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Research article

DOXORUBICIN-TRANSFERRIN CONJUGATE SELECTIVELY OVERCOMES MULTIDRUG RESISTANCE IN LEUKAEMIA CELLS

DOROTA ŁUBGAN[1,*], ZOFIA JÓŹWIAK[2], GERHARD G. GRABENBAUER[1] and LUITPOLD V. R. DISTEL[1]

[1]Department of Radiation Oncology, Friedrich-Alexander-University Erlangen-Nuremberg, Universitätsstr. 27, D-91054 Erlangen, Germany, [2]Department of Thermobiology, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland

Abstract: Neoplastic cells frequently have an increased number of transferrin receptors. Coupling transferrin to an anti-neoplastic drug has the potential to overcome multidrug resistance (MDR). The purpose of this study was to examine the distribution and action of doxorubicin-transferrin conjugate (DOX-TRF) in a leukaemia cell line (HL60), a multidrug-resistant leukaemia cell line (HL60ADR) and a normal tissue cell line (human fibroblasts). The intracellular accumulation of DOX and DOX-TRF was monitored by direct fluorescence. More DOX-TRF than free DOX was delivered to the tumour cells, and consecutively the levels of DNA double-strand breaks and apoptosis increased even in the multidrug-resistant cell line. In the normal tissue cell line, DOX-TRF did not accumulate, and therefore, the levels of DNA double-strand breaks and apoptosis did not increase. Cell viability was determined using the MTT assay. The IC_{50} for DOX-TRF was lower than the IC_{50} value for the free drug in both leukaemia cell lines. The IC_{50} values for the HL60 cells were 0.08 μM for DOX and 0.02 μM for DOX-TRF. The IC_{50} values for HL60ADR cells were 7 μM for DOX and 0.035 μM for DOX-TRF. In conclusion, DOX-TRF was able to overcome MDR in the leukaemia cell lines while having only a very limited effect on normal tissue cells.

* Author for correspondence: e-mail: Dorota.Lubgan@uk-erlangen.de, tel.: +49 9131 853 2312, fax: +49 9131 853 9335

Abbreviations used: DOX – doxorubicin; DOX-TRF – doxorubicin-transferrin conjugate; MDR – multidrug resistance; MTT – 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; SD – standard deviation; SDS – sodium dodecyl sulfate

Key words: Doxorubicin-transferrin, Promyelocytic leukaemia, Apoptosis, DNA double-strand breaks, Multidrug resistance

INTRODUCTION

The anthracycline antibiotic doxorubicin (DOX, adriamycin) is a commonly used chemotherapeutic agent in cancer therapy [1]. Its cardiotoxicity [2] and the resistance of malignant cells to it [3] are serious problem that severely limit the success of DOX-based treatment.

It has been demonstrated that DOX coupled to various molecules such as epidermal growth factor, tumor-specific antibodies and transferrin could eliminate many of the toxicities of the free drug [4]. DOX covalently linked to carrier proteins can be specifically delivered to cancer cells [5]. Transferrin has several adventages over other drug carriers. It is not an immunogenic, but is a commercially available protein [6]. The transferrin receptor (known as CD71), a type II transmembrane glycoprotein found on the surface of cells, is a protein involved in iron uptake and the regulation of cell growth [7]. The expression of transferrin receptors is significantly upregulated in a variety of malignant cells [7], so the conjugation of anticancer drugs with transferrin might aid in delivering the therapeutics directly to neoplastic cells (via receptor-mediated endocytosis) with reduced injury to normal cells [4-6].

A number of studies have indicated that the conjugation of doxorubicin with the serum protein transferrin significantly increased the cytotoxic effect against tumour cells both *in vitro* [6, 8, 9] and *in vivo* [10]. DOX-TRF is toxic against a variety of human cell lines including HL60-promyelocytic leukemia [11], K562-erythroleukemia [11], Hep2-liver carcinoma [10], HeLa-cervical adenocarcinoma [12], SBC-3-small cell lung cancer [13] and cell lines resistant to DOX: KB-oral carcinoma [14], MCF-7-breast cancer [9], and SBC-3/ADM [13].

In this study, we used a transferrin-doxorubicin conjugate to compare the effect of the free drug and the conjugate on human promyelocytic cell lines HL60 and the doxorubicin-resistant HL60ADR, both overexpressing the TRF receptor. A fibroblast cell line from a healthy individual was also used. The question was whether it is possible to overcome drug resistance using the DOX-TRF conjugate and introduce an increased effect by means of DNA damage, apoptosis and cell death. An additional issue was whether the fibroblast cell line would sustain a reduced effect.

MATERIALS AND METHODS

Cell culture

The human promyelocytic cell lines HL60 (parental) and HL60ADR (prepared by treating the HL60 cell line with gradually increasing concentrations of DOX) were obtained from Prof. G. Bartosz (Department of Molecular Biophysics, University of Lodz, Poland). HL60 originated from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia [15]. The HL60ADR

cells isolated for resistance to DOX overexpress the MRP1 protein and represent the non P-glycoprotein MDR type [16]. HL60ADR cells were cultured in continuous presence of 0.2 μM DOX. One day prior to the experiments, the DOX was replaced with an equivalent volume of medium. Both cell lines were grown in suspension in RPMI 1640 with Glutamax® (Gibco BRL, Karlsruhe, Germany, No. 72400-021) supplemented with 10% heat inactivated foetal calf serum (Biochrom, Berlin, Germany), 100 units cm^{-3} penicillin and 10 μg cm^{-3} streptomycin (Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. The fibroblast cell line named SBL3F (not immortalized) was derived from a healthy donor. The SBL3F cells were cultured in F12 medium (Gibco BRL, Karlsruhe, Germany, No. 21765-037) supplemented with Glutamax®, 15% heat inactivated foetal calf serum, 100 units cm^{-3} penicillin and 10 μg cm^{-3} streptomycin under standard cell culture conditions.

Conjugation procedure

Doxorubicin was coupled to transferrin using the conjugation procedure described by Berczi *et al.* [11]. The DOX-TRF was chromatographed on a 0.8 x 33-cm column of Sepharose CL-4B (Sigma). The optical spectrum of each fraction was determined between 210 nm and 600 nm using a Ultraspec 2000 spectrophotometer UV/VIS Lambda 2 (Perkin, Elmer, Überlingen, Germany). The concentrations of TRF and DOX were calculated from standard curves for the two substances (at 280 and 495 nm). The collected fractions were analyzed by SDS-PAGE [17]. The gel was calibrated using a HMW-SDS marker (Batch, England), run in 0.5 x TBE for 1 h at 120 V, and the protein was stained for 1 h using CoomassieFluor™ (Molecular Probes, Eugene, USA). The fluorescence was excited at 312 nm and images were acquired by a CCD camera (CF 20 DXC, Kappa, Gleichen, Germany).

Transferrin receptor (CD71) measurement using immunostaining and Western Blotting

Immunostaining. HL60 and HL60ADR cells were grown in cell culture flasks (Greiner Bio-One, Frickenhausen, Germany), and after reaching a density of $5x10^5$ cells cm^{-3}, the cells were fixed on 24 x 60-mm cover slips and permeabilised using a 4% formaldehyde solution (0.1% Triton X-100, 4% formaldehyde in phosphate buffered saline-PBS). The SBL3F cells were seeded and grown for 48 h on 24 x 60-mm cover slips (300,000 cells/cover slip in 5 cm^3 medium) in quadriperm culture dishes (In Vitro Systems, Göttingen, Germany), fixed, and permeabilised. After washing with PBS, the cells were incubated for 12 h with a mouse monoclonal antibody against CD71 (US Biological, Swampscott, Massachusetts), and for 5 h with an Alexa Fluor 488 donkey anti-mouse antibody (Molecular Probes, Karlsruhe, Germany) After incubation with 0.1 μl DAPI 1.0 cm^{-3} 4 x SSC/Tween for 1 min, the samples were embedded in VectaShield and analysed under a fluorescence microscope (Zeiss,

Axioplan, Oberkochen, Germany). The images were processed with an image analysis system (Biomas, Erlangen, Germany).

Western blotting. HL60, HL60ADR and SBL3F cells were lysed in RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl 0.5% deoxycholate; 1.0% NP40; 0.1% SDS), and the protein concentration was fixed to 3.5 µg/µl using a BCA Protein Assay Kit (Pierce Bio Science). Electrophoresis was done with a Mini-Protean 3 Electrophoresis Cell (BioRad). The protein samples were mixed in a ratio of 1:1 with 2 x SDS sample loading buffer and denatured for 10 min at 95ºC. 17.5 µg protein was loaded per lane on polyacrylamide gels (10%). 3.5 µl Precision Plus Protein^{TM} Standards (BioRad) was loaded as a running control. Electrophoresis was started at 50 V (20 mA) for 30 min and then continued for 90 min at 120 V (40 mA). Blotting was performed with Mini Trans-Blot Transfer Cell (BioRad) using PVDF membranes (Schleicher and Schüll, Germany) for 2 h at 80 V (350 mA). Afterwards, the membranes were blocked in blocking buffer (1 x TBS, 10% BSA, 0.1% Tween) for 1 h with rocking at room temperature, and then washed in 1 x TBS for 5 min. The membranes were probed with mouse monoclonal antibody against CD71 (US Biological, Swampscott, Massachusetts) diluted in the primary antibody dilution buffer (1 x TBS, 5% BSA, 0.1% Tween) overnight at 4ºC. The secondary antibodies were horseradish peroxidase-conjugated donkey anti-mouse (Abcam, Cambridge, UK). Blots were visualized using Pierce SuperSignal (Pierce Bio Science) chemoluminescence and were exposed to BioMax X-ray films (Kodak).

Drug and DOX-TRF treatment

HL60 and HL60ADR cells (5 x 10^5 cells cm^{-3}) were treated with 1 µM DOX (Adriablastin® 50 mg HL, doxorubicin hydrochloride solution in a phosphate buffered saline-PBS, Pharmacia Upjohn, Germany) and DOX-TRF. At different times up to 120 h of incubation under standard conditions, the cells were washed twice with PBS (180 g, 24ºC, 10 min) and used for further experiments. DOX and DOX-TRF (1 µM) were added to a monolayer of SBL3F-cultured cells. After 0 to 120 h incubation under standard conditions, the cells were trypsinised, washed twice with PBS, centrifuged (180 g, 24ºC, 10 min) and used for further experiments.

Fluorescence measurements of the free drug and DOX-TRF distribution

Fibroblasts were seeded on cover slips in quadriperm culture dishes (In Vitro Systems, Göttingen, Germany) and allowed to adhere for 48 hours prior to the experiments. DOX and DOX-TRF (1 µM) were added to the monolayer of SBL3F cells and suspension of HL60 and HL60ADR (100,000 cells cm^{-3}). After up to 72 h of incubation in standard conditions, the cells were fixed for 15 min in 4% paraformaldehyde and visualized using a fluorescence-microscope (Zeiss, Axioplan, Oberkochen, Germany) with a suitable filter combination (Fluorescein/Texas Red) at a 600x magnification. The data was analyzed with

the Biomas image analyzing software (Erlangen, Germany) [18]. The values are given as grey values per nucleus [10, 11, 19, 20] after incubation with DOX and DOX-TRF.

DNA double-strand break analysis using constant field gel electrophoresis

Cells (1.25 x 10^6 cm^{-3} cells) were embedded in agarose and cut into plugs with 40,000 cells per plug. The protein was digested in Proteinase K (1 mg cm^{-3} Proteinase K, 2% SLS, 100 mM EDTA, 10 mM dm^{-3} Tris, 50 mM NaCl, pH 8) for 24 h at 50ºC. The plugs were inserted into the wells of a 0.5% agarose gel. The gel was run for 16 h at 0.85 V cm^{-1} and stained in ethidium bromide. The gels were transilluminated with 312 nm UV light. Images were acquired with a CCD camera (CF 20 DXC, Kappa, Gleichen, Germany). The luminescence (grey values) of the stained DNA is proportional to the amount of DNA. The luminescence of the plugs and lane were analyzed using Biomas (Erlangen, Germany) and the fraction of DNA released was calculated from the amount of DNA that migrated in comparison to the total amount of DNA [21].

Apoptosis assessment using the TUNEL assay

Apoptotic cells were identified using an Apopdetect Fluorescein Kit (Quantum Appligene, Heidelberg, Germany), as suggested by the manufacturer. Cells were stained with propidium-iodide. At least 1,000 cells per slide were counted blind with a fluorescence-microscope (Zeiss, Axioplan, Oberkochen, Germany) with a suitable filter combination (Fluorescein/Texas Red) at a 400x magnification. The data was analyzed with Biomas (Erlangen, Germany) [18]. The results are given as percentages (apoptotic cells/total cells x 100).

Cytotoxicity assay

Cytotoxicity was measured using a modified MTT assay previously described by Mosmann [22]. The two HL60 cell lines (in suspension) and the fibroblast cell line were seeded into 96-well plates at a density of 5,000 cells per well.

After 24 h (SBL3 cells were allowed to adhere), the cells were treated with different concentrations of DOX and DOX-TRF (0 to 15 µM). After 72 h of incubation at 37ºC in a humidified atmosphere containing 5% CO_2, MTT was added with an end concentration of 0.5 mg cm^{-3}, and the cells were incubated for a further 4 h. The cell viability after the elution of the dye with 99.5% DMSO was assessed by scanning the plates with an Perkin Elmer HTS 7000 Bio Assay Reader (Perkin Elmer, Überlingen, Germany) using a 600 nm filter. Cell viability was calculated as the percentage ratio of the sample absorbance to the reference control. IC_{50} represents the drug concentration showing a lethal effect on 50% of the cells.

Statistical analysis

Statistical significance was assessed using the two-tailed Student's t-test for unpaired samples. $P < 0.05$ was accepted as statistically significant. The results

are presented as the means ± SD of 3 to 6 independent experiments. The graphs were plotted with Origin 7SR2 (OriginLab Corp., Northampton, USA).

RESULTS

One of the major problems in cancer chemotherapy is the potential for severe side effects due to anticancer drugs' lack of selectivity between tumour and normal cells. This limits the usable dose of such drugs. This study was performed to analyse the influence of DOX-TRF conjugate compared to the free drug itself on promyelocytic leukaemia HL60 and HL60ADR cells and skin fibroblasts. We studied intranuclear DOX concentrations and the resulting induction of DNA damage, apoptosis and cytotoxicity in those cells.

DOX-TRF conjugate

After the conjugation procedure, DOX-TRF was chromatographed through Sepharose CL-4B. A spectroscopic analysis of each fraction for protein at 280 nm and for doxorubicin at 495 nm revealed two peaks (Fig. 1A). At peak 2, the position of the 280 nm maximum corresponded to the position of the 495 nm maximum for doxorubicin, indicating the conjugation of DOX to transferrin. Similar results were obtained using SDS-PAGE analysis. After the conjugation procedure and chromatographic clean-up, the DOX was shifted in the transferrin band (Fig. 1B). No free DOX was detectable, and a high amount of DOX was conjugated to TRF.

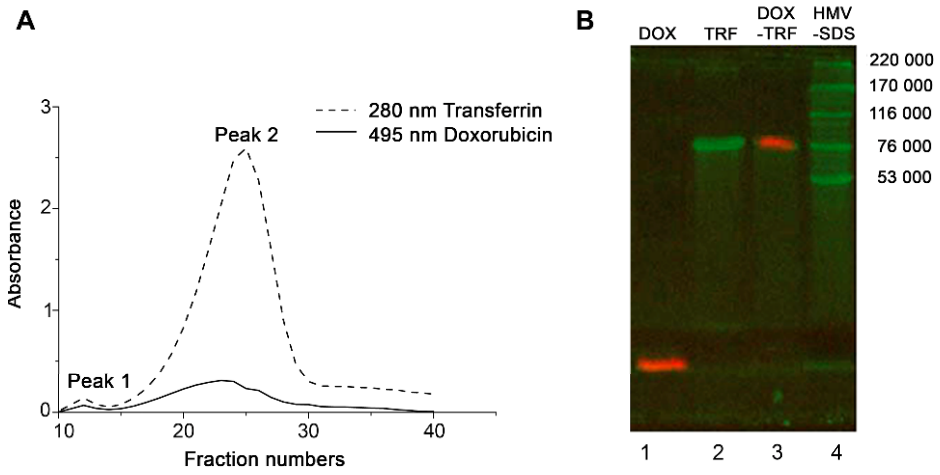

Fig. 1. Chromatography and SDS-PAGE of the DOX-TRF conjugate. A – Column chromatography of DOX-TRF conjugates. The absorbance of each fraction was determined for transferrin at 280 nm (dashed line) and for doxorubicin at 495 nm (solid line). B – SDS-PAGE of the DOX-TRF conjugate (lane 3) eluted in fractions 24 and 25. The positions of the DOX-TRF are shown relative to monomeric (76,000) human TRF (lane 2) and DOX (lane 1). The gel was calibrated by using the standard protein marker HMW-SDS (lane 4).

Transferrin receptor (CD71)

The levels of transferrin receptor on the HL60, HL60ADR and SBL3F cells were quantified by immunostaining and western blotting. The transferrin receptors (CD71) were numerous on the HL60 und HL60ADR cells (Fig. 2B, C). Transferrin receptors were not detectable in the fibroblast cell line SBL3F (Fig. 2A, C).

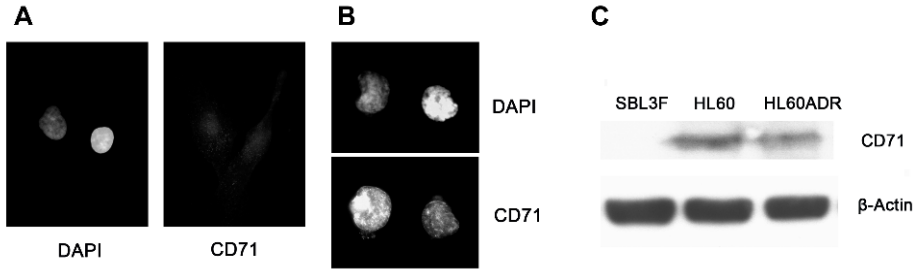

Fig. 2. The immunocytochemistry of the transferrin receptor (CD71) in: A – SBL3F and B –HL60ADR cells. The DNA of the cell nuclei was stained with DAPI. The CD71 were observed using a suitable fluorescence filter (Fluorescein). C – Western Blot analysis of the transferrin receptor (CD71) in SBL3F, HL60 and HL60ADR cells. Representative blots showing CD71 and β-actin levels.

Content of the free drug and DOX-TRF in the cells

Doxorubicin fluorescence in HL60 cells reached a maximal level following 1 h incubation and then rapidly decreased. However, after a prolonged time (24-72 h), the fluorescence of the drug once again slowly increased (Fig. 3B). The average fluorescence of the free DOX in the HL60ADR cells was markedly lower than in the sensitive HL60 cell line (Fig. 3C). In the same experiments, the fluorescence of DOX-TRF conjugate was significantly higher than that of free DOX both in HL60 and HL60 ADR cells (after 6 to 48 h). By contrast, the average fluorescence of the conjugate in SBL3F fibroblasts was slightly diminished as compared to the drug alone. These results clearly demonstrate that conjugation of doxorubicin to transferrin significantly increased the intracellular drug accumulation in the HL60 and resistant HL60ADR cells.

DNA double-strand break analysis

After treatment with DOX and DOX-TRF, DNA double-strand breaks (DSBs) were measured by constant field gel electrophoresis. Both forms of DOX contributed to the DNA double-strand breaks in the HL60 and HL60ADR cells. As shown in Fig. 4 A, B, the pronounced differences in the percentage of DNA released in DOX- and conjugate-treated HL60 and HL60 ADR cells were respectively observed between 6 and 12 h and between 24 and 48 h. In both cell lines, DOX-TRF conjugate induced far more DNA double-stand breaks than the free drug. In the fibroblast cell line, no DNA DSBs were detectable after incubation of the cells with 1 μM DOX or its conjugate (Fig. 4 C, E).

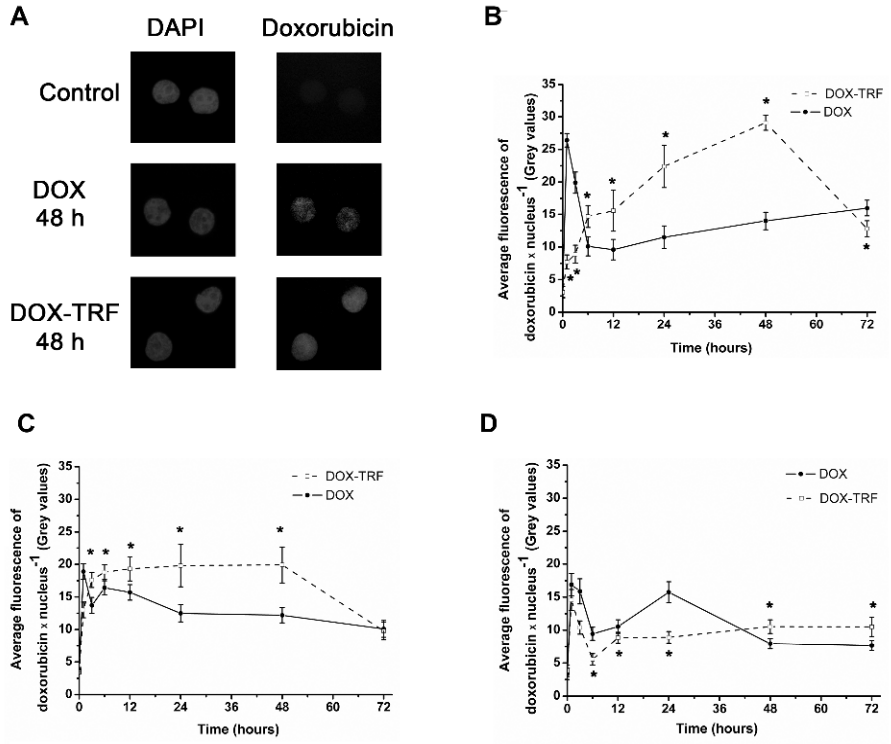

Fig. 3. The concentration of DOX (—●—) and DOX-TRF (--□--) in the nucleus, given as fluorescence grey values per nucleus. A –The DNA of the cell nuclei was stained using DAPI. The DOX concentration in the region of the nuclei was quantified using a fluorescence filter (Texas Red). The concentrations were quantified in: B – HL60, C –HL60ADR and D – SBL3F cells. Each point represents the mean values of 5 separate experiments with SDs. *Values statistically significant in comparison to free doxorubicin ($p < 0.0001$).

Induction of apoptosis after DOX and DOX-TRF treatment

TUNEL staining of DOX- and DOX-TRF-treated cells showed that the extent of apoptosis was dependent on the cell line and incubation time. The percentage of apoptotic cells in both promyelocytic cell lines increased with the time of incubation. In the HL60 cells, a maximal increase in the number of apoptotic cells (about 95%) was observed 120 h after exposure to DOX and 72 h after conjugate treatment (Fig. 5A). The level of apoptosis in the HL60ADR cell line was markedly lower in comparison to that for the HL60 cells. At 120 h, the extent of apoptosis in the DOX- and DOX-TRF-treated cell lines respectively reached 30% and 55% (Fig. 5B). No apoptotic cells were detected in the SBL3F cell line (data not shown). These findings strongly demonstrate that DOX-TRF conjugate was able to induce apoptosis in parental HL60 and HL60 ADR resistant cells. However, the extent and rate of apoptosis in this last cell line

were decreased compared with HL60, and the conjugate was more effective than the free drug at inducing apoptosis.

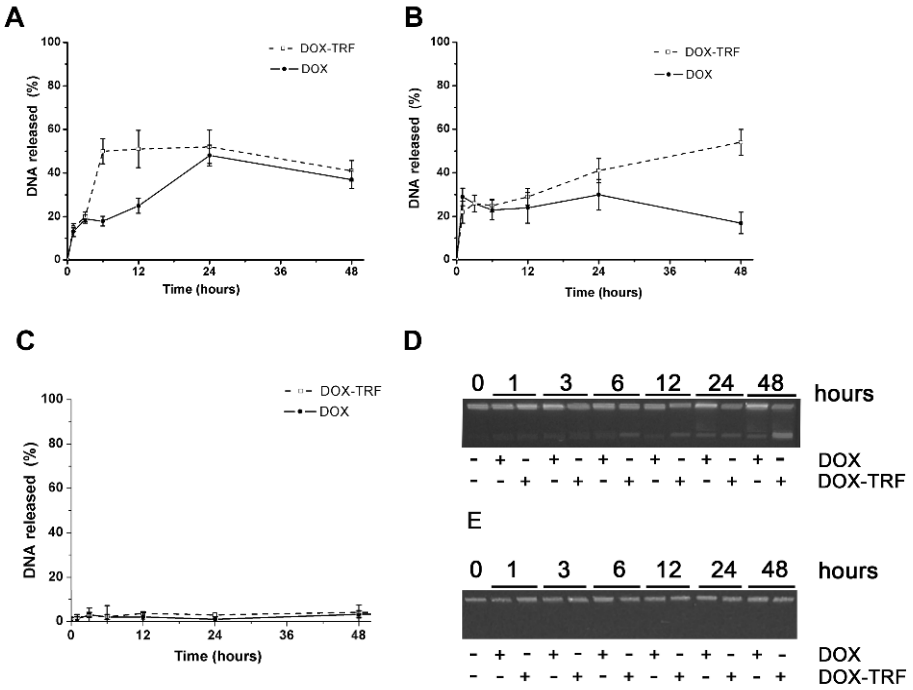

Fig. 4. DNA DSBs in: A – HL60, B – HL60ADR and C – SBL3F. Using constant field electrophoresis, the initial and remaining DNA DSB damage after 0-72 h of treatment with DOX (—●—) and DOX-TRF (--▫--). The mean values of 4 separate experiments with SDs show the percentage of migrating DNA. *Values statistically significant in comparison to the free doxorubicin ($p < 0.0001$). D, E – Sections of an electrophoresis gel showing initial DNA DSBs and repaired DNA DSBs 0-48 h after treatment with DOX and DOX-TRF in HL60 (D) and SBL3F (E) cells.

Cell viability measurement

Cell viability was estimated 72 h after treatment, using the MTT assay. The respective DOX and DOX-TRF IC_{50} values for HL60 cells were 0.08 μM and 0.02 μM, whereas 7 μM doxorubicin and 0.035 μM DOX-TRF were required to cause 50% growth inhibition in the doxorubicin-resistant cell line (Tab. 1, Fig. 6A). The resistance to DOX of HL60ADR cells was approximately 90 times higher than that of the parental cell line. However, the IC_{50} for DOX-TRF for the doxorubicin resistant cell line was only 1.5 times higher than for the HL60 cells. In the fibroblast cell line (Fig. 6B), increased doses of DOX and DOX-TRF were needed to obtain an overall IC_{50} of 1.8 μM for the free drug and 2.8 μM for the conjugate (Tab. 1). DOX-TRF reduced the viability of all the leukaemia cell lines more efficiently than the free drug.

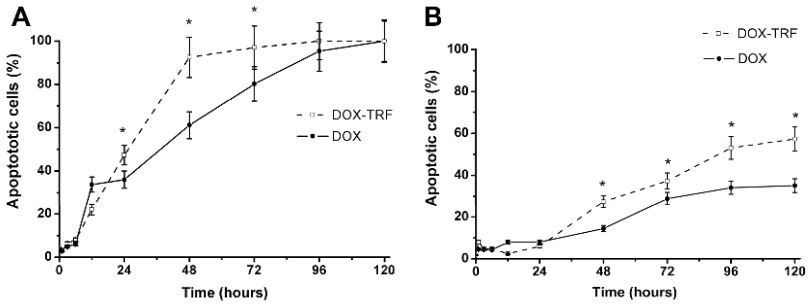

Fig. 5. Apoptotic rates measured using the TUNEL assay after treatment with DOX (—●—) and DOX-TRF (--▫--) at different time points. The increases in the level of apoptosis observed in: A – the parental cell line and B – HL60ADR were significantly higher after treatment with DOX-TRF conjugate in comparison to the results with free DOX ($p < 0.001$). Each point represents the average ± SD of six independent experiments. *Values statistically significant in comparison to the free DOX ($p < 0.0001$).

Tab. 1. The IC_{50} parameter for the HL60, HL60ADR and SBL3F cells. Mean values of 6 independent experiments, each with SDs. *Values statistically significant in comparison to the IC_{50} for doxorubicin ($p < 0.0001$).

	HL60	HL60ADR	SBL3F
IC_{50} DOX (μM)	0.08 ± 0.007	7.0 ± 840	1.8 ± 0.370
IC_{50} DOX-TRF (μM)	0.02 ± 0.002*	0.035 ± 0.002*	2.8 ± 0.170*

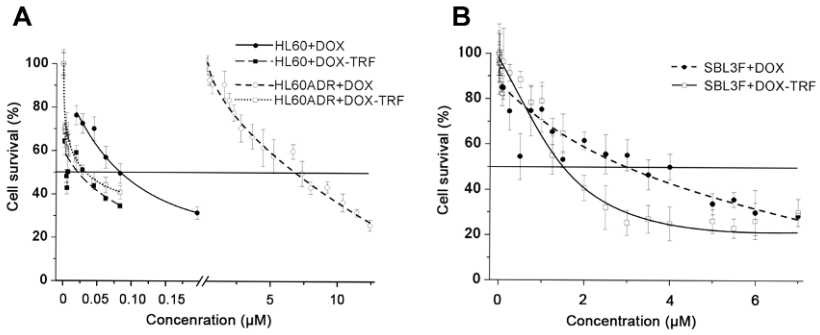

Fig. 6. The IC_{50} parameter for: A – HL60 and HL60ADR cells and B – SBL3F cells. Cell viability was estimated using the MTT-test. Mean values of 6 separate experiments, each with SDs.

DISCUSSION

The anthracycline antibiotic doxorubicin (DOX) is a commonly used chemotherapeutic agent in the treatment of leukaemia, breast cancer and many other cancers. The cytotoxic action of DOX is mainly due to its intercalation into DNA and blocking of the enzymatic activity of topoisomerase II [23]. It is well known that cancer chemotherapy remains largely non-specific, and agents like DOX are toxic for normal cells as well as for tumour cells. Furthermore, the potential benefits of DOX may be blocked by the development of drug-resistant cancer cells. Neoplastic cells have increased numbers of receptors for transferrin (150,000-1,000,000 per cell) compared to normal cells like fibroblasts [24]. Therefore, transferrin can deliver drugs directly to neoplastic cells with a reduced injury to normal tissue cells [6, 25]. Coupling doxorubicin to transferrin may alter the drug disposition, improve the therapeutic index and overcome resistance to conventional therapy [14]. In this study, we used a DOX-TRF conjugate and compared its ability to induce DNA damage, apoptosis and viability in three cell lines with that of the free drug. The cell lines were HL60, the multidrug-resistant HL60ADR, and a human fibroblast line. We investigated in one setting the whole cause-and-effect chain from the uptake and accumulation in the cell nuclei of free DOX and DOX-TRF, through acquired DNA double-strand breaks, up to the induction of apoptosis and cell death.

In this study, we showed that a multidrug-resistant leukaemia cell line and its parental cell line had similar amounts of transferrin receptors, while in the fibroblast cell line in our setting, the transferrin receptors were not detectable. In contrast to the uptake of the free drug by diffusion, the DOX-TRF conjugate undergoes receptor-mediated endocytosis [4, 13, 26]. It is likely that these differences are responsible for the higher toxicity of the conjugates for the sensitive and resistant tumour cells [27]. Moreover, overexpression of transferrin receptors on the membranes of tumour cells may play an important role in DOX-TRF delivery.

The uptake of DOX and DOX-TRF can be determined by the accumulation of the free drug in the cell nuclei. In the study of Barabas *et al.* [28], DOX and DOX-TRF (10 μM) were incubated with K562 cells for 1 h. The free DOX localised to the cell nuclei and DOX-TRF to the cell membrane. No DOX-TRF fluorescence was observed in the cell nuclei after this period.

In our study, DOX and DOX-TRF (1 μM) were added to the HL60, HL60ADR and SBL3F cells and incubated for up to 72 h (0, 1, 3, 6, 12, 24, 48, 72 h). Both leukaemia cell lines and the fibroblasts were able to increase the efflux of DOX very efficiently. DOX fluorescence in the HL60 cells reached a maximal level following 1 h incubation (as in the study of Barabas *et al.*) and then rapidly decreased. However, after a prolonged time (24-72 h), the fluorescence of DOX-TRF once again slowly increased (Fig. 3B). The average fluorescence of free DOX in the HL60 ADR cells was markedly lower than in the sensitive HL60 cell line (Fig. 3C). In the same experiments, the fluorescence of DOX-TRF

conjugate was significantly higher than the free DOX, both in the HL60 and HL60 ADR cells (after 6 to 48 h).

In the multidrug-resistant HL60ADR cells, DOX-TRF was delivered to the cells whereas the free drug was released from the cell (Fig. 3B), probably by MDR-associated proteins [14]. In the fibroblasts [29], the conjugated DOX remained at low concentrations for up to 72 h. Our results demonstrate that the conjugation of doxorubicin to transferrin significantly increased the intracellular drug accumulation in HL60 and resistant HL60ADR cells after a longer incubation time.

The dissimilarity of uptake of DOX and its TRF conjugate should be reflected in the interactions with the DNA [19]. DNA double strand breaks (DSBs) were not detectable in the fibroblasts. We found DNA-DSBs in leukemia HL60 and HL60ADR cells after treatment with DOX or DOX-TRF. In the multidrug resistant cell line, DOX-TRF induced more DNA-DSBs compared to the free drug. It shows that DOX-TRF induced DSBs more efficiently than free DOX, probably because of its specific delivery system (receptor-mediated endocytosis) and the higher intracellular accumulation of DOX-TRF.

Our *in vitro* study revealed that in the resistant cell line HL60ADR, the increase in the apoptotic rates was triggered by DOX-TRF to a much higher extent than in the parental cell line. The influence of DOX-TRF conjugate on DSBs formation and apotosis in DOX-resistant leukaemia cells was not previously demonstrated, although it was demonstrated that apoptotic rates after doxorubicin therapy correlate with theraputic success [30].

DOX-TRF is toxic against a variety of human cell lines including HL60 [10, 11, 19, 20] and cell lines resistant to DOX: KB [14], HL60ADR [19], and MCF-7 [9]. Due to the targeted delivery of DOX into tumour cells, the conjugate was 3- to 10-fold more cytotoxic than the free drug, depending on the cell line [27]. DOX-TRF conjugate showed an increase of growth inhibition, as evaluated by the MTT-assay, in both of the leukaemia cell lines, HL60 and HL60ADR, compared to free drug [19, 20]. Based on the cytotoxicity MTT-Assay, Berczi *et al.* [11] found that free DOX at a concentration of 0.05 μM had little effect on HL60 cells, while DOX-TRF used at this concentration inhibited 49% of cellular activity. Fritzer *et al.* [14] examined the cytotoxicity of DOX-TRF conjugate and compared it with DOX in sensitive (KB-3-1) and in multidrug-resistant KB cell lines (KB-8-5, KB-C1, and KB-V1). In the clonogenic assay, DOX exhibited respective IC_{50} concentrations of 0.03 and 0.12 μM in the sensitive (KB-3-1) and resistant (KB-8-5) cell lines, whereas DOX-TRF conjugate was more effective, with respective IC_{50} concentrations of 0.006 and 0.028 μM. In highly multidrug-resistant KB-C1 and KB-V1 cells, DOX up to 1 μM did not cause any cytotoxic effects, while the DOX-TRF conjugate inhibited the colony formation of these cells with IC_{50} levels of 0.2 and 0.025 μM respectively. These results demonstrate that DOX-TRF is effective against multidrug-resistant tumour cells. Moreover, the DOX-TRF conjugate was 4 times more active against resistant cell line HL60 compared to the sensitive parental cell line [10].

Our cytotoxicity study showed that the IC_{50} for DOX-TRF was lower compared to the IC_{50} value for the free drug, for both leukaemia cell lines. The IC_{50} values for HL60 cells were 0.08 μM for DOX and 0.02 μM for DOX-TRF. The respective IC_{50} values for HL60ADR cells were 7 μM and 0.035 μM. Most impressive was the cytotoxic effect in the leukaemia cells, where the MDR cell line was 90 times more resistant to DOX than the parental cell line; however, using DOX-TRF, the ratio was only 1.5, so DOX-TRF could nearly completely overcome the multidrug resistance.

Expression of the transferrin receptors is significantly upregulated in a variety of malignant cells [7], and the conjugation of anticancer drugs with transferrin might deliver the therapeutics directly to the neoplastic cells (by receptor mediated endocytosis) with a reduced injury to normal cells [4-6]. Normal peripheral blood mononuclear cells were tested against DOX-TRF conjugates, and the 50% inhibitory concentration was found to be 1.4-1.7 μM [31]. Our results confirmed that DOX-TRF has only a limited effect on normal tissue cells without overexpression of transferrin receptors (the IC_{50} for DOX-TRF is 2.8 μM). Our studies demonstrate that transferrin can be successfully used as an ligand for directing anticancer drugs to human tumour cells, and the conjugation of transferrin to doxorubicin significantly increased not only the intracellular accumulation of drug but it also effectively potentiated the doxorubicin damage to DNA and remarkably elevated the extent of apoptosis in the resistant HL60ADR cells. The data presented suggests that the transferrin can successfully be used as carrier molecule for the targeted delivery of doxorubicin to resistant leukaemia cells. Moreover, our results show that the conjugation of doxorubicin to transferrin has only a limited effect on normal tissue cells without overexpression of the transferrin receptor, and simultaneously can overcome multidrug resistance in receptor-positive cancer cells.

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