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# Distribution of daunorubicin, a potent in vitro trypanocide which lacks in vivo activity, in the blood of trypanosome-infected mice

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Daunorubicin is a highly potent trypanocide in vitro but lacks significant in vivo activity. Distribution of the drug in the blood components and in trypanosomes from mice infected with *Trypanosoma rhodesiense* was therefore examined. Drug is accumulated in the trypanosomes in a similar manner to, but to a greater extent than, its accumulation in platelets; it is, however, taken up by erythrocytes to a much smaller extent. Drug concentrations in trypanosomes, platelets and erythrocytes declined with its decline in plasma whereas in white blood cells the drug was retained at a constant level. Daunorubicin concentrations in plasma were several orders of magnitude higher than the in vitro trypanocidal concentration. Fluorescence microscopy showed that although daunorubicin reaches the trypanosome nucleus, its concentration decreases rapidly so that adequate levels of drug may not be sustained in the bloodstream form of the organism for a sufficient length of time to give a trypanocidal effect in vivo.

There is currently an urgent need for new drugs to combat African trypanosomiasis not only in man but also in domestic animals where resistance to current drugs is widespread (Williamson 1970, 1975; Ormerod 1979). The antitumour drug daunorubicin has recently been shown to be a potent trypanocide (Williamson & Scott-Finnigan 1975). In in vitro tests, this drug at $1 \times 10^{-10}$ M, gives a permanent loss of infectivity in *Trypanosoma rhodesiense*, however, it shows no trypanocidal activity in infected mice at intraperitoneal doses up to 60 mg $kg^{-1}$ (Williamson & Scott-Finnigan 1978). This result is unexpected since work with mice suggested that the plasma concentration of the drug would be many orders of magnitude higher than that necessary to give a trypanocidal effect (Finkel et al 1969; Bachur et al 1970; DiFronzo et al 1971; Andersson et al 1979; Baurain et al 1979). To investigate this anomalous lack of in vivo trypanocidal activity, we have examined the distribution of daunorubicin and its metabolites in the plasma, blood cells and trypanosomes of infected mice.

## MATERIALS AND METHODS

### *Isolation of blood fractions from infected mice treated with daunorubicin*

Daunorubicin hydrochloride (25 mg $kg^{-1}$) was administered intraperitoneally to specific-pathogen-free Parkes mice infected two days previously with a monomorphic strain of *T. rhodesiense* (Yorke et al 1929). At the time of treatment, microscopic examination of coverslip preparations of tail blood showed 5–20 organisms per field (objective × 40). The mice had free access to food and water and one mouse was killed for each datum point (see Results and Discussion). Blood samples were obtained by dissection of the thoracic cavity and cardiac puncture with addition of citrate saline (1·5 ml). The citrated blood was centrifuged at 1750 *g* for 10 min, and the plasma decanted. The buffy coat trypanosome layer was removed and the erythrocytes collected. The buffy coat was fractionated by centrifugation on a sucrose density gradient (Williamson & Cover 1966). The upper platelet layer, the trypanosome layer, the erythrocyte layer and the white blood cell (granulocyte) pellet were recovered separately, the erythrocyte fraction being bulked with that obtained from the first centrifugation. The identity of each fraction was confirmed by microscopic examination and all samples were then frozen in liquid nitrogen until analysed.

### *Fluorescence microscopy*

Unfixed thin films of blood from treated infected mice were taken at the time of blood sampling. The films were examined with a Zeiss Standard 16 fluoresence microscope fitted with a IVFI epifluorescence condenser, green exciter filter BP546, FT580 chromatic splitter and LP590 red barrier filter, as

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used for selective rhodamine excitation. Comparable detection of daunorubicin fluorescence was also obtained with a fluorescein-type filter unit. Fluorescence was estimated semi-quantitatively by a subjective system (i.e. ±, +, ++).

*High performance liquid chromatography assay of daunorubicin in plasma and in blood cell samples*

The samples were rapidly thawed by immersion in water at 37 °C. In the case of plasma, 0·5 ml was pipetted into a silanized centrifuge tube. With cell samples, 1 ml of distilled water was added and the cells suspended by vortex mixing; 0·5 ml was then pipetted into a silanized centrifuge tube and sonicated for 15 min to disrupt the cells (confirmed by microscopic examination). Plasma and cell samples were then treated in an identical manner according to Brown et al (1981). First, 0·1 ml of 0·1 M $H_3PO_4$ and 0·4 ml $CH_3CN$ were added to precipitate protein. After mixing and centrifugation at 2200 *g* for 10 min, a 20 µl aliquot was injected on to a Lichrosorb RP-2 (5 µm) 250 × 46 mm i.d. reverse phase h.p.l.c. column and eluted isocratically with 35% $CH_3CN$ in 0·01 M $H_3PO_4$ at 1·0 ml $min^{-1}$. The eluate was monitored in a 25 µl flow cell using fluorimetric detection with the excitation monochromator at 475 nm (15 nm slit) and the emission monochromator at 557 nm (20 nm slit). The concentration of daunorubicin was determined by comparison with standards of daunorubicin hydrochloride in citrated human plasma (ranging from 20 to 600 ng $ml^{-1}$) treated in an identical manner. Samples of daunomycinone and daunorubicinol were prepared by acid hydrolysis (DiMarco et al 1964) and borohydride reduction (Jolles & Ponsinet 1977) respectively, and their retention times determined in order to allow identification of these known metabolites if present in the samples.

*Determination of protein in plasma and in blood cell samples*

Protein was determined by the Coomassie blue method (Bradford 1976) with bovine serum albumin as the protein standard in concentrations from 1 mg $ml^{-1}$ to 200 µg $ml^{-1}$. Each sample was diluted to give a reading on this linear region of the calibration curve, and assayed in triplicate. The daunorubicin concentration in each sample was expressed in terms of ng daunorubicin $mg^{-1}$ protein.

*Plasma binding of daunorubicin*

Solutions (4 ml) of daunorubicin in M/15 phosphate buffer (pH 7·4), ranging in concentration from 100 ng $ml^{-1}$ to 1 µg $ml^{-1}$, were dialysed against citrated human plasma (4 ml) for 3 h at 37 °C in a Dianorm equilibrium dialysis apparatus with Spectrapor 2 membrane (mol. wt. cut-off 12 000–14 000) at 20 rev $min^{-1}$. The plasma and the buffer samples were then assayed for daunorubicin by the h.p.l.c. method described above.

## RESULTS

*Availability of daunorubicin to trypanosomes*

Samples of citrated plasma from mice infected with *T. rhodesiense* and treated with daunorubicin were assayed for drug and the values corrected to allow for dilution of the plasma with citrated 0·9% NaCl. The results (Fig. 1) show that daunorubicin injected i.p. is rapidly absorbed, reaching its peak in plasma within 1 h. There is then a first order decay with a t½ (calculated on the 1–6 h period) of 2·4 h. The plasma peak from a 25 mg $kg^{-1}$ i.p. dose of daunorubicin, was found to be 6 × $10^{-7}$ M (350 ng $ml^{-1}$) declining

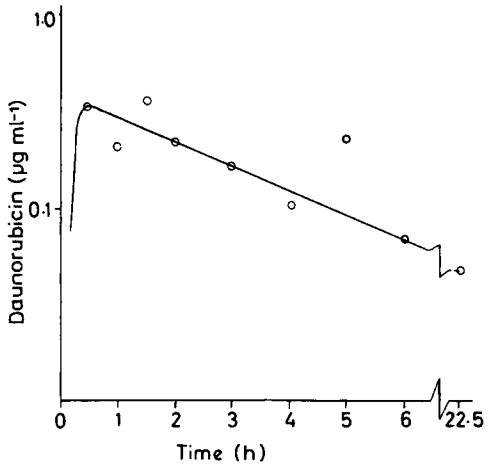


FIG. 1. Plot of log plasma concentration of daunorubicin vs time after i.p. injection of 25 mg $kg^{-1}$ daunorubicin into mice infected with a monomorphic strain of *T. rhodesiense*.

to 1 × $10^{-7}$ M after 23 h. This shows that the curve of log plasma drug concentration against time must be multiphasic, the initial phase (t½ = 2·4 h) being succeeded by one or more slower elimination phases. Levels of daunomycinone (the aglycone of daunorubicin) were generally low and erratic but significant levels of daunorubicinol (the 13-dihydro metabolite of daunorubicin) were present. In the initial period after drug administration, the levels were lower than those of daunorubicin but gradually increased such that from 4 h onwards the levels of daunorubicinol were approximately equivalent to,

but never exceeded, those of daunorubicin. Protein-binding studies of daunorubicin in human plasma at concentrations ranging from 100 ng $ml^{-1}$ to 1 μg $ml^{-1}$ showed that daunorubicin is 64% protein bound (s.d. = 7%, n = 20). Levels of free drug would thus be about 1/3 the total drug concentration. Since protein-bound drug is assumed to be unavailable to the organism, the free drug concentration can be regarded as the effective drug concentration.

*Uptake of daunorubicin into blood cells*

The blood cell pellet from each mouse was fractionated to yield principally erythrocyte, trypanosome and platelet fractions. A white blood cell fraction was also isolated but was found to be heterogeneous by microscopic analysis. The erythrocyte fraction consisted mainly of packed cells, hence the concentration of daunorubicin in erythrocytes can be directly compared with the concentration in plasma: it was found to be 60–90% of that in plasma showing that binding of drug in erythrocytes is lower than that in plasma. Other cell samples were then assayed but owing to the problem of obtaining cells free of contaminating fluid after density gradient centrifugation, the concentration of drug is expressed relative to the concentration of cellular protein. The values for erythrocytes, trypanosomes and platelets are given in Fig. 2. Both platelets and trypanosomes appear to concentrate drug to a significant extent.

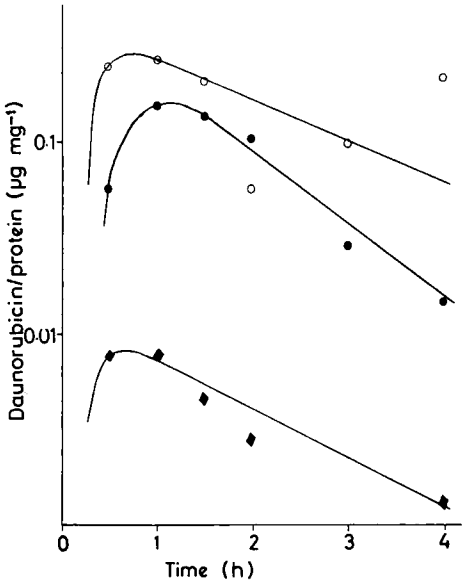


FIG. 2. Concentration of daunorubicin in blood cells after i.p. injection of 25 mg $kg^{-1}$ daunorubicin into mice infected with a monomorphic strain of *T. rhodesiense*. The symbols (○), (●) and (◆) represent, respectively, trypanosomes, platelets and erythrocytes.

In platelets, the levels of daunorubicinol were slightly lower than those of daunorubicin itself, but in trypanosomes and erythrocytes only low levels of this metabolite were found: the levels of aglycone were generally negligible.

Fluorescence microscopy showed that drug uptake into trypanosomes peaked at 2 h (with drug being increasingly concentrated in the cell nucleus) (Fig. 3). The level then decreased such that only slight fluorescence was detectable at 5 h. With white blood cells, however, the rate of uptake of drug was slower than that for trypanosomes, but once the peak was reached, it was maintained at a plateau for 23 h (the longest time monitored).

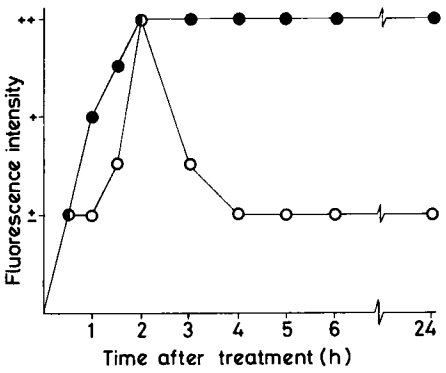


FIG. 3. Fluorescence detected in trypanosomes and white blood cells isolated from mice treated with daunorubicin (25 mg $kg^{-1}$). The symbols (○) and (●) represent trypanosomes and white blood cells respectively.

## DISCUSSION

To investigate the lack of activity of daunorubicin against trypanosomes in vivo it was first necessary to determine if adequate levels of drug are available to the parasite. After an i.p. dose of 25 mg $kg^{-1}$, the highest detected plasma concentration of daunorubicin in mice infected with *T.rhodesiense* was 600 nM, declining to 100 nM at 23 h (a rebound was noted at 5 h (see Fig. 1) in accord with previous studies of doxorubicin administered by the i.p. route (Shinozawa et al 1980)). Taking into account protein binding, the level of daunorubicin is above 35 nM for 23 h after i.p. injection of the drug, yet nanomolar concentrations have been shown to trypanocidal in vitro (Williamson & Scott-Finnigan 1978). Hence the plasma values found here are *adequate* in this respect and would be maintained above the nanomolar value for several days. From 4 h after injection onwards, the metabolite daunorubicinol is present at a similai

concentration to the parent drug. The effects of this compound on trypanosomes have not yet been reported: any activity would be additive to that expected from the daunorubicin thus exaggerating the anomalous lack of trypanocidal activity in vivo.

Since an adequate concentration of daunorubicin is present in the plasma surrounding the trypanosomes, it was next necessary to determine if drug was reaching the intracellular target within the organisms. Assay of daunorubicin in the trypanosome fraction from blood showed that drug is concentrated in a manner similar to (but greater than) its concentration in platelets, which is far greater than in erythrocytes (Fig. 2). The results with platelets, a 25-fold concentration over erythrocytes, is in line with the 20-fold concentration reported by Garattini (1978); similar results are reported for doxorubicin in rats (Broggini et al 1980). The time course of the drug in trypanosomes parallels that of drug in plasma.

In the blood cell analysis there is always some contamination with other cell types and so complementary studies by fluorescence microscopy were made. Drug was found to be taken up by the trypanosome but the fluorescence rapidly declined, in contrast to the white blood cells where uptake was slower but drug was retained in the cells. With trypanosomes, drug was found to concentrate in the cell nucleus, but only for a short period. Nevertheless, in the in vitro test for trypanocidal activity (Williamson & Scott-Finnigan 1978) parasites were exposed to drug for only 4 h, yet this exposure to nanomolar concentrations of drug leads to loss of infectivity. The lack of in vivo activity, when drug is present at 500 times this level for over 6 h, remains an anomaly. A similar effect has been noted where daunorubicin has lower antitumour activity in vivo than predicted on the basis of in vitro studies (Balconi et al 1973; Martin & McNally 1979). In the in vitro test for trypanocidal activity, the trypanosomes do not divide and are not truly typical of the forms in the blood stream; this is the most likely reason for the difference in activity of daunorubicin against the in vivo and in vitro forms. The bloodstream form may eliminate drug more effectively or repair DNA damage more efficiently than the static in vitro form. The drug may delay cell division rather than inhibit its initiation as occurs with the in vitro form (the in vitro test evaluates the infectivity of the organism after treatment with drug). Although drug reaches the cell nucleus of the blood-stream form, it remains there for only a short period. Increasing the length of retention of drug (e.g. by sustained release) might thus lead to an in vivo trypanocidal effect.

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