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COMPARATIVE CARDIOTOXICITY AND ANTITUMOUR ACTIVITY OF DOXORUBICIN (ADRIAMYCIN) AND 4′-DEOXYDOXORUBICIN AND THE RELATIONSHIP TO *IN VIVO* DISPOSITION AND METABOLISM IN THE TARGET TISSUES

JEFFREY CUMMINGS,*† NEVILLE WILLMOTT,‡ IAN MORE,§ DAVID J. KERR,* J. GILMOUR MORRISON* and STANLEY B. KAYE*

*Department of Medical Oncology, University of Glasgow, 1 Horselethill Road, Glasgow G12 9LX, ‡Department of Pharmacy, University of Strathclyde, Glasgow G1 1XW, and §Department of Pathology, Western Infirmary, Glasgow G12, U.K.

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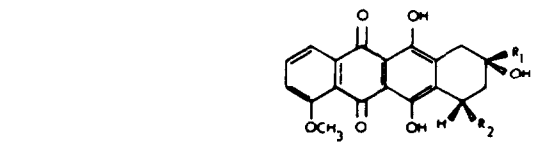
Abstract—4′-Deoxydoxorubicin (4′-DOX) is an analogue of the anticancer drug Adriamycin (ADR) believed to lack its cardiotoxicity. Bioreduction to a semi-quinone free radical has been implicated in the etiology of ADR induced cardiotoxicity. We have studied (in a rat model) acute cardiotoxicity (after 16 mg/kg i.v. of both drugs), antitumour activity (after 5 mg/kg i.v.) and the relationship to disposition and metabolism in the target tissues (after 5 mg/kg i.v.). 7-Deoxyaglycones, which are considered inactive lipophilic metabolites derived from ADR semi-quinone, were utilised as markers of *in vivo* tissue free radical generation. Both drugs produced toxicity of equal severity to hearts after 24 hr, associated with high cardiac levels of 7-deoxyaglycones in the case of ADR ($AUC_{0\text{-}48\,hr}$, μg/g × hr: ADR, 47; ADR 7-deoxyaglycone (ADR-DONE), 24; and adriamycinol 7-deoxyaglycone (AOL-DONE), 35) compared to low cardiac levels of 7-deoxyaglycones but a times five higher peak cardiac concentration of parent drug in the case of 4′-DOX ($AUC_{0\text{-}48\,hr}$, μg/g × hr: 4′-DOX, 68; 4′-DOX-DONE, 3.8; and 4′-DOL-DONE, 0.8). 4′-DOX displayed superior antitumour activity to ADR against the MC 40A sarcoma growing sub-cutaneously, achieving higher concentrations of parent drug in tumour ($AUC_{0\text{-}48\,hr}$, μg/g × hr: 4′-DOX, 150; ADR, 60). There was an absence of 7-deoxyaglycones of both drugs in the tumour. These data suggest that drug bioreduction is involved principally only in ADR induced cardiotoxicity and that the level of unchanged parent drug achieved in the tumour is the most important pharmacokinetic determinant of antitumour activity for both ADR and 4′-DOX.

Adriamycin (ADR) is clinically the most useful member of the large group of anthracycline anticancer drugs [1]. It is subject to specific dose-dependent cardiotoxicity which limits its administration to patients to a recommended safe cumulative dose of 550 mg/m^2 [2]. Well defined morphological and ultrastructural changes accompany ADR induced cardiotoxicity characterised by nucleolar segregation and loss, swelling and degeneration of mitochondria and sarcoplasmic reticulum, appearance of intramitochondrial dense inclusion bodies and mitochondrial vacuolation [3–5]. The exact mechanism of this cardiotoxicity is unknown. A number of theories exist based on observation of drug-induced biochemical lesion in heart tissue such as: inhibition of oxidative phosphorylation, inhibition of Na^+–Ca^{2+} exchange, inhibition of Na^+–K^+ ATPase function and specific binding to membrane phospholipids [6–9]. Several pieces of circumstantial evidence suggest that the active toxic form of the drug is not ADR itself but the products of drug biotransformation: ADR semi-quinone free radical, the ADR hydroquinone intermediate and reactive species derived from molecular oxygen [10–14]. Products of lipid peroxidation have been identified in the hearts of mice and have correlated with ADR-induced cardiotoxicity [15]. 7-Deoxyaglycone metabolites of ADR are detected as stable bi-products of *in vitro* semi-quinone generation and their appearance in urine of cancer patients has been cited as proof of free radical formation occurring *in vivo* [16–18]. We have recently positively identified in serum and described the pharmacokinetics of ADR 7-deoxyaglycone metabolites in a group of 25 cancer patients [19]. The mechanism of action of ADR toxicity to heart appears to be distinct from its mode of action of cytotoxicity to tumour cells [15], the latter being generally considered not to involve drug bioreduction.

Numerous anthracycline analogues have been synthesised in an attempt to improve on the therapeutic index of ADR. 4′-Deoxydoxorubicin (4′-DOX) is structurally identical to ADR differing only by the absence of an oxygen atom at position 4 on the daunosamine sugar group (Fig. 1). Removal of the oxygen increases basicity and lipophilicity [20]. In preclinical pharmacology studies performed in Italy on several animal species 4′-DOX appeared to be non-cardiotoxic [20, 21] and preliminary clinical trials suggest that this is also the case in man [22, 23]. 4′-DOX has been shown not to be converted to a semi-quinone free radical under conditions which produced the ADR semi-quinone [24], providing

† To whom correspondence should be addressed, at present address: I.C.R.F. Medical Oncology Unit, Western General Hospital, Edinburgh, EH4 2XU, UK.



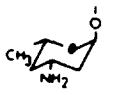
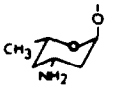
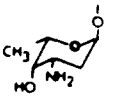
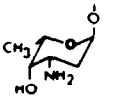
COMPOUND	R_1	R_2
4'- Deoxydoxorubicin (4'- DOX)	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	
4'- Deoxydoxorubicinol (4'- DOL)	$-\underset{H}{\overset{OH}{\overset{\|}{C}}}-CH_2OH$	
Adriamycin (ADR)	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	
Adriamycinol (AOL)	$-\underset{H}{\overset{OH}{\overset{\|}{C}}}-CH_2OH$	
4'- Deoxydoxorubicin aglycone (4'-DOX-ONE), also Adriamycin aglycone (ADR-ONE)	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	OH
4'- Deoxydoxorubicinol aglycone (4'- DOL-ONE), also Adriamycinol aglycone (AOL-ONE)	$-\underset{H}{\overset{OH}{\overset{\|}{C}}}-CH_2OH$	OH
4'- Deoxydoxorubicin 7 - deoxyaglycone (4'-DOX-DONE), also Adriamycin 7 - deoxyaglycone (ADR-DONE)	$-\overset{O}{\overset{\|}{C}}-CH_2OH$	H
4'- Deoxydoxorubicinol 7-deoxyaglycone (4'- DOL-DONE), also Adriamycinol 7 - deoxyaglycone (AOL-DONE)	$-\underset{H}{\overset{OH}{\overset{\|}{C}}}-CH_2OH$	H

Fig. 1. Molecular structures of adriamycin, 4'-deoxydoxorubicin and all their major metabolites.

a biochemical rationale for reduced cardiotoxicity, despite the close structural similarity.

We considered 4'-DOX an ideal candidate to continue our studies relating *in vivo* drug biotransformation of anthracyclines to activity and toxicity because of its proposed lack of cardiotoxicity and bioreduction to free radicals.

MATERIALS AND METHODS

Drug and metabolite standards and other chemicals. ADR-HCl, 4'-DOX-HCl, adriamycinol-HCl (AOL), ADR 7-deoxyaglycone (ADR-DONE) and AOL 7-deoxyaglycone (AOL-DONE) (see Fig. 1 for all structures) were generous gifts from Dr S. Penco, Farmitalia, Milan, Italy. 4'-Deoxydoxorubicinol (4'-DOL), ADR aglycone and AOL aglycone were synthesised as previously described [25]. ADR and 4'-DOX share an identical aglycone structure, consequently their aglycone metabolites are identical (Fig. 1). All solvents and chemicals were of the highest grade available commercially and water was de-ionised and double distilled in a quartz glass still.

Drug analysis techniques. Chromatographic separations of ADR, 4'-DOX and their respective metabolites by HPLC are described in detail elsewhere [25, 26]. ADR, 4'-DOX and metabolites were extracted from rat serum using the rapid method as reported previously [25] and from rat heart and the MC 40A tumour using the same technique after pretreatment of homogenates with silver nitrate (33% w/v $AgNO_3$, 200 μl per 1 ml homogenate [27]). Daunorubicin was the internal standard. All samples which were stored at −20° were thawed at room temperature and analysed immediately.

Acute cardiotoxicity. Rats were administered a high dose of drug (16 mg/kg i.v. ADR and 4'-DOX under halothane anaesthesia), control animals received buffered saline administered in the same way. Twenty-four to 48 hr later they were sacrificed and the apex of the heart was collected. One mm cubes of tissue were fixed in freshly made up 2.5% phosphate buffered glutaraldehyde (pH 7.3), post fixed in isotonic osmium tetroxide and embedded in araldite glue. Sections were prepared on a Reichert

Ultracut, double stained with uranyl acetate and lead citrate and examined in a Phillips EM 301 G electron microscope. Electron micrographs were scored for frequency and severity of ultrastructural damage compared to controls.

Antitumour activity. Rats (male Nottingham Wistars) were from an inbred colony based in the Department of Medical Oncology and the MC 40A tumour (a 3-methylcholanthrene induced sarcoma) was maintained by serial sub-cutaneous passage of 100 mg fragments or a suspension of disaggregated cells. Tumour bearing animals were randomised into three treatment groups of ten rats each. One group received no drug treatment and acted as controls, another received 5 mg/kg i.v. ADR and the last 5 mg/kg i.v. 4'-DOX. Tumour size was measured with calipers taking two diameters at right angles. Weight in grams was calculated from the formula: $\frac{1}{2} \times$ shorter diameter $\times$ longer diameter. Inhibition of tumour growth was evaluated by comparing against the control group using Students *t*-test.

Pharmacokinetic studies. Rats bearing 2–5 g tumours were used in the pharmacokinetic studies. Drugs (5 mg/kg ADR and 4'-DOX) were delivered i.v. under halothane anaesthesia via a tail vein. Two animals per time point were killed by ether overdose before drug administration and at 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 16 hr, 24 hr and 48 hr after drug administration. At each time an aliquot of blood (1 ml approximately) was removed from the vena cava with a syringe and hypodermic needle; the whole heart and tumour were removed, washed with buffered saline (pH 7.4) and frozen to −60° with solid CO_2. Blood was allowed to clot, serum was separated by centrifugation and stored at −20°. Tissues were also stored at −20°. Prior to extraction heart was chopped into small fragments and further washed to remove blood.

Rat pharmacokinetic data were not computer fitted or modelled. Area under each concentration/time profile (representing total drug exposure) for both drugs and their metabolites in serum, heart and tumour (AUC) was calculated by the trapezoidal rule and half-lives were determined by a graphical method. The amount of a metabolite present in serum, heart and tumour relative to the parent drug was calculated as a percentage taking individual AUCs over the total AUC of the parent plus all the metabolites present.

RESULTS AND DISCUSSION

Comparative serum pharmacokinetics, heart and tumour disposition and metabolism of ADR versus 4'-DOX are summarised in Tables 1–3. Two main differences emerged between the two drugs. ADR was extensively biotransformed to up to three major metabolites identified as adriamycinol, ADR 7-deoxyaglycone and AOL 7-deoxyaglycone (techniques described [27]) whilst only trace amounts of 4'-DOX metabolites were detected, and 4'-DOX was cleared faster than ADR from serum, heart and tumour.

Relationship of acute drug induced cardiotoxicity to in vivo *cardiac drug disposition and metabolism*

Heart morphology was examined between 24 and 48 hr after drug treatment to coincide with the time scale of the pharmacokinetic studies. Both ADR and 4'-DOX induced a number of toxicological changes characterised by mitochondrial swelling, disruption of cristae, vacuolation and appearance of dense inclusion bodies (not shown). There was no significant difference in the severity and frequency of ultrastructural lesions produced by the two drugs. On the other hand cardiac disposition and metabolism varied (Table 2). Large quantities of ADR 7-deoxyaglycones were measured in hearts ($AUC_{0-48\,hr}$, $\mu g/g \times$ hr: ADR, 47; ADR-DONE, 24; and AOL-DONE, 35), with AUCs several times greater than serum profiles and showing a distinct pattern (Tables 1 and 2): ADR-DONE was not detected in serum. 4'-DOX achieved greater parent drug levels in hearts but was cleared faster (half lives: ADR, 34 hr; and 4'-DOX, 27 hr) and only trace amounts of its 7-deoxyaglycones were detected (Table 2).

It is interesting that ADR induced acute cardiotoxicity was accompanied by 7-deoxyaglycone metabolite formation. Although themselves non-cardiotoxic or cytotoxic, they have been identified as stable bi-products of *in vitro* bioreductive activation of ADR to a semi-quinone free radical and an ADR hydroquinone intermediate [18]. In the presence of molecular oxygen the semi-quinone free radical can continuously redox cycle electrons to generate the series of toxic oxy radicals strongly implicated in ADR cardiotoxicity [13, 28], degrading to only a small fraction of 7-deoxyaglycone molecules [18]. Under anaerobic conditions 7-deoxyaglycone metabolites are formed rapidly in almost 1:1 stoichiometry with the disappearance of native ADR [29, 30]. Here 7-deoxyaglycones are formed via a linear sequential pathway where the eventual end product is AOL-DONE [29]. The quantitative relationship between 7-deoxyaglycone concentration and free radical formation is, therefore, uncertain being dependent on conditions that prevail at the active site of formation, and the significance of their appearance *in vivo* is not fully understood. In this report we have demonstrated that ADR induced toxicity to heart is directly associated with the appearance of high levels of 7-deoxyaglycones specifically in heart. AOL-DONE, which also appeared in serum, may be a potentially important clinical marker of ADR cardiotoxicity. Lack of 7-deoxyaglycones of 4'-DOX in heart (and low amounts in serum) correlates with the observation that it is not as readily bioreduced to a semi-quinone free radical [24] and indicates a mechanism of toxicity not involving free radicals.

Peak blood concentrations are believed to be important in determining toxicity of anthracyclines to patients [31] and whilst the peak concentration of 4'-DOX in heart exceeded that of ADR by almost 5-fold (10.3 $\mu g/g$ versus 2.3 $\mu g/g$) both drugs produced the same degree of toxicity.

Relationship of antitumour activity to in vivo *tumour disposition and metabolism*

Antitumour activity was compared seven days after drug administration (Table 4). At that time (and up to 21 days) ADR did not cause a significant

Table 1. Serum pharmacokinetics of adriamycin, 4′-deoxydoxorubicin and all their major fluorescent metabolites after administration of 5 mg/kg i.v. of each drug

Compound	Adriamycin PC* (μg/ml)	Adriamycin AUC (μg/ml × hr)	Adriamycin % (%)	Adriamycin $t_{\frac{1}{2}}$ (hr)	4′-Deoxydoxorubicin PC (μg/ml)	4′-Deoxydoxorubicin AUC (μg/ml × hr)	4′-Deoxydoxorubicin % (%)	4′-Deoxydoxorubicin $t_{\frac{1}{2}}$ (hr)
Parent drug	3.4	0.67	65	38	1.1	0.76	96	21
Alcohol†	0.1	0.23	22			ND		
Parent drug 7-deoxyaglycone		ND‡				ND		
Alcohol 7-deoxyaglycone	0.6	0.13	13		0.1	0.03	4	

* PC, peak serum concentration; AUC, area under serum concentration/time profiles between 0 and 48 hr; %, percentage of total AUC; and $t_{\frac{1}{2}}$, half life.
† Adriamycinol or 4′-deoxydoxorubicinol.
‡ ND, not detected in serum.

Table 2. *In vivo* disposition and metabolism of adriamycin and 4′-deoxydoxorubicin in *heart* after 5 mg/kg i.v. of each drug

Compound	Adriamycin PC* (μg/g)	Adriamycin AUC (μg/g × hr)	Adriamycin % (%)	Adriamycin $t_{\frac{1}{2}}$ (hr)	4′-Deoxydoxorubicin PC (μg/g)	4′-Deoxydoxorubicin AUC (μg/g × hr)	4′-Deoxydoxorubicin % (%)	4′-Deoxydoxorubicin $t_{\frac{1}{2}}$ (hr)
Parent drug	2.3	47	44	34	10.3	68	94	27
Alcohol†		ND‡				ND		
Parent drug 7-deoxyaglycone	0.8	24	23		0.3	3.8	5	
Alcohol 7-deoxyaglycone	0.7	35	33		0.1	0.8	1	

* PC, peak heart concentration; AUC, area under heart concentration/time profiles between 0 and 48 hr; %, percentage of total AUC; and $t_{\frac{1}{2}}$, half life.
† Adriamycinol or 4′-deoxydoxorubicinol.
‡ ND, not detected in heart.

Table 3. *In vivo* disposition and metabolism of adriamycin and 4′-deoxydoxorubicin in the MC 40A tumour after 5 mg/kg i.v. of each drug

Compound	Adriamycin PC* (μg/g)	Adriamycin AUC (μg/g × hr)	Adriamycin % (%)	Adriamycin $t_{\frac{1}{2}}$ (hr)	4′-Deoxydoxorubicin PC (μg/g)	4′-Deoxydoxorubicin AUC (μg/g × hr)	4′-Deoxydoxorubicin % (%)	4′-Deoxydoxorubicin $t_{\frac{1}{2}}$ (hr)
Parent drug	2.4	60	99.8	50	4.5	150	99.85	33
Alcohol†		ND‡				ND		
Parent drug 7-deoxyaglycone	0.01	0.07	0.15		0.003	0.13	0.1	
Alcohol 7-deoxyaglycone	0.001	0.02	0.05		0.002	0.05	0.05	

* PC, peak tumour concentration; AUC, area under tumour concentration/time profiles between 0 and 48 hr; %, percentage of total AUC; and $t_{\frac{1}{2}}$, half life.
† Adriamycinol or 4′-deoxydoxorubicinol.
‡ ND, not detected in tumour.

Table 4. Antitumour activity of adriamycin versus 4′-deoxydoxorubicin

	Tumour weight (g) ± SD Day 1	Day 7
No drug* treatment	2.7 ± 1.4	8.2 ± 4.1
5 mg/kg adriamycin	2.8 ± 0.8	7.5 ± 2.0
5 mg/kg 4′-deoxydoxorubicin	2.6 ± 0.6	1.6 ± 0.6†

* Each group, N = 10.
† $P < 0.01$ compared to Day 7 control and Adriamycin treated (Students *t*-test).

delay in tumour growth. In contrast, 4′-DOX not only delayed tumour growth significantly ($P < 0.01$) but actually caused a small reduction in tumour size (Table 4).

Peak tumour levels of 4′-DOX were almost double that of ADR (Table 3) and its AUC was almost treble that of ADR (Table 3). Neither drug appeared to be converted to 7-deoxyaglycone metabolites in tumour, as only trace amounts were detected (Table 3).

Little is known about the pattern of 4′-DOX biotransformation in animals or man [32]. Formelli

and co-workers who investigated tissue distribution and toxicity of 4'-DOX and ADR in mice used only a crude technique to assay tissue levels which was unable to distinguish between parent drug and fluorescent metabolites [33, 34]. The fluorescence of tissue extracts was read directly without prior chromatography and results were expressed in total drug equivalents. The pattern of tissue distribution they observed was broadly consistent with our findings: higher heart and tumour concentrations of 4'-DOX and more rapid clearance of 4'-DOX. We have employed two new HPLC methods to enable us to separate, identify and quantitate ADR, 4'-DOX and all their principal metabolites. Such methodology proved to be necessary: in the case of the heart where the total drug content of ADR and its metabolites combined (106 $\mu g/g \times hr$) was greater than that of 4'-DOX (72 $\mu g/g \times hr$), the peak concentration of unchanged 4'-DOX was almost .ive times greater than that of ADR.

Our results have demonstrated a lack of metabolism of both drugs in tumour. From a pharmacokinetic viewpoint it would be safe to conclude that in the case of antitumour activity the parent drug is the active (or inactive) agent rather than a biotransformed product such as a free radical. Therefore, any process (such as metabolism) which results in less parent drug being available to interact with the tumour should be considered a pathway of inactivation. Supporting this contention it has been shown that inducing ADR metabolism in mice with phenobarbitone results in a reduction in antitumour activity [35] and inhibiting ADR 7-deoxyaglycone formation with the xanthine oxidase inhibitor allopurinol in DBA/2 mice results in an enhancement in antitumour activity [36].

In apparent contradiction to the above results we have recently reported that increased antitumour activity of ADR-loaded albumin microspheres over ADR is associated with increased production of 7-deoxyaglycones in tumour tissue of rat [37]. In explanation of these findings, *in vivo* 7-deoxyaglycone formation may be considered from two different view points: (1) inactivating metabolism in non target tissues like the liver (stimulated by phenobarbitone, inhibited by allopurinol) resulting in less active parent drug being able to interact with the tumour, and (2) bioreductive activation in tumour tissue itself (utilising the drug targeting properties of the microspheres) resulting in the local evolution of toxic semiquinone free radicals, ADR hydroquinone and other related reactive species. A similar process is envisaged being responsible for ADR cardiotoxicity.

Finally, the data we have obtained from the rat suggest that the reduced biotransformation of 4'-DOX to 7-deoxyaglycones has been partly responsible for both its lower cardiotoxicity (per unit concentration of parent drug in cardiac tissue) and increased antitumour activity over ADR. Our results would lead one to conclude that if 4'-DOX was not converted to 7-deoxyaglycones in patients it should have a better therapeutic index than ADR. Our ongoing clinical pharmacokinetic studies with 4'-DOX have shown that it is not as readily converted to 7-deoxyaglycones as ADR. However, results from recent clinical trials have shown 4'-DOX to be less active than ADR in breast cancer and inactive in colorectal cancer and hormone resistant prostate cancer [32, 38]. Also, using sensitive tests of heart function, 4'-DOX appears to produce significant reductions in ejection fraction after as low a cumulative dose as 140 mg/m^2 [38]. These data highlight the need for caution when extrapolating from animal experiments to man.

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