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THE CARBOXYLIC IONOPHORE MONENSIN INHIBITS ACTIVE DRUG EFFLUX AND MODULATES *IN VITRO* RESISTANCE IN DAUNORUBICIN RESISTANT EHRLICH ASCITES TUMOR CELLS

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Abstract—Acquired cellular resistance to anthracycline and vinca alkaloid drugs (pleiotropic resistance) is commonly associated with reduced drug accumulation, a phenomenon which is thought to be partly due to increased energy-dependent drug efflux. We have previously detected increased plasma membrane traffic to, and content of, the acid endosomal compartment in pleiotropic resistant Ehrlich ascites and P388 leukemia cells. This suggested that the endosome could be associated with the pleiotropic resistance phenotype by a mechanism of vesicular drug trapping and transport. The present study was undertaken in order to test the effects of the carboxylic ionophores monensin and nigericin, which are known to both disrupt intracellular vesicular traffic and to raise intravesicular pH, with relation to the pleiotropic resistance phenotype.

Both monensin and nigericin increased daunorubicin (DNR) accumulation in daunorubicin resistant Ehrlich ascites tumor cells (EHR2/DNR+) in a dose-dependent manner. Further, monensin inhibited glucose induced DNR efflux from EHR2/DNR+ cells loaded with drug by energy deprivation. On the other hand, monensin had only negligible effect on DNR accumulation and efflux in wild-type Ehrlich ascites tumor cells (EHR2). In a clonogenic assay system, monensin reduced resistance to DNR in EHR2/DNR+, whereas only an additive effect was obtained in EHR2. However, both ionophores proved too toxic in *in vivo* experiments.

These results, showing that drugs known to disrupt endosomal functions also inhibit the pleiotropic resistance phenotype, support the suggested link between the endosome and pleiotropic resistance.

Cellular resistance to cancer chemotherapy is considered to be a major obstacle to successful clinical treatment. Acquired resistance to one of the anthracycline drugs usually also confers resistance to the structurally and functionally unrelated vinca alkaloids and vice versa (pleiotropic resistance) [1]. The pleiotropic resistance phenotype is commonly associated with decreased drug accumulation, which is thought to be partly due to an increased energy dependent drug efflux mechanism(s) [2, 3], the nature of which is presently undetermined. One of the unresolved questions concerning the pleiotropic resistance phenotype is whether acidic intracellular compartments are involved [4]. The carboxylic ionophores monensin and nigericin are known to disrupt acidic vesicular traffic and to elevate intravesicular pH [5]. In order to study this question, we therefore tested the effect of these ionophores on anthracycline drug accumulation, efflux and sensitivity in wild-type and anthracycline resistant cells.

MATERIALS AND METHODS

Cells. Wild-type Ehrlich ascites tumor cells (EHR2)‖ and the daunorubicin (DNR) resistant subline (EHR2/DNR+) have previously been described [6]. Both sublines were maintained as ascitic tumors in first-generation hybrids of female Swiss mice and male inbred DBA/2 mice by weekly transplantation of 1.5×10^7 cells per mouse. Resistance was maintained in EHR2/DNR+ by IP treatment with a dose of DNR corresponding to LD10 as previously described [6]. Cells from the sixth to eighth day after transplantation were used for experiments. No drug was administered in the last transplantation before experiments. Cell viability was checked by trypan blue exclusion and was >95% before and after *in vitro* experiments with ionophores.

Drugs. DNR as hydrochloride was obtained from Farmitalia, Milan, monensin and nigericin both from Calbiochem, Behring-Diagnostics (San Diego, CA). Monensin and nigericin were both dissolved in 99% ethanol to make 20 mM stock solutions.

Media. These were as previously described for drug uptake studies [2]. Standard medium was a phosphate buffer containing 57.0 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 9 mM NaH_2PO_4, 51 mM Na_2HPO_4, and 10 mM glucose (pH 7.45). Apart from measurements of the initial rate of uptake, 5% (v/v) calf serum was added to the medium. As incubation with either monensin or nigericin entailed that ethanol was added to the medium, comparable amounts of ethanol, up to 0.12% (v/v), were added

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‖ Abbreviations used: EHR2, wild-type Ehrlich ascites tumor cell; EHR2/DNR+, daunorubicin resistant Ehrlich ascites tumor cell; DNR, daunorubicin.

to controls. This amount of ethanol had only negligible effect on DNR uptake, efflux and cell viability. A final cell concentration of 2×10^6 cells/ml was used in all experiments.

Tissue culture medium used in the clonogenic assay was as previously described [7], namely RPMI-1640 supplemented with 10% fetal calf serum, L-glutamine (0.29 g/l), streptomycin (50 mg/l) and penicillin (200,000 IU/l). Medium and serum were obtained from GIBCO Ltd. (Paisley, Scotland).

The pH of tumor bearing ascitic fluid was measured immediately after the animals were killed by neck dislocation on the sixth to eighth day after transplantation and found to be 7.0.

Determination of initial DNR uptake, cellular DNR content, DNR efflux and preparation of isolated nuclei. These procedures were performed as previously described [2, 9, 10]. Briefly, initial DNR uptake was measured in standard medium without glucose containing 10 mM sodium azide [2]. Cellular DNR content was measured in an Aminco-Bowmann spectrofluorometer by measurement of the total drug fluorescence extracted from the drained cell pellet by 0.3 N HCl:50% ethanol according to Bachur *et al.* [8], and the drug concentration determined by comparison with spectrophotometrically adjusted standards [9]. DNR efflux was measured by first loading cells in standard medium without glucose containing 10 mM sodium azide and subsequently adding 10 mM glucose [2]. In parallel efflux experiments, medium pH was measured at 30, 35 and 60 min. Preparation of isolated nuclei was performed after lysing cells in a buffer containing 250 mM sucrose, 5 mM $CaCl_2$, Nonidet P40 0.1% (v/v) and 25 mM Tris–HCl (pH adjusted to 7.45) [10].

Clonogenic assay. Cell survival was assessed by colony formation in soft agar [7]. A single cell suspension was exposed to increasing doses of either DNR alone or DNR plus 2.5 μM monensin for 1 hr, washed twice and plated in soft agar on top of a feeder layer consisting of sheep red blood cells and mercaptoethanol [7]. Colonies were counted after 3 weeks using a dissecting microscope, and surviving fractions calculated by dividing the number of colonies on the treated plates with the number of colonies on the untreated control plates.

Animal experiments. The IP LD_{10} values for monensin and nigericin were calculated according to the method of Cornfield and Mantel [11] on 13 groups of 5–10 mice. Both DNR and ionophores were administered IP once daily for four consecutive days starting on day 1. For therapy experiments, mice were inoculated with 1.5×10^7 cells IP on day zero and randomized to either control groups treated with drug vehicle alone or experimental groups treated with combinations of LD_{10} doses of DNR and/or monensin or nigericin.

Electron microscopy. Two millilitres of cell suspension (2×10^6 cells/ml) were incubated in standard medium with or without monensin (25 μM) for 30 min at 37°, fixed in equal volumes of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 60 min at 24° and thereafter pelleted. Cell pellets were dehydrated in ethanol and Epon embedded as previously described [14].

RESULTS

DNR initial uptake and accumulation

Figure 1 shows the effect of various concentrations of monensin and nigericin on the time-course of DNR uptake in EHR2 and EHR2/DNR+. While both ionophores increased DNR uptake in resistant cells, their effect was negligible in EHR2. Figure 2 demonstrates the dose-dependent effect of monensin on accumulation of DNR in EHR2/DNR+. That monensin is without effect on the initial rate of DNR influx in EHR2/DNR+ is shown in Fig. 4.

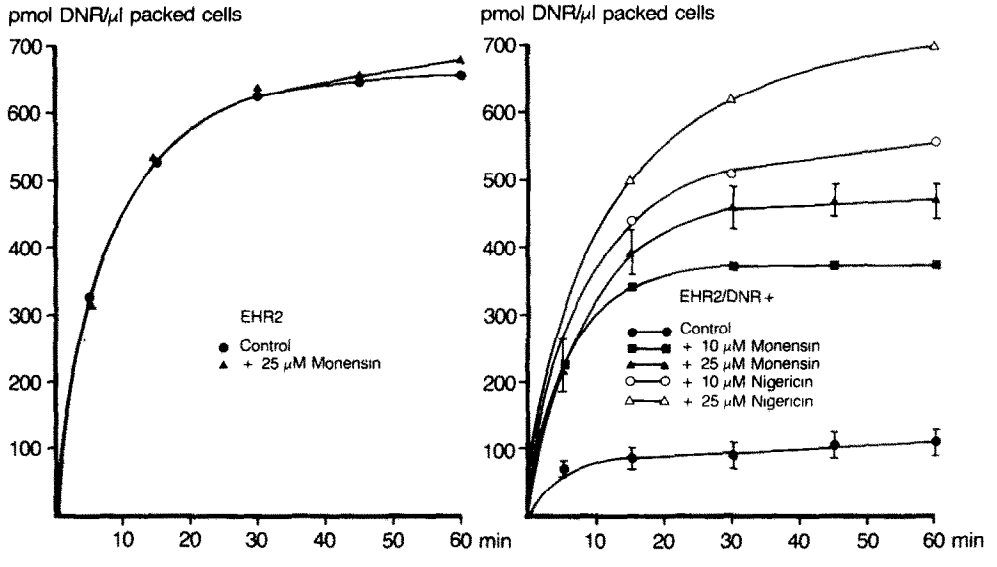

Fig. 1. Effect of monensin and nigericin on time-course of DNR uptake in whole cells. Cells (2×10^6/ml) were incubated in standard medium, pH 7.45 at 37°. DNR (5 μM) as well as monensin and nigericin (at the indicated concentrations) were added at time zero. Bar = SD of 3 experiments.

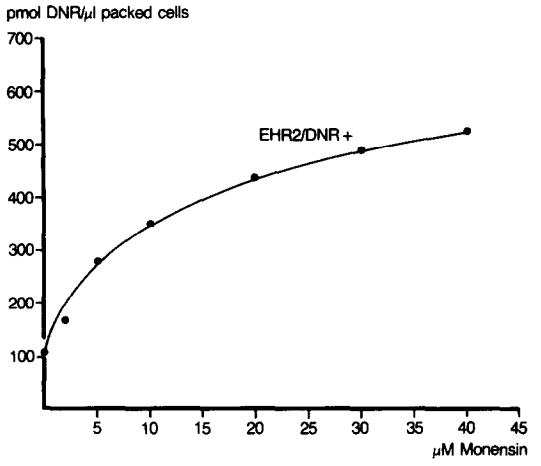

Fig. 2. Effect of monensin on uptake approaching steady-state of DNR in whole cells from the resistant subline. Cell suspensions (2×10^6/ml) were incubated for 60 min in standard medium at 37° with 5 μM DNR and monensin at the indicated concentrations.

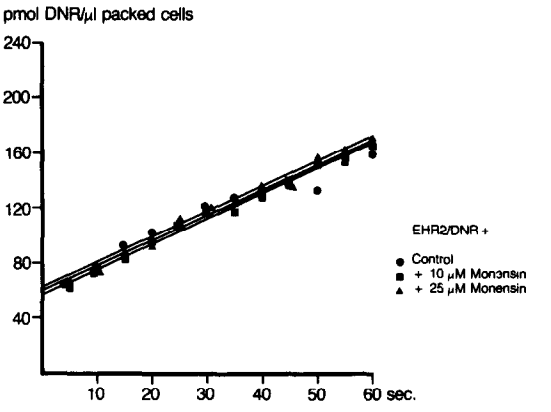

Fig. 4. Effect of monensin on initial uptake of DNR in resistant cells. Monensin (either 10 or 25 μM) and DNR (5 μM) were added at time zero to cell suspensions (2×10^6/ml) preincubated for 10 min in standard medium without glucose or calf serum but containing 10 mM sodium azide. Serial samples of 2000 μl were withdrawn at 5 sec intervals.

Furthermore, monensin had no effect on DNR binding to isolated nuclei of EHR2 or EHR2/DNR+ (data not shown).

DNR efflux

After cells were loaded with DNR by energy deprivation, efflux was induced by addition of glucose (Fig. 3). Addition of monensin 1 min prior to glucose inhibited DNR efflux in EHR2/DNR+ in a dose-dependent manner. Medium pH showed only a minimal decrease after addition of glucose in resistant cells, namely from 7.42 at 30 min to 7.40 at 35 min and 7.35 at 60 min, while no change was observed in sensitive cells. Similar figures were obtained when monensin (25 μM) was present.

Clonogenic assay

Figure 5 shows dose–response curves to DNR combined with monensin in relation to simultaneously performed dose–response curves to DNR alone. It appears that the addition of monensin only results in a parallel displacement of the dose–response curve in EHR2, indicating that the combined effect is additive. However, in EHR2/DNR+ the addition of monensin results in a dose–response curve with an increased slope. This indicates that monensin modulates the action of DNR on EHR2/DNR+ cells in a synergistic fashion.

Animal experiments

The LD_{10} of monensin and nigericin were cal-

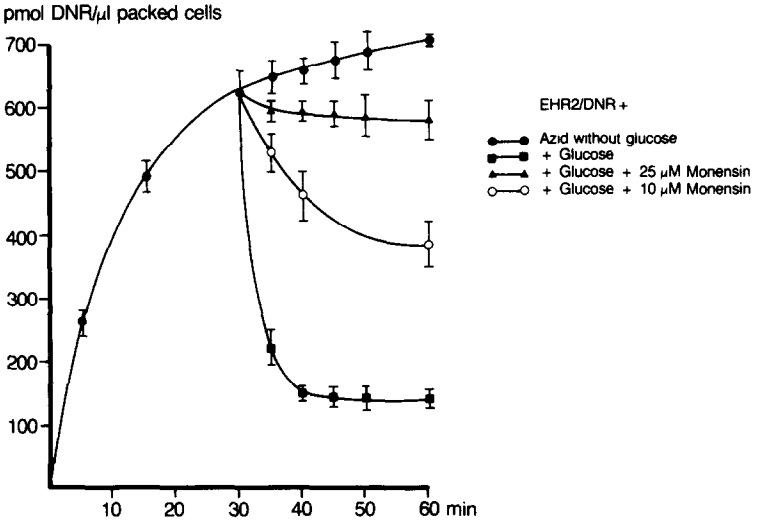

Fig. 3. Efflux of DNR induced by glucose in the resistant subline. Cell suspensions (2×10^6/ml) were incubated in standard medium without glucose and containing 10 mM sodium azide. At time zero DNR was added (to 5 μM). At 30 min, either 10 mM glucose, 10 mM glucose plus 10 μM monensin or 10 mM glucose plus 25 μM monensin was added as indicated.

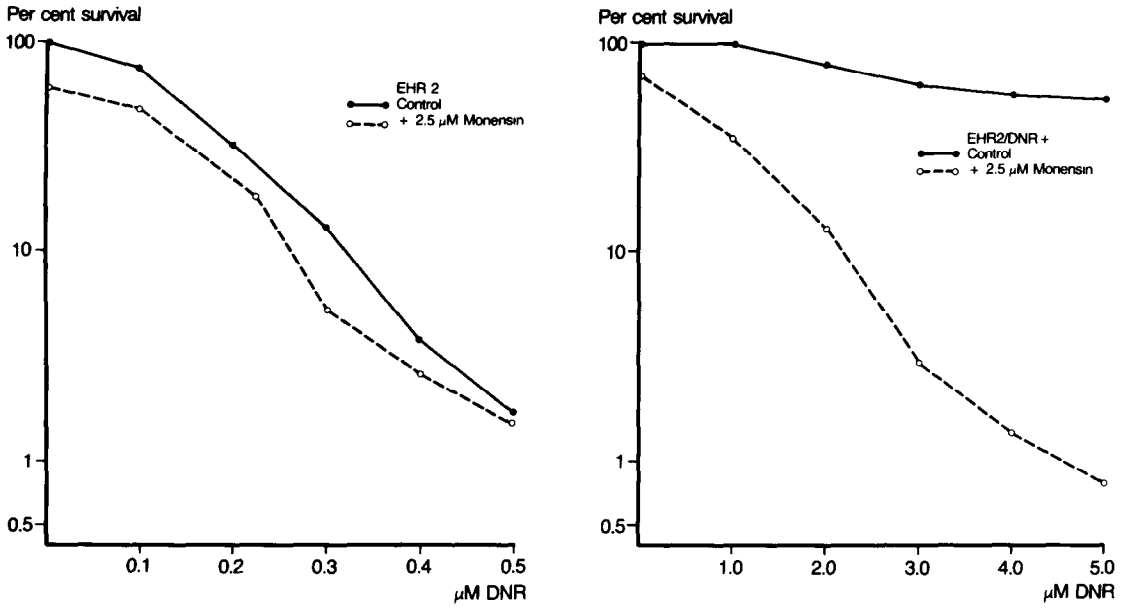

Fig. 5. Dose–survival curves for 1 hr exposure to DNR obtained for EHR2 (left) and EHR2/DNR+ (right). The broken lines represent the survival of cells simultaneously exposed to 2.5 μM monensin at the indicated DNR concentrations. Note that EHR2/DNR+ cells were tested at DNR concentrations which were 10 times that used for EHR2 cells.

culated to be 13.4 mg/kg (95% confidence limits 10.8–16.63 mg/kg) and 5.6 mg/kg (95% confidence limits 4.1–7.55 mg/kg) IP daily for 4 consecutive days, respectively. Neither ionophore, by itself, in an LD_{10} dose increased the life span of EHR2 or EHR2/DNR+ bearing animals. When LD_{10} doses of monensin or nigericin were administered together with a LD_{10} dose of DNR (1.6 mg/kg × 4) in EHR2/DNR+ bearing animals, no modulation of DNR resistance was observed. No additive toxicity was observed when nigericin and DNR were administered concurrently in LD_{10} doses, but a slight additive toxic effect was seen between monensin and DNR in LD_{10} dosage, wherefore an additional experiment was carried out with a reduced monensin dose of 8 mg/kg together with an LD_{10} dose of DNR. In this experiment no additive toxicity was observed, but no modulation of resistance either.

Electron microscopy

Cells incubated with monensin demonstrated the characteristic vacuolization of the Golgi complex [5], without morphological signs of damage to other cellular organelles (Fig. 6).

DISCUSSION

A common characteristic of the pleiotropic resistance phenotype is the ability of the resistant cell to maintain a reduced intracellular drug concentration. This is thought to be partly due to an energy-dependent efflux mechanism(s) in resistant cells [2, 3, 12], though other explanations such as altered rates of passive diffusion have been advocated [13].

The question which the present study has addressed is whether intracellular acidic vesicular compartments (endosomes and lysosomes) play a role in the pleiotropic resistance phenotype. We have previously shown that the plasma membrane to endosome flow as well as endosomal content is significantly increased in pleiotropic resistant Ehrlich ascites tumor and P388 sublines [14, 15]. Numerous studies have shown that various exogenous amines such as the calcium channel blocker verapamil, the phenothiazine tranquilizers, the lysosomotropic agents chloroquine and amantadine, as well as the antiarrythmic drugs propanolol and quinidine, have the ability to both inhibit active drug efflux in pleiotropic-resistant cells and to modulate resistance itself in both *in vitro* and *in vivo* assays [16–21]. Exogenous amines are known to raise the pH of acidic intracellular compartments, presumably by being trapped by protonation, and to perturb intracellular vesicular traffic, possibly by modifying the extent of membrane fusion [22]. The carboxylic ionophores monensin and nigericin, which are not amines, lead to similar effects on intravesicular pH and traffic as exogenous amines by inserting into membranes and facilitating the exchange of sodium (monensin) and potassium (nigericin) ions for protons [5, 23]. If therefore, the effect of exogenous amines on drug efflux and resistance modulation in pleiotropic resistant cells were to be, at least partly, due to their action on intracellular acidic compartments, then one would expect that the carboxylic ionophores would also be able to inhibit drug efflux and modulate resistance. This has been shown to be the case in the present study where both ionophores were able to increase DNR uptake in EHR2/DNR+ (Figs. 1 and 2), and where monensin not only inhibited energy dependent drug efflux in EHR2/DNR+ (Fig. 3), but also modulated *in vitro* resistance to DNR in EHR2/DNR+ (Fig. 5). That

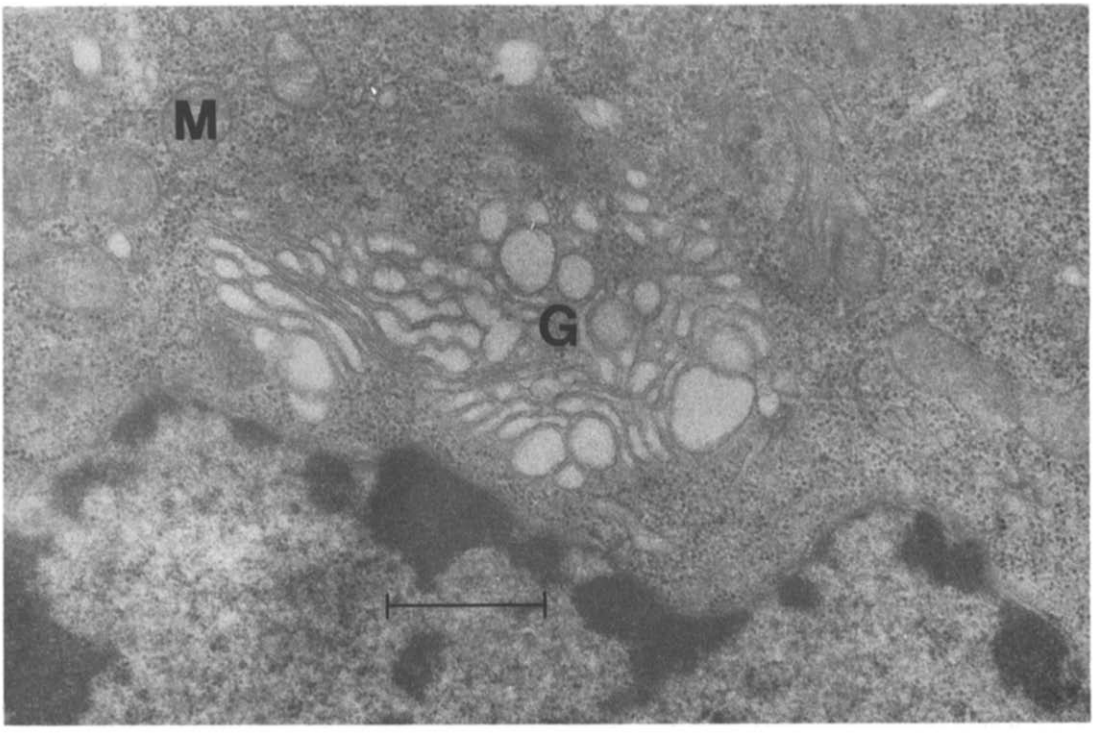

Fig. 6. Ultrastructure of EHR2/DNR+ cell incubated in standard medium containing 25 μM monensin for 30 min at 37° demonstrating typical vacuolization of Golgi complex (G). Note normal appearance of mitochondria (M). Bar = 1 μm.

this is due to effects on intracellular vesicular acidic compartments rather than to pH changes in the cytosolic compartment is explained by the different cationic specificities of monensin and nigericin. Because of the differences in intra- and extracellular levels of sodium and potassium, monensin which has a greater specificity for sodium would be expected to increase cytosolic pH, while nigericin, which has a greater specificity for potassium, should decrease cytosolic pH [24]. These considerations have been confirmed by Grinstein *et al.* in rat lymphocytes [25, 26].

Though the LD_{10} value found in the present study for monensin of 13.4 mg/kg daily for 4 days is somewhat higher than the IP LD_{50} in mice of 10 mg/kg reported by Gad *et al.* [27], the tolerated doses of both monensin and nigericin were presumably too low to be able to modulate resistance *in vivo*. In comparison, the LD_{10} of verapamil in identical schedules was found to be 50 mg/kg, or four times that of monensin [18], while the effect of monensin and verapamil on *in vitro* drug uptake were similar on a molar basis. Thus, though of theoretical interest in the study of the pleiotropic resistance phenotype, carboxylic ionophores are too toxic to play a role in the modulation of clinical drug resistance. Interestingly, nigericin has recently been suggested as a prototype of anticancer agent which might achieve selective killing of tumor cells by interfering with intracellular pH regulation, and the drug was found to be cytotoxic *in vitro* when extracellular pH was below 6.5 [28]. However, an LD_{10} dose of nigericin *per se* did not increase life span in tumor bearing mice in our *in vivo* assay. This fits with our observation that extracellular pH in tumor cell bearing ascites fluid is 7.0.

We conclude that the present study supports a role for acidic intracellular compartments such as endosomes and lysosomes in pleiotropic resistance. Our results would thus fit with the hypothesis recently outlined by Beck [4] linking the acidic intracellular compartments with the resistance associated glycoprotein P-170 originally described by Juliano and Ling [29] in the pleiotropic resistance phenotype.

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