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Metabolism of 4′-Modified Analogs of Doxorubicin. Unique Glucuronidation Pathway for 4′-Epidoxorubicin

H. WEENEN,*† J. M. S. VAN MAANEN,* M. M. DE PLANQUE,‡ J. G. MCVIE‡ and H. M. PINEDO*
*Department of Oncology, Free University, van de Boechorststraat 7, 1081 BT Amsterdam, The Netherlands and
‡The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Abstract—*The metabolism of doxorubicin (A), 4′-epidoxorubicin (E) and 4′-deoxydoxorubicin (D) was studied* in vitro *by incubating the analogs with rat liver subcellular fractions and* in vivo *by chromatographic analysis of human urine. Metabolites were identified by high-pressure liquid chromatography, fluorescence spectroscopy and enzymatic conversion. Human urine contained unchanged drug as well as the corresponding alcohol metabolites in all cases; however, urine of patients who received E also contained two glucuronides which could not be detected in the urine of patients who received A or D. We have identified these glucuronides as 4′-epidoxorubicin glucuronide (E-Glu) and 4′-epidoxorubicinol glucuronide (Eol-Glu). It was concluded that the glucuronide moiety is linked to the daunosamine sugar at the* C4′-OH *position. A hypothesis is proposed that this glucuronidation pathway may explain the differences in pharmacokinetics and toxicity between E and A. Rat liver microsomes were found to convert all three drugs to the 7-deoxyaglycones at the same rate. Rat liver* 100,000 *g supernatant was found to be capable of converting these drugs to their respective alcohol metabolites, doxorubicinol (Aol) being formed somewhat slower than 4′-epidoxorubicinol (Eol) and 4′-deoxydoxorubicinol (Dol).*

INTRODUCTION

4′-EPIDOXORUBICIN and 4′-deoxydoxorubicin, although only slightly different in their molecular structure from doxorubicin, have been proposed as being less toxic than it but equally or more active. 4′-Epidoxorubicin showed reduced toxicity in mice [1, 2], while both 4′-epidoxorubicin and 4′deoxydoxorubicin were less cardiotoxic in the rat [1, 3] than doxorubicin. Both 4′-modified doxorubicin analogs showed equal antitumor activity against L1210 in mice, 4′-epidoxorubicin being equally potent and 4′deoxydoxorubicin being more potent than doxorubicin [1]. Phase I and II clinical trials have indicated that 4′-epidoxorubicin has a pattern of acute toxicity including acute cardiotoxicity qualitatively similar to that of doxorubicin, but at equal doses, quantitatively lower gastrointestinal toxicity, alopecia and myelosuppression, and possibly a broader spectrum of antitumor activity [4–7]. As for chronic cardiotoxicity, to date only two mild-to-moderate and reversible congestive heart failures have been observed at doses of $\geqslant$1100 mg/m^2 in about 700 treated patients [7]. The difference in biological activity between 4′-epidoxorubicin and doxorubicin is not thought to be due to a difference in interaction with DNA [8], but may be caused by altered metabolic or pharmacokinetic properties [9]. Comparison of the pharmacokinetic and metabolic profiles of 4′-epidoxorubicin and doxorubicin in man revealed that 4′-epidoxorubicin is eliminated faster than doxorubicin, possibly due to a difference in metabolic conversion [10]. To allow for accurate comparison of the metabolism of the three 4′-analogs, we have studied two major metabolic pathways, reductive deglycosidation and carbonyl reduction, by *in vitro* incubations with rat liver subcellular fractions. In addition we have analyzed urine samples of patients who received any one of the three drugs as single-agent therapy.

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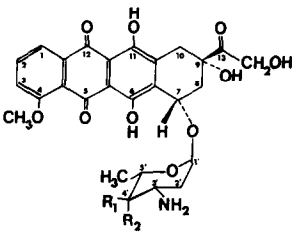

A: $R_1 = H; R_2 = OH$

E: $R_1 = OH; R_2 = H$

D: $R_1 = H; R_2 = H$

MATERIALS AND METHODS

E, Eol, D, Dol, Aol, 7-deoxydoxorubicinone (7d-Aone) and 7-deoxydoxorubicinol aglycone (7d-Aol) were kindly provided by Farmitalia Carlo Erba (Milan, Italy), doxorubicin (A), NADP, glycuronyl transferase (type II), glucuronidase (type VII) and arylsulfatase (type V) were obtained from Sigma Biochemicals (St. Louis, MO, U.S.A.) and all other chemicals were of analytical grade. Patients were treated in EORTC phase II clinical trials with A (60 mg/m^2), E (75 and 90 mg/m^2) or D (30 and 35 mg/m^2) as a single agent by an i.v. push injection. Urine aliquots were collected six-hourly during 48 or 96 hr and stored at −20°C.

High-pressure liquid chromatography

A 3-μm spherical particles C-18 reverse-phase column (Chrompack, CPtm microspher C 18) was used with KH_2PO_4 (0.05 M, pH 4.0)/CH_3CN, 70:40 (v/v), as the eluent for analysis of the incubations (system I)[11] and with KH_2PO_4 (0.02 M, pH 4.0)/CH_3CN, 75:30 (v/v), as the eluent for analysis of urine samples (system II). The capacity factors in system I were 1.56 (Aol), 1.76 (Eol), 2.00 (Dol), 2.18 (A), 2.5 (E), 3.0 (7d-Aol), 3.25 (D) and 5.6 (7d-Aone), and in system II 2.7 (Eol-glucuronide), 4.4 (E-glucuronide), 4.7 (Aol), 5.7 (Eol), 6.0 (Dol), 9.2 (A), 10.7 (E) and 12.3 (D). A fluorescence detector (Perkin Elmer, model 3000) was used at excitation wavelength 470 nm and emission wavelength 580 nm, in combination with an integrating system (Perkin Elmer, Sigma 10), for quantitation. Incubation mixtures (200 μl) were extracted with 5 ml chloroform/isopropanol (4:1, v/v), the organic layer was evaporated to dryness with air at 35°C and the residue redissolved in 100 μl methanol prior to injection (injection volume, 30 μl). Urine samples were not extracted but heated to 37°C while shaking, to carefully dissolve urine precipitates, and injected into the HPLC system as such. All glassware was treated with dichlorodimethylsilane to minimize adsorption effects.

Quantitation was based on integrated peak areas using external standards before and after each single series of measurements. Linear calibration curves were obtained for A, Aol, E, Eol, D and Dol between 5×10^{-9} and 5×10^{-6} M with the HLPC fluorescence assay used in this study. The percentages of Aol, Eol and Dol formed were calculated for each run and the mean values determined for each time interval ($n = 4$), thus making the use of an internal standard unnecessary. Fluorescence of E-Glu and Eol-Glu were assumed to be equal to E and Eol respectively, based on the similarity of their fluorescence spectra and the proposed molecular structure of their chromophores. The extraction, chromatographic and fluorescent properties of the 7 deoxyaglycones are different than for the glycosides but equivalent for both 7d-Aol and 7d-Aone [11], and do not allow linear calibration over a large enough concentration range. Their quantitation was therefore based on the parent drug concentration decrease and the relative integrated peak areas of 7d-Aol and 7d-Aone. For Example:

$$C(t)_{7d\text{-Aol}} = \{C(0)_{A,\,E\text{ or }D} - C(t)_{A,\,E\text{ or }D}\} \times \left\{ \frac{C(t)_{7d\text{-Aol}}}{C(t)_{7d\text{-Aol}} + C(t)_{7d\text{-Aone}}} \right\}.$$

The extraction efficiencies of E and metabolites were found to be 72 ± 11%, while extraction of the glucuronides was only 9 ± 5% efficient. The rather low recovery of the glucuronides can be explained by the polarity of the glucuronide moiety, and hence hydrophilicity of the glucuronide conjugate.

Isolation and identification of E-glucuronide (E-Glu) and Eol-glucuronide (Eol-Glu)

E-Glu and Eol-Glu were isolated from human urine by gradient elution on a silanized silica gel gravitation column, followed by preparative HPLC separation on a reverse phase C-18 column. Silanized silica gel 60 (Merck, 70-230 mesh ASTM) was packed into a glass column as a slurry in methanol + water (1:2, v/v). After dilution with methanol (urine: methanol, 9:1, v/v) large volumes of urine of up to 2 l could be brought into the column. After most impurities were washed away with methanol + water (9:1, v/v) the glucuronides could be eluted selectively with a gradient mixture, starting with methanol + water (8:2, v/v) and changing into pure methanol. The fractions which contained the glucuronides were pooled and evaporated to dryness under vacuum. The residue was redissolved in methanol, injected onto a semipreparative reverse-phase column (Chrompack, Lichrosorb 10 RP 18, 250 ×

12 mm) and eluted with water + acetonitrile (70:30, v/v). The capacity factor for E-Glu in this system was 5.3. The isolated glucuronides were pure on HPLC but decomposed on silica gel TLC (eluent: acetone + *n*-butanol + water, 5:4:1 v/v). Incubation of these metabolites with β-glucuronidase and arylsulfatase revealed that both compounds were glucuronides.

HLPC analysis of both the glucuronides and their hydrolysis products showed that the more polar of the two is converted into Eol while the less polar glucuronide is hydrolyzed to E upon incubation with β-glucuronidase (1000 U/ml, 37°C). The β-glucuronidase reaction was completed within 1 hr at pH 6.8 and was inhibited by glucaro-1,4-lactone, a specific β-glucuronidase inhibitor [12], at a concentration of 10^{-3} M. Incubating with arylsulfatase had no effect. The fluorescence spectra of both glucuronides as well as their β-glucuronidase hydrolysis products had an emission maximum at 585 mm and were identical with the fluorescence spectra of E and Eol. The nmr spectrum (Bruker, 250 MHz) of E-Glu in DMSO-d6 showed a doublet at 1.3 ppm (C5'-CH_3) and a sharp singlet at 4.0 ppm (C4-OCH_3) in addition to other peaks characteristic for E.

In vitro *experiments*

Adult male rats of the inbred strain R-Amsterdam were used (R/A). Their livers were homogenized in 1.15% KCl–0.01 M K_2HPO_4, pH 7.4 (6 ml/g liver). Microsomes were prepared by centrifugation of the homogenate at 10,000 *g* for 20 min and recentrifugation of the supernatant at 100,000 *g* for 1 hr [13]. The microsomal pellet was washed by resuspension in fresh extraction buffer and recentrifugation at 100,000 *g* for 1 hr. Incubations were carried out with 100,000 *g* supernatant and microsomes which were suspended in Hanks salt solution, pH 7.4, to obtain a protein concentration of 2.5 mg/ml, as determined by the Bio Rad protein assay [14]. As a cofactor system glucose-6-phosphate (4 mM), NADP (4.4 mM) and glucose-6-phosphate dehydrogenase (0.4 U/ml) was used. All experiments were carried out in four- or five-fold, and were stopped by cooling to 0°C and addition of the extraction medium. The total incubation volume was 200 μl per incubation and the concentration of anthracyclines was 2.5×10^{-6} M. Microsomal incubations were carried out under N_2, since reductive glycosidic cleavage of anthracyclines takes place only in the absence of O_2 [15, 16]. Enzymatic reduction of the carbonyl moiety was mediated by the 100,000-*g* supernatant under O_2 [17].

RESULTS

Anthracyclines containing a carbonyl moiety at the 12 position are metabolized in mammals and by cell extracts to yield the corresponding alcohol metabolites. This enzymic reduction takes place in the cytosol and requires NADPH as a cofactor [17]. All three compounds studied showed significant ketone–alcohol metabolism in the *in vitro* rat liver incubation system used. Doxorubicin was converted into the corresponding alcohol 1.5 times slower than 4'-epidoxorubicin and 4'-deoxydoxorubicin (see Table 1).

Reductive glycosidic cleavage of anthracyclines is also a major metabolic pathway in mammalian systems [18], but the enzymes responsible for this metabolic conversion are mainly localized in the microsomal subcellular fraction. This conversion is NADPH-dependent and requires anaerobic conditions. Thus rat liver microsomes were incubated with doxorubicin, 4'epidoxorubicin and 4'-deoxydoxorubicin in the presence of an NADPH generating system and in the absence of oxygen. Reductive glycosidic cleavage was quite significant, resulting in the formation of 7-deoxydoxorubicinone and 7-deoxydoxorubicinol (Table 2). No carbonyl-reduced glycosides could be detected, so 7-deoxydoxorubicinol must have been formed from 7-deoxydoxorubicinone rather than from the alcohol glycosides. Standard deviations were higher in these incubations than in the carbonyl reduction experiments, most likely because of very small amounts of oxygen still present in the system.

HPLC analysis of urine of seven patients who received 4'-epidoxorubicin showed that in addition to the parent drug, three major metabolites were present, while in urine of patients who received doxorubicin or 4'-deoxydoxorubicin (five samples each, $t = 6$–12 hr) only one major metabolite could be detected when analyzed in the same way (Fig. 1). The two compounds that were present in urine samples of all patients were identified as parent drug and the corresponding alcohol metabolite by chromatographic comparison with standards. The two extra compounds

Table 1. Formation of doxorubicinol, 4'-epidoxorubicinol and 4'-deoxydoxorubicinol after incubation of the 100,000-g supernatant of a rat liver homogenate with 2.5×10^{-6}M doxorubicin, 4'-epidoxorubicin and 4'-deoxydoxorubicin respectively

Incubation time (min)	Aol	Eol	Dol
15	6.0 ± 0.2*	8.7 ± 0.1	9.5 ± 0.5
30	11.1 ± 0.1	14.4 ± 0.4	15.8 ± 0.6
60	20.1 ± 0.2	29.5 ± 0.3	29.0 ± 2.0

*Standard deviation ($n = 4$).

Table 2. Formation of 7-deoxydoxorubicin, and 7-deoxydoxorubicinol after incubation of rat liver microsomes with doxorubicin, 4'-epidoxorubicin and 4'-deoxydoxorubicin respectively, for 60 min under nitrogen

Compound	Percentage of total of drug plus metabolites		
	Parent drug	7d-Aone	7d-Aol
Doxorubicin	5.1 ± 2.0*	40.1 ± 13.7	54.8 ± 13.7
4'-Epidoxorubicin	6.2 ± 2.5	45.2 ± 16.4	48.6 ± 27.0
4'-Deoxydoxorubicin	6.2 ± 1.3	43.0 ± 11.8	50.8 ± 14.0

*Standard deviation ($n = 5$).

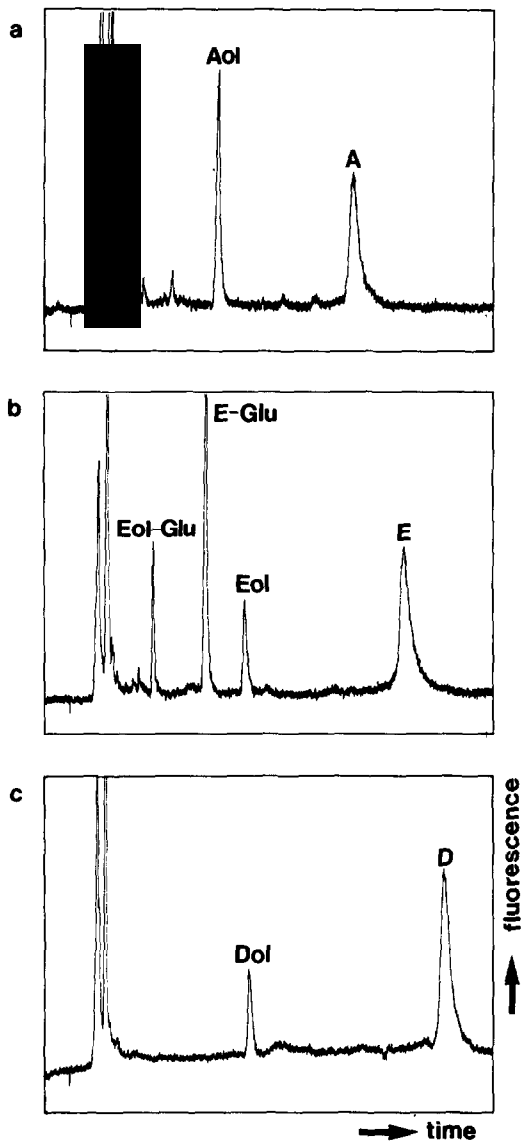

Fig. 1. Chromatograms of urine (t = 6–12 hr) of patients who received A (a), E (b) and D (c). Explanation of the abbreviations and the chromatographic conditions are in the text.

in urine of patients who received 4'-epidoxorubicin were isolated by reverse-phase gravitation column chromatography followed by reverse-phase HPLC. They were not stable on silica gel TLC but were pure by analytical reverse-phase HPLC. The nmr spectrum of the least polar of the two indicated that the C4–OCH_3 and the C5'–CH_3 moieties were still present in the isolated compound.

Incubation with β-glucuronidase converted the compound with $k' = 2.7$ (system II) to 4'-epidoxorubicinol and the compound with $k' = 4.4$ (system II) to 4'-epidoxorubicin, as shown by HPLC analysis. It was concluded that the metabolite with $k' = 4.4$ is the glucuronic acid conjugate of 4'-epidoxorubicin (E-Glu) and the metabolite with $k' = 2.7$ the glucuronic acid conjugate of 4'epidoxorubicinol. This assessment was in agreement with the fluorescence spectral identity of these glucuronides, their β-glucuronidase hydrolysis products, E and Eol. The glucuronidation pathway of 4'-epidoxorubicin is apparently different from doxorubicin, which has been reported to be converted to demethyl-deoxydoxorubicinol aglycone 4-*O*-β-glucuronide in man [19]. Incubations of rat liver microsomes or the 100,000-*g* fraction with 4'-epidoxorubicin (10^{-7} M) in the presence of UDPGA (0.3–1.6 mM) and a NADPH generating system, with or without glucuronyl transferase (Sigma, type II, from rabbit liver, 1–10 × 10^{-3} U/ml) and/or glucaro-1,4-lactone, did not give glucuronides in any significant amount. However, this may be due to differences in specificity depending on the species from which the enzyme originates.

DISCUSSION

Doxorubicin and the two 4'-analogs studied here are metabolized to the corresponding alcohols both *in vitro* (Table 1) and *in vivo* (Fig. 1 and Table 3). Enzymes present in the rat liver cytosol convert 4'-deoxydoxorubicin and 4'-epidoxorubicin into Dol and Eol rspectively 1.5 times faster than doxorubicin into Aol. Higher proportions of Eol were also seen in the rat *in vivo* [9] but not in man, as reflected in the relative areas under the plasma concentration curves and cumulative urine excretion of patients receiving E or A (Table 3 and [10]). The apparent discrepancy

Table 3. Cumulative urine excretion (0–48 hr) as a percentage of the administered dose of patients receiving doxorubicin, 4'epidoxorubicin and 4'-deoxydoxorubicin respectively

Compound	Percentage of the administered dose			
	Parent drug	Aol, Eol and Dol	E-Glu	Eol-Glu
Doxorubicin ($n = 10$)*	7.5 ± 2.5†	1.8 ± 0.8	-	-
4'-Epidoxorubicin ($n = 7$)	5.9 ± 2.3	0.8 ± 0.2	3.2 ± 1.1	0.8 ± 0.5
4'-Deoxydoxorubicin ($n = 6$)	6.5 ± 2.3	1.2 ± 0.6	-	-

*Data from Oosterbaan *et al.* [28].
†Standard deviation.

*Table 4. Relative areas under the plasma concentration vs time curve of 13 patients receiving 4'-epidoxorubicin (t = 15 min–48 hr)**

Eol-Glu	E-Glu	Eol	7d-Aol a/o Aone	E	7d-Aone
20%	25%	13%	12%	25%	6%

*Unpublished observations from this laboratory; see also [10].

between rat and human metabolism may be due to differences in species-dependent specificity of the enzymes, in relative bile excretion rates and other metabolic routes complicating a direct comparison of the metabolism and pharmacokinetics of laboratory animals and man.

Also, the large interindividual variations in patient studies may mask small differences in metabolic conversion rates.

All three compounds studied here are metabolized to 7d-Aone and 7d-Aol at the same rate under the conditions described. Unfortunately only limited clinical data [10] are available as yet to compare with these *in vitro* results, But it is obvious that reductive deglycosidation of anthracyclines is quite significant *in vivo* as well (Table 4). The formation of 7-deoxyaglycones is especially interesting since they are supposed to be formed via the semiquinone radical [20, 21], which may in turn be responsible for the (cardio)toxicity of anthracyclines [22]. Thus the fact that all three analogs are converted to the 7-deoxyaglycones and thus to the semiquinone radical at the same rate implies that the cardiotoxicity of these analogs should be directly proportional to the intracellular concentration of cardiac cells. Uptake of 4'-epidoxorubicin into L1210 cells *in vitro* is 14 times higher than for doxorubicin while 4'-epidoxorubicin is taken up 2.4 times more than doxorubicin [1]. However, uptake of the 4'-analogs into mouse cardiac cells is practically equal in the *a* phase but is significantly less at 24 and 48 hr [2, 23]. After 24 hr concentrations of E and D in the heart are approximately 1.6 times less than for A [2,23], while the relative minimal cardiotoxic doses in rats [1, 3] are 1.4–1.5 times higher for E and D than for A, suggesting that the cardiotoxicity of the 4'-analogs is indeed directly proportional to the intracellular concentrations in the heart. However, the data compared here are from different animal model systems and different studies, and correlations based on such comparisons must be considered with great caution.

Analysis of urine samples of at least five patients for each drug studies (Fig. 1) showed that only 4'-epidoxorubicin and 4'-epidoxorubicinol are directly glucuronidated to E-Glu and Eol-Glu. We propose that the glucuronic acid moiety is connected to the daunosamine sugar moiety via a C4'-*O*-glucuronide linkage (Fig. 2). The fact that 4'-deoxydoxorubicin does not form similar glucuronides makes the C3'-NH_2 an unlikely position as the site of conjugation. We attribute the lack of direct glucuronidation of doxorubicin to the steric hindrance of the 3'-NH_2 and 2'-CH_3 groups, which are both on the same side of the hexose ring as the 4'-OH moiety in the case of A but on opposite site of the six-membered ring in the case of E. We have not seen other polar doxorubicin metabolites to any significant extent, such as demethyldeoxydoxorubicinol aglycone-4-*O*-β-glucuronide, which was reported by Takanashi and Bachur [19]. However, it is conceivable that such a polar metabolite was not detected in our chromatographic system, due to interference with other fluorescent urine compounds. The unique glucuronidation pathway for 4'-epidoxorubicin may have clinical significance, since the formation of very polar glucuronides usually enhances excretion processes. Glucuronidation is generally a major

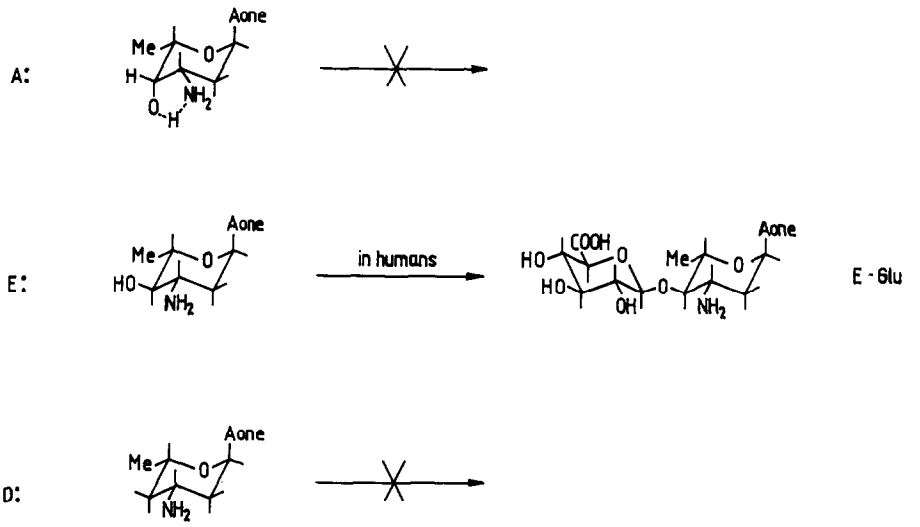

Fig. 2. Glucuronidation pathway of E in relation to the structures of A, E and D.

metabolic pathway not only in the liver but also in the kidney, spleen, adrenal glands, lung, gastrointestinal tract and heart [24]. Thus if glucuronidation of 4'-epidoxorubicin occurs in these organs, it may enhance drug excretion in general, but also locally. Both the favorable gastrointestinal toxicity of 4'-epidoxorubicin when compared with doxorubicin [4, 6] and its shorter terminal half-life $t_{\frac{1}{2}}\gamma$ (Table 5) seem to be in agreement with this hypothesis.

When addressing the question if a quantitative correlation between the extent of glucuronidation and the shorter $t_{\frac{1}{2}}$ for E may exist, the following pharmacokinetic calculations should be considered. First of all there is the question as to what the extent of glucuronidation may amount to, with respect to total drug elimination. Cumulative urine excretion of E-Glu is 3.2 ± 1.1% of the administered dose, or 33% of the total of drug and metabolites excreted via the urine (Table 3). Based on the contribution of the AUCs of drug and glycosidic metabolites to the total AUC (Table 4), an estimated contribution of 25% to the overall drug excretion due to glucuronidation seems reasonable. Assuming three-compartment linear pharmacokinetics, the following equation describes the relationship between the elimination rate constant k_{Cel} (total body clearance) and the terminal time constant τ_3: $K_{Cel} = D/(A\tau_1 + B\tau_2 + C\tau_3)$[25]. Since glucuronidation can be considered as elimination of the parent compound, k_{Cel} will increase because of glucuronidation. This implies that if glucuronidation amounts to about 25% of drug elimination,

Table 5. Pharmacokinetic parameters for 4'-epidoxorubicin* [10], *compared with doxorubicin parameters derived from* [28]

	E†	No. of patients	A‡	No. of patients
A (10^{-6} M)	9.7 ± 2.9§	8	16.0 ± 4.5	11
B (10^{-7} M)	1.2 ± 0.3§	8	0.9 ± 0.4	11
C (10^{-8} M)	3.0 ± 1.4§	8	2.8 ± 0.7	11
$t_{\frac{1}{2}}$ (min)	4.8 ± 1.8	8	4.6 ± 0.1	11
$t_{\frac{1}{2}}$ (hr)	2.6 ± 1.0	8	2.5 ± 1.3	11
$t_{\frac{1}{2}}$ (hr)	38 ± 14	8	70 ± 25	8
$t_{\frac{1}{2}}$ (hr)¶	27 ± 9	7	50 ± 5	11

*The parameters for A and E in this table were obtained from patients in the same institutes (Free University and Netherlands Cancer Institute) and should therefore be well comparable, although analyzed at different laboratories [10, 28].

†For eight patients receiving a mean dose of 75 mg/m².

‡For eight patients receiving a mean dose of 60 mg/m², from ref. [28].

§Unpublished data from this laboratory.

¶Based on urine excretion rates, $P < 0.001$.

($A\tau_1 + B\tau_1 + C\tau_3$) will increase by 25% as well. Since $C\tau_3$ accounts for about half of ($A\tau_1 + B\tau_2 + C\tau_3$) (see Table 5), τ_3 can be predicted to be reduced by about 40%, assuming all other parameters are unchanged (Table 5). In our comparative study on the pharmacokinetics of 4′-epidoxorubicin in man [10], $t_{\frac{1}{2}}\gamma$ for E and A were found to be 38 ± 14 and 70 ± 25 hr respectively (Table 5). This means that τ_3 for A is 46% longer than for E, which compares reasonably well with the above-calculated 40% considering the large variance of the parameters.

One can only speculate about the antitumor properties and toxicity of these glucuronides. Structure–activity relationships [26, 27] do not rule out their antitumor activity, but even if they are cytotoxic, they will probably not add significantly to the overall antitumor properties of the parent drug, since glucuronides are generally quickly eliminated.

It must be emphasized that in addition to the three metabolic pathways investigated in the present study (Fig. 3), other metabolic routes are available to anthracyclines such as hydrolytic cleavage and demethylation [19]. However, the metabolic pathways investigated in this study are of special importance since (i) carbonyl reduction leads to a product that still has antitumor activity; (ii) reductive deglycosidation is indicative for semiquinone radical formation [20, 21]; and (iii) direct glucuronidation is unique for 4′-epidoxorubicin and 4′-epidoxorubicinol.

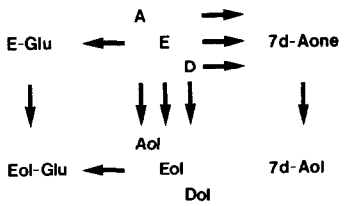

Fig. 3. Major metabolic pathways of A, E and D investigated in this study.

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