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Cellular pharmacology of cerulenin analogs that inhibit protein palmitoylation

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Abstract

S-Palmitoylation is a dynamic post-translational modification of certain proteins, which helps determine membrane association and may function to enhance the interactions of signaling molecules with their activated receptors and effector systems. Unlike enzymes that catalyze other protein lipidation reactions, e.g. farnesylation and *N*-myristoylation, protein palmitoyltransferase is virtually uncharacterized biochemically. We have described previously the synthesis of cerulenin analogs including *cis*-2,3-epoxy-4-oxononadecanamide (16C) and *cis*-2,3-epoxy-4-oxododecanamide (9C) that inhibit protein palmitoylation (Lawrence et al., J Med Chem 1999;42:4932–41), most likely through covalent alkylation of protein palmitoyltransferase. [^{3}H]9C and [^{3}H]16C were prepared by catalytic incorporation of $^3H_2$ into unsaturated precursors for further study of their cellular pharmacology. After 4 hr, T24 bladder carcinoma cells in the absence of serum accumulated a 4-fold higher intracellular level of [^{3}H]16C than of [^{3}H]9C. Uptake of [^{3}H]9C and [^{3}H]16C was reduced by the presence of serum in the medium, suggesting their binding to serum proteins. [^{3}H]9C and [^{3}H]16C alkylated unique patterns of proteins in T24 cells, with proteins of approximately 80 and 31 kDa being labeled by each compound. A panel of human tumor cell lines demonstrated half-maximal proliferation inhibition at concentrations of 7–30, 4–16, and 8–36 μM, for cerulenin, 9C, and 16C, respectively, indicating that the cell lines have approximately equal sensitivity to these compounds. Different cell lines have similar patterns of protein alkylation by [^{3}H]9C or [^{3}H]16C, with labeling intensity related to cytotoxicity of the compounds. Since both 9C and 16C inhibit palmitoylation, the commonly labeled proteins are candidates for human protein palmitoyltransferase.

Keywords: Cerulenin; Palmitoylation; Ras

1. Introduction

Palmitoylation is a post-translational lipid modification of certain proteins, which plays a key role in determining membrane association. During protein *S*-palmitoylation, palmitate is connected to the thiol group of cysteine residues through a thioester linkage, a process also referred to as thioacylation. Whereas protein prenylation and *N*-myristoylation are irreversible linkages of prenyl groups and myristate groups through stable bonds, palmitoylation is reversible, and, in fact, is highly dynamic within cells [1]. Since palmitoylation markedly affects the intracellular distribution and activities of proteins, it is important that the enzymology of this process be characterized.

While a variety of proteins are palmitoylated, and it appears that these proteins are limited to a subset of signaling molecules that are associated with the plasma membrane, the most common targets of palmitoylation include nonreceptor tyrosine kinases, G-protein-linked receptors, subunits of heterotrimeric G-proteins, endothelium nitric oxide synthase (eNOS), adenylyl cyclase, and Ras [2,3]. For many palmitoylated proteins, the site of palmitoylation is found near other lipid modifications, which include myristate or prenyl groups or near hydrophobic stretches of amino acids [4–6]. The presence of the first lipid modification provides a sufficient increase in protein hydrophobicity to allow a transient membrane association [7]. Therefore, since palmitoylation occurs after membrane association, the protein palmitoyltransferase activity probably resides in the plasma membrane [8].

The determinant of protein-membrane association due to palmitoylation provides a variety of functions for palmitoyl-

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Abbreviations: FT-IR, Fourier transform infrared; THF, tetrahydrofuran; FBS, fetal bovine serum; FPTase, farnesyl protein transferase; PPTase, protein palmitoyltransferase; FTI, farnesyl transferase inhibitor; DTT, dithiothreitol.

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ation, including the control of protein distribution either between subdomains of the plasma membrane or between membranes and the cytoplasm. For example, many signaling molecules are enriched in plasma membrane specializations called caveolae [9]. A role for palmitoylation in targeting signaling molecules to caveolae was identified when the mutagenesis of palmitoylatable Cys residues of eNOS prevented compartmentation to caveolae [10,11]. By localizing signaling molecules to specific regions of the plasma membrane, palmitoylation results in efficient and rapid coupling of receptors and their effector systems.

The activity of certain Ras proteins is dependent upon palmitoylation, which is included in a series of post-translational modifications at the C-terminus that targets the protein to the plasma membrane. After Ras is synthesized in the cytoplasm as pro-p21, it becomes farnesylated at the C-terminal CAAX box (C: Cys; A: aliphatic amino acid; X: another amino acid) by the enzyme FPTase. While farnesylation provides a transient membrane association, further modifications must occur for stable membrane association [12]. The subsequent post-translational modifications include cleavage of the three amino acids located C-terminal to the farnesylated Cys [6], followed by carboxy methylation of the C-terminus [13]. The final modification of H- and N-Ras is reversible palmitoylation of cysteine residues upstream from the farnesylated cysteine by the enzyme PPTase [14]. K-Ras, which is not palmitoylated, has increased localization to the plasma membrane due to the presence of a polybasic stretch of lysine residues. Once Ras is localized to the plasma membrane, it is properly positioned to mediate the signals transmitted from tyrosine kinase receptors [15].

It has been shown previously that the natural product cerulenin ([2*R*,3*S*]-2,3-epoxy-4-oxo-7,10-*trans*,*trans*-dodecadienamide) inhibits fatty acid synthase [16] and protein palmitoylation [17]. To increase the selectivity of cerulenin for the latter activity, analogs were synthesized and shown to inhibit protein palmitoylation [18]. Two of the analogs, herein called 9C (*cis*-2,3-epoxy-4-oxododecanamide) and 16C (*cis*-2,3-epoxy-4-oxononadecanamide), have shown interesting biological activity, in that 9C inhibits both fatty acid synthase and protein palmitoylation, whereas 16C selectively inhibits only protein palmitoylation. These two compounds have been radiolabeled, and we in this study describe their cellular pharmacology, including cytotoxicity, intracellular accumulation, and alkylated protein targets.

2. Materials and methods

2.1. Materials

Cerulenin was obtained from the Sigma Chemical Co. at 95% purity and stored as a stock solution of 5 mg/mL in ethanol at 4°. No noticeable decrease in activity occurred over several months. 9C and 16C, unlabeled analogs of cerulenin, were synthesized as described [18]. It should be noted that 9C and 16C were denoted as 7a and 7e, respectively, in that previous publication.

2.2. Synthesis of [^{3}H]9C and [^{3}H]16C

[^{3}H]9C and [^{3}H]16C were prepared by catalytic reduction of cerulenin and *cis*-2,3-epoxy-4-oxo-nonadec-14-enamide, which was synthesized as follows. Infrared spectra were measured on a Nicolet 250 FT-IR, and values are expressed in wavenumbers (cm^{-1}). ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker 300 WB spectrometer. All chemical yields are un-optimized and generally represent the result of a single reaction.

2.3. Ethyl 4-hydroxynonadec-14-en-2-ynoate (1)

Ethyl propiolate (4.61 mmol, 0.469 mL) in THF (14 mL) was added dropwise to a stirred solution of lithium diisopropylamide (4.61 mmol, 2.31 mL of a 2.0 M solution in heptane/THF/ethyl benzene), deaerated with nitrogen and chilled to −78°. After stirring for 30 min, *cis*-11-hexadecenal (1.00 g, 4.19 mmol, in 1 mL THF) was added, and the reaction mixture was stirred for another 1.5 hr. The reaction was quenched by the addition of acetic acid (2 mL in 10 mL THF), and the mixture was allowed to warm over 2 hr to room temperature. Dilution with diethyl ether, followed by washing with saturated $NaHCO_3$, H_2O, and brine, drying over $MgSO_4$, and evaporation of volatiles gave 1.13 g of an orange oil (after vacuum desiccation). Purification by radial chromatography (silica, 15% ethyl acetate/hexanes) gave 513 mg of product as a yellow oil (1.52 mmol, 36%). IR (neat): 3400 cm^{-1} (OH stretch), 3000 (alkene CH), 2950–2850 (aliphatic CH), 2240 (C ≡ C), 1720 (C = O), 1250 (carbonyl C − O); ^{1}H-NMR ($CDCl_3$): δ 5.36–5.30 (m, 2H, H_{14}/H_{15}), 4.48 (m, 1H, H_4), 4.24 (q, 2H, J_{AB} = 7.1 Hz, OCH_{2-}), 2.02 (bs, 4H, H_{13}/H_{16}), 1.80–1.73 (m, 2H, H_5), 1.46 (bs, 4H, H_{12}/H_{17}), 1.40–1.29 (m, 19H, CH_2 and ester $-CH_3$), 0.91 (bt, 3H, CH_3).

2.4. cis-*Nonadec-2,14-dienoic acid γ-lactone (2)*

Alkynoate ester (**1**) (513 mg, 1.52 mmol) was dissolved in methanol (1 mL) and pyridine (0.122 mL). Lindlar catalyst (41 mg) was added, and the reaction mixture was deaerated with nitrogen. Evacuation of the inert gas was followed by hydrogen bubbling, with stirring, for 7.5 hr. The reaction mixture was filtered through Celite to remove the catalyst, diluted catalyst, diluted with diethyl ether, and then washed with 1 N HCl, H_2O, and saturated $NaHCO_3$. The ether layer was then stirred with a catalytic amount of *p*-toluenesulfonic acid at room temperature while monitoring ring closure by TLC (silica, 25% ethyl acetate/hexanes). After approximately 48 hr, the diethyl ether solution was washed with 5% $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to give 425 mg of crude product as an orange oil.

Purification by radial chromatography (silica, 25% ethyl acetate/hexanes) gave 323 mg of product as a yellow oil (1.10 mmol, 73%). IR (neat): 3000 cm^{-1} (alkene CH), 2950–2850 (aliphatic CH), 1760 (C = O), 1160 (carbonyl C − O); ^{1}H-NMR ($CDCl_3$): δ 7.45 (d, 1H, J_{AB} = 5.6 Hz, H_3), 6.10 (d, 1H, J_{AB} = 5.6 Hz, H_2), 5.35 (t, 2H, J_{AB} = 4.75 Hz, H_{14}/H_{15}), 5.04 (m, 1H, H_4), 2.02 (bm, 4H, H_{13}/H_{16}), 1.91–1.53 (m, 2H, H_5), 1.43–1.28 (m, 18H, CH_2), 0.90 (t, 3H, J_{AB} = 6.7 Hz, CH_3).

2.5. cis, cis-2,3-Epoxynonadec-14-enoic acid γ-lactone (3)

Butenolide (**2**) (100 mg, 0.342 mmol) was dissolved in *N*,*N*-dimethyl formamide/diethyl ether (1:1, 10 mL), chilled to 0°, and treated with sodium hypochlorite solution (~4% aqueous, 2 equiv., 1.273 mL) added dropwise. Reactions were stirred for 1 hr, while monitoring by TLC (silica, 15% ethyl acetate/hexanes). When no further change in the product to starting material ratio was observed, the reaction mixture was worked up by dilution with diethyl ether, separation of layers, washing the organic layer with 10% $NaSO_3$, 5% $NaHCO_3$, H_2O, and brine, followed by drying over $MgSO_4$ and evaporation of volatiles to give the crude product as a clear oil. Purification by radial chromatography (silica, 15% ethyl acetate/hexanes) gave 21 mg of product as a clear oil (0.068 mmol, 20%) along with 68 mg (0.233 mmol, 68.0%) of recovered starting material. Re-treatment of recovered starting material resulted in an overall yield of 32%, IR ($CDCl_3$): 3025 cm^{-1} (alkene CH), 2925–2875 (aliphatic CH), 1790 (C = O); ^{1}H-NMR ($CDCl_3$): δ 5.35 (m, 2H, H_{14}/H_{15}), 4.57 (t, 1H, J_{AB} = 6.5 Hz, H_4), 3.965 (d, 1H, J_{AB} = 2.4 Hz, H_3), 3.775 (d, 1H, J_{AB} = 2.3 Hz, H_2), 2.01 (m, 4H, H_{13}/H_{16}), 1.67 (m, 2H, H_5), 1.33 (m, 18H, CH_2), 0.90 (t, 3H, J_{AB} = 6.7 Hz, CH_3).

2.6. cis-2,3-Epoxy-4-hydroxynonadec-14-enamide (4)

Epoxy lactone (**3**) was dissolved in methanol (5 mL) and chilled to 0°. Concentrated ammonium hydroxide (15 M, 0.15 mL) was added slowly, and the solution was stirred for 90 min when TLC (silica, 5% methanol in methylene chloride) showed the reaction to be complete. Dilution with methylene chloride, drying over Na_2SO_4, evaporation of volatiles, and vacuum desiccation gave 85 mg of crude product as a white powder. Purification by radial chromatography (silica, 5% methanol/methylene chloride) gave 22 mg of pure product as a white solid (0.068 mmol, 61%). IR (KBr) 3425–3150 cm^{-1} (NH/OH stretches), 3000 (alkene CH), 2950–2850 (aliphatic CH), 1680 (C = O), 1460 (carbonyl C − N); ^{1}H-NMR ($CDCl_3$): δ 6.20 (bs, 1H, NH), 6.04 (bs, 1H, NH′), 5.35 (bs, 2H, H_{14}/H_{15}), 3.55 (bs, 1H, H_2), 3.13 (bs, 1H, H_4), 2.74 (bs, 1H, H_3), 2.02 (bs, 4H, H_{13}/H_{16}), 1.29 (bs, 18H, CH_2), 0.90 (bs, 3H, CH_3).

2.7. cis-2,3-epoxy-4-oxo-nonadec-14-enamide (5)

Epoxy-hydroxyamide (**4**) (21 mg, 0.065 mmol) was oxidized by dissolving in dry methylene chloride (2 mL) and deaeration with argon. After addition of 4Å molecular sieves (powdered, 32.5 mg) and *N*-methyl morpholine oxide (0.098 mmol, 11.3 mg), the mixture was stirred for 10 min at room temperature under an argon atmosphere. The reaction was initiated by the addition of [tetra(propyl)ammonium]perruthenate (5 mol%, 3.25 μmol, 1.1 mg). After 90 min of stirring, TLC (silica, 5% methanol/methylene chloride) showed the reaction to be nearly complete. Filtration through a plug of silica with methylene chloride washed with methylene chloride/diethyl ether (1:1), followed by flash chromatography [silica, methylene chloride/diethyl ether (1:1)], gave 6 mg of product as an off-white solid (0.019 mmol, 28%). IR (KBr) 3500 cm^{-1} (asym. NH stretch), 3400 (symm. NH), 3000 (alkene CH), 2950–2850 (aliphatic CH), 1720 (ketone C = O), 1700 (amide C = O); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.34 (s, 1H, NH), 5.51 (s, 1H, NH′), 5.38–5.32 (m, 2H, H_{14}/H_{15}), 3.885 (d, 1H, J_{AB} = 5.31 Hz, H_3), 3.735 (d, 1H, J_{AB} = 5.27 Hz, H_2), 2.64–2.53 (m, 2H, H_5), 2.03–2.00 (m, 4H, H_{13}/H_{16}), 1.32–1.27 (m, 18H, CH_2), 0.90 (t, 3H, J_{AB} = 6.8 Hz, CH_3).

2.8. [^{3}H]-cis-2,3-epoxy-4-oxo-nonadecanamide ([^{3}H]16C and [^{3}H]9C)

Alkenamide (**5**) (3 mg) and cerulenin were catalytically tritiated separately by NEN Life Science Products in ethanol with PtO_2 following the basic methodology of Omura [16]. Exact conditions were worked out with cerulenin using hydrogen gas and HPLC [C_{18} reverse-phase, acetonitrile/water (65:35), 0.5 mL/min, 215 nm] to monitor the progress of the reaction. Radiolabeled products were analyzed by TLC, eluting with either methylene chloride/diethyl ether (1:1) or 5% methanol in methylene chloride. Both [^{3}H]9C and [^{3}H]16C co-eluted with authentic samples of the unlabeled analogs previously synthesized. NEN reported a yield of 369 mCi (39.8 Ci/mmol, if negligible mass loss) and 50 mCi (2.2 Ci/mmol) for [^{3}H]16C and [^{3}H]9C, respectively.

2.9. Cell lines

The following cell lines were obtained for these studies from the American Type Culture Collection: T24 human bladder carcinoma (ATCC HTB 4), MCF-7 human breast adenocarcinoma (ATCC HTB 22), MDA-MB-231 human breast adenocarcinoma (ATCC HTB 26), MDA-MB-157 human breast medulla carcinoma (ATCC HTB 24), Panc-1 human pancreatic epitheloid carcinoma (ATCC CRL 1469), HT-29 human colon adenocarcinoma (ATCC HTB 38), A498 human kidney carcinoma (ATCC HTB 44), and HepG2 human hepatocellular carcinoma (ATCC HB 8065). All cells were grown in RPMI-1640 medium containing

10% FBS (GibcoBRL) and 50 μg/mL of gentamycin sulfate.

2.10. Serum alkylation

Solutions were made that contained equal concentrations and equal specific activities (2.27 Ci/mmol) of [^{3}H]9C and [^{3}H]16C. Five nanomoles of [^{3}H]compound was incubated with aliquots of RPMI-1640 medium containing 10% calf serum at 37° for up to 48 hr. The incubations were terminated by the addition of a concentrated sample buffer, and samples were electrophoresed on 12.5% SDS-PAGE gels. The gels were fixed, soaked with Amplify (Amersham), dried, and exposed to Hyperfilm MP for fluorography.

2.11. Cellular accumulation of [^{3}H]compound

Cells were plated into 12-well plates and grown to approximately 90% confluency. Medium was removed and replaced with RPMI-1640 medium containing 0, 10, or 50% calf serum. [^{3}H]9C or [^{3}H]16C was added to a final concentration of 10 μM, and incubations proceeded for up to 10 hr. At the end of the incubation, extracellular drug was removed by washing the cells twice with cold PBS. Cells were then dissolved in 1% SDS, and the accumulated [^{3}H]compound was quantified by liquid scintillation counting. A parallel culture not treated with drug was used to assess cell number with a hemocytometer.

2.12. Cellular protein alkylation

The panel of cell lines grown in RPMI-1640 medium containing 10% FBS were plated into 100-mm tissue culture dishes and allowed to grow for 24 hr, after which the medium was replaced with RPMI-1640 medium containing 0% FBS. Drug solutions containing an equal concentration and an equal specific activity of [^{3}H]9C or [^{3}H]16C (5 nmol at 35 μCi) were added, and the cells were incubated for 24 hr. Then the cells were washed once with cold PBS, collected, and centrifuged at 1000 *g* for 10 min at 4°. The supernatants were removed, and the pellets were resuspended in lysis buffer [10 mM Tris (pH 7.5), 10 mM NaCl, 1.0 mM EDTA, protease inhibitor (Sigma, P8340), and 1 mM DTT] containing 0.1% TX100, and 50 units of DNase I. The samples were then incubated at 37° for 30 min after which the post-nuclear supernatants were created by centrifugation at 620 *g* for 5 min at 4°. The post-nuclear supernatants were resolved by electrophoresis on 12.5% SDS-PAGE gels and analyzed by fluorography, as indicated above. Parallel cultures not treated with drug were used to assess cell number with a hemocytometer.

2.13. Immunoprecipitation of albumin and IgG

Anti-bovine serum albumin (Sigma) and Protein G/agarose (Santa Cruz) were used to immunoprecipitate albumin and IgG from both serum and T24 lysates. In these assays, after alkylation with [^{3}H]16C, both T24 post-nuclear supernatants and 0.1% FBS were incubated with an overloaded concentration of anti-bovine serum albumin (>1:20) for at least 2 hr at 4°, followed by an incubation with 50 μL of Protein G/agarose overnight at 4°, after which the samples were centrifuged at 620 *g* for 5 min at 4°. The supernatants were collected, the pellets were washed at least one time with cold PBS, and resuspended in lysis buffer (see above), and all samples were then analyzed by fluorography after electrophoresis on 12.5% SDS-PAGE gels.

2.14. Serum albumin and IgG alkylation

Solutions of 19 mg/mL of human serum albumin (Sigma) or 10 mg/mL of IgG (Sigma) in RPMI-1640 medium containing 50 μg/mL of gentamycin sulfate were prepared. Serum albumin solutions were incubated in 1.84 μM [^{3}H]16C, and IgG solutions were incubated in 9.2 μM [^{3}H]16C for up to 24 hr. The labeling was terminated by the addition of 6× concentrated SDS-PAGE sample buffer, after which the samples were boiled for 5 min at 95°, resolved by electrophoresis on 12.5% SDS-PAGE gels, and then analyzed by fluorography.

2.15. Cytotoxicity assay

The toxicity of cerulenin, 9C, and 16C toward the panel of cell lines was determined following previously published procedures. Briefly, cells were plated into 96-well plates and grown for 24 hr to reach about 15% confluency. Cells were then treated with various concentrations of drugs for up to 72 hr, after which the percentage of cell death was determined by the sulforhodamine B (SRB) binding assay [19].

3. Results

3.1. Effects of serum on 9C and 16C uptake and cytotoxicity

To determine the effect of serum on the amount of drug that is taken up by T24 cells, the cells were incubated with 10 μM [^{3}H]9C or [^{3}H]16C in serum concentrations of 0, 10, and 50%, and intracellular accumulation was determined at various times. The accumulation of [^{3}H]9C reached a plateau at approximately 20 min (Fig. 1B), whereas [^{3}H]16C continued to accumulate for approximately 10 hr (Fig. 1A). In the absence of serum, [^{3}H]16C accumulation was 4-fold higher than that of [^{3}H]9C accumulation after 4 hr. However, as the concentration of serum increased, the accumulation of [^{3}H]9C and [^{3}H]16C decreased, such that at 50% serum, both compounds reached steady-state levels of approximately 10 pmol/10^6 cells. Therefore, although [^{3}H]16C accumulates to higher levels than [^{3}H]9C in the

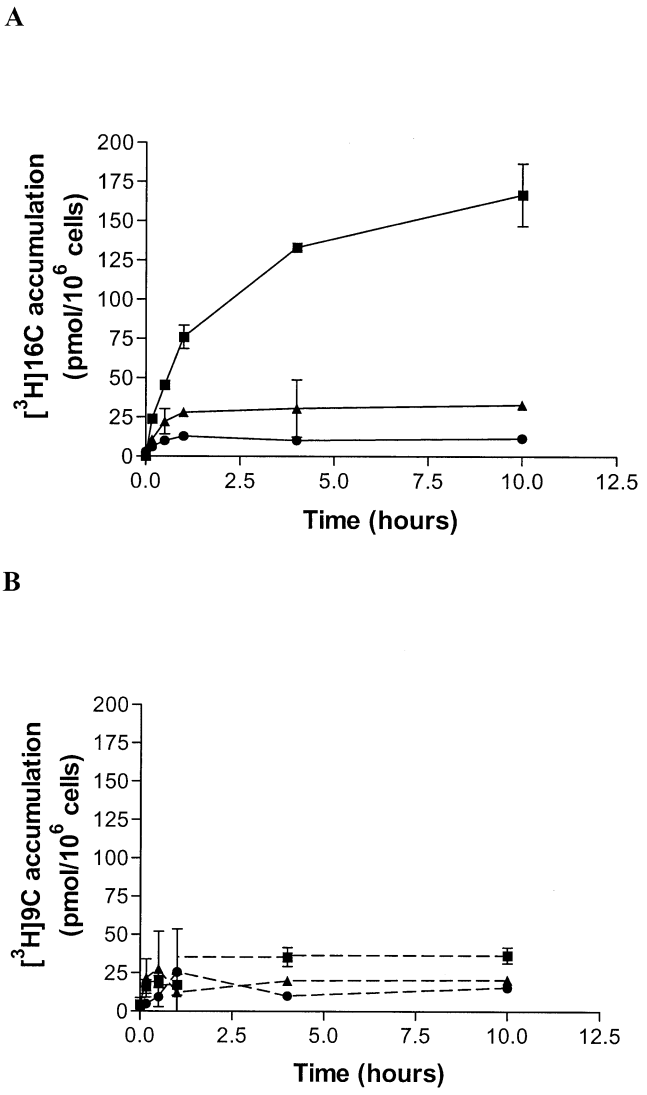

Fig. 1. Effects of serum on the accumulation of [^{3}H]9C and [^{3}H]16C. T24 cells in RPMI-1640 medium containing 0 (■), 10% (▲), or 50% (●) calf serum were treated with a final concentration of 10 μM [^{3}H]16C (panel A) or [^{3}H]9C (panel B) for the indicated times. Cell lysates were collected, and the amount of accumulated compound was determined by liquid scintillation counting as described in "Materials and methods." Values indicate the means ± SD for triplicate samples in one of four similar experiments.

absence of serum, both compounds appear to interact with proteins in the serum.

To determine the effect of serum concentration on the cytotoxic effects of 9C and 16C, SRB assays were performed on T24 cells grown in 10, 20, 30, or 50% total serum (10% FBS plus 0–40% calf serum). Fig. 2 shows that as serum concentrations increased the IC_{50} values for each of the compounds increased, indicating a decreased cytotoxic effect. The cytotoxicity of cerulenin was rather unaffected by increased serum concentration, whereas 9C and 16C both demonstrated increased IC_{50} values with increased serum concentration. Specifically, the IC_{50} for 16C shifted from 1.3 to 11.3 μM in going from 10 to 50% serum, while the IC_{50} for 9C shifted from 0.7 to 7.7 μM (Fig. 2D). The decreased cytotoxicity of both 9C and 16C with increasing serum concentration is consistent with reduced uptake of the compounds, again suggesting that the two compounds bind to proteins in the serum, which decreases the concentration of compound available to the cell.

3.2. Alkylation of serum proteins by [^{3}H]9C and [^{3}H]16C

To determine if the compounds alkylate serum proteins, 10% FBS was incubated in 10 μM solutions of [^{3}H]9C or [^{3}H]16C for up to 48 hr. After varying times, the samples were dissolved in an SDS buffer, boiled for 5 min at 95° and separated on 12.5% SDS-PAGE gels. Fluorography of the gels (Fig. 3) shows that both [^{3}H]9C and [^{3}H]16C labeled serum proteins of approximately 80 and 31 kDa [possibly representing serum albumin and light chain immunoglobulin (IgG), respectively]. These proteins remained labeled after denaturation with SDS and heat, indicating that [^{3}H]9C and [^{3}H]16C had covalently modified the proteins. Fig. 3 also shows that [^{3}H]9C and [^{3}H]16C modified the proteins in a time-dependent manner.

To directly determine if [^{3}H]16C labels serum albumin and IgG, serum albumin (19 mg/mL) and IgG (10 mg/mL) were incubated separately with [^{3}H]16C for 24 hr. Fluorography after electrophoresis demonstrated significant labeling of each purified protein (data not shown), indicating that these compounds will alkylate IgG and serum albumin.

3.3. Alkylation of cellular proteins by [^{3}H]9C and [^{3}H]16C

To determine if 9C and 16C alkylate cellular proteins, T24 cells were incubated with 10 μM [^{3}H]9C or [^{3}H]16C. After various times, the cells were collected and dissolved in a lysis buffer containing Triton X-100 and DNase I. The post-nuclear supernatants were created by centrifuging the samples at 620 *g* for 5 min at 4° to remove cellular debris, including nuclei and cytoskeleton, and the labeled proteins were detected by fluorography of an SDS-PAGE gel (Fig. 4). There was a time-dependent labeling of cellular proteins, with distinct labeling patterns by [^{3}H]9C and [^{3}H]16C. [^{3}H]16C labeled only a small number of proteins, whereas [^{3}H]9C labeled many more, especially a variety of >80 kDa proteins. Since both 9C and 16C inhibit protein palmitoylationase, the commonly labeled proteins of approximately 80 and 31 kDa are candidates for the PPTase.

Since the cell samples described above were centrifuged to remove debris, nuclei, and cytoskeleton, it is possible that labeled proteins were removed as well. Therefore, T24 cells were incubated again with [^{3}H]9C and [^{3}H]16C, this time in 0.44 μM [^{3}H]9C and 0.18 μM [^{3}H]16C in order to maximize protein labeling. The cell samples were incubated with DNase I, and the entire cell lysate was analyzed by SDS-PAGE and fluorography. Prominent new [^{3}H]16C labeled proteins appeared at approximately 40 and 19 kDa (data not

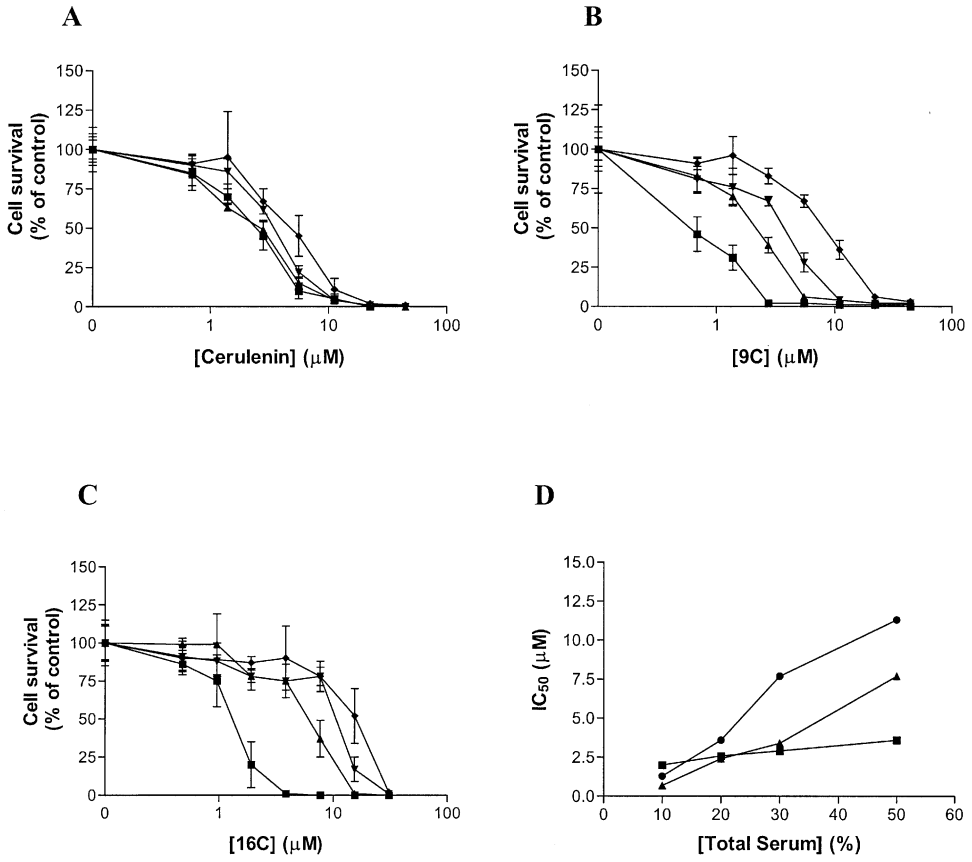

Fig. 2. Effects of serum on the cytotoxicity of cerulenin, 9C, and 16C. T24 cells in RPMI-1640 medium containing 10% FBS plus 0 (■), 10% (▲), 20% (▼), or 40% (◆) calf serum were treated with cerulenin (panel A), 9C (panel B), or 16C (panel C) for 48 or 72 hr. Cell survival was determined using the SRB assay as described in "Materials and methods." Values indicate the means ± SD for triplicate samples in one of two similar experiments. Panel D indicates the average IC_{50} for cerulenin (■), 9C (▲), and 16C (●) at the indicated total concentration of serum.

shown). Therefore, proteins in the nuclear and/or cytoskeletal fraction appear to be alkylated by these compounds.

The labeling patterns of [^{3}H]9C and [^{3}H]16C with serum proteins and the post-nuclear supernatant proteins are very similar, with common alkylated proteins of approximately 80 and 31 kDa. To determine if the labeled proteins of the cell samples were actual cellular proteins or simply residual serum proteins, a series of modifications to the assay were performed. First, T24 cells were labeled in the absence of serum. The cells were then grown in 10% FBS, but were washed once with PBS and then grown in 0% serum during the course of the assay. The distinct labeling patterns of cellular proteins by [^{3}H]9C and [^{3}H]16C were identical in both the presence and absence of serum (data not shown).

Second, any residual serum albumin and IgG were immunoprecipitated from the labeled cell lysates before SDS-PAGE as follows. T24 cells were again incubated in the absence of serum with 0.18 μM [^{3}H]16C. Serum albumin was immunoprecipitated after incubating the samples with antibodies against bovine albumin, and IgG was immunoprecipitated by the addition of Protein G conjugated to agarose beads. The Protein G/agarose pulled out both IgG and anti-albumin after a brief centrifugation. All samples, including the pellets obtained during centrifugation, were analyzed by fluorography after electrophoresis on 12.5% SDS-PAGE gels. For comparison, immunoprecipitation from [^{3}H]16C-labeled serum also was performed. Nearly all of the labeled serum proteins were removed during the immunoprecipitation (Fig. 5A), indicating that the labeled serum proteins were indeed albumin and IgG. Furthermore, the removal of serum albumin and IgG from the T24 post-nuclear supernatant did not remove the prominently labeled 80- and 31-kDa proteins (Fig. 5B), indicating the presence of actual cellular proteins that were not albumin or IgG.

3.4. Cell line specificity of alkylation and cytotoxicity

Since we showed that [^{3}H]9C and [^{3}H]16C alkylate distinct cellular proteins of T24 cells, we wanted to determine if similar proteins are alkylated in other cell lines. Therefore, a cell panel was selected to represent a variety of tissues, and each type of cell was incubated with 5 μM [^{3}H]9C or [^{3}H]16C in the absence of serum for 24 hr. The samples were boiled for 5 min at 95°, and then the labeled proteins were separated on SDS-PAGE gels (Fig. 6). The labeling patterns of each cell line were similar, with the

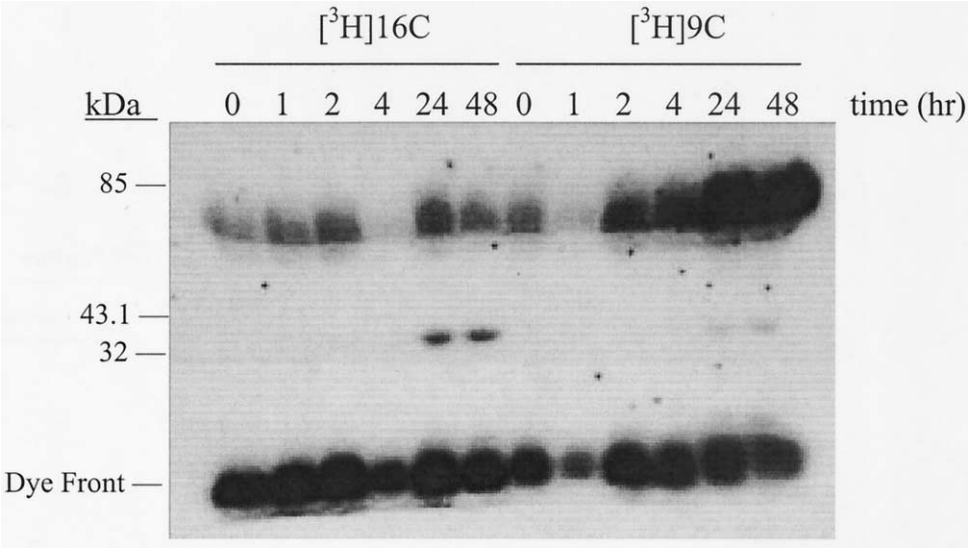

Fig. 3. Alkylation of serum proteins with [^{3}H]9C and [^{3}H]16C. RPMI-1640 medium containing 10% FBS was incubated with 10 μM [^{3}H]9C or [^{3}H]16C for the indicated time. Samples were then analyzed by SDS-PAGE and fluorography as described in "Materials and methods." Similar results were obtained in four independent experiments.

radiolabeled proteins of approximately 80 and 31 kDa visible in each cell line, although the intensity of radiolabeling differed widely in different cells. Comparing the alkylation patterns of [^{3}H]9C (Fig. 6A) and [^{3}H]16C (Fig. 6B) again indicated that [^{3}H]16C produced a more limited number of alkylated proteins than did [^{3}H]9C. Fig. 7 shows that when the accumulation of [^{3}H]compound was normalized for cell number, [^{3}H]16C accumulation was greater than [^{3}H]9C in most cell lines. This is in agreement with the intracellular accumulation data shown in Fig. 1. Fig. 7 also indicates that Panc-1 cells have the highest concentration of accumulated [^{3}H]9C and [^{3}H]16C.

The cytotoxicities of cerulenin, 9C, and 16C against the cell panel were determined. As described previously, SRB assays were used to determine IC_{50} values for each compound against each cell line. Table 1 shows the IC_{50} values ± SEM, as collected from three independent assays. These values show that each individual compound was relatively equally toxic for each cell line (vertical values). It was also seen that each cell line had approximately equal sensitivity to each compound (horizontal values). The most sensitive cell line was Panc-1, which is consistent with the accumulation data seen in Fig. 7.

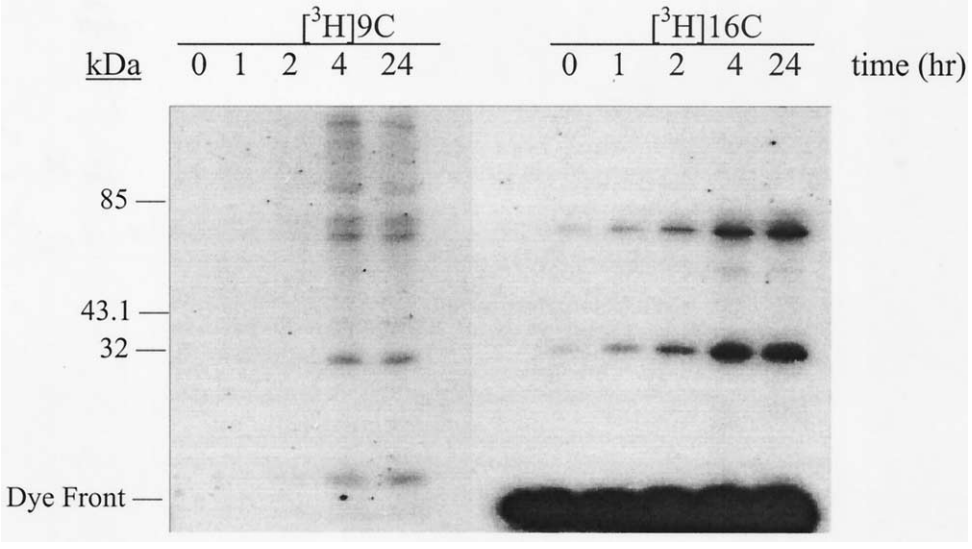

Fig. 4. Alkylation of cellular proteins with [^{3}H]9C and [^{3}H]16C. T24 cells in RPMI-1640 medium without serum were incubated with 1 μM (35 μCi) [^{3}H]9C or [^{3}H]16C for the indicated times. Post-nuclear supernatants were prepared by centrifugation at 620 *g* for 5 min at 4°, and the samples were then analyzed by SDS-PAGE and fluorography as indicated above. Similar results were obtained in multiple independent experiments.

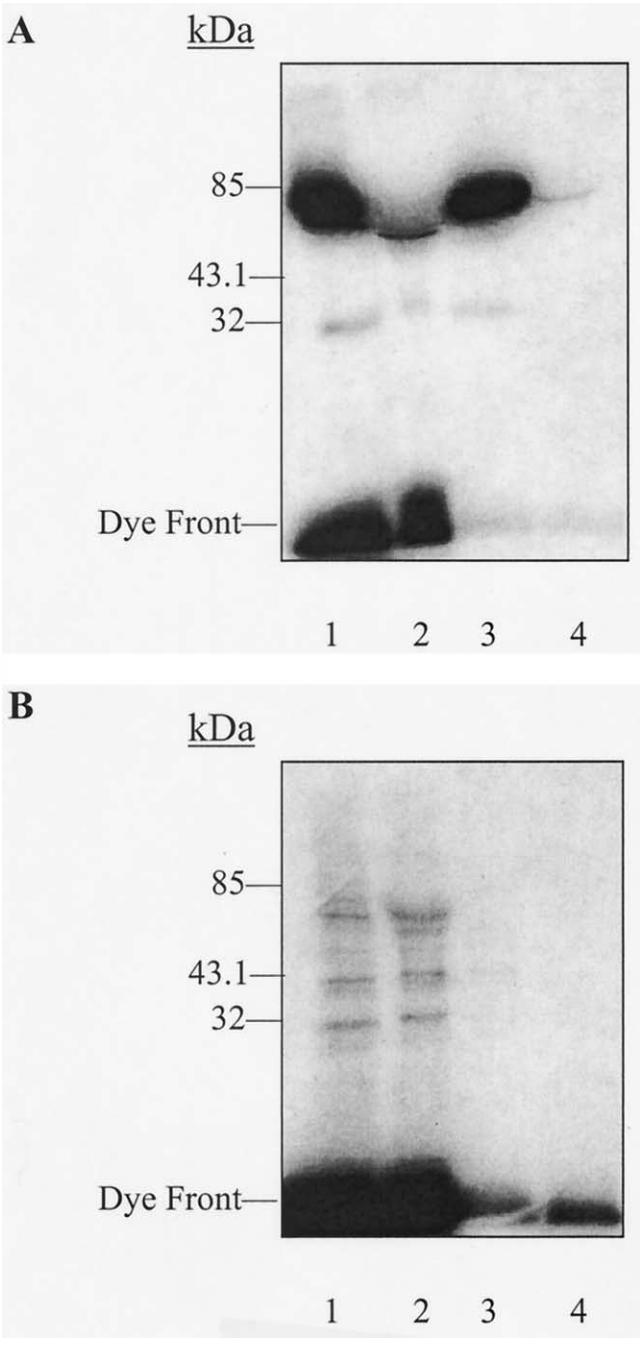

Fig. 5. Immunoprecipitation of albumin and IgG from [^{3}H]16C-labeled serum and T24 cells. Serum (0.1%) (Panel A) or T24 cells in RPMI-1640 medium without serum (panel A) or with 0.1% FBS (panel B) were incubated with [^{3}H]16C for 24 hr. Post-nuclear supernatants were prepared, and albumin and IgG were immunoprecipitated as described in "Materials and methods." The samples were then analyzed by SDS-PAGE and fluorography. Lane 1: total sample; lane 2: immunoprecipitation supernatant; lane 3: immunoprecipitated pellet; and lane 4: immunoprecipitation wash. Similar results were obtained in multiple independent experiments.

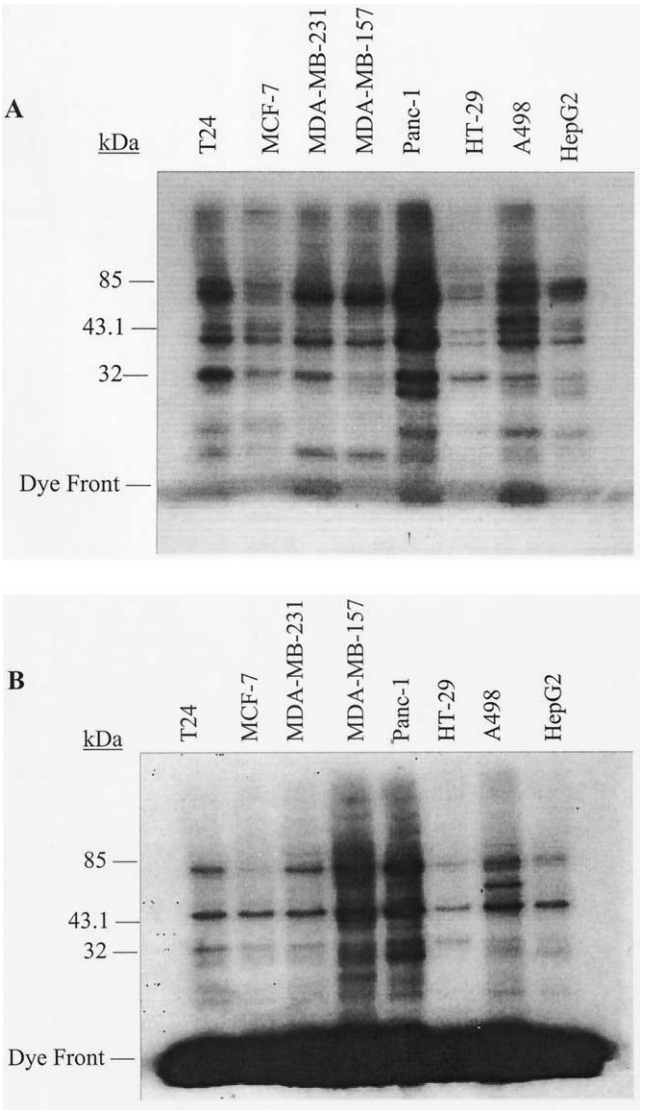

Fig. 6. Cell line specificity of protein alkylation by [^{3}H]9C and [^{3}H]16C. The indicated cell lines growing in RPMI-1640 medium without serum were incubated with 5 nmol (35 μCi) of [^{3}H]9C (panel A) or [^{3}H]16C (panel B) for 24 hr. Total cell lysates were prepared and analyzed by SDS-PAGE and fluorography as indicated above. Representative results from multiple experiments are shown.

4. Discussion

PPTase has not yet been biochemically characterized due to the lability of the protein. Currently, it is unclear whether a single enzyme modifies all known palmitoylated proteins, or whether multiple enzymes recognize different palmitoylation signals [20]. Berthiaume and Resh [21] characterized an acyl transferase activity for the nonreceptor tyrosine kinase Fyn. Later, an acyl-CoA transferase activity for G-protein α-subunits was characterized and found to be enriched in membranes [8]. Both of these activities use palmitoyl CoA as the preferred acyl donor. A 70-kDa protein, palmitoyl acyl transferase, that modifies spectrin has been isolated [22]; however, this modification occurs at non-farnesyl-containing sites. A suspected PPTase that palmitoylated a farnesylated N-Ras peptide [23] was later identified as peroxisomal 3-oxoacyl-CoA thiolase A, making the relevance of this enzyme to Ras processing doubtful. Since

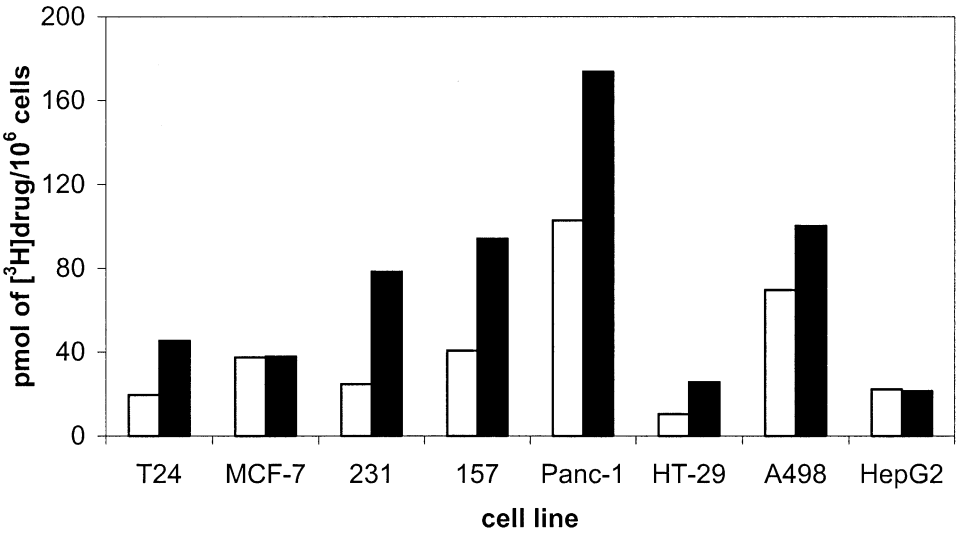

Fig. 7. Cell line specificity of [^{3}H]9C and [^{3}H]16C accumulation. The indicated cell lines were incubated with [^{3}H]9C (open bars) or [^{3}H]16C (closed bars) as indicated in Fig. 6. The intracellular accumulation of [^{3}H]9C and [^{3}H]16C was determined by liquid scintillation and normalized by cell number as indicated in "Materials and methods." Results are from the same experiment shown in Fig. 6.

it is likely that a family of PPTases exists, each with different substrate specificity and subcellular localizations, PPTase remains an interesting biochemical problem and an attractive target for developing inhibitors of specific signaling molecules.

One of the important targets of palmitoylation is Ras because Ras transformation requires membrane localization, which is achieved by a series of post-translational modifications at the C-terminus. The inhibition of membrane localization by mutation produces proteins that are defective in transformation abilities [24]. Based on this observation, the post-translational processing of Ras is a target for potential new anti-cancer drugs under the hypothesis that by inhibiting various steps of the post-translational pathway, transforming activity will be diminished.

The post-translational processing of Ras includes farnesylation at a CAAX sequence, proteolysis, methylation, and palmitoylation (for H- and N-Ras). Much attention has centered on inhibiting the enzyme FPTase [25–27], since it is the first step of the post-translational process. However, farnesylation is an irreversible process, whereas palmitoylation is dynamic [1], with palmitate turnover demonstrating a half-time of only 20 min. Therefore, the inactivation of Ras by inhibiting palmitoylation may be more effective than inhibiting farnesylation, since mature proteins can also be inactivated. The biochemistry and pharmacology of these two enzymatic reactions are still under investigation.

Several compounds inhibit farnesylation of Ras, which prevents transient membrane association, thereby preventing the functional activation of Ras. But the farnesyl group is not an absolute requirement for palmitoylation [28]. Also, H- and N-Ras proteins can be geranylgeranylated in the presence of FTIs [29,30], which allows the membrane association needed for palmitoylation. Therefore, tumor cells can avoid the effects of FTIs by incorporating other lipids, such as geranylgeranyl, for increasing hydrophobicity.

The molecular mechanisms for inhibition of tumor growth by FTIs are unclear. These drugs are not specific anti-Ras drugs, since the molecular target is FPTase, and there are at least 18 mammalian proteins that are known to be farnesylated [31]. It is unclear whether the biological effects of FTIs result from inhibiting these other proteins. The concentrations of FTI that will achieve a cellular effect are often 1000 times greater than the IC_{50} values for FTIs *in vitro* [32,33]. This suggests that FTIs have difficulty penetrating the cell or that other nonspecific actions may be occurring. It is possible that inhibiting farnesylation of proteins other than Ras may be cytotoxic to the cell, and in fact RhoB appears to be a prime candidate [34]. Therefore, the development of additional classes of anti-Ras drugs is desired.

Table 1
Cytotoxicities of cerulenin, 9C, and 16C

Cell line	IC_{50} (μM)		
	Cerulenin	9C	16C
T24	19 ± 3	12 ± 2	30 ± 8
MCF-7	25 ± 2	16 ± 1	34 ± 9
MDA-MB-231	12 ± 2	7 ± 1	20 ± 6
MDA-MB-157	10 ± 3	4 ± 1	9 ± 2
Panc-1	7 ± 1	5 ± 1	8 ± 3
HT-29	30 ± 3	15 ± 2	35 ± 12
A498	9 ± 4	7 ± 2	14 ± 4
HepG2	21 ± 3	12 ± 5	23 ± 5

The indicated cell lines were exposed to various concentrations of cerulenin, 9C, or 16C, and cell survival was determined after 48 hr as indicated in "Materials and methods." Values are means ± SEM for three independent experiments.

Although K-*ras* is the most commonly mutated *ras* gene in human cancer, all forms of mutated *ras* can transform mammalian cells in culture, as well as create transgenic animals that develop spontaneous malignancies [35,36]. Furthermore, cancers driven by increased expression or activity of growth factor receptors are dependent upon functional Ras for mitogenic and survival signals. Recent work has demonstrated that H-Ras in particular is important for mediating anti-apoptotic signaling through PI3-kinase and AKT [37]. Therefore, while palmitoylation inhibitors may not directly inhibit all forms of transformed Ras, their biological effect should be beneficial for many human cancers. Since there is a small variety of signaling molecules that are palmitoylated, the utility of palmitoylation inhibitors as therapeutic agents may be mediated by inhibiting palmitoylated proteins other than Ras.

The natural product cerulenin has long been known to inhibit fatty acid synthase [16], and this was assumed to be the mechanism of antitumor activity [38,39]. Recently, we have synthesized more than 30 cerulenin analogs, and it was demonstrated that these compounds inhibit protein palmitoylation of H-Ras in parallel with inhibition of cell proliferation [18]. Proliferation inhibition studies indicated that the α-keto-epoxide moiety is critical for cytotoxicity and that the compounds containing unsubstituted carboxamides inhibit the incorporation of [^{3}H]palmitate into H-Ras. It was also shown that the compounds with side chains longer than 12 carbons were selective inhibitors of protein palmitoylation, whereas the compounds with shorter side chains also inhibited fatty acid synthase [18]. Therefore, these cerulenin analogs have the potential to be selective inhibitors of protein palmitoylation.

Two of the cerulenin analogs, 9C and 16C, were selected for further investigation. 9C contains a saturated side chain whose length is equivalent to that of cerulenin, while the chain length of 16C is equivalent to that of palmitoyl-CoA, the putative substrate for PPTase. Both fatty acid synthase and protein palmitoylation are inhibited by 9C, whereas 16C selectively inhibits only the latter activity [18]. For the current studies, the two compounds were radiolabeled with tritium so that their cellular pharmacology could be investigated.

Intracellular accumulation studies indicated that both [^{3}H]9C and [^{3}H]16C can penetrate the cell membrane. In the absence of serum, 16C accumulated to greater levels in the cell than did 9C, with a 4-fold difference after 4 hr. But the presence of serum decreased the accumulation of both compounds, suggesting that the compounds are binding to proteins in the serum, such as albumin and IgG. Cytotoxicity assays support the conclusion that 9C and 16C are binding to serum proteins, since the presence of increasing serum concentrations decreased the cytotoxicity of the compounds. Serum did not markedly affect the cytotoxicity of cerulenin, but the cytotoxicities of both 9C and 16C were decreased up to 10-fold with increasing serum concentrations. Therefore, based on intracellular accumulation and cytotoxicity assays, the abilities of 9C and 16C to be effective palmitoylation inhibitors are reduced by the presence of serum.

Although 9C and 16C can bind to and alkylate serum albumin and IgG, it is clear that these compounds also alkylate cellular proteins that are distinct from these serum components, as demonstrated by the immunoprecipitation assays. The identity of these alkylated proteins is unknown; however, we postulate that 16C competes for the binding site of PPTase because it has a structure similar to that of palmitate. This hypothesis is supported by competition of the radiolabeled proteins by palmitoyl-CoA (data not shown), the putative substrate of PPTase. Although the radiolabeling of fatty acid synthase at 250 kDa is possible by 9C, the identity of the labeled proteins at 80 and 31 kDa remains unknown. Because of the reactivity of the epoxide moiety, it is likely that PPTase becomes covalently modified by the inhibitors and permanently inactivated. Therefore, these compounds provide a unique approach toward the identification and purification of this important enzyme.

A panel of cell lines was tested for sensitivity to 9C and 16C and protein alkylation by the radiolabeled compounds, in an attempt to correlate these properties. Although Panc-1 cells were the most sensitive to the cytotoxic effects of cerulenin, 9C, and 16C, the IC_{50} values for each compound were relatively similar for each cell line. The fact that the Panc-1 cell line was the most sensitive to 9C and 16C is consistent with the high level of accumulation of the compounds in these cells. Similarly, Panc-1 cells showed the highest intensity protein labeling by both [^{3}H]9C and [^{3}H]16C. Since 9C and 16C do not show selectivity towards any one cell type, the target of these compounds appears to be essential for cell proliferation.

As stated earlier, PPTase has not been purified yet, and the cerulenin analogs are the only selective inhibitors currently described in the literature. It is likely that additional inhibitors of PPTase will be difficult to define until the biochemistry of the enzyme has been better elucidated. The radiolabeled compounds described here provide important tools for the identification of PPTase. We are currently isolating the radiolabeled proteins alkylated by [^{3}H]9C and [^{3}H]16C. This should allow their sequencing and biochemical characterization. By synthesizing PPTase selective inhibitors, it will be possible to define the potential utility of these inhibitors as palmitoylation inhibitors.

Acknowledgments

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