APOFERRITIN-MEDIATED DOXORUBICIN INTERNALIZATION THROUGH TRANSFERRIN RECEPTOR 1

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Abstract: This work is aimed at the possibilities of targeted drug delivery into the tumour tissue. This approach can greatly reduce the otherwise serious side effects of conventional treatment – systemic toxicity. For this purpose, ubiquitous protein cage apoferritin was employed as a carrier of cytotoxic drugs. Its molecule size of 10–12 nm allows it to employ the effect of increased permeability and retention as well as to avoid renal clearance. The cellular uptake of this carrier is known to be mediated *via* the transferrin receptor 1 (TfR1), which is overexpressed on metabolically highly active cells, such as cancer cells. Therefore, apoferritin's ability to deliver drug molecules to site-of-action was tested using cell lines with high, medium and low expression of TfR1. The optimal conditions for studying the expression of TfR1 using western blotting were as follows: lysate of 50 000 cells applied in non-reducing non-denaturing buffer and the concentration of the primary antibody of 1.0 µg/ml. The properties of encapsulated doxorubicin were not affected by apoferritin, thus preserving its toxicity for cells with high level of TfR1expression (30% growth inhibition of these cells after 24 h of treatment). The suitable usage of apoferritin as a nanocarrier for chemotherapeutic delivery was confirmed in this work.

Key Words: apoferritin, cancer, doxorubicin, nanomedicine

INTRODUCTION

Even with advances in health care, more and more people die of cancer every year. The main guilt lies on unhealthy life style, the environment we live in, and also on genetic predisposition. Every 5th woman and every 4th man in developed countries dies of cancer. Although these figures are alarming, not only the mortality itself but also the quality of life of patients with diagnosed and treated cancer should raise the question. Although we are able to treat various types of cancer, each of the administered treatment is accompanied by many side effects that negatively affect not only the physical but also the psychological aspects of patients' lives.

Chemotherapy can be divided into groups based on various factors including their chemical composition and function. This work is focused on anthracycline doxorubicin, one of the most commonly used chemotherapeutics (Erkekol 2011). Doxorubicin has been used for the treatment of cancer for over 30 years. Although its ability to kill fast-dividing cells and to slow development of disease is known for several decades, its use is limited by its high toxicity. Like most drugs, doxorubicin enters the cells through passive diffusion. It mainly accumulates in the liver, most likely due to the role of liver in drug metabolism. Up to 40% of patients with this treatment suffer from some form of liver damage. One of the other reasons why dose of doxorubicin should be limited is its

cardiotoxicity. Patients are negatively affected to varying degrees from chronic to acute conditions. Doxorubicin is responsible for structural changes in cardiomyocytes in the heart, especially their magnification (Carvalho et al. 2009).

Systemic toxicity of anti-cancer treatment can be limited by its targeting to tumour cells only. This can be limited by encapsulation of chemotherapeutic drugs into a suitable carrier that can be targeted to specific glycoproteins overexpressed on the membranes of tumour cells. One type of such antigens is the transferrin receptor 1(TfR1). This work deals with the employment of natural antigen for these receptors – apoferritin, which uses these transferrin receptors for internalization in cells. Apoferritin is suitable for use as nanocarrier of anti-tumour drugs to tumour cells, due to its properties. Unlike other, artificial nanocarriers, apoferritin's particles are homogeneous and have a uniform size of approximately 12 nm (Iwahori et al. 2005). Its main advantage is its ability of reversible dissociation and association depending on the surrounding pH, whereby small-molecule drugs can be easily and efficiently encapsulated into the internal cavity of the apoferritin without use of any organic solvents that are needed for encapsulation in other nanocarriers. Its safety and biodegradability are also ensured, due to its natural presence in organisms (Zang et al. 2017).

In this study, the conditions for the study of TfR1 expression in various tumour cell lines were optimized. The results obtained were verified by short- and long-term *in vitro* testing of the anti-tumour effect of apoferritin loaded with doxorubicin.

MATERIAL AND METHODS

Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Transferrin receptor 1 expression study

Quantitative expression of transferrin receptor 1 (TfR1) in cell lysates was studied using western blot. 50 000 cells were lysed with RIPA buffer and 10 µg of the lysate proteins was separated on 12.5% SDS PAGE. The proteins were transferred to the Immun-Blot® PVDF membrane (Bio-Rad, Hercules, CA, USA). Anti-Transferrin Receptor antibody 13E4 (ab38171, Abcam, Cambridge, UK) was used, diluted in antibody buffer [1 mg/ml BSA in phosphate buffered saline (PBS)] in 1 : 2000, 1 : 1500, 1 : 1000 and 1 : 500 ratio, respectively.

Study of the short-term effect of apodox on cells with varying levels of TfR1 expression

The encapsulation of doxorubicin into apoferritin (creating apodox) and doxorubicin concentration measurements were performed according to previous publication (Dostalova et al. 2017). Internalization of doxorubicin / apodox into cells and their short-term effects on them were monitored by ambient and fluorescence microscopy using the IX 71S8F-3 (Olympus, Tokyo, Japan). 1×10^5 UKF-NB4 (high TfR1 expression), PC-3 (medium TfR1 expression), and MDA-MB-231 (low TfR1 expression) cells in 1 ml of IMDM (for UKF-NB4) or RPMI 1640 (for PC-3 and MDA-MB-231) medium was seeded in a 12-well culture plate and incubated for 21 h. After the incubation, the medium was replaced with 200 µl of fresh medium containing 34 µM doxorubicin / apodox. The doxorubicin / apodox-treated cells were incubated for additional 2 h. Living cells were stained with CellRox® Green (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Cell morphology was monitored under ambient light, fluorescein isothiocyanate filter (excitation of 460–495 nm, emission of 510–550 nm, dichroic mirror at 505 nm) was used to visualize oxidative stress, and fluorescence of doxorubicin was monitored using the Texas Red filter (excitation of 545–580 nm, emission of 610 nm, dichroic mirror at 600 nm) at 200-times magnification. All photos were uploaded and edited using the Stream Basic software.

Study of the long-term effect of apodox on cells with variying levels of TfR1 expression

To monitor the long-term effect of doxorubicin and apodox on tumour cell lines with varying levels of TfR1 expression, the XCELLigence RTCA DP (Roche Diagnostics, GmbH, Basel, Switzerland) was used. The background impedance was measured with 100 µl of culture media with doxorubicin / apodox. Then, 1.5×10^4 cells in 100 µl of culture medium were plated in 16-well

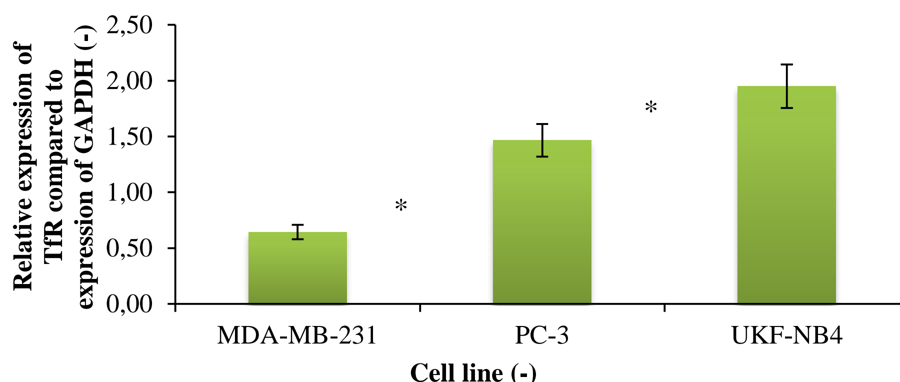
culture plate (Roche Diagnostic GmbH). Incubation was carried out at 37 °C in an atmosphere containing 5% CO_2. Cell proliferation was monitored every 30 s for 10 min, then every 30 min for 24 h. The experiments were performed in triplicates.

RESULTS AND DISCUSSION

Apoferritin is a protein cage composed of 24 subunits self-assembled by ferritin subunits in medium that does not contain iron. Ferritins are present in almost all living organism where their main function is the storage and transfer of iron (Zang et al. 2017). Ferritin employs TfR1 to internalize into cells (Suzumoto et al. 2012). According to literature, TfR1 is extensively expressed by some neoplastic cells on the surface of their cytoplasmic membrane to satisfy their higher metabolic needs (Peer et al. 2007).

To test the possibility of TfR1 use in nanomedicine, its expression in various cancer cell lines was evaluated (Figure 1). For this purpose, following cell lines were used: MDA-MB-231 (breast cancer cell line), PC-3 (prostate cancer cell line) and UKF-NB4 (neuroblastoma cell line). Significant differences in TfR1 expression were observed among these cell lines. UKF-NB4 showed the highest TfR1 expression (relative TfR1 expression of 1.9). PC-3 showed medium levels of TfR1 expression (1.4). The lowest levels of TfR1 expression were found in MDA-MB-231 cells (of 0.6).

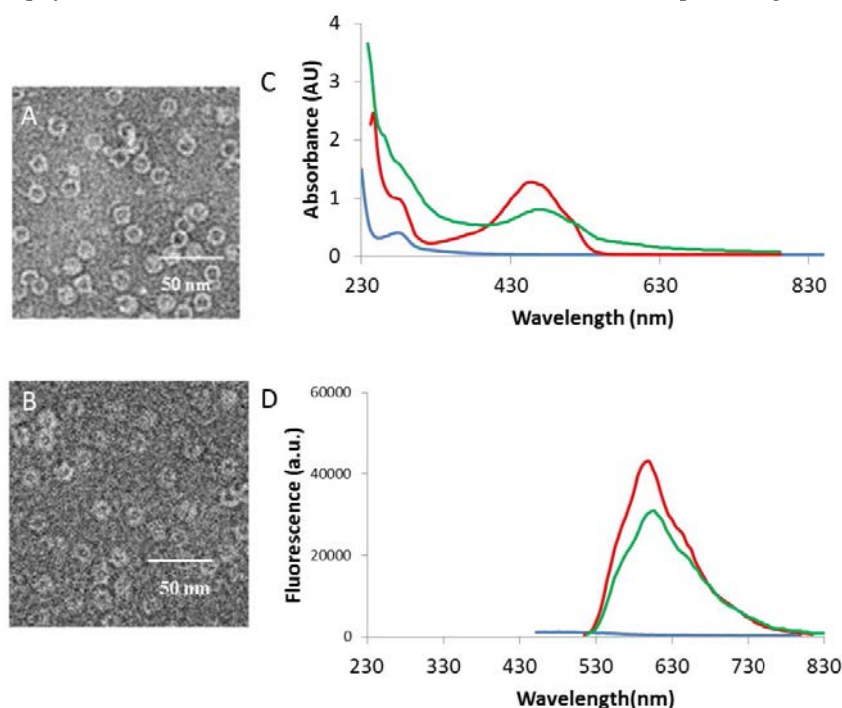
*Figure 1 Relative expression of TfR1 in different cell lines. MDA-MB-231- breast tumor; PC-3 - prostate cancer androgen independent; UKF-NB4 - neuroblastoma. * Determines a statistically significant difference ($p < 0.05$) in TfR1 expression between UKF-NB4 cells and other cell lines*



Even though apoferritin's cavity is naturally used to store iron ions, it can be artificially used to store and transfer any molecule of suitable molecular weight. Nanoparticles with a diameter below 100 nm can use the enhanced permeability and retention (EPR) effect in tumour tissues, so they can easily get into irregular, leaky tumour vessels with relatively large pores. In contrast, nanoparticles below 10 nm can be removed by normal vascular extravasation and renal clearance (Svenson 2013). Due to its size, apoferritin can employ the EPR effect of tumours for accumulation in cancerous tissue (Suzumoto et al. 2012) and TfR1 can then be employed in order to penetrate inside individual tumour cells (Peer et al. 2007). Moreover, apoferritin is suitable as a nanocarrier also due to its biocompatibility, high symmetry, solubility and stability, uniformity and ease of genetic and chemical manipulation (Zang et al. 2017).

The structure of the hollow protein cage was verified using a transmission electron microscope (Figure 2A). The size of the molecule was proven to be as expected, 10–12 nm in diameter (Suzumoto et al. 2012). The size did not increase after encapsulation (Figure 2B) and apoferritin retained its structure of icosahedral cage, although it can be seen that its cavity was filled by doxorubicin molecules, compared to apoferritin which showed empty cavity. However, the increase of its negative surface charge from -19.8 mV to -26.0 mV demonstrated that some doxorubicin molecules were probably bound to the surface of apoferritin during encapsulation and were not only encapsulated in the cavity. The amount of these doxorubicin molecules on the outer surface of apoferritin was low enough to not change the overall size of apoferritin (10–12 nm).

Figure 2 (A) – TEM apoferritin; (B) – TEM apodox; (C) – Absorbance spectra of apoferritin (blue colour), doxorubicin (red colour) and apodox (green colour); (D) – Fluorescence spectra of apoferritin (blue colour), doxorubicin (red colour) and apodox (green colour)



Furthermore, the optical properties of apoferritin, doxorubicin and apodox were characterized. The absorption spectrum (Figure 2C) clearly showed that apoferritin molecules absorbed only in UV area (with a specific absorbance of aromatic amino acids at 280 nm). Doxorubicin showed specific absorbance with maximum at 480 nm. Apodox showed absorbance spectrum containing both peaks characteristic for apoferritin and doxorubicin, although absorbance of encapsulated doxorubicin was lower than that of free doxorubicin. After filtration of non-encapsulated doxorubicin molecules, encapsulation efficiency was measured as 77%.

Doxorubicin also showed fluorescence after excitation at 480 nm (Figure 2D). It retained its fluorescent properties after encapsulation in apoferritin (creating apodox), which could be useful for detection of doxorubicin distribution *in vivo*. Due to the conditions for detection of doxorubicin, apoferritin fluorescence was examined at excitation wavelength of 480 nm. It is clear that apoferritin under these conditions showed no fluorescence.

Study of the short-term effect of apodox on cells with varying levels of TfR1 expression

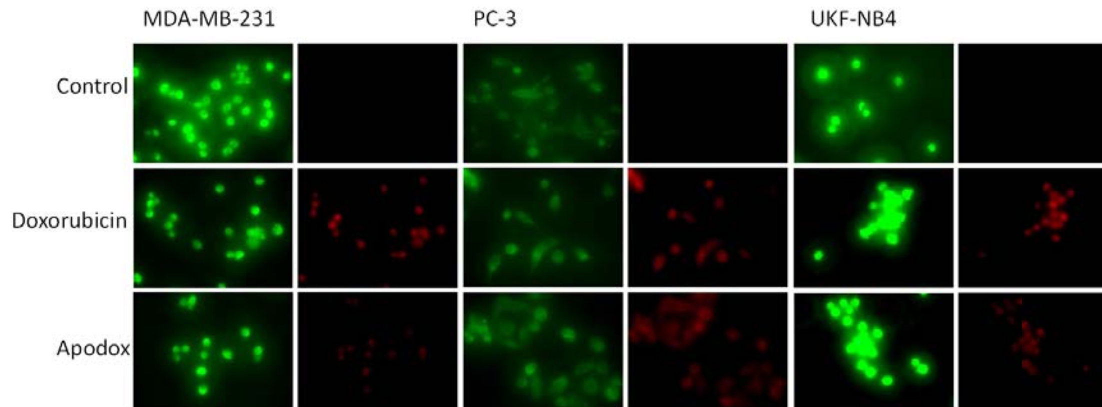
Thanks to the optical properties of doxorubicin, fluorescence microscopy was employed to evaluate the internalization of doxorubicin and apodox into tumour cells with different expression of TfR1 and their influence on oxidative stress of these cells (Figure 3). Oxidative stress is one of the main mechanisms by which doxorubicin and therefore apodox eliminate tumour cells (Shafiei-Roudbari et al. 2017).

No autofluorescence after excitation at 480 nm was detected in control sample of untreated cells. Free doxorubicin fluorescence differed among the cell lines. The highest fluorescence (39 a. u.) was observed in the tumour cell line with high TfR1 expression (line UKF-NB4). Medium doxorubicin fluorescence (35 a. u.) was observed in cell line PC-3 and lowest fluorescence (31 a. u.) was observed in cell line MDA-MB-231.

Oxidative stress is produced as a by-product of metabolism, by oxidation, degradation and detoxification of reactive intermediates. Therefore, this condition was observed not only in treated cells, but also untreated ones. After application of apodox and free doxorubicin, an increased oxidation stress level was expected. The level of oxidative stress caused by free doxorubicin was highest in case of UKF-NB4 (32 a. u.). Medium level of oxidative stress was observed in MDA-MB-231 cell line

(8 a. u.), while PC-3 cell line showed similar level of oxidative stress after doxorubicin treatment as in untreated cells.

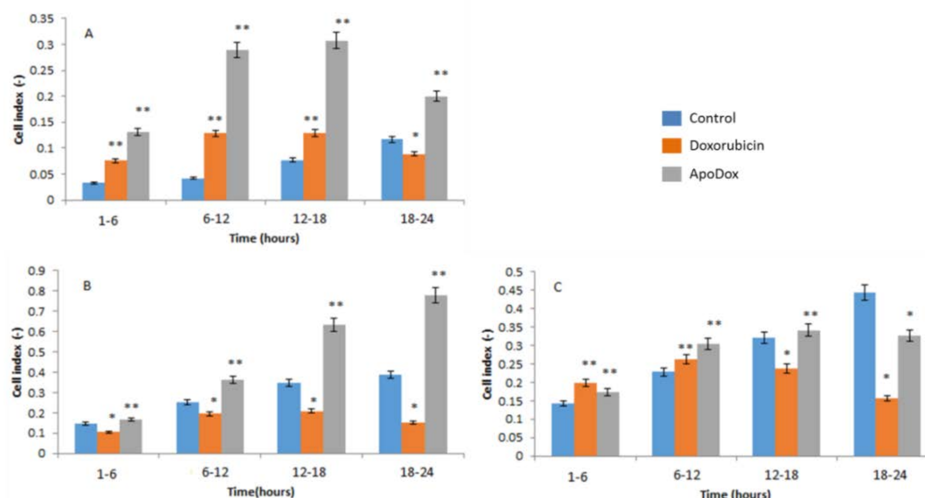
Figure 3 Fluorescence microscopy showing fluorescence (red colour) of doxorubicin and doxorubicin encapsulated in apoferritin (apodox) internalized into tumour cell lines with low (MDA-MB-231), medium (PC-3) and high (line UKF-NB4) expression of TfR1. Fluorescence microscopy showing the degree of oxidative stress (green colour) after treatment with doxorubicin and apodox



In the case of apodox treatment, the dependence of internalization of apodox into the cells was assumed to be higher. The difference between the cancerous cell lines with the highest and lowest TfR1 expression was statistically significant. The higher the expression of TfR1, the higher was the observed apodox fluorescence (33 a. u. for UKF-NB4, 29 a. u. for PC-3 and 27 a. u. for MDA-MB-231). However, oxidative stress showed different trend where highest level was observed in UKF-NB4 cells (26 a. u.), followed by MDA-MB-231 cells (4 a. u.), while PC-3 cells showed no level of oxidative stress when compared to untreated cells.

Study of the long-term effect of apodox on cells with varying levels of TfR1 expression

*Figure 4 Cell proliferation rates at 6 h intervals at the MDA-MD-231 (A); PC-3 (B) and UKF-NB4 (C) cell line untreated and treated with doxorubicin and apodox. * Determines a statistically significant ($p < 0.05$) decrease in the growth of the treated cells as compared to that of control cells. ** determines a statistically significant increase ($p < 0.05$) in the growth of treated cells compared to that of control cells*



Since the internalization of free doxorubicin and apodox showed the same trend in the short-term study, and the fluorescence of doxorubicin at its high concentrations may not be reliable, the long-term effect of doxorubicin and apodox on these cells was further studied (Figure 4).

The growth of all of the tested cell lines was inhibited after 24 h of doxorubicin treatment (25% for MDA-MB-231, 63% for PC-3 and 66% for UKF-NB4). However, their growth during treatment with apodox was significantly dependent on their TfR1 expression. Both MDA-MB-231 (Figure 4A)

and PC-3 (Figure 4B) cell lines showed increase in their growth after treatment with apodox. This was probably caused by hormetic effect, increase in cellular growth caused by adaptive response of cells to low concentration of toxic molecules (Mattson 2008). However, cells with high TfR1 expression were apparently able to internalize enough apodox to inhibit their growth by 30% after 24 h treatment. The significant differences caused by different TfR1 expression show reliable natural targeting of apoferritin nanocarrier and therefore its suitability for use in nanomedicine.

CONCLUSION

The experiment presented in this work dealt with the evaluation of the ability of naturally occurring and versatile protein apoferritin to deliver anti-tumour drugs selectively to cancer cells *via* transferrin receptors. Overall, the presented nanocarrier showed the optimal size in the range of 10–12 nm, required for passive targeting to tumours *via* EPR effect, while avoiding removal from the body through renal clearance. The various degrees of internalization of apodox into cells with different TfR1 expression and its long-term effect on these cells have been observed. This has confirmed the presumption of transferrin receptor function as a mechanism by which the nanocarrier penetrates metabolically highly active cancer cells.

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