Effect of probucol and desferroxamine against adriamycin toxicity in cardiac and renal tissues of rats

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Adriamycin (ADR) is an anthracycline glycoside with a broad spectrum of therapeutic activity against various tumors; however, its clinical use has been limited due to its cardiac and renal toxicity. Production of free radicals is involved in the development of ADR-induced toxicity. This study investigated the effect of pre-treatment with probucol (PROB, a hypolipidemic drug with a powerful antioxidant property) and desferroxamine (DFO, an iron chelator) against ADR-induced oxidative stress in the cardiac and renal tissues. Forty male Wistar rats were divided into four groups: Group I rats received ADR (3 mg/kg, b.w i.p.) over a period of 2 weeks for a cumulative dose of 18 mg/kg, b.w; Group II or ADR + PROB group rats were given PROB i.p. in a cumulative dose of 120 mg/kg, b.w. divided into twelve equal injections over a period of 4 weeks starting 2 weeks before ADR administration; Group III or ADR + DFO group rats were given DFO i.p. in six equal doses each 50 mg/kg, b.w. over a period of 2 weeks given 30 min before ADR injection; and Group IV rats were kept without treatment and served as a control. Results showed that ADR administration caused a significant increase in malondialdehyde (MDA) level in serum, heart and kidney tissues along with lowered activities of cardiac and renal glutathione peroxidase (GPx) and glutathione-S-transferase (GST). A significant decrease in cardiac glutathione (GSH) level and xanthine dehydrogenase (XD)/xanthine oxidase (XO) ratio, serum creatine kinase (CK) and renal glutathione reductase (GR) activities was also observed. Cytotoxic damage was evident from the histopathological examination in heart and kidney specimens. Pre-treatment with either PROB or DFO restored the cardiac, renal and serum MDA levels and renal GR and cardiac GST activities. They also caused significant elevation in serum CK activity and renal XD/XO ratio. PROB normalized the activity of cardiac GPx, whereas DFO restored activity of GPx in both cardiac and renal tissues. It can be concluded that pre-treatment with either PROB or DFO counteracts the state of oxidative stress associated with ADR treatment by modulating the antioxidant status of the animals.

Keywords: Adriamycin, Probucol, Desferroxamine, Oxidative stress, Heart, Kidney, Rat

Adriamycin (ADR, doxorubicin hydrochloride) is an anthracycline antibiotic with a wide spectrum of antitumor activities. However, its therapeutic usefulness is seriously limited by its toxicity to various organs mainly heart and kidneys. The development of cardiomyopathy and ultimately congestive heart failure are the main chronic side effects of ADR, which are critical and life-threatening that limit its clinical usefulness. In some patients, a delayed cardiomyopathy, several years (6-19 yrs) after the completion of therapy has also been reported. Thus, the risk of developing heart failure remains a life-long threat. ADR-induced changes in the kidney of rats include increased glomerular capillary permeability and tubular atrophy. Purine metabolic cycle and related enzymes such as xanthine oxidase (OXO) are reported to be one of the putative effectors of ADR nephrotoxicity.

Despite the use of ADR in the treatment of cancer, its mechanism of action is still not clearly understood and often been a subject of controversy. Several mechanisms seem to account for the effects of ADR, in terms of anticancer action, as well as its cardiac and other organs toxicity. These include inhibition of synthesis of nucleic acid and protein, alterations in calcium handling in the cell, iron-dependent oxidative damage to macromolecules, free radical generation and lipid peroxidation.

The oxidative stress and production of free radicals are the most important mechanisms involved in ADR action. Generally, the cytotoxic activity of ADR is partly related to its quinone structure. The drug is converted to a semiquinone free radical by NADPH-cytochrome P-450, which then leads to the generation of superoxide anion and hydroxyl radicals causing membrane lipid peroxidation. In addition, iron can...
react with ADR in a redox reaction, forming iron-drug free radical complex that can reduce oxygen, resulting in the formation of highly reactive oxygen species. Oxidative damage to membrane lipids and other cellular components is believed to be major factor in the toxicity of ADR and other anthracyclines.

The different free radical scavengers and antioxidants have been used to prevent or minimize these adverse effects of ADR. To prevent or minimize the toxic side effects of ADR, it is essential to preserve its anti-neoplastic efficacy. Probucol (PROB), a hypolipidemic drug possess a powerful antioxidant property attributed to the presence of two phenolic groups, instead of one as in vitamin E, a well-known antioxidant. This has opened a whole new perspective for the use of this drug in protection against ADR toxicity. The protection may also be offered by chelation of transient metals, especially iron such as that exerted by desferroxamine (DFO). This may reduce ADR cytotoxicity by slowing the radical chain reaction. Thus, the present study has been undertaken to investigate the effect of pre-treatment with PROB or DFO (an iron chelator) on ADR-induced oxidative stress in heart and kidney of male rats.

**Material and Methods**

**Animals**

Male wistar rats weighing 250 ± 10 g and female Swiss albino mice weighing 20 ± 2 g obtained from the Laboratory Animal Breeding Service, Faculty of Medicine, Cairo University were used in the study. They were kept under controlled environmental and nutritional conditions and allowed free access to water and standard diet throughout the experimental work.

**Drugs and chemicals**

Adriamycin (ADR) from Pharmacia Itallia, S.P.A. Co. Italy, probucol (PROB) from Sigma Chemical Co., St. Louis, MO, USA and desferroxamine (DFO) from Novartis Pharma AG Basle, Switzerland were obtained. All other chemicals were of pure analytical grade.

**Experimental design**

To study the protective effect of PROB and DFO on the toxic action of ADR, forty rats were divided into four groups: Group I or ADR-group: ADR was administered i.p. in six equal doses each of 3 mg/kg b.w. over a period of 2 weeks for a cumulative dose of 18 mg/kg b.w. Group II or ADR + PROB group: PROB was administered for a cumulative dose of 120 mg/kg b.w. i.p. divided into twelve equal injections (each of 10 mg/kg b.w.) over a period of 4 weeks starting 2 weeks before ADR administration and 2 weeks alternating with ADR. Group III or ADR + DFO group: DFO was injected i.p. to rats in six equal doses each of 50 mg/kg b.w. over a period of 2 weeks given 30 min before ADR injections; and Group IV or control group received only normal saline in the same regimen as the ADR group.

The effects of PROB and DFO on the antitumor activity of ADR were evaluated in mice bearing solid Ehrlich carcinoma (SEC). In brief, a total of sixty female mice were used and $2 \times 10^6$ Ehrlich ascitis carcinoma (EAC) cells were transplanted subcutaneously into the right thigh of the lower limb of each mouse. The mice with a palpable tumor mass (100 mm$^3$) that developed within 7 days after implantation were divided into four groups and received the same dosage protocol as previously mentioned.

At the end of treatment and 24 h after the last injections, rats were killed by decapitation, blood was collected and the serum was separated and used for the measurement of malondialdehyde (MDA) level and creatine kinase (CK) activity. Heart and kidney of different groups were rapidly isolated, washed by ice-cold normal saline and blotted dry. The whole heart and kidney were homogenized in 0.1 M Tris buffer (pH 8.1) using Potter-Elvejhem glass homogenizer and the homogenate was divided into three portions. A portion of the homogenate was centrifuged at 10,500 × g at 4°C for 40 min using Dupont Sorvall ultracentrifuge to obtain the cytosolic fraction used for the determination of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-s-transferase (GST) and xanthine dehydrogenase (XD)/xanthine oxidase (XO) activities. The second portion of the homogenate was mixed with 2.3% KCl and centrifuged at 3000 rpm for 15 min and the resulting supernatant was used for the determination of MDA level as an index of lipid peroxides. The remaining portion was precipitated with $m$-phosphoric acid and centrifuged at 3000 rpm for 15 min and the protein-free supernatant obtained was used for the estimation of glutathione (GSH) level.

Finally, the heart and kidney of other treated groups were removed, fixed in 10% formalin, embedded in paraffin and subjected to histopathological examination using hematoxylin and eosin (H and E) stain.
Biochemical assays

Levels of GSH and MDA were determined according to the previously described methods\(^{26,27}\). Serum CK activity was measured using commercially available kits (Stanbio, San Antonio, TX, USA)\(^{28}\). Measurement of cytosolic enzyme activities of GPx\(^{29}\), GR\(^{30}\), GST\(^{31,32}\) and XD/XO\(^{33}\) was carried out. Protein content in tissue fractions was measured by the method of Lowry et al\(^{34}\).

Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M). For statistical analysis of data, group means were compared by one-way analysis of variance (ANOVA), followed by Tukey-Kramer test, which was used to identify differences between groups. A value of \(P<0.05\) was considered as significant.

Results

The effect of PROB and DFO pre-treatment on ADR-induced changes of MDA level and XD/XO ratio, GSH level, activities of GPx, GR and GST in cardiac and renal tissues, as well as serum CK activity and MDA level of rat is shown in Table 1.

### Table 1—Effect of PROB and DFO pre-treatment on ADR-induced changes of MDA level and XD/XO ratio, GSH level, activities of GPx, GR and GST in cardiac and renal tissues, as well as serum CK activity and MDA level of rat

<table>
<thead>
<tr>
<th></th>
<th>ADR (Group I)</th>
<th>ADR + PROB (Group II)</th>
<th>ADR + DFO (Group III)</th>
<th>Control (Group IV)</th>
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<tbody>
<tr>
<td><strong>Heart</strong></td>
<td></td>
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<tr>
<td>XD/XO (a)</td>
<td>0.116 ± 0.004</td>
<td>0.063 ± 0.02</td>
<td>0.087 ± 0.036</td>
<td>0.18 ± 0.015</td>
</tr>
<tr>
<td>MDA (µmol/g tissue)</td>
<td>85.0 ± 5.2</td>
<td>57.5 ± 3.8</td>
<td>62.0 ± 5.12</td>
<td>51.75 ± 2.21</td>
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<tr>
<td>GSH (µg/g)</td>
<td>154.78 ± 11.6</td>
<td>179.48 ± 7.6</td>
<td>163.9 ± 7.8</td>
<td>202.86 ± 5.29</td>
</tr>
<tr>
<td>GPx (µmol NADPH/min/mg protein)</td>
<td>0.1 ± 0.019</td>
<td>0.152 ± 0.01</td>
<td>0.159 ± 0.009</td>
<td>0.157 ± 0.0087</td>
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<tr>
<td>GR (µmol NADPH/min/mg protein)</td>
<td>15.4 ± 1.25</td>
<td>12.05 ± 1.6</td>
<td>16.95 ± 1.22</td>
<td>15.1 ± 1.25</td>
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<tr>
<td>GST (µmol CDNB conjugated/min/mg protein)</td>
<td>8.64 ± 0.37</td>
<td>11.98 ± 0.4</td>
<td>10.23 ± 0.52</td>
<td>12.75 ± 1.25</td>
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<tr>
<td><strong>Kidney</strong></td>
<td></td>
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<tr>
<td>XD/XO (a)</td>
<td>0.033 ± 0.006</td>
<td>0.08 ± 0.007</td>
<td>0.067 ± 0.013</td>
<td>0.057 ± 0.0047</td>
</tr>
<tr>
<td>MDA (µmol/g tissue)</td>
<td>56.47 ± 2.9</td>
<td>33.47 ± 2.86</td>
<td>33.58 ± 4.37</td>
<td>39.9 ± 2.17</td>
</tr>
<tr>
<td>GSH (µg/g)</td>
<td>273.6 ± 17.2</td>
<td>316 ± 12.02</td>
<td>246 ± 7.6</td>
<td>275 ± 13.7</td>
</tr>
<tr>
<td>GPx (µmol NADPH/min/mg protein)</td>
<td>0.52 ± 0.053</td>
<td>0.67 ± 0.05</td>
<td>0.78 ± 0.055</td>
<td>0.74 ± 0.02</td>
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<tr>
<td>GR (µmol NADPH/min/mg protein)</td>
<td>9.17 ± 0.72</td>
<td>12.68 ± 1.22</td>
<td>13.5 ± 0.43</td>
<td>13.19 ± 0.93</td>
</tr>
<tr>
<td>GST (µmol CDNB conjugated/min/mg protein)</td>
<td>8.12 ± 0.68</td>
<td>9.075 ± 0.48</td>
<td>8.39 ± 0.49</td>
<td>10.68 ± 0.708</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
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<tr>
<td>MDA (µmol/dl)</td>
<td>3.077 ± 0.33</td>
<td>1.86 ± 0.105</td>
<td>1.84 ± 0.14</td>
<td>1.49 ± 0.073</td>
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<tr>
<td>CK (U/L)</td>
<td>110.77 ± 10.17</td>
<td>477.18 ± 35.37</td>
<td>287.07 ± 26.3</td>
<td>569 ± 40.63</td>
</tr>
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</table>

\(^{a}\)Significant difference from control group at \(p<0.05\); \(^{b}\)significant difference from ADR group at \(p<0.05\)

### MDA and XD/XO ratio in cardiac and renal tissues

Chronic administration of ADR caused a significant increase in MDA level in cardiac and renal tissues, as compared to the control group. This was accompanied by a significant decrease in the XD/XO ratio in the cardiac tissue, with no significant decrease in the renal tissues. Pre-treatment with PROB and DFO significantly reduced the MDA levels to near the normal values in both tissues. On the other hand, both pre-treatments caused further decrease in XD/XO ratio in cardiac tissue as compared to the ADR group, but were effective in increasing this ratio in renal tissues to near or above normal values.

### GSH and GSH-dependent enzymes in cardiac and renal tissues

ADR administration caused a significant decrease in the cardiac GSH, while no significant change was observed in its renal content. This decrease in cardiac GSH was accompanied by significant reduction in GPx and GST activities and no significant change in GR activity, compared with control group. In case of renal tissues, the activities of these three enzymes were reduced in response to ADR treatment.
PROB was able to normalize GPx activity in the cardiac tissue with no significant increase in the renal tissues. It also caused significant increase and normalization of renal GR and cardiac GST activities, whereas no change was detected in cardiac GR and renal GST. DFO caused significant increase and normalization of GPx activity in the cardiac and renal tissues. It caused a significant increase and normalization of renal GR, whereas no change was observed in cardiac GR. No change was observed in GST activity in the cardiac or renal tissues in DFO-treated group, as compared to the ADR-treated group.

**CK activity and MDA level in serum**

ADR administration caused a significant increase in the MDA level and decrease in CK activity in serum. Both PROB and DFO effectively reduced the elevated serum MDA to normal values. Both treatments also caused significant increase in the CK activity, as compared to ADR group, but only PROB was able to normalize CK activity.

**Effect of PROB and DFO on antitumor action of ADR against growth of solid Ehrlich carcinoma**

The results in Fig. 1 show that the size of tumors was significantly reduced by ADR to about 24% of control values at day 17th from the start of experiment. Moreover, pre-treatment of ADR-treated animals with PROB or DFO did not change significantly the efficacy of ADR in reducing the growth of solid Ehrlich carcinoma, since no significant difference was observed in the tumor size between ADR-treated and pre-treated groups.

**Histopathological examination**

Normal cardiac muscle fibres were seen in the heart of the control group. Vaculation of cardiac muscles with pyknotic nuclei (small dark nuclei) was observed in the ADR-treated group. Cardiac muscle fibres with elongated nuclei and with focal site of degeneration were observed in ADR + PROB and ADR + DFO treated groups respectively (Fig. 2). The kidney of control rats showed normal renal corpuscle, proximal and distal collecting tubules. Complete sloughing of glomerular capillary basement membrane with presence of tubular degeneration casts was found in kidney of ADR-treated group. Nearly normal cortical architecture showing proximal collecting tubules with slight vaculation and vesicular nuclei and less tubular casts was observed in the ADR + PROB treated group. Shrunken sclerotic glomeruli with degenerating tubules, some pyknotic nuclei and tubular casts were observed in the ADR + DFO treated group (Fig. 3).

**Discussion**

ADR is an anti-tumor drug, whose clinical use frequently results in lethal cardiac and renal toxicity. The present study revealed that repeated administration of ADR resulted in a state of oxidative stress. This was evident by a significant increase in
Fig. 3—Microscopic sections in kidney of a control rat following hematoxylin and eosin staining (X400) showing normal renal corpuscle, proximal and distal tubule (A), complete sloughing of glomerular capillaries with casts in ADR group (B), nearly normal renal architecture showing proximal tubules with less casts in PROB-treated group (C) and shrunken glomerulus with extensive casts in DFO-treated group (D).

the MDA level in the cardiac and renal tissues as well as the serum, together with a significant decrease in the antioxidant reserves (GSH, GPx and GST in cardiac and renal tissues) compared with the control values. These findings were consistent with other previous studies in cardiac and renal tissues.

It is widely accepted that oxidative stress and production of free radicals are involved in ADR toxicity. Two different ways of free radical formation by ADR have been described: i) the formation of a semiquinone free radical by the action of NADPH-dependent reductases that produce one-electron reduction product of ADR to the corresponding ADR-semiquinone. In the presence of oxygen, redox cycling of ADR-derived quinone-semiquinone yields superoxide radicals; and ii) ADR free radicals generated from a non-enzymatic mechanism that involves reaction with iron. Fe$^{3+}$ reacts with ADR in a redox reaction, after which the iron accepts an electron and a Fe$^{2+}$-ADR free radical complex is produced. This iron-ADA complex can reduce oxygen to hydrogen peroxide and other active oxygen species. In addition, the increase in myocardial and renal XO activity, as evidenced by low XD/XO may exacerbate the state of oxidative stress due to the generation of reactive oxygen species (ROS) in tissues of ADR-treated animals.

Xanthine oxidoreductase (XOR) has been implicated as a source of ROS generation in cardiac and renal tissues. In the present study, the decrease in XD/XO ratio suggested a conversion of XD to XO, following ADR administration. This observation was consistent with the earlier findings. XOR conversion occurs via two different routes either irreversibly by proteolysis or reversibly by oxidation of thiol groups. Chronic ADR treatment induces cardiomyopathy which is characterized by myocardial energy failure. This state of energy starvation may promote cytosolic calcium overloads after ADR treatment. The elevated cytosolic Ca$^{2+}$ triggers the conversion of NAD-reducing XD to the oxygen radicals-producing XO by proteolysis. In addition, conversion of XD to XO is sensitive to the redox state of cellular GSH. In the present study, the significant decrease in myocardial GSH level might have resulted in low GSH/GSSG ratio that could shift XD toward XO as reported in an earlier study.

A significant decline of serum CK activity in ADR-treated group may be due to the decreased cardiac CK activity. The reduced level of ATP, a marker of energy failure has been reported to be insufficient to sustain the myocardial function and may be partly responsible for ADR-induced cardiac myopathy.

In the present study, the ADR-induced increase in ROS generation was potentially exacerbated by a significant reduction in antioxidant reserve in cardiac and renal tissues. This might have caused a state of redox imbalance that enhanced the susceptibility of biological membranes to reaction with ROS, as indicated by the marked elevation of MDA and decrease of GPx in the cardiac and renal tissues. These results were in agreement with the previous studies in cardiac and renal tissues.

The high MDA level in plasma of ADR-treated rats may reflect the ADR-induced oxidative stress in the cardiac and renal tissues, as plasma oxidant/antioxidant status may reflect the extra-cellular response to the external agents or the tissue status. The decrease in GPx activity could be either due to inhibitory effect on protein synthesis by ADR and/or increased ROS production. Earlier study has shown that decrease in GPx activity after repeated ADR administration might be caused by the parallel decrease in the enzyme protein. The free radicals excessively formed in ADR-treated animals may
inhibit the activity of selected enzymes by oxidation of reduced thiols groups\textsuperscript{58}. MDA, a by-product of lipid peroxidation may also diminish the enzyme activity by oxidizing the active site or by forming protein cross-links\textsuperscript{57}. A significant decrease in GST activity in the cardiac and renal tissues may be attributed to the formation of a conjugate of ADR and GSH (GSH-ADR) which exerts non-competitive inhibition of GST activity\textsuperscript{58,59}.

The pre-treatment with PROB attenuated the ADR-induced increase in oxidative stress as well as a decrease in antioxidant status. This was clearly demonstrated by enhanced cardiac and renal GPx and GST activities and reduction in the MDA level in serum, heart and kidney. PROB also significantly restored the reduced renal GR and XD/XO and serum CK activity. These results may be explained in the light of other previous studies\textsuperscript{23,55,60-62}. PROB inhibits lipid peroxides due to the presence of two phenolic groups in its molecule. It also offers significant cardioprotection due to its relative high distribution in the heart tissue and its binding ability to cardiolipin\textsuperscript{63}. Moreover, the present study demonstrated that PROB might be providing protection by acting as an antioxidant as well as promoting endogenous antioxidant reserve. Besides, the beneficial effect of PROB may also influence gene function\textsuperscript{55}. Moreover, ADR-induced apoptosis that is mediated by oxidative stress is also prevented by PROB\textsuperscript{64}.

On the other hand, pre-treatment with DFO could counteract the ADR-induced decrease in cardiac and renal GPx and serum CK activities, along with an elevation in cardiac, renal and serum MDA level. It also improved renal GR significantly. Although the XD/XO ratio was improved in the renal tissues by DFO, but it failed to reach statistical significance. No change was observed in cardiac and renal GSH levels in DFO-treated group. In an earlier study\textsuperscript{12} also, DFO substantially reduced the acute peroxidative damage in cardiac and renal tissues of ADR-treated rats. The beneficial effects of DFO could be attributed to its iron-chelating properties, as it quickly and efficiently removes iron( III) from its complex with ADR. Thus, DFO greatly reduced hydroxyl radical production by iron(III)-ADR complex in the XD/XO superoxide generating system\textsuperscript{42}. Moreover, these results also suggested that DFO might protect against ADR-induced oxidative damage, not only by displacing iron bound to ADR, but also by chelating free or loosely bound iron, thus preventing site-specific iron-based oxygen radical damage\textsuperscript{42}. PROB and DFO did not offer any protection against ADR-induced decrease in cardiac XD/XO ratio, but caused further decrease in XD/XO ratio in the cardiac tissue. These unexpected results suggested the incomplete protection by these agents. Thus, further studies are needed to explore the exact mechanism of the inability of these agents in counteracting the decrease in XD/XO ratio.

In conclusion, the results indicate that repeated administration of ADR causes increased oxidative stress in the heart and kidney and that PROB and DFO counteracts most of these effects. In addition, histopathological examination reveals that the cardiac toxicity of ADR is more evident, as compared to its effect on the kidney. It is suggested that a carefully planned combined therapy of ADR with PROB or DFO should be clinically considered to counter the cardiotoxic and nephrotoxic effects of this useful drug. Interestingly, in addition to the beneficial effects in counteracting the ADR toxicity, PROB and DFO do not affect the anti-tumor property of this drug.

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References