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Cytotoxic Effects of a Doxorubicin-Transferrin Conjugate in Multidrug-Resistant KB Cells

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ABSTRACT. Cancer chemotherapy is often limited by the emergence of multidrug-resistant tumor cells. Multidrug resistance (MDR) can be caused by amplification of the MDR genes and overexpression of the P-glycoprotein, which is capable of lowering intracellular drug concentrations. A doxorubicin-transferrin conjugate has been synthesized and exerts its cytotoxic effects through a transmembrane mechanism. We have examined the cytotoxicity of this conjugate and compared it with doxorubicin in sensitive (KB-3-1) and in multidrug-resistant KB cell lines (KB-8-5, KB-C1, and KB-V1). In the clonogenic assay, doxorubicin exhibited IC_{50} concentrations of 0.03 and 0.12 μM in the sensitive (KB-3-1) and resistant (KB-8-5) cell lines, respectively, whereas, doxorubicin-transferrin conjugate was more effective with IC_{50} concentrations of 0.006 and 0.028 μM, respectively. In highly multidrug-resistant KB-C1 and KB-V1 cells, doxorubicin up to 1 μM did not cause any cytotoxic effects, while the doxorubicin-transferrin conjugate inhibited colony formation of these cells with IC_{50} levels of 0.2 and 0.025 μM, respectively. These results demonstrate that doxorubicin-transferrin is effective against multidrug-resistant tumor cells. BIOCHEM PHARMACOL 51;4:489–493, 1996.

KEY WORDS. doxorubicin–transferrin conjugate; multidrug resistance; transferrin receptor sites; clonogenic cytotoxicity; human epidermoid carcinoma KB cells

Development of resistance towards cytotoxic drugs like doxorubicin (Adriamycin®) constitutes a major problem in cancer chemotherapy [1, 2]. Resistance towards doxorubicin treatment can be caused by overexpression of the MDR-1¶ gene resulting in the synthesis of P-glycoprotein, which functions as a pump and is capable of removing the drug from the cytoplasm [1]. The mechanism of action of doxorubicin–transferrin conjugate is through the cell membrane [3, 4]. Therefore, doxorubicin–transferrin action may not be mediated by P-glycoprotein.

It was shown earlier that the number of transferrin receptors on the cell surface is increased significantly in tumor cells and other rapidly proliferating cells compared with slowly growing non-malignant cell populations [5]. Hence, Yeh and Faulk [6] proposed the use of the transferrin receptors as tumor specific targets for drug therapy. Subsequently, a doxorubicin–transferrin conjugate was synthesized by coupling doxorubicin to a protein through glutaraldehyde, resulting in a Schiff base-bonding between the components [6]. The bond is acid stable and is not hydrolyzed during endocytosis [7]. This conjugate was demonstrated to be selectively cytotoxic to leukemic cells and not to normal human leucocytes[6]. More recently, a preliminary clinical study demonstrated the therapeutic usefulness of this conjugate in the treatment of certain leukemias [8]. As this conjugate seems to exert its cytotoxic effects without entering the cell cytoplasm, we examined the effects of doxorubicin–transferrin conjugate in well established MDR-1 expressing KB cell lines [9]. We compared the cytotoxic effects of doxorubicin and the doxorubicin–transferrin conjugate by utilizing a clonogenic assay in the doxorubicin–sensitive (KB-3-1) and -resistant (KB-8-5, KB-C1 and KB-V1) cell lines.

MATERIALS AND METHODS

Materials

Doxorubicin was obtained from the Sigma Chemical Co., St. Louis, MO. [6-^{3}H]Thymidine was purchased from NEN (Boston, MA). All other chemicals were commercially available and of the highest purity. Diferric transferrin (Provivo, Finland) was iodinated using the iodogen method [10].

Cell Lines

The KB-3-1, KB-8-5, KB-C1 and KB-V1 cell lines were provided by Dr. M. M. Gottesman, National Cancer Institute, Bethesda, MD. The KB-3-1 cell line, which is sensitive to

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doxorubicin, was used as a control cell line. The MDR cell lines were grown in the presence of the selecting drug (colchicine at 1 μg/mL and 1 ng/mL for the KB-C1 and the KB-8-5 cells, respectively; and vincristine at a concentration of 1 μg/mL for the KB-V1 cells).

Cell Culture

The KB cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, NY), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were incubated at 37° in a humidified atmosphere of air with 5% CO_2.

For subculture, monolayer cells were dispersed with 0.25% trypsin plus 1 mM EDTA at 37° for 30 min. After centrifugation, the cells were resuspended in fresh medium.

Conjugate

Doxorubicin–transferrin conjugates were prepared using glutaraldehyde as coupling agent according to published methodology [6]. The average conjugation number (average of molecules of doxorubicin per molecule of transferrin) was 2.4, if not indicated otherwise. The synthesis of the conjugate was monitored by spectrophotometry.

Clonogenic Efficiency

For the clonogenic assay, exponentially growing cells were seeded in 24-well plates in medium containing 10% fetal bovine serum at a density of 200 cells/well. Six hours after seeding, various concentrations of the drugs were added; the cells were then maintained in the medium for 10 days. Triplicate samples were used for each drug concentration. Colonies consisting of more than 50 cells were counted [11].

[6-^{3}H]*Thymidine Incorporation*

Cells (2×10^5/mL) were incubated with doxorubicin or doxorubicin–transferrin conjugate at the specified concentrations in 96-well microtiter plates for 7 days in complete DMEM. The 1 μCi of [6-^{3}H]thymidine was added, and cells were incubated further for 20 hr. The cells were collected on filters using a cell harvester (Skatron, Trauby, Norway), the filters were transferred into scintillation vials, and the radioactivity was determined by scintillation spectrometry using a Packard liquid scintillation counter.

Estimation of Total Transferrin Receptor Concentration

Cells were seeded in 6-well plates for 24 or 48 hr to obtain logarithmically growing cells for the experiments. Transport experiments were carried out with DMEM containing 1% BSA. The cells were washed several times at 37° with this medium to remove endogenous transferrin.

Cells were then incubated with medium containing 2.7 nM [^{125}I]diferric transferrin and unlabeled diferric transferrin (0–350 nM) for 2 hr at 37°. This was sufficient to saturate recycling pathways of the cells, i.e. to occupy all active transferrin receptors in the cells. The medium was then removed and cells were washed several times with ice-cold medium, dissolved in 1 mL of 1 M NaOH per well and transferred to counting vials. The radioactivity was determined as described above.

The concentrations expressed in the figures refer to the levels of doxorubicin. In the case of doxorubicin–transferrin conjugate, the concentration of doxorubicin was calculated on the basis of the number of conjugates. The number of binding sites occupied under the individual conditions in each well were calculated from displacement of the radioactive transferrin by the unlabeled transferrin. The values obtained were then transformed into a Scatchard plot to calculate the maximal binding and the apparent binding affinity.

Cellular protein was determined with the micro-Bio-Rad protein assay [12].

RESULTS

Clonogenic Cytotoxicity Assay

Cytotoxicity studies were carried out using a clonogenic assay. The cells were incubated for 10 days with various doxorubicin concentrations. The clonogenic efficiency of the sensitive KB-3-1 cell line is shown in Fig. 1A. The IC_{50} value for doxorubicin was 0.028 μM, whereas a concentration of 0.006 μM of the conjugate was capable of reducing clonogenicity by 50%.

The KB-8-5 cells, which are partially multidrug resistant, yielded an IC_{50} of 0.12 μM for doxorubicin, whereas the conjugate showed a 4-fold lower IC_{50} (0.028 μM) (Fig. 1B).

When KB-C1 and KB-V1 cells were incubated with various concentrations of the two drugs for 10 days, doxorubicin did not exert any cytotoxic effect, whereas the doxorubicin–transferrin conjugate reduced colony formation with IC_{50} concentrations of 0.2 and 0.025 μM, respectively (Fig. 1, C and D).

Thymidine Incorporation

Incorporation of [6-^{3}H]thymidine into doxorubicin-sensitive and -resistant cells in the presence or absence of the title compounds was examined next. A chemosensitive response was observed primarily with doxorubicin–transferrin conjugate and not with doxorubicin alone. Thus, when highly multidrug-resistant cells (KB-V1 and KB-C1) were incubated with various drug concentrations (0.06 to 2 μM) for 7 days, doxorubicin exhibited IC_{50} values of >2 μM for these two cell lines, whereas the conjugate showed IC_{50} levels of 0.18 and 0.44 μM for the KB-V1 and KB-C1 cells, respectively.

Uptake of Transferrin

The Scatchard analysis of the transferrin binding to KB-3-1 and KB-C1 cells is shown in Fig. 2. Cells were exposed to radiolabeled transferrin for 2 hr at 37° in order to saturate all accessible binding sites to estimate their number and affinity. Based on the specific activity of radiolabeled transferrin and cellular protein, a total receptor number of 1.46×10^{11}/mg

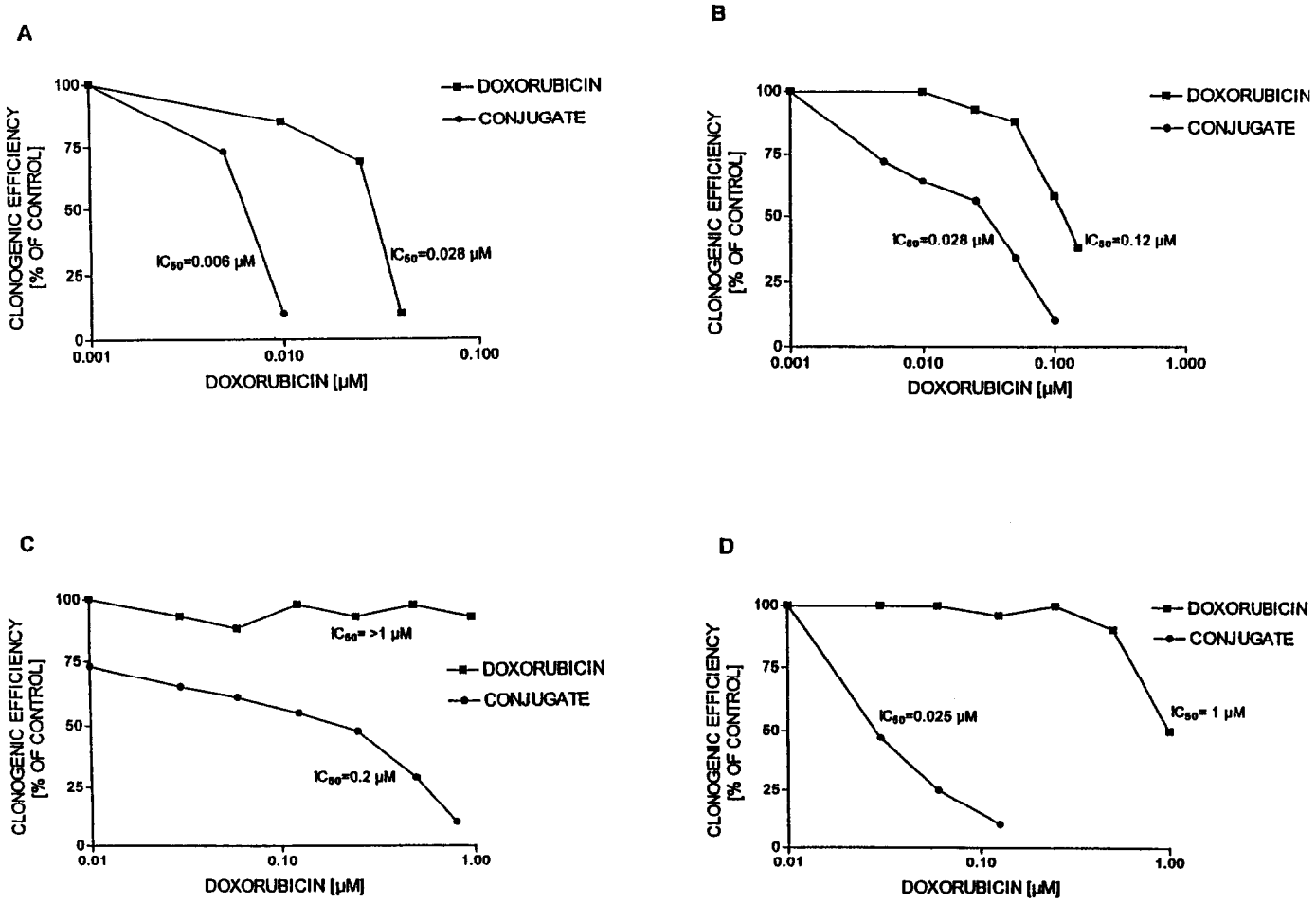

FIG. 1. Effect of doxorubicin and doxorubicin–transferrin conjugate on the clonogenicity of KB cells. Clonogenic efficiency was examined in the sensitive KB-3-1 cell line (panel A), partially multidrug-resistant KB-8-5 cells (panel B) and in the highly multidrug-resistant cell lines KB-C1 (panel C) and KB-V1 cells (panel D). The cells at a density of 200 cells/well were incubated with various concentrations of doxorubicin or conjugate for 10 days as described in Materials and Methods. The concentration of doxorubicin in the conjugate or of the parent compound is denoted on the abscissa.

protein for KB-3-1 and 1.80×10^{11}/mg protein for KB-C1 cells, and an apparent dissociation constant of 2.5 nM for the KB-3-1 cells and 3.44 nM for the KB-C1 cells were calculated. The concentrations of transferrin receptors were very similar in KB-3-1 and in KB-C1 cells with comparable affinities. Receptor binding assays in KB-V1 cells did not yield conclusive results due to very weak adherence of these cells to the dish surface in the incubation medium and during the washing procedure. These studies suggest that the higher sensitivity of KB-C1 cells to the conjugate was not due to a higher expression level of transferrin receptors. Also, there was no correlation between the sensitivity of the cells towards the conjugate with the total number of cellular transferrin receptors that participate in the endocytosis-recycling process mediating iron uptake.

DISCUSSION

The mechanism of the cytotoxic action of doxorubicin is thought to be due to intercalation with nuclear targets, free

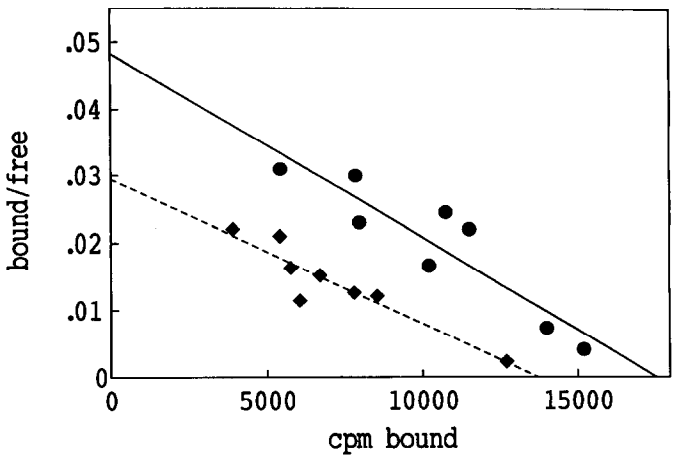

FIG. 2. Scatchard analysis of total transferrin binding sites in doxorubicin sensitive KB-3-1 (●) and resistant KB-C1 (◆) cells. Cells were saturated with radiolabeled transferrin for 2 hr at 37° and analyzed as detailed in Materials and Methods.

radical generation, and/or interaction with the cell membrane ensuing cell death [13–15]. However, treatment of cancer patients with doxorubicin is limited by its cardiotoxicity and the development of drug resistance. Multidrug resistance is caused by amplification of the MDR-1 gene and overexpression of the P-glycoprotein, which is capable of pumping out cytotoxic drugs from cellular cytoplasm [1, 16]. Moreover, development of drug resistance by overexpression of the P-glycoprotein constitutes a major problem, as shown *in vitro* and in patients [17].

Yeh and Faulk [6] synthesized a doxorubicin–transferrin conjugate specifically to target rapidly proliferating malignant cells, because malignant cells have a higher iron requirement than normal cells and overexpress the transferrin receptor on their cell surface. However, erythropoietic progenitor cells also require high amounts of iron and were shown to express higher numbers of transferrin receptors. Therefore, the effect of doxorubicin–transferrin conjugate on human progenitor cells has to be tested before determining its clinical usefulness.

Barabas *et al.* [3] have shown by fluorescence microspectrometry that the cell membrane rather than the cellular DNA is the target of doxorubicin–transferrin conjugate. Furthermore, the conjugate is acid-stable, and thermo-stable for at least 2 weeks at 37° at pH 3.0, without appearance of dialyzable free doxorubicin (data not shown), and the doxorubicin conjugate does not enter the cytoplasm, but remains bound to the transferrin protein; in contrast, doxorubicin can freely enter the cell nucleus. Thus, it was proposed that the mechanism of action of doxorubicin–transferrin conjugate related to a transmembrane mechanism [3, 7]. The conjugate after binding to the cell membrane was shown to dissociate slowly, and since transferrin receptor sites are bound, the endocytic recycling was also decreased [4, 18, 19]. This suggests the possibility of a prolonged effect of doxorubicin on the membrane resulting in subsequent membrane damage. Therefore, the P-glycoprotein pump should not be able to circumvent the action of the doxorubicin–transferrin conjugate. To test this hypothesis, we investigated the cytotoxic effects of doxorubicin–transferrin conjugate on well established KB cell lines expressing MDR-1 gene. Effectiveness of the conjugate against drug-resistant K562 or HL-60 cells has been demonstrated [7, 20], but in these cell lines the drug resistance is believed to be caused by a complex set of mechanisms [21–23]. However, the mechanism of MDR in KB cell lines was shown to be due to overexpression of P-glycoprotein [9]. Utilizing sensitive and multidrug-resistant KB cells, we have demonstrated that the doxorubicin–transferrin conjugate is capable of circumventing the multidrug-mediated resistance mechanisms.

To examine whether the cytotoxic effects of doxorubicin–transferrin conjugate are due to an increased number of transferrin receptors, we determined the number of transferrin receptors in doxorubicin-sensitive KB-3-1 and -resistant KB-C1 cells. However, the total amount of transferrin receptors accessible to the conjugate via the recycling pathway, and the apparent affinity of the receptors in resistant KB-C1 cells were similar to those found in sensitive cells.

These results highlight a crucial difference between transferrin conjugates and antibody conjugates of doxorubicin. Antibody conjugates require hydrolytic deconjugation to be effective [24–26], whereas doxorubicin–transferrin conjugate is stable and binds to the cell membrane. The glycosidic bond between the aminosugar (which is actually coupled to the protein) and the anthracycline base is not susceptible to hydrolysis at a pH expected in the endocytic compartment [3], leaving the conjugate stable for at least several weeks.

The higher overall efficacy of the conjugate compared with doxorubicin may be due to the concentrating effect of the covalent coupling at sensitive membrane sites, resulting in greater cytotoxicity. Small differences in the levels of transferrin receptors might not be enough to account for the increased sensitivity of cells towards doxorubicin–transferrin conjugate.

Recent studies have demonstrated that various doxorubicin-resistant KB cells express different levels of protein kinase C [27]. The highly resistant KB-C1 cell line had the most enzyme activity [27] and showed the highest IC_{50} value with the conjugate. Thus, there may be other factors not apparent at this time that can contribute towards differential sensitivity of the conjugate to the resistant lines.

Our studies indicate that doxorubicin–transferrin conjugate is active against multidrug-resistant cells *in vitro*, thus suggesting the possibility of utilizing this compound in conjunction with P-glycoprotein inhibitors [28] for treatment of cancer patients who develop multidrug resistance. Towards this goal, and to clarify the role of interaction of the conjugate with P-glycoprotein, future studies are planned to examine the cytotoxicity of the combination of doxorubicin–transferrin conjugate with inhibitors of P-glycoprotein.

Novel aspects of the present investigation include the following: (1) a doxorubicin–transferrin conjugate was shown to exert a cytotoxic effect in partially (KB-8-5) or in highly multidrug-resistant (KB-C1 and KB-V1) cells that are MDR-1 positive and express P-glycoprotein; and (2) the doxorubicin–transferrin conjugate exhibited a lower IC_{50} concentration than doxorubicin in all KB cell lines examined.

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