1970; Hall et al., 1977; Lee et al., 1977). The α-methylene γ-lactone moiety has also been shown to react with exposed sulfhydryl groups on the enzymes phosphofructokinase (Hanson et al., 1970) and glycogen synthetase (Smith et al., 1972). For both enzymes

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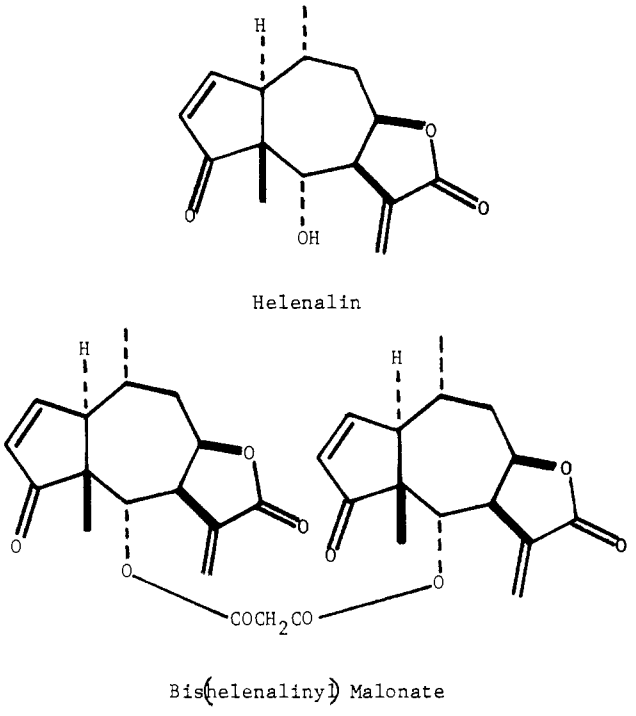

FIGURE 1: Structure of helenalin and bis(helenalinyl) malonate.

tested, the loss of catalytic activity could be correlated with the alkylation of critical sulfhydryl groups. Finally, the growth inhibitory properties of several sesquiterpenes have been prevented by sulfhydryl compounds such as cysteine or 2,3-dimercaptopropanol (Thimann et al., 1945; Hauschka et al., 1945; Sequeira et al., 1978). Thus, sesquiterpene lactones are generally considered to be relatively nonspecific sulfhydryl alkylating agents.

Helenalin, a sesquiterpene lactone of the pseudoguaianolide class (Figure 1), has been shown to possess significant antitumor activity against Ehrlich ascites, Walker-256 carcinosarcoma, and P-388 lymphocytic leukemia tumors (Lee et al., 1971; Hall et al., 1977, 1978). It also possesses significant antiinflammatory activity (Hall et al., 1979). Bis(helenalinyl) malonate (Figure 1) has been prepared synthetically from helenalin (Lee et al., 1981) and has been shown to possess comparable antitumor (Lee et al., 1981) and antiinflammatory (Hall et al., 1979) activities.

For both helenalin and bis(helenalinyl) malonate, their cytotoxicity closely correlates with inhibition of both DNA and protein synthesis (W. L. Williams et al., unpublished results). The mechanism of protein synthesis inhibition by helenalin has been studied in some detail. Helenalin specifically inhibits at the level of initiation in both P-388 (Liou et al., 1983) and rabbit reticulocyte (Williams et al., 1983) systems. With use of fractionated systems, it appears likely that eIF-3 is the only initiation factor directly sensitive to inactivation by helenalin (Liou et al., 1983; Williams et al., 1983). Thus, helenalin appears to possess a remarkable degree of selectivity as a sulfhydryl reagent.

The previous experiments were not, however, designed to measure the possible activation of eIF-2α kinase by helenalin or similar sesquiterpene lactones. Previous experiments have shown that eIF-2α kinase can be activated by a wide range of sulfhydryl reagents, including *N*-ethylmaleimide, sodium selenite, and oxidized glutathione (Gross & Rabinowitz, 1972; Clemons et al., 1975; Safer et al., 1980a,b). The active eIF-2α kinase can in turn rapidly inactivate eIF-2 by phosphorylating the α-subunit (Farrell et al., 1977; Jagus, et al., 1981). In addition, none of the previous experiments were designed to compare the mechanism of protein synthesis inhibition by sesquiterpene lactones other than helenalin. The purpose of this study was to compare the mechanism of protein synthesis inhibition by helenalin and bis(helenalinyl) malonate. In addition, the study was aimed at measuring the effects of these two compounds on eIF-2α kinase.

Experimental Procedures

Materials

[^{35}S]Methionine (>600 Ci/mmol), [^{3}H]leucine (60 Ci/mmol), and [^{14}C]phenylalanine (>450 mCi/mmol) were obtained from Amersham/Searle. [^{35}S]Methionyl tRNA$_f$ (2×10^4 dpm/pmol) was prepared from brewer's yeast tRNA (Boehringer-Mannheim) by the method of Takeishi et al. (1968). GMPPCP was obtained from Boehringer, and CTAB was obtained from Sigma. Helenalin (Hall et al., 1977) and bis(helenalinyl) malonate (Lee et al., 1981) were prepared as described previously. Stock solutions of each drug were prepared by suspending the compound in 0.05% Tween 80[1] [poly(oxyethylene)sorbitan monooleate, Sigma] at a concentration of 4 mM. *N*-Ethylmaleimide (Sigma) was dissolved in water just prior to use. Edeine was the kind gift of Dr. Zofia Kurylo-Borowska (Rockefeller University, New York, NY).

Rabbit reticulocyte lysates were prepared as described previously (Williams et al., 1983). New Zealand Minikin rabbits weighing 1–2 kg were used. The lysates were stored frozen in liquid nitrogen in 1-mL aliquots. Isolation of "runoff" ribosomes (Schreier & Staehelin, 1973), 40S subunits (Schreier & Staehelin, 1973), and pH 5 enzymes (Kruh, 1968) from rabbit reticulocytes were prepared essentially as described previously except that the final storage buffer for the 40S subunits contained only 100 μM dithiothreitol and the final storage buffer for runoff ribosomes and pH 5 enzymes contained no added thiol compound. The reticulocyte initiation factor preparations used in these experiments were prepared as described by Williams et al. (1983).

Methods

For routine measurement of endogenous protein synthesis in rabbit reticulocyte lysates, an assay containing 120 μL of lysate/260-μL total assay volume (low-lysate assay) was carried out as described by Williams et al. (1983). To obtain more linear incorporation in the time course experiments (Figures 5 and 6), the assay was modified to contain 175 μL of lysate, 100 mM KCl, 0.5 mM magnesium actate, and no added ATP or GTP per 260-μL assay volume (high-lysate assay). Incubation and sample analysis were as described previously (Williams et al., 1983). Poly(U)-directed polyphenylalanine synthesis and ternary complex formation were measured as described by Williams et al. (1983). The ternary complex formation assay contained 30–60 μg of initiation factors and 25–30 pmol of [^{35}S]methionyl-tRNA$_f$ (2×10^4 dpm/pmol). The 48S preinition complex formation was directly measured by sucrose gradient fractionation of edeine-treated lysates as described by Safer et al. (1979). The 48S preinitiation complex formation was also measured in a nitrocellulose filter binding assay similar to the one described by Gupta et al. (1973). This assay contained 30–60 μg of initiation factors and 10–15 pmol of [^{35}S]methionyl-tRNA$_f$ (2×10^4 dpm/pmol) and was carried out as described by Williams et al. (1983).

[1] Abbreviations: Tween 80, poly(oxyethylene)sorbitan monooleate; cAMP, adenosine cyclic 3′,5′-monophosphate; GMPPCP, 5′-guanylyl methylenediphosphate; CTAB, cetyltrimethylammonium bromide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

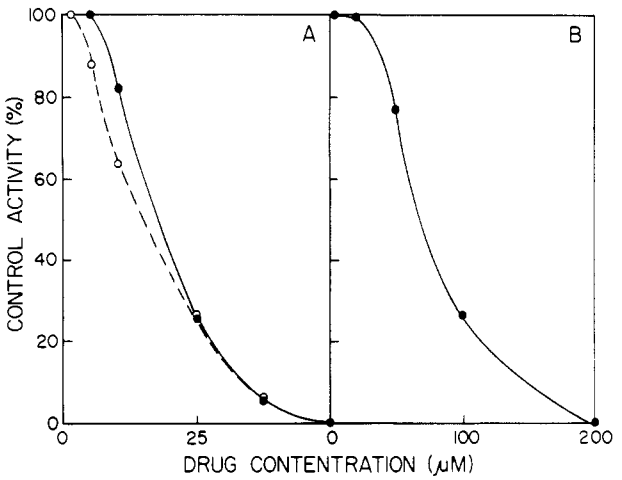

FIGURE 2: Inhibition of endogenous protein synthesis in rabbit reticulocyte lysates by helenalin and bis(helenalinyl) malonate. Lysates were preincubated for 30 min at 30 °C with the indicated concentration of drug or an equivalent volume of 0.05% Tween 80. The preincubation mixture contained 7.2 mM creatine phosphate, 10 μM hemin, and either 120 (A) or 175 (B) μL of lysate in a total volume of 230 μL. Following preincubation, the samples were cooled to 4 °C and additional components added to give the final assay composition described under Methods. The incubation was as described under Methods. The drugs used were helenalin (●) and bis(helenalinyl) malonate (○).

Phosphorylation of eIF-2α was measured in a two-stage assay essentially as described by Jagus & Safer (1981). In the first stage, partially purified hemin-regulated eIF-2α kinase (kindly supplied by Dr. B. Safer) or a rabbit reticulocyte lysate containing 20 μM hemin was preincubated for 15–30 min at 32 °C with either the drug to be tested or an equivalent volume of 0.05% Tween 80. In the second stage, 2 μL of the preincubation mixture was then added to 1 μg of purified eIF-2 (kindly supplied by Dr. B. Safer) and 16 μL of a kinase coctail designed to generate [γ-^{32}P]ATP of constant specific activity (Jagus & Safer, 1981a). The kinase cocktail mixture was preincubated for 10 min at 32 °C. Incubation of the complete assay mixture was for 15 min at 32 °C. The reaction was terminated by removing a 15-μL aliquot and adding it to 15 μL of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% sodium dodecyl sulfate, 20% glycerol, 40 mM disodium ethylenediaminetetraacetic acid, 0.01% bromphenol blue, and 10% 2-mercaptoethanol). After being heated for 5 min at 90 °C, the samples were electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gels (Laemmli, 1970) and autoradiographed.

Results

Our previous experiments have shown that optimal inactivation of rabbit reticulocyte lysates by helenalin occurs when the lysate is preincubated with helenalin for 30 min at 30 °C in the absence of any added thiol compound (Williams et al., 1983). The lysates are also supplemented with 7.2 mM creatine phosphate and 10 μM hemin for optimal stability during the preincubation. With these preincubation conditions, helenalin and bis(helenalinyl) malonate appear equally effective protein synthesis inhibitors in the rabbit reticulocyte system (Figure 2A).

The exact amount of helenalin required to give 100% inhibition is strongly dependent on the amount of lysate used (compare panels A and B of Figure 2). In the low-lysate assay (120 μL of lysate/260-μL assay volume, Figure 2A), reduced glutathione levels are usually about 0.8 mM. At this concentration, reduced glutathione offers very little protection against inactivation by helenalin. While the exact amount of

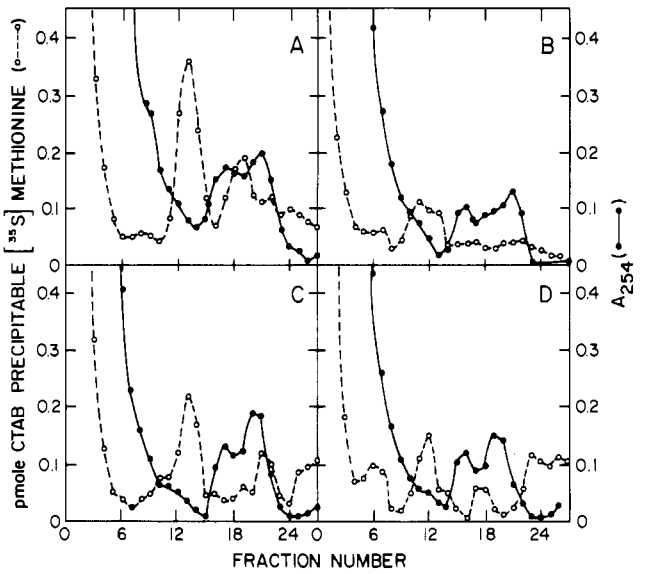

FIGURE 3: Inhibition of 48S preinitiation complex formation in lysates by helenalin and sodium selenite. Lysates were preincubated 15 min at 37 °C with the indicated concentrations of drug or an equivalent volume of 0.05% Tween 80. The preincubation mixture contained 9 mM creatine phosphate, 14 μM hemin, 90 μM of each of 20 amino acids, 100 mM KCl, 1.1 mM $MgCl_2$, and 80 μL of lysate in a total volume of 110 μL. Following preincubation, the samples were cooled to 4 °C and additional components added to give the final assay composition described by Safer et al. (1979). The final assay included 40 μM edeine and 14 pmol of [^{35}S]methionyl-tRNA (6.55 × 10^4 dpm/pmol) and was carried out for 5 min at 37 °C. The reaction was stopped by dilution into 2 volumes of ice-cold 10 μM GMPPCP. The samples were then layered on a 10–35% sucrose gradient in 10 mM Tris-HCl (pH 7.5), 95 mM KCl, 2.5 mM magnesium acetate, and 10 μM GMPPCP and centrifuged for 4.5 h in a SW40 rotor at 40000 rpm. CTAB precipitable counts were determined as described by Safer et al. (1979). (●) A_{254}; (○) CTAB precipitable counts; (A) preincubation with Tween 80; (B) preincubation with 15 μM sodium selenite; (C) preincubation with 50 μM helenalin; (D) preincubation with 100 μM helenalin.

helenalin and bis(helenalinyl) malonate needed to produce 100% inhibition varies slightly from lysate to lysate (due to small variations in endogenous glutathione levels), 100 μM drug was chosen for most subsequent experiments in the low-lysate assay since that level was always effective. The level of 100 μM helenalin and bis(helenalinyl) malonate is also the level used in previous studies with P-388 cell-free protein synthesizing systems (Liou et al., 1983). In experiments with higher lysate concentrations (175 μL of lysate/260-μL assay volume, Figure 2B), 200 μM drug concentrations were used to obtain a similar level of inhibition (higher drug concentrations were precluded by solubility limitations).

Our previous experiments had suggested that helenalin interferes with formation of the 48S preinitiation complex (Williams et al., 1983). However, these experiments were carried out with the filter binding assay of Gupta et al. (1973) and did not actually measure formation of the 48S preinitiation complex directly. Safer et al. (1979) have shown that it is possible to directly assay for the 48S preinitiation complex in an unfractionated lysate treated with the antibiotic edeine and to demonstrate inhibition of this complex by sodium selenite and other inhibitors. Using this assay, we were able to directly demonstrate the inhibition of 48S preinitiation complex formation by helenalin. One such experiment is shown in Figure 3. The controls demonstrate the formation of the 48S preinitiation complex in an edeine-treated lysate (Figure 3A) and the inhibition of 48S complex formation by 15 μM sodium selenite (Figure 3B) as described by Safer et al. (1979). Clearly, helenalin also inhibits 48S complex formation in a

Table I: Effect of Helenalin and Bis(helenalinyl) Malonate on Fractionated Protein Syntheses Assays from Rabbit Reticulocytes[a]

	pmol formed	activity remaining (%)
(Assay I) Poly(U)-Directed Poly(Phe) Synthesis		
(preincubation) ribosomes + pH 5 enzymes		
control	5.24	
+100 μM helenalin	5.64	107
+100 μM bis(helenalinyl) malonate	4.40	84
(Assay II) Ternary Complex Formation		
(preincubation) initiation factor prepn		
control	3.41	
+100 μM helenalin	2.81	82
+100 μM bis(helenalinyl) malonate	2.89	85
(Assay III) 48S Preinitiation Complex Formation		
(preincubation) 40S + initiation factor prepn		
control	1.11	
+100 μM helenalin	0.338	31
+100 μM bis(helenalinyl) malonate	0.701	63
(preincubation) initiation factor prepn		
control	1.34	
+100 μM helenalin	0.312	23
+100 μM bis(helenalinyl) malonate	0.999	74
(preincubation) 40S		
control	1.81	
+100 μM helenalin	1.68	93
+100 μM bis(helenalinyl) malonate	1.83	101

[a] For the poly(U)-directed polyphenylalanine synthesis assay, the preincubation mixture contained 3 A_{260} of runoff ribosomes, 15 μg of pH 5 enzymes, 65 mM Tris-HCl, pH 7.6, 138 mM KCl, 1.3 mM ATP, 0.06 mM GTP, 10 mM magnesium acetate, and 100 μM drug or an equivalent volume of 0.05% Tween 80 in a total volume of 42 μL. For the ternary complex formation assay, the preincubation mixture contained 60 μg of crude initiation factor, 50 μM dithiothreitol (from the initiation factor preparation), and 100 μM drug or an equivalent volume of 0.05% Tween 80 in a total volume of 20 μL. For the 48S preinitiation complex formation assay, the preincubation mixture contained 30 μg of crude initiation factors and/or 0.4 A_{260} unit of 40S ribosome, 44 mM Tris-HCl, pH 7.6, 159 mM KCl, 4.0 mM magnesium acetate, 0.5 mM GTP, 25–50 μM dithiothreitol (from the initiation factor and 40S ribosome preparations), and 100 μM drug or an equivalent volume of 0.05% Tween 80 in a total volume of 49 μL. All preincubations were for 30 min at 30 °C. Following preincubation, the samples were cooled to 4 °C, and additional components were added to give the final assay compositions described under Experimental Procedures. The incubations and sample analysis were carried out as described under Experimental Procedures. Background controls for both assays II and III were determined in parallel reaction mixtures lacking GTP. Background was typically 10–15% of the total incorporation.

dose-dependent manner (Figure 3C,D). These data help verify the data obtained with the filter binding assay.

The mechanism of protein synthesis inhibition by helenalin and bis(helenalinyl) malonate was compared in partially fractionated rabbit reticulocyte systems with the filter binding assays described under Methods (Table I). Dithiothreitol concentrations were kept at 50 μM or below in these assays, and helenalin and bis(helenalinyl) malonate were added at 100 μM. These data should be directly comparable to those obtained in low-lysate assay since neither 50 μM dithiothreitol nor 0.8 mM reduced glutathione offers significant protection against 100 μM helenalin. As observed previously (Liou et al., 1983; Williams et al., 1983), helenalin does not significantly inhibit poly(U)-directed polyphenylalanine synthesis or ternary complex formation—but does interact with the initiation factor preparation to inhibit the conversion of the ternary complex to the 48S preinitiation complex. This suggests, and subsequent studies have verified (Williams et al., 1983), that the initiation factor eIF-3 is selectively inactivated by helenalin. The pattern of inhibition observed with bis(helenalinyl) malonate is very similar. However, the extent of inhibition of

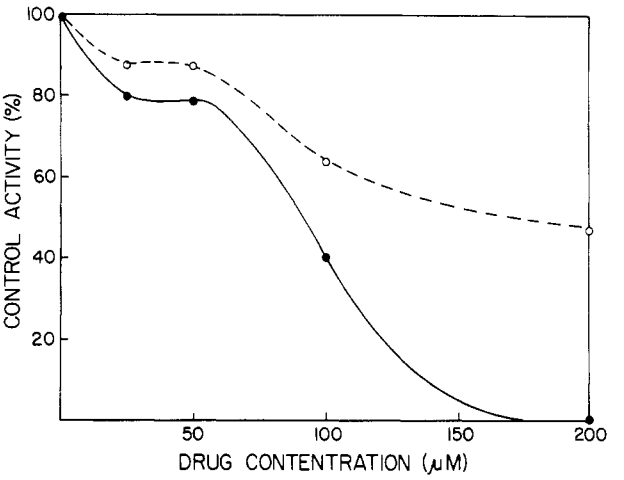

FIGURE 4: Inhibition of 48S preinitiation complex formation by helenalin and bis(helenalinyl) malonate. The 40S ribosomes and a partially purified initiation factor preparation (Materials) were preincubated for 30 min at 30 °C with the indicated concentrations of helenalin (●), bis(helenalinyl) malonate (○), or an equivalent volume of 0.05% Tween 80 as described in Table I. Following preincubation, the samples were cooled to 4 °C and additional components added to give the final assay composition described under Methods. The incubation was as described under Methods for the filter binding assay.

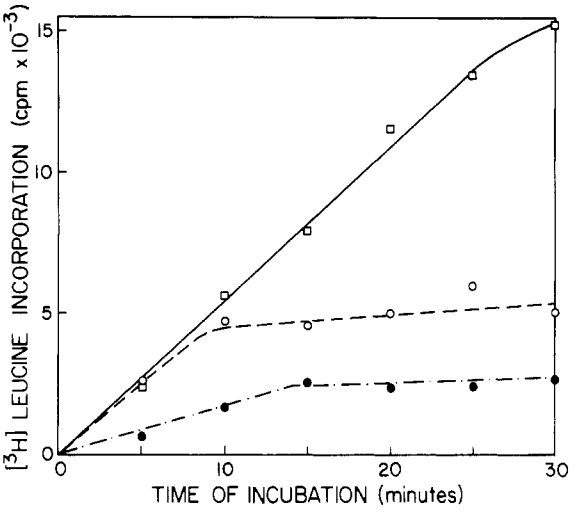

FIGURE 5: Time course of inhibition of endogenous protein synthesis by helenalin and bis(helenalinyl) malonate. Each incubation mixture (520 μL) contained 350 μl of lysate, 200 μM drug or an equivalent volume of 0.05% Tween 80, and all the other components required for protein synthesis as described under Methods. Incubation was at 30 °C. At the indicated times, 50-μL aliquots were removed, and hot trichloroacetic acid insoluble ^3H was determined by standard methods: (□) control; (●) helenalin; (○) bis(helenalinyl) malonate.

48S complex formation appears to be much less.

The differences in effectiveness of helenalin and bis(helenalinyl) malonate as inhibitors of the 48S preinitiation complex formation are even more apparent when one compares the concentration curves for inhibition by the two drugs (Figure 4). Clearly, bis(helenalinyl) malonate does inhibit 48S complex formation, but it is a less effective inhibitor than helenalin at all concentrations tested. Interestingly enough, neither bis(helenalinyl) malonate nor helenalin would appear to be strong enough inhibitors of the 48S preinitiation complex formation to fully explain their effectiveness as protein synthesis inhibitors in unfractionated reticulocyte lysates (compare Figure 2A and Figure 3).

The possible differences in mechanism of action of helenalin and bis(helenalinyl) malonate as protein synthesis inhibitors

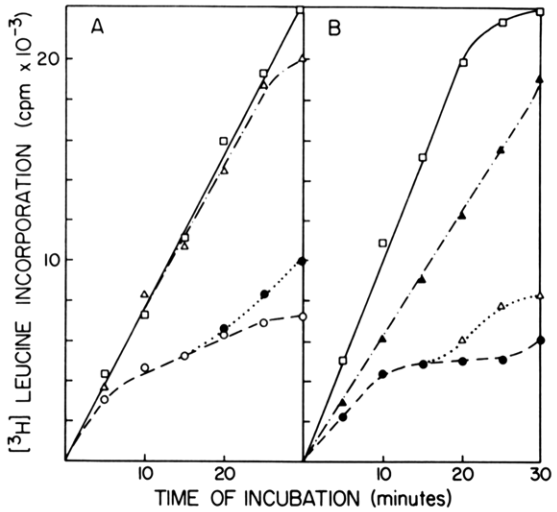

FIGURE 6: Effect of cAMP on inhibition of endogenous protein synthesis by helenalin and bis(helenalinyl) malonate. The assay was carried out essentially as described in Figure 5 except that 5 mM cAMP was added at either time zero or at 15 min after the start of the incubation. (A) Inhibition by bis(helenalinyl) malonate: (□) control; (○) 200 μM bis(helenalinyl) malonate; (△) 200 μM bis(helenalinyl) malonate plus 5 mM cAMP added at time zero; (●) 200 μM bis(helenalinyl) malonate plus 5 mM cAMP added at 15 min. (B) Inhibition by helenalin: (□) control; (●) 200 μM helenalin; (▲) 200 μM helenalin plus 5 mM cAMP added at time zero; (△) 200 μM helenalin plus 5 mM cAMP added at 15 min.

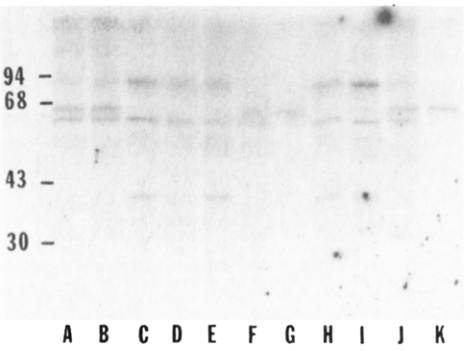

FIGURE 7: Effect of helenalin and bis(helenalinyl) malonate on eIF-2α kinase activity in rabbit reticulocyte lysates. Lysates containing only 20 μM hemin were preincubated for 30 min at 30 °C with either helenalin, bis(helenalinyl) malonate, *N*-ethylmaleimide, or an equivalent volume of 0.05% Tween 80. A total of 2 μL of this preincubation mixture was then added to 2 μg of purified eIF-2, 5 mM (final concentration) cAMP or an equivalent volume of water, and 16 μL of kinase cocktail (Methods). Incubation and analysis of samples was as described under Methods. Autoradiography was on Kodak XAR-5 film for 21 days: (A) Tween 80 present during preincubation and no eIF-2 added during incubation; (B) Tween 80 present during preincubation; (C) 200 μM *N*-ethylmaleimide present during preincubation; (D) 50 μM *N*-ethylmaleimide present during preincubation; (E) 200 μM helenalin present during preincubation; (F) 50 μM helenalin present during preincubation; (G) 200 μM helenalin present during preincubation and 5 mM cAMP present during incubation; (H) 200 μM *N*-ethylmaleimide present during preincubation; (I) 200 μM bis(helenalinyl) malonate present during preincubation; (J) 50 μM bis(helenalinyl) malonate present during preincubation; (K) 200 μM bis(helenalinyl) malonate present during preincubation and 5 mM cAMP present during incubation.

was explored by comparing their kinetics as protein synthesis inhibitors in reticulocyte lysates (Figure 5). In these experiments, higher concentrations of reticulocyte lysate had to be used in order to obtain linear incorporation rates over a sufficient length of time (30 min) to distinguish the inhibition kinetics of the two drugs. In these high-lysate assays (175 μL of lysate/260-μL assay volume), reduced glutathione levels are around 1.2 mM, and consequently, 200 μM helenalin and bis(helenalinyl) malonate were needed to give a comparable degree of inhibition (Figure 2B). From the data in Figure 5, some important differences in the kinetics of inhibition by helenalin and bis(helenalinyl) malonate are apparent. With bis(helenalinyl) malonate, there is a 10-min lag before effective inhibition is observed. With helenalin, there is also a comparable lag before full inhibition is observed. However, partial inhibition is observed even at very early times. Thus, the mechanism of inhibition of protein synthesis by helenalin appears to be complex. The data suggest that helenalin might inhibit protein synthesis by two distinct mechanisms—one of which is unique to helenalin and one of which is identical with or very similar to the mechanism of protein synthesis inhibition by bis(helenalinyl) malonate.

We already knew that helenalin and bis(helenalinyl) malonate differ greatly in their effectiveness as inhibitors of the 48S preinitiation complex formation (Table I, Figure 4). At the levels of reduced glutathione present in reticulocyte lysates, bis(helenalinyl) malonate would likely have little or no effect on 48S complex formation. Thus, the unique mechanism of helenalin action probably involves the inactivation of eIF-3. However, what could be the common mode of action for both helenalin and bis(helenalinyl) malonate? One possibility is that both compounds might activate eIF-2α kinase. eIF-2α kinase is known to be activated by a wide variety of sulfhydryl reagents (Gross & Rabinowitz, 1972; Clemons et al., 1975; Safer et al., 1980a,b). Activation of eIF-2α kinase results in the phosphorylation and eventual indirect inactivation of eIF-2 by a mechanism that is not completely understood at present (Farrell et al., 1977; Jagus et al., 1981). None of the previous assays with partially or completely fractionated systems were designed to measure activation of eIF-2α kinase or the subsequent phosphorylation of eIF-2α.

One way to assess eIF-2α phosphorylation is to measure the effects of 5 mM cAMP on the inhibition of protein synthesis by helenalin and bis(helenalinyl) malonate. cAMP, adenosine, 2-aminopurine, and several other purine compounds have been shown to prevent the phosphorylation and inactivation of eIF-2 by activated eIF-2α kinase (Farrell et al., 1977). The effects of cAMP are shown in Figure 6. cAMP (5 mM) added at time zero completely reverses the inhibition by bis(helenalinyl) malonate (Figure 6A) but only partially reverses the inhibition by helenalin (Figure 6B). When added 15 min after the drug, cAMP only allows partial reversal of inhibition in either case. These data strongly suggest that activation of eIF-2α kinase is of primary importance in the inhibition of protein synthesis caused by bis(helenalinyl) malonate and is responsible for a significant portion of the inhibition caused by helenalin.

This hypothesis was tested directly in a series of two-stage kinase experiments similar to those described by Jagus & Safer (1981). In the first stage, rabbit reticulocyte lysate or partially purified eIF-2 kinase is preincubated with either helenalin, bis(helenalinyl) malonate, *N*-ethylmaleimide, or an equivalent volume of 0.05% Tween 80. In the second stage, these "activated" lysates are added to a cocktail containing purified eIF-2, excess dithiothreitol (to scavenge any remaining sulfhydryl reagent), and a kinase mixture designed to generate [γ-^{32}P]ATP of constant specific activity. Each reaction mixture is then fractionated on sodium dodecyl sulfate–polyacrylamide gels, and the ^{32}P-labeled protein bands are identified by autoradioagraphy. The initial experiment with rabbit reticulocyte lysate in stage 1 of the assay is shown in Figure 7. Several phosphorylated protein bands are evident in this autoradiogram. The most important ones to observe, however, are the 38K band, which is eIF-2α, and the 94K band, which

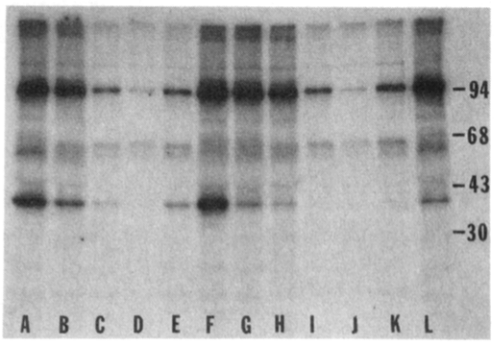

FIGURE 8: Effect of helenalin and bis(helenalinyl) malonate on phosphorylation of eIF-2α and eIF-2α kinase. The assay was carried out essentially as described in Figure 7 except that instead of lysate the preincubation mixture contained 0.5 μL of partially purified eIF-2α kinase in buffer A (20 mM Tris-HCl, pH 7.5, 25 mM KCl, 10 mM NaCl, and 1 mM $MgCl_2$). During the incubation phase, samples A–F contained 2 μg of eIF-2 while samples G–L did not. Autoradiography was on Kodak XAR-5 film for 5 days: (A and G) 200 μM bis-(helenalinyl) malonate present during preincubation; (B and H) 200 μM helenalin present during preincubation; (C and I) 200 μM bis-(helenalinyl) malonate present during preincubation and 5 mM cAMP present during incubation; (D and J) 0.05% Tween 80 present during preincubation; (E and K) 200 μM helenalin present during preincubation and 5 mM cAMP present during incubation; (F and L) 200 μM *N*-ethylmaleimide present during preincubation.

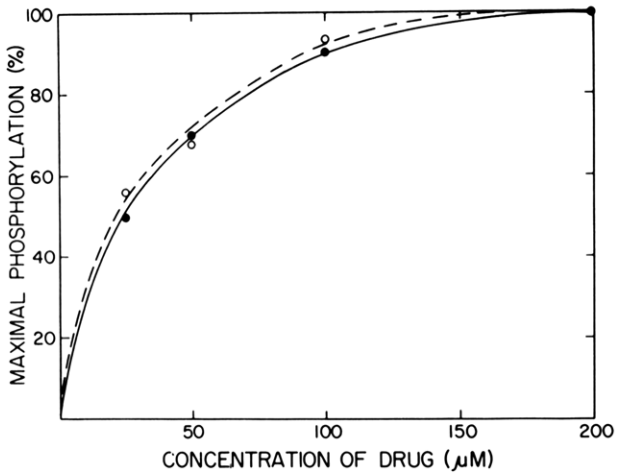

FIGURE 9: Concentration dependence of helenalin and bis(helenalinyl) malonate stimulated phosphorylation of eIF-2α. The assay was carried out essentially as described in Figure 7 except that the lysate was diluted 1:1 with buffer A prior to the preincubation. The eIF-2α band of the acrylamide gel was cut out and counted by Cerenkov radiation. The ^{32}P incorporation into the samples preincubated with 200 μM helenalin or bis(helenalinyl) malonate agreed very well and was considered to represent maximal phosphorylation under these assay conditions. (●) Helenalin; (○) bis(helenalinyl) malonate.

probably represents autophosphorylation of the eIF-2α kinase. Clearly, both bands are phosphorylated when the lysate is pretreated with 200 μM helenalin, bis(helenalinyl) malonate, or *N*-ethylmaleimide. Some phosphorylation is observed even at 50 μM drug concentration. When 5 mM cAMP is included in the second stage of the assay, phosphorylation of both bands is essentially blocked. These data demonstrated the activation of a protein kinase that phosphorylates eIF-2α in response to helenalin, bis(helenalinyl) malonate, and *N*-ethylmaleimide but do not unambiguously identify the protein kinase. Figure 8 shows the comparable experiment with partially purified hemin-regulated eIF-2α kinase (kindly supplied by B. Safer) in place of the reticulocyte lysate. These data confirm that helenalin and bis(helenalinyl) malonate do activate the hemin-regulated eIF-2α kinase and strongly suggest that the phosphorylation of the eIF-2α seen in the unfractionated lysate is due to the activation of this kinase.

The data in Figure 7 were obtained by using a lysate at full strength in stage one of the assay. The fact that some phosphorylation was observed even at 50 μM drug concentration suggested that the activation of eIF-2α kinase might be significantly more sensitive to helenalin and bis(helenalinyl) malonate than the inactivation of eIF-3. To confirm this observation, the experiment in Figure 7 was repeated with the low-lysate concentration (120 μL/260-μL assay volume) and several different drug concentrations. The results are shown in Figure 9. Clearly, the activation of eIF-2α kinase occurs at low enough concentrations of both helenalin and bis(helenalinyl) malonate to account for their effectiveness as protein synthesis inhibitors in whole lysates.

Discussion

In comparing the effects of sulfhydryl reagents such as sesquiterpene lactones as inhibitors of individual steps of protein synthesis, it is very important to be aware of the type and concentration of low molecular weight sulfhydryl compounds in the assay. This is especially true since various sulfhydryl compounds differ greatly in their ability to protect the protein synthetic machinery against sulfhydryl reagents (Williams et al., 1983). In some previous experiments, we have used gel-filtered lysates [prepared as described by Jagus & Safer (1981)] to remove endogenous sulfhydryl compounds entirely. However, we did not feel that this was entirely satisfactory since gel-filtered lysates were less stable in storage and since the filtration itself may result in alteration of the eIF-2 and/or eIF-2α kinase (Jagus et al., 1981). We have previously shown that if the lysate composes less than 50% of the total assay volume, levels of endogenous-reduced glutathione in the unfiltered lysates are low enough that they do not interfere with inhibition by helenalin at 100 μM or greater concentrations (Williams et al., 1983). In fractionated systems, we can obtain similar results by keeping 2-mercaptoethanol or dithiothreitol levels at 100 μM or less. Thus, the data in Figures 2A, 4, and 9 and Table I should be very comparable. The kinetic studies could only be carried out at higher lysate concentrations, but 200 μM helenalin was sufficient to achieve comparable levels of inhibition (compare panels A and B of Figure 2B). Since we were looking at kinetics only in these experiments (Figures 5 and 6) and not trying to produce comparable concentration curves, the fact that both reduced glutathione and drug levels are slightly different should not affect the interpretation of these experiments significantly.

The data clearly show that helenalin and bis(helenalinyl) malonate activate eIF-2α kinase (Figure 8) and indirectly cause phosphorylation of eIF-2 (Figures 7 and 8) at low enough concentrations to explain their effectiveness as protein synthesis inhibitors (Figures 2A and 9). Of course, these data do not prove phosphorylation of eIF-2 directly leads to inactivation of protein synthesis. The linkage between the phosphorylation of eIF-2 and the inhibition of protein synthesis is not completely clear at present. Phosphorylated eIF-2 is fully active in most in vitro assays (Trachsel & Staehlein, 1979; Benne et al., 1980). However, phosphorylation of eIF-2 in whole lysates does correlate with the inactivation of protein synthesis in several different situations (Farrell et al., 1977; Levin et al., 1976; Ranu et al., 1976; Cooper & Farrell, 1977). The best hypothesis at present seems to be that phosphorylation of eIF-2α inhibits its interaction with one or more factors required for recycling of eIF-2 (Ranu et al., 1978; Jagus et al., 1981; Konieczny & Safer, 1983; De Benedetti & Baglioni, 1983; Safer, 1983).

Activation of the eIF-2α kinase appears to be the primary

mode of action of bis(helenalinyl) malonate as a protein synthesis inhibitor. While helenalin is clearly more effective as an activator of eIF-2α kinase, it can inhibit eIF-3 also. In fact, at the concentrations routinely used in these assays both modes of action seem to be operable (Figures 5 and 6). The relative effectiveness of these two mechanisms of action in other eukaryotic cells is not known at present. Helenalin and bis(helenalinyl) malonate are equally effective as protein synthesis inhibitors in the P-388 system (Williams et al., 1983). Helenalin does selectively inhibit the 48S preinitiation complex formation in P-388 cells with a concentration dependence that is sufficient to explain its effectiveness as a protein synthesis inhibitor (Liou et al., 1983). The effectiveness of bis(helenalinyl) malonate as an inhibitor of 48S preinitiation complex formation or of either drug as an activator of eIF-2α kinase in the P-388 system is not known. Present evidence suggests that most other eukaryotic cells probably contain an eIF-2α kinase and that eIF-2 may be phosphorylated under some conditions (Gupta, 1979; Delauney et al., 1977; Wong et al., 1982). However, the physiological significance of this phosphorylation is not known at present.

These data extend our knowledge of the selectivity of sesquiterpene lactones such as helenalin and bis(helenalinyl) malonate. Helenalin has previously been shown to selectively inactivate eIF-3, although eIF-2, eIF-3, eIF-4B, and probably eIF-5 are all equally sensitive to the sulfhydryl reagent *N*-ethylmaleimide (Williams et al., 1983). While the reactive centers of helenalin and *N*-ethylmaleimide are quite similar, helenalin is larger and more hydrophobic than *N*-ethylmaleimide. Thus, we felt that the selectivity of helenalin probably involved steric hindrance or hydrophobic interaction with the enzyme in the region of the essential sulfhydryl groups. Bis(helenalinyl) malonate is a simple dimer of helenalin. One might expect it to show very similar selectivity. Yet on the basis of reactions with partially purified components, a somewhat different picture emerges. While it appears to be equally unreactive to most initiation factors, it is much less reactive than helenalin against eIF-3 (Table I, Figure 4).

Helenalin and bis(helenalinyl) malonate appear to be equally effective in the activation of eIF-2α kinase (Figure 9). Furthermore, the sensitivity of the eIF2α kinase to helenalin and bis(helenalinyl) malonate appears to be significantly greater than that of eIF-3 to either compound (Figures 4 and 9). It is difficult to say to what extent steric hindrance and hydrophobicity play a role in determining this sensitivity, since eIF-2α kinase also appears to be more sensitive to *N*-ethylmaleimide [compare Figure 9 in this paper with Figure 4B of Williams et al. (1983)]. It may well be that a critical sulfhydryl group(s) for this enzyme lies (lie) in a very accessible location and may be sensitive to a wide range of sulfhydryl reagents without much regard to steric hindrance or hydrophobic interactions. In any case, it would appear to be highly significant that, out of all of the protein synthesis enzymes reported to be highly sensitive to sulfhydryl reagents such as *N*-ethylmaleimide (Bermek et al., 1971; Fresno et al., 1976; Merrick, 1979), the sesquiterpene lactones helenalin and bis(helenalinyl) malonate are capable of selectively inactivating only one or two enzymes. This may help explain the relatively high therapeutic effectiveness and low toxicity of these compounds in some antitumor screens. We are currently investigating the specificity of these compounds further.

Acknowledgments

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Registry No. Helenalin, 6754-13-8; bis(helenalinyl) malonate, 68322-91-8.

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Carbon-13 Nuclear Magnetic Resonance Study of Microtubule Protein: Evidence for a Second Colchicine Site Involved in the Inhibition of Microtubule Assembly†

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ABSTRACT: A ^{13}C nuclear magnetic resonance study of bovine microtubule protein was carried out at 43 kG in the presence and absence of colchicine ^{13}C labeled at the tropolone methoxy. Analysis indicated that tubulin has at least two colchicine binding sites: a quasi-irreversibly bound, high-affinity site (i.e., the $K_D < 5$ μM site generally accepted as the site of colchicine action) as well as a low-affinity site(s) ($K_D \sim 650$ μM) with which free colchicine rapidly exchanges (>100 s^{-1}). The methoxy resonance is broadened to different apparent extents as a result of binding at these two sites (50- vs. 150-Hz broadening for the high- and low-affinity sites, respectively) but undergoes no change in chemical shift upon binding. The low-affinity sites are interpreted to be analogous to the sites deduced by Schmitt and Atlas [Schmitt, H., & Atlas, D. (1976) *J. Mol. Biol. 102*, 743-758] from labeling studies using bromocolchicine. These sites are likely to be the sites responsible for the abrupt halt in microtubule assembly ("capping") observed at high colchicine concentrations (>20 μM)—a qualitatively different behavior from that observed at low colchicine concentrations [Sternlicht, H., Ringel, I., & Szasz, J. (1983) *Biophys. J. 42*, 255-267]. Carbon-13 spectra from the aliphatic carbons of microtubule protein consist of narrow resonances—many with line widths < 30 Hz—superimposed on a broad background. The narrow resonances were assigned to flexible regions in nontubulin proteins [microtubule-associated proteins (MAPs)], in accord with an earlier ^{1}H nuclear magnetic resonance study of microtubule protein [Woody, R. W., Clark, D. C., Roberts, G. C. K., Martin, S. R., & Bayley, P. M. (1983) *Biochemistry 22*, 2186-2192]. This assignment was supported by ^{13}C NMR analysis of phosphocellulose-purified (MAP-depleted) tubulin as well as heat-stable MAPs. Aliphatic carbons in the MAP preparations were characterized by narrow resonances indicative of carbons with considerable motional freedom whereas the aliphatic regions of phosphocellulose-purified tubulin were, for the most part, characterized by broad resonances indicative of carbons with restricted mobility. However, a moderately narrow resonance (≲50-Hz line width) coincident with the C_γ resonance of glutamate was detected in ^{13}C NMR spectra of tubulin which indicated that a fraction of the glutamic acid residues is relatively mobile. These mobile residues are likely to be at the carboxy-terminus ends, regions rich in glutamates, where secondary structure considerations suggest flexibility.

Colchicine, a potent inhibitor of microtubule assembly, disrupts a variety of microtubule-dependent cellular processes (Dustin, 1978; Oppenhein et al., 1973; Reaven & Reaven, 1975). Inhibition is generally attributed to a 1:1 tight-binding complex of tubulin and colchicine (TC)[1] with an apparent dissociation constant (K_D) of ~0.1-3 μM (McClure & Paulson, 1977)[2] and a dissociation half-life of ~12 h at 37 °C (Wilson, 1970). Furthermore, it is apparent at substoichiometric concentrations of TC relative to tubulin (Olmsted

† From the Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106 (H.S.), and the Hebrew University Hadassah Medical School, Jerusalem, Israel (I.R.). *Received March 19, 1984.* This work was supported in part by American Cancer Society Grants CH-99C and CH-99D to H.S. A preliminary report of several aspects of this study was presented at the July 1981 meeting of the American Society of Biological Chemists (Ringel & Sternlicht, 1981).

[1] Abbreviations: TC, 1:1 tight-binding complex of tubulin and colchicine; tc, low-affinity form of tubulin–colchicine complex(es); MAPs, microtubule-associated proteins; MTP, microtubule protein consisting of ~85% tubulin and ~15% MAPs; PC-tubulin, MAP-depleted tubulin preparations obtained by phosphocellulose chromatography; [^{13}C]-colchicine, colchicine whose tropolone methoxy is ^{13}C labeled; T_1, spin-lattice relaxation time; NOE, nuclear Overhauser enhancement factor; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MS-2.5 M glycerol, microtubule protein stabilizing buffer (pH 6.7) consisting of 0.1 M MES, 2 mM EGTA, 0.1 mM EDTA, 2 mM mercaptoethanol, 0.5 mM $MgCl_2$, and 2.5 M glycerol; HMPA, hexamethylphosphoramide; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; BSA, bovine serum albumin.

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