Cancer Chemother Pharmacol (1999) 44: 422–426

ORIGINAL ARTICLE

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Pharmacokinetics and metabolism of doxorubicin after short-term infusions in lymphoma patients

Received: 30 November 1998 / Accepted: 16 March 1999

Abstract *Purpose/Methods*: Twenty-four patients (17 males and 7 females with a mean age of 54 years) with malignant lymphoma participated in a study of doxorubicin pharmacokinetics after 50 mg/m^2 as 10-min infusions. In addition to plasma samples, serial leukocyte samples and – in one subject – serial biopsy specimens from lymphoma infiltrates were obtained. The samples were analysed by reversed-phase high-performance liquid chromatography. *Results*: In contrast to several previous studies, the data suggested that 7-deoxydoxorubicinolone, and not doxorubicinone, is a metabolite of doxorubicin in humans. Doxorubicin, but no metabolites, was present in significant and fairly constant concentrations in circulating leukocytes. These levels may reflect the drug levels in lymphoma infiltrates. The data further suggest that metabolism to 7-deoxydoxorubicinone is subject to large interindividual variation, possibly due to a genetic polymorphism, and that significant levels of a metabolic product which may be a doxorubicin glucuronide can be recovered from plasma of patients treated with doxorubicin.

Key words Doxorubicin · Pharmacokinetics · Metabolism

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Introduction

The anthracycline doxorubicin is used in the treatment of a variety of malignancies. The pharmacokinetic-pharmacodynamic relationships at play during doxorubicin therapy have not been resolved, and optimal dose schedules remain a matter of debate. Pharmacokinetic investigations in patients treated with doxorubicin have so far given inconclusive results [1, 8, 14]. However, in vitro studies have demonstrated a relationship between cellular doxorubicin levels and cytotoxicity [13, 14]. The quantification of anthracycline drugs in blood and tissues has encountered methodological difficulties, which in our view are largely due to a combination of a failure to achieve chromatographic resolution of distinctly different metabolites, the high affinity of these drugs to cellular constituents and their chemical instability. Both fundamental and clinical studies of the pharmacology of anthracyclines have been hampered by problems associated with assay performance.

Herein, we report the results from a study of doxorubicin pharmacokinetics in 24 consecutive lymphoma patients who were administered short term infusions. In addition to the demonstration of a considerable interindividual variation in plasma pharmacokinetics and cellular drug concentrations, the results suggest that the metabolism of doxorubicin is subject to interindividual differences which may be due to a genetic polymorphism. We also demonstrate the presence of a metabolite of doxorubicin, tentatively identified as a doxorubicin glucuronide, in the plasma of these patients.

Materials and methods

Drugs and chemicals

Doxorubicin, doxorubicinol (13(S)-dihydroadriamycin), doxorubicinone, doxorubicinolone (13-dihydroadriamycinone), 7-deoxydoxorubicinone (7-deoxyadriamycinone) and 7-deoxydoxorubicinolone (7-deoxy-13-dihydroadriamycinone) were kind gifts from Dr. A. Suarato, Farmitalia Carlo Erba, Milan, Italy. Proteinase K

was obtained from Boehringer Mannheim, Mannheim, Germany. DNase I, β-glucuronidase type H-2 from *Helix pomatia* (G-0876; 3300 sulfatase units per ml), β-glucuronidase type VIII-A from *Escherichia coli* (G-7771), D-saccharic acid-1,4-lactone and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemicals, St. Louis, Mo., USA. Formic acid, HPLC grade acetone and HPLC grade isopropanol were obtained from E. Merck, Darmstadt, Germany. All other reagents were of analytical grade. Aqueous reagents and mobile phases were made up in water purified by reversed osmosis followed by polishing with a Milli-Q UF-PLUS system (Millipore, Bedford, Mass., USA).

Apparatus

Chromatographic equipment was produced by Shimadzu, Tokyo, Japan. The solvent delivery system consisted of a DGU-3A on-line degasser coupled to an LC-9A quarternary gradient pump. Column temperature was maintained using a CT0-6A column oven with an on-line solvent preheater. Samples were injected with a SIL-9A autoinjector maintained at ambient temperature. An RF-551 scanning fluorescence detector was used. Plotting and integration were performed by a Chromatopac C-R6A integrator or a Class-VP 4.2 computer-based integration system.

Chromatography

HPLC was performed on a Supelcosil LC18 column (4.6 × 150 mm, particle size 3 μm; Supelco, Bellefonte, Pa., USA) protected by a 20-mm Supelguard column. The mobile phase consisted of a 0.28-*M* sodium formate buffer (pH 3.55, 24 °C):acetone:isopropanol mixture (72.5:25:2.5, v/v). The mobile phase was delivered at a rate of 1.2 ml/min and the column temperature maintained at 40 °C. The fluorescence detector was operated at an excitation wavelength of 500 nm and an emission wavelength of 580 nm. One hundred microlitres of sample was injected.

Standard solutions

Standards of doxorubicin and the five metabolites were made up in methanol, aliquoted and stored at −70 °C until use. Concentrations in the standards were verified by measurement of absorption at 495 nm with a Shimadzu UV-1201 spectrophotometer. These solutions were subsequently diluted in citrated plasma to make standard curves.

Samples

Blood from patients undergoing doxorubicin therapy was obtained in citrated vacuum tubes (Becton Dickinson, Rutherford, N.J., USA) and the plasma separated by centrifugation. Mononuclear blood cells were isolated by sodium metrizoate-Ficoll density gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway), counted and washed. Cells from lymphoma nodules were obtained by needle biopsy and sampled into ice-cold phosphate-buffered saline (PBS), counted and washed. The analytical procedures for measuring doxorubicin and metabolites in plasma and in cells are both described in detail elsewhere [2, 3].

Enzyme treatment of plasma

One volume of plasma was mixed with one volume of ammonium acetate buffer (200 m*M*, pH 5.0) containing 5 m*M* $MgCl_2$ before addition of 18 U of β-glucuronidase per μl plasma. Parallel samples were incubated with 5 m*M* of the specific β-glucuronidase inhibitor D-saccharic acid-1,4-lactone for 2 min before the enzymes were added. After incubation at 55 °C for 1–2 h the samples were deproteinized as for plasma samples [2] and analysed. Some samples were extracted with 3 volumes of chloroform after enzyme treatment. The aqueous layer was subsequently treated as plasma samples and the organic phase evaporated to dryness, reconstituted in 50% methanol and analysed.

Results

Twenty-four patients, 17 males and seven females aged from 29 to 79 (mean ± SD 54 ± 13) years, participated in the study. Informed consent was obtained from all subjects. The patients suffered from a malignant lymphoma. The protocol specified that each patient should receive a 50-mg/m^2 dose of doxorubicin as a 10 min infusion prior to blood samples at 5, 10 min and 30 min and 1, 2, 6 h and 24 h. In some patients, additional samples were obtained. Mononuclear cells were prepared from 1 to 7 blood samples from a total of ten of the 24 individuals, and needle biopsy specimens from superficial lymphoma infiltrates were obtained from a few of the subjects.

Plasma pharmacokinetics of doxorubicin

The pharmacokinetic profiles of doxorubicin, doxorubicinol, 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone are shown in Fig. 1. Doxorubicin pharmacokinetics during the initial 24 h are described by biphasic elimination curves with half-lives of 5 min and 17 h, respectively. Significant levels of both 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone were measured in all the individuals examined. The putative doxorubicin metabolite, doxorubicinone, was not detected in any of the patient samples.

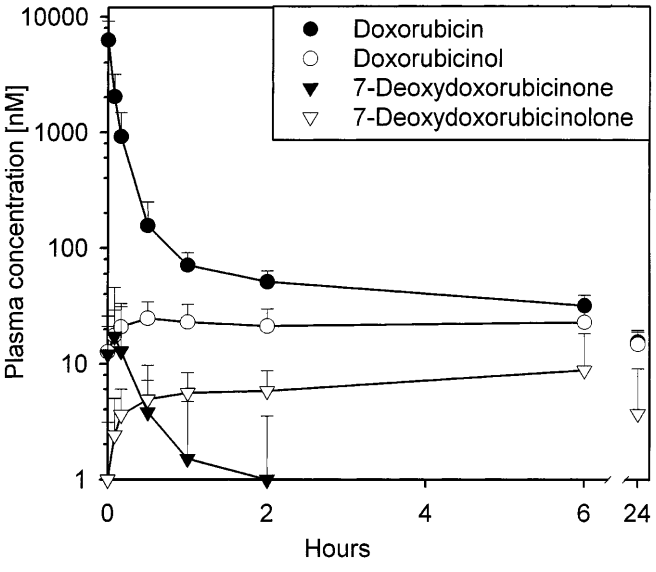

Fig. 1 Pharmacokinetic profiles of doxorubicin, doxorubicinol, 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone from 24 patients after administration of doxorubicin (50 mg/m^2) as 10-min infusions (mean + 1 SD). *SD* standard deviation

Cellular pharmacokinetics of doxorubicin

Twenty-four hour mononuclear cell doxorubicin levels at 5.5 pmol/10^6 cells (mean SD 1.3) were measured in ten subjects. For comparison with plasma concentrations, it may be assumed that 10^9 cells equals 1 ml [11, 12]. This implies that the mononuclear doxorubicin concentration is 5500 nM at 24 h, about 350 times higher than the plasma concentration. As is evident from Fig. 2, cellular doxorubicin levels were fairly constant during the entire sampling period. In a few patients, samples from lymphoma infiltrates were obtained and analysed. The results suggest that biopsy material from lymphoma infiltrates contained doxorubicin levels of the same magnitude as mononuclear blood cells (Fig. 3).

Interindividual differences in the metabolism of doxorubicin

The measured levels of 7-deoxydoxorubicinone demonstrated great interindividual variability. One subject had very high levels of this metabolite with an area under the concentration-time curve (AUC) of 173 n$M \times$ h, four subjects had intermediate levels with AUCs in the 14–51 n$M \times$ h range (mean $\pm$ SD 27.5 $\pm$ 16), whereas the remaining 19 individuals had 7-deoxydoxorubicinone AUCs below 10 n$M \times$ h (Fig. 4). The coefficient of variation (CV) for the AUC was 247%. No such major differences were evident for the metabolites doxorubicinol or 7-deoxydoxorubicinolone. CVs were 34% and 100%, respectively.

Metabolism of doxorubicin in a subject with renal dysfunction

One of the patients studied had moderate renal insufficiency, with serum creatinine and urea levels of 187 μM (2.1 mg/dl) and 11.3 mM (32 mg/dl), respectively. In agreement with [14], this did not bring about a reduction of the doxorubicin dose administered. In this particular subject, chromatography of plasma samples showed a prominent peak eluting ahead of doxorubicinol at approximately 4 min (Fig. 5). Although smaller than in this single subject, the peak also appeared in chromatograms from a majority of the plasma samples taken at 6 h and 24 h from the remaining 23 patients. Based on characteristics which include its behaviour after exposure to glucuronidase and its partition in liquids of different polarity, the peak has been tentatively identi-

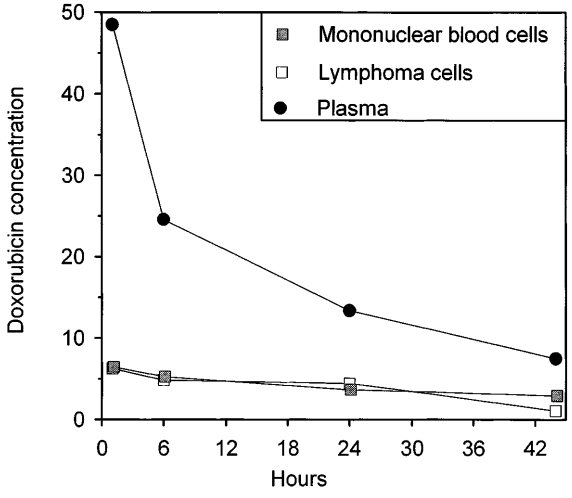

Fig. 3 Pharmacokinetic profiles of doxorubicin in plasma, mononuclear blood cells and cells obtained by needle biopsies from superficial lymphoma infiltrates. Cell concentrations are given as pmol/10^6 cells, plasma concentration as nM. The samples were from a single subject

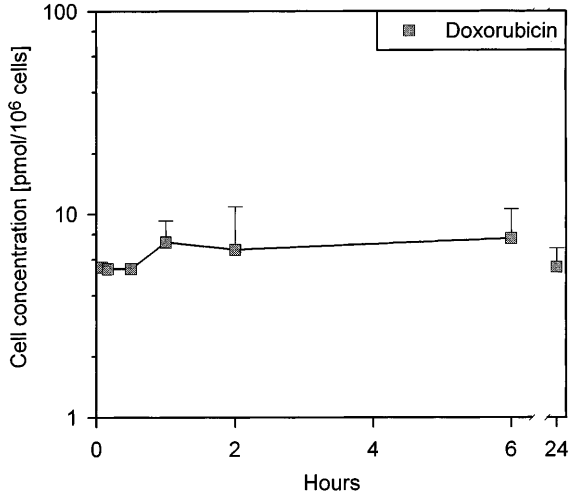

Fig. 2 Mononuclear blood cell doxorubicin levels in ten of the 24 individuals

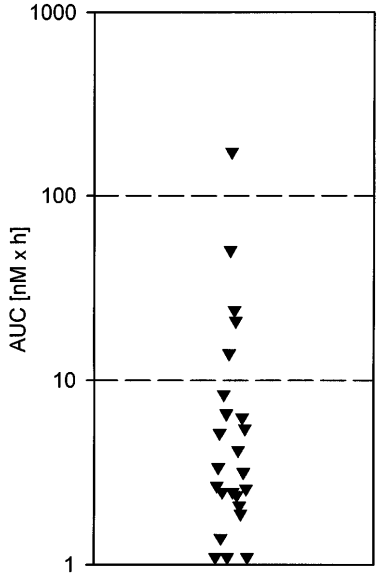

Fig. 4 Interindividual differences of 7-deoxydoxorubicinone AUCs in the 24 patients studied. *AUC* area under the concentration-time curve

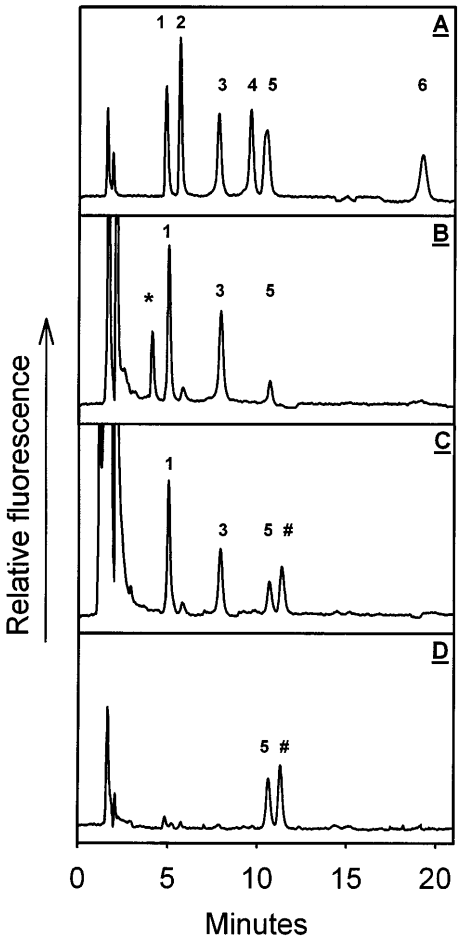

Fig. 5 Chromatograms of (**A**) plasma spiked with 25 n*M* each of (1) doxorubicinol, (2) doxorubicinolone, (3) doxorubicin, (4) doxorubicinone, (5) 7-deoxydoxorubicinolone and (6) 7-deoxydoxorubicinone; (**B**) a plasma sample obtained 6 h post-infusion from a patient with renal dysfunction, with the peak (*) tentatively identified as demethyl-7-deoxydoxorubicinolone 4-O-β-glucuronide eluting at 4 min; (**C**) the same patient sample after treatment with β-glucuronidase. The peak at 4 min has disappeared and a new peak (#), tentatively identified as demethyl-7-deoxydoxorubicinolone has appeared; and (**D**) a chloroform extract of the C sample, the peak (#) at 11.5 min is present together with the non-charged aglycone 7-deoxydoxorubicinolone

fied as a doxorubicin glucuronide, and possibly demethyl-7-deoxydoxorubicinolone 4-O-β-glucuronide.

Discussion

The investigation of baseline pharmacokinetic properties of doxorubicin revealed the presence of the doxorubicin metabolites doxorubicinol, 7-deoxydoxorubicinone and 7-deoxydoxorubicinolone in all subjects (Fig. 1). Cummings et al. [6] detected 7-deoxyaglycones in only about half of the patients. This discrepancy is probably due to the lower limit of detection in our method [2]. Previously published methods have to a large extent been unsuccessful in separating doxorubicinone and 7-deoxydoxorubicinolone. Of 13 pharmacokinetic investigations summarized in [14], six studies reported the occurrence of the metabolite doxorubicinone in patient plasma. We have not detected this metabolite, but have, on the other hand, measured 7-deoxydoxorubicinolone in patient samples. In reversed-phase chromatographic systems, these two compounds are prone to coelution. We believe, as suggested by Cummings [6], that reports on doxorubicinone in patient plasma are most likely to be methodological artefacts. Our data suggests that 7-deoxydoxorubicinolone, and not doxorubicinone, is a metabolite of doxorubicin in humans.

The cellular pharmacokinetics of doxorubicin in white blood cells were in agreement with previous investigations. Speth et al. have reported stable intracellular white blood cell levels of doxorubicin [11, 13] and daunorubicin [12] of a magnitude similar to the levels measured in the present study. It may also be noteworthy that we detected no doxorubicin metabolites intracellularly in leukocytes isolated from doxorubicin-treated patients. To investigate the technical feasibility of this approach, we obtained needle biopsy material from superficial lymphoma infiltrates in a few patients. It should be noted that these data are not the result of systematic investigations, but constitute single observations. Nevertheless, it may be of interest that in the single subject where serial samples were obtained over a prolonged period (Fig. 3), the levels of doxorubicin were of the same magnitude as those measured in nucleated blood cells.

An interindividual comparison of 7-deoxydoxorubicinone levels in the 24 patients revealed considerable differences. The subjects could be divided into three groups on the basis of 7-deoxydoxorubicinone biotransformation (Fig. 4). In a single individual, relatively extensive metabolism to this moiety was evident. Four persons demonstrated an intermediate degree of biotransformation to 7-deoxydoxorubicinone, whereas the remaining 19 subjects did not produce appreciable levels of this metabolite beyond 1 h post-infusion. To our knowledge, there is only one previous report on variability in 7-deoxydoxorubicinone formation in humans after administration of doxorubicin [6]. The reason for this variability is not known. In some of the subjects studied by us, samples were obtained during additional courses of doxorubicin treatment, with little between course variability in 7-deoxydoxorubicinone levels. It is tempting to speculate about whether this biotransformation pathway is subject to genetic polymorphism. At present, the relative contribution of the two possible metabolic pathways from doxorubicin to 7-deoxydoxorubicinolone in humans is not known, and the variability in biotransformation rates has not been linked to distinct genotypes. In this context, the association between 7-deoxydoxorubicinolone formation and cardiotoxicity proposed by Cummings and Smyth [5] and others (for review, see [10]) should also be noted. Basic studies aimed at identifying the mechanism responsible for anthracycline-induced cardiotoxicity have advocated that

the formation of aglycone metabolites may indeed be of major importance in causing oxidative stress and disruption of structural integrity in heart muscle tissue [7, 9, 10].

Chromatographic analyses of patient samples gave information suggestive of additional doxorubicin metabolites in plasma. The chromatographic peak at 4 min (Fig. 5, panel B) has been tentatively identified as the demethyl-7-deoxydoxorubicinolone 4-O-β-glucuronide [4, 15]. The evidence for this is circumstantial, and can be outlined as follows: As shown in Fig. 5 (panel C), addition of glucuronidase to plasma made the 4-min (*) peak disappear concomitant with the appearance of a new peak (#) at 11.5 min. This result was seen both with the addition of crude glucuronidase/sulfatase solution from *Helix pomatia* and of the purified glucuronidase from *E. coli*. Secondly, the emission intensity at 545 nm was 1.6 times higher than the emission intensity at 580 nm for the peak eluting at 4 min. For doxorubicin, doxorubicinol and 7-deoxydoxorubicinolone the difference in response between the two emission wavelengths was minor [15]. Thirdly, after chloroform extraction of the enzyme treated plasma, the resulting aglycone was present exclusively in the organic phase, together with the non-charged aglycone 7-deoxydoxorubicinolone. Doxorubicin and doxorubicinol, which are positively charged at pH 5, remained in the aqueous phase (Fig. 5, panel D). Experiments with spiked plasma samples further verified that all uncharged aglycones were recovered from the chloroform phase, while doxorubicin and doxorubicinol remained in the aqueous phase (data not shown). Finally, no conversion to the aglycone was observed after treatment of plasma with the crude glucuronidase/sulfatase mixture from *Helix pomatia* in the presence of the specific glucuronidase inhibitor D-saccharic acid-1,4-lactone, suggesting that the peak of interest is a conjugate with glucuronic acid. In the absence of the enzymes, no conversion was observed (data not shown).

The putative glucuronide was present in the majority of samples, but high levels of the compound were only observed in an individual with renal failure. Previous investigators have identified the demethyl-7-deoxydoxorubicinolone 4-O-β-glucuronide in urine after doxorubicin therapy [4, 15]. The present study suggests that this metabolic product may also be present in appreciable quantities in plasma.

To summarize, the present study suggests that 7-deoxydoxorubicinolone, and not doxorubicinone, is a metabolite of doxorubicin in humans. The parent compound is present in significant and fairly constant concentrations in circulating leukocytes, which may possibly reflect the drug levels in lymphoma infiltrates. Our data further suggest that metabolism to 7-deoxydoxorubicinone is subject to large interindividual variability, and that a metabolic product which is most likely to be a glucuronide can be recovered from plasma of patients treated with doxorubicin.

Acknowledgements This study was supported financially by the Norwegian Cancer Society. The authors wish to thank the staff at the Central Laboratory and at the Department of Oncology (B2), The Norwegian Radium Hospital, for participating in this study.

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