Anti-Cancer Drugs 1999, **10**, pp. 171–178

Research paper

Synergism of energy starvation and dextran-conjugated doxorubicin in the killing of multidrug-resistant KB carcinoma cells

Wing Lam, Hingleung Chan, Mengsu Yang, Shukhan Cheng and Wangfun Fong
Bio-active Product Research Group, Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong.

Here we report that 2-deoxyglucose/Na azide treatment and free/conjugated doxorubicin are synergistic in cell killing. As demonstrated by fluorescence confocal microscopy, KB-V1 cells retained more conjugated doxorubicin than free doxorubicin. Verapamil or 2-deoxyglucose/Na azide enhanced only the retention of the free drug and the small (<70 kDa) conjugates, indicating that P-glycoprotein (P-gp) is not effective against large conjugates. Conjugated doxorubicin was excluded from nuclei. Initially both free and conjugated doxorubicin accumulated in cytoplasmic organelles. Upon 2-deoxyglucose/Na azide treatment, fluorescence labeling of organelles dissipated. Prolonged (24 h) incubation of conjugate-preloaded cells resulted in redistribution of some of the organelle-associated fluorescence to nuclei, suggesting decoupling. The appearance of free doxorubicin was confirmed by capillary electrophoresis. 2-Deoxyglucose/Na azide treatment also retarded decoupling. Our results suggest that energy starvation, in addition to increasing cellular retention of P-gp substrates, may affect cellular fate of conjugated drugs with a possible enhancing effect in cancer cell killing. [© 1999 Lippincott Williams & Wilkins.]

Key words: Delayed decoupling, dextran-conjugated doxorubicin, multidrug resistance, 2-deoxyglucose/Na azide.

Introduction

The anthracycline antibiotic doxorubicin (adriamycin) is a wide spectrum anti-cancer drug,[1] but its use has been restricted by its *in vivo* cardiotoxicity[2] and drug resistance.[3] There have been many attempts to improve the action of doxorubicin by linking it to macromolecules, such as transferrin,[4,5] dextran,[6] antibodies,[7] antibodies through dextran,[8] microspheres[9,10] and polymers.[11] These modifications have led to various beneficial effects. A number of doxorubicin derivatives, such as annamycin, daunorubicin, 8-(*s*)-fluoroidarubicin, idarubicin[12–14] or doxorubicin conjugated to transferrin,[5] human serum albumin[15] and bovine serum albumin[16] have been studied for their ability to reverse the multidrug resistance (MDR) phenotype with varying results.

In previous studies[17] we showed that in KB-3-1 cells cytotoxicity of doxorubicin decreases 15- to 40-fold after conjugation to dextran of various sizes. In the multidrug-resistant KB-V1 sub-line, which is over 300-fold more resistant to doxorubicin, dextran conjugation results in a relative little decrease in cytotoxicity. We have also shown that dextran-conjugated doxorubicin can interact with DNA *in vitro*. Both the association and dissociation rate constants for the DNA-conjugates are more than three magnitudes smaller than those for free doxorubicin. On the other hand, the equilibrium constants of the conjugate-DNA complexes are only about 10 times smaller than that of the free-doxorubicin-DNA complex. Furthermore, we showed that in multidrug-resistant KB-V1 cells, the dextran-conjugated doxorubicin shows partial synergism with free doxorubicin and colchicine.[18]

The issue of energy starvation in the killing of multidrug-resistant cells has attracted much attention. Some multidrug-resistant cells, such as doxorubicin-resistant Ehrlich ascites cells,[19] exhibit an elevated ATP production rate. There are also reports that glucose deprivation may induce cytotoxicity in a number of drug-resistant cells, including KB cells.[20,21] Here we show that 2-deoxyglucose/Na azide treatment is synergistic with doxorubicin,

Correspondence to WF Fong, Bio-active Product Research Group, Department of Biology and Chemistry, City University, Tat Chee Avenue, Kowloon, Hong Kong.
Tel: (+852) 2788-9789; Fax: +852) 2788-7406;
E-mail: bhwffong@cityu.edu.hk

vinblastine and colchicine, as well as dextran-conjugated doxorubicin. To determine the mechanism of this synergism, we investigated the effects of 2-deoxyglucose/Na azide treatment on the cellular fate of dextran-conjugated doxorubicin in terms of accumulation, retention, redistribution and decoupling. It is suggested that in addition to increasing drug retention, other mechanisms, such as delayed decoupling, may be involved in the action of energy starvation in enhancing the cell killing activity of conjugated doxorubicin.

Materials and methods

Cell lines

The parental KB-3-1 cell line and the multidrug-resistant variant KB-V1 cell line, obtained by step selections for vinblastine resistance, were generously provided by Dr Michael Gottesman (NIH, Bethesda).[22] Cells were grown as monolayers in minimal essential medium (MEM-Eagle; Sigma, St Louis, MO) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY), and 1% of antibiotic and antimycotic solution (A-7292; Sigma). KB-V1 cells were maintained in the presence of 1 mg/ml of vinblastine. Cells were grown at 37°C in 5%CO_2/air. P-glycoprotein activity was inhibited by 10 μM verapamil or energy starvation (high dose for short incubation, 1 g/l 2-deoxyglucose/10 mM Na azide; low dose for long incubation, 0.2 g/l 2-deoxyglucose/0.2 mM Na azide).

Dextran–doxorubicin conjugates

Free drug was conjugated to dextran of various molecular sizes (200–275 kDa, BDH, Dorset, UK; 70 and 500 kDa, Pharmacia, Uppsala, Sweden) through Schiff base covalent bond formation as previously reported,[18] except that the dextran-doxorubicin conjugates were precipitated in 4 volumes of acetonitrile. Products were collected by centrifugation (4000 *g* for 15 min) and were extensively dialyzed against double-distilled water. The coupling efficiency was estimated by absorbance at 480 nm ($\varepsilon = 11500\ M^{-1}\ cm^{-1}$). The purity of dextran-doxorubicin conjugates was checked by thin layer chromatography using chloroform:methanol:water (13:6:1) as the mobile phase. The w/w ratio of doxorubicin/dextran for the 70, 200 and 500 kDa dextran conjugates was 2.3, 3.4 and 2.4%, respectively. The conjugates were stable at pH 7.2.

Cytotoxicity

Cytotoxicity was determined by the sulforhodamine B (SRB) method.[23] Briefly, cells were incubated in 96-well microtiter plate at 5000 cells/well overnight. Drug treatment lasted for 72 h and cells were fixed by 20% trichloroacetic acid for 2 h at 4°C. Cells were rinsed with water and then 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 un-buffered 10 mM Tris base. Cell number was estimated by correlating to OD at 515 nm.

Determination of combination indices (CI)

First, the median dose, or D_m (LD_{50}) of each treatment was determined according to the median-effect plot:

$$\log(\text{fraction}_{\text{affected}}/\text{fraction}_{\text{unaffected}}) = \log(\text{dose}/\text{dose}_{\text{median}})^m$$

The combined effect of two chemical treatments was studied with mixtures containing varying total amounts but a constant concentration ratio, which was similar to the ratio of the respective LD_{50}s, of the two treatments. CI were calculated at various killing rates (fraction affected, f_a) according to Chou and Talalay:[24,25]

$$CI = (D_{comb})_1/(D_{alone})_1 + (D_{comb})_2/(D_{alone})_2 + (D_{comb})_3/(D_{alone})_3$$

Where $(D_{alone})_n$ is the dose of drug n alone required to achieve a certain cell killing rate (f_a) and $(D_{comb})_n$ is the dose of drug n in the mixture to achieve a certain cell killing rate (f_a).

A CI greater than 1 indicates antagonism and smaller than 1 indicates synergism. In the case of an additive effect the CI equals 1. All experiments were repeated 4 times. All the CI curves or D_m were analyzed by two-way ANOVA (GraphPad Prism 1994) or *t*-test.

ATP assay

Cellular ATP content was measured by ATP assay kit (Calbiochem, San Diego, CA) according to the procedure provided by the manufacturer.

Drug accumulation and retention of pre-loaded drugs

To study drug accumulation, cells were grown on 22 mm × 0.13–0.17 mm coverslips and were washed

twice by 1 ml Hank's balance salt solution (HBSS: in g/l $MgSO_4.7H_2O$ 0.1, KCl 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). Cells were then incubated in HBSS containing 10 μM of a drug for 2 h, washed 3 times with 1 ml of 0.1 M phosphate buffer, pH 7.4, dried under an electric fan for 1 h, and studied by fluorescence microscopy.

To study drug retention, cells were preloaded with a drug as above for 1 h, washed and incubated drug-free for an additional 1 h, then washed and dried.

For measuring cellular fluorescence intensity, 30 cells were randomly selected and their fluorescence was measured using a Phocal Fluorescence System (Life Science Resources, Cambridge, UK) connected to a Nikon Diaplot-TMD inverted fluorescence microscope ($\times$40 oil immersion objective, dichroic mirror 510 nm and barrier filter 520 nm). Each experiment was repeated 3 times.

Fluorescence quenching by intact cells

A modified procedure of Bogush and Robert[12] was used. First, 2 ml of a 2×10^6 cells/ml suspension in HBSS was pre-treated with DNase I (50 μg/ml, cell culture grade; Boehringer Mannheim, Mannheim, Germany) for 1 h and then loaded into a chamber with a coverslip bottom. Cells were kept at 37°C and were kept suspended by a paperclip wire rotating at about 180 r.p.m. Doxorubicin was added to cell suspensions to a final concentration of 2 μM. Fluorescence intensity of the cell suspension was measured.

Fluorescence and confocal microscopy

Cells attached on coverslips were washed twice with 1 ml HBSS, fixed with 1 ml fresh fixing solution (0.25 M sucrose, 1 mM EGTA, 0.5 mM EDTA, 20 mM HEPES, 0.01% NaN_3, 4% formaldehyde, 0.5% glutaraldehyde) for 20 min at 37°C and washed 3 times with 1 ml cold TBS (20 mM Tris-HCl, 4.5% sucrose, 150 mM NaCl, 0.05% sodium azide, pH 7.5) for 5 min. Coverslips were mounted on glass slides with a mounting solution (1:9 PBS:glycerol, 1% phenylenediamine) and sealed with nail-polish. Cells were studied with a Nikon Diaplot-TMD inverted fluorescence microscope and photographed using Fuji ASA800 Super G plus negative color film. Cells were also studied in optical sections by a Zeiss LSM510/Axiovert 100 M confocal imaging system.

Free doxorubicin measurement by capillary electrophoresis

Cell extracts in $CHCl_3$:MeOH (4:1) were vacuum-dried and re-constituted in electrophoresis running buffer (100 mM boric acid, 50 mM sodium dodecyl sulfate, pH 9.08). Capillary electrophoresis was performed at 25 kV on uncoated fused-silica (75 μm $\times$ 45 cm) capillary in a Beckman P/ACE 5000 CE system equipped with 5.0 mW argon-ion laser at an excitation/emission of 488/520 nm. Pressure injection mode was employed. Free doxorubicin in growth medium was similarly extracted and vacuum-dried but reconstituted in phosphate-buffered saline. Doxorubicin was estimated by fluorescence at an excitation/emission of 485/590 nm.

Results

Synergism between 2-deoxyglucose/Na azide and cancer drugs

Medium-effect analysis of drug mixtures in both KB-3-1 and KB-V1 cells demonstrated that there was strong synergism between 2-deoxyglucose/Na azide and drugs (Figure 1). 2-Deoxyglucose or Na azide alone was also effective but less powerful. Cellular ATP content reduced gradually to about 10% after 24 h treatment in low-dose 2-deoxyglucose/Na azide.

Drug accumulation in multidrug-resistant cells

KB-V1 cells accumulated much less free doxorubicin than KB-3-1 cells (Figure 2). Energy starvation or verapamil treatment, which inhibits P-gp, increased free drug accumulated, particularly in KB-V1. However, the accumulation of conjugates in the two cell lines was more comparable, suggesting conjugates are not good substrates for P-gp. Direct comparison of the accumulation of free and conjugated doxorubicin was not possible because of the different emission intensities of the two.

To estimate drug retention, cells were pre-loaded with free or conjugated doxorubicin and cellular fluorescence was measured after 1 h further incubation in the absence of drug in medium. As expected, fractional retention of conjugates was higher than that of the free drug (Table 1). This was particularly noticeable in the multidrug-resistant KB-V1 cells.

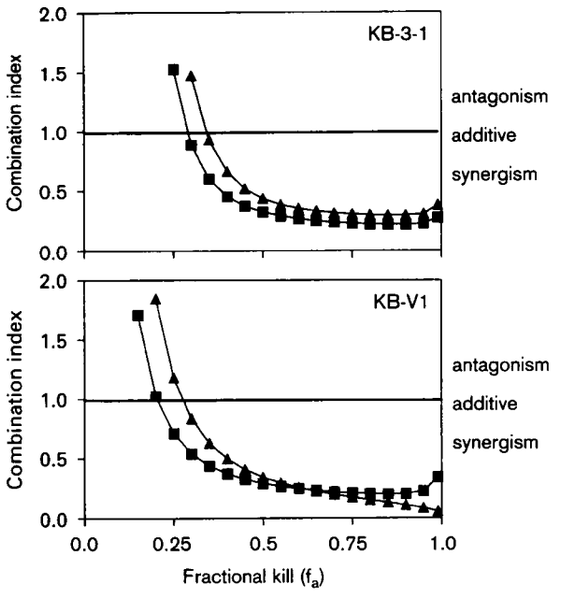

Figure 1. Medium-effect analysis of interactions between energy starvation and free/conjugated doxorubicin on killing of KB-3-1 and KB-V1 cells. Cells were grown in MEM–10% FCS and exposed to drug mixtures for 72 h. Experiments were designed and data were analyzed according to the medium-effect method of Chou and Talalay,[25] assuming a mutually exclusive model. Each data point represents the average of four experiments; each experiment was performed in triplicate. (■) Doxorubicin + 2-deoxyglucose/Na azide, 1:162/2500 for KB-3-1 cells, 1:1.63/25 for KB-V1 cells. (▲) D70 + 2-deoxyglucose/Na azide, 1:126/400 for KB-3-1 cells, 1:2.6/40 for KB-V1 cells.

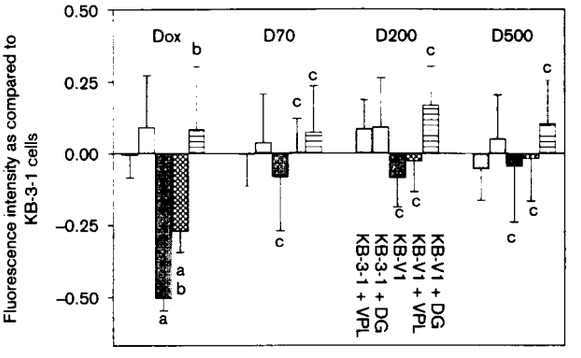

Figure 2. Accumulation of free and dextran-conjugated doxorubicin in KB-3-1 and KB-V1 cells. The intracellular fluorescence intensity was determined in cells incubated for 2 h with 10 μM free doxorubicin (Dox) or dextran-conjugated doxorubicin (D70, D200 and D500: doxorubicin conjugated to 70, 200 or 500 kDa dextran) with equivalent doxorubicin content. To inhibit P-gp activity some cells were treated with 10 μM verapamil (VPL) or energy starved (DG). Results are presented as fractions of fluorescence intensity emitted by KB-3-1 cells. In each experiment 30 cells were randomly selected and cellular fluorescence measured. Results represent the averages and standard errors of means of data obtained from three experiments. [a]Significantly different ($p<0.05$) from KB-3-1 cells receiving the same treatment. [b]Significantly different ($p<0.05$) from same cells of which P-gp was not inhibited. [c]Significantly different ($p<0.05$) from free doxorubicin.

When P-gp was inhibited by verapamil or energy starvation, fractional retention of the free drug and the 70 kDa conjugate, but not the larger (200 and 500 kDa) conjugates, significantly increased.

Nuclear presence of free doxorubicin but not conjugates

Cellular DNA-doxorubicin interaction can be monitored by fluorescence quenching in cell suspensions.[4,12,26] Fluorescence of free doxorubicin was quenched when added to suspensions of KB-3-1 cells (Figure 3a), indicating free doxorubicin is entering nuclei and interacting with DNA. Fluorescence quenching could be increased further by energy starvation. Quenching was not detectable when free doxorubicin was added to KB-V1 cells unless the cells were energy starved (Figure 3a).

Fluorescence of conjugates was not quenched by intact cells. However, in cell lysates conjugates are capable of interacting with DNA and fluorescence quenching can be observed (data not shown).

Decoupling

In cells preloaded with conjugates, on prolonged incubation cellular fluorescence increased gradually (Figure 3b). Since the fluorescence emission of free doxorubicin is more intense than conjugated doxorubicin, these results suggest that there might be a decoupling of doxorubicin from dextran. Free doxorubicin was detected in cell extracts and medium by capillary electrophoresis. In cells preloaded with conjugates, the amount of intracellular and medium free doxorubicin increased with time (Figure 4).

Decoupling may be catalyzed by an acidic environment, such as in the interior of organelles. When solutions of conjugates were made acidic, fluorescence intensity (Figure 3d) and the amount of free doxorubicin (data not shown) gradually increased, mimicking events taken place in intact cells.

Both the increase in fluorescence (Figure 3b) and increase in free doxorubicin could be reduced by energy starvation (Figure 3b and 4). Presumably upon

energy starvation, the acidic interior of organelles can not be maintained.

Subcellular distribution and redistribution

Doxorubicin, being basic, is in the first place trapped in acidic organelles, i.e. mitochondria and lysosomes[27,28] (Figure 5a and b), on which P-gp are not detected.[29] Under conditions of energy starvation, intracellular proton gradients could not be maintained and doxorubicin would be re-distributed. Conceivably some doxorubicin would be eliminated by membrane pumps and inside cells the majority would be found in nuclei where it is immobilized by drug-DNA interaction (Figure 5c and d).

Table 1. Retension of free and dextran-conjugated doxorubicin in KB-3-1 and KB-V1 cells

Drugs	KB-3-1	KB-V1	KB-3-1+VPL[a]	KB-V1+VPL[a]	KB-3-1+DG[b]	KB-V1+DG[b]
Dox[c]	0.767±0.017	0.520±0.042	1.113±0.019*	0.803±0.086*	1.170±0.029*	1.113±0.129*
D70[d]	1.024±0.090#	0.647±0.008#	0.860±0.025	0.979±0.098*	0.813±0.063	1.020±0.049*
D200[d]	0.797±0.089	0.770±0.064#	0.865±0.068	0.813±0.042	0.860±0.067	1.010±0.064
D500[d]	0.875±0.091	0.743±0.067#	0.673±0.056	0.817±0.038	0.757±0.020	0.810±0.032

Fluorescence intensity was measured in cells preloaded with drugs (incubated with 10 μM drugs at 30°C for 1 h) and in the same cells after a further 1 h of incubation without drugs. Fractional retention is the ratio between final and initial fluorescence. To inhibit P-gp activity, cells were treated with 10 μM Verapamil (VPL) or 1 g/l deoxyglucose+10 mM Na azide (DG). In each experiment, 30 cells were randomly selected for fluorescence measurement. Results represent averages±SEM of data obtained from three experiments.
[a]10 μM verapamil (VPL) treated.
[b]Energy starvation.
[c]Free doxorubicin.
[d]Doxorubicin conjugated to 70 (D70), 200 (D200) or 500 (D500) kDa dextrans.
#Significantly different ($p<0.05$) from the retention of free doxorubicin.
*Significantly different ($p<0.05$) from same cells (KB-3-1 or KB-V1) of which the P-gp pump was not inhibited.

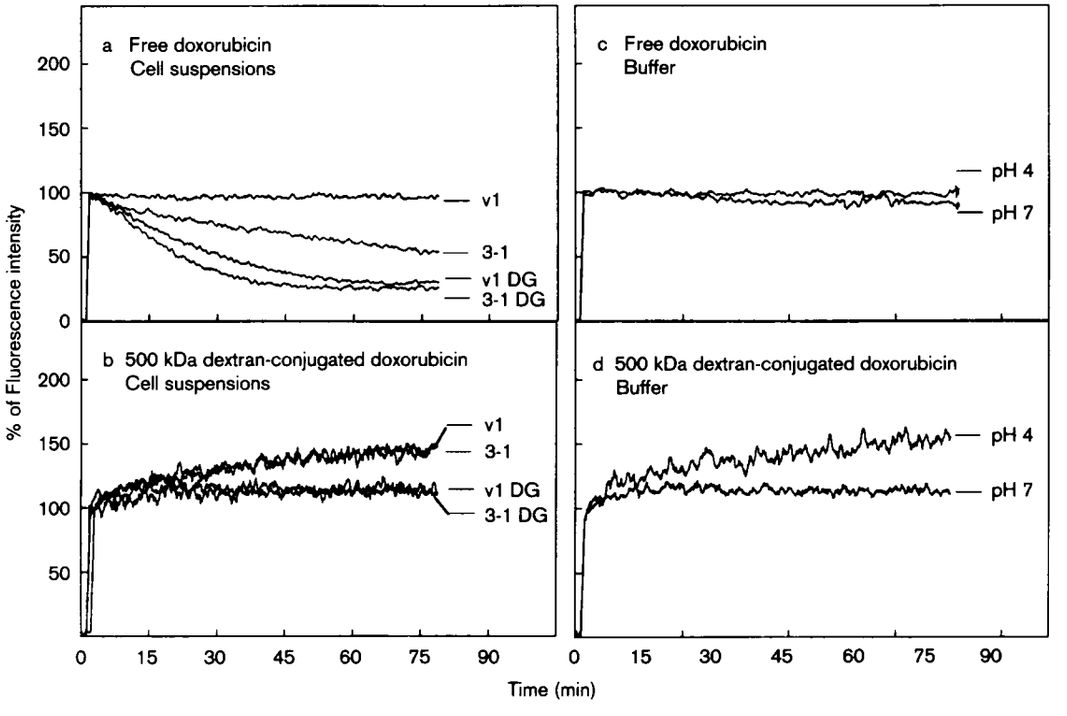

Figure 3. Changes in drug fluorescence in cell suspensions (a and b) and in acidic buffer (c and d). For panels (a) and (b), free or conjugated doxorubicin (final concentration 2 μM) was added to 2×10^6 cells in 2 ml suspensions under normal or energy starved (DG) conditions.

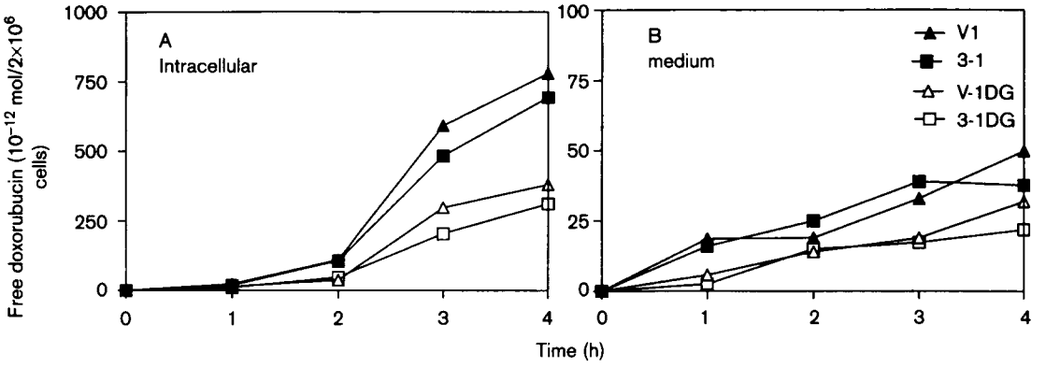

Figure 4. Decoupling of doxorubicin from dextran in KB-3-1 (■, □) and KB-V1 (▲, △) cells. Cells were preloaded with doxorubicin conjugated to 500 kDa dextran and were incubated under normal (solid symbols) or energy-starved (DG) (open symbols) conditions. Intracellular and medium free doxorubicin was measured by capillary electrophoresis or fluorescence, respectively.

Conjugated doxorubicin was also initially and predominantly found in cytoplasmic organelles (Figure 5e and f). In contrast to free doxorubicin, upon pretreatment with 2-deoxyglucose/Na azide, the conjugates re-distributed to cytoplasm but were largely excluded from nuclei (Figure 5g and h).

In cells preloaded with the 200 kDa conjugate, after prolonged (24 h) incubation some fluorescence began to appear in nuclei (Figure 5i). The nuclear presence of fluorescence was less in the multidrug-resistant KB-V1 cells (Figure 5j). Presumably prolonged incubation resulted in decoupling and the elevated P-gp pump activity in drug-resistant cells eliminated some of the decoupled free drug. With energy starvation, nuclear fluorescence increased in both cell lines, especially in KB-V1 cells (Figure 5k and l). The exact amount of doxorubicin in nuclei could not be determined by the techniques used in our studies.

Discussion

Cancer chemotherapy is often hindered by adverse side effects and drug resistance. The use of biological macromolecules and related substances as carriers of therapeutic agents could relieve some of the undesirable side effects. Dextran-conjugated doxorubicin is one such example.[30]

Our previous studies have shown that dextran conjugation alters drug action in multidrug-resistant KB-V1 cells[17,18] which over-express P-gp. Energy starvation may curtail multidrug resistance through inhibiting pump activities. Here we have shown that strong synergism occurs when 2-deoxyglucose/Na azide are applied with free/conjugated doxorubicin.

DNA interaction and the subsequent stabilization of topoisomerase II cleavage complexes are primary cell-killing mechanisms for doxorubicin,[1,26] although other sites of action are also likely.[4] Subcellular drug distribution may influence drug-DNA interaction and thus may have a critical effect on drug action. We have shown that dextran-conjugated doxorubicin, although capable of interacting with DNA *in vitro* with only a small decrease in the equilibrium binding constant,[17] has a much reduced cytotoxicity which can be attributed to its inability to enter nuclei. Large molecules bigger than 20-40 kDa are known to be excluded from nuclei.[31,32]

We have also clarified the relationship between drug size and P-gp activity. In the first 1-2 h of exposure, both free and conjugated doxorubicin tend to concentrate in cytoplasmic acidic organelles. Free doxorubicin, however, may slowly find its way to nuclei but most of the free doxorubicin is not available to nuclei of drug-resistant KB-V1 cells, unless P-gp is inactivated. Large conjugates of over 200 kDa, on the other hand, are excluded from nuclei but they are not good substrates for P-gp and their retention is improved. P-gp seems to be only effective on the free drug and smaller (<70 kDa) conjugates, and is not effective against conjugates larger than 200 kDa. Our interpretation is in line with the observation that the size of the central pore of P-gp is about 5 nm,[33] whereas the estimated effective size of 200 kDa dextran is greater than 9 nm.[32]

Upon prolonged incubation, conjugated doxorubicin may be released as free drug due likely to an acidic pH-dependent decoupling. This results in an intracellular delayed-release effect. This is analogous to cases where bovine serum albumin or poly-L-lysine drug

conjugates are degraded in the lysosomes before doxorubicin can interact with nuclear DNA.[16] It would be of great interest to delineate the relationship between the kinetics of drug release and the cytotoxicity of these conjugates in multidrug-resistant cells.

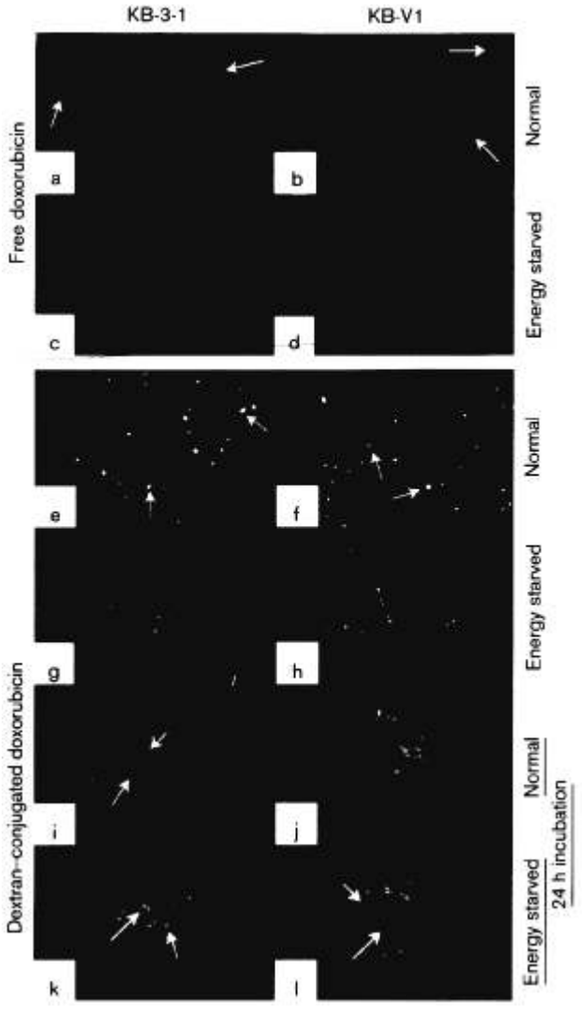

Figure 5. Drug distribution in KB-3-1 and KB-V1 cells as observed by fluorescence (a–d) or laser scanning confocal microscopy (e–l). KB-3-1 cells (a and c) and KB-V1 cells (b and d) were pre-incubated with 10 μM free doxorubicin at 37°C for 2 h. Bright spots (arrows in a and b) are presumed acidic organelles where doxorubicin was highly concentrated. Fluorescence in these spots dissipated in energy-starved cells (c and d) and partially redistributed to nuclei. In D200 conjugate-loaded cells (e to f), the bright cytoplasmic spots (arrows) indicate the organelle location of the conjugate and the lack of fluorescence in nuclei. In energy-starved cells these bright spots dissipated (g and h). Upon prolonged (24 h) incubation (i and j), the cytoplasmic organelle location of the conjugate remained, but some fluorescence started to appear in nuclei of KB-3-1 cells (arrows in i), but less was found in nuclei of KB-V1 cells (j). Upon prolonged energy starvation, nuclear fluorescence increased (arrows in k and l).

Conclusion

Energy metabolism and glucose transport may be altered in multidrug-resistant cells.[19–21] Energy starvation can cause apoptotic death of drug-resistant cells, an action which may be mediated by oxidative stress and c-*myc* expression.[34,35] Our results suggest additional mechanisms for the action of energy starvation. These include increasing the retention of free doxorubicin and small conjugates, facilitating a drug redistribution from cytoplasmic organelles to cytoplasm and nucleus, and delaying the decoupling of doxorubicin from conjugates. All of these could be potentially beneficial for cytotoxic drug action.

References

1. Cummings J, Anderson L, Willmott N, Smith JF. The molecular pharmacology of doxorubicin *in vivo*. *Eur J Cancer* 1991; **27**: 532-5.
2. Jaenke RS, Deprez-DeCampeneere D, Trouet A. Cardiotoxicity and comparative pharmacokinetics of six anthracyclines in the rabbit. *Cancer Res* 1980; **40**: 3530-6.
3. Nielsen D, Maare C, Skovsgaard T. Cellular resistance to anthracyclines. *Gen Pharmac* 1996; **27**: 251-5.
4. Barabas K, Sizensky JA, Faulk WP. Transferrin conjugates of doxorubicin are cytotoxic without intercalating nuclear DNA. *J Biol Chem* 1992; **267**: 9437-42.
5. Fritzer M, Szekeres T, Szuts V, Jarayam HN, Goldenberg H. Cytotoxic effects of a doxorubicin-transferrin conjugate in multidrug-resistant KB cells. *Biochem Pharmacol* 1996; **51**: 489-93.
6. Ueda Y, Munechika K, Kikukawa A, Kanoh Y, Yamanouchi K, Yokoyama K. Comparison of efficacy, toxicity and pharmacokinetics of free adriamycin and adriamycin linked to oxidized dextran in rats. *Chem Pharm Bull* 1989; **37**: 1639-41.
7. Hurwitz E, Levy R, Maron R, Wilchek M, Arnon R, Sela M. The covalent binding of daunomycin and adriamycin to antibodies, with retention of both drug and antibody activities. *Cancer Res* 1975; **35**: 1175-81.
8. Sheldon K, Marks A, Baumal R. Sensitivity of multidrug resistant KB-C1 cells to an antibody-dextran-doxorubicin conjugate. *Anticancer Res* 1989; **9**: 637-42.
9. Jeannesson P, Trentesaux C, Gerard B, Jardillier JC, Ross KL, Tokes ZA. Induction of erythroid differentiation in human leukemic K-562 cells by membrane-directed action of adriamycin covalently bound to microspheres. *Cancer Res* 1990; **50**: 1231-6.
10. Rogers KE, Carr BI, Tokes ZA. Cell Surface-mediated cytotoxicity of polymer-bound adriamycin against drug-resistant hepatocytes. *Cancer Res* 1983; **43**: 2741-8.

11. Yokoyama M, Miyauchi M, Yamada N, *et al.* Characterization and anticancer activity of the micelle-forming polymeric anticancer drug adriamycin-conjugated poly(-ethylene glycol)-poly(aspartic acid) block copolymer. *Cancer Res* 1990; **50**: 1693–700.
12. Bogush T, Robert J. Comparative evaluation of the intracellular accumulation and DNA binding of idarubicin and daunorubicin in sensitive and multidrug-resistance human leukaemia K562 cells. *Anticancer Res* 1996; **16**: 365–8.
13. Consoli U, Priebe W, Ling, YH, *et al.* The novel anthracycline annamycin is not affected by P-glycoprotein-related mutidrug resistant: comparison with idarubicin and doxorubicin in HL-60 leukemia cell lines. *Blood* 1996; **88**: 633–44.
14. Mankhetkorn S, Dubru F, Hesschenbrouck J, Fiallo M, Garnier-Suillerot A. Relation among the resistance factor, kinetics of uptake, and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(*s*)-fluoroidarubicin, and idarubicin in multidrug-resistant K562 Cells. *Mol Pharmacol* 1996; **49**: 532–9.
15. Hatano T, Ohkawa K, Matsuda M. Cytotoxic effect of the protein–doxorubicin conjugates on the multidrug-resistant human myelogenous leukemia cell line, K562, *in vitro*. *Tumour Biol* 1993; **14**: 288–94.
16. Takahashi N, Asakura T, Ohkawa K. Pharmacokinetic analysis of protein-conjugated doxorubicin (DXR) and its degraded adducts in DXR-sensitive and -resistant rat hepatoma cells. *Anti-Cancer Drugs* 1996; **7**: 687–96.
17. Yang M, Chan HL, Lam W, Fong WF. Cytotoxicity and DNA binding characteristics of dextran-conjugated doxorubicins. *Biochim Biophys Acta* 1998; **1380**: 329–35.
18. Fong WF, Lam W, Yang M, Wong JTF. Partial synergism between dextran-conjugated doxorubicin and cancer drugs on the killing of multidrug resistant KB-V1 cells. *Anticancer Res* 1996; **16**: 3773–8.
19. Miccadei S, Fanciulli M, Bruno T, Paggi MG, Floridi A. Energy metabolism of adriamycin-sensitive and -resistant Ehrlich ascites tumor cells. *Oncol Res* 1996; **8**: 27–35.
20. Kaplan O, Jaroszewski JW, Clarke R, *et al.* The multidrug resistant phenotype: ^{31}P nuclear magnetic resonance characterization and 2-deoxyglucose toxicity. *Cancer Res* 1991; **51**: 1638–44.
21. Bentley J, Bell S, Quinn DM, Kellett GL, Warr JR. 2-Deoxy-D-glucose toxicity and transport in human multidrug-resistant KB cancinoma cell lines. *Oncol Res* 1996; **8**: 77–84.
22. Shen DW, Cardarelli C, Hwang J, *et al.* Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, doxorubicin, or vinblastine show changes in expression of specific proteins. *J Biol Chem* 1986; **261**: 7762–70.
23. Skehan P, Storeng R, Scudiero D, *et al.* New colorimeteric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; **82**: 1107–12.
24. Chou TC, Talalay P. Quantitive analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enz Reg* 1984; **22**: 27–55.
25. Chou J. Quantitation of synergism and antagonism of two or more drugs by computerized analysis. In: Chou TC, Pideout DC, eds. *Synergism and antagonism in chemotherapy*. New York: Academic Press 1991: 223–44.
26. Neidle S, Pearl LH, Skelly JV. DNA structure and perturbation by drug binding. *Biochem J* 1987; **243**: 1–13.
27. Moriyama Y. Membrane energization by proton pumps is important for compartmentization of drugs and toxins: a new type of active transport. *J Exp Biol* 1996; **199**: 1447–54.
28. Willingham MC, Cornwell MM, Cardarelli CO, Gottesman MM, Pastan I. Single cell analysis of daunomycin uptake and efflux in multidrug-resistant and -sensitive KB cells: effects of verapamil and other drugs. *Cancer Res* 1986; **46**: 5941–6.
29. Willingham MC, Richert ND, Cornwell MM, *et al.* Immunocytochemical localisation of P170 at the plasma membrane of multidrug-resistant human cells. *J Histochem Cytochem* 1987; **35**: 1451–6.
30. Danhauser-Riell S, Hausmann E, Schick HD, *et al.* Phase I clinical and pharmacokinetic trial of dextran conjugated doxorubicin (AD-70, DOX-OXD). *Invest New Drugs* 1993; **11**: 187–95.
31. Forbes DJ. Structure and function of the nuclear pore complex. *Annu Rev Cell Biol* 1992; **8**: 495–527.
32. Lang I, Scholz M., Peters R. Molecular mobility and nucleocytoplasmic flux in hepatoma cells. *J Cell Biol* 1986; **102**: 1183–90.
33. Rosenberg MF, Callaghan R, Ford RC, Higgins CF Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *J Biol Chem* 1997; **272**: 10685–94.
34. Lee YJ, Galoforo SS, Berns CM, *et al.* Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. *J Biol Chem* 1998; **273**: 5294–599.
35. Lee YJ, Galoforo SS, Berns CM, Tong WP, Kim HR, Corry PM. Glucose deprivation-induced cytotoxicity in drug resistant human breast carcinoma MCF-7/ADR cells: role of c-*myc* and *bcl*-2 in apoptotic cell death. *J Cell Sci* 1997; **110**: 681–6.

(Received 29 September 1998; revised form accepted 22 October 1998)