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Report

Toxicity and DNA binding of dextran–doxorubicin conjugates in multidrug-resistant KB-V1 cells: optimization of dextran size

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We previously showed that conjugating doxorubicin to very large 70–500 kDa dextran decreased its removal rate from P-glycoprotein (P-gp) over-expressing, multidrug-resistant KB-V1 cells. Furthermore these conjugates could act synergistically with other cancer drugs. In the drug-sensitive 3-1 clone, but not in the V1 subclone which was 300-fold more resistant to free doxorubicin, conjugation led to a size-related decrease in toxicity. Here we identified the optimal size of dextran for avoiding P-gp-mediated efflux and yet preserving as much as possible doxorubicin toxicity. Chemically reduced, intracellularly stable 3.4–10 kDa conjugates were prepared. Confocal microscopy and fluorescence quenching experiments showed that these conjugates entered nuclei and interacted with DNA. In 3-1 cells, but not in V1 cells, cytotoxicity of conjugates decreased 14- to 45-fold linearly related to log size of the carrier (r=0.95). In V1 cells toxicity of the 10 kDa conjugate exceeded that of free doxorubicin. After conjugation the equilibrium binding constant of the DNA–drug complex (K_A) decreased only by up to 3-fold. In 3-1 cells, but not in V1 cells, DNA binding kinetics was an important factor and toxicity could be linearly correlated to $1/K_A$ of conjugate (r=0.94). Drug accumulation decreased with an increase in dextran size but drug removal was decreased only in V1 cells. It appeared that drug uptake was also sensitive to dextran conjugation. In V1 cells drug removal was sensitive to the P-gp inhibitor verapamil or energy starvation. Ratios of V1/3-1 toxicity, drug accumulation and drug removal correlated linearly with log dextran size. When these ratios equaled 1, dextran sizes were estimated to be 32, 103 and 21 kDa, respectively. [© 2000 Lippincott Williams & Wilkins.]

Key words: Dextran conjugates, DNA binding, doxorubicin, multidrug resistance.

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Introduction

Doxorubicin conjugated to 70 kDa dextran has been studied by Ueda *et al.*[1] who showed that although being 3-fold less toxic than free doxorubicin in acute tests, the conjugate had a higher activity against Walker carcinoma 256 in rats. The conjugate tends to achieve higher concentrations in plasma and tumor tissues than in the heart tissue.[2] It was clinically tested in human beings and encouraging results were obtained for the 70 kDa conjugate.[3]

Over-expression of the membrane 170 kDa P-glycoprotein (P-gp), thought to be an ATP-driven xenobiotic pump, is one of the best known mechanisms leading to multidrug resistance in cancer cells.[4] We have demonstrated that by conjugating doxorubicin to 70, 200 and 500 kDa dextran carriers, cellular drug removal could be decreased, particularly in P-gp over-expressing KB-V1 cells.[5] Although doxorubicin's toxicity towards the drug-sensitive KB-3-1 clone decreased proportionally up to 40-fold with an increase in the size of the dextran carrier, interestingly such correlation was not observed in the V1 subclone. For the largest 500 kDa conjugate tested, toxicity was comparable in both 3-1 and V1 cells. During acute toxicity tests, dextran conjugates did not affect the expression of P-gp mRNA or protein (Fong *et al.*, unpublished observation).

V1 cells remove intracellular xenobiotics such as rodamine-123, a substrate for P-gp, at a higher rate. However, in conjugate-loaded V1 cells the removal of such P-gp substrate decreases.[6] This may partially explain the observed synergism between these conjugates and some cancer drugs. In addition, energy starvation is synergistic with these conjugates in killing multidrug-resistant cells.[5] It is thought that energy starvation, by depleting cellular ATP, attenuates P-gp

activity and also releases free and conjugated doxorubicin from acidic organelles of which the pH gradient requires ATP to maintain.

We have previously considered and investigated three likely mechanisms by which dextran carrier might affect cytotoxicity, i.e. drug removal from cells, subcellular drug distribution and drug–DNA interaction.[5,6] V1 cells were found to be active in extruding free doxorubicin and were partially effective in extruding dextran conjugates smaller than 70 kDa.[5] It appeared that these cells had no added advantage in extruding 200 kDa conjugate, which has an effective diameter of 9 nm. These observations are consistent with the ATP-dependent pump theory since the P-gp is thought to posses a central pore estimated to be 5 nm in diameter.[7] It was also shown that in the first 1–2 h of exposure, both free and conjugated doxorubicin tended to concentrate in cytoplasmic vesicles.[5] After prolonged (hours) incubation, free doxorubicin could slowly appear in nuclei of drug-sensitive 3-1 cells. However, in P-gp over-expressing V1 cells nuclei were poorly labeled by free doxorubicin—presumably most of the free drug had been removed from cells. Conjugates larger than 200 kDa were too large for the nuclear pore and were excluded from nuclei.[5,8,9]

We have also considered the possible effect of altered DNA binding affinity. DNA interaction and the subsequent stabilization of topoisomerase II cleavage complex are thought to be the most important cell-killing mechanisms for doxorubicin.[10,11] Our previous studies involving large 70–500 kDa dextran carriers showed that their association (k_{ass}) and dissociation (k_{diss}) rate constants for DNA–conjugate complex were more than three orders of magnitudes smaller than that of free doxorubicin.[12] However, there was only a 10-fold decrease in the equilibrium constant (K_A). Furthermore, the K_A's for 70, 200 and 500 kDa conjugates are similar, and changes in K_A alone cannot fully explain the changes in cytotoxicity.

Thus it would appear that dextran carrier primarily alters drug uptake and removal. The effect on DNA binding kinetics is only of secondary importance. Here we attempted to answer the question whether there is an optimum size for the dextran carrier, particularly in the killing of P-gp over-expressing cancer cells.

In our previous studies involving dextran with sizes of 70, 200 and 500 kDa, conjugates were not chemically reduced, and doxorubicin may be decoupled from the dextran carrier under cell-free acidic conditions and also intracellularly.[5,6] In the present study, chemically reduced and intracellular stable conjugates of 3.4–10 kDa were investigated. From these studies it appeared that the minimum size of the dextran carrier for negating multidrug resistance due to P-gp over-expression is 32 kDa. The critical size of the dextran carrier for equalizing drug removal rates in 3-1 and V1 cells is 21 kDa; and for drug accumulation (net result of the combined effect of drug uptake and removal) the critical size is 103 kDa.

Materials and methods

Dextran–doxorubicin conjugates

Free drug was conjugated to dextran of various sizes (0.34, 0.5, 1.2, 3.6 and 10 kDa; American Polymer, Mentor, OH) through Schiff base formation as previously reported.[5,13] This linkage was unstable under acidic conditions and free doxorubicin may be released intracellualrly.[6] In the present study Schiff base was stabilized by reducing to the secondary amide bond with $NaCNBH_4$. Conjugates were purified by a Sephadex G10 column eluted with phosphate-buffered saline, pH 7.4. The coupling efficiency was estimated by absorbance at 480 nm (ε=11 500 M^{-1} cm^{-1}). Purity was assessed by thin layer chromatography using chloroform:methanol:water (13:6:1) as the mobile phase.

To check linkage stability, conjugates were incubated in 0.1 M phosphate buffer at pH 4 for 24 h. Free doxorubicin was separated by HPLC on a reversed-phase column (Beckman, Fullerton, CA; Ultrasphere ODS 5 μM 4.6 mm × 25 cm) under the following conditions: mobile phase 0.06 M KH_2PO_4 buffer, pH 4.0:acetonitrile 70:32.5, v/v; flow rate 1 ml/min; detection at excition 480 nm/emission 590 nm. Intracellular stability was assessed by incubating cells with reduced conjugates (containing 10 μM doxorubicin) overnight. Cells were lysed in 0.3 N HC1–50% methanol and free doxorubicin was extracted by $CHCI_3$:MeOH (4:1) and separated by HPLC as above. No free doxorubicin was detected.

Cell lines

The parental drug-sensitive 3-1 and the multidrug-resistant variant V1 cell lines were obtained from Dr Michael Gottesman (NIH, Bethesda, MD).[14] Cells were grown as monolayers in minimal essential medium (MEM-Eagle; Sigma, St Louis, MO) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), and 1% of antibiotic and antimycotic solution (Sigma; A-7292). V1 cells were maintained in the presence of 1 mg/ml of vinblastine. Cells were grown at 37°C in 5% CO_2/air. In some experiments P-gp activity was inhibited by 10 μM verapamil or by energy starvation (short incubation 1 g/l 2-deoxyglucose/10 mM sodium azide;

long incubation 0.2 g/l 2-deoxyglucose/0.2 mM sodium azide).

Cytotoxicity assay

Cell survival was estimated by the Sulforhodamine B (SRB) assay[15] which stained cellular protein. Color intensity at 515 nm can be positively correlated to the number of cells. Cells were incubated in 96-well microtiter plates at 5000 cells/well overnight and treated by drugs for 72 h. Cells were fixed by 20% trichloroacetic acid for 2 h at 4°C, rinsed with water and stained with 0.4% SRB in 1% acetic acid for 30 min. Unbound dye was removed by 1% acetic acid and the protein-bound dye was dissolved in unbuffered 10 mM Tris base and measured by a microtiter plate reader.

Fluorescence titration studies of DNA binding

Fluorescence intensity (excitation 480 nm; emission 590 nm) of mixtures containing 2 μM of free or conjugated doxorubicin and various concentrations of calf thymus DNA (ctDNA; Sigma type I) were obtained on an *f*MAX fluorescence microtiter plate reader (Molecular Devices, Eugene, OR) at ambient temperature. The equilibrium binding constants were obtained by Scatchard analysis[16] as outlined below.

The concentration of the free drug complex was determined using the equation:

$$C_F = C_T(I/I_0 - P)/(1 - P)$$

where C_T is the total concentration of the conjugate, C_F is the concentration of the free conjugate, and I and I_0 are fluorescence intensities in the presence and in the absence of DNA, respectively. P is the ratio of the fluorescence quantum yields of the bound to that of the free conjugate and was obtained from a plot of I/I_0 versus 1/[DNA]. The amount of bound conjugate (C_B) at any concentration was equal to $C_T - C_F$. A plot for r/C_F versus r, where r is equal to C_B/[DNA], was constructed and K_A determined according to McGhee and von Hippel.[17]

Cellular accumulation and removal of drugs

To study drug accumulation, 2×10^6 cells were incubated in 1 ml of Hank's balanced salt solution (HBSS; in g/l: $MgSO_4 \cdot 7H_2O$ 0.1, KC1 0.4, KH_2PO_4 0.06, NaCl 4, $NaHPO_4$ 0.046, $NaHCO_3$ 0.55, $CaCl_2$ 0.144, glucose 1, pH 7.4) containing 10 μM of a drug for 2 h at 37°C. Cells were washed twice with ice-cold 0.1 M phosphate buffer and collected by centrifugation at 1000 *g* for 1 min. Cellular free or conjugated doxorubicin was extracted with 0.3 N HCl:50% methanol and fluorescence measured by an *f*MAX fluorescence microplate reader (Molecular Devices) with excitation at 480 nm and emission at 590 nm.

Drug accumulation is a net result of the combined effect of drug uptake and drug removal. To study drug removal, cells were incubated with a drug for 1 h, then washed and incubated in drug-free medium for an additional 1 h. Cellular content of free and conjugated doxorubicin was extracted and measured as above. Drug removal was assessed by the fraction of drug retained inside cells.

Results

Subcellular distribution of reduced conjugates

Compared to non-reduced conjugates previously studied,[5] reduced conjugates are stable under acidic conditions and remained intact inside cells for hours (see Materials and methods).

Fluorescence microscopy showed that nuclei of both 3-1 and V1 cells were brightly labeled by dextran–doxorubicin conjugates (results not shown here), indicating these conjugates (0.34–10 kDa) can enter nuclei. Some conjugates are trapped in cytoplasmic vesicles. Conjugate–DNA interaction was confirmed by the quenching of doxorubicin fluorescence.[18] Fluorescence quenching was observed when these conjugates were added to whole cell suspensions (Figure 1). Since P-gp is ATP dependent, if cells were energy starved by glucose depletion and metabolic inhibition (simultaneous treatment by 2-deoxyglucose and Na-azide), P-gp activity could be attenuated.[5] Indeed further fluorescence quenching was observed in energy starved cells, indicating an increase of the nuclear presence of these drugs. Since no free doxorubicin is released from these reduced conjugates, fluorescence quenching of conjugates by intact cells implies conjugates are moving into nuclei and making contact with chromatin materials.

Cytotoxicity of conjugates

These results are shown in Table 1. Cytotoxicity was assessed by the median toxic dose (D_m, equivalent to LD_{50}). Drug dose in these experiments referred to the final amount of doxorubicin present, disregarding the amount of the dextran carrier, and was calculated based on the contents of doxorubicin per dextran molecule (Table 1). In drug-sensitive 3-1 cells, cytotoxicity of conjugates decreased 14- to 45-fold

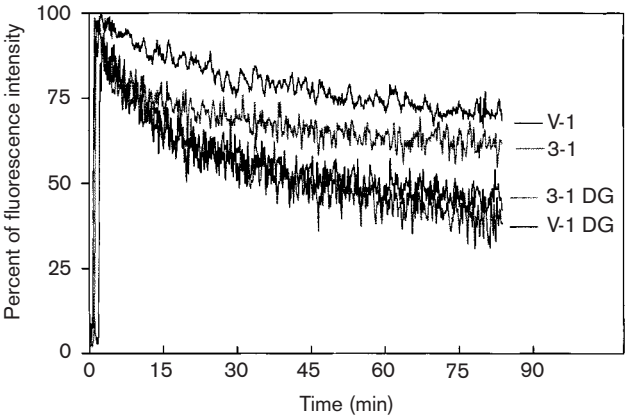

Figure 1. Quenching of dextran-conjugated doxorubicin fluorescence in cell suspensions. Doxorubicin was conjugated to 10 kDa dextran and was added to 3-1 or V1 cells to make 2 ml suspensions each containing 2×10^6 cells and 2 μM doxorubicin. Increased quenching was observed in energy starved (DG) cells.

Table 1. Cytotoxicity of free and 0.34–10 kDa dextran-conjugated doxorubicin in 3-1 and multidrug-resistant V1 cells

Dextran conjugates	N[a]	D_m[b] (μM)		Relative D_m (V1/3-1)
		3-1	V1	
Free doxorubicin	–	0.06 ± 0.02	20.8 ± 2.2	359
0.34 kDa	1.3	0.83 ± 0.18[c]	163.8 ± 8.8[c]	196
0.5 kDa	1.4	0.57 ± 0.04[c]	43.6 ± 8.3[c]	77
1.2 kDa	2.5	0.61 ± 0.05[c]	23.7 ± 4.1	39
33.6 kDa	2.9	1.81 ± 0.17[c]	26.5 ± 2.1	14.5
10 kDa	3	2.58 ± 0.24[c]	11.2 ± 1.4[c]	4.3

[a]Number of doxorubicin molecules per dextran molecule.
[b]Median dose values (D_m) were determined according to Chou and Talalay[5,6,28] and are equivalent to LD_{50}. For conjugates D_m's are shown in terms of doxorubicin contents. Values shown are means ± SEM.

compared to free doxorubicin and was linearly related to log size of the dextran carrier (r=0.95).

On the contrary, in the P-gp over-expressing V1 cell line, which is 300-fold more resistant to free doxorubicin, cytotoxicity of conjugates slightly increased with an increased size of dextran carriers. With the 10 kDa dextran carrier, the conjugate was more toxic than free doxorubicin. The relative toxicity (D_m ratio of V1 versus 3-1 cells) reduced from 359 for free doxorubicin to 4 for the 10 kDa conjugate.

Equilibrium binding constants, K_A, of drug–DNA complexes

The K_A values of conjugate–DNA binding in solution were determined by the fluorescence titration method.[19] As shown in Figure 2 (inset), the emission intensity of the 10 kDa conjugate decreased as the concentration of ctDNA increased. K_A's of conjugate–DNA complexes were obtained by Scatchard analyses[16] and results for the 10 kDa conjugate are shown in Figure 2 as an example. Results for conjugates of various sizes are summarized in Table 2.

Table 2. Intrinsic binding constants of DNA–conjugate complexes for 3.4–10 kDa doxorubicin conjugates determined by fluorescence titration experiments

Conjugates[a]	K_A (M^{-1})[b]
Free doxorubicin	$7.91 \pm 0.21 \times 10^5$
0.34 kDa	$7.01 \pm 1.25 \times 10^5$
0.5 kDa	$4.90 \pm 1.84 \times 10^5$
1.2 kDa	$4.65 \pm 1.24 \times 10^5$
3.6 kDa	$2.83 \pm 0.36 \times 10^5$
10 kDa	$2.47 \pm 0.09 \times 10^5$

[a]Free doxorubicin or doxorubicin conjugated to dextran carriers with sizes shown.
[b]K_A: intrinsic binding constant.

In cell-free mixtures $1/K_A$ is linearly proportional to log size (in kDa) of conjugates (r=0.97) (Figure 3, left panel). In drug-sensitive 3-1 cells D_m's of conjugates are also closely correlated to $1/K_A$ (r=0.94) (Figure 3, right panel). However, such correlation could not be observed in drug-resistant V1 cells.

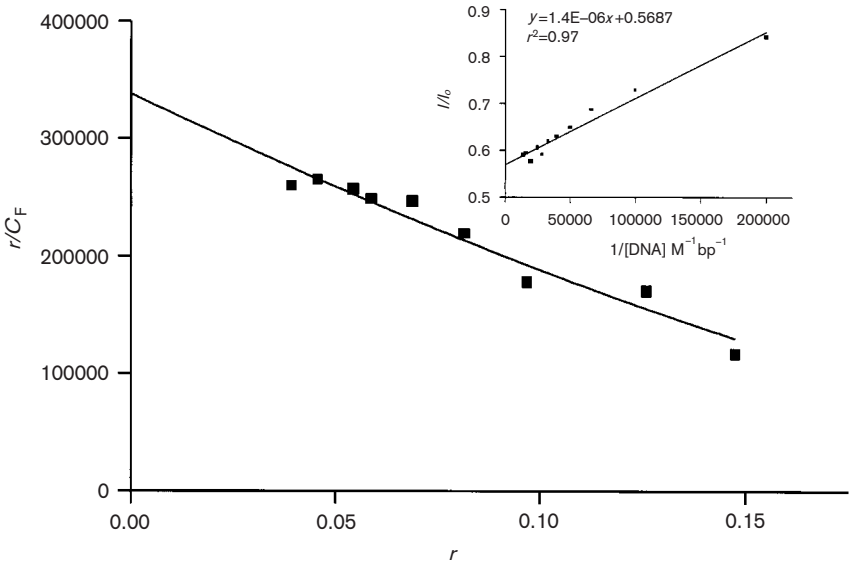

Figure 2. Scatchard analysis of conjugate–DNA binding. Inset shows changes in fluorescence emission of mixtures containing 10 kDa conjugate (containing 2 μM doxorubicin) and various amount of ctDNA. Scatchard analysis was performed as described in the text.

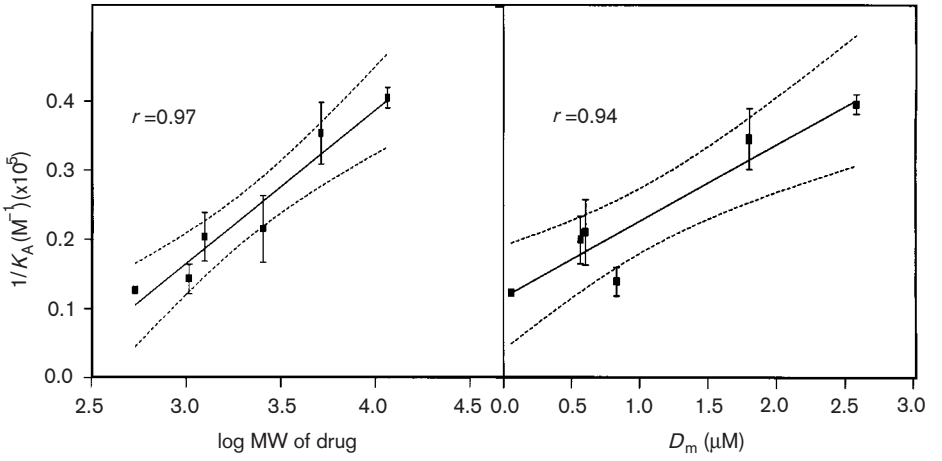

Figure 3. Correlation analysis between K_A and the size of dextran carrier (left panel), and between K_A and toxicity (D_m) (right panel).

Drug accumulation, removal and cytotoxicity

Drug accumulation is defined as the total amount of a drug found associated with cells after a period of incubation in drug-containing medium, and is a net result of the combined effect of drug uptake and drug removal. The removal rate of intracellular drug when drug-loaded cells are incubated in drug-free medium is assessed by the fraction of drug retained ('fractional retention'). These results are summarized in Table 3.

In drug-sensitive 3-1 cells, drug accumulation decreased with an increase of dextran size (r=0.92). However, the relationship between the size of the dextran carrier and drug removal was not so clear (r=0.85). It seems that in drug-sensitive cells uptake of conjugates is much more sensitive to the increased size of the dextran carrier than drug removal is. In this drug-sensitive cell line drug accumulation and removal had little correlation with cytotoxicity (r=0.74 and 0.69, respectively).

Very different results, however, were obtained in P-gp over-expressing V1 cells. Drug removal was much decreased with an increased dextran size. Drug

Table 3. Accumulation and removal of free and dextran-conjugated doxorubicin (0.34–10 kDa) in 3-1 and V1 cells

Size of dextran	Accumulation of Drug (10^{-10} mol/2×10^6 cells)						Fraction retained					
	3-1	3-1/VPL	3-1/DG	V1	V1-VPL	V1/DG	3-1	3-1/VPL	3-1/DG	V1	V1/VPL	V1/DG
Free	66.9 ± 3.7	67.0 ± 6.0	71.6 ± 8.6	44.4 ± 6.2[a]	58.3 ± 3.6[b]	78.3 ± 5.5[b]	0.53 ± 0.05	0.55 ± 0.04	0.63 ± 0.06	0.25 ± 0.05[a]	0.43 ± 0.06[a,b]	0.59 ± 0.05[b]
0.34 kDa	62.2 ± 3.5	59.9 ± 5.7	56.5 ± 7.3	40.1 ± 2.4[a]	67.7 ± 3.7[b]	54.4 ± 3.2[b]	0.52 ± 0.05	0.54 ± 0.05	0.68 ± 0.05	0.28 ± 0.05[a]	0.40 ± 0.06[a,b]	0.56 ± 0.04[b]
0.5 kDa	56.3 ± 3.5[c]	52.1 ± 3.1	61.4 ± 8.6	37.0 ± 3.7[a]	55.7 ± 4.0[b]	53.4 ± 8.5[b]	0.58 ± 0.06	0.68 ± 0.05	0.71 ± 0.05	0.38 ± 0.05[a,c]	0.49 ± 0.05[a,b]	0.74 ± 0.05[b]
1.2 kDa	41.9 ± 4.6[c]	37.7 ± 4.2	44.4 ± 5.3	31.4 ± 3.8[a,c]	32.2 ± 1.7	40.1 ± 2.3[b]	0.60 ± 0.06	0.55 ± 0.06	0.70 ± 0.06	0.46 ± 0.04[a,c]	0.46 ± 0.07	0.66 ± 0.06[b]
3.6 kDa	43.7 ± 5.9[c]	40.8 ± 2.7	41.1 ± 4.5	31.3 ± 4.4[a,c]	41.7 ± 2.5[b]	34.3 ± 4.5	0.58 ± 0.05	0.75 ± 0.05[b]	0.67 ± 0.05	0.43 ± 0.04[a,c]	0.49 ± 0.05[a]	0.78 ± 0.06[b]
10 kDa	39.4 ± 5.7[c]	41.9 ± 2.3	40.4 ± 5.7	32.5 ± 5.5[c]	36.4 ± 1.8	40.0 ± 3.0	0.63 ± 0.06	0.80 ± 0.05	0.80 ± 0.04	0.54 ± 0.06[c]	0.61 ± 0.05	0.61 ± 0.06

Drug accumulation was measured in cells incubated for 2 h with 10 μM free doxorubicin or with conjugates containing equivalent amounts of doxorubicin. Fraction retained was fraction of doxorubicin remaining associated with cells after an additional 1 h of incubation in drug-free medium. To inhibit P-gp activity some cells were treated with 10 μM verapamil (VPL) or energy starved (DG).

[a]Significantly different ($p < 0.05$) from 3-1 cells that received the same treatment.

[b]Significantly different ($p < 0.05$) from the same cells of which P-gp was not inhibited.

[c]Significantly different ($p < 0.05$) from free doxorubicin.

removal could be further attenuated by the P-gp inhibitor verapamil or energy starvation, which had relatively little effect on drug-sensitive 3-1 cells.

Discussion

Factors affecting cytotoxicity

Dextran conjugation affected drug accumulation (a net result of the combined effects of drug uptake and removal) and kinetics of drug–DNA binding. In both drug-sensitive and -resistant KB cells dextran conjugation decreased doxorubicin accumulation despite that the removal rates of these small (0.34–10 kDa) conjugates were either unchanged (in 3-1 cells) or decreased (in V1 cells) (Table 3). Our data imply that uptake, presumably through endocytosis, is more affected by macromolecular carriers than drug extrusion.

In drug-sensitive 3-1 cells the major determining factor of toxicity of conjugates of various sizes is not drug accumulation but DNA binding kinetics. For this group of small conjugates, a positive correlation between drug accumulation and cytotoxicity (D_m) could not be established (r=0.74). However, it appeared that D_m could be correlated to K_A's of drug–DNA complexes (r=0.94).

Previous reports have shown that poly-intercalators of DNA have higher K_A values than those of mono-intercalators.[20–23] In our study, however, the K_A of conjugates decreased with an increase in the size of the dextran carrier. One plausible explanation is that in our studies doxorubicin contents were not strictly proportional to dextran sizes. Steric hindrance might also play a role since doxorubicin was directly linked to dextran. We are now considering adding a long linker between doxorubicin and the dextran carrier.

Plausible mode of action of P-gp

Compared to drug-sensitive 3-1 cells, drug-resistant V1 cells remove more free doxorubicin and conjugates smaller than 3.6 kDa (Table 3). This is probably due to the activity of P-gp since removal of these molecules could be inhibited by verapamil and energy starvation. However, the accumulation of the 10 kDa conjugate was similar in the drug-sensitive and -resistant cells, indicating that conjugates larger than 10 kDa are not good substrates for P-gp. Furthermore, verapamil and energy starvation did not affect the removal of the 10 kDa conjugate in either cell lines.

Various models have been proposed for the action of P-gp. P-gp has been labeled as an efflux pump, a hydrophobic vacuum cleaner or a flippase.[24–26] A

proposed three-dimensional structure derived from the amino acid sequence of P-gp revealed a channel structure with a central pore of 5 nm.[7] The effective sizes of 10–150 kDa dextran, estimated from fluorescence isothiocyanate-labeled dextran, range from 5.66 to 18.14 nm.[27] Our data on conjugate removal seem to agree with this channel model.

Is there an optimum size for the dextran carrier?

One way to interpret the relevance of dextran size in overcoming multidrug resistance is to look at the relative D_m of drug-resistance and -sensitive cells. For free doxorubicin this value is 359 and for the 10 kDa conjugate this value is 4.3 (Table 1). When relative (V1/3-1) D_m was plotted against log conjugate size, a linear relationship can be demonstrated (r=0.99) (Figure 4, top panel). When the relative D_m is unity, i.e. when multidrug resistant cells lose their survival advantage over drug-sensitive cells, Y=0 and the size of the dextran carrier can be calculated to be 32 kDa. Unfortunately at this large size the toxicity of the conjugate was also decreased by several hundred fold. One might consider various means to increase the toxicity of the conjugate. On possibility is to increase the number of doxorubicins linked per unit length of dextran.

Using this method it can be calculated that the size of the dextran carrier which could completely equalize

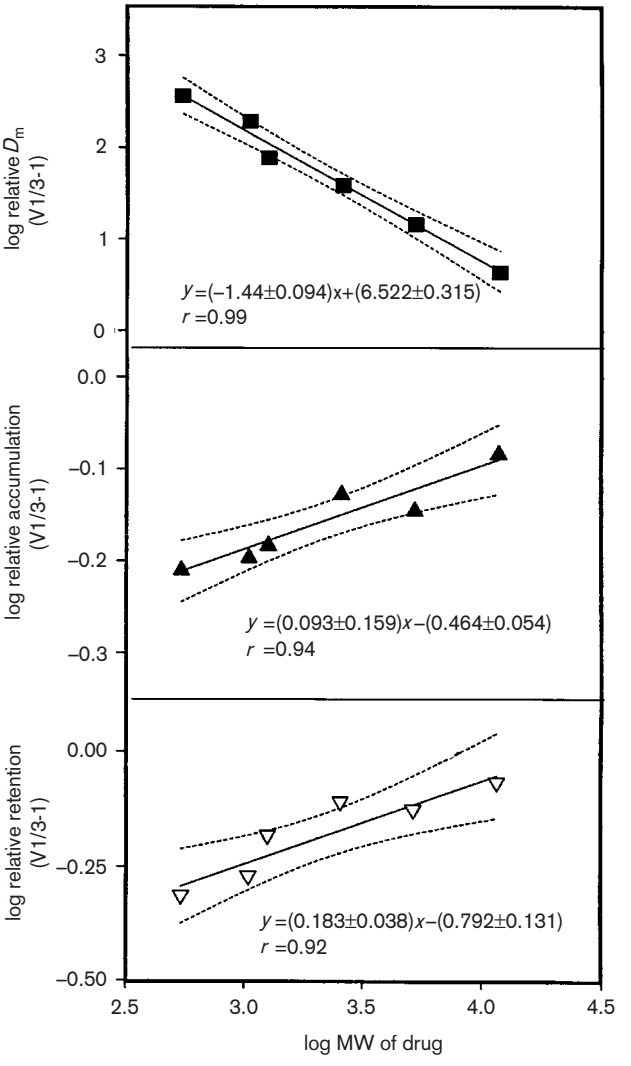

Figure 4. Regression analyses of influences of carrier size on relative (V1/3-1 cells) toxicity (D_m), drug accumulaton and drug retention. Areas between dotted lines represent 95% confidence intervals.

drug accumulation (a net result of the combined effects of drug uptake and removal) in drug-sensitive and -resistant KB cells is 103 kDa (Figure 4, middle panel). For drug removal this critical size is 21 kDa (Figure 4, bottom panel).

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