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Bai-Tang Lai · Jian-Ping Gao · Karl W. Lanks

Mechanism of action and spectrum of cell lines sensitive to a doxorubicin–transferrin conjugate

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Abstract A transferrin–doxorubicin conjugate exhibited greatly increased cytotoxicity relative to unconjugated doxorubicin toward a variety of cultured tumor cell lines. An L929 cell line selected for doxorubicin resistance was as sensitive to the transferrin–doxorubicin conjugate as was the parental unselected line. Quantitative measurements of doxorubicin fluorescence in single L929 cells showed that uptake was similar in amount when cells were exposed to equivalent concentrations of doxorubicin presented either free or as the transferrin–doxorubicin conjugate. However, unconjugated drug fluorescence was distributed in membranes, cytoplasm and nucleus, whereas conjugate fluorescence was confined mainly to the cytoplasmic compartment. In as much as NADPH-dependent free radical formation is a known mechanism of doxorubicin cytotoxicity, localization in the vicinity of NADPH production might facilitate this cytotoxic pathway. Neither cytotoxicity nor uptake of the conjugate quantified by doxorubicin fluorescence was significantly blocked by excess free transferrin, and the conjugate was not concentrated in the plasma membrane at 4 °C. These findings suggest that conjugate internalization is not entirely dependent on transferrin receptor binding.

B.-T. Lai · J.-P. Gao · K.W. Lanks (✉)
Department of Pathology and Laboratory Medicine,
Staten Island University Hospital,
475 Seaview Avenue, Staten Island,
N.Y. 10305 USA
Tel. +1 718-226-9367

K.W. Lanks
Department of Medicine,
Staten Island University Hospital,
475 Seaview Avenue, Staten Island,
N.Y. 10305, USA

B.-T. Lai
Department of Cell Biology,
Beijing Thoracic Tumor Institute,
Tongxian, Beijing 101149,
People's Republic of China

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Introduction

Although doxorubicin (DOX) is an effective and widely used cancer chemotherapeutic agent, cardiotoxicity and emergence of resistant tumor cell lines significantly limit its utility in clinical practice. Various approaches have been devised in order to circumvent these limitations including liposome encapsulation, photoactivation and coupling to various carriers such as monoclonal antitumor antibodies [1], albumin [2, 3] and transferrin (TRF) [4–7]. Carriers that exhibit tumor cell specificity can, in principal, be effective at concentrations that do not cause significant systemic toxicity.

Many elegant studies [4–6, 9, 10] have focussed on TRF-DOX conjugates because of the potential to target the conjugate to tumor cells that express TRF receptors. The available evidence has favored a model in which the conjugate is internalized after binding to TRF receptors leading to cytotoxicity at somewhat lower doses than are effective using the unconjugated drug [4]. Because of technical difficulties, much of this work employed conjugates that were labeled on the TRF moiety. However, recent advances in imaging technology make feasible visualization and quantification of the DOX itself. Therefore, we undertook a reexamination of the uptake and distribution of TRF-DOX conjugates in a cultured cell system with the specific objective of quantifying the DOX component.

Materials and methods

Doxorubicin–transferrin conjugate

This glutaraldehyde-linked adduct was prepared essentially as described by Berczi et al. [4] using TRF (98% iron-saturated, from Sigma, St. Louis, Mo.). DOX (Doxorubicin HCl for injection, USP, from Choron, Emeryville, Calif., glutaraldehyde (Ernest

F. Fullam, Latham, N.Y.), and ethanolamine (Eastman Kodak). Peak fractions from Sepharose CL-4B chromatography were pooled and the conjugate stoichiometry determined from the absorbancies of DOX and TRF at 488 nm and 280 nm, respectively. TRF which had been subjected to glutaraldehyde conjugation (TRF-G) and purification protocol in the absence of DOX was prepared in an identical manner. TRF conjugated to fluorescein (TRF-F) was obtained from Sigma.

Cells and culture conditions

The murine L929 cell line, a human breast adenocarcinoma cell line (MCF-7) and a human bladder transitional cell carcinoma cell line (RT-4) were used in the present study. All cell lines were obtained from MA Bioproducts (Bethesda, Md.) and were routinely maintained in high-glucose Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) and 10% newborn calf serum (L929) or 10% fetal calf serum (MCF-7 and RT-4). The DOX-resistant cell line L929/DOX was prepared by treating the L929 cell line with gradually increasing concentrations of DOX in vitro until the concentration of 50 μg/ml was attained. A resistant subclone was then treated once with 100 μg/ml DOX and the surviving resistant population used in subsequent experiments. The L929/DOX cells were maintained in DMEM medium supplemented with 10% fetal calf serum and lacking DOX. Drug efflux data (not shown) indicated that DOX was pumped out of the resistant cells much faster than out of the wildtype cells, consistent with a multidrug-resistant phenotype.

Cytotoxicity assays

Cells from the stock cultures were plated in 35-mm plastic tissue culture dishes at a density of 200 cells/dish 24 h in advance of treatment. DOX or TRF-DOX were added directly into the culture medium from concentrated stock solutions: 2 mg/ml DOX and 0.027 mg/ml TRF-DOX. After incubation for 3 h at 37 °C in an atmosphere containing 10% CO_2, the dishes were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) to remove extracellular drugs. Fresh medium was added and the dishes were incubated for determination of clonogenic survival as previously described [8]. All experiments were performed from two to four times with similar results. The clonogenic survival assay was routinely accurate to ±20%. IC_{50} was defined as the concentration of cytotoxic agent that inhibited clonogenic survival by 50%.

Fluorescence microscopy and image analysis

Cells were grown on 0.5 inch diameter microscope coverslips in 24-well tissue culture plates. DOX or TRF-DOX was added to the wells and incubated for 1–3 h at either 37 °C or 4 °C. The wells were then rinsed two times with PBS and fresh medium was added. After incubation for 2 h, the coverslips were extracted from the plates, rinsed with PBS, mounted on slides in Quantafluor mounting medium, and examined by epifluorescence using a Zeiss microscope equipped with a 63× immersion oil objective. The combination of G5465 excitation filter, FT580 dichroic mirror, and LP590 emission filter allowed efficient detection of DOX fluorescence at 590 nm. The fluorescence was detected by a CCD72S

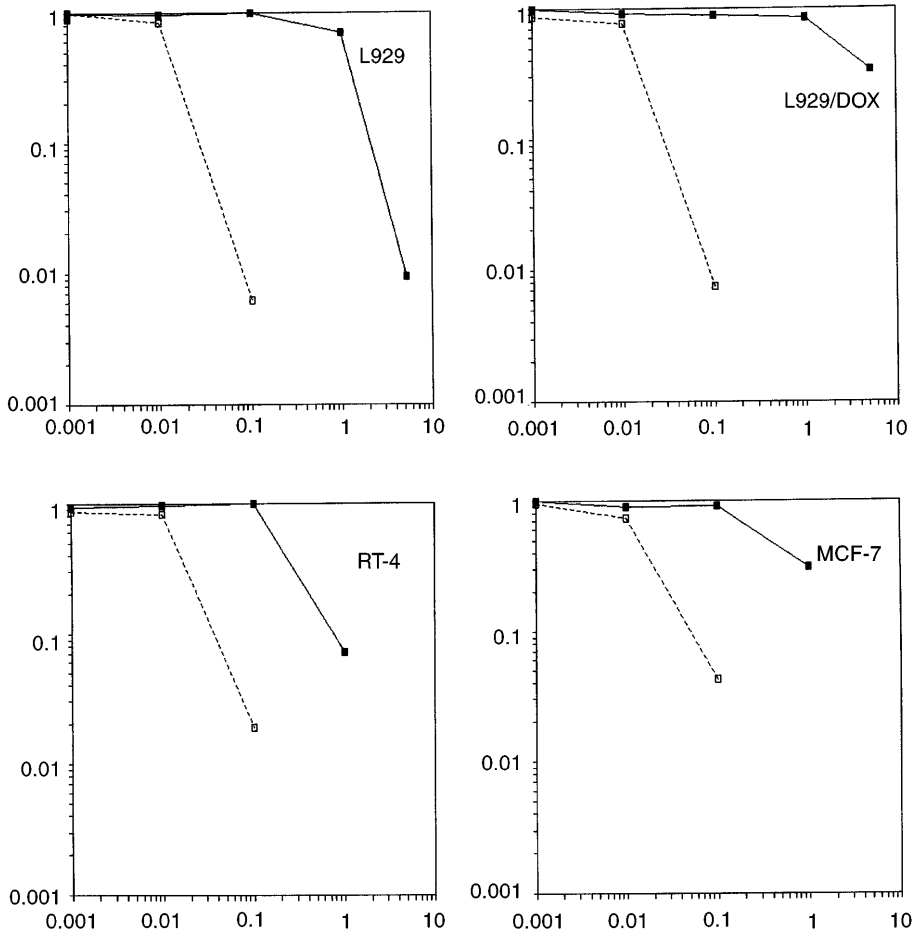

Fig. 1 Effect of DOX on clonogenic survival of cultured cell line. Low density cultures were exposed to free drug (*solid line*) or to conjugate (*dashed line*) containing equivalent drug concentrations for 3 h at 37 °C and treated as described in Methods. Graphs depict surviving fraction (*ordinate*) *vs.* μg/ml DOX as the free drug or in conjugate (*abscissa*).

camera (Dage-MTI, Michigan City, Ind.) and images were acquired and analyzed using OPTIMAS software (Optimas Corp., Seattle, Wash.). The fluorescence images were digitized on a 255-level gray scale which was linear with respect to fluorescence intensity and, therefore, proportional to the quantity of doxorubicin. Area (A) per fluorescent image, i.e. per cell, and mean gray per image (M_1) were measured for 23 to 75 cells. Mean gray for a background area containing no cell images (M_B) was also determined. Fluorescence units (FU) per cell were calculated as:

$$FU = \frac{\sum [(M_1 - M_B) \cdot A]}{\text{number of cells analyzed}}$$

Competition by TRF and TRF-G

The ability of excess TRF lacking any bound DOX to compete with the drug-containing complex was determined by simultaneously adding the desired concentration of TRF or TRF-G along with TRF-DOX. Clonogenic survival and subcellular distribution of DOX fluorescence were determined after periods of incubation ranging from 10 min to 3 h.

Results

The elution profile of monomolecular TRF-DOX conjugates was essentially as reported by Berczi et al. [4]. The DOX/TRF stoichiometry of conjugates used in the present experiments was 4:1.

The cytotoxicity of this conjugate toward four cultured cell lines was determined at low cell density in 35-mm culture dishes in order to ensure that uptake by the cells did not significantly deplete extracellular drug. It is readily apparent from Fig. 1 that the conjugate was more cytotoxic than DOX toward all of the cell lines. The IC_{50} of the conjugate relative to free DOX was reduced 57-, 21-, and 14-fold for L929, MCF-7 and RT4, respectively. The L929/DOX cell line, which had been selected for DOX resistance, was indeed resistant to the free drug. However, the IC_{50} for TRF-DOX was similar to that of the unselected L929 cells. As a result, the IC_{50} of the conjugate for these cells was 130-fold lower than that of free DOX.

The distribution of fluorescent DOX taken up either as the free drug or in conjugates is shown in Fig. 2. Under the conditions of this experiment (3 h, 37 °C), fluorescence of the free drug was located in cell membranes, cytoplasm, and nuclei while fluorescence of the conjugate was predominantly cytoplasmic. Regions of paranuclear concentration, possibly representing Golgi localization were present in both systems. The presence of nonfluorescent nuclear outlines in the conjugate-treated cells was interpreted as cytoplasmic rather than plasma membrane binding because the latter localization would result in relatively uniform fluorescence over the entire cell image or in peripheral concentration without a nuclear outline. The reduced nuclear localization of TRF-DOX in relation to DOX was confirmed by examining cells after the cytoplasm had been removed by nonionic detergent treatment. Distribution at concentrations producing equivalent cytotoxicity could not be determined because fluorescence was nearly undetectable at 10- and 100-fold lower concentrations of the conjugate.

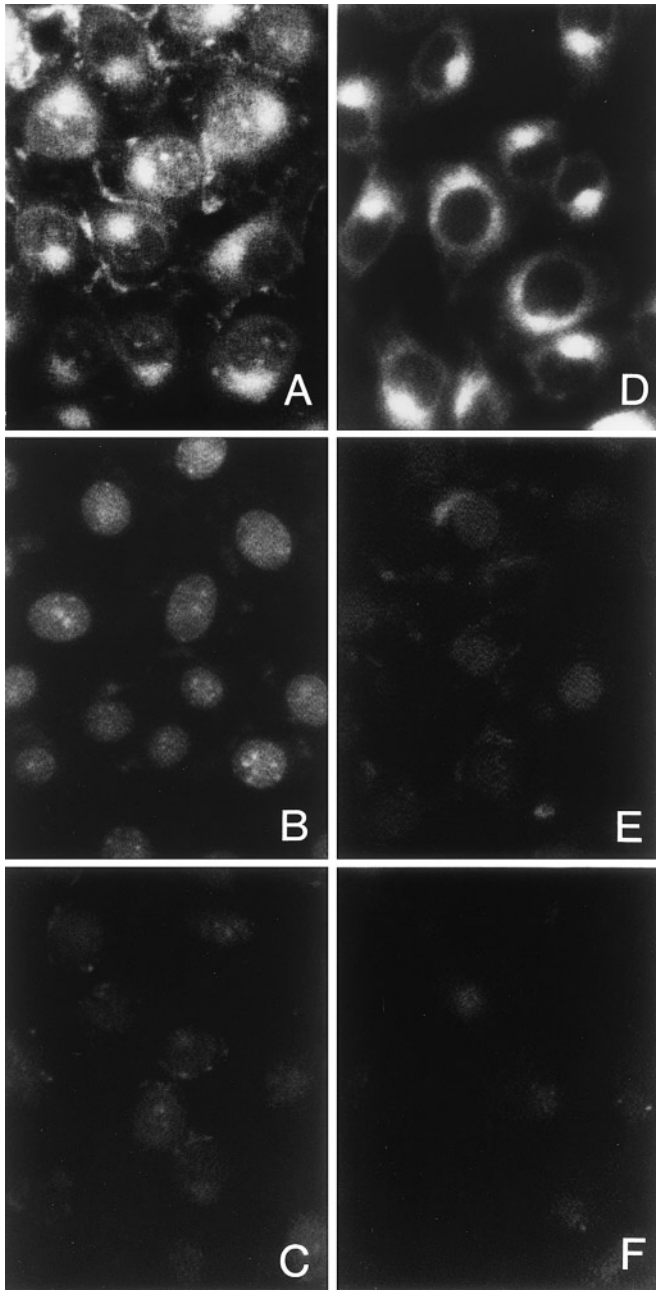

Fig. 2A–F Intracellular distribution of DOX fluorescence. L929 cells were exposed to 3 μg/ml DOX either as the free drug (**A**, **B**, **C**) or as TRF-DOX conjugate (**D**, **E**, **F**). Parallel cultures were exposed to 0.5% Triton X-100 for 1 min (**B**, **E**) or incubated for 2 h in drug-free medium (**C**, **F**) prior to examination by fluorescence microscopy and capture of digitized images

The results of quantitative measurements performed on such fluorescent images are shown in Table 1. Nuclear fluorescence is determined after detergent treatment to remove any overlying cytoplasmic or membrane-bound drug to yield a value that is proportional to nuclear drug uptake. Under the conditions used for the experiment shown in Figure 1, i.e. 3 h and 37 °C,

Table 1 Uptake of DOX and TRF-DOX conjugate by L929 cells after exposure to 3 μg/ml of drug for 3 h at 37 °C. Values are mean fluorescence units per cell

Treatment conditions	DOX	TRF-DOX
TRF-DOX or DOX (3 μg/ml)	77 ± 26	75 ± 37
+0.1% Triton X-100	23 ± 6.8	5.9 ± 2.9
+3 h drug-free medium	7.1 ± 4.7	3.1 ± 1.8

Table 2 Effect of TRF competition on uptake of TRF-DOX conjugate and L929 cell clonogenic survival (*FU* fluorescence units)

TRF-DOX exposure	FU per cell[a]	Surviving fraction[b]
1 h at 37 °C	200 ± 160	0.092
+10 mg/ml TRF	180 ± 110	0.023
1 h at 4 °C	21 ± 7.4	0.107
+10 mg/ml TRF	36 ± 16	0.065

[a] Fluorescence experiments employed conjugate containing 100 μg/ml TRF and 3 μg/ml DOX
[b] Survival experiments employed conjugate containing 1 μg/ml TRF and 0.03 μg/ml DOX

the total fluorescence did not depend on the form of administration. However, the subcellular distributions were clearly different such that the intranuclear concentration of TRF-DOX was nearly fourfold lower than that of unconjugated DOX. After a 3-h chase in drug-free medium, both TRF-DOX and unconjugated DOX were largely eliminated from both the nucleus and cytoplasm of L929 cells (Fig. 2C,F).

Two strategies were employed to determine whether uptake was mediated by TRF receptor binding and translocation. A large excess (100-fold) of either TRF or TRF-G was used to compete with and block binding to TRF receptors. The results with TRF showed that such competition experiments neither blocked TRF-DOX uptake nor diminished conjugate cytotoxicity (Fig. 3, Table 2). In fact, excess TRF significantly increased conjugate uptake and cytotoxicity in most experiments. Results with TRF-G were similar in all respects (data not shown). Experiments were also done with incubation times as short as 10 min to circumvent the possibility that, even though TRF-DOX internalization was greatly reduced by excess TRF, slow residual rates of both internalization and expulsion of the complex could lead to significant toxicity. Excess TRF did not reduce cytotoxicity of TRF-DOX in any of these experiments.

A low temperature (4 °C) was used in an attempt to block TRF-DOX uptake by inhibiting receptor translocation. Figure 2 shows that this treatment did reduce the uptake of both free DOX and TRF-DOX to nearly undetectable levels, did not appear to block TRF-DOX internalization as expected and did not lead to accumulation of noninternalized TRF-DOX-receptor complexes on the cell surface. Cytotoxicity was diminished at the low temperature, but was not further inhibited by excess TRF (Table 2).

Distribution of TRF-F was examined to determine whether the aberrant behavior of TRF-DOX was a consequence of the presence of the DOX ligand. Figure 4 shows that at 37 °C TRF-F displayed a punctate cytoplasmic distribution, possibly corresponding to localization in lysosomes or other vacuoles, which was quite different from that of TRF-DOX. The TRF-F conjugate was excluded from nuclei as was TRF-DOX. At 4 °C, a typical plasma membrane distribution was seen, as expected for receptor binding without internalization. While excess TRF did not appear to block TRF-F uptake at 37 °C, plasma membrane binding at 4 °C was greatly diminished. The image analysis system did not yield acceptable intensity measurements of these images.

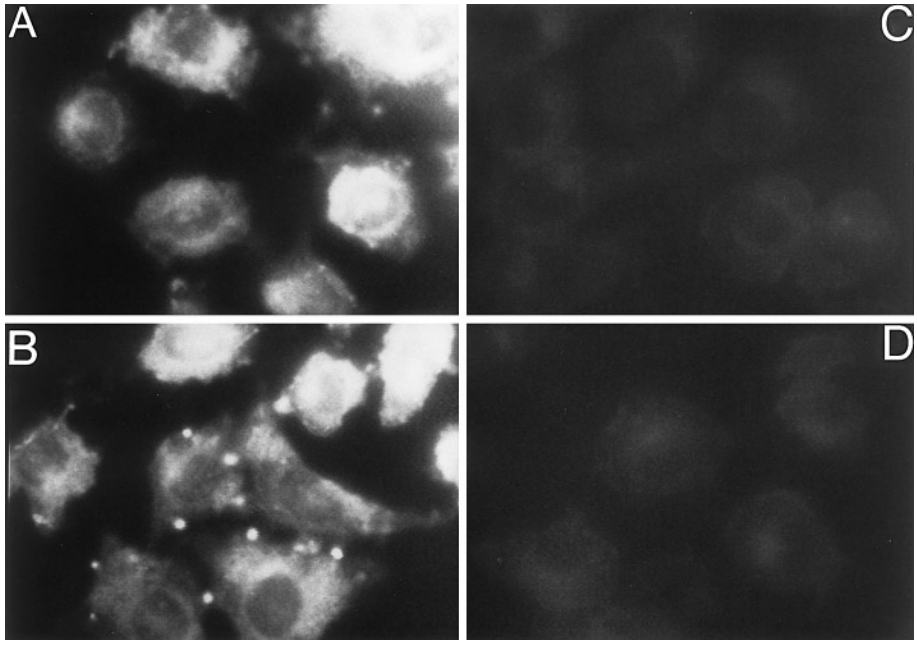

Fig. 3A–D Intracellular distribution of DOX fluorescence. L929 cells were exposed for 1 h to TRF-DOX (containing 3 μg/ml DOX and 100 μg/ml TRF) at 37 °C (**A**, **B**) or at 4 °C (**C**, **D**). Parallel cultures were simultaneously incubated with 10 mg/ml TRF (**B**, **D**) prior to examination by fluorescence microscopy and capture of digitized images

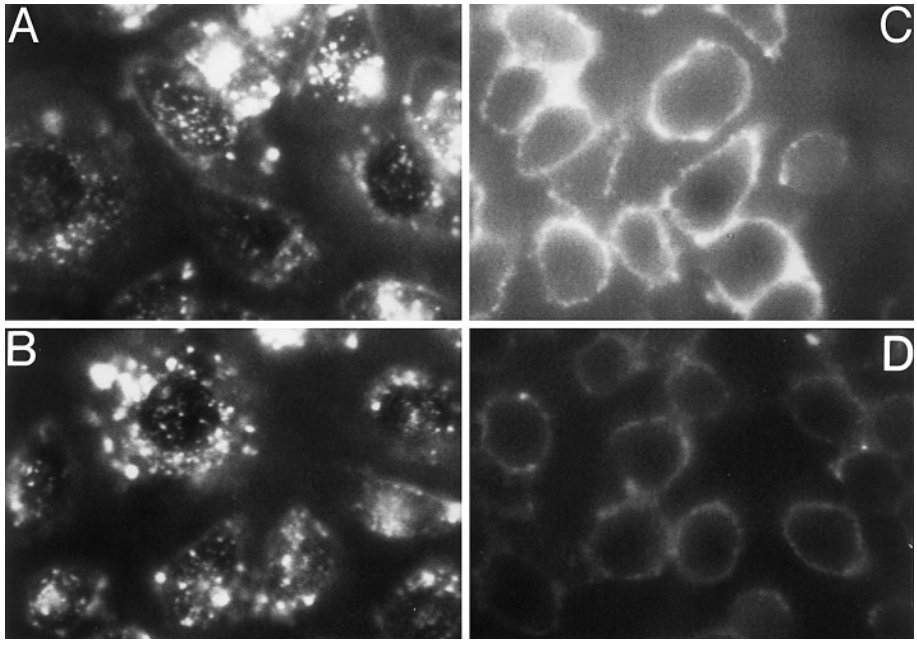

Fig. 4A–D Intracellular distribution of fluorescein fluorescence. L929 cells were exposed for 1 h to 100 μg/ml TRF-F at 37 °C (**A**, **B**) or at 4 °C (**C**, **D**). Parallel cultures were simultaneously incubated with 10 mg/ml TRF (**B**, **D**) prior to examination by fluorescence microscopy and capture of digitized images

Discussion

Chromatographic behavior of the TRF-DOX conjugate and the resulting TRF:DOX stoichiometry indicate that the preparative method of Berczi et al. [4] is readily reproducible. Similarly, the observations that cytotoxicity of the conjugate was greatly enhanced relative to the free drug, and that cells selected for DOX resistance were still as sensitive to the conjugate as the unselected parental line largely confirm previous observations. Although confirmatory of the results obtained by Fritzer et al. [5], the results are encouraging because entirely different methods were used to assess cell survival and drug uptake in the present experiments.

However, our data suggest different mechanisms of conjugate uptake and action from those previously proposed. Based on the ability of excess TRF to block uptake of [^{125}I]-TRF-DOX [4] or FITC-TRF-DOX [10], other workers have concluded that TRF-DOX binds to TRF receptors. This conclusion was reached even though excess TRF did not block TRF-DOX cytotoxicity [10]. In contrast, none of our experiments points to this uptake mechanism. Specifically, excess TRF did not block TRF-DOX uptake or cytotoxicity and low temperature did not result in plasma membrane localization of noninternalized TRF-DOX–receptor complexes.

We suggest that this discrepancy arises because we quantified DOX fluorescence while earlier studies followed the labeled TRF moiety. Since TRF lacking any conjugated DOX is almost certainly present in the TRF-DOX preparations, the distribution of labeled unconjugated TRF might not reflect the distribution of DOX which is conjugated to other labeled TRF molecules. Competition of excess TRF only with unconjugated labeled TRF would lead to the appearance of reduced binding, but unimpaired cytotoxicity observed by others [4, 10].

Our data also suggest that conjugation to TRF does not increase cytotoxicity by increasing uptake. At equivalent concentrations of DOX, either free or as TRF-DOX, similar amounts of drug were taken up and at equivalent cytotoxic doses intracellular TRF-DOX concentrations were below the limit of detection by fluorescence. Our findings are consistent with those of other workers with the exception of Hatano et al. [2] who used conjugates of serum albumin.

Although our data do not explain how TRF-DOX enters cells or whether the entry mechanism is different from that of free DOX, two findings may help to explain why TRF-DOX is more cytotoxic than the free drug. A predominantly cytoplasmic localization potentially exposes the conjugate bioreductive processes that are known to play an important role in DOX cytotoxicity [11]. Drug in an intranuclear location could still be cytotoxic, but not as readily by a mechanism that is dependent on reduction. Moreover, cytoplasmically located drug would generate short-lived reactive oxygen species in the vicinity of cytoplasmic membranes which are highly susceptible to free radical damage. Nuclear components could also be damaged by free radicals, but not as readily as the unsaturated lipids in cytoplasmic membranes.

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