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Award Number: DAMD17-99-1-9100

TITLE: Mechanism of Action of Substituted Indanones in Multidrug Resistant Breast Cancer

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REPORT DATE: October 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
Distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual Summary (30 Sep 99 - 31 Aug 00)

4. TITLE AND SUBTITLE
Mechanism of Action of Substituted Indanones in Multidrug Resistant Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9100

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color graphics.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES 18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

Mechanism of Action of Substituted Indanones in Multidrug Resistant Breast Cancer

Our laboratory has recently synthesized a series of novel substituted indanones that are selectively toxic to multidrug resistant cancer cells, including breast cancer cell lines. In this application we proposed to characterize the mechanism of action of indanocine and to assess the in vivo anti-tumor activity of indanocine.
During the first year we:

- published the first report on the biological activity of indanocine (J Natl Cancer Inst 2000, 92:217-224)
- generated an indanocine-resistant stable cell line
- synthesized a water-soluble analog of indanocine (indanocine-phosphate)
- tested the anti-angiogenic activity of indanocine in *in vivo* animal models
- demonstrated the selective activity of indanocine against primary chronic lymphocytic leukemia cells

The results shown in this annual summary demonstrate that indanocine is a very promising new anti-cancer agent that we hope will be soon tested in clinical trials.

Body

Task 1

In the first aim of our application, we proposed to characterize further the mechanism of action and the molecular targets of indanocine and related compounds in multidrug resistant breast cancer cell lines. Specifically, we planned to:

1. Develop indanocine resistant breast cancer cell line mutant and assess its pattern of cross resistance to other agents (months 1-24)
2. Characterize biochemically the resistant phenotype, emphasizing cytoskeletal and apoptotic regulatory proteins (months 7-18)
3. Investigate differentially expressed genes in the resistant mutants by human cDNA expression chips (months 12-24)
4. Test the role of the differentially expressed genes by transfection in the wild type cell lines (months 24-32)

Point #1

Establishment of an indanocine resistant cell line

We obtained an indanocine-resistant clone (CEM-178) derived from the human T lymphoblastoid cell line CEM. The CEM-178 cells can proliferate in presence of up to 300nM of indanocine. The resistant phenotype persisted when the mutant cells were cultured in drug-free medium for at least 9 months. The doubling time of the CEM-178 was 24 hours as compared to 18 hours of the parental cells. Major morphological alterations, for example, size or shape, were not observed. Quantitative analysis of DNA content by flow cytometry did not reveal any change. Karyotyping also showed no difference between CEM and CEM-178 (data not shown).

The drug sensitivity of parental WT CEM and indanocine-resistant clone CEM-178 is shown in Table 1.

Table 1: Profile of chemosensitivity of the indanocine-resistant cells CEM-178

Drugs	CEM IC_{50}	CEM-178 IC50	Fold
indanocine	1.79 nM	206 nM	115
paclitaxel	0.5 nM	0.5 nM	1
colchicine	2.0 nM	62.5 nM	31
vinblastine	0.1 nM	4.0 nM	40
fludarabine	5.8 μM	13.3 μM	2.3
doxorubicin	0.2 μM	0.4 nM	1.9
cytochalasin B	7.1 μM	4.6 μM	0.6

The CEM-178 cells displayed cross-resistance to other tubulin-depolymerizing drugs, such as vinblastine (40 fold) and colchicine (31 fold) but not to the tubulin-polymerizing agent paclitaxel. No cross-resistance to other anti-tumor drugs like fludarabine, doxorubicin and cytochalasin B was observed.

Point #2

Biochemical characterization of the resistant cells

We performed an extensive biochemical characterization of the CEM-178 cells, by measuring the expression of the several proteins involved in the apoptotic regulation.

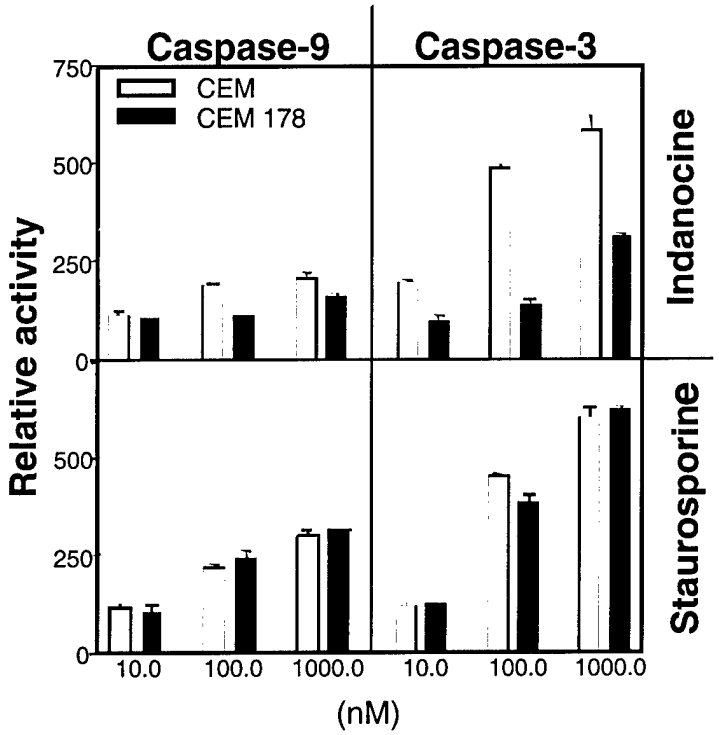

Figure 1: Activity of caspase-3 and –9 in indanocine and staurosporine-treated wild type CEM and CEM-178 cells.

Figure 1 show the pattern of caspase activation of the CEM-178 as compared to CEM treated with indanocine and staurosporine. The results indicate that the CEM-178 cells cells displayed a lower level of caspase-3 and –9 activity when treated with indanocine, but retain a functional apoptotic machinery as indicated by activation of caspases when treated with staurosporine. Indanocine failed to induce apoptosis in the CEM-178 cells, as indicated by the lack of cytosolic cytochrome c release (Fig 2)

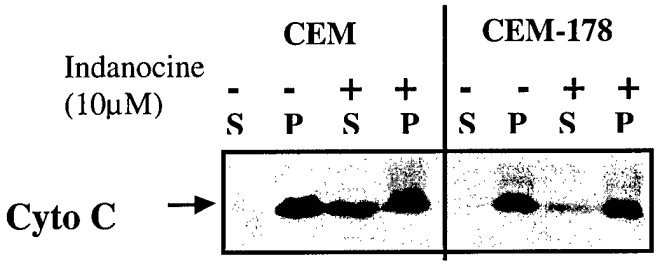

Figure 2: Indnanocine-induced cytochrome c cytosolic release in indanocine-treated CEM and CEM-178 cells

Additional biochemical assays, that can not be detailed for reason of space, suggested that the CEM-178 expressed tubulin with altered polymerization/depolymerization properties. In order to investigate the tubulin alterations on the CEM-178 cell we sequenced the 2 predominant isotypes of tubulin, M40 and b2, in both parental WT CEM and CEM-178 cells.

One point mutation was identified in the cDNA of M40 isotype, with a G to T substitution at nucleotide 1050, which converts amino acid 350 from lysine (AAG) to asparagine (AAU). This substitution was present at a single peak suggesting that there was no expression of the wild type sequence. Molecular modeling experiments performed in collaboration with Rick Gussio at the National Cancer Institute suggests that the observed tubulin mutation (K→D) may hinder the binding of indanocine (and cochicine) to tubulin, without affecting the capacity of tubulin to polymerize or depolymerize. Detailed studies on the modeling of the indanocine/tubulin interactions are currently underway, but it is already clear the CEM-178 cells will be very helpful in defining the molecular details of the mechanism of action of several other clinically used anti-cancer drugs.

Point 3 and 4

We are currently preparing an experiment to look at the differences in gene expression between the CEM and CEM-178 cells using gene arrays. We also are cloning the mutated tubulin isoform found in the CEM-178 cells into a mammalian expression vector. We plan to produce stable cells expressing the altered tubulin to verify its role in indanocine resistance.

Task 2

In the second aim of the application we proposed to assess the in vivo antitumor activity of indanocine in nude mice bearing multidrug resistant human breast cancer cell lines. Specifically, we planned to:

1. Determine the acute and chronic toxicity of indanocine and related compounds in nude mice.
2. Establish human tumor models in nude mice using wild type, multidrug resistant, and indanocine resistant breast cancer cell lines.
3. Test indanocine and related compounds in the established in vivo tumor models.

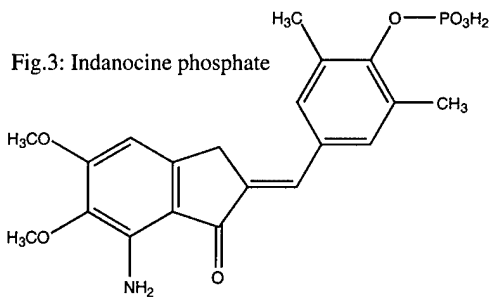

Fig.3: Indanocine phosphate

The main problem we encountered when testing indanocine in animals was the poor water solubility of the compound. Indanocine can only be prepared in DMSO solutions at a concentration of up to 20 mM concentrations. The highest concentration achievable in acquesous solutions is 100 μM. In order to overcome this problem we generated an indanocine analog that can be solubilized in water and other acqueous solutions (Fig. 3).

We used indanocine-phosphate in all the experimenst performed in animals.
Recent studies suggest that tubulin-binding drugs may display in vivo anti-cancer activity by reducing the blood supply to the cancer cells (anti-vascular activity), and by selectively killing cells on the blood vessels that feed the tumot cells (anti-angiogenic activity).

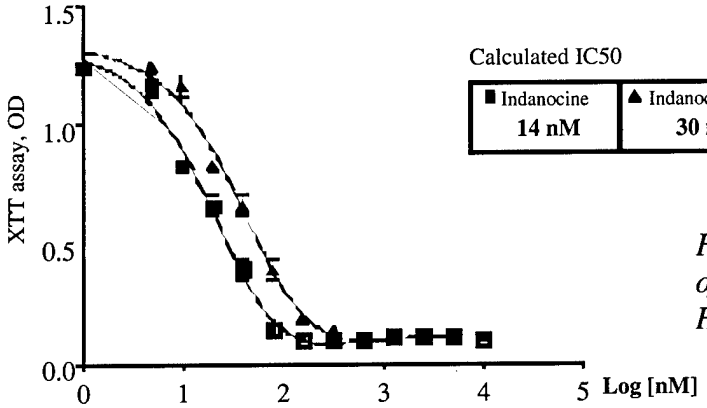

Figure 4: Cytotoxic activity of indanocine-phosphate in HUVEC cells.

We first tested the cytotoxic activity of indanocine and indanocine-phosphate against human umbilical vein endothelial cells (HUVEC), using the MTT proliferation assay (Figure 4). We obtained an IC50 of 14 nM for indanocine and 30 nM for indanocine-phosphate.

In collaboration with Judy Varner at UCSD Dept. Medicine, we then tested the in vivo anti-angiogenic activity of indanocine in the the chick chorioallantoic membrane (CAM) model (Figure 5).

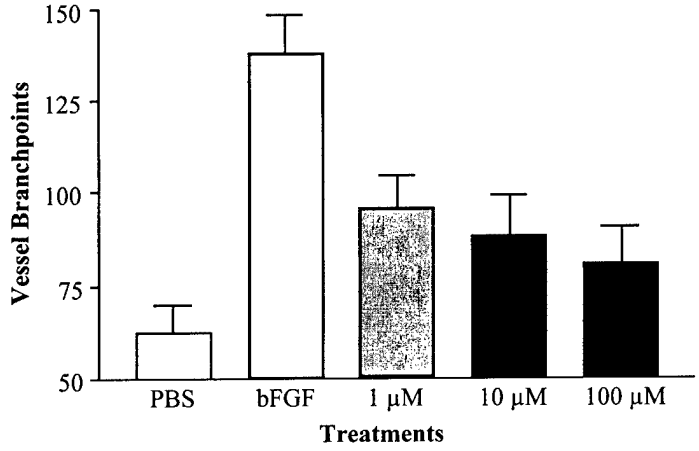

Figure 5: Anti-angiogenic activity of indanocine-phosphate tested in the CAM assay

Indanocine-phosphate was able to reduce the number of vessel branchpoints induced by fibroblast growth factor beta (bFGF), without displaying any visible toxicity to the chicken embryo. The results obtained clearly indicate that indanocine possess anti-angiogenic activity.

In order to further investigate the in vivo activity of indanocine, we then initiated a collaboration with Dr. Charles Parkins at the Tumour Microcirculation Group at the Gray Laboratory Cancer Research Trust, Mount Vernon Hospital, Northwood, England. We used the very well established murine breast adenocarcinoma CaNT tumour-bearing CBA female mice model. Indanocine-phosphate is very well tolerated by the animals even at very high concentrations (>500 mg/kg). The data we obtained show that there is no significant cytotoxicity by indanocine-phosphate against cultured CaNT tumour cells and very slight cytotoxicity against tumours treated in vivo (assayed using excision assay at 24h after treatment).

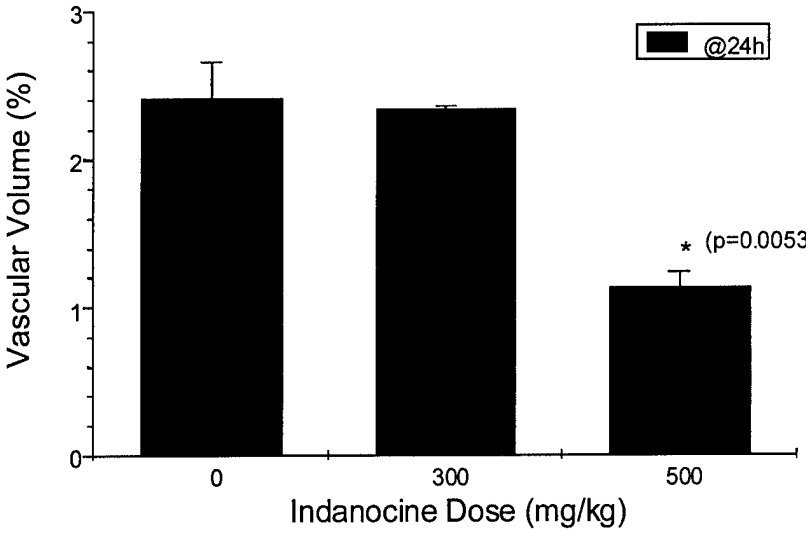

Figure 6: Anti-vascular activity of indanocine-phosphate

There was evidence for a significant anti-vascular action of indanocine using the vascular volume assay at 500mg/kg after 24 hours (**Fig X+3**). In order to confirm the anti-vascular activity of indanocine we performed histological analysis **(Fig X+4).** The pictures show that only a viable rim of tumor cells survive, all cells within the central part of the tumour have been destroyed by indanocine-phosphate. The results obtained are very smilar to those observed with other anti-vascular agents, such as combretastatin A-4.

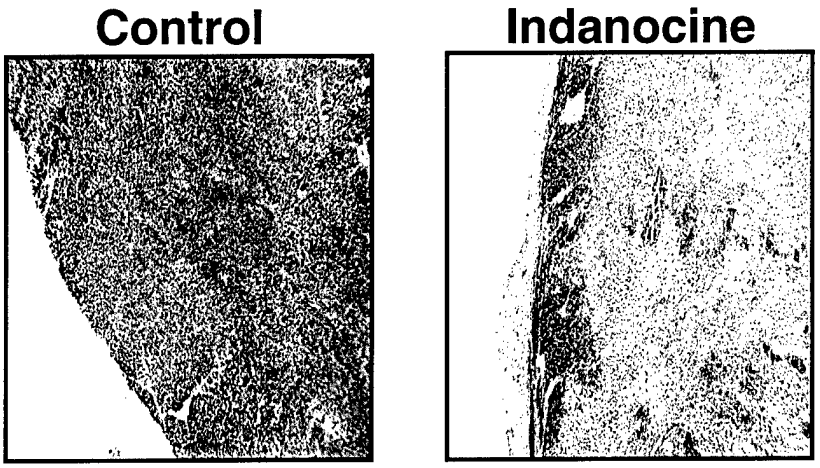

Figure 7: Indanocine induces selective tumor cell death in vivo

Taken together these preliminary results indicate that further detailed investigations on the anti-angiogenic and anti-vascular activity of indanocine are warranted. The major problems that are expected are the bioavailability of the drug and its delivery route. Preliminary assays suggest that most of the drug seem to be protein-bound, and that the half-life of the drug in vivo is very short. We plan to address these issues by a medicinal chemistry approach and by combination of the drug with carrier molecules. Synthesis of novel analogs with a better pharmacological profile and co-delivery of indanocine with lipids will be performed within one year.

Reportable Outcome

1. Manuscript JNCI

Leoni, Lorenzo M.; Hamel, Ernest; Genini, Davide; Shih, Hsiencheng; Carrera, Carlos J.; Cottam, Howard B.; Carson, Dennis A. Indanocine, a microtubule-binding indanone and a selective inducer of apoptosis in multidrug-resistant cancer cells. In: Journal of the National Cancer Institute (Bethesda) Feb. 2, 2000. 92 (3): 217-224.

2. Patent on Indanocine

09/148,576 Carson, Leoni, Cottam and Shih: Novel Anticancer Agents. Claims indanocine and related agents. US Patent Application, European Patent Application # 98958023.8-2112

Appendix
Funding # DAMD17-99-1-9100
Lorenzo M. Leoni Ph.D.

Indanocine, a Microtubule-Binding Indanone and a Selective Inducer of Apoptosis in Multidrug-Resistant Cancer Cells

Lorenzo M. Leoni, Ernest Hamel, Davide Genini, Hsiencheng Shih, Carlos J. Carrera, Howard B. Cottam, Dennis A. Carson

Background: **Certain antimitotic drugs have antitumor activities that apparently result from interactions with nontubulin components involved in cell growth and/or apoptotic cell death. Indanocine is a synthetic indanone that has been identified by the National Cancer Institute's Developmental Therapeutics Program as having antiproliferative activity. In this study, we characterized the activity of this new antimitotic drug toward malignant cells.** ***Methods:*** **We tested antiproliferative activity with an MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, mitochondrial damage and cell cycle perturbations with flow cytometry, caspase-3 activation with fluorometry, alterations of the cytoskeletal components with immunofluorescence, and antimicrotubule activity with a tubulin polymerization assay.** ***Results/Conclusions:*** **Indanocine is a cytostatic and cytotoxic indanone that blocks tubulin polymerization but, unlike other antimitotic agents, induces apoptotic cell death in stationary-phase multidrug-resistant cancer cells at concentrations that do not impair the viability of normal nonproliferating cells. Of the seven multidrug-resistant cell lines tested, three (i.e., MCF-7/ADR, MES-SA/DX5, and HL-60/ADR) were more sensitive to growth inhibition by indanocine than were their corresponding parental cells. Confluent multidrug-resistant cells (MCF-7/ADR), but not drug-sensitive cancer cells (MCF-7) or normal peripheral blood lymphocytes, underwent apoptotic cell death 8–24 hours after exposure to indanocine, as measured by sequential changes in mitochondrial membrane potential, caspase activity, and DNA fragmentation. Indanocine interacts with tubulin at the colchicine-binding site, potently inhibits tubulin polymerization *in vitro*, and disrupts the mitotic apparatus in dividing cells.** ***Implications:*** **The sensitivity of stationary multidrug-resistant cancer cells to indanocine suggests that indanocine and related indanones be considered as lead compounds for the development of chemotherapeutic strategies for drug-resistant malignancies. [J Natl Cancer Inst 2000;92:217–24]**

Antimitotic drugs are a major group of antitumor agents, whose varied mechanisms of action have been only partly elucidated *(1)*. Derivatives of natural products, such as the vinca alkaloids, colchicine, cryptophycin, the combretastatins, and related compounds *(2,3)*, as well as several different synthetic heterocyclic compounds *(4,5)*, inhibit tubulin polymerization and prevent microtubule assembly. The taxanes, on the other hand, prevent the depolymerization of tubulin, resulting in the rearrangement of the microtubule cytoskeleton *(6)*. Although some of the antimitotic agents have broad-spectrum cancer chemotherapeutic activity, others, such as colchicine and nocodazole, have no selectivity toward malignant cells. In general, antimitotic agents take advantage of kinetic abnormalities of cancer cells, such as their increased proliferation rate or loss of mitotic checkpoints. Many newer antineoplastic agents focus on biochemical abnormalities that differentiate malignant tumors from most normal tissues *(7–9)*.

The multidrug-resistant phenotype, although not strictly specific for cancer cells, is an attractive target for anticancer drugs because it develops during chemotherapy with bulky hydrophobic antineoplastic agents, limiting their efficacy *(10)*. Several mechanisms may contribute to intrinsic and acquired cross-resistance to multiple antineoplastic agents (clinical drug resistance). They include decreased drug accumulation due to overexpression of the P-glycoprotein drug efflux pump encoded by the mdr1 gene *(11,12)*, the multidrug resistance-associated protein (MRP) *(13)*, and the p110 major vault glycoprotein *(14)*. In addition, multidrug resistance has been linked to decreased expression of topoisomerase IIα *(15)*, to altered expression of drug-metabolizing enzymes and drug-conjugate export pumps *(16,17)*, and to modification of the apoptotic machinery *(18,19)*.

Various hydrophobic drugs with low toxicity for tumor cells can partially reverse multidrug resistance *in vitro* and *in vivo*. In contrast, cytotoxic compounds that preferentially target multidrug-resistant cells are not well described, but such agents should be very useful in the treatment of cancer. The National Cancer Institute's Developmental Therapeutics Program has identified indanocine, a newly synthesized indanone, as a compound with antiproliferative activity.

In this article, we investigate the action of indanocine on cultured multidrug-resistant cancer cells and their corresponding parental (wild-type) cells.

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See "Notes" following "References."

Materials and Methods

Materials

Indanocine, NSC 698666 (Fig. 1, A), is one of a series of synthetic indanones with antiproliferative activity (Shih H, Deng L, Carrera CJ, Adachi S, Cottam HB, Carson DA: unpublished data). Solid idanocine is a white powder that is stable when stored dry at room temperature or when dissolved in dimethyl sulfoxide or in water containing cyclodextrins. Paclitaxel, vinblastine sulfate, and nocodazole were from Calbiochem (San Diego, CA). Electrophoretically homogeneous bovine brain tubulin was prepared as described previously *(20)*. Media and tissue culture supplies were purchased from Irvine Scientific (Santa Ana, CA) and Fisher Scientific (San Diego, CA). All radiochemicals were from NEN-Dupont (Boston, MA). Unless otherwise indicated, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Cell lines from the American Type Culture Collection (Manassas, VA), propagated according to the instructions of the supplier, were as follows: MES-SA (human uterine sarcoma) and its multidrug-resistant variant MES-SA/DX5 raised against doxorubicin *(21)*, monkey COS-1, and Hep-G2 (human hepatocellular carcinoma). KB-3-1 (human carcinoma) and KB-GRC-1 (a transfectoma expressing high levels of the MDR1-encoded 170-kd P-glycoprotein) were provided by Dr. Stephen Howell (University of California San Diego, La Jolla) and have been described previously *(22)*. Dr. Michael J. Kelner (University of California San Diego) provided the following cell lines: MV522 (human metastatic lung carcinoma) and MV522/Q6 (a transfectoma expressing high levels of the MDR1 gene-encoded 170-kd P-glycoprotein); MCF-7/ADR, a human breast adenocarcinoma multidrug-resistant line selected against doxorubicin (expressing both gp170 and the embryonic glutathione transferase π isoform), and MCF-7/wt, the parental (wild-type) line; MDA-MB-231, a human breast adenocarcinoma line, and MDA3-1/gp170+, the doxorubicin-resistant daughter line expressing the 170-kd P-glycoprotein; and HL-60, a human acute promyelocytic leukemia line, and HL-60/ADR, the multidrug-resistant variant line selected against doxorubicin and expressing the MRP/gp180 protein. Dr. William T. Beck (Cancer Center, University of Illinois at Chicago) provided CEM, a human lymphoblastoid line, and CEM/VLB100, a multidrug-resistant line selected against vinblastine and expressing the 170-kd P-glycoprotein.

We incubated cells for 72 hours in 96-well plates with the test compounds and then measured cell proliferation by reduction of the yellow dye MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to a blue formazan product. The cleavage is performed by the "succinate-tetrazolium reductase" system, which belongs to the respiratory chain of the mitochondria and is active only in viable cells. Therefore, the amount of formazan dye formed is a direct indication of the number of metabolically active cells in the culture. The optical density of the blue formazan product was measured at 570 nm with a Thermo-Max (Molecular Devices, Sunnyvale CA) and analyzed with the Vmax Program (BioMetallics, Princeton, NJ).

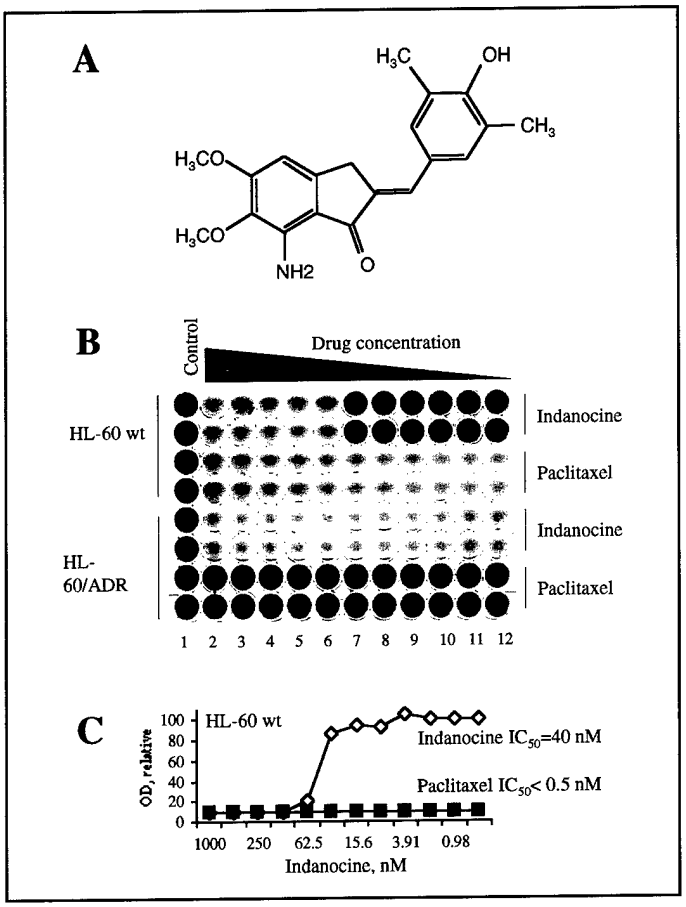

Fig. 1. A) Structure of indanocine. In **panels B** and **C,** HL-60 and HL-60/ADR cells display collateral sensitivity to indanocine; i.e., the multidrug-resistant cell line was substantially more sensitive to the growth-inhibitory effects of indanocine than the original parental cell line. **B)** Cytotoxic MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay of HL-60 cells. Parental wild-type HL-60 and HL-60/ADR multidrug-resistant cells were plated at low density (<5000 cells per well), and indanocine and paclitaxel were added at 1 : 2 serial dilutions from **column 2. Column 1** is the control without drugs. The initial concentrations were 1 μM for indanocine and 10 μM for paclitaxel, and the concentrations in **column 12** were 1 n*M* and 10 n*M*, respectively. After 3 days, the MTT assay was used to quantitate viable cells. The picture of the plate was obtained by optically scanning the 96-well plate. **Dark wells** represent metabolically active cells, and **clear wells** represent metabolically inactive cells. **C)** Graphic representation of the scanned plate for parental wild-type HL-60 cells, with the calculated 50% inhibitory concentrations (IC_{50}) shown (*see* Table 1).

Cell Cycle Analysis

Cells were harvested, fixed in ice-cold 70% ethanol, treated with ribonuclease A at 100 μg/mL, and stained with propidium iodide at 50 μg/mL for 1 hour at 37 °C. The DNA content of the cells was analyzed by flow cytometry (FACScalibur; Becton Dickinson Immunocytometry Systems, San Jose, CA), and the cell cycle distribution was calculated with the ModFit LT 2.0 Program (Verity Software House, Topsham, ME).

Caspase Analysis

Extracts were prepared by the suspension of 5×10^6 cells in 100 μL of a lysis buffer (i.e., 25 m*M* Tris–HCl [pH 7.5], 150 m*M* KCl, 5 m*M* EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate), incubation on ice for 10 minutes, and then centrifugation at 14 000*g* for 5 minutes at 4 °C. The resulting supernatants were collected and frozen at –80 °C or used immediately. Lysates (20 μL containing 5–10 μg of total protein) were mixed with 30 μL of assay buffer [50 m*M* piperazine-*N,N'*-bis(2-ethanesulfonic acid), 50 m*M* KCl, 5 m*M* ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and 0.1 m*M* phenylmethylsulfonyl fluoride], containing 100 μM of Z-DEVD-AFC (where DEVD is Asp–Glu–Val–Asp, Z is benzyloxycarbonyl, and AFC is 7-amino-4-trifluoromethyl coumarin). Caspase-3-like protease activity was measured at 37 °C with a spectrofluorometric plate reader (LS50B; The Perkin-Elmer Corp., Foster City, CA) in the kinetic mode with excitation and emission wavelengths of 400 nm and 505 nm, respectively. Activity was measured by the release of 7-amino-4-methyl coumarin (AMC) from the synthetic substrate Z-DEVD-AFC (Biomol, Plymouth Meeting, PA).

Mitochondrial Analysis

Cells were treated with the indicated amount of drug and 10 μM Ac-DEVD-fmk (*N*-acetyl-Asp–Glu–ValAsp–fluoromethylketone; Enzyme System Products, Livermore, CA), the cell-permeable caspase-3/caspase-7-selective inhibitor. Cells were then incubated for 10 minutes at 37 °C in culture medium containing 40 n*M* 3,3'-dihexyloxacarbocyanine iodide (DiOC6; Molecular Probes, Inc., Eugene, OR), followed by immediate analysis in a FACScalibur cytofluorometer. Fluorescence at 525 nm was recorded.

Immunofluorescence Assays

Hep-G2 (human hepatocellular carcinoma) cells were grown on glass coverslips in the presence or absence of drugs for 16 hours. Cells were fixed in

paraformaldehyde, permeabilized in Triton X-100, and stained with an anti-β-tubulin monoclonal antibody, followed by tetramethyl rhodamine B isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG). To visualize filamentous actin filaments, we stained the cells with fluorescein isothiocyanate-conjugated phalloidin, as described previously *(23)*. The type 1 nuclear mitotic apparatus protein was detected with monospecific human autoantibodies, as described previously by Andrade et al. *(24)*. The secondary antibody was fluorescein isothiocyanate-labeled goat anti-human IgG (Tago, Burlingame, CA). Nuclei were stained with the DNA-binding dye 4′,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes, Inc.) according to the manufacturer's instructions.

Tubulin Assays

Assessment of the inhibition of tubulin polymerization and the evaluation of the inhibition of [^{3}H]colchicine binding to tubulin were performed as described previously *(25)*. In all experiments, tubulin without microtubule-associated proteins *(20)* was used. In brief, for inhibition of assembly, 10 μ*M* (1.0 mg/mL) tubulin was preincubated with various concentrations of drug (4% [vol/vol] dimethyl sulfoxide as drug solvent) and 0.8 *M* monosodium glutamate for 15 minutes at 30 °C. The reaction mixture was placed on ice, and guanosine 5′-triphosphate (0.4 m*M*) was added. Reaction mixtures were transferred to cuvettes at 0 °C in Gilford 250 spectrophotometers (Beckman-Gilford, Fullerton, CA), baselines were established, and the temperature was increased to 30 °C with electronic temperature controllers (over a period of about 60 seconds). The IC_{50} value is the drug concentration required to inhibit 50% of the assembly, relative to an untreated control sample, after a 20-minute incubation. It should be noted that the bulk of polymer formed in the presence of glutamate consists of sheets of parallel protofilaments. The drug effects in this system are similar to those observed with a preparation containing tubulin and microtubule-associated proteins (i.e., microtubule proteins). The chief advantage of the glutamate system is that it unambiguously establishes tubulin as the drug target. For the colchicine-binding assay, reaction mixtures contained 1.0 μ*M* (0.1 mg/mL) tubulin and 5.0 μ*M* [^{3}H]colchicine and were incubated for 10 minutes at 37 °C before filtration through a stack of two DEAE-cellulose filters. At this time in reaction mixtures without inhibitor, binding is 40%–50% of maximum, so that the inhibition of the rate of colchicine binding to tubulin can be measured accurately.

Results

Inhibition of Cell Growth

In an initial screen performed by the National Cancer Institute's Developmental Therapeutics Program, the mean 50% growth-inhibitory concentration (GI_{50}) of indanocine was less than or equal to 20 n*M*. In 29 of 49 cell lines, including a doxorubicin-resistant breast cancer line, the GI_{50} for indanocine was less than the lowest concentration tested (10 n*M*). Because the indanone is hydrophobic, its activity toward the multidrug-resistant cells was surprising. To confirm this result, we compared the effects of indanocine on the growth of the following pairs of parent and corresponding multidrug-resistant lines (Table 1): MCF-7 and MCF-7/ADR, MES-SA and MES-SA/DX5, MDA-MB-321 and MDA3-1/GP170+3-1, HL-60 and HL-60/ADR, CEM and CEM/VLB100, KB-3-1 and KB-GRC-1, and MV522 and MV522/Q6 cells. The multidrug-resistant cell lines have different multidrug resistance mechanisms, including alterations of gp170 (mdr1 gene), gp180 (MRP gene), and the glutathione transferase π isoform. In several of the cell lines tested, the antiproliferative concentrations of indanocine were equivalent or lower in the multidrug-resistant cells than in the corresponding parent cells. Three of the cell lines tested (i.e., MCF-7, MES-SA, and HL-60) showed collateral sensitivity; i.e., the multidrug-resistant cell line was substantially more sensitive to the growth-inhibitory effects of indanocine than the parental cell line. An example of collateral sensitivity is shown in Fig. 1, where HL-60 and HL-60/ADR cells were plated in a 96-well plate and then treated for 3 days with decreasing (1 : 2 dilutions) concentrations of indanocine (from 1 μ*M*) or paclitaxel (from 10 μ*M*). The MTT assay was then performed at day 3. To prove that P-glycoprotein expression did not confer resistance to the indanone, we compared its effects on two carcinoma cell lines, KB-3-1 and MV522, and their corresponding transfectoma clones that overexpressed P-glycoprotein (the mdr1 gene product), KB-GRC-1 and MV522/Q6 *(22)*. These transfectomas were resistant to paclitaxel, as expected, but retained complete sensitivity to indanocine (Table 1).

Table 1. Growth-inhibitory concentrations of indanocine and paclitaxel in seven multidrug-resistant cell lines*

Cell line	GI_{50} (indanocine), n*M*		GI_{50} (paclitaxel), n*M*	
	Wild type	Mutidrug resistant	Wild type	Multidrug resistant
MCF-7	20 ± 5	4 ± 1†	50 ± 6	>10 000
MES-SA	85 ± 6	12 ± 3†	<1	>1000
MDA-MB-321	10 ± 3	25 ± 2	50 ± 2	>1000
HL-60	40 ± 3	2 ± 0.2†	<1	>1000
CEM	12 ± 2	20 ± 1	<1	606 ± 20
KB-3-1	7 ± 2	7 ± 3	7 ± 3	>1000
MV522	13 ± 3	8 ± 2	15 ± 4	358 ± 58

*The cells were treated with various concentrations of indanocine or paclitaxel for 72 hours. Cell proliferation was assessed by the MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. The results represent the 50% growth-inhibitory concentrations (GI_{50}) (mean ± standard deviation; n > 5). The human breast adenocarcinoma MCF-7/ADR cell line was selected against doxorobucin and expresses both gp170 and the embryonic glutathione transferase π isoform. The human uterine sarcoma line MES-SA/DX5 line was selected against doxorobucin *(21)*. The doxorubicin-resistant human breast adenocarcinoma cell line MDA3-1/gp170+ expresses the 170-kd P-glycoprotein. The human acute promyelocytic leukemia line HL-60/ADR was selected against doxorobucin and expresses the MRP/gp180 protein. The human lymphoblastoid CEM/VLB100 line was selected against vinblastine and expresses the 170-kd P-glycoprotein. The transfectoma KB-GRC-1 expresses high levels of the MDR1-encoded 170-kd P-glycoprotein *(22)*. The metastatic human lung transfectoma MV522/Q6 expresses high levels of the MDR1-encoded 170-kd P-glycoprotein.

†$P<.001$ versus wild-type value by Wilcoxon signed rank test.

Effect of Indanocine on Stationary-Phase Cells

The results obtained with the actively growing parent and multidrug-resistant cell lines led us to test indanocine in stationary-phase cell lines. As determined by flow cytometry after propidium iodide staining, up to 81% of stationary MCF-7/ADR cells (1 week in confluent culture) were in the G_1 phase of the cell cycle (Fig. 2, B; middle panel). Remarkably, indanocine treatment of stationary-phase multidrug-resistant cells, but not parental cells, resulted in cell death (IC_{50} = 32 n*M*) (Fig. 2, A). The cytotoxic effect of indanocine in noncycling MCF-7/ADR cells was confirmed by the detection of an apoptotic sub-G_0/G_1 population by flow cytometry and by the activation of caspase-3 (Fig. 2, B; left panel). Parental (wild-type) MCF-7 cells were similarly growth arrested but did not show apoptotic features (data not shown). In addition, normal peripheral blood lymphocytes exposed to 1000-fold higher concentrations of indanocine for 72 hours showed no loss of viability (data not shown).

Apoptosis

The study described above demonstrated that stationary MCF-7/ADR cells, but not wild-type MCF-7 cells, were sensitive to treatment with indanocine. To test this observation in

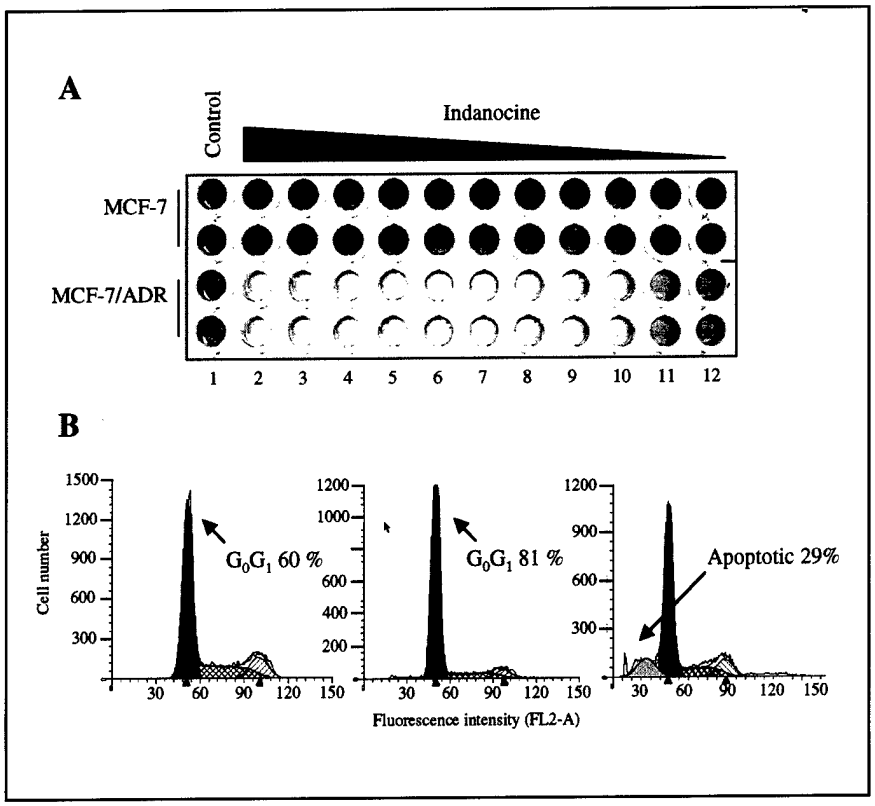

Fig. 2. Effect of indanocine on resting multidrug-resistant cells. **A**) Toxicity of indanocine on resting MCF-7 cells. Parental wild-type MCF-7 and MCF-7/ADR multidrug-resistant cells were maintained in a confluent state for 7 days by daily replacement of the medium. Indanocine was then added at 10 μM in wells in **column 2** and serially diluted 1 : 2 in each successive column (reaching 10 nM in **column 12**). **Column 1** is the control, without indanocine. After 3 days, the MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to quantitate viable cells. **B**) DNA content and caspase activity of indanocine-treated cells. MCF-7/ADR multidrug-resistant cells were harvested, permeabilized, and stained with propidium iodide. The DNA content of cells was established by flow cytometry. **Solid peak** = cells in the G_0/G_1 phase; **cross-hatched peak** = cells in the S phase; **hatched peak** = cells in the G_2/M phase; **shaded peak** (present only in the **right panel**) = hypodiploid, apoptotic cells, as modeled with the ModFit Program. At the **left** are normally proliferating cells, in the **middle** are confluent cells (7 days), and at the **right** are indanocine-treated, growth-arrested confluent MCF-7/ADR cells. The percentage of cells that are in G_0/G_1 phase or that are apoptotic is indicated. The relative caspase activity is the fluorometric measurement of the caspase-3-like activity normalized to the control cells (**left**). For normally proliferating MCF-7/ADR cells, the relative caspase activity is 100; for 7-day confluent MCF-7/ADR cells, it is 100; for growth-arrested, confluent MCF-7/ADR cells treated with indanocine, it is 450.

another cell line pair that displayed collateral sensitivity to indanocine in the multidrug-resistant derivative line, we selected HL-60 and HL-60/ADR cells because of the exquisite sensitivity of HL-60/ADR cells to indanocine. In the experiment shown in Fig. 3, we tested the ability of indanocine to activate caspase-3 in parental and multidrug-resistant HL-60 cells. Caspase-3, considered an "executioner" caspase, is implicated in the last and irreversible phase of the apoptotic caspase pathway and is activated by upstream "initiator" caspases, such as caspase-8 and caspase-9 [reviewed in *(26)*]. Caspase activity was measured by use of the fluorogenic caspase-3-specific substrate DEVD-AMC. HL-60/ADR cells incubated with 10 nM indanocine showed a time-dependent increase in caspase-3 activity compared with untreated cells, reaching a maximum at 24 hours. In contrast, wild-type HL-60 cells showed only a slight increase in caspase-3 activity, about 25% of that obtained in the multidrug-resistant cells (Fig. 3, A).

To determine the effect of indanocine treatment on the mitochondrial transmembrane potential of multidrug-resistant and wild-type cells, we used the fluorochrome DiOC6 (Fig. 3, B). In apoptosis induced by various stimuli, a decrease in the mitochondrial transmembrane potential has been shown to precede nuclear DNA fragmentation *(27)*. The flow cytometry results showed a visible reduction in DiOC6 fluorescence for HL-60/ADR cells incubated with indanocine for 8 hours, indicating that the mitochondrial transmembrane potential in these cells was reduced. After 16 hours, the percentage of cells with reduced DiOC6 fluorescence had reached 44%. HL-60 wild-type cells incubated with the same amount of indanocine did not display the same strong reduction in DiOC6 fluorescence.

Effect on Tubulin Polymerization

Indanocine did not change the flow cytometry profile of stationary-phase cells stained for DNA, other than to cause the appearance of hypodiploid apoptotic cells in multidrug-resistant cultures (Fig. 2). However, concentrations of the drug that inhibited cell proliferation caused a rapid increase in the number of cells in G_2/M phases in growing cultures (data not presented).

Antimitotic drugs usually interfere with cellular microtubules by interacting with tubulin *(3)*. Using glutamate-induced assembly of purified tubulin (containing no microtubule-associated proteins) as our assay, we found (Fig. 4, A) that indanocine inhibited tubulin assembly in a manner comparable to that of nocodazole rather than inducing polymerization as would paclitaxel. This observation led us to perform a quantitative analysis (Fig. 4, B), in which we found that indanocine was nearly as potent as combretastatin A-4 (a gift of Dr. G. R. Pettit, Arizona State University, Tempe, AZ) as an inhibitor of tubulin assembly. We measured the extent of tubulin assembly after a 20-minute incubation at 30 °C and determined that the IC_{50} of combretastatin A-4 was 1.20 ± 0.03 μM (mean ± standard deviation; n = 4) and that the IC_{50} of indanocine was 1.7 ± 0.1 μM (n = 3). Both compounds practically eliminated the binding of 5 μM [^{3}H]colchicine to 1 μM tubulin when present at 5 μM—combretastatin A-4 inhibited 98% ± 4% of colchicine binding (n = 4), and indanocine inhibited 95% ± 2% of colchicine binding (n = 4). The effects of various concentrations of the two drugs on colchicine binding are shown in Fig. 4, C. Neither agent inhibited the binding of [^{3}H]vinblastine to tubulin (single experiment).

Cytoskeletal Effects of Indanocine

COS-1 and Hep-G2 cells were grown on glass coverslips and treated with various concentrations of indanocine, nocodazole, or vinblastine sulfate. The microtubule network was then visualized by indirect immunofluorescence with an anti-β-tubulin antibody, and the microfilament network was stained with fluorescein isothiocyanate-coupled phalloidin. COS-1 and Hep-G2

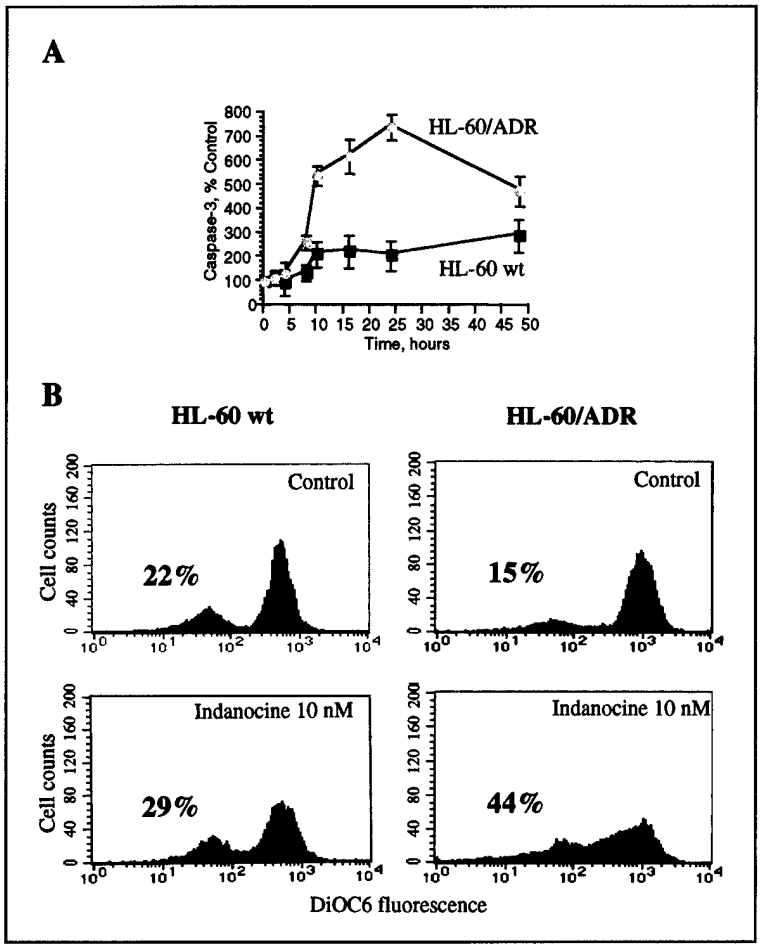

Fig. 3. Indanocine induces apoptosis in multidrug-resistant cells. **A**) Activation of caspase-3 by indanocine. HL-60 wild-type (wt) and multidrug-resistant (ADR) cells were treated with 10 n*M* indanocine; at the indicated times, caspase-3-like activity was measured with the specific fluorogenic substrate DEVD-AMC (Asp–Glu–Val–Asp coupled to 7-amino-4-methyl coumarin). The results are expressed as the means ± standard deviation (**error bars**) and are representative of up to four experiments. **B**) Reduction of mitochondrial transmembrane potential by indanocine. HL-60 cells were treated with and without indanocine at 10 n*M*. After 16 hours of incubation, cells were incubated with 40 n*M* 3,3′-dihexyloxacarbocyanine iodide (DiOC6), followed immediately by flow cytometry in a FACScalibur. The ***x*-axis** represents the DiOC6 fluorescence. The ***y*-axis** represents the number of cells. The percentage of low-DiOC6 fluorescence-gated cells is indicated.

cells were used for these studies because of their clearly defined microtubule and microfilament networks, respectively. Untreated cells had extensive microtubule systems with perinuclear organizing centers (Fig. 5, a and b) and had microfilament bundles and stress fibers that were predominantly aligned with the major axis of the cell (Fig. 6, a and b).

Treatment of COS-1 cells with 0.5 or 5 μ*M* indanocine for 1 hour depleted the cells of microtubules, resulting in diffuse cytoplasmic staining with anti-β-tubulin antibody (Fig. 5, c and d). Treatment with 0.55 μ*M* (0.5 μg/mL) vinblastine sulfate (Fig. 5, f) for 1 hour had a similar effect. Modifications induced by 3.3 μ*M* (1 μg/mL) nocodazole (Fig. 5, e) for 1 hour were less pronounced, and some of the perinuclear organizing centers were still visible.

Indanocine-treated Hep-G2 cells had a rounded shape, and the microfilament cytoskeleton in these cells was disorganized, characteristic of treatment with a depolymerizing agent (Fig. 6, c). After a 1-hour incubation in 0.5 μ*M* indanocine, some cells

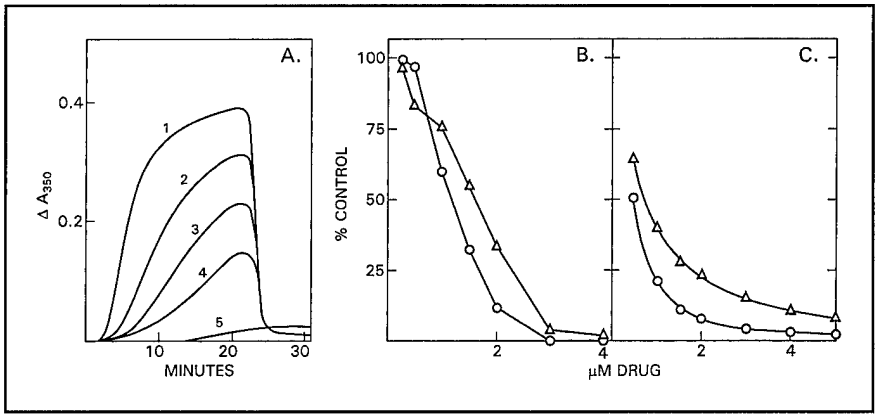

Fig. 4. Effect of indanocine on tubulin polymerization and binding of [^{3}H]colchicine to tubulin. Absorbance was measured at 350 nm (A_{350}). Inhibition of tubulin polymerization (**A** and **B**) and inhibition of [^{3}H]colchicine binding to tubulin (**C**) by indanocine and by combretastatin A-4. **Panel A:** For the tubulin polymerization assay, tubulin (10 μ*M*) was incubated with indanocine at the following concentrations: 0 for **curve 1,** 1.0 μ*M* for **curve 2,** 1.5 μ*M* for **curve 3,** 2.0 μ*M* for **curve 4,** and 3.0 μ*M* for **curve 5.** At 0 time, the temperature controller was set at 30 °C for 20 minutes to measure polymerization; at 20 minutes, the temperature controller was set at 0 °C to measure the amount of cold-reversible tubulin polymer formation. **Panels B** and **C:** Concentrations of indanocine (△) and combretastatin A-4 (○) were as indicated. **Panel B:** Values obtained for the inhibition of tubulin polymerization in all experiments were averaged. For 1.0, 1.5, and 2.0 μ*M* drug, there were four values for combretastatin A-4 and five for indanocine. There were fewer experimental values for the other concentrations. The mean control A_{350} value in these experiments was 0.382. **Panel C:** Indanocine inhibition of [^{3}H]colchicine binding to tubulin. In this assay, indanocine was added at various concentrations to a reaction mixture containing purified tubulin and [^{3}H]colchicine and incubated for 10 minutes at 37 °C. The amount of [^{3}H]colchicine bound to tubulin was then measured after filtration on DEAE-cellulose. The experiment shown in panel C was performed once with duplicate samples for each data point.

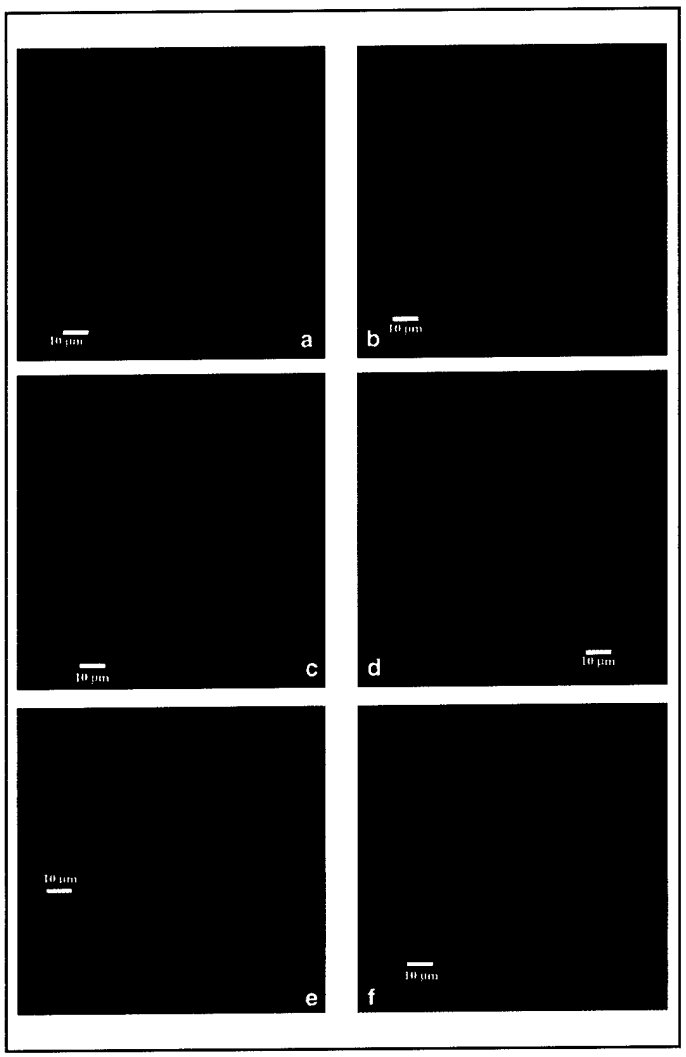

Fig. 5. Effect of indanocine on the microtubular network. COS-1 cells were incubated for 2 hours with dimethyl sulfoxide (**a**) or ethanol (**b**), 5 μM and 500 nM indanocine (**c** and **d,** respectively), 3.3 μM (1 μg/mL) nocodazole (**e**), or 0.55 μM (0.5 μg/mL) vinblastine sulfate (**f**). The cells were fixed, microtubules were labeled (**red**) with an anti-β-tubulin antibody, and nuclei were stained (**blue**) with 4′,6-diamidino-2-phenylindole dihydrochloride.

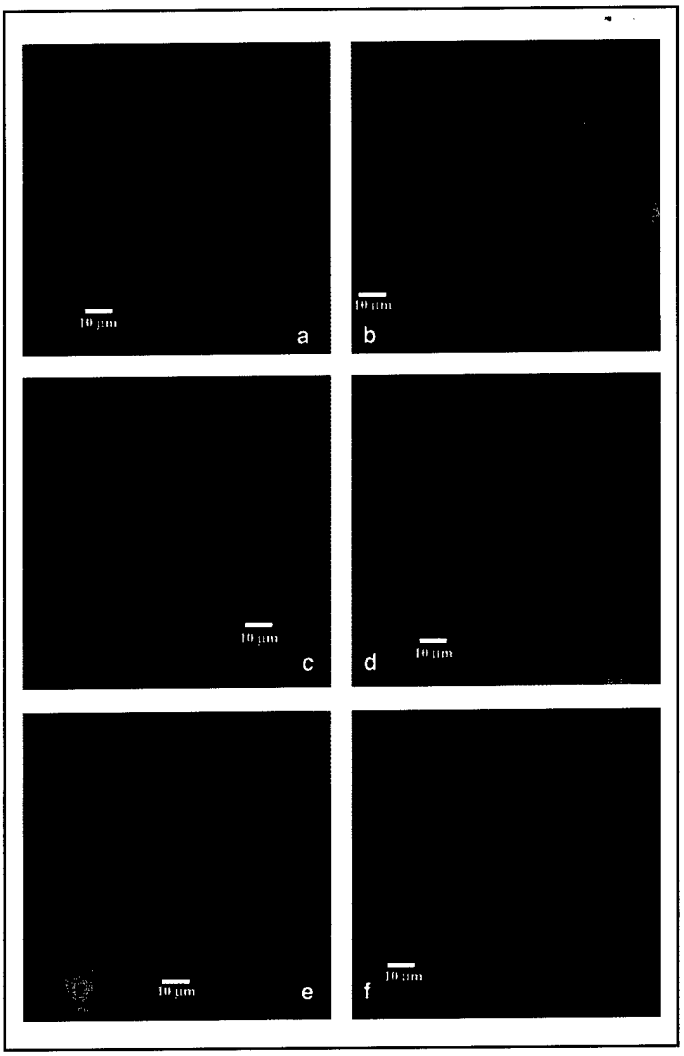

Fig. 6. Effect of indanocine on the microfilament network. Hep-G2 cells were incubated for 2 hours with dimethyl sulfoxide (**a**) or ethanol (**b**), 5 μM and 500 nM indanocine (**c** and **d,** respectively), 3.3 μM (1 μg/mL) nocodazole (**e**), and 0.55 μM (0.5 μg/mL) vinblastine sulfate (**f**). Cells were fixed, microfilaments were stained with fluorescein isothiocyanate-coupled phalloidin (**green**), and nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (**blue**).

had a characteristic rounded shape, but other cells had normal microfilament bundles (Fig. 6, d). A similar effect was observed after exposure to nocodazole (Fig. 6, e) or vinblastine sulfate (Fig. 6, f). This is probably an indication that the microfilament breakdown observed at 5 μM indanocine is not a direct effect but rather is a consequence of the rapid and potent disruption of the microtubule network.

After treatment of Hep-G2 cells with 100 nM indanocine, the subcellular localization of the mitotic apparatus (as shown by human autoantibodies against the type 1 nuclear mitotic apparatus protein) was determined by immunofluorescence. In control cells undergoing mitosis, the type 1 nuclear mitotic apparatus protein was localized at the poles of the mitotic spindle (Fig. 7, a). In cells exposed to 100 nM indanocine and arrested in the M phase, type 1 nuclear mitotic apparatus protein was distributed in spots scattered over the nucleus (Fig. 7, b). A similar effect was observed with 3.3 μM nocodazole (Fig. 7, c). Paclitaxel treatment did not interfere with the subcellular distribution of the type 1 nuclear mitotic apparatus protein, although it affected the formation of a functional mitotic spindle (Fig. 7, d).

Discussion

Indanocine is a derivative of indanone with antiproliferative activity (Shih H, Deng L, Carrera CJ, Adachi S, Cottam HB, Carson DA: unpublished data). An initial screen of malignant cell lines performed by the National Cancer Institute's Developmental Therapeutics Program and the COMPARE Program *(28)* suggested that these compounds had tubulin-binding properties. In this screening, the indanones, including indanocine, retained activity toward multidrug-resistant breast cancer cells. Indanocine interacts with tubulin at the colchicine-binding site, and it inhibits tubulin polymerization with an IC_{50} value equivalent to values obtained with podophyllotoxin and combretastatin A-4 *(29)*. Consistent with these biochemical effects, in intact cells, indanocine disrupts intracellular microtubules including those of the mitotic spindle and leads to redistribution of the components of the nuclear mitotic apparatus. The discrepancy between the micromolar concentration of drug required for *in vitro* inhibition of tubulin polymerization and the nanomolar concentrations of drug that blocked cell proliferation has been

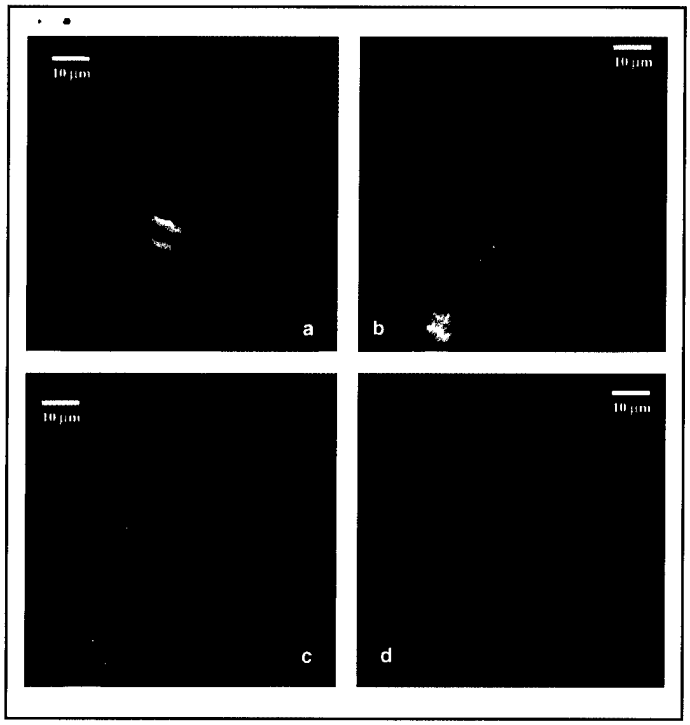

Fig. 7. Effect of indanocine on type 1 nuclear mitotic apparatus protein. Hep-G2 cells were incubated for 16 hours with dimethyl sulfoxide (**a**), 0.1 μ*M* indanocine (**b**), 3.3 μ*M* (1 μg/mL) nocodazole (**c**), or 0.1 μ*M* paclitaxel (**d**). Cells were fixed and labeled with monospecific human autoantibodies to type 1 nuclear mitotic apparatus protein.

observed with other antitubulin agents *(5,25,29)*. This discrepancy may indicate that indanocine interacts with other nontubulin cellular components to produce a cytotoxic response. This hypothesis is supported by the ability of short-term treatment with indanocine to trigger apoptosis in stationary multidrug-resistant cancer cells (whose survival should not depend on DNA synthesis or an intact mitotic apparatus) but not in control cells.

Modification of the apoptotic machinery has been proposed as an explanation for the *de novo* and acquired cross-resistance to multiple antineoplastic agents. It has been shown that the Bcl-2 protein may protect cancer cells from drug-induced apoptotic cell death *(18,30,31)*. Microtubule-disrupting drugs, such as vincristine, vinblastine, and colchicine, and microtubule-stabilizing drugs, such as paclitaxel and doxetaxel, induce growth arrest, which is followed by phosphorylation and inactivation of Bcl-2, which eventually leads to apoptotic cell death in the G_2/M phase of the cell cycle *(32–34)*. In contrast, cells in the stationary phase are generally resistant to many of these agents, and phosphorylation of Bcl-2 in the G_0/G_1 phase is generally not observed. This property limits the utility of tubulin-binding drugs for the treatment of malignant tumors containing only a few proliferating cells (i.e., tumors with a low S-phase fraction).

As with other microtubule-damaging drugs, indanocine arrested the growth of multidrug-sensitive cancer cells at the G_2/M boundary and induced apoptotic cell death. The nanomolar concentrations of indanocine that induced apoptosis in multidrug-resistant cells did not kill wild-type G_1-phase cancer cells or quiescent normal peripheral blood lymphocytes. Nanomolar concentrations of indanocine forced stationary, multidrug-resistant cells into the apoptotic program. That these cells were arrested in the G_0/G_1 phase of the cell cycle was confirmed by cytofluorometric analysis. Apoptosis, in these cells, was confirmed by the appearance of a subdiploid-DNA flow-cytometry peak and by caspase-3 activation. Compared with their respective parental lines, five multidrug-resistant cell lines displayed higher or indistinguishable sensitivity to indanocine toxicity.

The cell lines hypersensitive to indanocine have modified various systems for multidrug resistance. MES-SA/DX5 cells overexpress P-glycoprotein, and MCF-7/ADR cells overexpress P-glycoprotein and also have an embryonic π isoform of glutathione transferase *(35)*. HL-60/ADR cells express the MRP/gp180 protein. The other two cell lines that we examined, with unaltered sensitivity to indanocine, only express P-glycoprotein.

The fact that sensitivity to indanocine was retained by all of the multidrug-resistant cells tested, including both KB-3-1 and MV522 transfectomas that overexpress the P-glycoprotein, suggests that this agent acts independently of the P-glycoprotein hydrophobic multidrug transporter and/or that the cytoskeletal disorganization induced by the indanone interfered with P-glycoprotein functions. In other experiments, indanocine did not alter the rate of rhodamine efflux from loaded cells (data not shown). Thus, it seems probable that the uptake and regulation of indanocine do not depend on or directly influence the P-glycoprotein multidrug transporter.

Several different microtubule-disrupting agents have been developed that do not depend on the 170-kd P-glycoprotein and that display antiproliferative activity against multidrug-resistant cancer cells *(23,36)*. It should be of interest to determine whether any of these agents, like indanocine, are cytotoxic to noncycling mdr1-expressing cells. Such comparative studies could answer the question whether the mechanism of action of indanocine is related only to inhibition of microtubule function. If indanocine-induced cell death involves another intracellular target, then other microtubule-disrupting agents with antiproliferative activity toward multidrug-resistant cells should not be able to induce apoptosis in the G_0/G_1 phase.

The observation that indanocine kills noncycling, multidrug-resistant cells has practical implications. The low percentage of cycling cells in many human solid tumors limits the potential of antimitotic drugs. The combination of a drug that is selectively cytotoxic to nondividing, multidrug-resistant cells and an antineoplastic agent that kills tumors with abnormalities of cell cycle checkpoints could represent an exceptionally effective approach to eradicating malignant cells while sparing most normal tissues. Thus, we suggest that indanocine and related indanones be considered lead compounds for the development of chemotherapeutic strategies for drug-resistant malignancies.

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NOTES

Supported in part by a grant from the Swiss National Science Foundation; by Public Health Service grants GM23200 (National Institute of General Medical Sciences) and CA78040-01 (National Cancer Institute), National Institutes of Health, Department of Health and Human Services; and by a grant from the Novartis corporation.

Manuscript received June 29, 1999; revised November 4, 1999; accepted November 18, 1999.