Tumor Cell Growth Inhibition by Several Annonaceous Acetogenins in an in Vitro Disk Diffusion Assay

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Abstract:

The cell inhibition activities of several Annonaceous acetogenins, covering the three major structural classes of bis-adjacent, bis-non-adjacent, and single tetrahydrofuran (THF) ring compounds and their respective ketolactone rearrangement products, were tested in an in vitro disk diffusion assay against three murine (P388, PO3, and M17/Adr) and two human (H8 and H125) cancerous cell lines as well as a non-cancerous immortalized rat GI epithelial cell line (I18). The results demonstrate a dose-dependent inhibition of cancerous cell growth, while non-cancerous cell growth is not inhibited by the same dosages. All of the acetogenins, irrespective of their various structural types, inhibit the growth of adriamycin resistant tumor cells and non-resistant tumor cells at the same levels of potency. These results show that the Annonaceous acetogenins are an extremely potent class of compounds, and their inhibition of cell growth can be selective for cancerous cells and also effective for drug resistant cancer cells, while exhibiting only minimal toxicity to 'normal' non-cancerous cells.

Keywords: Acetogenin | Disk diffusion assay | Annonaceae

Article:

1. Introduction

The potent biological activity of the Annonaceous acetogenins continues to encourage our examination of their anticancer effects in vitro, as well as in vivo. Over 12 years have elapsed since Joland et al. [8] reported uvaricin as a new, in vivo active, antitumor agent; since then over 160 of these natural compounds have been reported, and all of these show significant biological activity [3,5,14]; yet, the acetogenins have undergone only minimal testing in animal cancer models. The recently developed natural product, taxol, has shown noted clinical success in the

treatment of ovarian and breast carcinomas [6,13]; however, numerous second and possibly even third generation taxol derivatives, with improved activities and milder side effects, might already be available if the development time of over 20 years for taxol had been shortened [15]. The Annonaceous acetogenins have a similar potential to become life-prolonging compounds, and their further examination in animal tumor models should be undertaken as soon as possible. We report herein preliminary results which demonstrate the selectivity of this highly potent class of compounds for cell growth inhibition of cancerous versus normal cells and demonstrate, furthermore, that this growth inhibition is unaffected by adriamycin drug resistance.

A major problem with regard to modern cancer chemotherapy stems from the emergence of cancerous cells which have developed resistance to the common chemotherapeutic agents such as adriamycin, vincristine, taxol, etc. Standard protocols of dose intensification and the use of alternative, structurally unrelated, drugs often fail because the cells have developed multidrug resistance (MDR). A common form of MDR has been characterized by an increased expression of a 170 kDa plasma membrane glycoprotein (the P-170 glycoprotein), which acts as a cellular 'pump' extruding the anticancer agents before they can accumulate in lethal concentrations within the MDR cell. Two homologous intracellular ATP binding sites are common to such transporter proteins, and they are believed to require the energy of ATP cleavage to effect active efflux [4].

The Annonaceous acetogenins have been shown to be potent inhibitors of complex I (NADH:ubiquinone oxidoreductase) of mitochondrial electron transport systems (ETS) [1,10,11]. We initially believed that they caused tumor cell inhibition by blocking oxidative phosphorylation, limiting the level of ATP, and, therefore, inducing a type of suffocation (ATP deprivation) at the cellular level. Recently, Morre et al. [12] have shown that the acetogenins also selectively inhibit the NADH oxidase activity of plasma membrane vesicles derived from HeLa (human cervical carcinoma), HL-60 (human promyelocytic leukemia), and HL-60/Adr (human promyelocytic leukemia resistant to adriamycin) cells while not affecting those derived from normal rat liver cells. This second mode of action also lowers intracellular ATP levels by blocking NAD regeneration and, thus, inhibiting glycolytic (substrate level) phosphorylation in the cytosol; these combined modes of action likely lead to apoptosis (programmed cell death) [16] and explain the extreme potency (cell inhibition ED_{50} values often less than 10^{-12} μg/ml) [3,5,14] of this class of compounds. The selectivity of the acetogenins for tumor cells could then be explained both by the higher activities of the NADH oxidase that are peculiar to tumor cells as well as the increased ATP demands that are inherent due to their uncontrolled growth.

We have previously hypothesized that since the Annonaceous acetogenins lower intracellular ATP levels, they may be effective in circumventing MDR [3,5]. This could either be in adjuvant dosings with standard therapeutics, or possibly as new, single agent, antineoplastics. In this paper we report that the acetogenins not only have potent inhibitory effects in 'normal' cancer cells, but they are also equipotent to adriamycm resistant cells; this is an important observation since such cells usually show cross resistance to structurally unrelated compounds [4]. Furthermore, the acetogenins showed significantly decreased inhibitory effects to immortalized, non-cancerous, rat GI epithelial cells. Taken together, these results suggest that the Annonaceous acetogenins show considerable promise for development as new antineoplastic agents, with excellent potential for usefulness in treating MDR tumors. It is our hope that these results will initiate both

additional in vitro examinations of their potential to circumvent MDR, as well as further in vivo experimentation so that human benefits can be conceived in a timely fashion.

2. Materials and methods

2.1. Mice, tumors, and cells

Inbred C57-BL/6 and C3H HENCR.MTV mice were obtained from the National Cancer Institute.

Pancreatic ductile adenocarcinoma 03 (PO3) and adriamycin resistant mammary adenocarcinoma 17 (M17/Adr), which are solid transplantable murine tumors, were used for the in vitro testing. These tumors are in the Developmental Therapeutics Program frozen tumor repository, maintained by the Biological Testing Branch, Frederick, MD. Each has a detailed description, code identification number, and list of references at the National Tumor Repository.

Human colon tumor (H8) and human lung tumor (H125) were grown in culture until plating was done. They were maintained in CMRL/Fischer's (50:50) media. The cells were passaged mechanically with a 1:3 dilution twice weekly. For the plating assay, the cells were mechanically dispersed and diluted in a CMRL/Fischer media mixture.

Rat GI epithelial cells were obtained from the American Type Culture Collection (ATCC# CRL 1589). Progeny from the seed stock are capable of a least 20 additional population doublings.

2.2. Soft-agar colony formation disk diffusion assay

For this assay, murine B-cell lymphoma (P388) and the aforementioned solid tumor cells were plated in soft agar. The drugs were placed on a 6.5 mm filter paper disk, which was then placed on top of the soft agar containing the tumor cells [2].

For the P388/solid tumor differential assay, a hard bottom layer (containing tryptic soy broth (0.8%), Nobel agar (0.8%), media (CMRL/Fischer's 50%/50%), and horse serum (11%) at 48°C) was poured into 60-mm plastic dishes (3 ml in each), allowed to solidify, and stored at 37°C in 5% CO_2 [2]. Bottom layers were used 4-10 days after preparation. A soft agar top layer, containing Nobel agar (0.44%), media (CMRL/Fischer's 50%/50%), horse serum (11%), and titered tumor cells, was poured on top and allowed to solidify.

2.3. Cell preparation

The mouse solid tumors were passaged s.c. in the appropriate inbred mice. Cells for the in vitro assay were derived directly from these s.c. passage tumors. The tumors (800-1500 mg) were cut into 200-300 mg fragments in 10-15 ml of cold Hanks' basal salt solution containing 10% horse serum. The tumor was disrupted using a Stomacher-SO for 15 s. This material was then poured through a 100 mesh sieve. Residual material was forced through (by finger with a sterile glove) and the sieve was rinsed with cold media. The material was then drawn up (rapidly) and pushed down (slowly) in a 5-ml glass syringe (without a needle) and again poured through a sieve

without forcing material through (with one rinse). The cell suspension was centrifuged twice at 150 × g for 5 min in cold CMRL/Fischer's media with 11% horse serum. Plating efficiencies varied from one tumor type to another and colonies arose from varying size clumps of cells as well as single cells. Titers were adjusted to produce 300-1000 colonies per 60-mm dish [2].

A volume of 0.05 ml of each drug dilution in ethanol was added to 6.5-mm disks (standard hole punch of Whatman No. 1 filter paper). The disks were allowed to dry and then placed one-third of the distance from the edge of the tumor-containing dish. The plates were incubated for 6-10 days and examined on an inverted microscope (×40). Depending upon the innate sensitivity of the cells for the drug (and the concentration of the drug) a zone of inhibition of colony formation occurred. The zone of inhibition (measured from the edge of the disk to the first colonies) was determined in units; 200 units = 6.5 mm (the size of the filter paper disk). Also, if a region beyond the clear zone existed in which sporadic cell growth was visualized, then a range is noted to where 'normal' cell growth begins. Adriamycin was run in parallel as a positive control and the results represent single experiment determinations. Although the primary goal of the disk diffusion assay set forth by Corbett et al. [2] was to select for compounds which have solid tumor selectivity, the objective of these experiments was to test the acetogenins on both MOR tumor cells and on 'normal' non-cancerous cells, neither of which has been thoroughly examined in the literature [3,5,14]. In such experiments, a zone unit difference of 250 units or more between the cancerous versus non-cancerous cells is predictive of in vivo activity [2].

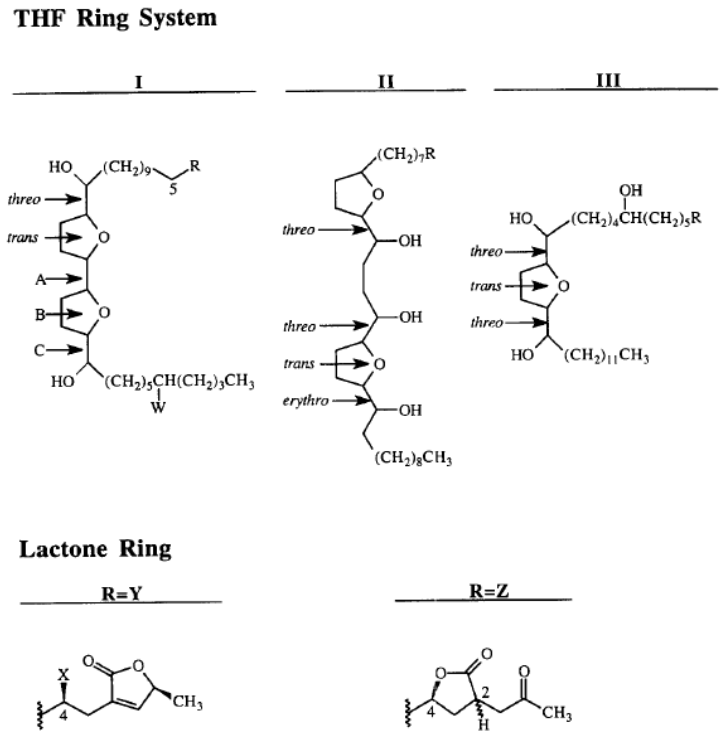

Fig. 1. Structures of the acetogenins tested.

2.4. Acetogenins

The acetogenins (Fig. I, Table 1) were isolated from various Annonaceous plants [3,5, 14] and were chromatographically (thin-layer and HPLC) and spectroscopically (^{1}H and ^{13}C NMR) pure.

The acetogenin compounds and reference adriamycin (as a positive control) were coded, and the disk diffusion cell inhibition experiments [2] were performed as a blind study.

Table 1. Structures[a] of the acetogenins tested

Compound	Ring system	A	B	C	R	W	X
Bullatacin **(1)**	I	*threo*	*trans*	*erythro*	Y	H	OH
4-Deoxybullatacin **(2)**	I	*threo*	*trans*	*erythro*	Y	H	H
Bullatacinones[b] **(3)**	I	*threo*	*trans*	*erythro*	Z	H	—
30-0H-Bullatacinones[b] **(4)**	I	*threo*	*trans*	*erythro*	Z	30-OH	—
Asimicin **(5)**	I	*threo*	*trans*	*threo*	Y	H	OH
Trilobacin **(6)**	I	*erythro*	*cis*	*threo*	Y	H	OH
Bullatalicin **(7)**	II	—	—	—	Y	—	OH
Bullatalicinones[b] **(8)**	II	—	—	—	Z	—	—
Annonacin **(9)**	III	—	—	—	Y	—	OH
Isoannonacins[b] **(10)**	III	—	—	—	Z	—	—

[a] For absolute stereochemistries see [3,5, I 4].

[b] The ketolactones are 2,4-*cis* and -*trans* mixtures.

3. Results

3.1. Cell growth inhibition with adjacent THF ring acetogenins

The disk diffusion assay results with bullatacin **(1)** are typical for the Annonaceous acetogenins. As shown in Fig. 2a, at the dose of 2.5 μg/disk, **1** is extremely inhibitory to the cell growth and is equipotent in both the cancerous and non-cancerous cell lines; such a result, at first examination, suggested a lack of selectivity. However, at the tenfold lower dose of 0.25 μg/disk, **1** showed greater efficacy than the positive control, adriamycin. Importantly, at this lower dose, **1** continued effectively to inhibit the adriamycin resistant mammary cell line (M17/Adr), and it was less inhibitory than ten times the concentration of adriamycin to the non-cancerous immortalized GI epithelial cells (I18). Very similar results (Fig. 2a) were obtained with the structurally related bis-adjacent ring compounds, asimicin **(5)** and trilobacin **(6),** with **6** being the least inhibitory to the I18 cells. It appears that the peculiar stereochemistry of the THF rings and flanking hydroxyls of bullatacin **(1)** may have, at least in this assay, the most potent inhibitory effects. Also, 4-deoxybullatacin **(2)** was less inhibitory than an equivalent concentration of bullatacin which again suggests the importance of a third hydroxyl group for potency [17].

3.2. Cell growth inhibition with ketolactone acetogenins

Bullatacinones **(3),** the ketolactone rearrangement products of bullatacin **(1),** showed similar, and possibly even more promising, results. At an initial dose of 12.50 μg/disk, **3** (Fig. 2b) was very inhibitory to all of the cancerous cell lines and was slightly more inhibitory to the immortalized GI epithelial cells (I18) than the positive control, adriamycin. As the dose was decreased, **3** continued to inhibit the growth of the cancerous cells, including the adriamycin resistant cells (Ml7/Adr); however, the bioactivity against the I18 cells decreased to near that of adriamycin. Interestingly, an analog of the bullatacinones, hydroxylated in the 30 position **(4),** had efficacy similar to that of the parent compound. This could indicate that such analogs, with improved water solubility, may also retain potency.

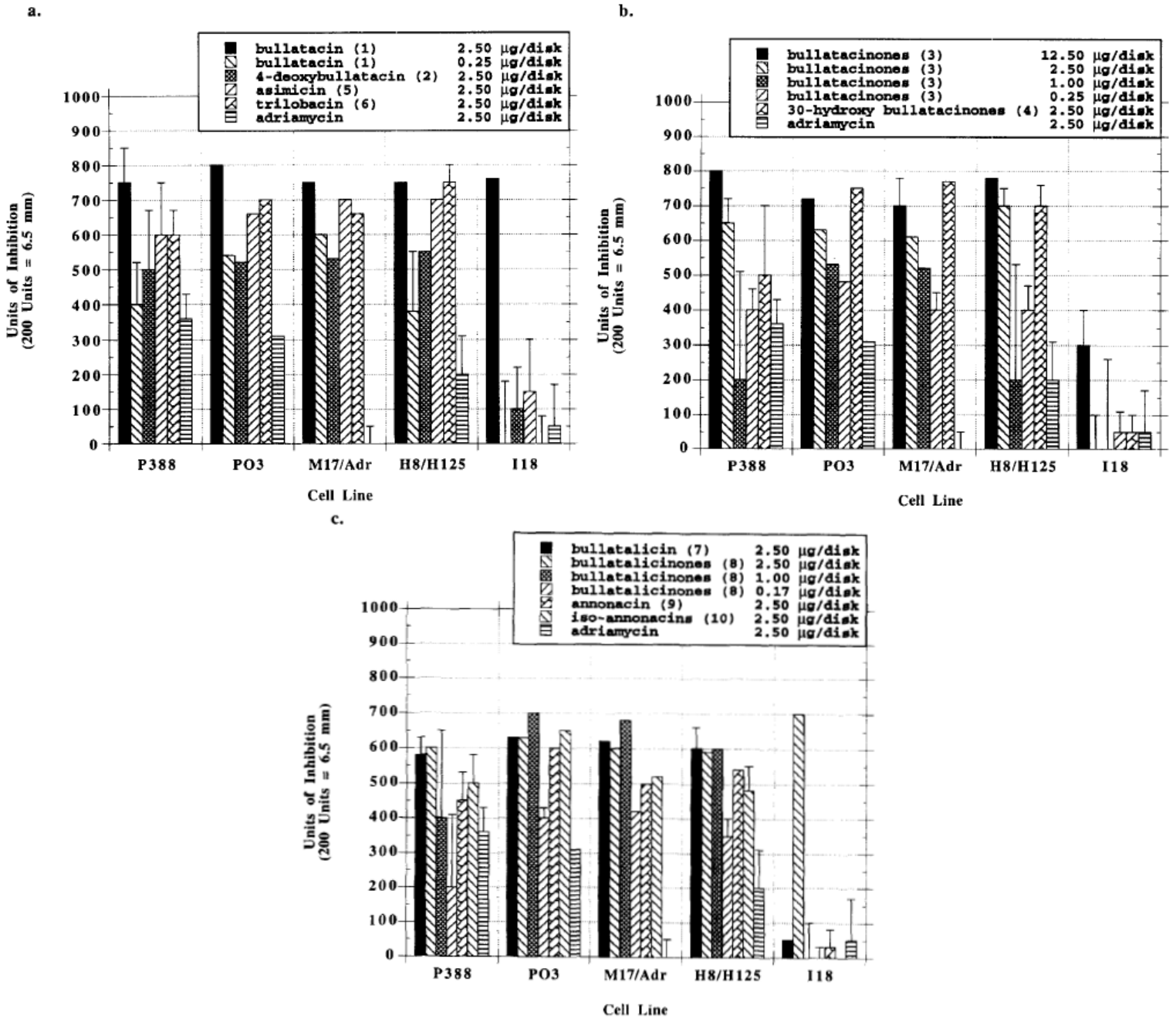

Fig. 2. Inhibition of cell growth was determined as described in Section 2. A clear zone, indicating no tumor cell growth up to a definite point, is given a number representing distance from the disk (200 units = 6.5 mm) and is illustrated by the height of the bars in the graph; beyond such a distance, 'normal' tumor cell growth exists. Further, if there is cell growth beyond the clear zone which is sporadic and fewer in number, an 'error' bar is drawn to illustrate how far such a region exists before 'normal' tumor cell growth commences. Note, not all determinations displayed sporadic growth, and, therefore, they do not all receive error bars. Murine tumor cells: P388, B cell lymphoma; PO3, pancreatic ductile adenocarcinoma; Ml7/Adr, adriamycin resistant mammary carcinoma. Human cells: H8/HI25, either human colon carcinoma 8 or human lung carcinoma 125; results with either are considered comparable [2]. 'Normal' cells: I18, immortalized rat GI epithelial cells. (a) Tumor cell growth inhibition of bis-adjacent THF ring acetogenins versus adriamycin and showing the reduced effectiveness when the third hydroxyl is missing (4-deoxybullatacin). (b) Tumor cell growth inhibition of ketolactone bis-adjacent THF ring acetogenins versus adriamycin and showing the typical dose response relationship with bullatacinones. (c) Tumor cell growth inhibition of non-adjacent THF ring and single THF ring acetogenins versus adriamycin and showing the typical dose response relationship with bullatalicinones.

3.3. Cell growth inhibition with non-adjacent and single ring acetogenins

Fig. 2c illustrates that both the non-adjacent ring compounds, bullatalicin (7) and its ketolactone rearrangement product, bullatalicinones **(8),** as well as the single ring compounds annonacin **(9)** and its ketolactones, isoannonacins **(10),** follow a similar trend of selective inhibition towards the cancerous cells versus the non-cancerous cells and retain their inhibitory action against the adriamycin resistant mammary cell line (M17/Adr).

4. Discussion

In the disk diffusion assay, the Annonaceous acetogenins are extremely inhibitory to cancer cells, more so than the standard antineoplastic agent, adriamycin, and they are, furthermore, equipotent to those cells which have developed resistance to adriamycin. The compounds with two THF rings are slightly more inhibitory than those with one THF ring; and, the bis-adjacent THF ring compounds appear to be more potent, although only slightly more so, than those with non-adjacent THF rings. Both of these observations follow previously published trends found in vitro, with cytotoxicities [3,5,14] and mitochondrial inhibition [9], and in vivo, with brine shrimp lethality [3,5,14], murine leukemia (L1210) in normal mice, and human ovarian carcinoma (A2780) xenografts in athymic mice [1].

This present study suggests that, with care, it is possible to find doses of the acetogenins which are selectively inhibitory to cancerous versus normal cells. This is a very important and, unfortunately, neglected point, especially when designing in vivo tests. For example, the recent work of Holschneider et al. [7] tested bullatacin **(1)** in a murine ovarian teratoma model in which they reported that it is too toxic, and, therefore, ineffective in a dose range for **1** of 20 mg/kg down to 75 μg/kg. However, earlier work by Ahammadsahib et al. [1] had reported an effective dose for **1** of 50 μg/kg which inhibited, by 67%, the growth of A2780 (human ovarian carcinoma) tumors in nude mice. It is unfortunate that the minimum dose tested by Holschneider et al. [7] is 1.5 times greater than the effective dose reported by Ahammadsahib et al. [1], and, thus, it is not possible to critically access the anticancer versus toxic effects of **1** in their murine ovary teratoma model; their work also failed to include a positive control to demonstrate the feasibility of treatment of such a stubborn tumor.

The second mode of action of the Annonaceous acetogenins, recently reported by Morre et al. [12], points to inhibition of NADH oxidase activity in the plasma membrane. This oxidase had previously been found to be overexpressed in cancerous cell lines and may be the key to tumor selectivity. Indeed, the results, reported herein with the disk diffusion assay, show a preference of inhibitory action towards both the 'normal' and the adriamycin resistant cancerous cells and substantiate the report of Morre et al. [12].

In the near future, the Annonaceous acetogenins should be subjected to thorough in vitro and in vivo testing which explores proper dosages and formulations. As shown in Fig. 2, most of the acetogenin compounds tested exhibit at least a 250 zone unit difference between activity against the cancerous versus non-cancerous cell lines, including the adriamycin resistant murine mammary cell line (Ml7/Adr), and such results are predictive of in vivo activity [2]. Furthermore, their ability to block the formation of ATP, both through the inhibition of complex

I in the ETS and the inhibition of NADH oxidase in the plasma membrane, represents a unique mode of action for antineoplastic agents and provides a logical foundation for future experiments and, hopefully, eventual treatment of MDR tumors that require ATP to energize the transporter mechanism.

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