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Differential Retention of Doxorubicin in the Organs of Two Strains of Rats

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Abstract—Fluorescence microscopy and high pressure liquid chromatography were used to study the decrease of doxorubicin (DXR) concentrations in the liver, spleen, heart, lung, kidney and skeletal muscle of two strains of rats at various times after a single intravenous injection of the drug (8 mg kg^{-1}). DXR was located within the cell nucleus and was mostly undegraded, it persisted, especially in heart, lungs and spleen where it was detectable 10 days after injection. The DXR/DNA ratio, was used as an index of nuclear fixation of the drug. A major difference in the DXR/DNA ratio between the two strains were observed in heart and spleen results; the DXR/DNA ratio was significantly higher in heart and spleen compared with lung, liver and kidney in both strains.

Doxorubicin (DXR; Adriamycin) is a major antineoplastic drug (Young et al 1981). The drug intercalates into the DNA molecule and inhibits DNA and RNA synthesis (Di Marco & Arcamone 1975; Di Marco et al 1981; Gabbay et al 1976). Nuclear localization of DXR is therefore required for the antimitotic activity and the toxicity of this drug. Numerous pharmacokinetic data are available on the high plasma clearance and the rapid distribution of DXR in human and animal organs (Benjamin et al 1977; Lee et al 1980; Oosterbaan et al 1984; Tavoloni & Guarino 1980; Terasaki et al 1982; 1984b). However, the persistence of DXR in those organs has been poorly studied and the variation of DXR persistence in different strains of an animal species has not been reported.

In the present paper, we have used fluorescence microscopy for localization and high pressure liquid chromatography (HPLC) to measure concentrations of DXR in the organs from two strains of rats at different times following a single intravenous injection of the drug.

Material and Methods

Doxorubicin (mol. wt 580) was obtained from Roger Bellon Laboratories (Neuilly, France) and dissolved in 0·9% NaCl immediately before intravenous injection. Daunorubicin (mol. wt 564), used as internal standard for HPLC assays and doxorubicinol, used as reference standard to detect the major metabolite, were kindly supplied by Specia Laboratories (Paris, France).

Male, syngeneic BDIX rats, 5–6 months, mean weight 340 g (s.d. 29 g) were bred in our laboratory. Male, non-syngeneic Sprague-Dawley (S.D.) 7–8 months, mean 534 g (s.d. 34 g) were obtained from Iffa Credo (L'Arbresle, France). Groups of three rats of each strain were killed, one, five and ten days after a single injection of DXR (8 mg kg^{-1}) in the femoral vein. A thin piece of organ or tissue was immediately examined by fluorescence microscopy, the remainder was stored at −20°C until HPLC analysis.

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Groups of three non-treated rats of the two strains were used as controls in fluorescence and HPLC studies.

Microscopic localization of DXR fluorescence

In preliminary studies, a similar partition of fluorescence was observed between the nucleus and cytoplasm whether the organs of rats were instantly frozen in liquid nitrogen or frozen at −20°C. Therefore, for routine procedure a thin piece of wet organ was inserted in embedding medium for frozen tissue specimens (Tissue Tek, Miles Scientific, Naperville, USA) then cooled to −20°C. Frozen sections of 3 μm were cut on a cryostat, then transferred on a glass cover slip and dried at room temperature. Cover slips were inverted and mounted in inclusion medium (Eukitt, Freiburg, RFA) on a microscopic glass slide. Preparations were observed under a fluorescence microscope (Dialux 20, Leitz, Wetzlar, Germany) equipped with a high pressure mercury lamp, an N-2 filter set, and a 1000 × oil immersion objective (Leitz, Wetzlar, RFA). Wavelength of excitation and emission were 450 and 520 nm, respectively. Counting of typical orange-red fluorescent nuclei in each microscopic field through the 100 × objective permitted a semi-quantitative appreciation of the DXR content in each organ. No fading of DXR nuclear fluorescence was observed if examinations and microphotographs were made in less than 3 min.

Doxorubicin quantitation

DXR present in tissue samples was assayed following a method derived from Israel et al (1978), Baurain et al (1979) and Robert (1980). A fraction of wet tissue was weighed and homogenized with 2 mL of distilled water containing 5 μg of the internal standard daunorubicin. To a 200 μL aliquot of the mixture were added 300 μL of borate buffer (pH 9·8) and 9·5 mL of chloroform–methanol (4:1, v/v). After mixing and centrifugation, the aqueous phase was discarded and the organic layer evaporated under nitrogen. The residue was dissolved in 200 μL of the HPLC mobile phase and centrifuged. A 50 μL aliquot of the supernatant was analysed. The chromatographic conditions were the following: the stationary phase was Microbondapak C18 (150 × 3·9 mm) 5 μm (Waters Associates, Milford, USA) and the

mobile phase was an isocratic mixture of acetonitrile and 0·1% ammonium formate pH 4 (35:65 v/v). The HPLC apparatus was a 6000 A pump with an U6K injector (Waters Associates, Milford, USA). Drugs were detected with a fluorometer (Jobin Yvon JY 3D, Longjumeaux, France) equipped with a microcell for HPLC. Excitation and emission wavelengths were 480 and 560 nm, respectively. The retention time of the compounds were 1·3 min for doxorubicinol, 1·8 min for doxorubicin, and 3·2 min for daunorubicin.

Tissue DNA concentration

Tissue DNA concentrations were determined using a method adopted from Schneider (1946). Wet organs were weighed, then homogenized in distilled water (5 mL g^{-1} of wet tissue). One mL aliquot of the homogenate was precipitated with 3 mL of 10% cold trichloroacetic acid (TCA). After centrifugation, the precipitate was shaken in 6 mL of ethanol–water mixture (80:20, v/v) and centrifuged again. The second precipitate was heated at 90°C for 15 min in 2 mL of 5% TCA. After centrifugation 2 mL of supernatant was combined with 4 mL of a diphenylamine reagent (1·5 g diphenylamine, 100 mL glacial acetic acid and 2·75 mL of 0·5 M sulphuric acid) and heated at 100°C for 10 min. Absorbance of the final solution was measured at 595 and 650 nm. Standard curves of DNA concentrations were established by dilution of herring sperm DNA (Sigma, St Louis, USA) heated in 5% TCA and combined with the diphenylamine reagent.

Statistical methods

All statistical computations were done using the one way or two way analysis of variance with replications. A comparison of DXR/DNA ratios between heart and other organs in the two strains of rats was performed by a method of linear regression: each value of DXR/DNA ratio for the heart in a rat strain was coupled on a graph with the value of DXR/DNA ratio of the compared organ. Regression lines were drawn from 9 data points (3 rats on 3 days) and slopes of straight lines were calculated; comparison of results of correlations has been effected by one way analysis of covariance.

Results

Fluorescence microscopy

In non-treated rats, we observed a greenish autofluorescence mainly in the cytoplasm of lung, heart, liver, muscle and tubular cells of the kidney. Spleen and glomerular cells of kidney were less autofluorescent. Cell nuclei were never autofluorescent.

In BDIX rats 24 h after intravenous injection of DXR, a bright orange fluorescence due to DXR was observed in the nuclei of all tissues except the nuclei of tubular cells of the kidney (Table 1). There was no orange fluorescence, related to DXR, in the cytoplasm of any tissue cells. Tissues from S.D. rats killed 24 h after intravenous injection of DXR, showed a less intense nuclear fluorescence than did the tissues of BDIX rats, except in the glomerular area of the kidney. In BDIX rat tissue at day 5 after DXR injection, a persistent nuclear fluorescence was observed, except in the liver and in the tubular area of the kidney. Under the same conditions, nuclear fluorescence was less intense in the organs of S.D. rats (Table 1). At 10 days after injection of DXR, a detectable fluorescence was observed only in the nuclei of the glomerular area of BDIX rats, whereas no nuclear fluorescence was observed in any tissue of S.D. rats (Table 1).

Table 1. Semi-quantitative appreciation of tissue fluorescence related to DXR. BDIX or S.D. rats were injected intravenously at Day 0 with 8 mg kg^{-1} DXR.

	Day killed					
	1		5		10	
	BDIX	S.D.	BDIX	S.D.	BDIX	S.D.
Heart	+++[a]	++	++	+	0	0
Lung	+++	++	++	0	0	0
Liver	++	+	0	0	0	0
Spleen	+++	++	+	+	0	0
Kidney:						
glomerular area	+++	+++	++	++	+	0
tubular	0	0	0	0	0	0
Muscle	+++	+	++	0	0	0

[a] Semi-quantitative appreciation of DXR-related nuclear fluorescence; +++ more than 2 brightly fluorescent nuclei in each microscopic field (objective × 1000); ++: 1 or 2 fluorescent nuclei in each microscopic field; +: one faintly fluorescent nucleus for several microscopic fields; 0: no nuclear fluorescence with only cytoplasmic autofluorescence.

HPLC analysis of DXR concentrations

DXR concentrations decreased with time in all tissue of the two strains of rats (Table 2). At day one after injection, tissue concentrations of DXR were higher in BDIX rats than in S.D. rats, in heart, kidney, liver and muscle. A significant difference in concentrations of DXR between the two strains persisted on day 5 after injection between kidney and heart, and at day 10 in heart only. The major metabolite, doxorubicinol, or other compound was not detected in any tissue of the two strains of rats at any time. Extractions without internal standard attested to the lack of interference by other metabolites.

DNA concentrations

The DNA concentrations of each tissue were of the same order in BDIX and S.D. rats (Table 3), except in the spleen.

DXR/DNA ratio

The evolution of the DXR/DNA ratio as a function of time in each organ of the two strains of rats is shown in Fig. 1. A significant correlation between the DXR/DNA ratio in heart and the DXR/DNA ratios of the other organs was observed in the two strains of rats (correlation coefficients ranged from 0·839 to 0·963). Slopes of correlation lines are indicated in Table 4. DXR/DNA ratios were statistically lower in kidney, lung and liver than in heart in both BDIX and S.D. rats.

Discussion

Fluorescence microscopy on frozen tissue sections and HPLC assays were used as complementary methods to appreciate DXR retention in different organs after a single intravenous injection. Fluorescence microscopy is a conve-

Table 2. Concentration of DXR (g net wt) in tissues from two strains of rats after a single i.v. injection (8 mg kg^{-1})[a].

	Day killed					
	Day 1		Day 5		Day 10	
	BDIX	S.D.	BDIX	S.D.	BDIX	S.D.
Heart	24·71[a] (2.05) (S)[b]	6·88 (0·32)	7·09 (0·28) (S)	3·91 (1·25)	2·74 (0·23) (S)	0·59 (0·47)
Lung	18·35 (5.93)	15·36 (1·14)	7·69 (2·39)	8·22 (1·03)	3·72 (0·70)	5·02 (1·27)
Liver	7·10 (1·13) (S)	3·48 (1·00)	1·67 (0·53)	1·44 (0·10)	N.D.	N.D.
Spleen	45·48 (19·92)	37·88 (4·34)	10·59 (0·70)	12·62 (4·35)	2·50 (1·16)	5·83 (0·99)
Kidney	22·01 (4·06) (S)	10·03 (0·76)	6·78 (1·03) (S)	3·21 (1·36)	1·68 (0·76)	0·33 (0·31)
Muscle	5·56 (0·97) (S)	4·24 (0·76)	2·56 (0·91)	1·38 (0·20)	N.D.	0·54 (0·46)

[a] Mean of 3 rats. Standard deviation in parentheses.
[b] S: indicates significant difference ($P < 0.05$, Student's t-test).
N.D: not detected.

Table 3. DNA concentration (mg g^{-1} wet tissue) in organs from two strains of rats[a].

	BD IX	S.D.
Heart	1·49 (0·14)[a]	1·17 (0·10)
Lung	5·85 (1·30)	5·05 (1·01)
Liver	2·80 (0·18)	2·40 (0·20)
Spleen	2·87 (1·00)	5·95 (1·25)
Kidney	3·90 (0·43)	3·80 (0·53)
Muscle	0·57 (0·06)	0·49 (0·09)

[a] Standard deviations in parentheses.

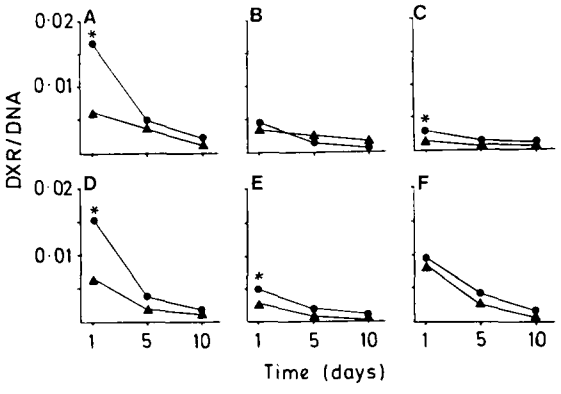

FIG. 1. Variation of DXR/DNA ratio as a function of time in the organs of BDIX rats (●) and Sprague-Dawley rats (▲). The statistical significance by the method of two ways analysis of variance is indicated by (*). A, heart; B, lung; C, liver; D, spleen; E, kidney; F, skeletal muscle.

nient but not quantitative method for localization of DXR in cultivated cells or in organs (Ozols et al 1979; Egorin et al 1980; Bigotte & Olsson 1982; Paschoud et al 1985). Quenching DXR fluorescence on binding to DNA could lead to an underestimate of the DXR concentration in the nucleus; however, quenching appears to be limited in our experiment and that of Egorin et al (Egorin et al 1980; Chauffert et al 1984). As did other authors, we observed that the characteristic orange-red fluorescence of DXR was restricted to the nucleus of tissue cells; however, it has been reported that DXR can also be localized in lysosomes of cells in culture (Noel et al 1978; Chauffert et al 1984). In the present work,

Table 4. Comparison of DXR/DNA ratio of heart with other organs from two strains of rats[a].

	Lung	Liver	Spleen	Kidney	Muscle
S.D.	0·35 (0·06)*	0·22 (0·02)*	0·95 (0·11)	0·40 (0·03)*	1·29 (0·13)
BDIX	0·20 (0·02)*	0·16 (0·01)*	0·91 (0·14)	0·34 (0·02)*	0·61 (0·05)*

[a] In this Table are indicated the slopes (s) of the correlation lines between DXR/DNA ratio of heart and each other tissue:

$\frac{DXR}{DNA}$ organ $= s \frac{DXR}{DNA}$ heart. Standard deviations in parentheses.

* indicates a statistical difference in the organ compared with heart by one way analysis of covariance ($P < 0.05$).

we did not observe DXR fluorescence in the cytoplasm of any tissue cells. The explanation for this could be the rapid redistribution of DXR from lysosomes, or other cytoplasmic structures to the nucleus after the death of the animal. However, we have not observed a cytoplasmic fluorescence after instant freezing by immersion in liquid nitrogen of organs removed from anaesthetized rats. A major advantage of fluorescence microscopy was the precise localization of DXR in the different organs, so a clearly different pattern of fluorescence persistence was observed in the two functional areas of the kidney; in the other organs fluorescence was homogeneously distributed.

Both the semi-quantitative appreciation of nuclear fluorescence by microscopy and the HPLC assays demonstrated a decrease of the DXR content in all organs of the two strains of rats as a function of time. A striking difference in the DXR concentration in many organs, particularly the heart, was observed between BDIX and S.D. rats. Interstrain variation of distribution and persistence of DXR in the organs of two strains of an animal species has not been reported before. Such differences could be under genetic control. The mechanisms of elimination of DXR from normal cells are not well established. One can exclude the metabolism in-situ since no metabolites of DXR were found in the organs. An active efflux of DXR, identical to that described in resistant cancer cells (Skovsgaard 1978; Inaba et al 1981; Chauffert et al 1984), could be hypothesized; for example, the difference of the efflux rate could explain the longer persistence of DXR in glomerular rather than in tubular cells of the kidney.

Diminution of the resistance of cancer cells can be achieved in-vitro by such inhibitors of the DXR efflux mechanism as verapamil (Tsuruo et al 1982) or amiodarone (Chauffert et al 1986). These inhibitors could also act on the efflux mechanism of the normal cells of the organs and increase the retention and the toxicity of DXR. Careful toxicological studies must be performed on animals before the clinical use of these inhibitors in association with DXR.

In this paper, the predominant location of DXR in the nucleus was confirmed by fluorescence microscopy. This fact has been discussed by many authors and is explained by the ability of the drug to bind tightly with DNA (Di Marco & Arcamone 1975; Gabbay et al 1976; Noel et al 1978; Egorin et al 1980; Terasaki et al 1984a). Therefore, we have hypothesized that the DXR concentrations in different organs could be equal to intranuclear concentrations and that the number of DNA sites occupied by DXR could be estimated from the DXR/DNA ratio. Calculation of this ratio allowed the comparison of the saturation of DNA sites by DXR in different organs with varying DNA content. At days 1 and 5 after DXR injection, the highest values of the DXR/DNA ratio were observed in heart, spleen and muscle compared with lower values in the other organs. Moreover we observed striking differences in the DXR/DNA ratio in the heart and spleen between the two strains of rats; Terasaki (1982, 1984b) used kinetic models for the calculation of a plasma to tissue partition coefficient (Kp); he concluded that a relation existed between DXR distribution and DNA content in some, but not all, organs in Wistar rats, rabbit and guinea-pigs. In our study, the absence of a relation between DXR and DNA tissue concentrations was more evident in BDIX rats than in Sprague-Dawley rats. This interstrain difference in retention of DXR could explain, in part, the discrepancies between our work and Terasaki's. Toxicity of DXR could be related to its long retention in the nucleus of organ cells. The well known cardiotoxicity of DXR (Von Hoff et al 1979) and the efficiency of DXR in lymphomas with spleen involvement (Young et al 1981) could be explained by the long retention of DXR and the high saturation of the DNA in the cells of these organs. Striking differences in the DXR/DNA ratio in the heart of the two strains of rats suggest that the cardiotoxicity of DXR should be compared in BDIX and S.D. rats to assess the DXR/DNA ratio as a valuable indicator for cardiotoxicity prediction.

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References

Baurain, R., Deprez De Campeneere, D., Trouet, A. (1979) Rapid determination of doxorubicin and its fluorescent metabolites by high pressure liquid chromatography, Anal. Biochem. 94: 112–116

Benjamin R. S., Riggs, C. E., Bachur, N. R. (1977) Plasma pharmacokinetics of Adriamycin and its metabolites in humans with normal hepatic and renal function, Cancer Res. 37: 1416–1420

Bigotte, L., Olsson, Y. (1982) Cytofluorescence localization of Adriamycin in the nervous system, Acta Neurophathol. 58: 193–202

Chauffert, B., Martin, F., Caignard, A., Jeannin, J. F., Leclerc, A. (1984) Cytofluorescence localization of Adriamycin in resistant colon cancer cells, Cancer Chemother. Pharmacol. 13: 14–18

Chauffert, B., Martin, M. S., Hammann, A., Michel, M. F., Martin, F. (1986) Amiodarone-induced enhancement of doxorubicin and 4'deoxydoxorubicin cytotoxicity to rat colon cancer cells in vitro and in vivo, Cancer Res. 46: 825–830

Di Marco, A., Arcamone, F. (1975) DNA complexing antibiotics: daunomycin, Adriamycin and their derivatives, Arzneimittel-Forsch. 25: 368–374

Di Marco, A., Arcamone, F., Zunino, F. (1981) Daunorubicin and Adriamycin and structural analogs: biological activity and mechanisms of action, in: J. W. Cockran, F. M. Mahn (eds) Mechanism of action of antimicrobial and antitumor agents, Springer Verlag, Berlin p. 101

Egorin, M. J., Clawson, R. E., Cohen, J. L., Ross, L. A., Bachur, N. R. (1980) Cytofluorescence localization of anthracycline antibiotics, Cancer Res. 40: 4669–4676

Gabbay, E., Grier, D., Fingerle, R. E., Reimer, R., Levy, R., Pearce, S. W., Wilson, W. D. (1976) Interaction specificity of the anthracyclines with deoxyribonucleic acid, Biochemistry 15: 2062–2070

Inaba, M., Fujikura, R., Sakurai, Y. (1981) Active efflux common to vincristine and daunorubicin in resistant P388 leukemia, Biochem. Pharmacol. 30: 1863–1865

Israel, M., Peff, W. J., Wilkinson, P. M., Garnick, M. B. (1978) Liquid chromatographic analysis of adriamycin and metabolites in biological fluids, J. Liqu. Chromatogr. 1: 795–809

Lee, Y. T. N., Chan, K., Harris, P., Cohen, J. L. (1980) Distribution of Adriamycin in cancer patients, Cancer 45: 2231–2239

Noel, G., Peterson, C., Trouet, A., Tulkens, P. (1978) Uptake and subcellular localization of daunorubicin and Adriamycin in cultured fibroblasts, Eur. J. Cancer 14: 363–368

Oosterbaan, M. J., Dirks, R. J. M., Vree, T. B., Van Der Kleijn, E. (1984) Pharmacokinetics of anthracyclines in dogs: evidence for structure-related body distribution and reduction to their hydroxy metabolites, Pharmacol. Res. 1: 33–39

Ozols, R. F., Locker, G. Y., Doroshow, J. H., Grotzinger, K. R., Myers, C. E., Young, R. C. (1979) Pharmacokinetics of Adriamycin and tissue penetration in murine ovarian cancer, Cancer Res. 33: 3209–3214

Paschoud, N., Bignell, L., Reinhold, H. (1985) A double fluorescence method for determining doxorubicin distribution and vascular supply in the mouse kidney, J. Histochem. Cytochem. 33: 73–76

Robert, J. (1980) Extraction of anthracyclines from biological fluids for HPLC evaluation, J. Liqu. Chromatogr. 3: 1561–1572

Schneider, W. C. (1946) Phosphore compounds in animal tissues. A comparison of methods for the estimation of nucleic acids, J. Biol. Chem. 164: 747–751

Skovsgaard, T. (1978) Mechanism of cross resistance between vincristine and daunorubicin in Ehrlich tumor cells, Cancer Res. 38: 4722–4727

Tavoloni, N., Guarino, A. M. (1980) Disposition and metabolism of Adriamycin in the rat, Pharmacology 21: 244–255

Terasaki, T., Iga, T., Sugiyama, Y., Hanano, M. (1982) Experimental evidence of characteristic tissue distribution of Adriamycin. Tissue DNA concentration as a determinant, J. Pharm. Pharmacol. 34: 597–600

Terasaki, T., Iga, T., Sugiyama, Y., Hanano, M. (1984a) Interaction of doxorubicin with nuclei isolated from rat liver and kidney, J. Pharm. Sci. 73: 524–528

Terasaki, T., Iga, T., Sugiyama, Y., Hanano, M. (1984b) Pharmacokinetic study on the mechanism of tissue distribution of doxorubicin: interorgan and interspecies variation of tissue to plasma partition coefficients in rats, rabbits, and guinea pigs, J. Pharm. Sci. 73: 1359–1363

Tsuruo, T., Iida, M., Tsukagoshi, S., Sakurai, Y. (1982) Increased accumulation of vincristine and Adriamycin in drug-resistant tumor cells following incubation with calcium antagonists and calmodulin inhibitors, Cancer Res. 42: 4730–4733

Von Hoff, D. D., Layard, M. W., Basa, P. (1979) Risk factors for doxorubicin-induced congestive heart failure, Ann. Intern. Med. 91: 710–717

Young, R. C., Ozols, R. F., Myers, C. E. (1981) The anthracycline antineoplastic drugs, N. Engl. J. Med. 305: 139–153