The Annonaceous Acetogenin Bullatacin Is Cytotoxic against Multidrug Resistant Human Mammary Adenocarcinoma Cells

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Abstract:

Cytotoxic effects of the Annonaceous acetogenin, bullatacin, were studied in multidrug-resistant (MDR) human mammary adenocarcinoma (MCF-7/Adr) cells vs. the parental non-resistant wild type (MCF-7/wt) cells. Bullatacin was effectively cytotoxic to the MCF-7/Adr cells while it was more cytostatic to the MCF-7/wt cells. ATP depletion is the mode of action of the Annonaceous acetogenins, and these agents offer a special advantage in the chemotherapeutic treatment of MDR tumors that have ATP-dependent mechanisms.

Keywords: Acetogenins | Bullatacin | Multidrug resistance | P-gp | Mammary adenocarcinoma

Article:

1. Introduction

In many cancer patients, the eventual cause of death is not from the original, chemotherapeutically responsive, tumor cells but from the surviving tumor cells that develop resistance to both the original antineoplastic agent and to new, mechanistically and structurally unrelated compounds [1,2]. This phenomena has been accurately coined multidrug resistance (MDR) and is often characterized by the increased expression of a 170 kDa phospholipid glycoprotein (P-gp). The P-gp forms a channel in the cell membrane which serves to extrude anticancer agents before antitumor efficacy can be realized. The active export of antineoplastic compounds requires energy supplied by ATP cleavage, and two ATP binding sites for this ATPase activity have been identified on the cytosolic side of the P-gp [3-7].

The Annonaceous acetogenins are a relatively new class of biologically active natural compounds [8-11] which act to decrease ATP production by inhibiting complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron transport system (ETS) [12-

14]. A second, related, mode of action is the inhibition of an ubiquinone-linked NADH oxidase, involved in substrate level phosphorylation, which is constitutively expressed in the plasma membranes of cancer cells and only transiently expressed in the membranes of 'normal' non-cancerous cells [15]. A recent study reported that a series of related acetogenin compounds significantly inhibited the growth of several different transformed cell types, including an adriamycin-resistant murine mammary cell line (M17/Adr), while only minimally affecting the growth of non-cancerous rat G.I. epithelial (118) cells [16]. Mean bar graphs, showing cytotoxicities in the NCI panel of tumor cells [17], revealed that MDR cell lines are often five times more susceptible to the acetogenins than the parent, non-MDR cell lines.

Numerous studies have explored the use of adjuvant compounds that serve to impede the action of the P-gp by competitively blocking the efflux channel in an effort to increase the exposure time of antineoplastic compounds within MDR cell types [6,18-23]. However, in vivo trials, using verapamil as an adjuvant, failed due to verapamil-induced hypotension [24]. Alternatively, we have approached the MDR problem by investigating the hypothesis that the biochemical difference between MDR and parental cancer cells, i.e. the ATP-dependent P-gp, results in a higher demand for ATP in the MDR cancer cells. Therefore, the Annonaceous acetogenins, because of their ability to decrease ATP levels [14], were investigated for their effect on the growth of MDR cells. We herein report that the adriamycin (MDR)-resistant human mammary adenocarcinoma (MCF-7/Adr) cell line is more susceptible than its parental human mammary adenocarcinoma (MCF-7/wt) cell line to treatment with the acetogenin bullatacin. Bullatacin was found to be cytotoxic to the MDR MCF-7/Adr cells, but it was only cytostatic to the MCF-7/wt cells.

2. Methods

2.1. Materials and reagents

Bullatacin was isolated and characterized in our laboratory as previously described and reviewed [8-11]. Adriamycin, vincristine, vinblastine, penicillin, streptomycin, poly-D-lysine, and Nonidet NP-40 were purchased from Sigma, St. Louis.

2.2. Culturing and plating of MCF-7 cell lines

The MCF-7/wt (wild type human mammary adenocarcinoma) and the MCF-7/Adr (adriamycin-resistant human mammary adenocarcinoma) cell lines were kindly provided by Craig Fairchild of NIH/NCI. Both were maintained in RPMI (Gibco; Grand Island, NY) with 10% heat-inactivated fetal calf serum (Intergen, Purchase, NY) and I% penicillin/streptomycin (PS) and were transferred twice weekly at a ratio of 1:6 for the former and 1:3 for the latter. The MCF-7/Adr cells were originally isolated from the MCF-7 /wt cells by growth in the presence of 10 µ,M adriamycin. They retain their adriamycin resistance for at least 6 months in its absence.

The 96-well microtitre plates (Falcon Labware, Oxnard, CA) were coated with poly-D-lysine in order to facilitate cell adherence [25]. A 50 µl aliquot of poly-D-lysine (100 µg/ml in distilled water filtered through a 0.2 µm filter) was added to each well and incubated at room temperature

for at least 30 min. The plates were rinsed twice with sterile water and allowed to dry overnight in a sterile environment. The plates can be stored for several weeks at 4°C.

For all of the experiments, MCF-7/wt and MCF-7/Adr cells were plated at 5 x 103 and 1.5 x 104 cells/ml, respectively, on the poly-D-lysine-coated 96-well microtitre plates, in a total volume of 200 μl of medium per well and incubated overnight in a humidified CO_2 incubator at 37°C. We previously determined that the cells could tolerate 1% by volume of 95% ethanol without significantly affecting their growth (data not shown); this facilitated the dilution and dispersion of bullatacin. Thus, 24 h after plating the cells, 100 μl of fresh medium was added to the test wells followed by the indicated concentration of bullatacin in a total volume of 3 μl of 95% ethanol; the plates were then incubated at 37°C in the humid atmosphere. Adriamycin was used as a positive control, while six wells per cell line were used as a standard vehicle control.

2.3. Determining the amount of cell growth using the bicinchoninic acid (BCA) protein assay

The amount of cell growth inhibition was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL) [26]. The cells were washed with an eight-channel plate washer (Flow Laboratories) with phosphate buffered saline (PBS). Ten μl of non-ionic detergent solution (1% by volume of Nonidet NP-40 in sterile water) was added to each well in order to solubilize the cells, followed by 200 μl of BCA working reagent (a 50: 1 mixture of base reagent/4% copper sulfate solution). The plates were incubated at 37°C for 30 min, and the absorbance was determined at 570 nm on a Dynatek MR 600 microplate reader.

2.4. Cell growth assay

For the standard 7-day assay (Figs. 1 and 2), the amount of cell growth inhibition was determined at the end of 6 days of exposure to the test compounds using the BCA assay described above. The average absorbance in the vehicle control wells was calculated initially so that all subsequent determinations could be normalized to this average; the abscissas on Figs. 1 and 2 represent percent of control. Each test compound was examined in duplicate on two different plates so that $n = 4$. The average normalized absorbance is plotted at each respective concentration (log[concentration]) with the error bars representing the standard deviation of the four determinations. Alternatively, the cell growth inhibition was determined every 24 h after the original plating of the cells and loading of test compound using the BCA assay described above (Fig. 3). In order to calculate an average absorbance and a standard deviation, six test wells were used for the vehicle-treated controls ($n = 6$), while four test wells were used in the bullatacin-treated wells ($n = 4$). The results are displayed such that the abscissa represents an absolute absorbance while the ordinate represents time in hours.

For the re-feeding experiments (Fig. 4), cell growth inhibition was determined every 24 h after the original planting of the cells and loading of test compound using the BCA assay described above. On day three, half of the remaining plates had their medium removed using a vacuum aspirated sterile syringe. and fresh medium was added followed by incubation. Thus, on days 4 through 7, two plates were examined each day (one standard and one re-feed). For these experiments, the average absorbance of six wells ($n = 6$) for both the control wells and the test

compound wells is plotted such that the abscissa represents the absolute absorbance; the error bars depict the standard deviation, and the 'R' shows when refeeding was initiated.

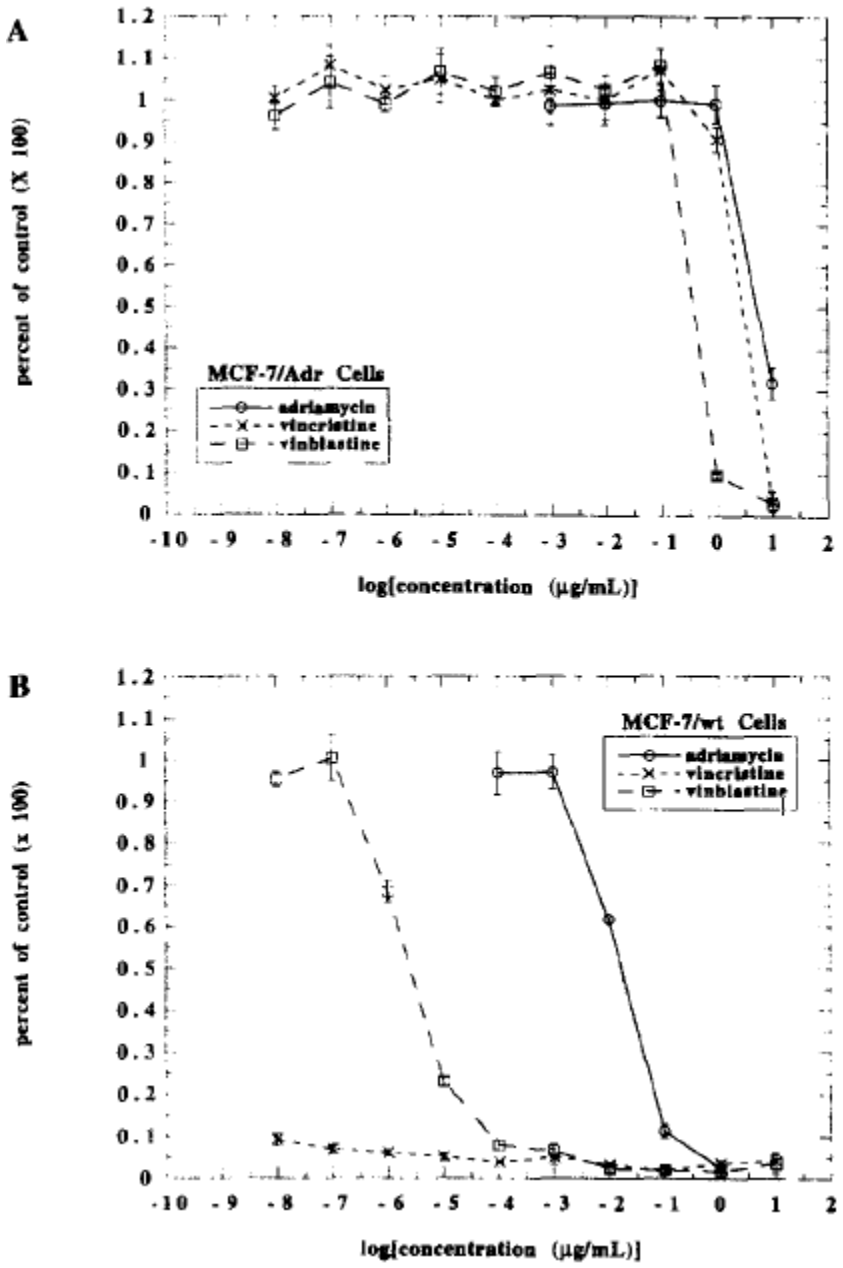

Fig. 1. The effect of 6 days of exposure to the standard antineoplastic drugs, adriamycin, vincristine, and vinblastine, against MCF-7/Adr (A) and MCF-7/wt (B) cells. Values are expressed as a percentage of the vehicle-treated controls with each point representing the normalized average of four values and the error bars representing the standard deviation about that average. The concentration values are log[dose] with units of µg/ml.

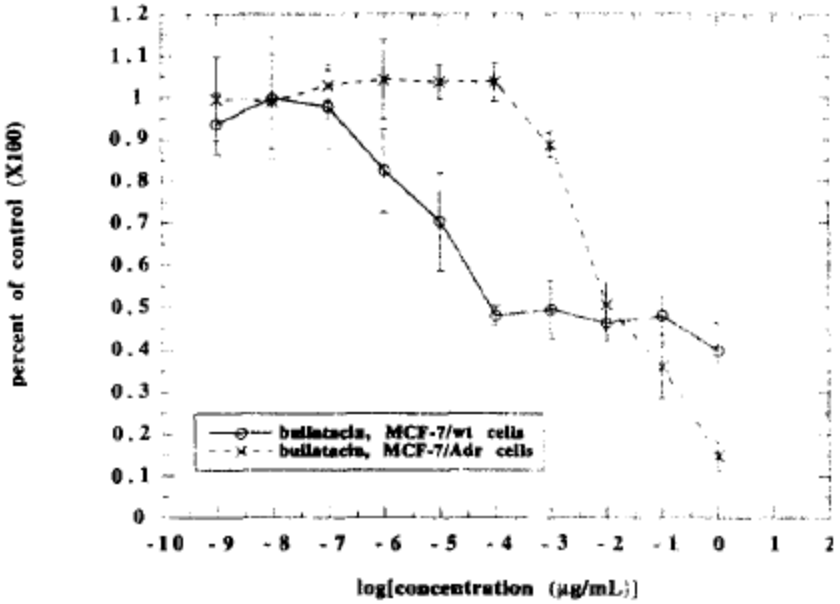

Fig. 2. Comparison of the effect of 6 days of exposure to bullatacin against the MCF-7/wt vs. MCF-7/Adr cells. Values are expressed as a percentage of the vehicle-treated controls with each point representing the normalized average of four values and the error bars representing the standard deviation about that average. The concentration values are log[dose] with units of µg/ml.

3. Results and discussion

Fig. 1 shows the effect of three standard antineoplastic compounds on the growth of adriamycin-resistant and parental (wild type) MCF-7 cells. The MCF-7/Adr cells were resistant to treatment with either adriamycin, vincristine or vinblastine (Fig. lA) as anticipated for these multidrug-resistant (MDR) cells [3-7,27]. The concentration of adriamycin that inhibited cell growth by 50% (IC^{50} value) in the MOR MCF-7/Adr cells was greater than 1 µg/ml, whereas, against the parental MCF-7/wt cells (Fig. lB), the IC_{50} value was 5 x 10-2 µg/ml. The differences in IC_{50} values between the two cell lines were even greater with vincristine and vinblastine and served to illustrate the MOR phenomenon.

In contrast to adriamycin, vincristine, and vinblastine, bullatacin was effective at inhibiting the growth of the MOR MCF-7/Adr cells and exhibited a linear dose-response curve over a concentration range of 1.0 µg/ml to 1.0 x 10-4 µg/ml (Fig. 2). However, over the same concentration range, there was a plateau near the IC_{50} value against the parental MCF-7/wt cells. At the most concentrated dose of 1.0 µg/ml, bullatacin inhibited nearly all of the growth of the MOR MCF-7/Adr cells but only 50% of the growth of the MCF-7/wt cells (Fig. 2). This observation was examined further by analyzing the growth of both cell lines periodically over the 7 days of the assay (Fig. 3). In the MOR MCF-7/Adr cells, bullatacin inhibited cell growth by varying amounts in a dose-dependent fashion, i.e. nearly zero cell growth at the most concentrated dose of 1.0 µg/ml vs. nearly 100% cell growth at the least concentrated dose of 1.0 x 10-4 µg/ml (Fig. 3A). Alternatively, the cell growth of the parental MCF-7/wt cells was only inhibited by 50%, relative to the growth of the vehicle-treated controls, regardless of the dose of bullatacin (Fig. 3B). The data in Figs. 2 and 3, therefore, illustrate that a linear dose-response

curve in the MCF-7/Adr cells after a 6 day treatment (Fig. 2) reflects dose-dependent cell growth when analyzed on a daily basis (Fig. 3A). Likewise, dose-independent cell growth was confirmed in the MCF-7/wt cells both at the end of 6 days of bullatacin exposure (Fig. 2) as well as over the 7 days of the assay (Fig. 3B).

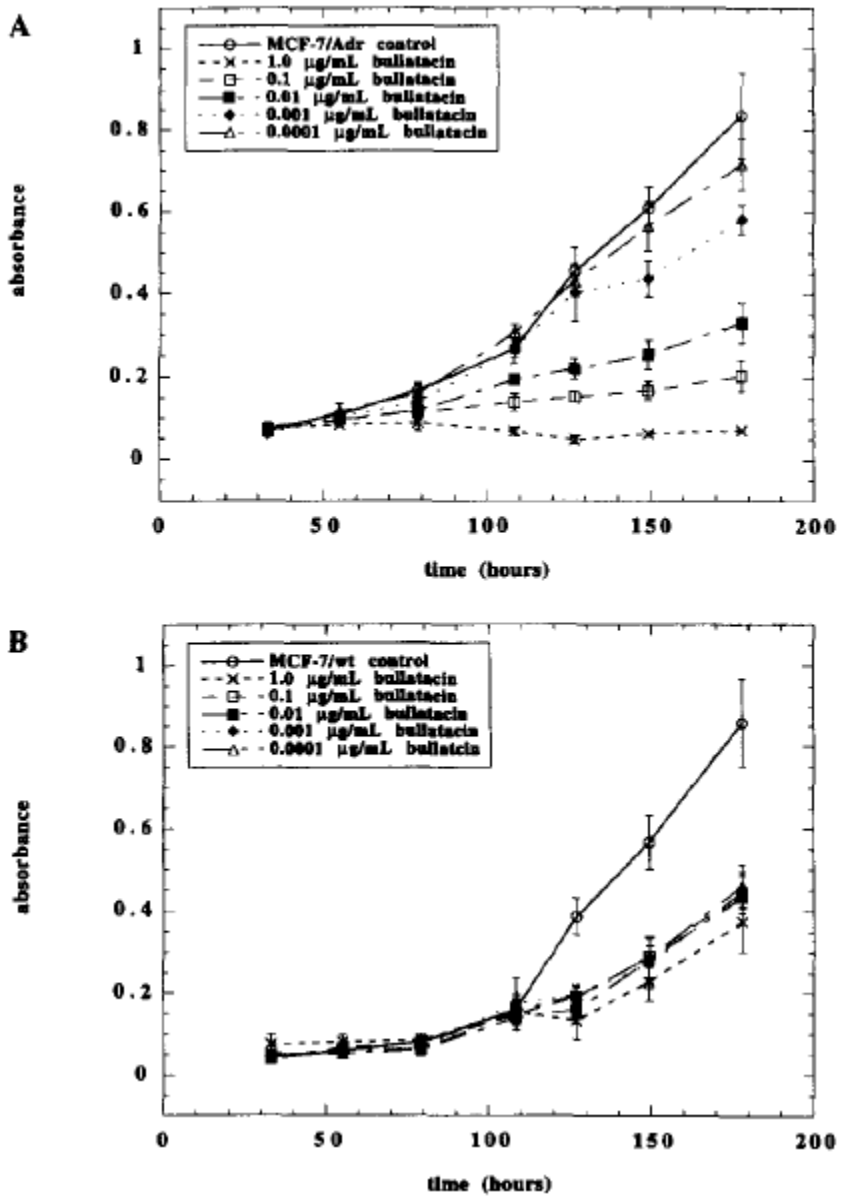

Fig. 3. Periodic analysis (every 24 h) of serial dilutions of bullatacin against MCF-7/Adr (A) vs. MCF-7/wt (B) cells. Values are expressed as an average absorbance (n = 6 for the vehicle treated control, n = 4 for the bullatacin-treated wells) and the error bars represent the standard deviation about that average. The concentration values are log[dose] with units of µg/ml.

Both cell lines were then analyzed to determine if they were still viable after bullatacin treatment (Fig. 4A,B). A 24 h exposure to bullatacin (1.0 µg/ml) was cytotoxic to the MOR MCF-7/Adr cells since these cells were not able to grow after being fed with fresh medium (Fig. 4A). However, the MCF-7/wt cells were able to grow to a level near that of the vehicle treated control

upon being fed fresh medium; thus, bullatacin was more cytostatic than cytotoxic to these wild type cells. A similar re-feeding experiment, using 1.0 μg/ml of adriamycin, showed opposite results; the MCF-7/Adr cells were completely unaffected by the antineoplastic agent (Fig. 4C), whereas the MCF-7/wt cells were no longer viable after Adriamycin treatment (Fig. 4D).

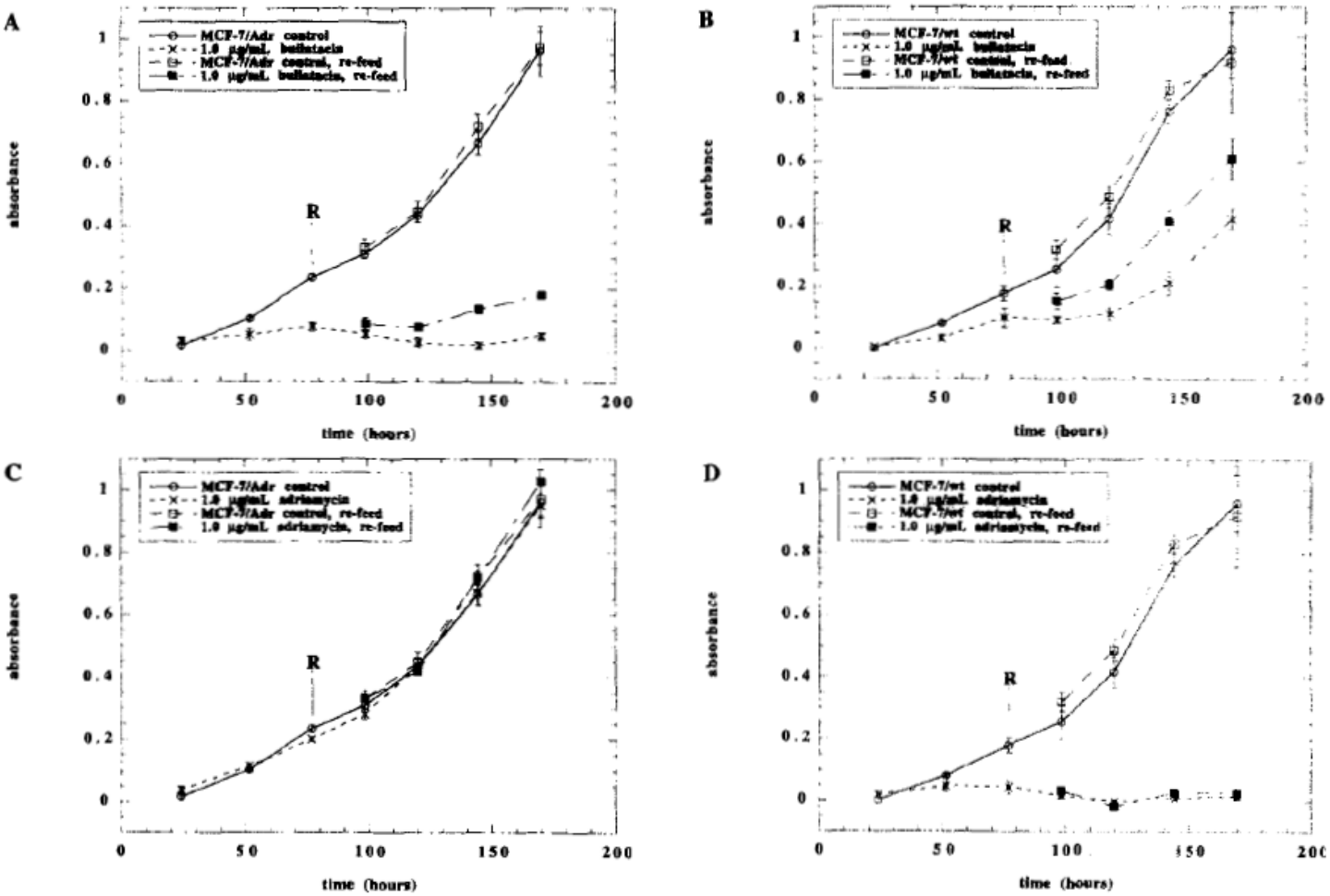

Fig. 4. Periodic analysis (every 24 h) of 1.0 μg/ml of bullatacin (A,B) vs. 1.0 μg/ml of adriarnycin (C,D) against MCF-71Adr lA.C) vs. MCF-7/wt (B,D) cells with re-feeding of fresh media in half of the plates. Values are expressed as an average absorbance (n = 6 for both control and drug-treated wells) and the error bars represent the standard deviation about that average. The 'R' depicts when re-feeding was initiated.

The acetogenins are potent inhibitors of ATP production via their interaction with complex I in the mitochondria [12-14] and an NADH oxidase at the plasma membrane [15]. In cells in which ATP requirements are elevated, such as in MDR MCF-7/Adr cells that require ATP to drive the P-gp transporter [3-7], bullatacin was cytotoxic. These cells were not viable after only 2 days of bullatacin exposure (Fig. 4A). Alternatively, in the parental MCF-7/wt cells, it appears that ATP production is limited by bullatacin treatment to induce only a static response in cellular growth and replication. These cells grew to nearly the same level as the vehicle-treated control after bullatacin was removed (Fig. 4B). Thus, such MOR cell types seem to be more susceptible to

ATP depletion than the parental cells from which they were derived, and this biochemical difference may be
exploitable by the chemotherapeutic use of the Annonaceous
acetogenins.

In conclusion, the Annonaceous acetogenins, such as bullatacin, may have a unique potential as chemotherapeutic agents. Earlier in vivo studies have shown bullatacin to be effective at only 50 μg/kg per day against Ll210 murine leukemia in normal mice and against A2780 human ovarian xenografts in athymic mice [14J. Furthermore, bullatacin was effective against multidrug resistance in both the previously reported adriamycin-resistant murine mammary model [16] and, now, in the Adriamycin-resistant MCF-7 human mammary adenocarcinoma model. The latter cell line was no longer viable after 2 days of exposure to 1.0 μg/ml of bullatacin. Bullatacin, therefore, may be useful as an adjuvant with standard chemotherapeutic regimes. In this regard, it could not only assist in hindering 'normal' cancerous cell growth as previously shown in in vivo models [14], but, more importantly, when MDR tumor types, induced by chemotherapy, begin to evolve, bullatacin may effectively eliminate them before they have a chance to become problematic, and, possibly, before they are even detected. The Annonaceous acetogenins, thus, present a unique approach to circumventing MDR in that most previous studies have explored the use of adjuvants such as verapamil, chloroquine, progesterone, and tamoxifen [6,18-23] which, in theory, only competitively block the efflux action of the P-gp so that standard antineoplastic agents can remain within the cell long enough to induce a toxic response. The use of bullatacin would attempt, instead, to eliminate MDR cell types directly by targeting their ATP production and their elevated requirement for ATP.

Current studies are exploring the structure-activity relationships among additional Annonaceous acetogenins in order to determine which regions of the molecules contribute maximally to this observed bioactivity; over 220 of these compounds have been isolated [8]. In vivo models of parental vs. MDR tumors must now be subjected to acetogenin treatment in order to demonstrate these proposed extrapolations from this promising in vitro data.

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