MODE OF ACTION OF BULLATACIN, A POTENT ANTITUMOR ACETOGENIN: INHIBITION OF NADH OXIDASE ACTIVITY OF HELA AND HL-60, BUT NOT LIVER, PLASMA MEMBRANES

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Abstract:
Bullatacin, a potential antitumor substance isolated from plants of the Annonaceae, and analogs of bullatacin, known collectively as acetogenins, have been reported previously to show potent activity in the inhibition of growth of murine tumors and human tumor xenografts grown in athymic mice as well as an ability to inhibit mitochondrial electron transport. In this report, we show activity of bullatacin in inhibition of NADH oxidase activity of plasma membrane vesicles isolated from HeLa cells and HL-60 cells but not with plasma membrane vesicles isolated from rat livers which, unlike the inhibition of mitochondrial activity, correlated with the ability of the acetogenins to kill tumor cells. Additionally, bullatacin is active against HL-60 cells that are resistant to adriamycin which may suggest utility for bullatacin in management of drug-resistant cells and cell lines.
Key Words: acetogenins, antitumor agents, growth inhibition, HeLa, NADH oxidase, plasma membranes, liver

Article:
Various members of the plant family Annonaceae have yielded a group of bioactive secondary metabolites known collectively as the annonaceous acetogenins. These compounds are variously cytotoxic, pesticidal, antimalarial, antiparasitic, antimicrobial, and antineoplastic (1-3). A characteristic of these compounds, which helps to explain their cytotoxicity, is activity in the inhibition of mitochondrial electron transport (4, 5). However, a more selective activity also was sought that might help to explain the ability of certain members of the series to kill selectively transformed (cancer) cells under conditions where normal cells would be unharmed.

Bullatacin is one of the most potent of the annonaceous acetogenins (4). This report concerns the response to bullatacin of a growth factor- and hormone-stimulated NADH oxidase activity of rat liver plasma membranes (6, 7). Several correlative studies have produced evidence for the involvement of this growth factor-responsive NADH oxidase in the control of cell proliferation (8). The activity in transformed cells and tissues was distinguished from that of liver in that the growth factor- and hormone-responsiveness was lost in plasma membranes of transformed liver tissues. These studies were with hyperplastic nodules of liver induced by the liver carcinogen, 2-acetylaminofluorene (9) and transplanted rat hepatomas (10).

The NADH oxidase activity of liver plasma membranes is unique among oxidoreductase activities not only in its response to growth factors and hormones but, also, in its response to inhibitors and activators other than growth factors and hormones (8, 11, 12). In order to characterize further this unusual NADH oxidase activity, we extended our studies to include responses to bullatacin.

The NADH oxidase activity of rat liver plasma membrane was largely unaffected by bullatacin, whereas the NADH oxidase activity of HeLa (human cervical carcinoma origin) and HL-60 (human promyelocytic leukemia origin) plasma membranes was strongly inhibited. The results indicate a fundamental difference in response to bullatacin between the NADH oxidase activity of liver and that of HeLa cells that may correlate with the previously reported potent inhibition of growth of transformed cells (1-3).

Materials and Methods

Growth of cells. HeLa (ATCC CCL2) cells were grown in 150 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37°C with 10% bovine calf serum (heat-inactivated), plus 50 mg/1 gentamycin sulfate (Sigma). Cells were trypsinized with Sigma IX trypsin for 1 to 2 min and harvested by scraping and taken up in 140 mM NaC1, 5 mM KC1, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4, to a final cell concentration of 0.1 g wet weight (gww) per ml.

HL-60 cells were grown in RPM1-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. Cells were concentrated from stock cultures by centrifugation at 1000 x g and washed twice with serum-free RPM1-1640 medium and resuspended for assay in 100 mM Tris-HC1, pH 7.4.

Cells were from the American Type Culture Collection (Rockville, MD). The adriamycin-resistant cell lines were provided courtesy of Dr. Page Faulk, Methodist Hospital, Indianapolis, IN. All chemicals were from Sigma unless otherwise specified. Diferric transferrin was human holo (iron saturated) from Boehringer-Mannheim (Lot #Fe_2TF BLA 108). The epidermal growth factor was from mouse, culture grade, from Upstate Biotechnology Inc. (Lake Placid, NY).

Purification of plasma membranes from rat liver. The 5000 x g pellet from the preparation of Golgi apparatus (13) was the starting material. The fluffy layer which contains the Golgi apparatus fraction was mixed, withdrawn and excluded from the plasma membrane preparations. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown upper part of the pellet was resuspended with a pen-brush, leaving the reddish tightly packed bottom part of the pellet undisturbed. The resuspended material was homogenized in aliquots of 5 ml each in a 30 ml stainless steel (Duragrind) homogenizer 20 times by hand. The homogenates were combined, diluted with cold 1 mM $NaHCO_3$ (1:1 dilution), and centrifuged at 6000 x g in a HB rotor for 15 min. The supernatant was discarded and the pellet was used for the two-phase separation (10).

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) polyethylene glycol 3350 (Fisher), and 5 mM potassium phosphate buffer (pH 7.2) (14). The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm (150 x g) in a Sorvall HB 4 rotor for 5 min. The upper phases were withdrawn carefully with a Pasteur pipette, divided in half and transferred into 40 ml plastic centrifuge tubes. The tube contents were diluted with cold 1 mM $NaHCO_3$ and collected by centrifugation at 10,000 x g in a HB rotor for 30 min. Plasma membrane pellets were resuspended in 50 mM Tris-Mes buffer (pH 7.2) and stored at -70°C. Proteins were determined using the bicinchoninic acid (BCA) assay (15) with bovine serum albumin as standard. Yields were approximately 3-5 mg plasma membrane protein per 10 g liver (16).

The plasma membrane preparations from rat liver have been characterized extensively based on both morphological and enzymatic criteria (13, 15). From morphometric analysis using electron microscopy, the preparations contain 90 ± 4 percent plasma membrane. Contaminants include mitochondria (4%) and endoplasmic reticulum (3%). Based on analyses of marker enzymes, the contamination by endoplasmic reticulum was estimated to be 3%, that of mitochondria 15% and that of Golgi apparatus 1%. The recovery of plasma membranes was estimated to average 18% based on recovery of enzyme markers.

Purification of plasma membranes from cultured cells. Cultured cells were collected by centrifugation for 6 to 15 min at 1,000 to 3,000 rpm. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron Homogenizer for 30 to 40 sec at 10,500 rpm using a PT-PA 3012/23 or ST-probe in 7 to 8 ml aliquots. To estimate breakage, the cells are monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1,000 rpm (175 g) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4 x 10^6 g•min (e.g., 1 h at 23,500 g) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approximately 1 ml per pellet from 5 x 10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis as described above for rat liver. The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate and the membranes are collected by centrifugation. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Spectrophotometric assay of NADH oxidase. NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity, and 150 µg NADH at 37°C with stirring. Activity was measured using a Hitachi U3210 spectrophotometer with continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity.

Results

NADH oxidation by HeLa plasma membranes was reduced markedly by increasing concentrations of bullatacin. Drugs were added as DMSO solutions (final DMSO concentrations 0.1%). The immediate response to bullatacin addition was a temporary (1 to 2 min) stimulation of the activity followed by steady-state inhibition. Values presented are of the steady-state inhibitions.

The dose response of HeLa plasma membranes to bullatacin summarized in Figure 1 indicated an ED_{50} of 5 to 10 nM with nearly complete inhibition of the activity by 1 µg. The dose response to bullatacin was log linear over the range of 1 nM to 100 nM.

In contrast to results with HeLa cell plasma membranes, plasma membranes of rat liver were unaffected by bullatacin over the concentration range of 1 nM to 10 µg (Fig. 2). At the highest concentrations tested of 1 and 10 µg there appeared to be a slight but insignificant stimulation of the NADH oxidase activity.

NADH oxidase activity of HL-60 cells also was inhibited by bullatacin. Of interest, however, was the ability of bullatacin to inhibit the NADH oxidase activity of HL-60 cells resistant to adriamycin (Fig. 3). More bullatacin was required to inhibit the NADH oxidase activity of the NADH oxidase from the adriamycin-resistant HL-60 cells than was required to inhibit the NADH oxidase of plasma membranes from HeLa. Yet, the NADH oxidase of the plasma membranes of the adriamycin-resistant HeLa cells was strongly inhibited by 1 MM bullatacin.

Other annonaceous acetogenins also inhibited the NADH oxidase of HeLa plasma membranes (Table 1). Asimicin inhibited with an ED_{50} of 5 nM and annonacin A which was the least growth inhibitory of the three (4) also exerted the smallest effect on the NADH oxidase (ED_{50} of 1 *µg).* Bullatacinone inhibited with an ED50 of 0.1 *µg* to 1 *µg.* Asimicin was without effect on NADH oxidation of liver plasma membranes and bullatacinone gave only slight stimulations or inhibitions. With annonacin, an inhibition of about 30% was observed at 1 *µg.*

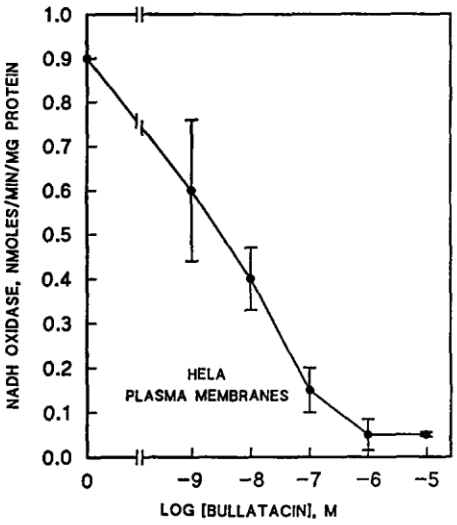

Fig. 1.
Dose response of NADH oxidase of HeLa cell plasma membrane vesicles to bullatacin. Values are of duplicate determinations ± mean average deviations.

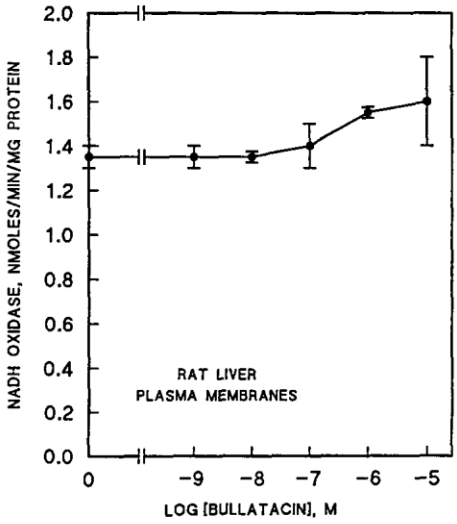

Fig. 2.
Dose response of NADH oxidase of vesicles of plasma membranes of rat liver to bullatacin. Values are of triplicate determinations ± standard deviations.

TABLE I

Summary of inhibition of NADH oxidase activity of HeLa and rat liver plasma membrane vesicles by annonaceous acetogenins.

Acetogenin	ED_{50} Rat liver	HeLa
Annonacin A	> 10 μM	1 μM
Asimicin	> 10 μM	5 nM
Bullatacin	> 10 μM	5 - 10 nM
Bullatacinone	> 10 μM	0.1 μM - 1 μM

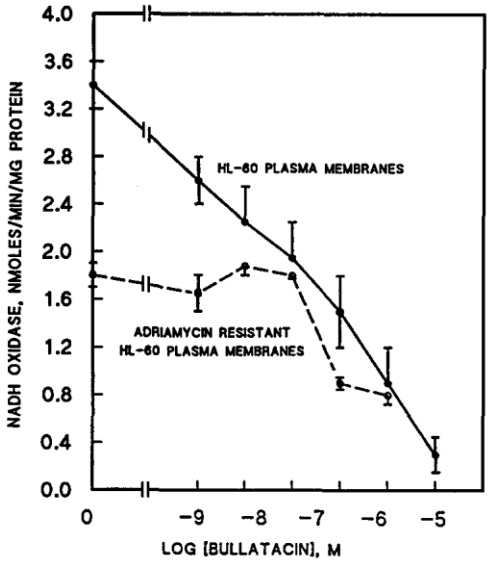

Fig. 3.
NADH oxidase activity of plasma membrane vesicles isolated from HL-60 cells and HL-60 cells resistant to adriamycin. Values are of duplicate determinations ± mean average deviations.

Discussion

In previous studies, the antitumor activity of annonaceous acetogenins has been investigated in *in vivo* tumor studies with normal mice (1-3). Promising antitumor activity of bullatacin and bullatalicin has been exhibited in athymic mice, and the mode of action has been investigated using Sf-9 (insect) cells and rat liver and beef heart mitochondria (4). The abilities to inhibit respiration and mitochondrial electron transport were reported (4), and the investigations have been extended to include all of the acetogenin subclasses (5). Bullatacin inhibited mitochondrial electron transport even in mitochondria from rat liver with IC-50 values down to 10 nM. The present report describes a new parameter, that of an NADH oxidase activity of the plasma membrane, which is resistant to bullatacin in preparations from normal rat liver but susceptible in HeLa and HL- 60 plasma membranes.

Also in previous studies, an NADH oxidase activity of the plasma membrane of rat liver (6-8), keratinocytes (11) and plant stems (17), that was stimulated by hormones and growth factors, has been identified. In cancer, the activity was constitutively activated and no longer hormone-responsive (9, 10). The NADH oxidase activity of rat liver plasma membranes required quinones for activity (13). In plants, stimulation or inhibition of the activity correlated closely with inhibition or stimulation of growth (18, 19).

In the present work, the NADH oxidase activity of HeLa cells (human cervical carcinoma derivation) was inhibited by bullatacin. In contrast to the NADH oxidase of HeLa plasma membranes, the NADH oxidase of plasma membranes from rat liver was not inhibited. Similarly, the NADH oxidase of HL-60 (human promyelocytic leukemia) cells resistant to adriamycin was inhibited by bullatacin.

Not only does bullatacin inhibit the NADH oxidase of plasma membrane vesicles from HeLa cells and not that of liver, but the substance exerts a response on growth (2) parallel to that observed with NADH oxidase activity. While comparable growth studies were not done with HeLa, bullatacin and other active acetogenins inhibit growth of susceptible cell lines in culture in the nanomolar range of drug concentrations (4).

These findings suggest that the inhibition of the activity of the NADH oxidase of the plasma membranes may correlate more closely with the antitumor activity of bullatacin, and, thus, the other acetogenins, than does the inhibition of mitochondrial electron transport. Furthermore, this new site of action, with its added vulnerability in transformed vs normal cells, may help to explain the *in vitro* and *in vivo* selectivity of the acetogenins toward transformed cells.

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