Biochemical Pharmacology, Vol. 52, pp. 1553–1560, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00557-6

Cucurbitacin E-Induced Disruption of the Actin and Vimentin Cytoskeleton in Prostate Carcinoma Cells

Kimberly L. K. Duncan,†*
Mark D. Duncan,‡ Michael C. Alley† and Edward A. Sausville†

†Laboratory of Biological Chemistry, Division of Basic Sciences, and Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis, and Centers, National Cancer Institute, NIH, Bethesda, MD; and ‡Surgical Service, VA Medical Center, Washington, DC, U.S.A.

ABSTRACT. Cucurbitacin E has been identified by an empiric screening strategy as a sterol with potent growth inhibitory activity *in vitro* directed against prostate carcinoma explants (IC_{50} of 7–50 nM in 2- to 6-day exposures). The mechanism of cucurbitacin cytoxicity has not been elucidated previously. In the present study, we observed that cucurbitacin E caused marked disruption of the actin cytoskeleton, and in a series of cucurbitacin analogues, anti-proliferative activity correlated directly with the disruption of the F-actin cytoskeleton. The distribution of vimentin was also altered in cells exposed to cucurbitacin E, as vimentin associated with drug-induced membrane blebs. The appearance of microtubules was unaffected. Western blot analysis of intracellular actin in cells exposed to cucurbitacins and quantitation of rhodamine-phalloidin binding support the hypothesis that cucurbitacin treatment leads to an inappropriate increase in the filamentous or polymerized actin fraction in prostate carcinoma cells. We conclude that cucurbitacins are potent disruptors of cytoskeletal integrity. Prostate carcinoma cells appear notably sensitive to growth inhibition by cucurbitacin E. BIOCHEM PHARMACOL 52;10:1553–1560, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. actin; cytoskeleton; steroid; prostate neoplasm drug treatment; vimentin

The cucurbitacins are cytotoxic sesquiterpinoid sterols derived from plants with medicinal properties known since antiquity [1]. Little is known about the mechanism of the cytotoxic effect of cucurbitacins. Cucurbitacin I has been reported to inhibit rapidly precursor incorporation into DNA, RNA, and protein [2]. Cucurbitacins also have been reported to bind to a glucocorticoid receptor [3], to modify prostaglandin and adrenocorticosteroid synthesis [4], and to cause altered cell morphology [5–7]. Cucurbitacins have been tested for antitumor activity in several tumor model systems *in vitro* and *in vivo* [8], but a low therapeutic index was observed when cucurbitacin D was administered to mice by intermittent injection. Doses of drug with antitumor activity led to a large increase in capillary permeability and decreased cardiac function in both *in vivo* and *in vitro* preparations [9, 10]. We report here that cucurbitacin E emerged through an empiric screening strategy to define agents with potent growth inhibitory activity in non-immortalized prostate carcinoma cell explants, which renewed interest in discerning the mechanism of its cytotoxic effect. We demonstrated that an early effect of cucurbitacin E is rearrangement of the actin and the vimentin cytoskeleton, and that the growth inhibitory actions of a series of cucurbitacin congeners correlate with these effects. Thus, the actin and vimentin cytoskeleton emerge as potential targets for this family of natural products.

MATERIALS AND METHODS

Materials

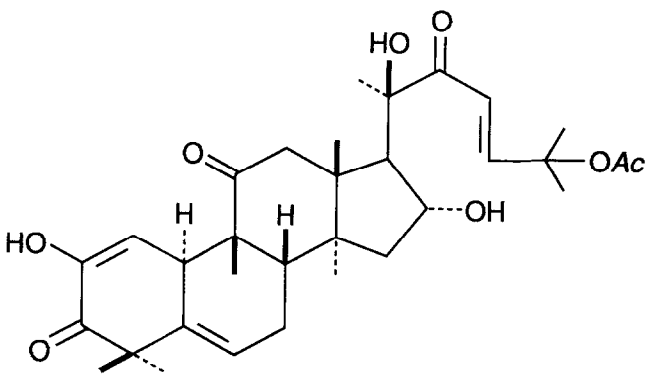
Cucurbitacin E (Fig. 1; NSC 106399) and other cucurbitacin analogues were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, Bethesda, MD. The PC-3 and LNCaP human prostate carcinoma cell lines were obtained from J. Trepel (Clinical Pharmacology Branch, NCI); the DU145 prostate cancer line was from the Biological Testing Branch, NCI. Rhodamine-phalloidin, fluorescein DNAse I, and calcein AM were purchased from Molecular Probes (Eugene, OR).

All tissue culture reagents were obtained from Gibco/BRL (Grand Island, NY), and other reagents including antibodies were from the Sigma Chemical Co. (St. Louis, MO) unless otherwise listed.

Screening Methods

The NCI cancer drug screen studies sixty human tumor cell lines in an *in vitro* semi-automated protein-based cytotox-

* Corresponding author: Dr. Kimberly Duncan, Department of Surgery, Johns Hopkins Bayview Medical Center, 4940 Eastern Ave., Baltimore, MD 21224-2780. Tel. (410) 550-0400; FAX (410) 550-1245.
Received 24 January 1996; accepted 6 June 1996.



Cucurbitacin E

FIG. 1. Cucurbitacin E.

icity assay using SRB[‖] to quantitate cell protein mass following drug exposure [11]. Compounds with evidence of anti-proliferative activity in the main screen have been studied further in a prostate carcinoma subpanel screen, which utilizes explants of tissue obtained from biopsies of patients with carcinoma of the prostate in the course of their routine diagnosis and treatment. Agents selected for study in the prostate subscreen were added in duplicate to prostate tumor cell strains plated 24 hr previously in 96-well microtiter plates for a 6-day incubation. Cucurbitacin E has been demonstrated to be stable in medium for this time period. Plates were stained with SRB as described previously [12, 13].

Cell Culture

For detailed mechanistic studies, PC-3, LNCaP, and DU145 cells were maintained in log phase culture in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. All three lines were derived from metastases of prostate carcinoma; both the PC-3 and DU145 are hormone refractory, while the LNCaP responds to some antiandrogens [14, 15]. For cytotoxicity assays, 2×10^3 cells/well were plated in 96-well plates 24 hr before drug exposure. Cells were exposed to fresh medium containing cucurbitacin E or its vehicle (0.02% ethanol) for the indicated times, and a viable cell number was determined with MTT [16]. Results were confirmed with both the SRB assay and manual cell counts. For studies of recovery following drug exposure, cells were plated 24 hr before use, then exposed to drug for 6, 12, 24, or 48 hr, following which the medium was replaced with cucurbitacin-free medium for the duration of the recovery period. Viable cell mass was estimated using the MTT assay 144 hr after initial drug addition.

[‖] *Abbreviations*: F-actin, filamentous actin; FITC, fluorescein isothiocyanate; G-actin, globular actin; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide; SRB, sulforhodamine B; and TTS, Tris–Tween–saline.

Fluorescent Microscopy

PC-3 cells, plated at 1×10^4 cells/well, were grown on 8-well glass chamber slides (Nunc, Naperville, IL) 24 hr before use. PC-3 cells were used as they grew well on glass slides (plastic growing surfaces utilized for tissue culture may autofluoresce). After drug exposure, slides were rinsed with Dulbecco's PBS, and fixed with freshly prepared 4% phosphate-buffered formaldehyde, pH 7.4, for 10 min at room temperature. After rinsing in PBS, cells were permeabilized with 0.1% Triton X-100 for 5 min and rinsed in PBS. Fluorescent probes and antibodies for direct fluorescent staining were diluted in PBS and added for 30 min: to detect F-actin, rhodamine-phalloidin (330 nM); for G-actin, fluorescein-DNAse I (10 μg/mL); for 1 hr: cytokeratin, anti-(pan) cytokeratin-fluorescein (clone PCK-2G, 1:25 dilution). Slides were then rinsed in PBS, and cover slips were mounted in glycerol vinyl acetate. For indirect immunofluorescence, primary antibodies used included anti-β-tubulin (clone TUB2.1, 1:200), anti-vimentin (Zymed, clone ZC64, undiluted), and anti-fibronectin (clone FN-15, 1:200). Slides were incubated for 1 hr, rinsed in PBS, and then incubated for 30 min with the appropriate fluorescein-conjugated secondary antibody: goat anti-mouse–FITC (1:20) for anti-tubulin and anti-vimentin; and goat anti-rabbit–FITC (1:80) for anti-fibronectin. Slides were protected from light after addition of fluorescent label, and examined using a Leitz Dialux 20 fluorescent microscope.

Rhodamine-Phalloidin Binding

To quantify changes in F-actin levels, bound rhodamine-phalloidin was extracted from stained cells [17]. PC-3 cells were plated at 2×10^4 cells/well in 24-well plates. Drug was added to cells 24, 6, and 1 hr before harvest. After drug incubation, wells were rinsed with PBS, fixed, permeabilized, and stained with rhodamine-phalloidin as described above. After staining, wells were rinsed thoroughly with PBS, and 2 mL of methanol was added for 30 min to extract F-actin-bound rhodamine. The fluorescence emission of the extracts at 575 nm was measured in a Perkin–Elmer LS-5B Fluorimeter during excitation at 550 nm, and compared against a standard curve of rhodomine-phalloidin in methanol. Nonspecific rhodamine-phalloidin binding was less than 10% as measured by pretreating fixed, permeabilized cells with unlabeled phalloidin prior to addition of rhodamine-labeled phalloidin. The presence of cucurbitacin during the addition of rhodamine-phalloidin did not alter the binding of rhodamine-phalloidin to fixed, permeabilized controls. Nonspecific binding was subtracted from the mean fluorescence measurement in all measurements. Protein content was determined in parallel plates using the BCA Protein Assay (Pierce, Rockford, IL). Results are expressed as femtomoles of bound rhodamine-phalloidin per milligram of protein. Student's *t*-test was used to discern significance.

Evaluation of G-Actin Content in Drug-Treated Cells

The amount of actin in the soluble globular form (G-actin) was assessed by ultracentrifugation of cell extracts. PC-3 cells plated at 1×10^6 cells/75 cm^2 flask were grown for 18 hr and, while still in log phase growth, exposed to cucurbitacin E (50, 100, and 200 nM) for 4 hr; control cells were untreated. Five milliliters of lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl, pH 7.6, 1 mM sodium EGTA, 2 mM sodium EDTA, 0.1% sodium azide, 0.2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 200 μM Na_3VO_4, 25 μg/mL aprotinin, 25 μg/mL leupeptin adjusted to pH 7.9) was added, and lysates were centrifuged at 5600 *g* for 10 min at 4°. A pre-centrifugation aliquot of the lysate (20 μL) was removed, and equivalent amounts of protein from each sample (2 mg) were ultracentrifuged at 180,000 *g* for 2 hr at 4° [18]. Twenty microliters of supernatant, which contained exclusively the G-actin fraction, was removed, and both pre- and post-centrifugation aliquots were boiled after addition to 20 μL loading buffer (Tris–HCl, β-mercaptoethanol, 2 times; Integrated Separations Systems) at 95° for 10 min; the aliquots were then subjected to SDS–PAGE on 4.5 to 20% gradient gels. Proteins were transferred to PVDF membranes and the blot was incubated for 16 hr in blocking solution [TTS (0.9% NaCl, 0.05% Tween, 200 mM Tris–HCl, pH 7.5) + 3% BSA], then probed by a 1:1000 dilution of anti-actin antibody MAB 1501 (Chemicon, Temecula, CA) in TTS + 0.3% BSA buffer for 60 min, washed twice with TTS, and then thrice with TTS + 0.3% BSA; the blot was re-probed for 30 min with a 1:1000 dilution of rabbit anti-mouse immunoglobulin in TTS buffer, washed, and then incubated for 30 min with a 1:1000 dilution of ^{125}I-Protein A conjugate in TTS buffer, washed, dried, and then placed on Kodak X AR-5 film for 48 hr of exposure at −80°.

RESULTS

Antiproliferative Activity of Cucurbitacin E

In the NCI *in vitro* cell line screen [13], cucurbitacin E inhibited the growth of a variety of carcinoma cell lines after 48 hr of exposure to drug with IC_{50} values in individual cell lines ranging from 13 to 295 nM (Table 1). However, when studied in a screen for growth inhibition of non-immortalized prostate carcinoma explants using 6 days of continuous exposure to drug, IC_{50} values ranging from 2.2 to 11 nM were observed in duplicate experiments in three independent explants (data not shown). Cucurbitacin E was also found to have the following potency (IC_{50} values for cell growth after 2 days of exposure) in immortalized prostate carcinoma cell lines: LNCaP, 7.1 nM; DU145, 9.8 nM; and PC-3, 51.3 nM. In assays that utilized 6 days of exposure to drug, essentially the same potency was observed. There was little recovery from drug effect when medium containing cucurbitacin was removed after a 48-hr exposure, and replaced with drug-free medium for four subsequent days. Thus, we can conclude that although cucurbitacin E appeared to inhibit the growth of many cultured cell lines (Table 1), prostate carcinoma explants and cell lines appeared more sensitive than many when evaluated in 2- or 6-day assays of growth inhibitory effect. We therefore elected to perform further mechanistic studies in prostate carcinoma cells.

Morphologic Changes

We noted a marked alteration of cell morphology in cells exposed to cucurbitacin E. In contrast to control cells (Fig. 2a), the cell membranes of cells exposed to cucurbitacin in many cases had submembranous inclusions or blisters (Fig. 2b). These structures were apparent as early as 30 min after exposure to cucurbitacin. These altered morphologic features did not contain recognizable cellular materials or subcellular structures. Following a 24-hr exposure, many cells contained two nuclei (Fig. 2b), suggesting that cucurbitacin may inhibit cytokinesis, but not karyokinesis. After longer exposures, cells frequently contained more than two nuclei. Cells demonstrating these morphologic changes continued to exclude trypan blue at 24 hr (0.4% in PBS) and ethidium homodimer, indicating intact cell membranes. Esterases remained active and intracellular as demonstrated by fluorescence of calcein AM (data not shown).

Effects of Cucurbitacin E on Cytoskeletal Elements

The prominent morphologic changes described above suggested that cucurbitacin E might affect cytoskeletal elements. Cytochalasin E, which disaggregates filamentous actin, is known to produce cells that undergo karyokinesis without cytokinesis [19], owing to the involvement of actin in the contractile furrow during mitosis to allow cytokinesis [20]. We therefore examined the effect of cucurbitacin on cytoskeletal elements using fluorescent staining. To study changes in actin distribution, cells were stained with rhodamine-phalloidin, a toxin derived from mushrooms which binds selectively to polymerized F-actin [21]. Figure 3c demonstrates F-actin in control cells, where the distribution reflects a delicate network extending continuously through the cytoplasm. Figure 3d demonstrates cucurbitacin E-induced changes after 24 hr. The distribution of F-actin dramatically changed from the homogeneous network in control cells to one or two large intensely fluorescent cytoplasmic accumulations located adjacent to the cell nucleus in treated cells. The intense rhodamine-phalloidin-staining material frequently corresponded to the location of the submembranous blebs observed by phase contrast microscopy (compare panels b and d of Fig. 3). The appearance of cucurbitacin-treated cells was quite different from that seen after cytochalasin treatment, where the actin network remained partially intact, but demonstrated a stippled appearance (data not shown). The cucurbitacin-induced actin accumulations appeared within 1 hr after drug addition, and as exposure was increased, they became larger and

TABLE 1. Growth inhibition by cucurbitacin E in the NCI screen

Cell line	IC_{50} (nM)	Cell line	IC_{50} (nM)
Leukemia/Lymphoma		Melanoma	
CCRF-CEM	32 ± 1	LOX IMVI	13 ± 3
HL-60 (TB)	33 ± 2	MALME-3M	39 ± 3
K-562	63 ± 18	M14	44 ± 4
MOLT-4	240 ± 63	M19-MEL	69 ± 7
RPMI-8226	54 ± 16	SK-MEL-2	24 ± 2
		SK-MEL-28	21 ± 1
Lung carcinoma (non-small cell)		SK-MEL-5	41 ± 2
A549	28 ± 2	UACC-257	35 ± 2
EKVX	69 ± 23	UACC-62	65 ± 8
HOP-62	107 ± 28		
HOP-92	48 ± 9	Ovarian carcinoma	
NCI-H226	27 ± 4	IGROV1	29 ± 3
NCI-H23	127 ± 31	OVCAR-3	51 ± 7
NCI-H460	26 ± 4	OVCAR-4	32 ± 6
NCI-H522	37 ± 4	OVCAR-5	295 ± 112
LXFL 529	34 ± 3	OVCAR-8	147 ± 27
		SK-OV-3	49 ± 1
Colon carcinoma			
COLO 205	34 ± 2	Renal cell carcinoma	
DLD-1	35 ± 3	786-O	36 ± 3
HCC-2998	44 ± 7	A498	27 ± 3
HCT-116	29 ± 8	ACHN	31 ± 3
HCT-15	25 ± 4	CAKI-1	22 ± 0.3
HT29	23 ± 4	RXF393	23 ± 2
KM20L2	32 ± 3	RXF-631	40 ± 9
SW-620	47 ± 6	SN12C	79 ± 8
		TK-10	35 ± 3
CNS		UO-31	38 ± 10
SF-268	27 ± 2		
SF-295	33 ± 6		
SF-539	24 ± 1		
SNB-19	54 ± 11		
SNB-75	21 ± 1		
SNB-78	43 ± 9		
U251	42 ± 12		
XF 498	32 ± 1		

The IC_{50} values shown (means ± SEM; three separate experiments) are the concentrations that inhibited growth of the indicated cell line by 50% in a 48-hr continuous exposure to drug.

more intense in fluorescence. After removal of cucurbitacin E from cells exposed to subtoxic levels of drug, the actin network appeared partially to re-form, although the cucurbitacin E-induced accumulations of F-actin persisted in these abnormally large cells, even 1 week after drug removal (data not shown). Conventional antitumor agents including vincristine, doxorubicin, 5-fluorouracil, cisplatin, camptothecin, and methotrexate at IC_{50} concentrations did not disrupt actin distribution of PC-3 cells.

To determine if cucurbitacin E disrupts microtubules, we stained cells for microtubules using an antibody to β-tubulin (Fig. 4), as well as with FITC-conjugated colchicine (data not shown). Cucurbitacin E did not disrupt the microtubule network, although the occurrence of double nuclei altered the general appearance of these preparations (Fig. 4, a and b). Cucurbitacin E did not alter the appearance of FITC-conjugated colchicine-treated cells.

We stained intermediate filaments using anti-vimentin and anti-pan cytokeratin. In control cells, the vimentin filaments paralleled the cell membrane and formed an extensive intracellular network (Fig. 4c). In cucurbitacin-treated cells, the vimentin filaments followed the outline of the submembranous blebs and protrusions, and the intracellular vimentin network was clearly disrupted (Fig. 4d). Cytochalasin did not disrupt the vimentin network, and no difference in cytokeratin distribution was noted (data not shown).

Effect of Cucurbitacin E on Actin Compartmentation

The morphologic changes described above suggested that the F-actin network is altered after exposure to cucurbitacin. If this hypothesis is correct, an alteration in the amount of F-actin and/or a change in the compartmentation of G-actin may be appreciated. To quantitate the changes observed in F-actin, we measured the fluorescence of rhodamine-phalloidin-stained material in cells exposed to cucurbitacin. Cucurbitacin E (50 nM) significantly in-

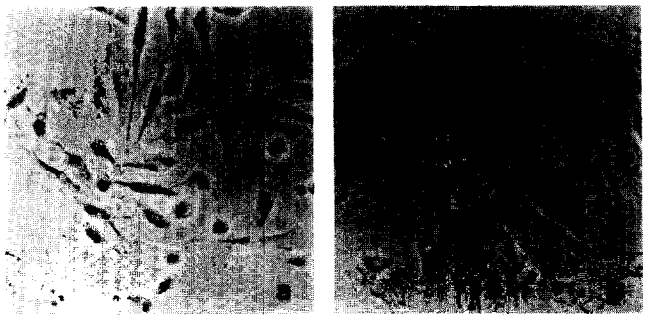


FIG. 2. Effect of cucurbitacin E on the morphology of PC-3 cells. Key: (a) phase contrast of untreated cells; and (b) phase contrast of cells exposed to 50 nM cucurbitacin E for 24 hr. Note abnormal structure below the cell membrane of some cells [open arrow] and double nuclei [closed arrow] in treated cells. Panels a and b, 200×.

creased rhodamine-phalloidin-stained material after 48 hr of exposure (Table 2). This effect was observed at 24 hr with 100 nM cucurbitacin. In contrast, cytochalasin E at 100 nM caused, as expected, an 83% decrease in rhodamine-phalloidin-stainable material at 48 hr (data not shown).

Following ultracentrifugation of cytosol, F-actin sediments to a poorly solubilized pellet, while G-actin remains in the supernatant [18]. Figure 5 shows a western blot which demonstrated no change in the total actin content, while in striking contrast the soluble G-actin was completely absent in cells exposed to concentrations of cucurbitacin E near the IC_{50} for growth for as little as 4 hr. This effect would be concordant with the increase in F-actin ultimately detectable at 48 hr by increased rhodamine-phalloidin binding (Table 2).

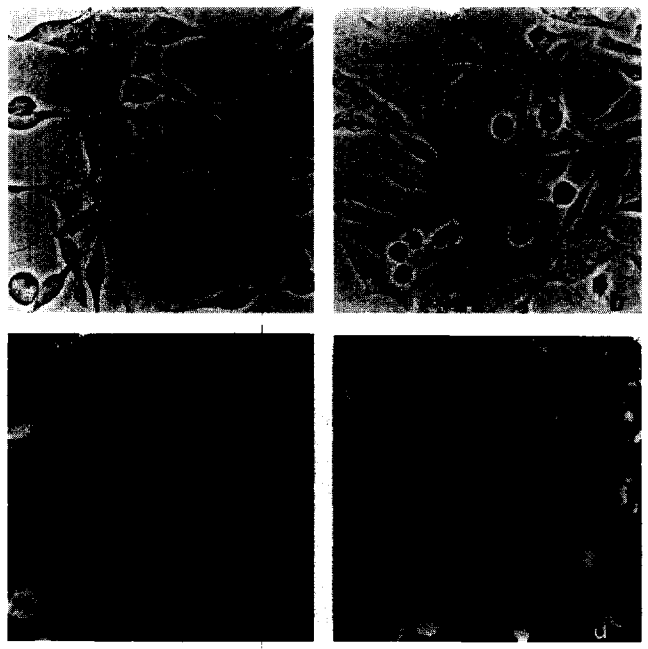


FIG. 3. Effect of cucurbitacin E on rhodamine-phalloidin staining of PC-3 cells Key: (a) control, phase contrast; (b) after 50 nM cucurbitacin E for 24 hr, phase contrast; (c) control, rhodamine-phalloidin staining for F-actin; and (d) 50 nM cucurbitacin E for 24 hr, rhodamine-phalloidin.

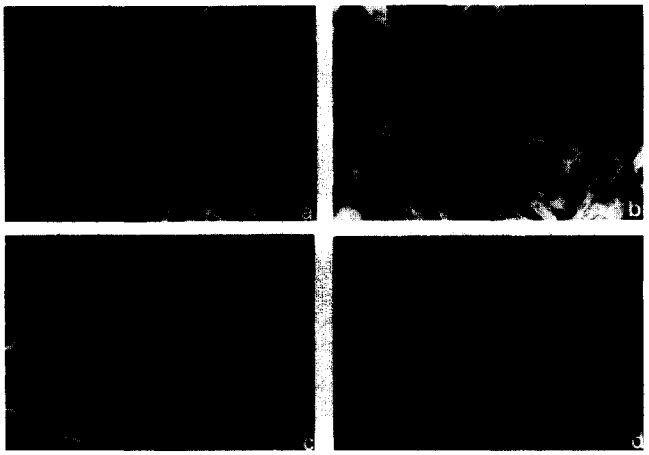


FIG. 4. Effect of cucurbitacin E on β-tubulin and vimentin in PC-3 cells. Key: (a) control, stained with antibody to β-tubulin; (b) 50 nM cucurbitacin E for 24 hr, β-tubulin antibody; (c) control, stained with antibody to vimentin; and (d) 50 nM cucurbitacin E for 24 hr, vimentin antibody. Panels a–d, 200×.

Structure-Activity Relationship for Growth and Actin Disruption

We compared a series of cucurbitacin congeners for cell growth inhibition and for actin microfilament disruption using rhodamine-phalloidin staining of cells exposed to drug for 48 hr. Table 3 demonstrates that the two activities are closely related. Perturbation of the side chain in particular (cucurbitacin I, cucurbitacin D) or C-3 hydroxylation (cucurbitacin Q) reduced but did not eliminate antiproliferative activity, and in parallel decreased actin-disrupting activity. C-23 hydroxylation and reduction of Δ^{22}, as represented in cucurbitacins K and P, abolished both activities completely. Quantitatively similar results were obtained with other cell lines studied. These data are consistent with the postulate that the growth inhibitory potential of the cucurbitacins is related to their effect on the actin cytoskeleton and point to the importance of the

TABLE 2. Rhodamine-phalloidin binding in PC-3 cells

Time of exposure (hr)	Bound rhodamine-phalloidin (fmol/mg protein)		
	CuE (nM)		
	0	50	100
1	12.1 ± 0.3	12.3 ± 0.5	13.4 ± 0.5
4	9.6 ± 0.6	11.1 ± 0.3	10.7 ± 1.0
24	9.0 ± 0.5	0.1 ± 0.5	13.7 ± 0.2*
48	10.5 ± 0.5	15.4 ± 0.7*	17.1 ± 1.2*

Values are means ± SEM, N = 6.

* $P < 0.01$ when compared with cucurbitacin ("0").

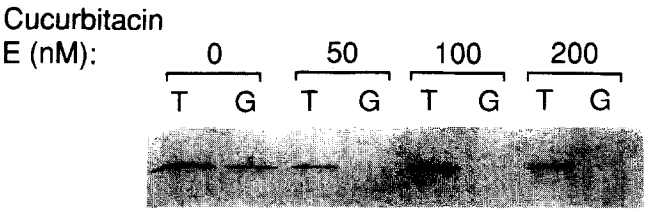


FIG. 5. Western blot analysis of actin following exposure to cucurbitacin. PC-3 cells were exposed to the indicated concentrations of cucurbitacin E for 4 hr, and the total (T) and unpolymerized globular (G) actin fractions were determined after ultracentrifugation as described in Materials and Methods.

unique side chain from C17 in mediating both the antiproliferative and actin-directed activities.

DISCUSSION

The experiments presented here have led to the following conclusions. First, cucurbitacin E potently inhibits cell growth of a variety of tumor cell lines, with notable activity in both primary prostate carcinoma explants and immortalized prostate carcinoma cells. A 48-hr exposure led to persistent growth inhibition of the prostate carcinoma cell lines even after drug removal. Drug exposure led to the development of morphologically abnormal, multinucleated cells. Second, a prominent effect of cucurbitacin E is the disruption of the F-actin cytoskeleton, with altered appearance of rhodamine-phalloidin-stainable material. Consistent with this observation, filamentous, polymerized actin appeared to accumulate in treated cells with the concomitant disappearance of G-actin. Third, cucurbitacin E also changes the distribution of vimentin in association with drug-induced membrane blebs. Cytokeratin filaments and the microtubule cytoskeleton were not altered. Fourth, disruption of the actin cytoskeleton correlates with antiproliferative effects in a series of cucurbitacin congeners.

TABLE 3. Structure-activity relationship of cucurbitacins

Compound (structural change from cucurbitacin E)	NSC	PC-3 IC_{50} (nM)	F-actin disruption (nM)
Cucurbitacin E	106399	50	20
Cucurbitacin B (Δ^1-dihydro)	49451	35	18
Cucurbitacin I (C-24-OH)	521777	223	55
Cucurbitacin D (Δ^1-dihydro, C-24-OH)	308606	367	100
Cucurbitacin Q (Δ^1-dihydro, C-3-OH)	135075	455	100
Cucurbitacin K (Δ^{22}-dihydro, C-23-OH, C-24-OH)	112166	> 1000	> 1000
Cucurbitacin P (Δ^1-dihydro, C-3-OH, Δ^{22}-dihydro, C-24-OH)	135074	> 1000	> 1000

The compounds are listed with their structural differences from cucurbitacin E [1, 8]. The IC_{50} refers to the concentration that inhibited PC-3 cell growth by 50% after 24 hr using the SRB assay as described in Materials and Methods. F-actin disruption refers to the drug concentration where F-actin distribution was detectably altered after 24 hr of exposure, using rhodamine-phalloidin staining.

The specific molecular target of the action of cucurbitacins on the cytoskeleton is unknown. The drug could directly affect actin filament polymerization, as with cytochalasin, phalloidin, and jasplakinolide [22, 23], or it could alter microfilaments via indirect effects on one or more actin-binding proteins [24]. The effects of actin disruption on the cellular economy would obviously be pleiotropic. Actin and actin-binding proteins have been implicated as critical intermediates in several signal transduction pathways controlling cell division [24–27]. Growth factors such as bombesin, which activates the actin-associated focal adhesion kinase, and epidermal growth factor induce demonstrable actin cytoskeleton remodeling [27, 28]. Agents that perturb these pathways in neoplastic cells could also interrupt proliferative signals. The appearance of numerous multi-nucleated cells following cucurbitacin treatment is consistent with a cytokinetic block; other actin-disrupting agents also demonstrate this effect [20]. These drugs disrupt the formation of the actin-derived contractile ring important for separation of cells during mitosis [19]. Cells in cytokinetic block have been reported to be more susceptible to the toxic effects of conventional antitumor therapies [29–32]. The structural relationship of cucurbitacins to cholesterol also suggests the possibility of a partially membrane-mediated effect, such as disruption of microfilament attachment to the membrane, or disruption of regulatory proteins in the membrane which may affect cytoskeletal architecture [33]. Apart from direct cytotoxic effects, the interaction of cucurbitacins with actin may also disrupt motility-based processes such as metastasis and angiogenesis that require an intact actin cytoskeleton. Cytochalasin has, in fact, been reported to inhibit metastasis in animal models [34, 35]. Actin changes analogous to those observed with the prostate carcinoma cells have also been seen in the immortalized human endothelial cell line ECV 304.

Prior to the current study, there had been no cell biological explanation for the mechanism of action of cucurbitacins; the mechanism of actin disruption described here may underlie the toxicological injury observed in *in vivo* models and with mammalian ingestion [1, 9]. The present study suggests that examination of cucurbitacin-treated animals for evidence of cytoskeletal changes in heart and endothelia would be warranted, as a basis for understanding the action of the drug.

In addition to its effects on actin, cucurbitacin E altered vimentin distribution. The expression of vimentin correlates with a more aggressive phenotype in prostate carcinoma [36, 37]. The tail domain of vimentin has been demonstrated to bind actin [38]. While this domain is not required for intermediate filament organization, this interaction with actin has been proposed as the mediator of

vimentin re-organization following actin disruption [38]. Elucidation of the molecular basis for the interaction of cucurbitacins with cytoskeletal elements will be required to address this issue adequately. The prominent effect on cucurbitacin-mediated cytotoxicity by minor changes in its C-20 side chain does, however, suggest a rather specific high-affinity interaction as a potential basis for its effects.

Although several active chemotherapeutic agents disrupt the microtubule cytoskeleton, agents that disrupt other cytoskeletal elements, such as microfilament or intermediate filament networks, remain relatively unexplored as potential therapeutics. Cucurbitacins disrupt these networks at concentrations that parallel their potent antiproliferative activity in immortalized prostate carcinoma cell lines. The experiments presented here direct our attention to the ability of cucurbitacins to affect these targets, and encourage further efforts to develop cucurbitacins as therapeutic agents for prostate carcinoma and other neoplasms.

We wish to acknowledge the assistance of Gurmeet Kaur.

References

1. Lavie D and Glotter E, The cucurbitanes, a group of tetracyclic triterpenes. *Fortschr Chem Org Naturst* **29:** 307–362, 1971.
2. Witkowski A, Woynarowska B and Konopa J, Inhibition of the biosynthesis of deoxyribonucleic acid, ribonucleic acid and protein in HeLa cells by cucurbitacins, glucocorticoid-like cytotoxic triterpenes. *Biochem Pharmacol* **33:** 995–1004, 1984.
3. Witkowski A and Konopa J, Binding of the cytotoxic and antitumor triterpenes, cucurbitacins, to glucocorticoid receptors of HeLa cells. *Biochim Biophys Acta* **674:** 246–255, 1981.
4. Dadayan MA, Panossian AG, Karaguezian KG and Gevorkian GA, Content of prostaglandins E_2, $F_{2\alpha}$ and 5-hydroxyeicosatetraenoic acid in blood of immobilized rats and effect of dihydrocucurbitacin D diglucoside. *Vopr Med Khim* **31:** 98–100, 1985.
5. Gitter S, Gallily R, Shohat B and Lavie D, Studies on the antitumor effect of cucurbitacins. *Cancer Res* **21:** 516–521, 1961.
6. Shohat B, Gitter S and Lavie D, Action of elatericin A on human leukemic and normal lymphocytes. *J Natl Cancer Inst* **38:** 1–9, 1967.
7. Shohat B, Gitter S and Lavie D, Antitumor activity of cucurbitacins: Metabolic aspects. *Cancer Chemother Rep* **23:** 19–24, 1962.
8. Blickenstaff RT, Sterols and cucurbitacins. In: *Antitumor Steroids* (Ed. Blickenstaff, RT) pp 187–191. Academic Press, New York, 1992.
9. Edery H, Schatzberg-Porath G and Gitter S, Pharmacodynamic activity of elatericin (cucurbitacin D). *Arch Int Pharmacodyn* **130:** 315–335, 1961.
10. Kupchan SM, Gray AH and Grove MD, Tumor inhibitors. XXIII. The cytotoxic principles of *Marah oreganus* H. *J Med Chem* **10:** 337–340, 1967.
11. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P and Vaigro-Wolff A, Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* **83:** 757–766, 1991.
12. Peehl D, Erickson E, Malspeis L, Orr J, Mayo J, Camilier R, Monks A, Cronise P, Paull K and Grever MR, Prostate cancer: NCI drug discovery efforts. *Proc Am Assoc Cancer Res* **34:** 369, 1993.
13. Peehl D, Culture of human prostatic epithelial cells. In: *Culture of Epithelial Cells* (Ed. Freshney RI), pp. 160–180. Wiley-Liss, New York, 1992.
14. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF and Jones LW, Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* **17:** 16–23, 1979.
15. Horoszewicz JS, Leong SS, Chu TM, Wajsman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK and Sandberg AA, The LNCaP cell line—A new model for studies on human prostatic carcinoma. *Prog Clin Biol Res* **37:** 115–132, 1980.
16. Mosmann TR and Fong TA, Specific assays for cytokine production by T cells. *J Immunol Methods* **116:** 151–158, 1989.
17. Howard TH and Oresajo CO, The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution and the shape of neutrophils. *J Cell Biol* **101:** 1078–1085, 1985.
18. Rubin RW, Warren RH, Lukeman DS and Clements E, Actin content and organization in normal and transformed cells in culture. *J Cell Biol* **78:** 28–35, 1978.
19. Inoue S, Dynamics of mitosis and cleavage. *Ann NY Acad Sci* **582:** 1–14, 1990.
20. Pollard TD, Satterwhite L, Cisek L, Cordern J, Sato M and Maupin P, Actin and myosin biochemistry in relation to cytokinesis. *Ann NY Acad Sci* **582:** 120–130, 1990.
21. Katsuma Y, Swierenga SHH, Marceau N and French S, Connections of intermediate filaments with the nuclear lamina and cell periphery. *Biol Cell* **59:** 193–203, 1987.
22. Cooper JA, Effects of cytochalasin and phalloidin on actin. *J Cell Biol* **105:** 1473–1478, 1987.
23. Bubb MR, Senderowicz AMJ, Sausville EA, Duncan KLK and Korn ED, Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J Biol Chem* **269:** 14869–14871, 1994.
24. Luna EJ and Hitt AL, Cytoskeleton-plasma membrane interactions. *Science* **258:** 955–964, 1992.
25. Aderem A, Signal transduction and the actin cytoskeleton: The roles of MARCKS and profilin. *Trends Biochem Sci* **17:** 438–443, 1992.
26. Ridley AJ and Hall A, The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70:** 389–399, 1992.
27. Zachary I and Rozengurt E, Focal adhesion kinase (p125FAK): A point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell* **71:** 891–894, 1992.
28. Peppelenbosch MP, Tertoolen LGJ, Hage WJ and deLaat SW, Epidermal growth factor-induced actin remodeling is regulated by 5-lipoxygenase and cyclooxygenase products. *Cell* **74:** 565–575, 1993.
29. Ren L, Zhang H, Yang J and Zhang Z, A sequential study on the use of the cytokinesis-block micronucleus method in mouse splenocytes. *Mutat Res* **301:** 223–227, 1993.
30. O'Neill FJ, Selective destruction of cultured tumor cells with uncontrolled nuclear division by cytochalasin B and cytosine arabinoside. *Cancer Res* **35:** 3111–3115, 1975.
31. Kolber MA and Hill P, Vincristine potentiates cytochalasin B-induced DNA fragmentation *in vitro*. *Cancer Chemother Pharmacol* **30:** 286–290, 1992.
32. Shibamoto Y, Streffer C, Fuhrmann C and Budach V, Tumor radiosensitivity prediction by the cytokinesis-block micronucleus assay. *Radiat Res* **128:** 293–300, 1991.
33. Fenton RG, Kung HF, Longo DL and Smith MR, Regulation of intracellular actin polymerization by prenylated cellular proteins. *J Cell Biol* **117:** 347–356, 1992.

34. Bousquet PF, Paulsen LA, Fondy C, Lipski KM, Loucy KJ and Fondy TP, Effects of cytochalasin B in culture and *in vivo* on murine Madison 109 lung carcinoma and on B16 melanoma. *Cancer Res* **50:** 1430–1439, 1990.
35. Hart IR, Raz A and Fidler IJ, Effect of cytoskeleton-disrupting agents on the metastatic behavior of melanoma cells. *J Natl Cancer Inst* **64:** 891–900, 1980.
36. Fong CJ, Sherwood ER, Sutkowski DM, Abu-Jawdeh GM, Yookoo H, Bauer KD, Kozloski JM and Lee C, Reconstituted basement membrane promotes morphological and functional differentiation of primary human prostatic epithelial cells. *Prostate* **19:** 221–235, 1991.
37. Ramaekers FC, Verhagen AP, Isaacs JT, Feitz WF, Moesker O, Schaart G, Schalken JA and Vooijs GP, Intermediate filament expression and the progression of prostatic cancer as studied in the Dunning R-2237 rat prostatic carcinoma system. *Prostate* **14:** 323–329, 1989.
38. Cary RB, Klymkowsky MW, Evans RM, Domingo A, Dent JA and Backhus LE, Vimentin's tail interacts with actin-containing structures *in vivo*. *J Cell Sci* **107:** 1609–1622, 1994.