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# Inhibition of membrane redox activity by rhein and adriamycin in human glioma cells

**Maurizio Fanciulli, Francesco P Gentile, Tiziana Bruno, Marco G Paggi, Marcello Benassi and Aristide Floridi[CA]**

M Fanciulli, T Bruno, MG Paggi and A Floridi are at the Laboratory for Cell Metabolism and Pharmacokinetics, and FP Gentile and M Benassi are at the Laboratory for Medical Physics, Center for Experimental Research, Regina Elena Institute for Cancer Research, Via delle Messi D'Oro 156, 00158 Rome, Italy. Tel: 39-6-4985562. Fax: 39-6-4180473.

**The effect of the combination of adriamycin (ADM) with the anti-inflammatory drug rhein (RH) on the membrane redox activity in human glioma cells was investigated. RH, although less effective than ADM, inhibits ferricyanide reduction by human glioma cells in a dose-dependent manner as well as ferricyanide-induced proton release. The inhibition of the plasma membrane redox system might represent a further mechanism by which RH, other than ATP depletion, affects cell survival. The analysis of the interaction between ADM and RH, performed with the isobolar method, demonstrates a strong synergic response, probably due to an effect on different sites of action. The synergism of the ADM–RH association allows us to achieve a pre-established extent of inhibition with ADM concentrations much lower than with ADM alone. RH might, therefore, represent a very useful tool to improve the therapeutic index of ADM and to lower its general toxicity.**

*Key words:* Adriamycin, combined treatments, human glioma cells, membrane redox activity, rhein.

## Introduction

A transplasma membrane redox enzyme system, which transfers electrons from reducing agents in the cytoplasm to external impermeable oxidants, such as ferricyanide, seems to be present in all cells.[1,2] Electron transport in this enzyme system is accompanied by proton release from the cell, which could be used as an energy source to drive molecular transport across the membrane.[3,4] Several studies demonstrate that the external ferricyanide can stimulate growth in melanoma cells, when growth factors present in serum are limiting, and ferric sulfate has also been shown to stimulate growth in 3T3 cells.[5–7] There is evidence that agents involved in tumor promotion, as well as selected antitumor drugs, act at the level of the plasma membrane.[8–11] Among these (e.g. adriamycin, bleomycin, actinomycin D and cisplatin), the most effective in impairing transplasma membrane ferricyanide reduction in transformed or tumor cells is adriamycin (ADM) which, by contrast, is much less effective against normal or untransformed cells.[12–14] Bleomycin and cisplatin do not inhibit ferricyanide reduction in isolated liver cells, whereas a significant inhibition in HeLa cells has been observed.[15] The observation that the antineoplastic drugs inhibit transplasma membrane redox activity at concentrations which affect cell survival strongly suggests that the transmembrane enzyme can play an important role in the control of neoplastic growth. This is further confirmed by the observation that the inhibition of transplasma redox activity is accompanied by a reduced proton extrusion and that an increased cytosolic proton concentration is associated with growth inhibition.[16,17]

It has been previously reported that rhein (RH), 4,5-dihydroxy-anthraquinone-2-carboxylic-acid, an anti-inflammatory drug,[18,19] affects the clonogenic activity of human glioma cells,[20,21] mainly by lowering ATP availability due to its effect on oxidative and glycolytic metabolism. The inhibition of oxidative metabolism is linked to a block of electron transfer from site 1- and 2-entering substrates to electron carriers of the respiratory chain,[22] whereas the inhibition of glycolysis is

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[CA] Corresponding Author

related to a reduced glucose uptake, due to an aspecific effect on the plasma membrane.[23] However, the possibility that RH affects cell clonogenicity by other mechanisms cannot be ruled out.[20] This drug not only has a quinonic structure similar to that of anthracyclines, but also interferes with the plasma membrane so that an effect on membrane redox activity might be involved in the inhibition of cell growth as well as in the enhancement of the cytotoxicity of other antineoplastic drugs.

The experiments described in this paper were undertaken to investigate the properties of the transmembrane electron transport system in human glioma cells, and to evaluate in this context the effect of RH and ADM, either alone or in combination.

## Materials and methods

### Cells

The cell line used throughout the experiments was established from a surgical specimen of a glioblastoma multiforme.[24] The cultures were maintained at 37°C in a humidified 5% $CO_2$–95% air in RPMI 1640 medium (Gibco) supplemented with 2 mM glutamine, antibiotics and 10% fetal calf serum (Gibco). In the exponential growth phase the plating efficiency ranged between 40 and 50%. Confluent monolayer cultures were prepared for study by harvesting the suspension treated with 0.02% EDTA at 700 *g*. The pellet was diluted with TNK medium [25 mM *N*-Tris[hydroxymethyl]-2-aminoethane sulfonic acid (TES), 137 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 2.5 g/l sucrose, pH 7.4] to a final concentration of 0.05 g cell wet weight/ml (g w.w/ml).

### Drugs

RH, kindly supplied by Dr Vittorio Behar, Proter Laboratories, Opera, Milan, Italy, and ADM, purchased from Farmitalia, Milan, were dissolved in sterile quartz bidistilled water.

### Ferricyanide reduction

Ferricyanide reduction in human glioma cells was evaluated by dual-wavelength spectrophotometry (Aminco DW-2a). The cuvette (10 mm), thermostated at 37°C, was provided with magnetic stirring. The reaction medium contained 2.6 ml of TNK medium and 0.05 g w.w/ml of cell suspension. After 2 min at 37°C drugs, RH, ADM or both, were added and the incubation was allowed to proceed for a further 5 min. Then ferricyanide was added by rapid injection from a microsyringe in such a way as to achieve the shortest possible mixing time. Absorbance changes were measured at 420–500 nm and recorded with a linear recorder. Blanks were run with ferricyanide plus RH, ADM or both and no cells. An extinction coefficient of 1.0 $cm^{-1}$ $mM^{-1}$ was used for ferricyanide. To check for ferricyanide reduction by mitochondria released from broken cells, the activity was measured in the presence of rotenone and antimycin A; no effects of these agents on either phase of ferricyanide reduction with these cells were observed.

### Determination of ferricyanide-induced proton generation

Ferricyanide-induced proton generation was measured at 37°C in a thermostated glass chamber of 2 ml capacity (Gilson Medical Electronics, WI) with a Beckman combination electrode connected to a Beckman 3500 pH meter. Cells (0.005 g w.w/ml) were suspended in a salt–sucrose medium (10 mM KCl, 10 mM $CaCl_2$, 0.1 M sucrose and 5% TES buffer, pH 7.0). The sample was stirred continuously and air was bubbled through the reaction mixture to remove $CO_2$. After the pH came to equilibrium, ferricyanide (0.3 mM) was added. The proton generation was measured by the change in pH over the range 7.0–7.5. Known amounts of HCl were added as internal standard to calibrate the pH electrode response in any single experiment.

### Cell survival studies

For these experiments, $1 \times 10^5$ cells were plated in 25 $cm^2$ tissue culture flasks (Corning). On the fifth day of culture, i.e. during the exponential phase of growth, an amount of freshly prepared RH solution to obtain the established concentration was added to the flasks. Exposure was always for 24 h at 37°C in a humidified atmosphere containing 5% $CO_2$–95% air. At the end of incubation the medium was discarded, cells were washed and harvested as a single cell suspension and counted (Coulter Counter, model ZM). Known aliquots of the cell suspension were dispersed into 80 mm Petri dishes, so that colonies would appear after 8–10 days. In

each experiment the plating efficiency of at least four controls was assayed simultaneously. The survival fractions for different drug concentrations were normalized with respect to the individual control for each experiment. All experiments were performed at least three times.

### Data analysis

The analysis of the interaction of RH with ADM on ferricyanide reduction was performed by the isobolar approach.[25] Briefly, this method, which is supposed to offer a general solution to the problem of interaction,[26] implies the construction of isoeffect curves or surfaces based on the dosage of the agents, given alone or in combination, to achieve the same effect. Furthermore, it enables the effect of non-interactive combinations to be calculated from experimental data, regardless of the particular types of exposition–effect relationships of the individual agents and, more generally, without invoking the mechanism of action.[26]

## Results

The time-course of ferricyanide reduction by human glioma cells is shown in Figure 1. The curve is biphasic; there is a rapid initial reduction of ferricyanide for about 1 min, followed by a steady state over several minutes. If the cells are preincubated for 5 min with 50 $\mu$M RH before the pulse of ferricyanide there is a significant inhibition of the slow rate of reduction (−57%), whereas the fast initial rate is unaffected. Figure 2 shows the

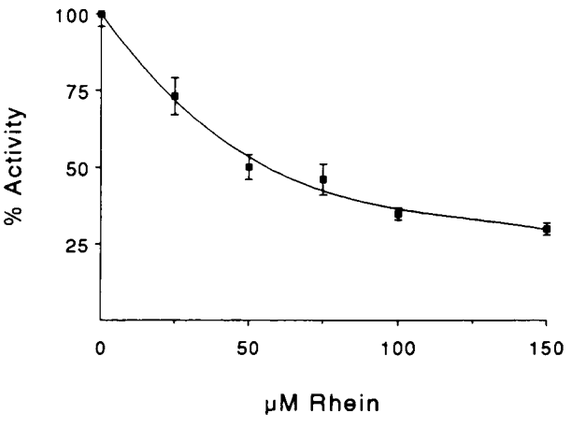


**Figure 2.** Dependence of ferricyanide reduction on RH concentration in human glioma cells. Each point ± SD was averaged from experiments performed on six different cell preparations.

effect of increasing RH concentrations on the slow rate of ferricyanide reduction. The rate decreases exponentially with drug concentration and the half-maximal inhibition is given by 48 $\mu$M RH.

Coupling of $H^+$ ejection in the medium to transmembrane redox activity was also found in human glioma cells. A time-course of proton release upon the addition of ferricyanide is shown in Figure 3. Cells were incubated in the medium and the suspension was bubbled with air to remove $CO_2$. After the pH became stable, a pulse of 0.3 mM ferricyanide induced a rapid initial $H^+$ ejection followed by a slower rate of proton release, which continued unaffected for several minutes. Preincubation of the cells with 50 $\mu$M RH strongly inhibited the slow rate of $H^+$ ejection (−42%)

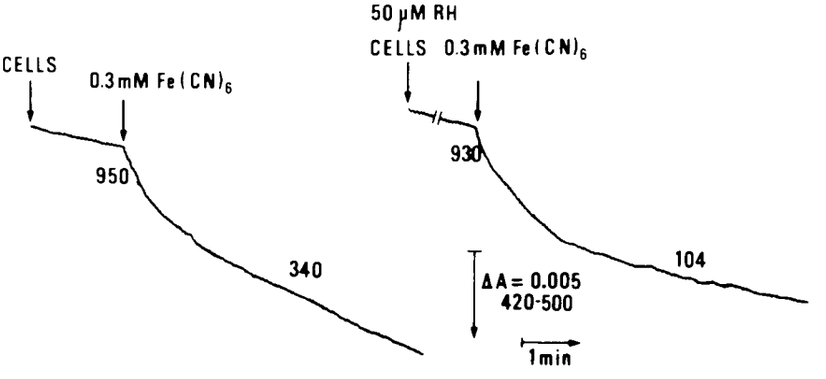


**Figure 1.** Typical traces showing the rate of ferricyanide reduction by human glioma cells and the effect of 50 $\mu$M RH. Cells (0.05 g/wet wt) were incubated for 5 min at 37°C before the addition of K-ferricyanide at 0.3 mM final concentration. The volume was always 3.0 ml. The numbers along the traces indicate the reduction rates expressed as nmol/min/g wet wt.

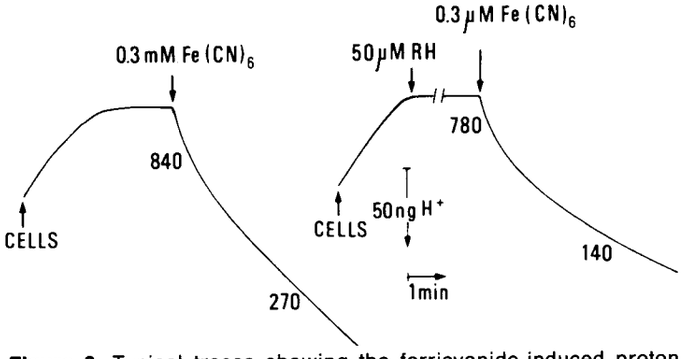


**Figure 3.** Typical traces showing the ferricyanide-induced proton release by human glioma cells and the effect of 50 $\mu$M RH. The numbers along the traces indicate the rate of proton release expressed as nmol $H^+$/min/g wet wt. Experiments were repeated four times and give comparable results ($\pm$8%). Assay as described in Materials and methods.

whereas the initial rapid phase was practically unaffected (−7%). Although the experiments of $H^+$ release were performed in a different medium, so that the rates of ferricyanide reduction and $H^+$ ejection are not comparable, it is worthy of mention that an almost similar extent of inhibition by 50 $\mu$M RH was observed.

These data clearly demonstrate that RH inhibits the transmembrane redox activity of human glioma cells; however, this does not mean that, under different experimental conditions, a similar effect might not have been observed. To verify this point the effect of RH on ferricyanide reduction was tested under the same experimental conditions in which RH inhibits the clonogenic activity of human glioma cells.[20,21] The cells, in the exponential phase of the growth, were treated with 65 $\mu$M RH for 24 h. At the end of the treatment, cells were washed, detached, counted and assayed for the ferricyanide reduction.

Table 1 shows that the 24 h RH treatment did not significantly affect the fast rate of ferricyanide reduction, whereas it inhibited the slower one by 48% (a value overlapping that obtained after 5 min treatment; Figure 2). It reduced the clonogenic activity by 66%, a value that is in excellent agreement with those previously reported.[20,21,27]

**Table 1.** Effect of 24 h treatment with RH on the reduction rate of ferricyanide and on the clonogenic activity of human glioma cells

| RH ($\mu$M) | Rate of $Fe(CN)_6$ reduction | | Relative survival |
|---|---|---|---|
| | fast | slow | |
| — | 980 ± 30 | 230 ± 18 | 100 |
| 65 | 930 ± 35 | 120 ± 13 | 34 ± 5 |

The rate of ferricyanide reduction is expressed as nmol/min/g w.w. Each value ± SD was averaged from three different experiments performed in triplicate.

Because of the striking structural similarities between RH and ADM, and since the latter inhibits transplasma membrane redox activity in neoplastic

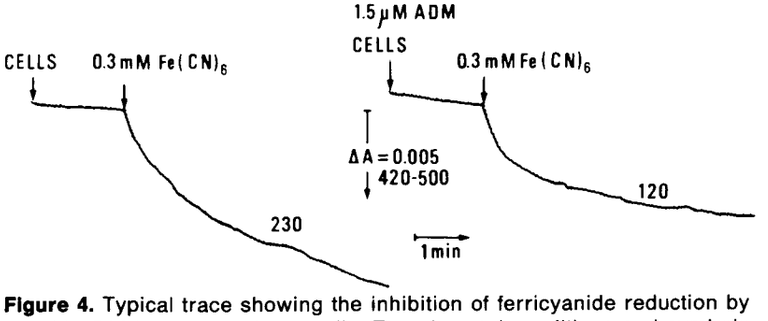


**Figure 4.** Typical trace showing the inhibition of ferricyanide reduction by 1.5 $\mu$M ADM in human glioma cells. Experimental conditions and symbols as in Figure 1.

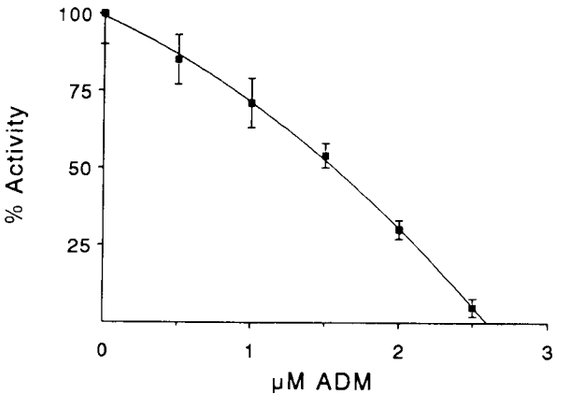


**Figure 5.** Dependence of ferricyanide reduction by human glioma cells on ADM concentration. Each point ± SD was averaged from five different experiments.

cells (see Crane *et al.*[1] for review), it was conceivable that RH might enhance the inhibition by ADM. Therefore, its effect, alone and in association with RH, on ferricyanide reduction was evaluated. ADM is more effective than RH in affecting transmembrane electron transport in human glioma cells as shown by the strong inhibition of the ferricyanide reduction rate brought about by 1.5 μM ADM (Figure 4). The effect of increasing ADM concentration is shown in Figure 5. Half-maximal inhibition was given by 1.5 μM ADM and an almost complete inhibition by 2.5 μM ADM.

The effect of combined ADM–RH on ferricyanide reduction by human glioma cells is shown in Figure 6. When given alone, the concentrations of ADM and RH able to obtain 50% inhibition were 1.5 and 50 μM, respectively. All combinations tested are strongly synergic because all experimental points are located below the straight line joining the concentration of ADM and RH which, when given alone, produce the same effect as that in combination. The interaction index is always much less than 1, ranging from 0.38 to 0.58. A similar response has been also found with different end-points, e.g. 40 and 30% inhibition (data not shown).

## Discussion

Our data demonstrate that the transplasma redox system is also present in human glioma cells, thus confirming once more that it can be considered an ubiquitary system which may play a role in the control of normal and neoplastic cell growth.[5,7]

The ferricyanide reductase system of these human glioma cells (Figure 1) shows the typical kinetics found in other cell types with an initial fast rate of reduction followed by a slower one. The electron transport is associated with a proton release in the medium which may be related to the control of cell duplication. It has been shown that an increase in the pH of the cytoplasm plays a key role in the enhancement of cellular activation and mitogenesis.[16,17,28] If the proton transport function associated with the plasma membrane redox system is involved in the stimulation of cell division, then inhibitors of this enzyme should inhibit cell growth. Such an effect has been found with adriamycin and bleomycin which, impairing the transplasma membrane electron transport, inhibit cytoplasmatic

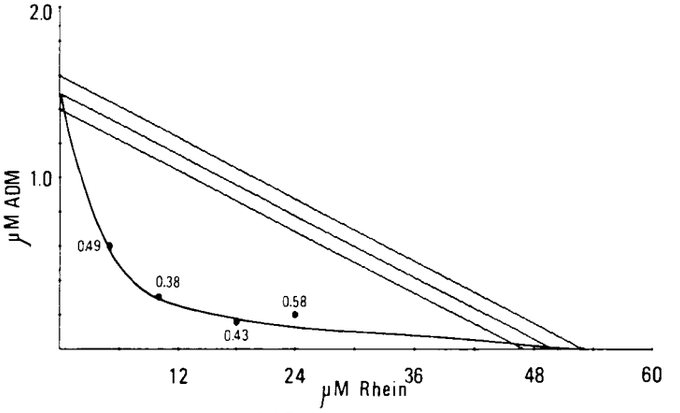


**Figure 6.** Concentration of ADM and RH alone or in combination to obtain a 50% inhibition of the rate of ferricyanide reduction. The upper and lower lines show the zero-interaction region, whereas the numbers indicate the interaction index calculated according to Berembaum.[25] Each combination was repeated at least four times with different cell preparations. Error bars are within closed circles.

pH increase, thus affecting HeLa cell growth.[2,12,15] A similar behavior has been observed in RH-treated human glioma cells in which the redox system is affected by RH at concentrations and under the same experimental conditions (i.e. 24 h treatment) at which it inhibits the growth (Table 1). RH inhibits the plasma membrane redox system (Figure 1) and the associated proton extrusion (Figure 3) with a lowering of the cytosolic pH which would inhibit cell division.

The effect on the transmembrane electron transport enzyme brought about by RH may be ascribed to an aspecific drug effect on the plasma membrane rather than to an interaction with the enzyme at the cell surface. By modulating its fluidity, this interaction might modify the activity of membrane-bound proteins. RH interacts with cell membranes and reduces glucose uptake[23] through a change in membrane fluidity which, in its turn, affects glucose transporter activity (Castiglione *et al.*, in preparation).

Moreover RH, in spite of its quinonic structure, does not seem to inhibit the transplasma membrane redox system through the generation of free radicals. If RH induces the formation of superoxide radicals which decompose to yield hydroxyl, peroxy radicals and hydrogen peroxide, an enhancement of the ferricyanide reduction rate should have been observed, due to the production of superoxide which reduces the ferricyanide. Moreover, the ferricyanide, by the oxidative removal of superoxide, would also tend to prevent further radical formation.

Therefore, we propose that RH affects cell survival by at least two complementary mechanisms: one involving ATP depletion by the inhibition of the oxidative and glycolytic metabolism;[23] the other involving an effect on the plasma membrane redox system (Figure 1, Table 1). The inhibition of the transmembrane electron transport system leads to a reduced proton release (Figure 3), so that the cytoplasmic pH becomes more acidic, thus inhibiting cell division.

An interesting feature for the potential clinical applications is the capacity of RH to enhance the inhibition of ferricyanide reductase by ADM. It has been reported that ADM can be cytotoxic without entering the cell, but solely by interacting with a transplasma membrane dehydrogenase involved in a plasma membrane redox system.[10–14] Yet, its cardiotoxic effects, which represent one of the major drawbacks for its use in tumor chemotherapy, may be at least partially related to the inhibition of this enzyme. Since cardiotoxicity is definitely related to the total dose of ADM administered, it is clear that the possibility of employing much lower ADM dosage, without impairing its therapeutic effectiveness, would delay the onset and reduce the extent of this and other side effects. In this respect RH could represent a very useful tool to improve the therapeutic index of ADM and lower its toxicity.

The data reported above clearly demonstrate that although ADM is by itself more active than RH in inhibiting ferricyanide reduction in human glioma cells, their combination results in a strong synergic effect that allows us to reach a pre-established level of inhibition with ADM concentrations much lower than those necessary when used alone. This synergic effect may be ascribed to the fact that these drugs have different sites of action. It has been demonstrated that ADM does not affect NADH-ferricyanide reductase by superoxide formation, but rather by a direct action on the enzyme.[12,14] On the contrary, RH does not act at a specific site, but modulating the membrane fluidity, could make the transmembrane electron transport enzyme more sensitive to ADM. The effect on the enzyme needs only a few minutes of incubation, so it does not require drug internalization.

Nevertheless, it should be kept in mind that a combination that results in a metabolic synergism, when tested in isolated systems *in vitro*, does not necessarily result in a therapeutic synergism *in vivo*. However, the concentrations required *in vitro* to obtain these effects provide an initial estimate of the concentrations which would be required *in vivo* to obtain comparable effects.

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