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The mitochondrial uncoupler dicumarol disrupts the MTT assay

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Abstract

Dicumarol is routinely added to the 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to study the role of NAD(P)H:quinone oxido-reductase in drug activation and detoxification. We assessed the direct impact of dicumarol (a mitochondrial uncoupler) on the MTT assay. Mouse mammary tumor (EMT6) and Chinese hamster ovary (CHO) cells were treated with media containing either 10 or 1% fetal bovine serum and dicumarol (0–1000 μM) mimicking standard assay conditions. MTT, clonogenic, total reactive oxygen species (ROS), and oxygen consumption assays were performed. Significant increases in the apparent viability of EMT6 and CHO cells were observed with MTT assays after short time periods with maximum effects at 2 hr. Reduced serum concentrations intensified this effect. Conversely, significant decreases in viability for both cell lines occurred after longer incubations and serum withdrawal enhanced this effect in both cell lines. Clonogenic assays provided contrasting results where viability increased significantly only in EMT6 cells (not CHO) and was smaller than that reported by MTT. Furthermore, greater dicumarol toxicity was observed in clonogenic assays. Significant toxicity compared to control occurred after 4-hr treatment (vs. 12 hr MTT) and serum withdrawal also increased the toxicity of dicumarol with extended culture. ROS production in EMT6 and CHO cells increased in a concentration-dependent manner with 20-min dicumarol administration and thereafter declined. The EC_{50} for dicumarol-induced oxygen consumption was 0.84 μM in CHO compared to 1.18 μM in EMT6 cells. Cell lines are differentially sensitive to the toxicity of dicumarol and cell survival data may be skewed by its inclusion, probably due to ROS production and mitochondrial uncoupling. Dicumarol is not recommended for inclusion in the MTT assay.

Keywords: MTT assay; Cytotoxicity; Dicumarol; Oxidative stress; NQO1; Mitochondria

1. Introduction

The MTT assay is a common assay for cell proliferation and viability, which functions by measuring intracellular reduction of yellow, water soluble MTT reagent to a purple formazan salt which is water insoluble and can be colorimetrically detected at 595 nm. The technique is particularly well liked due to its low cost, rapidity, and high-throughput format.

Although MTT reduction is considered to be a measure of mitochondrial viability, the precise location of the enzymes responsible is unknown. The widely accepted explanation that reduction of MTT is primarily *via* succinate-dependent conversion in the mitochondrial electron transport chain stems from original research by Slater *et al.* [1] who used rat liver homogenates and studied the effect of respiratory inhibitors on the electron transport chain. Subsequent research has demonstrated that succinate dehydrogenase-dependent reduction of MTT accounts for some of the formazan produced but NADH- and NAD(P)H-dependent formazan production also occurs. Nicotinamide-dependent MTT reduction occurs outside of the mitochondrial inner membrane and is responsible for around 90% of the total cellular reduction of MTT [2]. Other studies have confirmed this by demonstrating that MTT reduction associated with the cytosolic compartment is equal to that associated with the mitochondria in rat livers [3] and that only 25–45% of reduced MTT reagent in the human cell line HepG2, was situated within the mitochondrial membrane [4]. This does not exclude ROS produced by the mitochondria as the cause of MTT reduction as the mitochondrial membrane is permeable to ROS.

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Abbreviations: CHO, Chinese hamster ovary cell line; EMT6, mouse mammary tumor cell line; NQO1, NAD(P)H:quinone oxido-reductase; NQO2, NRH:quinone oxido-reductase; MTT, 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species.

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Of further interest is the report that MTT can be used as a direct substrate for the enzyme NRH:quinone oxido-reductase (NQO2, K_m 12 μM) and, with menadione as a co-substrate, for both NQO2 and NAD(P)H:quinone oxido-reductase (NQO1, K_m 2.3 and 2.7 μM, respectively), perhaps accounting for the extra-mitochondrial MTT reduction observed [5–7].

Dicumarol (3,3′-methylenebis[4-hydroxycoumarin]) is a coumarin derivative with anti-coagulant activity that also acts intracellularly to uncouple mitochondrial oxidative phosphorylation at the NADH-cytochrome b5 site. It is an inhibitor of the NQO1, NQO2, and uridine diphosphate glucuronosyltransferase enzymes [7–9] and is routinely added to *in vitro* activity assays of NQO1 for selective enzyme inhibition. Conversely, dicumarol appears to increase the activity of the enzyme xanthine dehydrogenase [10].

While previous studies on the effects of respiratory chain inhibitors (such as rotenone, antimycin, and oligomycin) on the MTT assay have been performed [11], none have concentrated on the effects of dicumarol. Thus, our research aimed primarily to evaluate the suitability of the MTT assay as a measure of cytotoxicity when dicumarol is included in cell cultures. Secondarily, the experiments may provide further insight into the cellular mechanisms of MTT reduction which have not been fully elucidated.

2. Materials and methods

EMT6 cells were a kind gift from Dr. Alan Sartorelli, Yale School of Medicine, and were originally generated by Dr. Sarah Rockwell, Yale School of Medicine [12]. CHO cells (AA8 wild type) were purchased from the American Type Culture Collection. All tissue culture supplies were from Fisher Scientific and all chemical reagents were from Sigma Chemical Company and of analytical grade or higher.

2.1. Cell culture and assays for cell viability

EMT6 and CHO cells were cultured and maintained in Waymouths Medium and α-Minimum Essential Medium, respectively, containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM glutamine plus with 10% fetal bovine serum (FBS). The MTT assay used was essentially that of Mosmann [13]. Briefly, cells were plated in 96-well plates with 100 μL of cells which had previously been resuspended to 1×10^5/mL. The cells were left to adhere overnight, then exposed to dicumarol (0–1000 μM) administered in media containing either 10 or 1% FBS, and returned to the incubator for 1–24 hr. Subsequently, MTT reagent (4 mg/mL in sterile PBS) was added directly to the wells or, dicumarol-containing medium was removed, cells were washed gently with 200 μL warm, sterile PBS, and media and MTT reagent added to the wells. Cells were returned to the incubator for 4 hr whereupon SDS (10% in sterile PBS, 100 μL) was added. The cells were subsequently re-returned to the incubator overnight and optical density was assessed at 595 nm. Clonogenic assays were performed as previously described [14]. Briefly, exponentially growing cells were exposed to dicumarol (0–1000 μM) administered in media containing either 10 or 1% FBS and returned to the incubator for 1–24 hr. Cells were then harvested and 100 cells plated in 6-cm dishes and left to form colonies for 10–12 days. Viability was measured by counting colonies and normalizing to control (zero treatment) values.

2.2. Measurement of total ROS

The method used for determining production of ROS *in vitro* has been described previously [15]. Briefly, 100 μL of cells (re-suspended to 1×10^5/mL) were plated in 96-well plates, left to adhere overnight, and then exposed to dicumarol (0–1000 μM) for 1–24 hr administered in media containing either 10 or 1% FBS. Fluorometric detection of ROS was subsequently performed by observing conversion of dichlorofluorescein diacetate (10 μM, added directly to cells) to dichlorofluorescein at 485 nm excitation and 535 nm detection. Results were transformed using standard curves prepared in triplicate from dichlorofluorescein (0.1–100 μM, $r^2 = 0.991 \pm 0.005$, mean ± SEM, N = 4).

2.3. Isolation of mitochondria and oxygen consumption analysis

Mitochondrial fraction preparation and oxygen consumption studies in EMT6 and CHO cell lines were performed with an YSI Oxygen Monitor and a Clarke electrode at 25° with a final volume of 1.5 mL as previously described [16]. Dicumarol (0.1–100 μM) was added and the subsequent rate of oxygen consumption observed.

3. Results

3.1. The effect of dicumarol on cell viability assays

Including dicumarol in cellular incubations during the MTT assay caused a transient increase, then a prolonged decrease in apparent cell viability (Fig. 1). Maximum viability was observed at 2 hr in cells treated with 10% FBS media/1 mM dicumarol and was 178 ± 11% and 212 ± 19% of control in EMT6 and CHO cell lines, respectively (mean ± SEM). Minimum viability was recorded at 24 hr with 1% FBS/1 mM dicumarol and was 22 ± 3% and 52 ± 5% of control for EMT6 and CHO cells, respectively (mean ± SEM), although significant toxic effects were first observed 12-hr post-dicumarol addition.

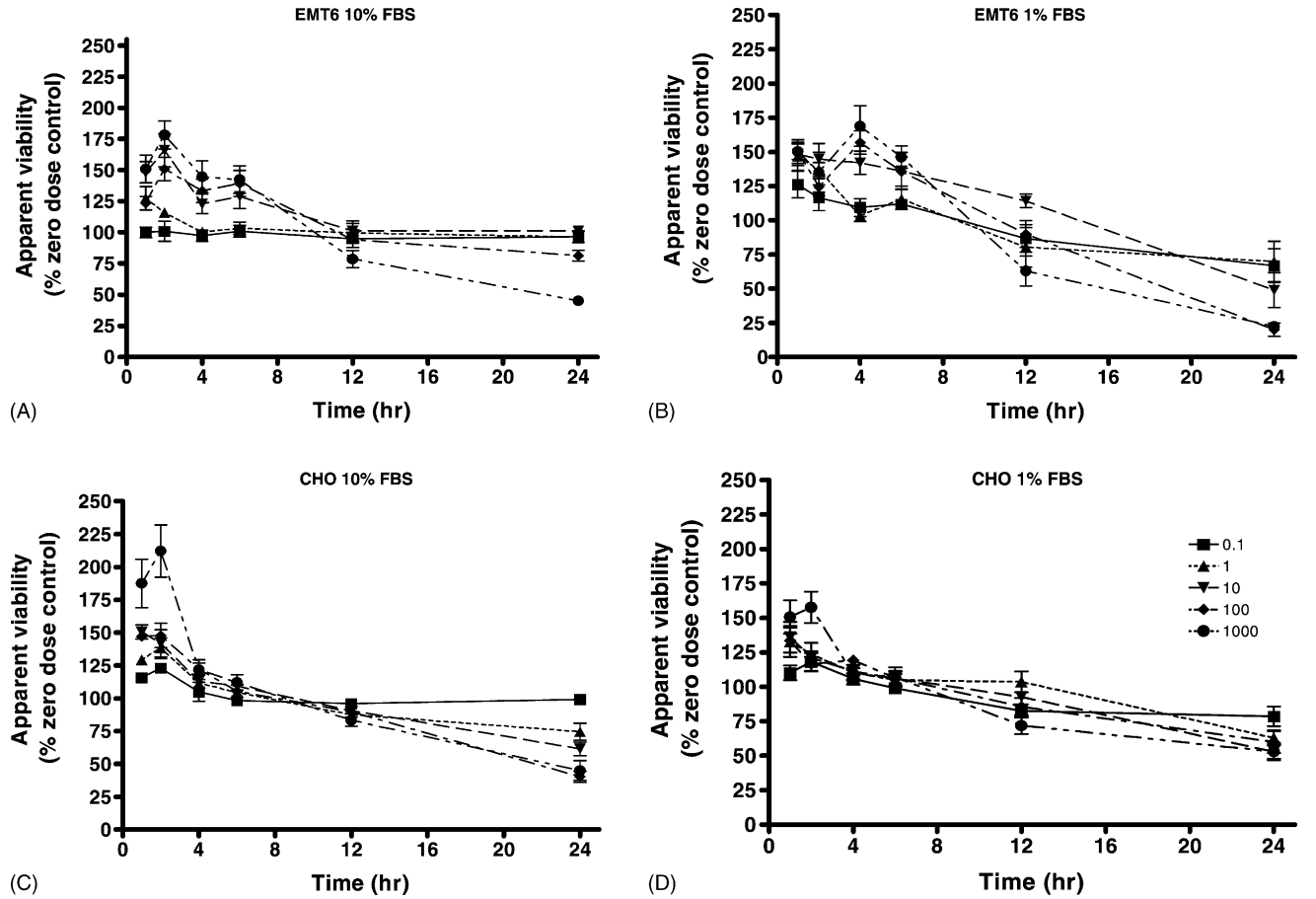

Fig. 1. Time course of MTT reduction in the cultured cell lines EMT6 and CHO exposed to dicumarol (0–1000 μM), then the MTT assay performed with dicumarol still in the wells of the microtiter plate. EMT6 cells were treated for 0–24 hr with dicumarol in media containing 10% FBS (A) or 1% FBS (B). CHO cells were likewise treated with dicumarol in media containing 10% FBS (C) or 1% FBS (D). Points are means of N = 3, performed in triplicate ± SEM.

Differences in the apparent cell viability of dicumarol-treated cells were observed under low serum conditions and between cell lines. After 2 hr, a significant increase in apparent viability compared to control was observed in EMT6 cells treated with 1 μM dicumarol in media with 10% FBS ($P < 0.05$, *t*-test). However, significance was achieved at 0.1 μM dicumarol with 1% FBS ($P < 0.05$, *t*-test). At 24 hr, significant decreases in viability were observed with 100 μM dicumarol in 10% FBS ($P < 0.01$, *t*-test) but at 10 μM dicumarol in 1% FBS ($P < 0.01$, *t*-test). In the CHO cell line, significant increases in MTT turnover occurred within 2 hr compared to control at 0.1 μM dicumarol regardless of the FBS concentration ($P < 0.05$, *t*-test). However, at longer time points (24 hr) FBS was protective such that when media contained 10% FBS, 1 μM dicumarol was required for significant reduction of apparent viability ($P < 0.01$, *t*-test) while when media contained 1% FBS, 0.1 μM dicumarol reduced viability significantly ($P < 0.001$, *t*-test).

To rule out a direct interaction between the MTT reagent and dicumarol, the time course was performed with a washout, whereby dicumarol-containing medium was removed from wells after the appropriate incubation and the cells were washed gently in 200 μL warm, sterile PBS before addition of 100 μL MTT reagent dissolved in media. These results, shown in Fig. 2, were similar to the MTT results obtained without washout.

Using a clonogenic assay for viability, a more marked concentration-dependent effect on cell viability is observed (Fig. 3A–D). The significant increase in viability in both EMT6 and CHO cells reported by the MTT assay at 2 hr is only apparent in the clonogenic assay in EMT6 cells and is considerably lower at 139 ± 25% and 126 ± 36% (0.1 μM dicumarol, 10 and 1% FBS, respectively). The maximum increase in viability caused by dicumarol does however occur at the same 2-hr time point.

At later time points, a greater decrease in viability after exposure is observed in both cell lines with the clonogenic assay compared to the MTT assay when cultured in 10% FBS-containing media ($P < 0.05$, 4 hr, 10–1000 μM dicumarol, Fig. 3A and B, respectively). When cells are treated in 1% FBS-containing media significant toxicity compared to control is also observed after 4-hr exposure but at lower concentrations of dicumarol (10 μM for CHO cells and 1 μM for EMT6 cells, $P < 0.05$, Fig. 3C and D). The contrast between the MTT and clonogenic assays is most

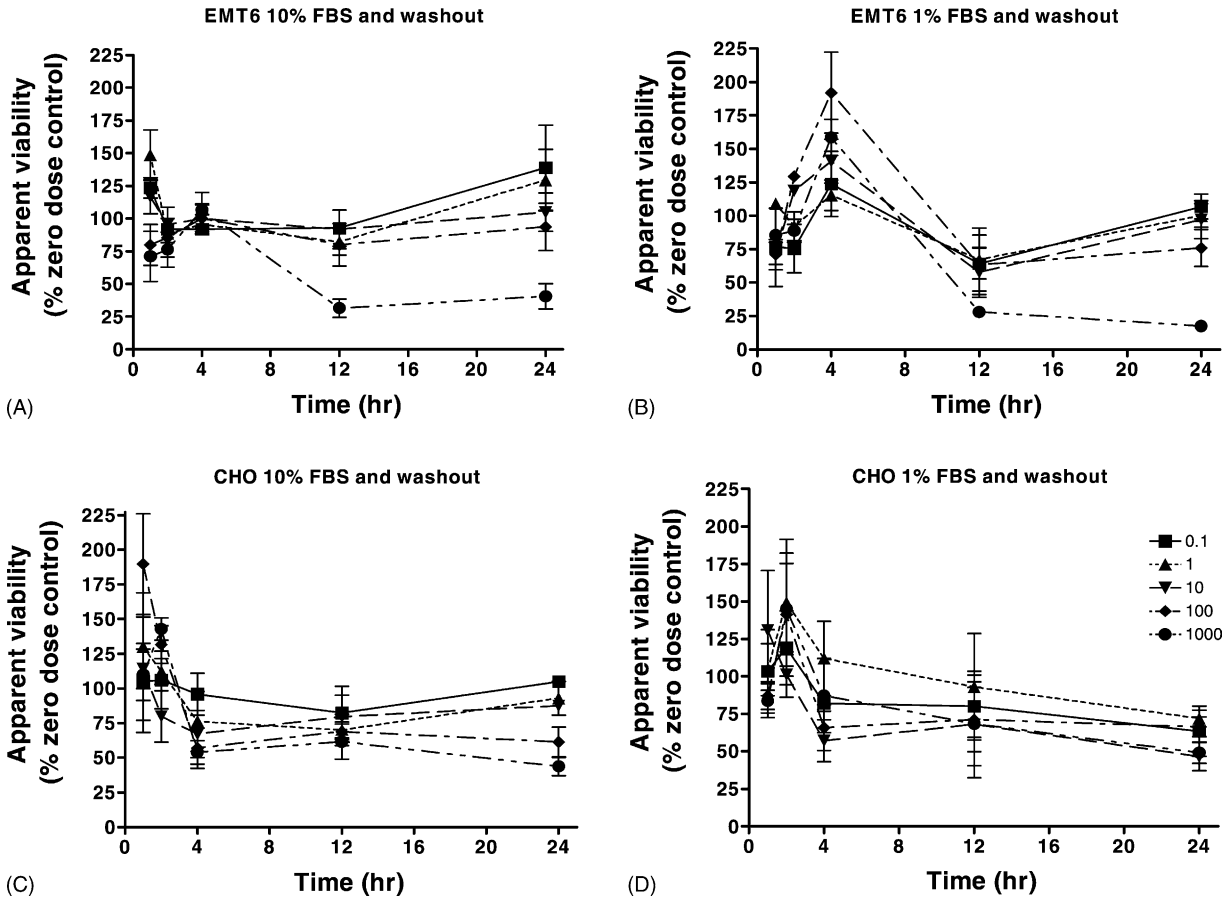

Fig. 2. Time course of MTT reduction in the cultured cell lines EMT6 and CHO exposed to dicumarol (0–1000 μM) which was washed out before addition of the MTT reagent. EMT6 cells were treated for 0–24 hr with dicumarol in media containing 10% FBS (A) or 1% FBS (B). CHO cells were likewise treated with dicumarol in media containing 10% FBS (C) or 1% FBS (D). Points are means of N = 3, performed in triplicate ± SEM.

noticeable with highest (1000 μM) and lowest dicumarol treatments. In clonogenic assays after 4 hr or more treatment with 1000 μM dicumarol, survival falls to 0–5%, compared to approximately 25% survival consistently reported by the MTT assay. With treatment periods of less than 4 hr, an increase in viability which is maximal with 0.1 μM dicumarol and 2-hr treatment is observed in the clonogenic assays which is in contrast to the maximum effect observed in the MTT assay which occurs with 1000 μM dicumarol treatment for 2 hr and is greater in magnitude.

3.2. ROS determination and oxygen consumption analysis

Using an assay for ROS, maximum rates of ROS production at 2 hr was observed in both cell lines with 100 μM dicumarol (Fig. 4). These data demonstrating increasing ROS production with increasing dicumarol concentration are consistent with data presented in Fig. 1 showing apparent decreased viability *via* the MTT assay in the presence of dicumarol at extended time points. While the initial apparent increase in viability occurs at the same time as the increase in ROS, it is postulated that the increased ROS causes decreased viability at later time points (post 2 hr). As ROS-dependant toxicity is a time-dependent process, decreases in viability cannot occur at precisely the same time as an increase in ROS but are more likely to happen at later time points. This suggests that dicumarol is affecting the cells, presumably through mitochondrial uncoupling, in such a way as to skew the MTT assay.

The mitochondrial uncoupling effect of dicumarol in these cell lines was confirmed in mitochondria isolated from EMT6 and CHO cells as an increase in oxygen consumption compared to baseline rates upon administration of the compound (Fig. 5). The EC_{50} was 1.18 μM in EMT6 cells and 0.84 μM in CHO cells (mean). The EC_{50} in CHO cells was 29% lower than that of EMT6 cells and while the slope of the dose–response curve is parallel, it is right-shifted and demonstrates lower threshold and maximum effect values.

4. Discussion

Dicumarol affects the MTT assay indirectly when applied to cell lines *in vitro* such that viability is exag-

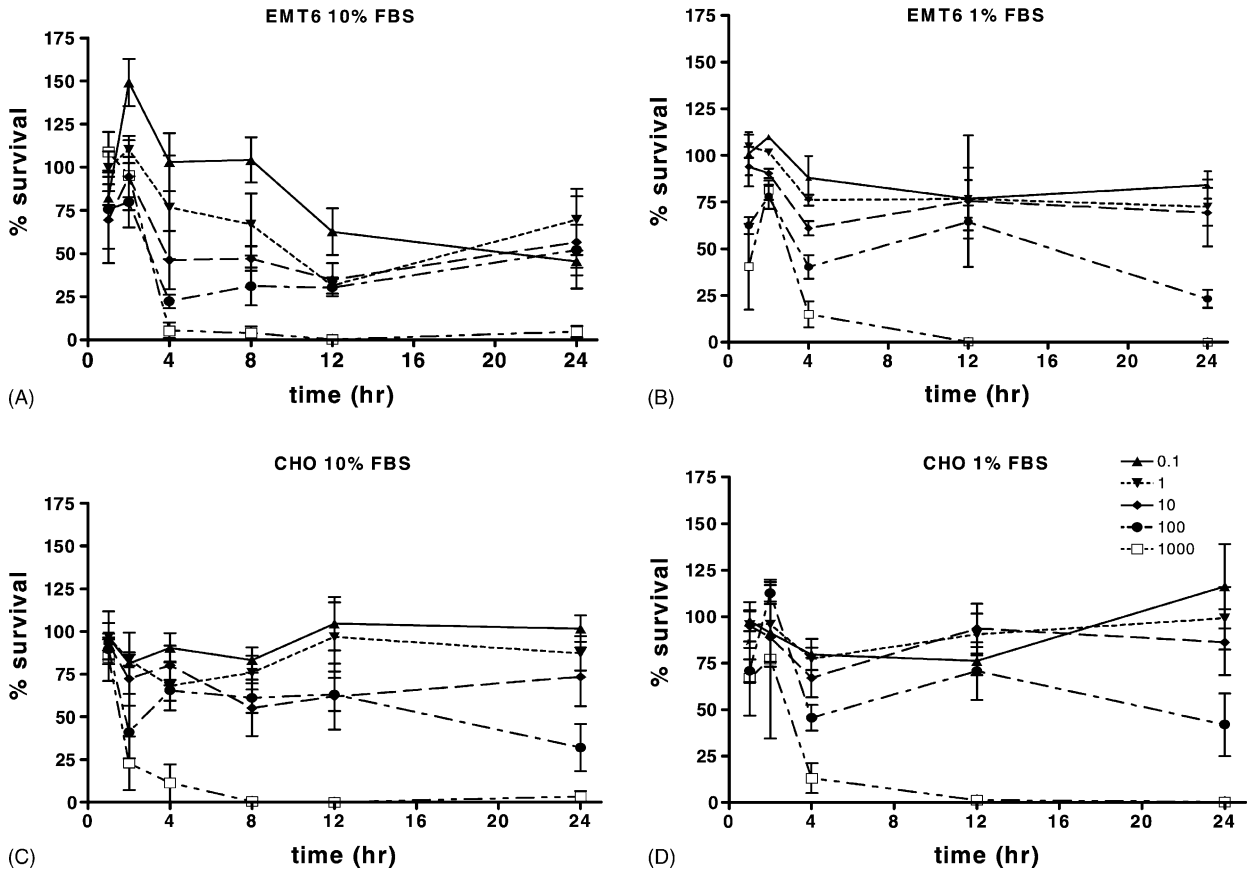

Fig. 3. Clonogenic assays of cell viability in EMT6 and CHO cells exposed to dicumarol (0–1000 μM). EMT6 cells were treated for 0–24 hr with dicumarol in media containing 10% FBS (A) or 1% FBS (B). CHO cells were also treated with dicumarol in media containing 10% FBS (C) or 1% FBS (D). Cells were then harvested, plated into 60-mm sterile cell culture dishes, and incubated in 8% CO_2/92% air for 10–12 days before colonies were counted. Points are means of N = 3, performed in triplicate ± SEM.

gerated within short periods of culture (≤2 hr) and is over-reported with extended exposure to the compound. This effect is considered indirect as we believe that the apparent over-reporting of cell viability is caused by dicumarol causing increased ROS. Subsequent ROS-associated toxicity is responsible for decreasing viability rather than direct cellular effects by dicumarol. These effects are likely to be due to changes in cellular energy production and

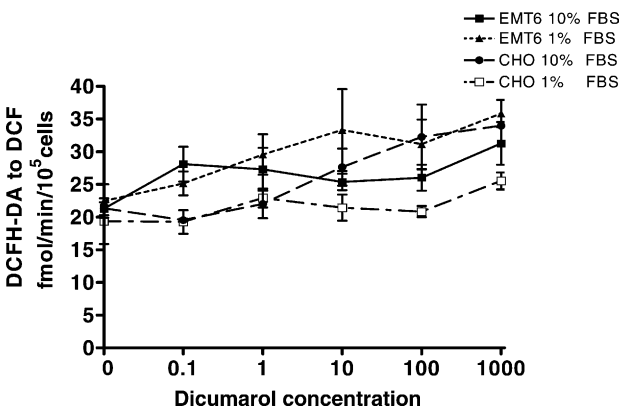

Fig. 4. Total ROS produced in the EMT6 and CHO cell lines plated at 1×10^5 cells/well and exposed to dicumarol (0–1000 μM) in culture for 2 hr. Basal rates of oxygen consumption for the two cell lines were similar. Fluorometric detection of ROS was performed by observing conversion of dichlorofluorescein diacetate (10 μM) to dichlorofluorescein at 485 nm excitation and 535 nm detection. Media contained either 10 or 1% FBS. Points are means of N = 4, performed in triplicate ± SEM.

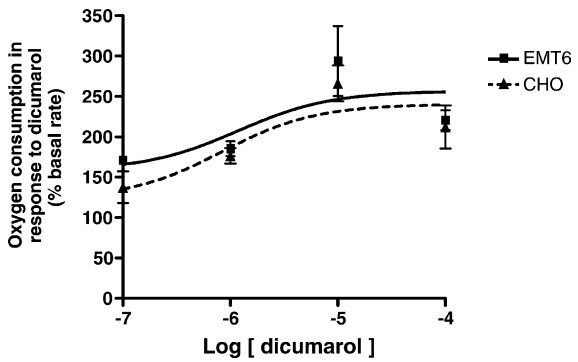

Fig. 5. Dose–response curves for the increase in oxygen consumption (% baseline control) in isolated mitochondria exposed to 0.1, 1, 10, or 100 μM dicumarol. Oxygen consumption was assessed in triplicate, and plotted as mean ± SEM, for each concentration of dicumarol. EC_{50} (the concentration causing 50% maximum response) was calculated using Graph Pad Prism 3.0 and fitting a non-linear, sigmoidal regression model to our data. This model defined baseline response, maximal response, and EC_{50} values. The fit was considered appropriate when convergence was reached (two consecutive iterations produced a sum of squares which differed by less than 0.01%).

metabolism caused by dicumarol's mitochondrial uncoupling and the associated physiological changes which subsequently take place.

Interestingly, with exposure up to 2 hr dicumarol appears to exert a proliferative effect on cells. This was evident in both clonogenic and MTT assays, although the magnitude of the effect was reported markedly higher in the MTT assay than the clonogenic assay. Although we have no current explanation for this phenomenon, our data are in agreement with a report by Goodwin *et al*. [6] who observed a consistent increase (10–20%) in apparent cellular viability when cells were treated with 1 mM dicumarol for 20 min and assessed with MTT assay.

Conversely, with extended culture dicumarol causes cell death. Significant toxicity was observed in clonogenic assays after 4-hr treatment with dicumarol concentrations as low as 0.1 μM, while with MTT toxic effects were not significant until at least 12 hr and with higher concentrations of 10–100 μM. The toxic effects observed were exacerbated by the removal of serum, particularly in the EMT6 cell line. Again, these indirect effects were underreported by the MTT assay which consistently returned higher viability rates than the clonogenic assay for cells treated for up to 24 hr (approximately 25% vs. 1% for MTT and clonogenic, respectively). The observation that dicumarol is indirectly toxic to cells is consistent with a report by Schulze-Osthoff *et al*. [11] detailing an increase in tumor necrosis factor α toxicity when respiratory chain inhibitors are co-administered which is above that seen by either compound alone. This is probably caused by increased ROS generation due to mitochondrial uncoupling shortly after administration and the associated disrupted respiration at later stages.

While dicumarol influences MTT results significantly in both CHO and EMT6 cell lines, it appears that the impact of reduced serum was more pronounced in the EMT6 cell line. This may be because CHO cells are more sensitive to dicumarol and maximum toxicity is reached earlier, masking changes attributable to serum withdrawal. Alternatively, serum proteins may compete with the MTT reagent to bind ROS and therefore higher protein concentrations may be protective through binding and inactivation. The data presented in the oxygen consumption assays (Fig. 5) correlate with the greater sensitivity of CHO cells to the mitochondrial effects, namely that the slope of the dose–response curve is parallel but right-shifted compared to EMT6 cells and demonstrates lower threshold, maximum effect, and EC_{50} values. Alternatively, as dicumarol is a highly protein bound drug, a direct effect of reduced serum in culture media may give rise to more unbound dicumarol and therefore increase its toxicity. Reducing media serum concentrations or substituting bovine gamma globulins for fetal calf serum is a common practice in many laboratories, particularly where the presence of serum proteins *in vitro* affects assay performance (such as ELISAs). Our results detailing reduced viability under reduced serum conditions concur with a report that there was no effect of dicumarol (20 μM for 24 hr) on HeLa cells cultured with 10% FBS (trypan blue exclusion and ^{3}H-thymidine incorporation) but reduction in viability to 40% of control occurred in cells cultured with 1% FBS [17].

The increases reported in apparent cell viability after short periods of culture appear to be over-estimated by the MTT assay compared to the clonogenic assay and similarly with extended culture viability is over-reported. This was shown not to be a direct chemical interaction but a result of the cellular effects exerted by dicumarol, most likely mitochondrial uncoupling and the over-production of ROS. Data presented show a significant increase in ROS when dicumarol is present in culture and this along with published studies on the cellular location of MTT turnover [4] suggest that oxidative processes other than mitochondrial electron chain-associated enzymes may be responsible at least in part for MTT turnover in competent cells. These ''other oxidative processes'' may prove even more critical in cells which are under stress. Although it is possible to include a control which contains dicumarol and normalize to its effects, researchers who do so risk masking the actual toxicity of their compound of interest due to over-reporting as was demonstrated in these studies. We therefore suggest that extreme caution should be exercised in the interpretation of MTT assay results where chemicals, especially mitochondrial poisons, are included in culture alongside the compound of interest. This is especially important with the use of dicumarol as significant interference with the MTT assay is observed at concentrations as low as 0.1 μM, 100- to 1000-fold below concentrations commonly used *in vitro* for blocking NQO1 activity. We also believe that compounds which readily generate oxygen radicals, such as doxorubicin, are likely to interfere with MTT oxidation and therefore make the MTT assay unreliable. Another significant factor to consider in the use of the MTT assay for toxicity studies is the length of time for which cells are cultured in the wells of a 96-well microtiter plate. Cells, such as EMT6 or CHO, have a doubling time of approximately 12 hr while other commonly used cells, such as HT-29, double in approximately 16 hr. This means that growth periods of several days after drug washout as are generally used in the clonogenic assay may not be appropriate as exponential cell growth can result in death due to media starvation or contact inhibition within the microtiter well.

In conclusion, the addition of dicumarol to cells being measured for viability with the MTT assay is not recommended when evaluating cytotoxins regardless of the use of washout period before introducing the MTT substrate.

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