Radicicol Binds and Inhibits Mammalian ATP Citrate Lyase*

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Six different biotinylated radicicol derivatives were synthesized as affinity probes for identification of cellular radicicol-binding proteins. Derivatives biotinylated at the C-17 (BR-1) and C-11 (BR-6) positions retained the activity of morphological reversion in v-src-transformed 3Y1 fibroblasts. Two radicicol-binding proteins, 120 and 90-kDa in size, were detected in HeLa cell extracts by employing BR-1 and BR-6, respectively. The 90-kDa protein bound to BR-6 was identified to be Hsp90 by immunoblotting. The 120-kDa protein bound to BR-1 was purified from rabbit reticulocyte lysate, and its internal amino acid sequence was identical to that of human and rat ATP citrate lyase. The identity of the 120-kDa protein as ATP citrate lyase was confirmed by immunoblotting. Interaction between BR-1 and ATP citrate lyase was blocked by radicicol but not by herbimycin A that interacts with Hsp90. These results suggest that radicicol binds the two proteins through different molecular portions of its structure. BR-1-bound ATP citrate lyase isolated from rabbit reticulocyte lysate showed no enzymatic activity. The activity of rat liver ATP citrate lyase was inhibited by radicicol and BR-1 but not by BR-6. Kinetic analysis demonstrated that radicicol was a non-competitive inhibitor of ATP citrate lyase with $K_i$ values for citrate and ATP of 13 and 7 μM, respectively.

Radicicol (also known as monorden), a 14-membered macrolide originally isolated from Monosporium bonorden as an antifungal antibiotic in 1953 (1), is a compound showing a variety of biological activities. It was reported again as a potent tranquilizer with low toxicity in 1964 (2). We rediscovered radicicol as a potent inducer of reversal of the transformed phenotype in v-src-transformed fibroblasts to the normal one (3, 4). We also showed that radicicol caused cell cycle arrest in G1 and G2 phases, and Oikawa et al. (5) demonstrated that it inhibited in vivo angiogenesis. Furthermore, radicicol was reported to induce morphological reversion of not only src but also ras, mos, raf, fos, and SV40-transformed cell lines and inhibited the expression of mitogen-inducible cycloxygenase in macrophages (6–8). Some of leukemia cell lines were differentiated in response to radicicol (4, 9). Recently, KF25706, a novel oxime derivative of radicicol, was reported to show potent antitumor activity and is currently under consideration as an anticancer drug (10). The in vivo inhibition of tyrosine kinases and MAP kinases has been suggested to be involved in these characteristic phenotypes elicited by radicicol (6, 7). Increased expression of gelsolin, an actin regulatory protein, has also been observed during the induction of morphological changes in various transformed cells (11). Recent studies showed that radicicol disrupted the Ras-activated signaling pathway by selectively depleting Raf kinase or reducing Ras/Raf molecular interaction (12, 13). The target molecule of radicicol was proposed to be Hsp90, because it strongly binds Hsp90 in a manner competitive with ATP and geldanamycin, a known Hsp90 inhibitor (14, 15). However, it is still unclear whether Hsp90 is the only protein targeted by radicicol.

To identify radicicol-binding proteins using affinity matrix, we synthesized several biotinylated radicicol derivatives and used them for purification of radicicol-binding proteins. This strategy has been successfully employed in identifying the target molecules of several natural products such as fumagillin (16), didemnin (17), rapamycin (18), and leptomycin (19). We show here that radicicol binds not only Hsp90 but also ATP citrate lyase (ACL), a key enzyme that produces acetyl-CoA in the cytosolic compartment. ACL is required for both fatty acid and sterol syntheses. These lipids and lipid-modified molecules play important roles in many cellular and tissue functions including signal transduction and protein membrane sorting. We suggest that the inhibition of ACL, in addition to Hsp90, contributes to some extent to a variety of biological effects of radicicol.

EXPERIMENTAL PROCEDURES

Synthesis of Radicicol Derivatives—A palmitoyl derivative of radicicol (Fig. 1, derivative 2): a solution of radicicol (105 mg, 0.29 mmol), 16-hydroxypalmitic acid (79 mg, 0.29 mmol), DCC (105 mg, 0.51 mmol), and DMAP (10 mg, 0.08 mmol) in dichloromethane was stirred at room temperature overnight. The reaction mixture was poured into water and extracted with chloroform. The extract was washed with brine, dried (MgSO4), and concentrated. A monoester (50 mg, 28%), diester (derivative 2; 40 mg, 16%) and unreacted radicicol (35 mg, 33%) in the residue were separated with preparative silica gel TLC as colorless powder.

Reduced radicicol (Fig. 1, derivatives 3 and 4): a mixture of radicicol (100 mg, 0.27 mmol) and 5% Pd(OH)2/C (10 mg) in ethyl acetate (3 ml) was stirred under hydrogen at room temperature for 2.5 h. After filtration and concentration, silica gel chromatography gave derivative 3 (84 mg, 83%) and derivative 4 (16 mg, 14%).

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† The abbreviations used are: ACL, ATP citrate lyase; DCC, dicyclohexylcarbodiimide; DMAP, 4-diimethylaminopyridine; HBTU, O-benzotriazol-1-yl-3,4,3',4'-tetramethyluronium hexafluorophosphate; DMF, dimethylformamide; HPLC, high performance liquid chromatography; THF, tetrahydrofuran; MAP, mitogen-activated protein.
Inhibition of ATP Citrate Lyase by Radicicol

Synthesis of BR-1—A solution of radicicol (35 mg, 0.096 mmol), biotinylbis-ε-aminocaproic acid (50 mg, 0.10 mmol), DCC (30 mg, 0.15 mmol), and DMAP (5 mg, 0.041 mmol) in pyridine (2 ml) was stirred at room temperature for 5 days. After concentration, preparative silica gel TLC gave BR-1 (25 mg, 49%) as a colorless powder and unreacted radicicol (17 mg, 49%).

Synthesis of BR-2—A suspension of radicicol (300 mg, 0.82 mmol), 1-bromo-3-iodopropane (700 mg, 2.81 mmol), and K₂CO₃ (470 mg, 3.43 mmol) in acetone was refluxed for 1.5 h. The reaction mixture was poured into saturated NH₄Cl solution and extracted with ethyl acetate. The mixture was stirred at 50 °C for 5 days. After concentration, silica gel chromatography gave an oxime of radicicol (52 mg, 0.21 mmol) in dichloromethane (2 ml) was dropped a solution of m-chloroperbenzoic acid (36 mg, 0.21 mmol) at −78 °C, and the mixture was stirred for 10 min. The reaction mixture was poured into 10% aqueous Na₂S₂O₄/saturated NaHCO₃ (1:1). After extraction with ethyl acetate, the extract was dried (MgSO₄) and concentrated. The residue was dissolved in benzene, and the mixture was stirred at 50 °C for 5 days. After concentration, silica gel chromatography gave an oxime derivative (119 mg, 98%) as colorless powder. To a solution of the oxime derivative (119 mg, 0.21 mmol) in dichloromethane (2 ml) was added 15-O-ethylmorpholine (5 ml) in methanol (8 ml), and 15-O-alkylated radicicol (217 mg, 54%) as a colorless powder. To a solution of the amine (14 mg, 0.032 mmol) in DMF (1 ml) were added HBTU (13 mg, 0.035 mmol) and N-ethylmorpholine (5 μl, 0.039 mmol). The reaction mixture was stirred at room temperature overnight, and poured into saturated NH₄Cl solution. After extraction with ethyl acetate, preparative silica gel TLC gave biotinylated BR-2 (4 mg, 14%).

Synthesis of BR-3 and BR-4—A solution of compound 3 (80 mg, 0.22 mmol), 6-biotinylaminocaproic acid hydrazide (Molecular Probes B-1600, 50 mg, 0.13 mmol) and acetic acid (500 μl) in methanol (8 ml) was stirred at room temperature overnight. After concentration, preparative silica gel TLC gave BR-3 (17 mg, 11%) as a colorless powder.

Synthesis of BR-5—A solution of radicicol (100 mg, 0.27 mmol) and 3-aminoxypropyl-1-azide (40 mg, 0.34 mmol) was refluxed for 2 h. After concentration, silica gel chromatography gave a 1,4-adduct (85 mg, 67%) as colorless powder. This compound (35 mg, 0.073 mmol) was dissolved in DMF (1 ml) and was added to biotinylbis-ε-aminocaproic acid (35 mg, 0.072 mmol) and tri-n-butylphosphine (18 μl, 0.072 mmol) at −78 °C. The reaction mixture was allowed to warm to room temperature and stirred overnight. After concentration, preparative silica gel TLC gave BR-5 (10 mg, 15%) as a colorless powder.

Synthesis of BR-6—To a solution of radicicol (150 mg, 0.41 mmol) in THF (3 ml) was added dropwise a solution of PhSLi in THF (0.2 ml, 2.0 mol, 0.40 mmol) at −78 °C under argon and stirred at −78 °C for 4 days. The reaction mixture was poured into saturated NH₄Cl solution and extracted with ethyl acetate. The same workup as above and silica gel chromatography gave a 1,4-adduct (130 mg, 67%) as colorless powder with a trace 1,6-adduct. This 1,4-adduct (100 mg, 0.21 mmol), 3-aminooxypropyl-1-azide (200 mg, 1.72 mmol) and acetic acid (100 μl) were dissolved in benzene, and the mixture was stirred at 50 °C for 5 days. After concentration, silica gel chromatography gave an oxime derivative (119 mg, 98%) as colorless powder. To a solution of the oxime derivative (119 mg, 0.21 mmol) in dichloromethane (2 ml) was dropped a solution of m-chloroperbenzoic acid (36 mg, 0.21 mmol) at −78 °C, and the mixture was stirred for 10 min. The reaction mixture was poured into 10% aqueous Na₂S₂O₄/saturated NaHCO₃, (1:1). After extraction with ethyl acetate, the extract was dried (MgSO₄) and concentrated. The residue was dissolved in ethyl acetate (2 ml) and refluxed in the presence of calcium carbonate (50 mg, 0.50 mmol) for 5 h. After filtration and concentration, silica gel chromatography gave an oxime of radicicol (52 mg, 57%) as a colorless powder. This compound (52 mg, 0.12 mmol) was dissolved in THF (5 ml) and added to 20% solution of triethylphosphine in toluene (80 μl, 0.14 mmol) at −78 °C. The reaction mixture was allowed to warm to room temperature and was stirred overnight. After concentration, preparative silica gel TLC gave an amine (30 mg, 58%) as a colorless powder. To a solution of the amine (14 mg, 0.032 mmol) and biotinylbis-ε-aminocaproic acid (15 mg, 0.031 mmol) in DMF (1 ml) were added HBTU (12 mg, 0.032 mmol) and N-ethylmorpholine (5 μl, 0.039 mmol). The reaction mixture was stirred at room temperature for 2 days and poured into saturated NH₄Cl solution. After extraction, the same workup as before and preparative silica gel TLC gave BR-6 (4 mg, 14%) as a colorless powder.

Detection of Radicicol-binding Proteins—HeLa cells were washed twice with phosphate-buffered saline and then homogenized with a glass teflon homogenizer in binding buffer (10 mM Tris-HCl pH 7.6, 50 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 0.1 mM Na₃VO₄). The cell lysate

![Fig. 1. Structures and biological activity of radicicol and its derivatives used in this study. Plus signs indicate a significant difference in the percentage of cells exhibiting a flat morphology in v-src-transformed 3Y1 cells at 24 h relative to control cells with 0.1% ethanol or 0.1% Me₂SO, and a minus sign alone indicates no significant difference from control. At least two independent experiments with triplicate samples were performed for each treatment.](Image 353x559 to 509x729)

![Fig. 2. Screening of radicicol-binding proteins. Two mg of total protein was incubated with 1 μg each of biotinylated radicicol derivatives, and bound proteins were precipitated with streptavidin beads. The proteins eluted were analyzed by 4–20% gradient SDS-polyacrylamide gel electrophoresis. Coomassie Brilliant Blue staining showed that BR-1 bound to a 120-kDa protein, whereas BR-6 bound to a 90-kDa protein.](Image 50x376 to 294x729)
streptavidin (Sigma Chemical Co., St. Louis, MO) for 60 min at 4 °C. The lysate (Promega) had been precleared by incubating with immobilized avidin-agarose. After the supernatant of HeLa cells or rabbit reticulocyte lysate (Promega) had been precleared by incubating with immobilized avidin-agarose, the supernatant was centrifuged at 50,000 × g for 30 min at 4 °C, and the supernatant was collected. After the supernatant of HeLa cells or rabbit reticulocyte lysate was centrifuged at 50,000 × g for 5 min, the cleared supernatants were incubated with biotinylated radicicol derivatives in the presence or absence of radicicol as a competitor. After incubation for 60 min at 4 °C, proteins associated with the biotinylated radicicol derivatives were precipitated with streptavidin-agarose. The pellet was washed with washing buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% (v/v) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 ng/ml aprotinin, 10 ng/ml leupeptin, 10 mM glycerophosphate, 1 mM NaF, and 0.1 mM Na3VO4. The bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer, separated by a 4–20% gradient polyacrylamide gel, and visualized by silver staining (Wako Pure Chemical Industries, Ltd.) or Coomassie Brilliant Blue staining.

**FIG. 3. Identification of Hsp90 as the BR-6-binding protein.** A, immunoblot analysis of the 90-kDa protein bound to BR-6. The 90-kDa protein bound specifically to a 90-kDa protein, which was reactive with an anti-Hsp90 antibody. B, competition of radicicol for BR-6 binding to the 90-kDa protein. Radicicol blocked the binding of BR-6 to Hsp90. Herbigycin A, a benzoquinone ansamycin antibiotic known to inhibit Hsp90, also competed for this binding.

**FIG. 4. Identification of ATP citrate lyase (ACL) as the protein bound to BR-1.** A, quantitative association of the 120-kDa protein (p120) with BR-1 in rabbit reticulocyte lysate. B, immunoblot analysis of p120 with an anti-ACL antibody. To confirm the identity of the protein with ACL, the immunoblot analysis was performed with a newly prepared anti-ACL antibody.

was centrifuged at 50,000 × g for 30 min at 4 °C, and the supernatant was collected. After the supernatant of HeLa cells or rabbit reticulocyte lysate (Promega) had been precleared by incubating with immobilized streptavidin (Sigma Chemical Co., St. Louis, MO) for 60 min at 4 °C followed by centrifugation at 500 × g for 5 min, the cleared supernatants were incubated with biotinylated radicicol derivatives in the presence or absence of radicicol as a competitor. After incubation for 60 min at 4 °C, proteins associated with the biotinylated radicicol derivatives were precipitated with streptavidin-agarose. The pellet was washed with washing buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% (v/v) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 ng/ml aprotinin, 10 ng/ml leupeptin, 10 mM glycerophosphate, 1 mM NaF, and 0.1 mM Na3VO4. The bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer, separated by a 4–20% gradient polyacrylamide gel, and visualized by silver staining (Wako Pure Chemical Industries, Ltd.) or Coomassie Brilliant Blue staining.

**FIG. 5. Inactivation of ACL by BR-1 binding.** A, specific binding of BR-1 to ACL in rat liver extract. ACL was enriched in rat liver by a high sucrose diet, and the specific association with BR-1 was detected. Lane 1, 10 μg of total protein of ACL-enriched liver extract; lane 2, proteins bound to streptavidin beads without biotinylated radicicol derivatives from 1 mg of total protein; lane 3, proteins bound to biotin-streptavidin beads; lane 4, proteins bound to BR-1-streptavidin beads; lane 5, proteins bound to BR-1-streptavidin beads in the presence of 50 μg/ml radicicol; and lane 6, proteins bound to BR-2-streptavidin beads. B, loss of ACL activity in the BR-1-bound protein. The ACL activity from each protein sample obtained as in A was determined by the malate dehydrogenase-catalyzed reduction of oxaloacetate by NADH as described under "Experimental Procedures." The purified BR-1/ACL complex (lane 4) showed no enzyme activity.

For enzymatic digestion and peptide sequence analysis, 2.4 g of protein of the rabbit reticulocyte lysate was incubated with 72 μg of BR-1 and 3.6 ml of streptavidin-agarose. The radicicol-binding protein was separated by SDS/7.5% polyacrylamide gel electrophoresis, and then electrophoresed onto nitrocellulose filters (Schleicher & Schuell) for 3 h. For efficient transfer of the 120-kDa protein, 0.05% SDS was added to the transfer buffer (15.6 mM Tris, 120 mM glycine). After the transfer, proteins were reversibly stained by 0.1% Ponceau S dye (Nacalai Tesque, Kyoto, Japan). Protein-containing regions thus detected were cut out, washed thoroughly, and incubated for 1 h at 37 °C in 1 ml of 0.5% polyvinylpyrrolidone-40 (Sigma Chemical Co., St. Louis, MO) dissolved in 100 mM acetic acid to prevent adsorption of the protease to the nitrocellulose during digestion. After removal of excess PVP-40, the protein on the nitrocellulose pieces was digested with 1 μg of lysyl endopeptidase (Wako Pure Chemical Industries, Ltd.) in 100 μl of protease buffer (20 mM Tris-HCl pH 9.0, 5% acetonitrile) at 37 °C overnight. After digestion, the whole reaction mixture was acidified by adding 4% (v/v) acetic acid and immediately loaded onto the HPLC column. The peptide mixture was separated with HPLC on a C8 (250 × 4.6-mm) reverse phase column (Shiseido Co.). The peptides were eluted with a linear gradient of acetonitrile, 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min using a Shimadzu HPLC system. Elution was monitored at 215 nm, and peaks were manually collected. The N-terminal sequence was determined on an Applied Biosystems protein sequencer.

**Immunodetection of Hsp90 and ACL—A polyclonal anti-ACL antibody was raised against a synthesized C-terminal peptide corresponding to Hsp90 antibody was raised against a synthesized C-terminal peptide corresponding to Hsp90. The rabbit antiserum and preimmune serum were used for immunoblotting. Protein samples were loaded and electrophoresed on SDS/12.5% polyacrylamide gels and transferred onto an Immobilon-P membrane (Millipore Co., Bedford, MA). After the membrane had been treated with the anti-ACL antibody or a monoclonal anti-Hsp90 antibody raised against a peptide of human Hsp90 (residues 586–732), which reacts with the C terminus of human, mouse, and rat Hsp90 (BD Transduction Lab., CA), the im-
mune complexes were detected with ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

**Preparation of Rat Liver ATP Citrate Lyase and Enzyme Assay—** Rats starved for 48 h and fed a high sucrose diet (63% sucrose, 30% casein, 4% salt mixture, 2% cellulose powder, 1% vitamin mixture, and 0.1% choline chloride) for 3 days were killed by cervical dislocation, and their livers were excised and quickly homogenized in 4 volumes of buffer containing 0.25M sucrose, 50 mM Tris-HCl, pH 8.2, 10 mM MgCl₂, 5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 17,500 \( \times g \) for 15 min at 4 °C, and the supernatant was filtered through four layers of gauze and recentrifuged for 1 h at 100,000 \( \times g \) at 4 °C. The supernatant thus obtained was divided into 1-ml portions, stored at \(-80^\circ\)C and thawed each time before use. ACL activity was measured by the malate dehydrogenase-catalyzed reduction of oxaloacetate by NADH (20). The standard reaction mixture (100 \( \mu \)l) contained 100 mM Tris-HCl, pH 8.5, 10 mM MgCl₂, 10 mM dithiothreitol, 0.33 mM CoA, 0.14 mM NADH, 2 units of malate dehydrogenase, 5 mM citrate, and 5 mM ATP. For kinetic analysis, a sample of the freshly thawed supernatant was preincubated with radicicol or biotinylated radicicol derivatives for 90 min at 4 °C. The reaction was started by the addition of 1 \( \mu \)l of the enzyme preparation to the reaction mixture, and the rate of NADH oxidation was measured at 340 nm with a Spectra Max spectrometer (Molecular Devices Co., Sunnyvale, CA). One unit of enzyme is defined as the amount of enzyme that oxidizes 1 \( \mu \)mol of NADH per min at 25 °C.

**RESULTS AND DISCUSSION**

Radicicol (Fig. 1, compound 1) is a macrocyclic antibiotic that contains an epoxide, a diene, and two phenolic hydroxy groups. To examine the role of these moieties in biological activity, we synthesized three novel radicicol analogs (compounds 2–4) and analyzed their activity to induce morphological reversion in \(v\)-\(src\)-transformed 3Y1 cells. Di(16-hydroxypalmitoyl) radicicol (compound 2) was as active as radicicol, whereas compound 3 lacking the diene structure showed about a half of the activity of radicicol. Reduction of the epoxide group of this analog (compound 4) fairly impaired the activity, suggesting that the epoxide is important for the activity of radicicol. On the basis of these observations, we introduced a biotin group into radicicol or these radicicol analogs. Radicicol was biotinylated at the C-17 (BR-1), C-9 (BR-5), or C-11 (BR-6) position. BR-2 and BR-3 were derivatives of compound 3 biotinylated at C-15 and C-11, respectively, whereas BR-4 was a derivative of compound 4 modified at C-11. Of these compounds, BR-1 and BR-6 were found to retain the activity of morphological reversion of \(src\)-transformed 3Y1 (Fig. 1), although they were less active than radicicol. The effective concentrations of BR-1, BR-6, and radicicol for the reversion of \(src\)-transformed cells were determined to be 1.35 \( \mu \)M, 13.5 \( \mu \)M, and 0.27 \( \mu \)M, respectively.

A HeLa cell extract was incubated with six biotinylated radicicol derivatives, and the bound proteins were precipitated with streptavidin beads and detected by Coomassie Brilliant Blue staining. As shown in Fig. 2, a 120-kDa protein was detected as a BR-1-binding protein, and a 90-kDa protein was detected as a BR-6-binding protein. On the other hand, no apparent proteins able to bind other biologically inactive derivatives (BR-2–BR-5) were detected.

Radicicol as well as benzoquinone ansamycin antibiotics, such as geldanamycin and herbimycin A, were shown to interact with Hsp90 (14, 15, 21). To test whether the 90-kDa protein bound to BR-6 is Hsp90, we transferred the proteins eluted from the beads onto a membrane and analyzed them by Western blotting using an anti-Hsp90 antibody. As shown in Fig. 3A, the 90-kDa protein bound to BR-6 was reactive with the...
anti-Hsp90 antibody, indicating the identity of the protein as Hsp90. To examine the specificity of this interaction, we tested whether radicicol can compete for the binding of BR-6 to Hsp90. Binding of BR-6 to Hsp90 was completely blocked by 10 μg/ml radicicol as well as 10 μg/ml herbijinyc A, one of the known inhibitors of Hsp90 (Fig. 3B). These results confirm the previous observation that radicicol binds Hsp90 in a manner similar to geldanamycin and herbijinyc A (14, 15).

The rabbit reticuocyte lysate abundantly contained the 120-kDa BR-1-binding protein (p120). The amount of p120 bound to BR-1 increased as the amount of total protein of the lysate was increased (Fig. 4A). We obtained a large amount of p120 from rabbit reticuocyte lysate by using the BR-1 affinity beads and then digested the preparation with lysyl endopeptidase and isolated a fragmented peptide by HPLC. The N-terminal amino acid sequence of the peptide was LVSSLTSGLTIGDGF-GALDAAK, which matched 100% with those (residues 920–944) of the human and rat ACL (22). To confirm immunologically the identity of p120 as ACL, we raised a polyclonal antibody against a peptide corresponding to the amino acid residues 1076–1095 in the C terminus of human ACL. The immunoblot analysis showed that p120 was ACL. Binding of ACL to BR-1 was specific because an excess of radicicol blocked this association (Fig. 4B). In contrast to the BR-6-Hsp90 interaction, however, herbijinyc A did not compete for this binding (data not shown).

To determine whether radicicol itself affects the enzyme activity of ACL, we assessed the effect of radicicol binding on the activity of ACL obtained from livers of rats starved for 48 h and fed with a high sucrose diet for 3 days. This diet resulted in about a 10-fold increase in the amount of the enzyme in the liver (23). We observed that BR-1 could also bind the 120-kDa protein in the rat liver extracts as well as in rabbit reticuocyte lysate in a radicicol-sensitive manner. There was no detectable enzyme activity in the BR-1-ACL complex (Fig. 5). These results imply that radicicol inhibits the ACL activity through binding. Consistent with this, BR-1 and radicicol could directly inhibit the enzyme activity, when added to the enzyme preparation (Fig. 6). On the other hand, BR-6, which does not bind ACL but does bind Hsp90, had no inhibitory effect on the ACL activity. The Lineweaver-Burk plot of 1/v versus 1/S (citrate−1) in the absence and presence of radicicol crossed at a fixed point on the 1/S axis, suggesting that radicicol did not affect the apparent Kₘ but decreased the Vₘₐₓ value. Thus, we conclude that radicicol acts as a noncompetitive-type inhibitor of ACL. The Vₘₐₓ values obtained with various concentrations of citrate and ATP were replotted for the determination of Kᵢ values. The Kᵢ values for citrate and ATP were 13 and 7 μM, respectively.

In this study, we showed that radicicol binds two different cellular proteins, Hsp90 and ACL. The Kᵢ values for ACL were higher than the effective concentration of radicicol to induce morphological changes in v-src-transformed cells, which may rule out the possibility that effects of radicicol on the morphological changes of transformed cells are caused by the inhibition of ACL. Although BR-1, which does not bind Hsp90, induced the reversal of morphology of v-src-transformed cells (Fig. 1), it is unclear whether BR-1 is stable in vivo, because the ester bond at C-17 could be cleaved in the cells, leading to the generation of free radicicol. Therefore, it seems probable that the activity of BR-1 to induce morphological reversion of src-transformed cells is because of radicicol released from BR-1. However, it is still possible that ACL inhibition is involved in other biological activities of radicicol.

We showed that BR-1 specifically binds ACL, whereas BR-6 does not. In contrast, BR-6 binds Hsp90, but BR-1 does not. These observations imply that a different part in radicicol is required for each specific binding and that unmodified radicicol can bind both proteins. It is likely that the introduction of the bulky biotin probe prevents these biotinylated compounds from access to one of these target proteins. In fact, recent crystal structure analysis demonstrated that radicicol acted as a nucleotide mimic and that the phenolic hydroxy group at C-17 of radicicol interacted with the water molecule tightly bound to Asp-79, Gly-83, and Thr-171 in the N-terminal ATP/ADP binding domain of Hsp90 (24). This water molecule forms a hydrogen bond to the adenine base of ATP/ADP in the absence of radicicol. Thus, the modification at C-17 of radicicol should impair the binding of radicicol to Hsp90. On the other hand, the carbonyl group at C-11 may be important for radicicol binding to ACL. Kinetic analysis suggests that radicicol does not compete with ATP for ACL binding, supporting the idea that radicicol binds and inhibits ACL in a different way. No competition of herbijinyc A for the binding of BR-1 to ACL also supports this idea. It is therefore possible to design a selective inhibitor of ACL by modifying radicicol at C-17. In the cytosolic compartment of mammalian cells, ACL generates acetyl-CoA by the ATP-driven conversion of citrate and CoA into oxaloacetate and acetyl-CoA. Because this is the first step for de novo synthesis of sterol and fatty acid, ACL is a potential target for hypolipidemic intervention (25). The high level expression of fatty acid synthase is widely observed in carcinoma of the colon, prostate, ovary, breast, and endometrium (26–28), and the growth of tumor cells with a high level of fatty acid synthase could be suppressed by inhibition of fatty acid synthase (29, 30). These observations suggest that ACL inhibitors bear potential as an anticancer agent by reducing the fatty acid synthesis. Thus, radicicol will be a novel lead compound for not only anti-Hsp90 drugs but also selective ACL inhibitors, which may be important for blocking both lipogenesis and oncogenesis.

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