0026-895X/06/6904-1158–1165
MOLECULAR PHARMACOLOGY
U.S. Government work not protected by U.S. copyright
Mol Pharmacol 69:1158–1165, 2006

Vol. 69, No. 4
18572/3098069
Printed in U.S.A.

2-Cyano-3,12-dioxooleana-1,9(11)-diene-28-oic Acid Disrupts Microtubule Polymerization: A Possible Mechanism Contributing to Apoptosis

Robin D. Couch, Neil J. Ganem, Ming Zhou, Veljko M. Popov, Tadashi Honda, Timothy D. Veenstra, Michael B. Sporn, and Amy C. Anderson

Department of Chemistry, Dartmouth College, Hanover, New Hampshire (R.D.C., V.M.P., T.H., A.C.A.); Departments of Biochemistry (N.J.G.) and Pharmacology and Toxicology (M.B.S.), Dartmouth Medical School, Hanover, New Hampshire; and Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, Maryland (M.Z., T.D.V.)

Received September 2, 2005; accepted January 6, 2006

ABSTRACT

The semisynthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) has several biological activities, including the induction of apoptosis in many cancer cell lines. To identify potential protein targets, immobilized biotinylated CDDO was used to screen the proteome of a human lymphoma cell line (U937) sensitive to CDDO-induced apoptosis. Tubulin was identified as one of several putative targets of CDDO. CDDO was shown to selectively bind to tubulin, with a dissociation constant of ~7 μM, and to disrupt microtubules both in vivo and in vitro. CDDO inhibits tubulin polymerization in vitro, possibly through interactions with a hydrophobic site on β-tubulin. The CDDO-tubulin interaction may also involve a reversible 1,4-addition with a protein sulfhydryl group. Unlike other known spindle poisons, CDDO does not result in a temporal increase in the mitotic index. Rather, CDDO seems to initiate apoptosis early in M phase.

Microtubules have long been recognized as the target for a large and chemically diverse group of chemotherapeutic agents (Cragg and Newman, 2004). Microtubules are dynamic protein polymers comprised of 12 to 13 protofilaments arranged to form a pipe-like structure (Downing, 2000). Protofilaments consist of α- and β-tubulin heterodimers combined in a head-to-tail arrangement. The GTP-dependent polymerization of microtubules occurs by a nucleation-elongation mechanism, in which a short microtubule nucleus is formed and then elongated by the noncovalent, reversible addition of tubulin heterodimers. Once formed, microtubules exist in a dynamic equilibrium, a feature that is crucial to their cellular function (Jordan, 2002). Because microtubules are intimately involved in the formation of the mitotic spindle, agents that interfere with microtubule dynamics (often called spindle poisons) typically inhibit cell cycle progression through mitosis and ultimately initiate apoptosis (Jordan et al., 1998). Spindle poisons are generally categorized into separate classes depending on their binding site; there are classes for agents that bind to tubulin at the colchicine site (e.g., colchicine, nocodazole), the vinca alkaloid site (e.g., vinblastine), the peptide site (e.g., cryptophycin 1), the paclitaxel (Taxol) site (e.g., epothilones), the tubulin sulfhydryl groups (e.g., stypoldione), or other/uncharacterized binding sites (e.g., lupeol) (Luduena and Roach, 1991; Jordan et al., 1998; Downing, 2000).

The semisynthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) was derived from oleanolic acid (Fig. 1) through iterative structure-activity relationship studies, employing an inducible nitric-oxide synthase (iNOS) assay (Honda et al. 1997, 1998, 2000a,b, 2002). CDDO at nanomolar concentrations suppresses the de novo synthesis of the inflammatory enzymes iNOS and cyclooxygenase-2 in activated macrophages (Suh et al., 1999). Additional studies with CDDO have revealed potent differentiating, antiproliferative, and apoptotic activity, suggesting the possibility of multiple targets (Wang et al., 2000). Micromolar concentrations of CDDO (ca. 1–10 μM) have been observed to induce apoptosis in several cancer cell lines, including myeloid (Ito

Financial support provided by the National Institutes of Health grant CA78814.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.018572.

ABBREVIATIONS: CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; iNOS, inducible nitric-oxide synthase; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid; DMSO, dimethyl sulfoxide; OD, optical density; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol.

et al., 2000; Stadheim et al., 2002) and lymphocytic leukemia cells (Pedersen et al., 2002; Inoue et al., 2004), osteosarcoma cells (Ito et al., 2001), nonsmall cell lung carcinoma cells (Kim et al., 2002), breast cancer cells (Lapillonne et al., 2003), and skin cancer cells (Hail et al., 2004). In general, the process of apoptosis can be divided into three phases (Kroemer et al., 1995): the induction phase (cells receive a stimulus that initiates programmed cell death), the effector phase (proteases and nucleases become systematically activated), and the degradation phase (numerous vital structures and functions are destroyed, ultimately leading to cytolysis). Several investigations have offered insights into the effector and degradation phases of CDDO-induced apoptosis (Ito et al., 2000, 2001; Pedersen et al., 2002; Stadheim et al., 2002; Lapillonne et al., 2003; Hail et al., 2004). However, a target protein of CDDO that could initiate the induction phase had not been previously identified.

In this report, we describe the identification and validation of β-tubulin as one target of CDDO; the inhibition of microtubule dynamics may contribute to the initiation of CDDO-induced programmed cell death. CDDO has been shown to exert its effects in vivo using immunofluorescent microscopy of osteosarcoma cells. In vitro, CDDO exhibits a dose-dependent effect on tubulin polymerization, more potent binding to microtubules than to soluble tubulin, and competitive binding with bis-ANS, a known microtubule poison that interacts with a hydrophobic site on tubulin.

Materials and Methods

Column Chromatography and Protein Identification. Chromatography resin was prepared by combining 0.3 mg of biotinylated CDDO (Honda et al., 2004) with 1 ml of streptavidin gel slurry (Pierce Biotechnology, Rockford, IL) and incubating for 1 h at 4°C. The resin was transferred to a chromatography column (13 mm i.d.), and unbound biotinylated CDDO was removed by washing three times with 1 ml of column buffer A [100 mM Tris, pH 7.5 and 1% Triton X-100 (all common reagents were obtained from Sigma-Aldrich, St. Louis, MO)]. Human lymphoma (U937) cells (Sundstrom and Nilsson, 1976) were lysed by resuspending 2.6 g of cells in 5 ml of lysis buffer [Cellytic M reagent (Sigma-Aldrich) supplemented with 1% Triton X-100, 100 units of DNase, 100 units of RNase, and 1 tablet of protease inhibitor cocktail (Roche, Indianapolis, IN)] and incubating for 1 h at 4°C. Differential centrifugation clarified the lysate, which was then passed through the chromatography column twice (all steps were performed at 4°C). The column was then washed with 10-column volumes of buffer A, 40-column volumes of 100 mM Tris, pH 7.5, and 10-column volumes of buffer A. Elution was accomplished with 6 × 1-ml aliquots of a 1.6 mg/ml CDDO solution in buffer A. Purified proteins were precipitated with 10% trichloroacetic acid, electrophoresed (8–16% SDS-PAGE; Bio-Rad, Hercules, CA), and visualized using a SilverQuest silver staining kit (Invitrogen, Carlsbad, CA). Protein bands were excised from the gel and analyzed by liquid chromatography-tandem mass spectrometry as described previously (Ory et al., 2003).

Secondary Screen. [^{14}C]CDDO (4.5 μM, 52.5 mCi/mmol; 0.21 μCi/μl, labeled in the cyano moiety, synthesized through the National Cancer Institute-Rapid Access to Intervention Development program) was incubated with either 5 μM tubulin (Cytoskeleton, Denver, CO), 5 μM bovine serum albumin (BSA), or water in general tubulin buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, and 0.5 mM EGTA) for 30 min at 25°C. Unbound [^{14}C]CDDO was removed by gel filtration (MicroSpin G50; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The amount of protein-bound radioactivity was quantified with 20 μl of each eluate in scintillation cocktail.

Indirect Immunofluorescent Microscopy. Human U2OS cells, treated with DMSO, CDDO (5–50 μM), or nocodazole (10 μM), were processed for immunofluorescence as described elsewhere (Ganem and Compton, 2004). Primary antibodies (tubulin antibody DM1α; Sigma-Aldrich) were detected using species-specific fluorescein-conjugated secondary antibodies (Vector Labs, Burlingame, CA), and DNA was detected with 0.2 μg/ml 4′-6-diamidine-2-phenyl indole (Sigma-Aldrich). Fluorescent images were captured with a Hamamatsu Orca II cooled charge-coupled device camera mounted on a Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Thornwood, NY). A series of 0.5-μm optical sections were collected in the *z* plane for each channel (4′-6-diamidine-2-phenyl indole and fluorescein) and deconvolved using Openlab software (Improvision Inc., Lexington, MA) to eliminate extraneous fluorescence background.

Mitotic Index. Osteosarcoma cells were treated with either 10 μM nocodazole, 5 μM CDDO, or DMSO alone, and mitotic indices were observed by phase contrast microscopy using a Nikon TE2000-E inverted light microscope equipped with a 40× objective (Nikon, Melville, NY). The mitotic index is reported as the percentage of mitotic cells per total cell number of cells.

Scintillation Proximity Assays. The dissociation constant was determined using 100-μl assays containing general tubulin buffer, 0.2 μM biotinylated tubulin (Cytoskeleton), and variable [^{14}C]CDDO concentrations. After a 15-min incubation at 37°C, 80 μg of streptavidin-coated yttrium beads (GE Healthcare) were added, and the mixture was further incubated for 50 min at 25°C. Tubes were then

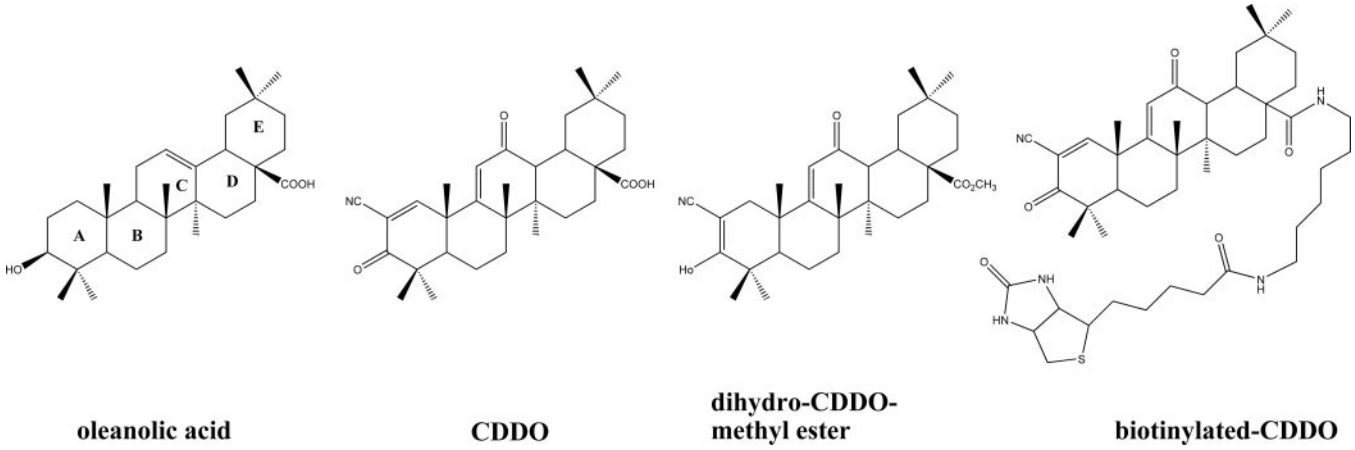

Fig. 1. Molecular structures of the natural product oleanolic acid, the semisynthetic derivatives CDDO and dihydo-CDDO-methyl ester, and the biotinylated CDDO derivative used to screen for protein targets. Rings A through E are identified in the structure of oleanolic acid.

centrifuged (16,100*g*, 2 min, 25°C) and quantified using a Tri-Carb scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). The dissociation constant was determined from nonlinear regression curve fitting using Prism software (ver. 4; GraphPad Software Inc., San Diego, CA).

Competitive binding studies were performed essentially as described above with the exception of preincubating the biotinylated tubulin with the indicated amount of competing ligand (1 h, 37°C) followed by the addition of 10 μM [^{14}C]CDDO. Competitive versus noncompetitive inhibition was graphically determined using variable concentrations of [^{14}C]CDDO with fixed concentrations of bis-ANS.

Tubulin Polymerization and Depolymerization Assays. Polymerization was monitored at 340 nm in a Shimadzu spectrophotometer using the Tubulin Polymerization Assay kit (Cytoskeleton) with a slightly modified protocol. In brief, 50 μl of tubulin (80 μM) was incubated in a microcentrifuge tube for 5 min on ice with the indicated concentration of CDDO, colchicine, vinblastine, or bis-ANS (each added in a 5-μl volume) and then was diluted to 40 μM with ice-cold general tubulin buffer. One-hundred-five microliters of the assay solution was then transferred to a prewarmed cuvette to begin the assay. Temperature was maintained at 37°C throughout the duration of polymerization, and the OD_{340} was measured every 4 min. A plot of percent tubulin polymerized (OD_{340} at 60 min) versus the log of CDDO concentration was used to determine the IC_{50} value. Microtubule depolymerization was evaluated by the addition of the indicated concentrations of CDDO at 60 min and following the OD_{340} thereafter.

Interactions of CDDO with Microtubules and Soluble Tubulin. Tubulin (40 μM) was polymerized for 1 h at 37°C in the presence of 0, 10, 33, or 55 μM [^{14}C]CDDO. Microtubules were collected by centrifugation (16,100*g*, 10 min, 25°C) and washed twice with 50 μl of 37°C general tubulin buffer, whereas soluble tubulin was transferred to a prechilled (4°C) MicroSpin G50 column to remove unbound [^{14}C]CDDO. The microtubule pellet was resuspended in 50 μl of 6 M urea. Protein concentrations were determined using Fluka's Protein Assay Reagent with γ-globulins (Sigma-Aldrich) as the standard. The amount of bound [^{14}C]CDDO was determined by liquid scintillation counting. This analysis was also performed with [^{14}C]CDDO added after the 1-h polymerization step. After an additional 30-min incubation (at 37°C), the assay was completed as described.

Screening for Covalent Binding of CDDO to Tubulin. Binding assays (55 μl total volume) containing general tubulin buffer, 5 μM tubulin, and 80 μM [^{14}C]CDDO were incubated for 2 h at 37°C. Unbound [^{14}C]CDDO was removed using a MicroSpin G50 column. Ten microliters of eluate was combined with either SDS-PAGE loading buffer containing DTT (+DTT) or without DTT (−DTT). The samples were electrophoresed using a 4 to 12% SDS-PAGE gel (Invitrogen). Control samples, processed as outlined above but without the addition of [^{14}C]CDDO, were visualized by Coomassie staining. Gels with radiolabeled protein were placed in fixing solution [isopropanol/water/acetic acid (25:65:10)] for 30 min at 25°C, soaked in Amplify reagent (GE Healthcare) for 30 min (at 25°C), and then vacuum-dried (at 80°C) and visualized by autoradiography.

Binding of CDDO after Preincubation with DTNB. Tubulin (5 μM) was combined with either 227 μM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich) or DMSO (control), incubated for 1 h at 37°C, and then passed through a MicroSpin G50 column to isolate the protein. Protein concentrations were determined as described, and 5 μM was mixed with 55 μM [^{14}C]CDDO and allowed to incubate for 1 h at 37°C. Unbound [^{14}C]CDDO was removed by gel filtration. Protein concentration and amount of bound radioligand were determined as described.

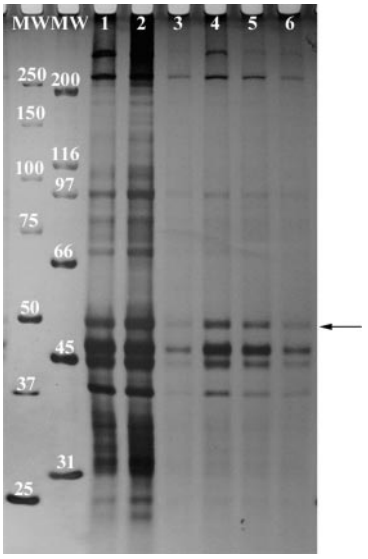

Fig. 2. Silver-stained protein gel showing the eluates obtained from affinity chromatography with immobilized biotinylated CDDO. CDDO was used as the eluant. Lanes 1 through 6 contain elution fractions 1 through 6, respectively. Lanes labeled MW contain molecular mass markers, and the corresponding molecular mass (in kilodaltons) are indicated. The arrow identifies the protein band containing tubulin.

Results

Protein Targets of CDDO. To search for a protein target of CDDO, immobilized biotinylated CDDO was used to screen

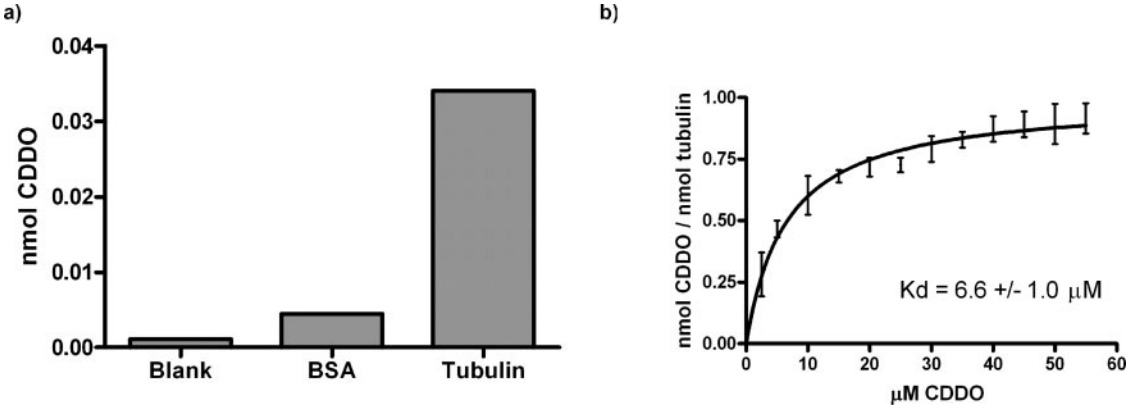

Fig. 3. CDDO selectively binds tubulin. a, radiolabeled CDDO (4.5 μM) was incubated either alone (blank), with 5 μM BSA, or with 5 μM tubulin. After removal of unbound [^{14}C]CDDO by gel filtration, the amount of protein-associated radioactivity was determined. b, a binding isotherm with tubulin and [^{14}C]CDDO yields a dissociation constant of ~7 μM.

the proteome of a human lymphoma cell line (U937) sensitive to CDDO-induced apoptosis [IC_{50} = 5 μM (Ikeda et al., 2003)]. The biotinylated CDDO construct (TP301; Fig. 1) was immobilized on streptavidin resin and used in affinity column chromatography. After extensive washing to disrupt nonspecific interactions, selective elution was performed with CDDO as the eluant. As seen in the silver-stained polyacrylamide gel shown in Fig. 2, several abundant protein bands were obtained, as might be expected with a pleiotropic agent. Liquid chromatography-tandem mass spectrometry analysis of these protein bands identified the individual protein constituents, and based upon peptide occurrence and a rational evaluation of the identified proteins, tubulin was selected as a likely protein target of CDDO that could contribute to the initiation of the induction phase of apoptosis. The remaining proteins will be validated and discussed in subsequent communications.

A Secondary Screen. To confirm the specificity of the CDDO-tubulin interaction, a secondary screen was performed by evaluating the direct binding of CDDO to purified tubulin. Radiolabeled CDDO was incubated with either BSA or tubulin, and after unbound [^{14}C]CDDO was removed by gel filtration, scintillation counting confirmed that CDDO selectively binds to tubulin (Fig. 3a). Furthermore, a binding isotherm with [^{14}C]CDDO and purified tubulin produced a dissociation constant of ~7 μM (Fig. 3b), which is comparable with the IC_{50} value for CDDO-induced apoptosis of U937 cells (5 μM). Thus, tubulin seemed to be a highly probable target of CDDO.

Indirect Immunofluorescent Microscopy. To evaluate whether CDDO would disrupt microtubules in vivo, osteosarcoma cells were treated with DMSO, CDDO, or nocodazole (a known spindle poison), and microtubules were examined at various time points by indirect immunofluorescent microscopy. The IC_{50} value for CDDO-induced apoptosis of osteosarcoma cells is ~6 μM (Ito et al., 2001). As expected, cells treated with DMSO (the solvent for nocodazole and CDDO) for either 1 h (Fig. 4a) or 24 h (not shown) exhibit normal microtubules. In contrast, a 1-h treatment of cells with 10 μM nocodazole results in the near complete depolymerization of microtubules (Fig. 4b). Similar in appearance to DMSO-

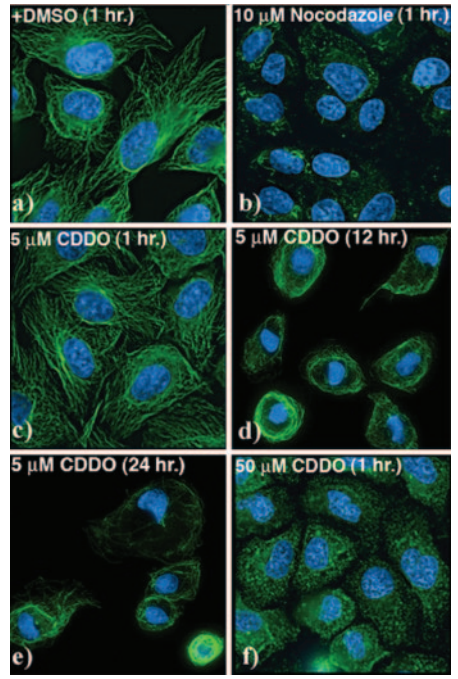

Fig. 4. CDDO disrupts microtubules in vivo. Human U2OS cells were treated with either DMSO (a), 10 μM nocodazole (b), 5 μM CDDO (c–e), or 50 μM CDDO (f) for the indicated time and then processed and examined by indirect immunofluorescent microscopy. Microtubules are colored green. DNA is colored blue.

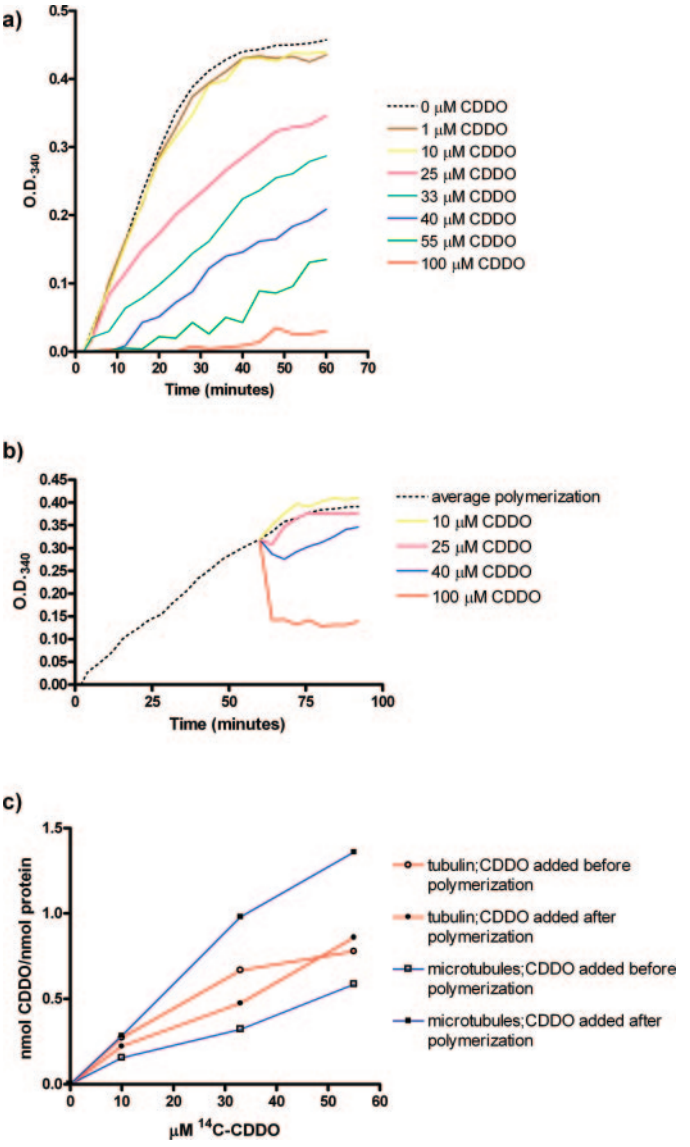

Fig. 5. CDDO interactions with tubulin and microtubules. a, the polymerization of 40 μM tubulin (in 1 mM GTP, 1 mM $MgCl_2$, 250 μM EGTA, and 40 mM PIPES, pH 6.9) was monitored spectrophotometrically at 340 nm in the presence of various concentrations of CDDO (0–100 μM). Polymerization was initiated by elevating the temperature of the assay mixture from 4–37°C, and the extent of polymerization was recorded at 4-min time intervals over the course of 1 h. A plot of percent tubulin polymerized (OD_{340} at 60 min) versus the log of CDDO concentration was used to calculate an IC_{50} value of 31 μM. b, the dose-dependent depolymerization of microtubules by CDDO. After 60 min of tubulin polymerization, CDDO was added at the indicated concentrations. For clarity, the average polymerization (OD_{340} value) is presented for the first 60 min of the assay. The average polymerization trace thereafter represents a control with 0 μM CDDO added at 60 min. c, radiolabeled CDDO was added either before or after a tubulin polymerization reaction, and the amount of radioactivity associated with unincorporated tubulin or microtubules was determined.

treated cells, the majority of cells treated for 1 h with 5 μM CDDO seemed to have unaffected microtubules (Fig. 4c). However, with prolonged incubation, microtubule depolymerization becomes apparent (Fig. 4, d and e). At higher concentrations of CDDO, rapid microtubule disruption is observed (Fig. 4f). Collectively, these results illustrate that CDDO does indeed influence microtubules in vivo, further supporting the possibility of tubulin as a target of CDDO. However, CDDO seems less potent than nocodazole, which may simply reflect the greater affinity of tubulin for nocodazole [K_d = 0.3–1.5 μM (Tahir et al., 2000; Xu et al., 2002)]. Alternatively, the relative potencies could be a consequence of the cellular permeability of each molecule (note that nocodazole is uncharged, whereas CDDO is anionic at physiological pH). Regardless, these results motivated further investigations to probe the nature of the CDDO-tubulin interaction.

The CDDO-Tubulin/Microtubule Interaction. Because CDDO caused the disruption of microtubules in vivo, we set out to evaluate its effects on microtubules in vitro. Cuvettes containing 4 mg/ml tubulin, 1 mM GTP, 1 mM $MgCl_2$, 250 μM EGTA, 40 mM PIPES, pH 6.9, and variable concentrations of CDDO (0–100 μM) were placed into a spectrophotometer and the polymerization of tubulin was monitored at 340 nm. Polymerization was initiated by elevating the temperature of the assay mixture from 4–37°C, and the extent of polymerization was recorded at 4-min time intervals over the course of 1 h. The dose-dependent inhibition of tubulin polymerization by CDDO is illustrated in Fig. 5a. When assayed with 40 μM tubulin, CDDO inhibits microtubule formation, with an IC_{50} value of 30 μM. To evaluate the relative efficacy of CDDO, the same assay was performed with the potent microtubule inhibitors colchicine (IC_{50} = 1.4 μM), vinblastine (IC_{50} = 1.2 μM), and bis-ANS (IC_{50} = 20 μM) (data not shown). Not surprisingly, the relative efficacies seem directly proportional to the affinity of tubulin for each of these inhibitors: CDDO, K_d = 7 μM; bis-ANS, K_d = 2 μM (Horowitz et al., 1984); colchicine, K_d = 0.3 to 1.4 μM (Williams et al., 1983; Tahir et al., 2000); and vinblastine, K_d = 0.5 μM (Safa et al., 1987).

Because CDDO was observed to depolymerize microtubules in vivo, we also tested the effects of adding CDDO to preformed microtubules in vitro. As seen in Fig. 5b, CDDO is capable of depolymerizing microtubules in a dose-dependent fashion. This observation raised the possibility that CDDO might be capable of directly binding to microtubules. To explore this possibility, radiolabeled CDDO was added to tubulin polymerization reactions either before the reactions were initiated or after they were complete. Examination of [^{14}C]CDDO-bound soluble-tubulin (i.e., tubulin that is not incorporated into microtubules) illustrates little difference in binding whether CDDO was added before or after microtubules were formed (Fig. 5c). On the other hand, examination of the protein polymer suggests that CDDO is indeed capable of directly binding to microtubules, and at elevated concentrations of CDDO, over 2-fold greater binding is observed when [^{14}C]CDDO is added to preformed microtubules. In fact, with 55 μM [^{14}C]CDDO, the molar ratio of CDDO to protein suggests that CDDO binds to multiple sites on tubulin in microtubules. Because tubulin hydrolyzes GTP to GDP when polymerized into microtubules, we tested the possibility of GTP hydrolysis influencing the binding of CDDO to tubulin. However, CDDO did not preferentially bind to either GTP- or GDP-tubulin (data not shown). Thus, although CDDO seems capable of binding to preformed microtubules and may do so at multiple sites on tubulin, the specific details of this binding remain unclear.

The CDDO Binding Site. To localize the CDDO binding site on the tubulin heterodimer, a competitive scintillation proximity assay was performed in the presence of vinblastine, colchicine, or bis-ANS. As seen in Fig. 6a, bis-ANS inhibits [^{14}C]CDDO binding to tubulin. A double reciprocal plot of CDDO binding in the absence and presence of 50 μM bis-ANS (Fig. 6b) indicates that bis-ANS is a competitive inhibitor of CDDO, implying that these two molecules share the same binding site. Because the bis-ANS binding site on tubulin is thought to be hydrophobic (Jordan et al., 1998), the CDDO-tubulin interaction probably also involves hydrophobic interactions.

The Role of Sulfhydryls. The structure of CDDO contains two electrophilic α,β-unsaturated carbonyl moieties, one in ring A and one in ring C (Fig. 1), raising the possibility that the CDDO-tubulin interaction may involve a conjugate nucleophilic addition. We have recently reported that molecules containing sulfhydryl groups, such as DTT or glutathione, can form reversible adducts with CDDO through pref-

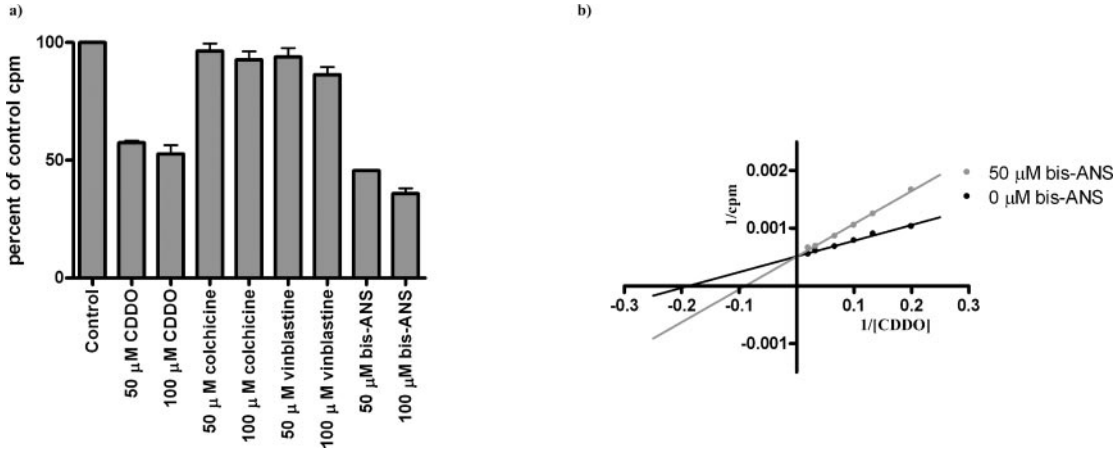

Fig. 6. Competitive binding to tubulin. After the preincubation of tubulin with either unlabeled CDDO, colchicine, vinblastine, or bis-ANS (at the indicated concentrations), tubulin was incubated with 10 μM radiolabeled CDDO, and the amount of [^{14}C]CDDO bound to tubulin was determined and compared with an uninhibited binding control (100% = 0.55 ± 0.05 nmol CDDO/nmol tubulin).

erential addition to the ring A enone moiety (Couch et al., 2005). Although the A-ring enone was found to be highly reactive toward nucleophilic additions, spectroscopic analysis revealed that the adducts were quite unstable and would quickly undergo elimination by ejection of the nucleophile. Specifically, NMR experiments intended to monitor the proton in ring A showed that simply elevating the temperature to 37°C caused the covalent thiol adduct to disintegrate. In addition, UV-visible spectroscopy experiments showed that the covalent adduct disintegrated over 30 min. In light of these observations, we first set out to evaluate if the CDDO-tubulin interaction might involve a sulfhydryl group from tubulin. As shown in Fig. 7a, the binding of [^{14}C]CDDO to DTNB-pretreated tubulin was significantly reduced relative to tubulin not pretreated with DTNB, supporting the possibility of the involvement of a sulfhydryl group in the CDDO-tubulin interaction. Then, to investigate if the ring A enone contributes to this interaction, a competitive scintillation proximity assay was performed with CDDO and its reduced analog, dihydro-CDDO-methyl ester (Fig. 1). As shown in Fig. 7b, the binding of dihydro-CDDO-methyl ester is reduced relative to that of CDDO (i.e., dihydro-CDDO-methyl ester is a poorer inhibitor of [^{14}C]CDDO binding than is CDDO), supporting the possibility that the ring A enone contributes to the CDDO-tubulin interaction. Together, the DTNB and dihydro-CDDO-methyl ester experiments imply the possibility of a conjugate addition between a sulfhydryl and the ring A enone. And finally, to determine whether the CDDO-tubulin interaction is irreversible (i.e., a nucleophilic addition results in a stable covalent bond), tubulin was incubated with [^{14}C]CDDO and then electrophoresed and visualized by autoradiography. As shown in Fig. 7c, radioactivity is not associated with protein electrophoresed in either the presence or absence of DTT (which could potentially disrupt the CDDO-tubulin adduct). Thus, it seems that CDDO interacts with a hydrophobic site on tubulin, and it may involve a 1,4-addition via a sulfhydryl group, but this interaction is probably reversible. Additional experiments will be necessary to further elucidate the details of the interaction.

CDDO and the Mitotic Index. Collectively, the aforementioned results clearly indicate an interaction between CDDO and tubulin and support the hypothesis that this interaction contributes to CDDO-induced apoptosis. Spindle poisons typically function by causing a cell cycle block at M phase, which eventually initiates apoptosis (Jordan and Wilson, 1998). To evaluate whether CDDO behaves as a typical spindle poison, the effects of CDDO on the mitotic index was determined. As shown in Table 1, whereas treatment with nocodazole results in the temporal accumulation of M phase osteosarcoma cells, the mitotic index of CDDO-treated cells is observed to decline over time. Interestingly, the number of apoptotic cells that appear in the CDDO-treated culture closely parallels the number of M phase cells observed in the nocodazole-treated sample. Thus, in contrast to a typical spindle poison, it seems that CDDO rapidly induces apoptosis in treated cells. Hence, the combination of CDDO binding to tubulin and to an additional protein target may collectively contribute to the mechanism of CDDO-induced apoptosis.

TABLE 1
The effects of CDDO on the mitotic index
The mitotic index was determined at several time points after exposure of osteosarcoma cells to either DMSO, 10 μM nocodazole, or 5 μM CDDO. Typical of a spindle poison, nocodazole increases the mitotic index by blocking cell progression in mitosis. In contrast, CDDO seems to diminish the number of mitotic cells.

	0 h	1 h	3 h	12 h
		%		
DMSO	3	3	3	3
Nocodazole	3	3.5	4.5	43
CDDO	3	2.4	1	N.D.

N.D., not detected.

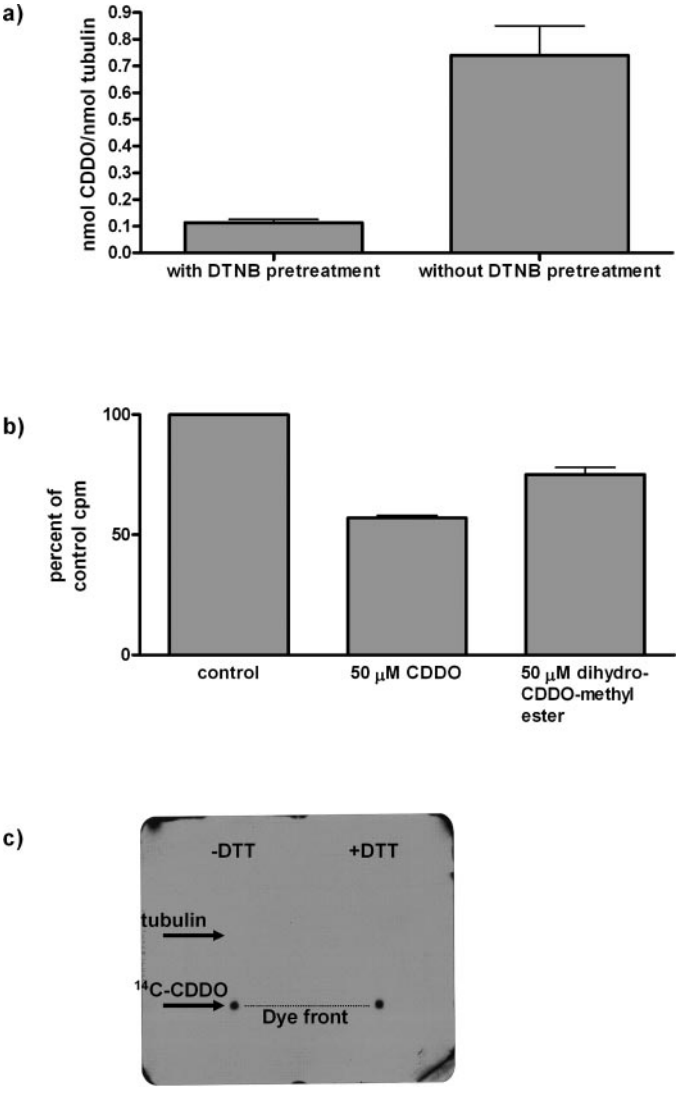

Fig. 7. The CDDO-tubulin interaction might involve a reversible 1,4 addition via a tubulin sulfhydryl group and the ring A enone. a, preincubation of tubulin (5 μM) with DTNB (227 μM) inhibits [^{14}C]CDDO (55 μM) binding, suggesting a role for the tubulin sulfhydryl groups in the CDDO-tubulin interaction. b, in a competitive scintillation proximity assay, dihydro-CDDO-methyl ester is a poorer inhibitor of [^{14}C]CDDO (10 μM) binding than is CDDO, suggesting a role for the ring A enone in the CDDO-tubulin interaction. c, an SDS-PAGE autoradiographic analysis of the CDDO-tubulin complex indicates that CDDO is not covalently bound to tubulin. Tubulin (5 μM) was incubated with 80 μM [^{14}C]CDDO for 2 h at 37°C. Unbound radiolabeled CDDO was removed by gel filtration, and the purified protein was electrophoresed and examined by autoradiography. −DTT and +DTT specifies that the protein was electrophoresed in the absence or presence of DTT, respectively. The location of tubulin (as determined by Coomassie staining) and [^{14}C]CDDO are indicated with arrows. The electrophoretic dye front is also identified.

Discussion

The semisynthetic triterpenoid CDDO is pleiotropic. CDDO suppresses the de novo synthesis of the inflammatory enzymes iNOS and cyclooxygenase-2 in activated macrophages, and because these enzymes have been implicated as possible enhancers of carcinogenesis (Hussain et al., 2003), CDDO has potential to be used as a chemopreventive agent. Furthermore, CDDO may also serve as a chemotherapeutic agent, because it has been shown to effectively induce differentiation of human myeloid leukemia cells (Suh et al., 1999), inhibit the proliferation of various human tumor cell types (Suh et al., 1999; Lapillonne et al., 2003), and induce apoptosis in several human cancer cell lines, including cell lines resistant to other chemotherapies. Affinity column chromatography, using immobilized CDDO and a human lymphoma cell line sensitive to CDDO-induced apoptosis, identified tubulin as a protein target (Fig. 2). Competitive binding studies suggest that CDDO is likely to bind to β-tubulin using a combination of hydrophobic interactions and a reversible 1,4-addition with a protein sulfhydryl group (Figs. 6 and 7). Thus, like many other sulfhydryl-binding compounds known to inhibit tubulin polymerization (Kuriyama and Sakai, 1974; Mellon and Rebhun, 1976; Deinum et al., 1981; Lee et al., 1981; Luduena and Roach, 1981; Roach and Luduena, 1984; Bai et al., 1989; Li and Broome, 1999; Shan et al., 1999; Combeau et al., 2000), CDDO may function in part by disrupting the regulatory role of a sulfhydryl group in the formation of the mitotic spindle (Jordan et al., 1998). Furthermore, because the binding site of CDDO on β-tubulin was localized to a region also bound by bis-ANS, which is thought to reside in a flexible area distal from the $\alpha\beta$-interface (Ward et al., 1994), CDDO may also inhibit tubulin polymerization in a manner analogous to bis-ANS (Mazumdar et al., 1992). Interestingly, three cysteine residues have been suggested to reside within the bis-ANS binding site on β-tubulin (Roychowdhury et al., 2000). The fact that CDDO does not seem to arrest the cell cycle at M phase (Table 1) could indicate that other protein targets of CDDO may be acting synergistically with tubulin to cause a rapid apoptotic event. Interestingly, this multitarget mode of action does not seem unique to CDDO, because the marine natural product stypoldione is thought to induce apoptosis by interacting with tubulin and other protein targets (O'Brien et al., 1983). Multiple targets have also been implicated in the spindle poisons lupeol (Jordan et al., 1998), estramustine (Speicher et al., 1994), and quercetin (Gupta and Panda, 2002).

Acknowledgments

We thank Charlotte Williams, Renee Risingsong, and Darlene Royce for culturing the U937 cells. Special thanks go to Duane Compton for access to the immunofluorescent microscope. We also thank Ashley Davis for fruitful discussions.

References

Bai R, Duanmu C, and Hamel E (1989) Mechanism of action of the antimitotic drug 2,4-dichlorobenzyl thiocyanate: alkylation of sulfhydryl group(s) of beta-tubulin. *Biochim Biophys Acta* **994:**12–20.

Combeau C, Provost J, Lancelin F, Tournoux Y, Prod'homme F, Herman F, Lavelle F, Leboul J, and Vuilhorgne M (2000) RPR112378 and RPR115781: two representatives of a new family of microtubule assembly inhibitors. *Mol Pharmacol* **57:** 553–563.

Couch RD, Browning RG, Honda T, Gribble GW, Wright DL, Sporn MB, and Anderson AC (2005) Studies on the reactivity of CDDO, a promising new chemopreventive and chemotherapeutic agent: implications for a molecular mechanism of action. *Bioorg Med Chem Lett* **15:**2215–2219.

Cragg GM and Newman DJ (2004) A tale of two tumor targets: topoisomerase I and tubulin. The Wall and Wani contribution to cancer chemotherapy. *J Nat Prod* **67:**232–244.

Deinum J, Wallin M, and Lagercrantz C (1981) Spatial separation of the two essential thiol groups and the binding site of the exchangeable GTP in brain tubulin. A spin label study. *Biochim Biophys Acta* **671:**1–8.

Downing KH (2000) Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. *Annu Rev Cell Dev Biol* **16:**89–111.

Ganem NJ and Compton DA (2004) The KinI kinesin Kif2a is required for bipolar spindle assembly through a functional relationship with MCAK. *J Cell Biol* **166:** 473–478.

Gupta K and Panda D (2002) Perturbation of microtubule polymerization by quercetin through tubulin binding: a novel mechanism of its antiproliferative activity. *Biochemistry* **41:**13029–13038.

Hail N Jr, Konopleva M, Sporn M, Lotan R, and Andreeff M (2004) Evidence supporting a role for calcium in apoptosis induction by the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO). *J Biol Chem* **279:**11179–11187.

Honda T, Finlay HJ, Gribble GW, Suh N, and Sporn MB (1997) New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* **7:**1623–1628.

Honda T, Gribble GW, Suh N, Finlay HJ, Rounds BV, Bore L, Favaloro FG Jr, Wang Y, and Sporn MB (2000a) Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages. *J Med Chem* **43:**1866–1877.

Honda T, Honda Y, Favaloro FG Jr, Gribble GW, Suh N, Place AE, Rendi MH, and Sporn MB (2002) A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. *Bioorg Med Chem Lett* **12:**1027–1030.

Honda T, Janosik T, Honda Y, Han J, Liby KT, Williams CR, Couch RD, Anderson AC, Sporn MB, and Gribble GW (2004) Design, synthesis and biological evaluation of biotin conjugates of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid for the isolation of the protein targets. *J Med Chem* **47:**4923–4932.

Honda T, Rounds BV, Bore L, Finlay HJ, Favaloro FG Jr, Suh N, Wang Y, Sporn MB, and Gribble GW (2000b) Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *J Med Chem* **43:**4233–4246.

Honda T, Rounds BV, Gribble GW, Suh N, Wang Y, and Sporn MB (1998) Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg Med Chem Lett* **8:**2711–2714.

Horowitz P, Prasad V, and Luduena RF (1984) Bis(1,8-anilinonaphthalenesulfonate). A novel and potent inhibitor of microtubule assembly. *J Biol Chem* **259:** 14647–14650.

Hussain SP, Hofseth LJ, and Harris CC (2003) Radical causes of cancer. *Nat Rev Cancer* **3:**276–285.

Ikeda T, Sporn M, Honda T, Gribble GW, and Kufe D (2003) The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance. *Cancer Res* **63:**5551–5558.

Inoue S, Snowden RT, Dyer MJ, and Cohen GM (2004) CDDO induces apoptosis via the intrinsic pathway in lymphoid cells. *Leukemia* **18:**948–952.

Ito Y, Pandey P, Place A, Sporn MB, Gribble GW, Honda T, Kharbanda S, and Kufe D (2000) The novel triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid induces apoptosis of human myeloid leukemia cells by a caspase-8-dependent mechanism. *Cell Growth Differ* **11:**261–267.

Ito Y, Pandey P, Sporn MB, Datta R, Kharbanda S, and Kufe D (2001) The novel triterpenoid CDDO induces apoptosis and differentiation of human osteosarcoma cells by a caspase-8 dependent mechanism. *Mol Pharmacol* **59:**1094–1099.

Jordan A, Hadfield JA, Lawrence NJ, and McGown AT (1998) Tubulin as a target for anticancer drugs: agents which interact with the mitotic spindle. *Med Res Rev* **18:**259–296.

Jordan MA (2002) Mechanism of action of antitumor drugs that interact with microtubules and tubulin. *Curr Med Chem Anticancer Agents* **2:**1–17.

Jordan MA and Wilson L (1998) Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr Opin Cell Biol* **10:**123–130.

Kim KB, Lotan R, Yue P, Sporn MB, Suh N, Gribble GW, Honda T, Wu GS, Hong WK, and Sun SY (2002) Identification of a novel synthetic triterpenoid, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells. *Mol Cancer Ther* **1:**177–184.

Kroemer G, Petit P, Zamzami N, Vayssiere JL, and Mignotte B (1995) The biochemistry of programmed cell death. *FASEB J* **9:**1277–1287.

Kuriyama R and Sakai H (1974) Role of tubulin-SH groups in polymerization to microtubules. Functional-SH groups in tubulin for polymerization. *J Biochem (Tokyo)* **76:**651–654.

Lapillonne H, Konopleva M, Tsao T, Gold D, McQueen T, Sutherland RL, Madden T, and Andreeff M (2003) Activation of peroxisome proliferator-activated receptor gamma by a novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. *Cancer Res* **63:** 5926–5939.

Lee YC, Yaple RA, Baldridge R, Kirsch M, and Himes RH (1981) Inhibition of tubulin self-assembly in vitro by fluorodinitrobenzene. *Biochim Biophys Acta* **671:**71–77.

Li YM and Broome JD (1999) Arsenic targets tubulins to induce apoptosis in myeloid leukemia cells. *Cancer Res* **59:**776–780.

Luduena RF and Roach MC (1981) Interaction of tubulin with drugs and alkylating agents. 1. Alkylation of tubulin by iodo[^{14}C]acetamide and *N,N'*-ethylenebis(iodoacetamide). *Biochemistry* **20:**4437–4444.

Luduena RF and Roach MC (1991) Tubulin sulfhydryl groups as probes and targets for antimitotic and antimicrotubule agents. *Pharmacol Ther* **49:**133–152.

Mazumdar M, Parrack PK, Mukhopadhyay K, and Bhattacharyya B (1992) Bis-ANS

as a specific inhibitor for microtubule-associated protein induced assembly of tubulin. *Biochemistry* **31:**6470–6474.
Mellon MG and Rebhun LI (1976) Sulfhydryls and the in vitro polymerization of tubulin. *J Cell Biol* **70:**226–238.
O'Brien ET, Jacobs RS, and Wilson L (1983) Inhibition of bovine brain microtubule assembly in vitro by stypoldione. *Mol Pharmacol* **24:**493–499.
Ory S, Zhou M, Conrads TP, Veenstra TD, and Morrison DK (2003) Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr Biol* **13:**1356–1364.
Pedersen IM, Kitada S, Schimmer A, Kim Y, Zapata JM, Charboneau L, Rassenti L, Andreeff M, Bennett F, Sporn MB, et al. (2002) The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood* **100:**2965–2972.
Roach MC and Luduena RF (1984) Different effects of tubulin ligands on the intrachain cross-linking of beta 1-tubulin. *J Biol Chem* **259:**12063–12071.
Roychowdhury M, Sarkar N, Manna T, Bhattacharyya S, Sarkar T, Basusarkar P, Roy S, and Bhattacharyya B (2000) Sulfhydryls of tubulin. A probe to detect conformational changes of tubulin. *Eur J Biochem* **267:**3469–3476.
Safa AR, Hamel E, and Felsted RL (1987) Photoaffinity labeling of tubulin subunits with a photoactive analogue of vinblastine. *Biochemistry* **26:**97–102.
Shan B, Medina JC, Santha E, Frankmoelle WP, Chou TC, Learned RM, Narbut MR, Stott D, Wu P, Jaen JC, et al. (1999) Selective, covalent modification of beta-tubulin residue Cys-239 by T138067, an antitumor agent with in vivo efficacy against multidrug-resistant tumors. *Proc Natl Acad Sci USA* **96:**5686–5691.
Speicher LA, Laing N, Barone LR, Robbins JD, Seamon KB, and Tew KD (1994) Interaction of an estramustine photoaffinity analogue with cytoskeletal proteins in prostate carcinoma cells. *Mol Pharmacol* **46:**866–872.
Stadheim TA, Suh N, Ganju N, Sporn MB, and Eastman A (2002) The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) potently enhances apoptosis induced by tumor necrosis factor in human leukemia cells. *J Biol Chem* **277:**16448–16455.
Suh N, Wang Y, Honda T, Gribble GW, Dmitrovsky E, Hickey WF, Maue RA, Place AE, Porter DM, Spinella MJ, et al. (1999) A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative and anti-inflammatory activity. *Cancer Res* **59:**336–341.
Sundstrom C and Nilsson K (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* **17:**565–577.
Tahir SK, Kovar P, Rosenberg SH, and Ng SC (2000) Rapid colchicine competition-binding scintillation proximity assay using biotin-labeled tubulin. *Biotechniques* **29:**156–160.
Wang Y, Porter WW, Suh N, Honda T, Gribble GW, Leesnitzer LM, Plunket KD, Mangelsdorf DJ, Blanchard SG, Willson TM, et al. (2000) A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma. *Mol Endocrinol* **14:**1550–1556.
Ward LD, Seckler R, and Timasheff SN (1994) Energy transfer studies of the distances between the colchicine, ruthenium red, and bisANS binding sites on calf brain tubulin. *Biochemistry* **33:**11900–11908.
Williams RF, Aivaliotis MJ, Barnes LD, and Robinson AK (1983) High-performance liquid chromatographic application of the Hummel and Dreyer method for the determination of colchicine-tubulin binding parameters. *J Chromatogr* **266:**141–150.
Xu K, Schwarz P, and Luduena RF (2002) Interaction of nocodazole with tubulin isotypes. *Drug Dev Res* **55:**91–96.

Address correspondence to: Amy C. Anderson, Department of Medicinal Chemistry, School of Pharmacy, University of Connecticut, 69 North Eagleville Rd., Storrs, CT 06269-3092. E-mail: amy.anderson@uconn.edu