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QUARTERLY

Metabolic activation of adriamycin by NADPH-cytochrome P450 reductase; overview of its biological and biochemical effects★✪

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NADPH-cytochrome P450 reductase (P450 reductase) is one of the enzymes implicated in the metabolism of adriamycin, a very important clinically used antitumour drug. However, apart from the enzyme involvement, so far little was known about the chemical route and biochemical effects of this process. We demonstrated that the application of P450 reductase simultaneously with adriamycin to tumour cells in culture significantly increased cytotoxicity of the drug. Under tissue culture conditions, we noticed also that, in the presence of P450 reductase, adriamycin metabolite(s), displaying an altered spectrum within the visible light range were formed. This observation was taken adavantage of to study the metabolism of adriamycin in cell-free systems, using initially the enzyme isolated from rat liver and the recently obtained recombinant human P450 reductase. The reductive conversion of the drug turned out to be a multi-stage process, which occurred only under aerobic conditions and was accompanied by excessive NADPH consumption. Further research carried out with the aid of radical scavengers and radiolabelled adriamycin revealed that the enhancement of biological activity of adriamycin by P450 reductase stemmed from the formation of alkylating metabolite(s) rather than from the promotion of redox cycling known to be induced in the presence of anthracyclines.

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Abbreviations: DMPO, 5,5-dimethyl-1-pyrrolidine *N*-oxide; DOX, doxorubicin; GSH, glutathione; MDA, malondialdehyde; NaCl/P_i, phosphate-buffered saline.

Adriamycin (doxorubicin) belongs to anthracycline antibiotics and is firmly established as a major therapeutic agent in the treatment of a wide variety of tumours. However, despite over 30 years of careful investigations, the mode of antitumor action of this drug remains unresolved. A critical review of the proposed mechanisms has appeared recently (Gerwitz, 1999). The biological effects displayed by adriamycin that may be responsible for the inhibition of tumour cell growth involve the interference with the synthesis of macromolecules, covalent DNA binding and DNA crosslinking, topoisomerase II inhibition, arrest of tumour cell cycle progression in G_2 phase, induction of apoptosis and generation of oxygen radicals. Two of the listed phenomena require enzymatic activation: covalent modification of macromolecules and redox cycling with molecular oxygen. Both these effects can cause cytotoxicity, though the latter is usually associated with cardiotoxicity of anthracyclines (e.g. Tolba & Deliargyris, 1999).

Numerous studies suggest that enzymes responsible for adriamycin activation belong to the oxidoreductase family. Nevertheless, so far the route of this activation has not been demonstrated unequivocally. It is presumed that it begins with the drug conversion to a semiquinone free radical *via* one-electron reduction. Such a reaction is catalysed by several enzymes, amongst which NADPH-cytochrome P450 reductase (P450 reductase) has been most extensively investigated (Bachur *et al.*, 1979). It was also shown that, in tumour tissue, adriamycin is metabolised mainly by this enzyme (Cummings *et al.*, 1994). Since semiquinone radical is capable of redox cycling with molecular oxygen (Sinha, 1989), and, moreover it has been postulated to be an intermediate form leading to the formation of alkylating metabolite(s) (Wallace & Johnson, 1987), it was interesting to find out whether this metabolic pathway influences the biological activity of adriamycin.

Studies aimed at establishing the relevance of metabolic conversion of adriamycin by P450 reductase for toxicity of this drug towards cultured human breast tumour MCF-7 cell line demonstrated that the cells treated with adriamycin in the presence of exogenously added purified rat P450 reductase and NADPH were about 6 times more sensitive than when incubated with this drug alone (Fig. 1). No potentiation of cell killing was observed when adriamycin was incubated with P450 reductase and NADPH for 1 h at 37°C prior to addition to the cells, which implicates the involvement of short-lived species (Bartoszek & Wolf, 1992). The enhancement of cytotoxicity was dependent on the drug dose and the amount of the enzyme (Fig. 2).

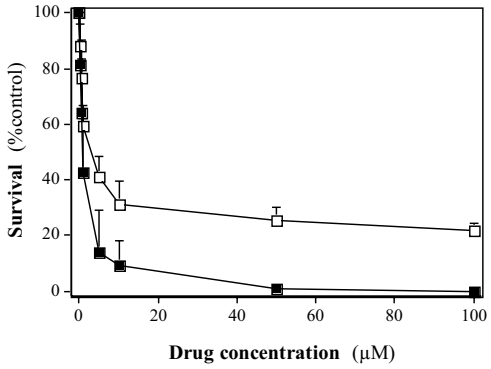

Figure 1. The influence of P450 reductase on adriamycin cytotoxicity towards human breast tumour MCF-7 cells.

The cells were treated for 3 h with the drug alone or drug applied concomitantly with P450 reductase (250 U/ml) and NADPH (1 mM). Cell survival was measured by MTT assay. Data adapted from (Bartoszek & Wolf, 1992).

The enhanced biological effect was not associated with altered drug uptake. Intracellular concentration of adriamycin, measured using its isotopically labelled form, was not affected by the presence of P450 reductase. Also, drug metabolites isolated from lysates of cells treated with and without the enzyme were identical. Since these metabolites were not detected in culture medium, they most probably

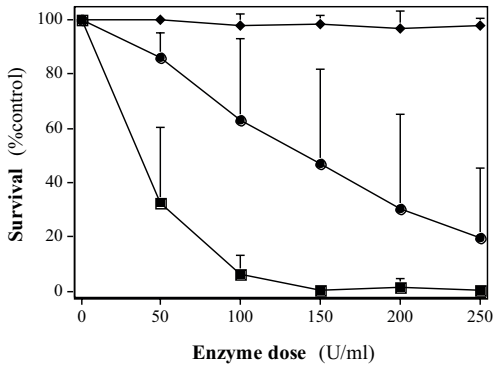

Figure 2. The influence of P450 reductase dose on the survival of human breast tumour MCF-7 cells treated with 1 μM (diamonts), 10 μM (circles) or 50 μM (squares) adriamycin in the presence of 1 mM NADPH.

The cells were treated for 3 h, then the culture medium was replaced and cell survival determined 72 h later by MTT assay. Data adapted from (Bartoszek & Wolf, 1992).

resulted from intracellular metabolism (Bartoszek & Wolf, 1992). However, the colour of culture medium from cultures treated in the presence of the enzyme, changed from bright orange due to adriamycin to purple red. These media were analysed and found to contain an unknown metabolite exhibiting similar chromatographic properties but quenched absorption in the UV-VIS range (Fig. 3) as compared to the parent compound (Bartoszek & Wolf, 1992; Cummings *et al.*, 1991). This

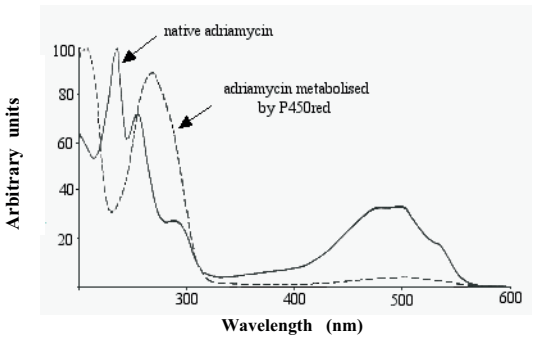

Figure 3. UV-VIS absorption spectra of adriamycin and its P450 reductase activation product taken during chromatography.

The methodological details have been described elsewhere (Cummings *et al.,* 1991).

metabolite was not detected in cell lysates; hence it was probably unstable under cellular conditions. Neither does it necessarily represent the biologically active form of the drug, but rather that effectively stabilised by culture medium components. The identity of this species remains unknown. Nonetheless, the lack of alteration of its retention time in HPLC system applied suggests that it contains a sugar moiety, while the major change in UV-VIS spectrum points to some rearrangements within the aglycon part of adriamycin molecule.

Most importantly, however, by taking advantage of the observed change of drug absorption in the visible light region, it became possible to monitor the kinetics of adriamycin reduction by P450 reductase in cell-free system. The reaction conditions were adjusted so that the reactant concentrations corresponded to the treatment conditions used in the case of cultured MCF-7 cells and the reduction was followed spectrophotometrically at 480 nm (Bartoszek & Wolf, 1992; and unpublished results). This approach enabled us to demonstrate, initially for the purified rat enzyme (Fig. 4) and recently for recombinant human enzyme (A. Bartoszek, Z. Mazerska, in preparation) that, upon P450 reductase catalysis, adriamycin underwent a multistage chemical transformation. As can be seen in Fig. 4, it was possible to discern five stages of the drug reduction, in which the subsequent metabolites displayed different, but always decreased, absorption at 480 nm compared to the parent compound (actually adriamycin absorption within the entire relevant visible light range fluctuated) which suggested that their electron configuration within aglycon moiety was also changing. During the last stage, the aglycon precipitation occurred. The multistage route of reduction could be clearly observed only for a narrow range of reaction conditions resembling those for which the enhancement of drug cytotoxicity in MCF-7 cells was determined, that is 50–100 μM adriamycin. For lower concentrations, a steady

slow decline of absorption at 480 nm occurred leading finally to aglycon precipitation; the particular stages of reduction were, however, not discernible (unpublished observations). To our knowledge, the decline of adriamycin absorption at 480 nm following one-electron type of reduction has been reported only once previously for the reaction catalysed by purified NADPH-cytochrome *c*-(ferrodoxin)-oxidoreductase from *Euglena gracilis* (Paur *et al.*, 1984).

Surprisingly, the adriamycin reduction by P450 reductase took place only under aerobic conditions (Bartoszek & Wolf, 1992) and was associated with excessive NADPH consumption (Fig. 4) indicative of redox cycling with molecular oxygen, a phenomenon known to be

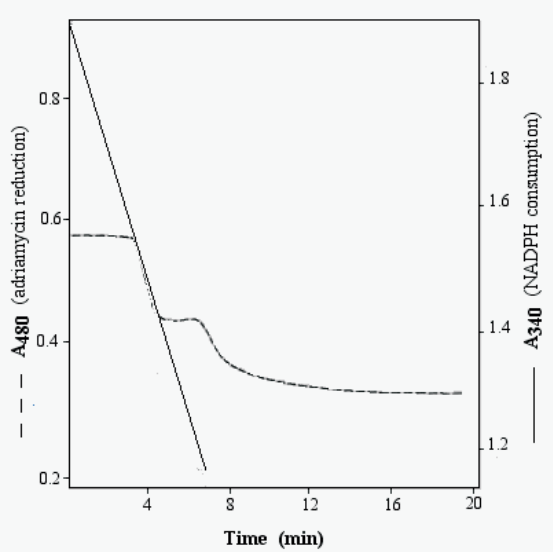

Figure 4. Kinetics of adriamycin reduction by P450 reductase and parallel NADPH consumption in cell-free system.

The reaction mixture consisted of 50 μM drug, 1 mM NADPH, 1000 U/ml purified rat enzyme in NaCl/P_i solution, pH 7.2. The sample was incubated at 37°C and the rate of drug reduction (- - -) and NADPH consumption (—) was followed spectrophotometrically at 480 nm and 340 nm, respectively.

induced by anthracyclines. Recently, it has been demonstrated by electron paramagnetic resonance (EPR), using 5,5-dimethyl-1-pyrrolidine *N*-oxide (DMPO) as a spin trap, that in cell-free system all five stages of adriamycin reduction by P450 reductase were accompanied by oxygen radical formation. Under hypoxia obviously no oxygen radicals could be detected by EPR, only a weak signal of adriamycin semiquinone radical was seen and no change of drug absorption in the visible light range was observed (A. Bartoszek, G. Bartosz, in preparation). The oxygen requirement for adriamycin reduction perhaps might be explained by quantum calculations published by Tempczyk *et al.* (1988) revealing that quinone containing anthracyclines can form complexes with singlet oxygen. Such a complex is a better electron acceptor than the free quinone; hence it may undergo reduction more easily than the uncomplexed drug.

The studies in cell-free system thus demonstrated that the metabolic activation of adriamycin by P450 reductase leads to the mentioned earlier events that might be responsible for the enhancement of cell killing seen in the presence of this enzyme: generation of toxic oxygen radicals and formation of drug-related alkylating species. Further research was aimed at establishing their relative importance for the biological outcome observed in cultured cells. The relevance of redox cycling was assessed by measurements of lipid peroxidation as well as by the analysis of influence of radical scavengers on cytotoxicity of P450 reductase-activated adriamycin. In MCF-7 cells incubated with the drug in the presence of the enzyme, malondialdehyde (MDA), a product of lipid peroxidation, was hardly detectable and its concentrations were only slightly elevated (without statistical significance) compared to cells incubated with the drug alone. Moreover, even at the doses causing 100% of cell killing, MDA levels were much lower than those detected in cells treated with cumene peroxide and $FeCl_2$ used as a positive control (Bartoszek & Wolf, 1992). Also, as compiled in Fig. 5, the correlation between the determined levels of MDA and cytotoxic effect was rather poor.

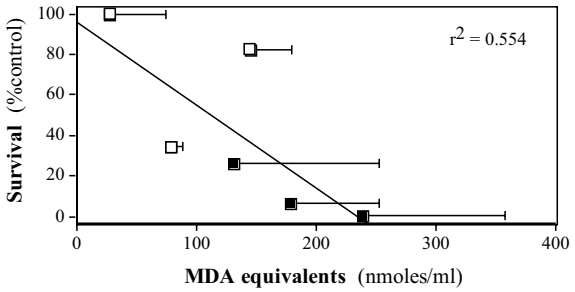

Figure 5. Correlation between malondialdehyde concentration used as a measure of cellular lipid peroxidation and the survival of human breast tumour MCF-7 cells treated for 3 h with adriamycin in the absence (□) or presence (■) of P450 reductase (250 U/ml) and NADPH (1 mM).

Malondialdehyde content was measured spectrophotometrically immediately after cell treatment following the extraction with appropriate solvents; cell survival was estimated by MTT assay 72 h after the application of fresh medium to the cells. Data adapted from (Bartoszek & Wolf, 1992).

In another series of experiments, the influence of a variety of scavengers, which had been previously shown to modulate adriamycin-induced production of oxygen radicals (Doroshow, 1986; Alegria *et al.*, 1989), on the survival of MCF-7 cells treated with this drug in the presence or absence of P450 reductase was assayed (Bartoszek & Wolf, 1992). Table 1 presents a summary of the results obtained. As can be seen, in the case of treatments with unmetabolised drug, the scavengers employed seemed to differ slightly in their impacts on the toxicity of the drug. However, when P450 reductase was included, most of the used oxygen radical scavengers did not prevent the potentiation of cell killing. The exception was 10 mM glutathione (GSH), which effectively protected the cells against the metabolised drug although in the incubations without the enzyme, it significantly enhanced the toxicity of adriamycin. It was the only agent which made P450 reductase-generated drug metabolite(s) cause less toxicity than did the parent compound. All these observations suggest that the protection by GSH does not result from scavenging oxygen radicals but rather from neutralisation of activated form(s) of adriamycin, perhaps owing to binding by GSH of electrophilic metabolites of various xenobiotics. Such a conclusion is in contrast with published results obtained with resistant MCF-7/DOX cells showing a 14-fold increase of glutathione transferase P1-1 expression, where no formation of GSH–adriamycin conjugates could be detected (Gaudiano *et al.*, 2000). However, there is one reservation concerning the methodology employed in the latter study. The cell lysates resolved by HPLC were monitored at 480 nm, that is at the wavelength quenched in P450

Table 1. The influence of radical scavengers on cytotoxicity of adriamycin towards human breast tumour MCF-7 cells in the absence or presence of NADPH-cytochrome P450 reductase

Radical scavenger	EC_{90} (μM)[a]	
	+P450 reductase	–P450 reductase
No additives	13.2 ± 5.2	92.0 ± 11.8
1 mM mannitol	16.5 ± 4.1	110.0 ± 2.5
1 mM DMPO	24.2 ± 3.0	128.6 ± 15.9
25 μM α-tocopherol	22.0 ± 4.4	61.6 ± 17.8
Cells preincubated 12 h with 25 μM α-tocopherol	17.3 ± 1.3	50.7 ± 6.4
50 μg/ml superoxide dismutase	16.7 ± 0.4	126.5 ± 21.3
1 mM glutathione	17.2 ± 16.5	21.4 ± 7.4
10 mM glutathione	60.1 ± 30.1	31.3 ± 16.4

[a]EC_{90} is drug concentration inhibiting cell growth by 90%; values given in the Table were calculated based on data published in (Bartoszek & Wolf, 1992).

reductase-generated adriamycin metabolites, hence most probably also in respective adriamycin metabolites produced by other enzymes capable of one-electron reduction. Indeed, our recent studies in cell-free system, carried out on human recombinant P450 reductase, showed that the products of adriamycin enzymatic reduction in the presence of GSH exhibited declined absorption in the visible light region (unpublished observations). If this property were displayed also by adriamycin-GSH conjugates, their detection at 480 nm would be rather difficult.

tal systems used so far: MCF-7 cells and *in vitro* incubations. The synopsis of experimental protocols used and results obtained is given in Table 2. The covalent modification of cellular proteins and DNA was estimated following the incubation of MCF-7 cells with [^{14}C]adriamycin in the presence or absence of P450 reductase (Bartoszek & Wolf, 1992). It was shown that the level of the drug irreversibly associated with cellular proteins was significantly higher in cells treated in the presence of the enzyme. Moreover, only in the digest of DNA isolated from cells exposed to the drug

Table 2. The levels of covalent binding of [^{14}C]adriamycin to DNA and proteins exposed to this drug in the absence or presence of P450 reductase.

Model system	Macromolecule type and the method of drug binding determination	[^{14}C]-adriamycin bound	
		–P450reductase	+P450reductase
MCF-7 cells treated for 3 h with: 50 μM drug 1 mM NADPH 250 U/ml P450 reductase	cellular proteins submitted to exhaustive extraction with organic solvents	1.43 ± 0.46 [a] nmoles/mg background level[a]	4.41 ± 0.95 [a] nmoles/mg 0.19 nmoles per 50 μg DNA[a]
	cellular DNA digested to nucleosides then resolved by reversed phase HPLC		
incubation for 1 h in cell-free system consisting of: 50 μM drug 1 mM NADPH 1000 U/ml P450 reductase NaCl/P_i solution, pH 7.2	bovine albumin submitted to exhaustive extraction with organic solvents	0.258 ± 0.039[b] nmoles/mg	0.497 ± 0.048[b] nmoles/mg
	heat-denatured calf thymus DNA resolved by anion-exchange HPLC	background level[c]	0.034 nmoles per 100 μg DNA[c]
	oligonucleotide (single-stranded) resolved by anion-exchange HPLC	not determined	0.68 nmoles per 100 μg oligonucleotide[c]

[a]Data taken from (Bartoszek & Wolf, 1992); [b]albumin (10 mg/ml) was incubated for 2 h with indicated concentrations of other reagents at 37°C, then it was submitted to exhaustive organic solvent extraction to remove irreversibly bound adriamycin as described for cellular proteins in (Bartoszek & Wolf, 1992); [c]data taken from (Cummings *et al.*, 1991)

In view of the lack of convincing evidence that P450 reductase-mediated enhancement of adriamycin cytotoxicity was brought about by the increased production of oxygen radicals, the ability of the generated drug metabolites to bind covalently with biologically important macromolecules was examined. These studies were carried out mainly with the aid of radioactively labelled drug in both experimen-

and P450 reductase, the radioactive peak could be separated by HPLC. This peak was eluted with the retention time corresponding to R_F's of known adriamycin metabolites, thus it might have represented drug–DNA adducts (Bartoszek & Wolf, 1992). Similarly, in the case of incubations in cell-free system, the addition of P450 reductase to the reaction mixtures increased the amount of the radioac-

tivity bound both with nucleic acids (Cummings *et al.*, 1991) and protein. However, in the case of DNA, it was necessary to use denatured or, even better single-stranded molecules since native DNA blocked adriamycin reduction by P450 reductase entirely. Probably, very strong physicochemical association with double-stranded DNA rendered adriamycin inaccessible for the enzyme.

The detection of covalent binding between adriamycin and DNA as a result of reductive metabolism is in accord with other researcher' findings, first of all from T. Koch's group who demonstrated binding of this drug with guanine both in cellular and cell-free systems (Taatjes *et al.*, 1998; Zeman *et al.*, 1998; Sharples *et al.*, 2000). Their investigations made possible to determine that the active site involved in DNA adduct formation was positioned within the sugar moiety. Also, formation of the so called "virtual crosslink" has been proposed (Fig. 6) which involves on one hand the covalent linkage between drug aminosugar and amino group in guanine *via* methylene bridge and, on the other hand, linking the opposite strand by hydrogen bonds between 9-OH group of the antibiotic and guanine (Zeman *et al.*, 1998; Podell *et al.*, 1999; Taatjes & Koch, 2001). To date, there have been no reports proposing the chemical structure of DNA adduct that would involve aglycon moiety of adriamycin, though such a type of binding has been suggested for a long time. Our data confirm that the reaction with biological nucleophiles can involve the aglycon part of adriamycin molecule. Taking these results together, it can be presumed that this drug is able to form simultaneously two covalent links with DNA, i.e., it may induce interstrand covalent DNA crosslinks. The investigations carried out for many years by the group headed by J. Konopa on the covalent DNA crosslinking by antitumour drugs demonstrated that adriamycin, similarly as other antitumour anthracyclines, induced covalent interstrand crosslinks in DNA of tumour cells after prior metabolic activation (Konopa, 1990). Moreover, the biological relevance of this phenomenon has been also well-documented (e.g. Składanowski & Konopa, 1994).

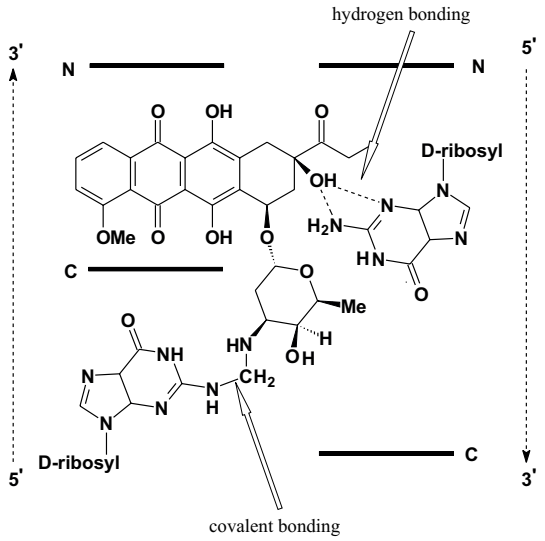

Figure 6. The structure of DNA adduct formed by epidoxorubicin-formaldehyde with guanine (after Taatjes *et al.*, 1998; Podell *et al.*, 1999).

Hydrogen bonds between 9-OH group of adriamycin and guanine on the opposite DNA strand are also depicted. This combination of covalent and hydrogen bonding was named "virtual crosslink" and was proposed to account for interstrand DNA crosslinking observed experimentally in tumour cells and with pBR322 plasmid in cell-free system.

To summarise, adriamycin is effectively reduced by P450 reductase, regardless of its origin, and this reaction, occurring only in the presence of oxygen, produces drug metabolites capable of covalent binding to cellular nucleophiles. It appears that such a metabolism is of biological relevance since it can enhance adriamycin cytotoxicity. Similar enhancement of cytotoxicity by P450 reductase was observed also in the case of another quinone containing antitumour drug, mitomycin C, also requiring reductive metabolism for biological activity. Moreover, the exogenously added P450 reductase restored the sensitivity of CHO cells resistant to mitomycin C while *Salmonella typhimurium* strain LR5000 expressing a rat liver variant

of this enzyme displayed increased vulnerability to the drug (Bligh *et al.*, 1990). It may be thus concluded that the biological importance of one-electron reduction for antitumour chemotherapy deserves further research, especially that on one hand numerous important antitumor drugs contain moieties susceptible to this type of metabolism, and, on the other hand, many cellular oxidoreductases found in tumour cells exhibit such activity.

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