Investigating the Affects of Cucurbitacin-I on Cellular Motility

Rebecca LaFleur

University of Connecticut - Storrs, rlafleur01@gmail.com

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Investigating the Affects of Cucurbitacin-I on Cellular Motility

Rebecca LaFleur
Honors Thesis

Major Advisor- Dr. David Knecht

Department of Molecular and Cell Biology
University of Connecticut

May 2008
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Abstract

Cellular migration is an integral component of many biological processes including immune function, wound healing and cancer cell metastasis. A complete model illustrating the mechanism by which cells accomplish movement is still lacking. Exploring the affects of various drugs on cell motility may be instrumental in discovering new proteins which mediate cell movement. This project aims ultimately to characterize the molecular target of the drug Cucurbitacin-I, a natural plant product. This drug has been shown to inhibit migration of epithelial sheets and may have anti-tumor activity.

In this paper, we show that Cucurbitacin-I inhibits the migration of MDCK and B16F1 cells. The drug also affects the integrity of the actin cytoskeleton of these cells by indirectly stabilizing filamentous actin. Cucurbitacin-I does not, however, have an effect on the motility or cytoskeletal morphology of the soil amoeba, Dictyostelium discoidium.

Introduction

Cellular motility is the basis for many important biological processes. Without it, life as we know it simply would not exist. Life’s dependence on cell migration begins during embryonic development. During this process, neurons travel along predetermined pathways within the embryo directed by chemical signals. In addition, mesenchymal cells disperse throughout the embryo to environments which allow them to differentiate into various tissues. Cell migration is also critical for immune function. Upon injury or infection, white blood cells are able to traverse the blood vessel endothelium and enter the surrounding tissue where they seek out and destroy bacterial invaders (1-3). On the other hand, cellular motility can have a pathological significance. Cancer cells are able to break away from the primary tumor and disseminate throughout the body. This process, known as metastasis increases the number of organs affected and greatly complicates treatment (4, 5). Because cell motility plays such a significant role both in health as well
as disease, it is important to understand how and why cells move. This knowledge may provide clues as to how cell motility can be inhibited or promoted.

Although research continues to make strides toward understanding how cells accomplish movement, a complete mechanism for cell migration is still lacking. Although the appearance of cell movement can differ slightly depending on cell type, current literature agrees on a basic model for migration. In this scheme, cells extend a thin membrane protrusion, known as a lamella, forward which is subsequently anchored to the extracellular matrix to provide the cell with traction. The rear of the cell is then retracted resulting in the translocation of the cell body. The efficacy of cell movement is based on how well a cell can couple protrusion and retraction (2, 4, 6, 7). An example of optimized coupling can be found in the fish epidermal keratocyte. This cell maintains a constant fan shape with a wide protruding lamella at the front while the cell body follows closely behind (8). During keratocyte locomotion, protrusion and retraction are closely coupled. In other cell types, such as fibroblasts, the front and the rear of the cell are not as closely coordinated. These cells accomplish movement through a slightly different mechanism. While keratocytes are mostly devoid of focal contacts, fibroblasts form focal adhesions in the front and rear of the cell. Fibroblasts first extend a lamella which is then anchored to the substrate through focal adhesions (8). Once the protrusion is stabilized, adhesions at the rear of the cell are disassembled to allow the cell body to move forward. If these adhesions are not removed, they will either be torn from the substrate as the front of the cell moves forward or the cell will stop moving forward (9).

Cell shape and motility are ultimately dictated by a meshwork of filaments known collectively as the cytoskeleton. The cytoskeleton is composed of three major classes of protein including microtubules, microfilaments and intermediate filaments. Of these three components, actin microfilaments are primarily responsible for the motility machinery. Within the cell, actin exists as monomeric (G-actin) which is polymerized into filamentous
(F-actin). Actin filaments have inherent polarity and contain both a barbed and a pointed end. Lengthening of the filament can take place at either end, however polymerization occurs at a much faster rate at the barbed end and barbed ends are usually near the plasma membrane (8). As filaments elongate, protrusive force is generated and the membrane is pushed outward from the cell body. At first it seems counterintuitive that a filament which needs to add subunits in order to grow would be able to maintain enough contact to push a membrane forward. One theory for the mechanism of this occurrence is known as the tethered ratchet model. This model states that the actin filament is only transiently attached to the membrane. It is also possible that the filament never becomes attached to the membrane. Brownian motion causes fluctuations in the membrane, and if it deforms far enough, a new G-actin monomer will be able to join the end of the filament. This prevents the membrane from flexing back to its original shape and must remain extended to accommodate the lengthened filament (10). The action of multiple filaments polymerizing in this manner results in membrane protrusion. Actin also provides the machinery for retraction of the rear of the cell. In some cell types, actin filaments in the rear of the cell are gathered into antiparallel bundles and slide past each other through the interaction with myosin II (8).

Actin filaments are capable of polymerizing in vitro in a solution of only adenosine triphosphate (ATP), actin monomers and salt. The filaments that form, however, are unable to interact with each other to form the elaborate actin structures found in the cell. This difference occurs because the cellular environment contains a number of proteins which regulate actin dynamics, known collectively as actin-binding proteins (ABPs). For example, ABPs can facilitate polymerization (profilin), sever filaments (cofilin), prevent severing (tropomyosin), bundle (alpha-actinin) or branch (Arp2/3) (4). The presence of different ABPs and different signaling pathways within the cell allow for the generation of vastly different actin structures within the same cell.
Actin and its binding proteins are controlled through various upstream signaling pathways. A number of small guanosine triphosphatases (GTPases) including Rac, Rho and cdc42 have been widely implicated in regulating cell shape and motility (11). Differential protein activation determines which cellular structures will form. For example, Rac promotes formation and extension of lamellae, Rho facilitates bundling of contractile actin bundles such as stress fibers and cdc42 is involved in filopod formation. These relationships have been derived mainly through overexpression experiments. For instance, overexpression of cdc42 results in the formation of numerous filopodia (6).

Small GTPases also regulate other cell processes such as cell growth, cell-cell adhesion and cell adhesion to the ECM (2). Rac has been implicated in the formation of focal complexes within the lamella. These complexes are smaller and more dynamic than focal adhesions formed by Rho in the rear of the cell (11). Differential regulation of cell shape and adhesion are crucial to successful cellular locomotion.

The plasticity of actin and its ABPs can be illustrated by comparing two major organelles of motility, the lamellipod and the filopod. Both structures are produced in the anterior portion of a cell’s leading edge during motility, however, they differ greatly in their underlying actin structures. The lamella is a thin protrusion, about 200nm thick, which is composed of a meshwork of polarized, branching actin filaments (4, 8). Barbed ends are oriented towards the anterior of the cell to facilitate protrusion. The branching of actin filaments within the lamella is due to the presence of the Arp2/3 complex which nucleates new filament branches off existing filaments. Arp2/3 not only induces polymerization of the new branch, but also caps the pointed end of the new branch to protect it from disassembly (4). Arp2/3’s role in actin nucleation has been supported by in vitro assays in which Arp2/3 is able to branch filaments and through immuno-electron micrographs of the lamella (7). In contrast, filopodia are thin hairlike membrane protrusions that extend past the cell edge and contain parallel bundles of actin held
together by actin bundling proteins such as fascin and fimbrin. Like lamellae, filopodia also harness the protrusive force of actin polymerization, but they do not contain Arp2/3 and thus they do not branch (7). Filopodia and have been implicated in providing the cell with adhesions to the extracellular matrix because adhesion molecules such as cadherin and integrin localize to their tips. Filopodia are also thought to be a site for signal transduction and may play a role in steering during directed cell movement such as chemotaxis (6). During chemotaxis cells sense and move towards a gradient of chemoattractant molecules.

Advances continue to be made in the study of cellular motility. These strides can be attributed, in part, to the development of innovative assays which overcome the difficulties of researching cell locomotion. Actin rich structures such as lamellapodia and filopodia are difficult, if not impossible, to isolate from the cell (4). Therefore scientists have developed several model systems which are analogous to the leading edge of a cell. Scientists have replicated motility in a test tube containing only actin, Arp2/3 cofilin and capping protein. In this assay, activated beads are placed in the motility solution and are moved through the solution powered by the force of polymerizing actin. The assay can be tailored to study the effects of different ABPs on actin dynamics (10).

Pathogens have also been exploited in the study of cellular migration. Intracellular bacteria such as Listeria and Shigella propel themselves through the cytoplasm by polymerizing a comet tail of cellular actin (10). Listeria expresses a protein, Act A, located on its surface which is able to activate the Arp2/3 complex.Activated Arp2/3 recruits actin to polymerize behind the bacteria and hence propel it forward. This is analogous to actin driven lamellar protrusion. Recent studies have shown that Listeria construct two structurally different actin tails depending on the bacteria’s position within the cell. Listeria which protrude beyond the cell edge have long linear tails whereas
Listera within the cell have branching tails. This difference is thought to be due to differential ABP recruitment (4).

Fluorescence microscopy has provided a way to study the dynamics of cellular motility in real time. Protein tags such as green fluorescent protein (GFP) and red fluorescent protein (RFP) can be conjugated to endogenous cellular proteins allowing researchers to track protein localization during locomotion. Multiple different proteins can be tracked at once if they are labeled with different tags. Light of a specific wavelength is used to excite each fluor. The images produced by each fluor can then be overlaid to determine how proteins localize in relation to one another. Fluorescence Speckle Microscopy (FSM) has been used to visualize treadmilling of actin filaments. During FSM, a small amount of fluorescently labeled actin is injected into a cell. The incorporation of the fluorescent monomers into F-actin is then tracked (8).

The experiments previously discussed have focused on the study of single cell locomotion. Researchers have also investigated the dynamics of cell sheet migration. A common example of this occurs during epithelial wound healing. Wound closure may provide insight into the mechanism of cell movement because wound healing is driven by cell protrusion and morphological change rather than proliferation (2). Dr. Fenteany’s lab in UConn’s Chemistry department has utilized a wound healing assay to study cell motility. This technique has been used to screen chemical compounds to find those which effect cell sheet migration. A confluent sheet of Mandin-Darby Canine Kidney (MDCK) was grown to confluence and a reproducible wound was made in the monolayer. The rate of wound closure in the presence of compound was monitored and compared to a control compound-free wound (2). Compounds which increase the rate of wound healing may have applications in treatment of slow healing wounds, especially those found in diabetic patients. Inhibitors of cell migration may be important in
preventing the metastasis of cancer cells (3). However, before a drug can be used in medicine, it is important to identify the compound’s molecular targets.

Using the wound closure assay, the Fenteany lab has identified Cucurbitacin-I as an anti-migratory compound, but a complete list of its molecular targets is lacking. Cucurbitacins are a family of toxic, naturally occurring compounds which have been isolated from several plant species. The Cucurbitacins are divided in to twelve groups, denoted A through T, based on differences in molecular structure (12). Past research has found that the Cucurbitacins have anti-tumor, anti-inflammatory and analgesic effects (12, 13). The anti-tumor activity of the Cucurbitacins was studied in the 1960’s, in hopes of finding a drug treatment for cancer, however, the cytotoxic affects of the drug seemed non-specific (12). Using various cell types as model systems, some Cucurbitacins have been found to inhibit proliferation, adhesion and to disrupt the actin cytoskeleton (14).

More recently, research has identified Cucurbitacin-I as an inhibitor of the Janus activated kinase/signal transducer and activator of transcription (JAK/STAT-3) signaling pathway. JAK is a non-receptor tyrosine kinase which regulates the activity of STATs. STAT is a transcription factor which, when phosphorylated by JAK, will translocate to the nucleus where it will bind DNA and regulate transcription (14). Although there are several different STATs expressed in mammalian cells, Cucurbitacin-I specifically affects STAT-3. The JAK/STAT-3 pathway has been shown to affect cell proliferation and dedifferentiation (15). Interestingly, certain cancer cells have constitutively activated STAT-3. Animal research has indicated that blocking STAT-3 activation will inhibit tumor growth. Cucurbitacin-I decreases cellular levels of activated JAK and STAT-3 therefore lowering STAT-3 DNA binding. This effect suggests the Cucurbitacin-I may be usable as an anti-cancer drug. However specific molecular targets need to be defined before the drug can be fully exploited. Because JAK and STAT levels are both lowered, it is unlikely
that either protein is the target of the drug. Scientists are now considering upstream effectors of JAK as possible targets (14). JAK/STAT-3 has also been implicated as a mediator of cell migration, yet discrete evidence is still lacking (15). Somewhat less defined is Cucurbitacin-I’s effect on the actin cytoskeleton. It is unclear whether the drug’s affects on the JAK/STAT-3 pathway are independent of its affects on the cytoskeleton (13).

My study utilizes several different cell types as model systems to investigate the effects of Cucurbitacin-I on cell migration and the cytoskeleton. MDCK epithelial cells were observed in sheets during wound healing and as single cells. B16-F1 motile mouse melanoma cells were used to study the drug’s effects on single migrating cells. The drug was also screened for effects on Dictyostelium discoidium, a eukaryotic amoeba. In nature Dictyostelium can be found in soil feeding on bacteria. Dictyostelium has been used as a model system for studying many aspects of cell biology, including cell motility. Axenic strains of Dictyostelium have a short generation time and are easily maintained in broth media. Therefore Dictyostelium may be useful to screen for drugs which affect cell motility in a high throughput assay.

Methods

Cell Culture

*Dictyostelium discoidium* strain Ax2 transformed with a GFP-ABP120 fusion protein was grown in HL5 nutrient media at 21°C. Cells were provided by Dr. Knecht.

Madin Darby Canine Kidney (MDCK) epithelial cells transformed to express RFP-actin were grown in Modified Eagle’s Medium containing 0.1% nonessential amino acids, ampicillin/streptomycin antibiotic and 10% FBS at 37°C and 5%CO2. Cells were provided by Dr. Dani Janzen.
B16F1 mouse melanoma cells transformed to express RFP-actin were grown in Delbucco’s Modified Eagles Medium with 0.1% D-glucose, 0.1% L-glutamine, amp/strep and 10% FBS at 37°C and 5% CO2. Cells were provided by Dr. Dani Janzen. All cells were passaged regularly upon reaching confluency.

*Drug Preparation and Storage*

A 10mM stock of Cucurbitacin dissolved in dimethlysulfoxide was obtained from the Fenteany lab. A 10uM working stock was prepared by adding 1ul drug to 1mL media. Drug was stored at -20°C. Each experimental control solution contained the same amount of DMSO as the drug dilution.

*Microscopy/Imaging*

During imaging, mammalian cells were maintained at 37 °C using a Bioptechs dish heater. Imaging was performed using a Zeiss widefield automated microscope. Time lapse images were acquired using Openlab software and subsequently compiled and analyzed using ImageJ. Motility was quantified using the ImageJ MTrackJ plug-in.

*Wound Healing Assay*

Bioptechs dishes were coated with 5ug/mL laminin. Five microliters 1ug/mL laminin solution was combined with 995ul phosphate buffered saline. 500ul of this solution was added to a single Biopechs dish. Dishes were then incubated at 37 degrees Celsius for one hour. A confluent plate of RFP-actin expressing MDCK cells were trypsinized and resuspended in 10mL MEM. One milliliter cell suspension containing 2 x 10^5 cells/mL was placed into the Bioptechs dish. Cells were allowed to attach and form a confluent sheet overnight. Before imaging, cells were placed in 500ul HAMS bicarb-free media containing 10% FBS. A single scratch wound was created in the monolayer
by scraping the surface of the dish with a 200ul pipette tip. The wound was then imaged for several hours. Then 500ul of medium containing 400nm Cucurbitacin-I was added to the dish to make a final concentration of 200nm Cucurbitacin-I. Images were taken for several more hours. Wound closure was evaluated based on rate of the wound over time before and after drug addition. Since the wound edge was not even, measurements from the top, middle and bottom of the wound were taken to evaluate degree of closure. The distance from one wound edge to the other at each of these three points were compared for closure during a two hour interval both with and without drug. These distances were converted to percentages by dividing the distance of wound closure by the width of the whole wound.

Random Motility Assay

Cells were plated on 5ul/mL laminin coated Bioptechs dishes at low density and allowed to settle overnight. Imaging was done before and after drug addition. A 2x drug solution was added to existing cell media to bring the drug to the final concentration.

Determination of G: F-actin ratio in vitro

Actin seeds were formed by incubating G-actin overnight on ice (40ul 5x ISAP buffer, 20ul 20uM G-actin, 140ul distilled water). Actin was polymerized the next morning at ambient temperature for one hour (75ul actin seeds, 15 ul 5x ISAP buffer, 7.5 ul 20uM G-actin, 52.5ul distilled water). Four sample tubes were then set up, one containing 200nm Jasplakinolide, one with 200nm Cucurbitacin-I and one with 1.6 uM Rhodamine Phalloidin stock and the last with no drug addition. Aliquots of 15ul polymerized actin were added to each tube and then the actin was diluted below the critical concentration by addition of depolymerization buffer (5mM Tris pH=7.8, 0.2mM CaCl2, 0.2mM ATP) to a final volume of 150ul. Tubes were incubated overnight at 4°C. Samples were
centrifuged in an airfuge for one hour, pellets resuspend in 20ul 2x sample buffer. Also, freshly polymerized actin was centrifuged in parallel. Samples were denatured in SDS sample buffer and loaded onto a 10% SDS-PAGE. The gel was stained with GelCode Blue G-250 and destained in deionized water.

**Determination of G:F-actin ratio in vivo**

A confluent p100 plate of MDCK cells was trypsinized and split into a six well plate. The cells were allowed to settle for three to four hours. Three of the six wells were treated overnight with DMSO, Cucurbitacin-I and Jasplakinolide as indicated in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>5% DMSO</th>
<th>10 uM Cucurbitacin</th>
<th>400x Jas</th>
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<tr>
<td>Control</td>
<td>80 ul</td>
<td>-</td>
<td>-</td>
<td>3.28 mL</td>
</tr>
<tr>
<td>200 nm Cucurbitacin</td>
<td>-</td>
<td>80 ul</td>
<td>-</td>
<td>3.28 mL</td>
</tr>
<tr>
<td>200 nm Jasplakinolide</td>
<td>80 ul</td>
<td>-</td>
<td>1 ul</td>
<td>3.28 mL</td>
</tr>
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Table 1

Media was aspirated from each well and two milliliters drug media were added to wells one through three. The remaining drug dilutions were stored at -20C overnight and used to treat wells four through six the next morning. Following incubation the cells were harvested into 1.5 mL ependorf tubes and spun down at 5,000 rpm for five minutes. Supernatant was aspirated and cells were resuspended in 250 ul lysis buffer (50mM PIPIES pH=6.9, 50mM NaCl, 5mM MgCl2 5mM EGTA, 5% glycerol, 1% Triton-X, 0.1% NP-40, 0.1% Tween 20, 0.1% 2-mercaptoethanol, 1mM ATP, 1% PMSF, 1% TAME, 1% TPCK, 1% Aprotinin, 1% Pepstatin, 1% Chymostatin). Cells were incubated on ice for 30 minutes. Samples were spun at 9,000 rpm for 10 minutes at 5°C. The supernatant (G
fraction) was removed and placed in a sterile 1.5 mL tube. The pellet (F fraction) was resuspended in 250ul lysis buffer. 250ul 2x loading buffer was added to each fraction. Samples were boiled at 100°C for 5 minutes on a heat block. 15 ul of each sample was fractionated using a 10% SDS-PAGE gel. The protein was then electroblotted onto a nitrocellulose membrane at 12V for 2.5 hours in toulbin buffer (0.51% Tris, 1.9 % glycine, 20% methanol). The membrane was blocked in non-fat dry milk for one hour, incubated in 1:500 mouse anti-actin antibody for one hour followed by one hour incubation in 1:5000 goat anti-mouse conjugated with alkaline phosphatase. Development was examined using 22.5 mL development buffer (12g Tris Base, 095g MgCl2, 5.85g NaCl in one liter, pH 9.5), 2.5mL NitroBlue tetrazolium (1mg/mL in development buffer) and 2.5 ul bcip (50mg/mL in DMSO).

Data and Results

Dr. Fenteany’s lab identified Cucurbitacin-I as an inhibitor of epithelial wound closure. His lab analyzed wounds for percent closure at low magnification. In order to gain more information about the nature of Cucurbitacin-I’s effect on the during wound closure, we wounded a monolayer of RFP-actin expressing MDCK cells and visualized the wound edges with a 40x oil objective. Using time lapse microscopy the wound could be seen closing slowly and steadily for two hours in the absence of drug. Upon drug addition, wound edge translocation was greatly inhibited within minutes. Cells at the wound edge seemed unable to move any further into the cleared area. As treatment continued, actin rich lamellae could still be seen extending from the cells on the wound edge into cleared zone. Over a two hour period in control media, about 21% wound closure occurred at a rate of about 0.45um/min. After the addition of 200nM Cucurbitacin-I, healing was visibly slowed within five minutes. During the two hour time interval following drug addition, the wound only closed by about 6% and the rate of
closure had been reduced 4.5x to 0.099um/min (Figures 1 and 2). Interestingly, after several hours incubation in drug the wound edge again began to migrate into the cleared area, however, instead of maintaining a confluent sheet, movement seemed uncoordinated. Cells capable of movement seemed to tear away from the sheet pulling the epithelial sheet behind them. These data support the observation that Cucurbitacin-I retards the rate of wound closure. The data also indicate that while cells can still protrude lamellae and some can still migrate, the cells are unable to coordinate wound closure.

We then explored the effects of Cucurbitacin-I on a different cell system and found that Cucurbitacin-I inhibits the translocation of B16F1 in a rapid and reversible manner (Fig. 1). When 250 nm Cucurbitacin-I was applied to migrating B16F1 cells, translocation ceased within five minutes. The cells appeared to freeze in place, while maintaining normal morphology. Although lamellae could be seen protruding from several cells, at low magnification we could not tell whether membrane ruffling was occurring during treatment. As drug incubation continued, the cells became increasingly rounded. Cells were left in drug for a total of 45 minutes. Upon removal of drug, cells continued to retract for about 30 minutes. The first cells began to regain movement one hour after drug removal. However, not all cells recovered at the same rate. Cells which were moving quickly and persistently before treatment were among the first to recover. Full recovery of the population occurred between four and five hours following drug wash out. Cucurbitacin-I does not affect the motility of Dictyostelium cells (data not shown). No difference in speed or morphology was seen at concentrations as high as 1.2uM.

Having shown that Cucurbitacin-I has a rapid and reversible effect on cellular translocation in B16F1 cells, we then looked more closely at the drug’s effect on the actin cytoskeleton. To do this, we imaged RFP-actin expressing B16F1 and MDCK cells in the presence of Cucurbitacin-I. We found that prolonged incubation in Cucurbitacin-I
caused actin aggregates to form MDCK cells (Fig. 3). Before drug addition, RFP-actin produces a diffuse signal throughout the cytoplasm. Areas of active actin polymerization, such as the lamellar edge produce a brighter signal; however no aggregation of actin is noted at this point. Upon addition of 200nM Cucurbitacin-I, cells cease translocation within minutes. However, cells are still able to extend lamellae during the first hour to hour and a half of treatment. This indicates that cells are still capable of actin polymerization in the early stages of drug treatment. After one and a half hours, cells cease lamellar extension, what appear to be stress fibers thicken and small punctate aggregates form throughout the cytoplasm. As treatment progresses, aggregates become larger while and cytoplasmic signal fades. By three and a half hours, cells have retracted all processes and the cells appear to have shrunken as they round up. Upon removal of drug, cells begin to spread out, extend processes and within 12 hours, recover movement. After recovery, aggregates remain in the cytoplasm for several days. Similar actin aggregation is seen B16F1 cells in response to Cucurbitacin-I treatment. Figure 4 shows B16F1 cells which were treated in only 25nm Cucurbitacin-I. Aggregates form at about one hour. Aggregation continues throughout treatment, but effects are less severe, probably due to the lower concentration of drug. These data show that concentrations of Cucurbitacin-I as low as 25nm are able to effect cells.

Interestingly, Cucurbitacin-I did not cause actin aggregation in Dictyostelium (Fig. 5). It has been shown that actin aggregates do form in Dictyostelium in during treatment with actin stabilizing drug, Jasplakinolide. Cells remained motile despite treatment, but moved at a much slower rate compared to untreated cells (17). We wondered if Cucurbitacin-I was also causing actin stabilization. In an attempt learn more about the mechanism by which Cucurbitacin-I causes actin aggregation, we compared its effects to those of Jasplakinolide. Using RFP-actin expressing cells we determined that Jasplakinolide also causes actin aggregation in MDCK cells (Fig. 6).
Since Jasplakinolide treatment caused similar actin aggregates to form in MDCK cells we then tested to see if this drug would inhibit cellular translocation in a similar manner as Cucurbitacin-I. When a population of migrating B16F1 cells was treated with Jasplakinolide cells ceased translocation within five minutes (Fig. 7). Upon drug addition, cells appeared to freeze in place. As incubation continued, cells became retracted all processes and became rounded. Cells were incubated in drug for a total of two hours. As with Cucurbitacin-I, translocation resumes after drug removal. The first cells begin to resume migration with four hours of drug wash out. Recovery of the entire population is seen between eight and nine hours. A shorter recovery time was noted following Cucurbitacin-I treatment. It is possible that the cells took longer to recover from Jasplakinolide than Cucurbitacin-I because the cells were treated with Jasplakinolide for twice as long.

Because treatment with Cucurbitacin-I and Jasplakinolide both caused similar effects on the actin cytoskeleton and gross motility, we wanted to determine if the two drugs were acting by the same mechanism. In order to explore this, we looked more closely at the effects of each drug on filamentous actin. Since Jasplakinolide is a known actin stabilizer, we wondered if Cucurbitacin-I was also stabilizing actin. To determine if this were true, we preformed an *in vitro* assay in which polymerized actin was treated with either Jasplakinolide or Cucurbitacin-I (Fig. 8). Each sample was then diluted in order to promote actin depolymerization. While Jasplakinolide treated actin was stabilized despite dilution, Cucurbitacin-I treated actin depolymerized such that the resulting F-actin band resembled the negative control which contained untreated actin. These results indicate that unlike Jasplakinolide, Cucurbitacin-I does not directly stabilize actin filaments.
Since fluorescence microscopy had shown that both drugs cause aggregation of F-actin, we explored the possibility that Cucurbitacin-I may be indirectly stabilizing actin filaments. We hypothesized that Cucurbitacin-I may acting on a protein within an actin regulatory pathway. To test this hypothesis, we performed an in vivo assay to looking at the distribution of filamentous and monomeric actin within cells both before and after drug treatment. Healthy cells contain both F and G-actin. We hypothesized that if Cucurbitacin-I was stabilizing F-actin within cells, the cellular ratio between G and F-actin pools would be shifted toward filamentous actin. Figure 9 shows the results of this experiment in which MDCK cells were incubated in growth medium, Jasplakinolide or Cucurbitacin-I. Cells were then assayed for their monomeric and filamentous actin content. Treated cells were lysed and the resulting suspension was centrifuged such that F-actin would precipitate while G-actin remained in suspension. F and G-actin fractions of cell lysate were run on a 10% acrylamine gel and actin bands were identified by western blot with an anti-actin antibody. Cells treated overnight with Cucurbitacin-I and Jasplakinolide both contained a higher ratio of F to G-actin than did the control cells. While control cells contained a ratio of 0.52 F to G-actin, Jasplakinolide and Cucurbitacin treated cells contained ratios of 3.34 and 2.26, respectively. This experiment was repeated on cells incubated for only two hours in Cucurbitacin-I to determine if the shift in F-actin occurred earlier or later in treatment. We found there was still a shift from G to F-actin even at the two hour time point (Fig. 10) Control cells and Cucurbitacin-I treated cells have F to G-actin ratios of 0.19 and 2.49, respectively. This indicates that although the aggregates are not very large at two hours, there is still an increased amount of F-actin within the cell.
Discussion

Previously published data has shown that Cucurbitacin-I is an inhibitor of the JAK/STAT signal transduction pathway (14). This pathway ultimately regulates transcription factor binding to DNA. We have shown that Cucurbitacin-I is a potent inhibitor of cellular motility which also disrupts the actin cytoskeleton. Researchers have wondered whether Cucurbitacin-I’s effect on transcription is independent or interdependent of its effects on the actin cytoskeleton. It is well known that altering transcription takes hours to effect the cell. Our data has shown that inhibition of motility and the drug’s effects on the cytoskeleton occur within minutes of drug addition. This suggests that Cucurbitacin-I’s effect on transcription does not cause motility inhibition or cytoskeletal abnormalities. It is possible that Cucurbitacin-I’s disruption of the cytoskeleton may lead to transcriptional effects. It is probable that the two effects are both caused by one upstream target because it is unlikely that Cucurbitacin-I binds to two different target molecules, one that controls transcription and one that regulates actin.

One of the most striking cellular abnormalities observed during treatment with Cucurbitacin-I is the formation of large cytoplasmic actin aggregates. After about one hour of drug incubation, actin structures appear thicker throughout the cytoplasm. Some of these structures look similar to stress fibers. As treatment continues these fibers seem to condense into aggregates. Once these aggregates have formed, they persist within the cell for several days after drug removal. It is tempting to think that these aggregates would cause a defect in cell motility, but that seems not to be the case. Interestingly, the inhibition of motility and formation of aggregates occur at drastically different time points during treatment. Inhibition of motility occurs within minutes while aggregate formation does not begin until at least one hour of treatment. This indicates that the aggregates themselves are not what cause the cells to cease translocation. In fact, our data has shown that cells will recover movement after drug removal while
cytoplasmic aggregates are still present. Thus the inhibition of motility is a short term and reversible effect whereas actin aggregation is a longer term effect which may be irreversible as complete clearance of aggregates was never observed.

The rate of recovery from drug treatment seems to be dependent on the length of time in drug. B16F1 cells that have been treated for 30 minutes in Cucurbitacin-I recover movement within several hours. B16F1 cells that have been incubated in drug for three to four hours begin to recover around 12 hours after drug removal. This suggests the rate of recovery is dependent on the length of treatment. This time difference may occur because after several hours of treatment most cellular actin in sequestered in actin aggregates and therefore can not be used by the cell. Perhaps after actin aggregation has occurred, recovery is dependent on the synthesis of new G-actin.

In an attempt to further characterize the effects of Cucurbitacin-I, we compare its action to that of actin stabilizing drug, Jasplakinolide. Aggregates caused by Jasplakinolide are visually indistinguishable from those induced by Cucurbitacin-I. We have found that although both of these drugs have similar downstream effects, they do not act by the same mechanism. Our data has confirmed that while Jasplakinolide directly stabilizes F-actin, Cucurbitacin-I does not. We have shown that in contrast to Jasplakinolide, Cucurbitacin-I is incapable of stabilizing F-actin filaments in vitro. Interestingly, both drugs cause an apparent stabilization of F-actin in vivo. This may imply that Cucurbitacin-I's target is a protein in a signaling pathway which is involved in actin regulation.

We have shown that cells are still capable of protrusion during the early stages of drug treatment. This suggests that Cucurbitacin-I does not inhibit actin polymerization. Conversely, it is possible that Cucurbitacin-I is interfering with the depolymerization of actin. Our in vivo data shows that Cucurbitacin-I causes an increase in the amount of F-actin and a decrease in G-actin within cells. This may be because Cucurbitacin-I
prevents the depolymerization of existing actin filaments. Since we know from *in vitro* data that Cucurbitacin-I does not directly interact with actin, it may be possible to conclude that the target of Cucurbitacin-I may lie somewhere within the regulatory pathway for depolymerization. Since the mechanism for actin depolymerization is not fully defined, it is difficult to speculate about which proteins in this mechanism may be affected.

Soil amoeba, Dictyostelium discoidium has provided us with further clues about Cucurbitacin-I’s action. Interestingly, Cucurbitacin-I does not have any apparent effect on Dictyostelium. Despite overnight incubation in 1.2uM drug, Dictyostelium do not show changes in motility or cytoskeletal morphology. Dictyostelium are often used as model systems for higher organisms because they contain many proteins which are homologous to mammalian proteins. The fact that Dictyostelium are not affected by Cucurbitacin-I suggests that the target of Cucurbitacin-I does not have a homolog in Dictyostelium. This information is consistent with our other data that Cucurbitacin-I does not directly stabilize actin because actin is a conserved protein in both cell systems.

**Acknowledgements**

I would like to thank Dr. David Knecht for inviting me to work in his lab and for his guidance and support throughout my project. Also, special thanks to Dr. Dani Janzen for all her help and for teaching me new laboratory techniques. Thank you to Dr. Carol Norris for the help using the microscopy facility. Finally thanks to all the members of the Knecht Lab; Dr. Dani Janzen, Dr. Fei Xue, Renee Gilberti, Charito Romeo, Garauv Joshi, Lindsey Costanitini, Ran-der Hwang, Chin-chi, Ross Bickford and Kate Grive.
References


Figure 1: Cucurbitacin-I Inhibits Epithelial Sheet Migration

RFP-actin expressing MDCK cells were plated and imaged as described in the materials and methods section. A single scratch wound was created in the monolayer and the wound edge was filmed for two hours without drug and then the media was changed to media containing 200nm Cucurbitacin-I. Cells were filmed for two additional hours. Images shown are a result of fluorescence imaging. Cells were exposed to Texas-red light and in response, fluorescence light was emitted from RFP fused actin.
Figure 2: Cucurbitacin-I Slows Rate of Wound Healing

Cells were prepped and imaged using same protocol as figure 1. This figure shows fluorescent images of MDCK RFP-actin cells during wound closure. There is a 4.5x difference in speed between control and drug conditions.
Figure 3: Cucurbitacin-I reversibly inhibits motility of migrating B16F1

B16-F1 cells were grown overnight on laminin coated Bioptechs dishes and then imaged at 37°C with a 10x objective. Cells were filmed for 45 minutes in the absence of drug and then drug was added and cells were filmed for an additional hour. Drug was then removed and cells were filmed until movement resumed. Tracks were added to images to follow movement during A) 45 minutes before drug B) 45 minutes drug treatment C) 2 hours after drug removal D) 5 hours after drug removal.
Figure 3: Cucurbitacin-I causes actin aggregation in MDCK cells

MDCK RFP-actin cells were plated at low density in MEM media in laminin coated bioptechs dishes and allowed at attach overnight. Immediately before imaging MEM was replaced with 500ul HAMs bicarb free media containing 10% FBS. Cells were imaged before addition of drug (0 hr) and then 500ul additional media containing a 2x concentration of Cucurbitacin-I was added to the cells. Images were collected every five minutes for 4.5 hours.
Figure 4: Cucurbitacin-I causes Actin Aggregation in B16F1 Cells

Followed same procedure as in Figure 3 except RFP-actin B16F1 cells were treated with 25nm Cucurbitacin-I. These imaged were captured using fluorescence microscopy. Aggregation begins at 1 hour and continues throughout imaging.
Figure 5: Cucurbitacin-I does not affect the morphology of the Dictyostelium actin cytoskeleton

Dictyostelium cells transformed to express RFP ABD-120 were plated in p35 dishes at low density in HL5 media and allowed to settle several hours. Cells were then placed in 1 ml of HL5 containing A) 0.25% DMSO B) 100nm Cucurbitacin 0.25% DMSO and C) 1250nM Cucurbitacin, 0.25% DMSO. Cells were incubated overnight at 21 degrees Celsius and imaged the following morning. Several still shots were taken of each plate and the cells pictured below are representative of each population.
Figure 6: Jasplakinolide causes similar cytoskeletal changes in MDCK cells as Cucurbitacin-I

Cells were prepared and imaged as in figure except 200nm Jasplakinolide was used instead of Cucurbitacin-I.
Figure 7: The actin stabilizer, Jasplakinolide, also reversibly inhibits cellular motility

B16-F1 cells were plated and imaged as indicated in Figure 2 except Jasplakinolide was used. A) Cells are spread and moving before drug addition B) Cells are rounded following one hour in drug C) Cells spread out again and resume movement 9 hours after drug removal. The results show that like Cucurbitacin-I, Jasplakinolide also rapidly and reversibly inhibits cellular motility in B16F1 cells.
Figure 8: Cucurbitacin-I does not directly stabilize actin filaments

Purified human actin was polymerized and then B) pelleted immediately without the addition of destabilization buffer C) incubated overnight in destabilization buffer only D) incubated overnight in destabilization buffer containing 200nM Jasplakinolide and E) incubated overnight in destabilization buffer containing 200nM Cucurbitacin-I. Pellets were solubilized in SDS sample buffer and run on 10% polyacrylamide gel. The gel was stained with GelCode Blue G-250 comasssie blue stain to visualize the actin band. Lane A contains molecular weight markers.
Figure 9: Cucurbitacin-I plays a role in the stabilization of actin filaments in vivo

Confluent sheets of RFP-actin MDCK cells were incubated in DMSO control media, 200nm Jasplakinolide or 200nm Cucurbitacin overnight. Cells were harvested, lysed and the lysate was spun down to separate G actin from filamentous actin. Band intensities were quantified using Image J.
Figure 10: A Shift to F-actin is seen after short term Cucurbitacin-I treatment

Followed same procedure as in Figure 9 except cells were incubated in 200 nm Cucurbitacin-I for two hours rather than overnight. A) Control cell G-actin B) control cell F-actin C) Cucurbitacin-I treated cells G-actin D) Cucurbitacin-I treated cells F-actin. This result shows that the shift to F-actin in Cucurbitacin-I treated cells occurs early in drug treatment.
References


