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Cytotoxicity, intracellular distribution and uptake of doxorubicin and doxorubicin coupled to cell-penetrating peptides in different cell lines: A comparative study

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ABSTRACT

One of the major obstacles which are opposed to the success of anticancer treatment is the cell resistance that generally develops after administration of commonly used drugs. In this study, we try to overcome the tumour cell resistance of doxorubicin (Dox) by developing a cell-penetrating peptide (CPP)-anticancer drug conjugate in aim to enhance its intracellular delivery and that its therapeutic effects. For this purpose, two cell-penetrating peptides, penetratin (pene) and tat, derived from the HIV-1 TAT protein, were chemically conjugated to Dox. The cytotoxicity, intracellular distribution and uptake were accessed in CHO cells (Chinese Hamster Ovarian carcinoma cells), HUVEC (Human Umbilical Vein Endothelial Cells), differentiated NG108.15 neuronal cell and breast cancer cells MCF7drug-sensitive or MDA-MB 231 drug-resistant cell lines. The conjugates showed different cell killing activity and intracellular distribution pattern by comparison to Dox as assessed respectively by MTT-based colorimetric cellular cytotoxicity assay, confocal fluorescence microscopy and FACS analysis. After treatment with 3 μM with Dox–CPPs for 2 h, pene increase the Dox cytotoxicity by 7.19-fold in CHO cells, by 11.53-fold in HUVEC cells and by 4.87-fold in MDA-MB 231 cells. However, cytotoxicity was decreased in NG108.15 cells and MCF7. Our CPPs–Dox conjugate proves the validity of CPPs for the cytoplasmic delivery of therapeutically useful molecules and also a valuable strategy to overcome drug resistance.

Introduction

Chemotherapy treatment is limited by the ability of cancer cells to become resistant to different drugs at the same time, a trait known as multidrug resistance (MDR). The mechanisms of drug resistance can happen at many levels, including alteration of the target protein, decreased membrane permeability and drug metabolism, increased processing of drug induced damage, or evasion of apoptosis [1,2]. Contrary to that, the principal mechanism of multidrug resistance is the active transport of drugs out of the cell by the ATP-binding cassette (ABC) transporters which are a family of proteins that mediate MDR via ATP-dependent drug efflux pumps.

In this study, we have concentrated on doxorubicin (Dox), one of the most effective anticancer drugs [3]. The major mechanism of Dox activity is the inhibition of topoisomerase II and stabilization of a ternary drug-topoisomerase II (TOPO II)-DNA complex, causing DNA damage and induction of apoptosis [4,5].

Dox resistance in tumor cells can arise in many ways and that resistance is often multifactorial [4]. The main mechanisms recognized so far are: (i) the classic MDR phenotype, which is due to the presence of P-glycoprotein (PGP) in plasma membrane, that is, a "pump" that can extrude a wide range of anticancer drugs [6]; (ii) rised GST content and detoxification mechanisms [4]; (iii) alteration in TOPO II which can be divided into two groups: (1) quantitative changes such as, decreased levels of TOPO II protein through downregulation of transcription or increased degradation or deletion of one allele and (2) qualitative changes such as mutations due to the alteration of ATP binding or drug-topoisomerase II-DNA interaction, or altered enzymatic function by posttranslational modification [4].

Conjugation of Dox, for improved efficiency and/or delivery has been developed in the last decade, for that, several approaches have been tested such as entrapping it in submicron carriers like

Abbreviations: Con A, concanavalin A; CPP, cell-penetrating peptide; Dox, doxorubicin; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate buffered saline; Pene, penetratin.

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liposomes [7], polymeric micelles [8] and nanoparticles [9] or coupled it to lactosaminated human albumin [10,11]. Recently, reports have focused on the subject of a new vehicle for intracellular delivery, namely cell-penetrating peptides (CPPs). CPPs are short peptides which are able to translocate a large variety of cargo molecules into a wide range of cell types [12]. Members of this interesting class of peptides include penetratin (pene), which constitutes the third helix of the Antennapedia homeodomain [13,14] or tat (48–60) which is derived from the HIV-1 TAT protein.

In this study, we have explored the efficiency of coupling Dox to tat and pene in five cell lines which differ from their sensitivity to Dox (CHO, HUVEC, MDA-MB 231, MCF7 and NG108 cells). It has been reported that chemosensitivity to Dox in MDA-MB 231 and MCF7 is due to the level of DNA double-strand break repair proteins, Rad51 which is overexpressed in MDA-MB 231 whereas MCF 7 display a decrease level in this protein [15]. It was also shown that in MDA-MB 231, the TOPO II catalytic activity in nuclear extract is reduced in MDA-MB 231 as compared to MCF 7 cells [16]. Also it was shown that MDA-MB 231 cells highly expressed PGP [17].

Intracellular delivery, subcellular distribution and cytotoxicity of free Dox or coupled to tat and pene were studied in all cell lines. The results obtained indicate that tat and pene are a good peptide vectors for the cell entry of Dox and that the coupling strategy enhance the Dox toxicity. In addition, coupling of Dox to the two CPPs permits to overcome the observed Dox resistance of CHO, HUVEC and MDA-MB 231 cells.

Materials and methods

Drugs and reagents. Dox was purchased from Alexis Biochemicals. Dox–tat and Dox–pene were synthesized as described earlier [18]. For cytotoxicity assay, MTT was purchased from CellQuanti-MTT™, Gentaur. Trypsin, culture media and supplements were from InVitrogen. Hypoxantin–Aminopterin–Thymidin (HAT) was from, Eurobio. FITC-conjugated concanavalin (Con A), dibutyrl-AMPc and dimethyl sulfoxide (DMSO) were from Sigma. All cells were purchased from ATCC.

Cells and cell culture. All cells were maintained at 37 °C, 5% CO_2 in a Hera cell 150 humidified incubator (Thermo). CHO cells, were grown in F-12 K medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 10,000 U/ml streptomycin and penicillin. HUVEC, MDA-MB 231 and MCF7 were cultured in DMEM medium supplemented as above with 10% (v/v) heat-inactivated fetal bovine serum and 10,000 U/ml streptomycin and penicillin and NG108.15 were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 10,000 U/ml streptomycin and penicillin 1/50 Hypoxantin–Aminopterin–Thymidin. NG108.15 cell were differentiated with 1% heat-inactivated foetal bovine serum supplemented with 1 mM dibutyrl-AMPc.

Cell toxicity assay. The cytotoxicity of Dox and Dox–CPPs against cells was determined by MTT assay. All cells were plated overnight in 96 well plates with a density of 1000 cells per well in 0.2 ml of appropriate growth medium with 10% FBS and 10,000 U/ml streptomycin and penicillin at 37 °C. In case of NG 108.15 cell toxicity was also tested after differentiating the cells for 8 days. Different concentrations of Dox or Dox–tat or Dox–pene up to a maximum of 10 μM were incubated with the cell for 2 h, removed the drug from media by replacing with fresh media and kept the cells in incubator for another 24 h. Cell culture medium alone or with cells, both without drug were included in each experiment as controls. After 24 h of incubation, 100 μl of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 3 h. Conversion of MTT into purple formazan by metabolically active cells indicates the extent of cell viability. The crystals of produced formazan were dissolved with DMSO and the optical density was measured at 570 nm using a microplate reader (Biotek ELx-800, Mandel Scientific Inc.) for quantification of cell viability. All assays were run in triplicates.

Confocal microscopy. The analysis of intracellular localization of doxorubicin was carried out on all cells grown in labtech overnight and incubated for 2 h with 3 μM Dox or Dox–tat or Dox–pene. Then the cells were washed twice with PBS and maintained in culture for another 24 h. After that, the plasma membrane was stained with 5 μg/ml FITC-conjugated Concanavalin A for 5 min. Cells were washed again with PBS. Live cells were then immediately analyzed by confocal laser scanning microscopy using a Leica TCS-SP2 operating system. Alexa-488 (FITC, 488 nm) and Dox (λ_{ex} = 470 nm λ_{em} = 590 nm) were sequentially excited and emission fluorescence were collected in *z*-confocal planes of 10–15 nm steps. Images were merged in Adobe Photoshop 7.0.

Flow cytometry. Innate fluorescence of Dox allowed us to use flow cytometry to study penetration and localization of free or conjugated-Dox. CHO, HUVEC, MDA-MB 231 and MCF7 cells were cultured in 24 well plates and than treated with or without free or Dox–CPPs for 2 h. The extracellular drugs were than removed and the cells were kept again in the incubator with fresh media. Next, cells were treated with 1 mg/ml trypsin for 10 min at 37 °C to remove remaining cell surface-bound drugs and detach cells from the dish surface. The cell suspension was centrifuged at 500*g* and resuspended in PBS. Flow cytometry analyses were performed using a FACS-Calibur flow cytometer, BD Biosciences. Live cells were gated by forward/side scattering from a total of 10,000 events. Data obtained were analyzed using the CellQuest software.

Results

Cell toxicity of the CPP vectors used

Before examining the cell toxicity of the CPP conjugates, the cell toxicity of free peptides was investigated on all cell lines (Fig. 1). As shown, incubation for 2 h of these cell lines with free tat, or pene up to a concentration of 5 μM produces no toxicity; the toxicity values observed were not significant from baseline and lower than 9%. However, above 5 μM, all peptides produced a limited cell toxicity that ranges between 11% and 22%. Interestingly, there was no significant difference observed among the two CPPs indicating that they should both represent equipotent vectors for the delivery of Dox into these cell types.

Comparative cell cytotoxicity between Dox and Dox–CPPs in five cells lines

The cytotoxic effects of Dox or Dox–CPPs on these five cells lines were investigated using the MTT test in the indicated conditions in material and methods (Fig. 2). Interestingly 3 μM of Dox alone produce almost no cell toxicity in CHO cells (5.9 ± 6%) (Fig. 2A). This cytotoxicity value is increased to 25.58 ± 3.5% and 68.5 ± 6.1% when using Dox conjugated to tat or pene, respectively, instead of Dox. This means that Dox–tat and Dox–pene are 3 and 7.19 time more effective in CHO cells. Likewise, in HUVEC cells (Fig. 2B), no cell toxicity was observed after 2 h of incubation with 3 μM of Dox (9.47 ± 1.8%). An increasing of the cytotoxic effect of Dox was observed when coupled to Dox–tat (22.48 ± 7.5%) and to Dox–pene (80.78 ± 3.5%) which means an improvement of toxicity up to 2.68 and 11.53. In MDA-MB 231 cells (Fig. 2C), 3 μM of Dox does not produce cell cytotoxicity (3.4 ± 2%) further confirming the cell resistance to Dox. This toxicity value was significantly enhanced to 34.1 ± 6.4% for Dox–tat. A similar enhancement was noticed for Dox–pene (37.2 ± 2.5%).

In contrast, a 2 h incubation of NG 108.15 cells with 3 μM Dox produces 90.54 ± 3.7% of cell killing (Fig. 2D). This value is

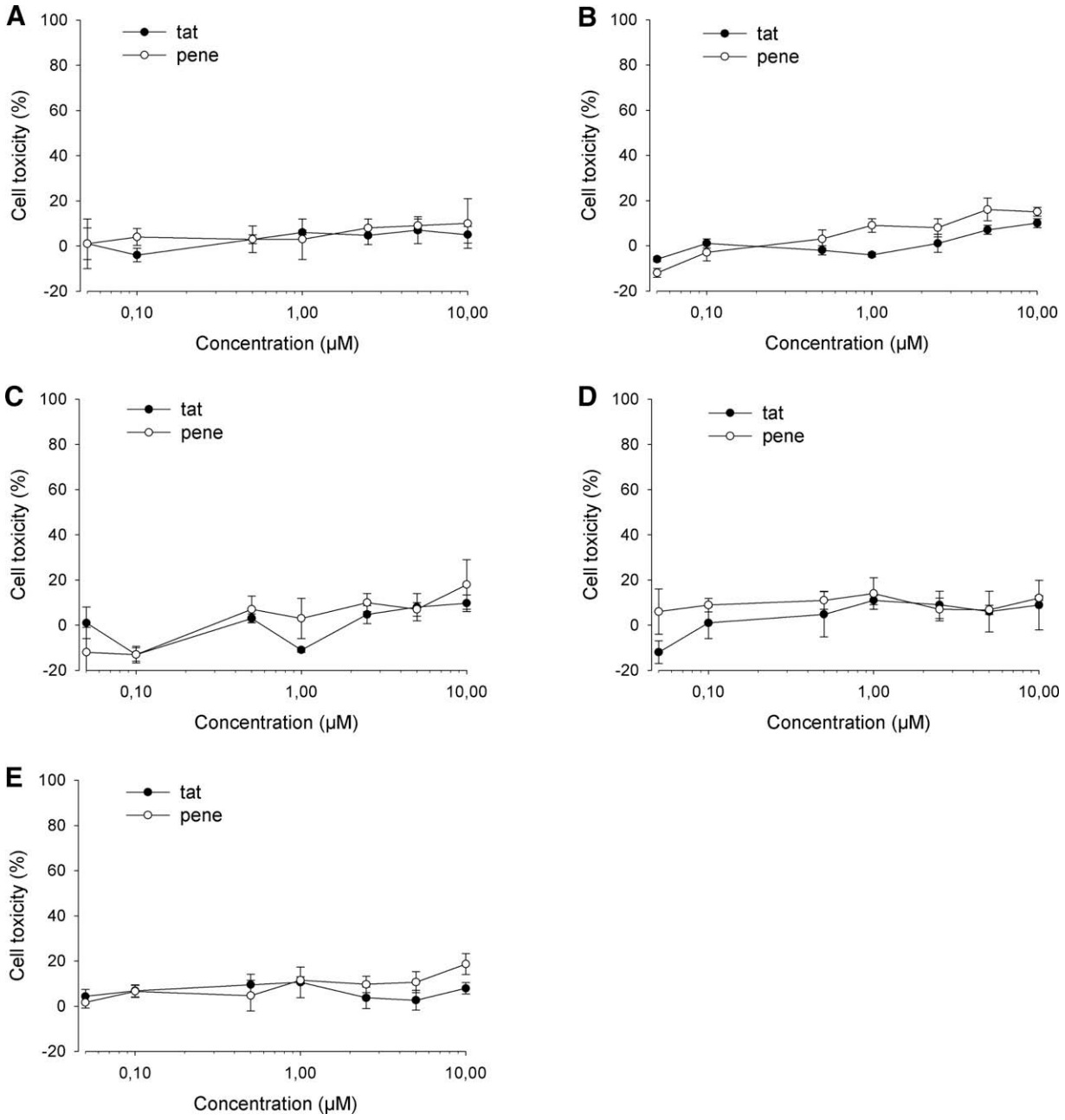

Fig. 1. Cell cytotoxicity of tat and pene peptides on CHO, HUVEC, MDA-MB 231, MCF7 and NG108.15 cells. Peptides were applied at various concentrations on CHO (A), HUVEC (B), MDA-MB 231 (C), MCF7 (D) and NG108.15 cells (E) for indicated time before performing the MTT assay, as described in Materials and methods.

decreased to 50.65 ± 8.6% and 62.65 ± 4.7 with Dox–tat and Dox–pene which shown that Dox–pene and Dox–tat, were found 1.44 and 1.78 less effective than the free drug in NG108.15 cells. The same decreasing cytotoxicity was observed in MCF7 cells. Our data indicate that NG108 and MCF7 are more sensitive to Dox and that using CPP conjugates of Dox does not improve cytotoxicity in these cells. These data seem to indicate that CHO, HUVEC and MDA-MB 231 cells are resistant to Dox and using CPP conjugates of Dox can reverse the cell resistance to Dox. This improve efficacy may be related to a reduced cell extrusion of drug when it is conjugate to these peptides [19].

Cell distribution pattern of Dox and Dox–CPPs in the cell lines

The effect of Dox conjugation on cell distribution was evaluated after short term exposure (2 h) (Fig. 3). A concentration of 3 μM was chosen to ensure Dox fluorescence detection by confocal microscopy (Fig. 3). As shown, the five cell lines did not exhibit similar patterns of cell distribution of Dox or Dox–CPP conjugates. After 24 h, Dox free was completely absent in CHO and MDA-MB 231 cells (Fig. 3A, C), slightly distribute in the cytoplasm of HUVEC cells (Fig. 3B) but still remained in both cell cytoplasm and nuclei of NG108.15 and MCF7 cells (Fig. 3D, E). It is important to note also that Dox–CPPs were found diffuse mainly into the cytoplasm of all five cell lines. Obviously, conjugation of Dox to tat or pene prevented the accumulation of Dox into the nuclei of the cells. Moreover, the greater apparent intensities in fluorescence observed in case of Dox–tat or Dox–pene seem to indicate that the peptides allow a greater accumulation of the drug in cells. However, since Dox fluorescence is intrinsically low, we cannot exclude that a fraction of Dox–CPPs reaches the nucleus but remains undetected.

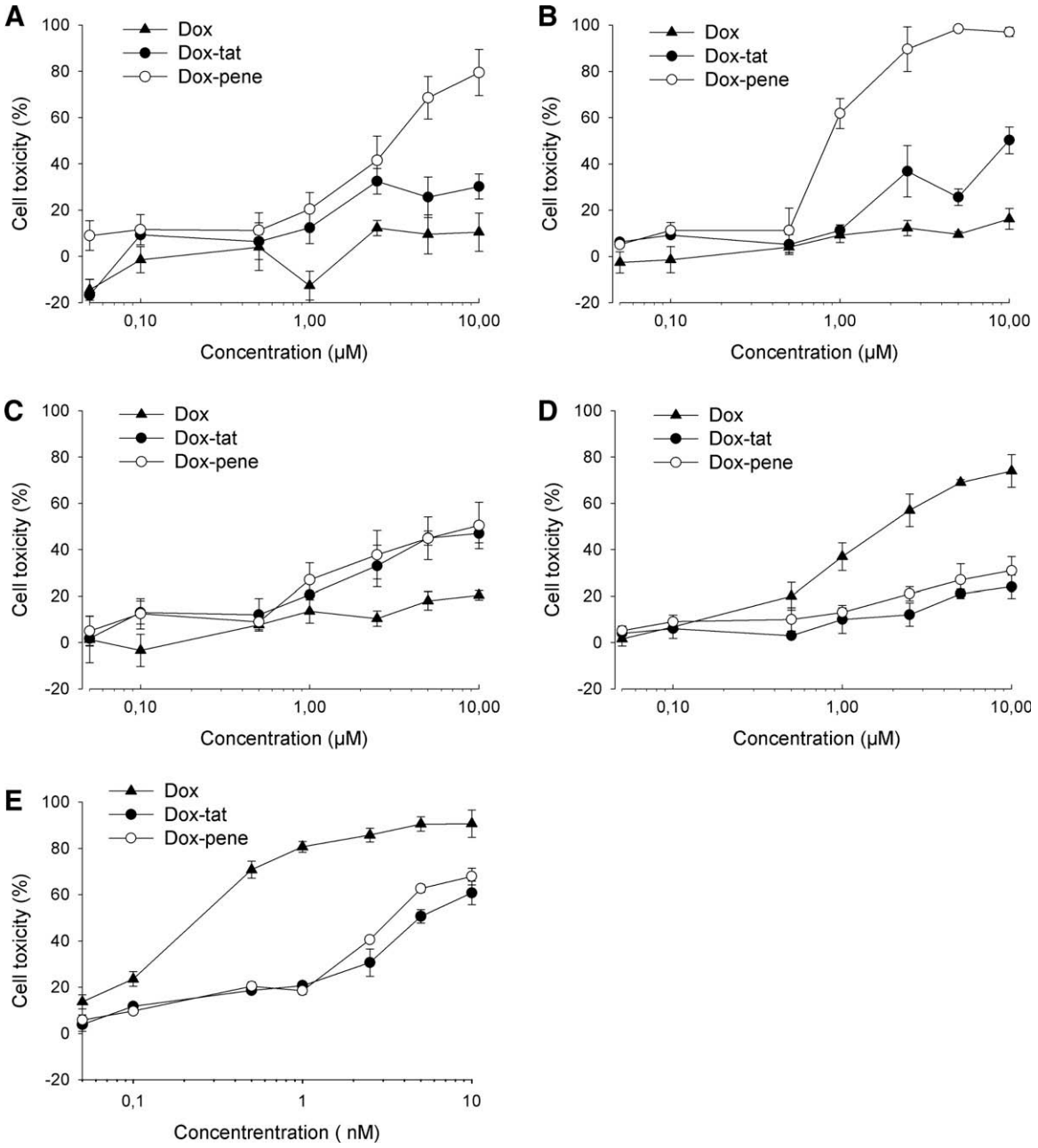

Fig. 2. Cell cytotoxicity of Dox–CPPs determined by MTT assays after indicated time with Dox conjugate. (A) CHO cells showed maximal toxicity values of 25.58 ± 3.5% (Dox–tat) and 78.5 ± 6.1% (Dox–pene). (B) HUVEC cells showed maximal toxicity values of 22.48 ± 7.5% (Dox–tat) and 80.78 ± 3.5% (Dox–pene). (C) MDA-MB 231 cells showed maximal toxicity values of 47.57 ± 2.5% (Dox–tat) and 51.15 ± 3.1% (Dox–pene). (D) MCF7 cells showed maximal toxicity values of 24 ± 5% (Dox–tat) and 31 ± 6% (Dox–pene). (E) NG108.15 cells showed a maximal toxicity values of 50.65 ± 8.6% (Dox–tat) and 62.65 ± 4.7 (Dox–pene).

Study of the uptake of both Dox and Dox–CPPs by FACS indicates that CHO, HUVEC and MDA-MB 231 cell lines accumulate significant amounts of Dox–CPPs over Dox alone since the fluorescence intensity was increased in the first case (Fig. 4). This means that both tat and pene markedly increased the uptake of Dox into CHO, HUVEC and MDA-MB 231 cells and reduced the extrusion of the drug. All the more it is clearly that Dox–pene was the most efficient Dox–CPP conjugate leading to a most important uptake similar to the finding in the studies of the cytotoxicity.

Such data of distribution or uptake are in accordance with cytotoxicity effect and could explain the greater cytotoxic effect of Dox–CPPs over Dox alone in CHO, HUVEC and MDA-MB 231 cells. This observed improvement in cytotoxicity observed for the conjugates over free Dox in CHO, HUVEC and MDA-MB 231 cells clearly indicate that (i) an extrusion phenomena of the free drug out of the cells is responsible on its reduced cytotoxic effect (ii) conjugation of the drug to tat or pene prevent its extrusion and (iii) this strategy of Dox delivery is an advantage in this three cell lines.

Discussion

Binding of low-molecular weight anticancer therapeutics to CPPs carriers results in drug delivery systems with numerous advantages, such as improved solubility, biodistribution and pharmacokinetic profiles. Furthermore, increased vessel permeability

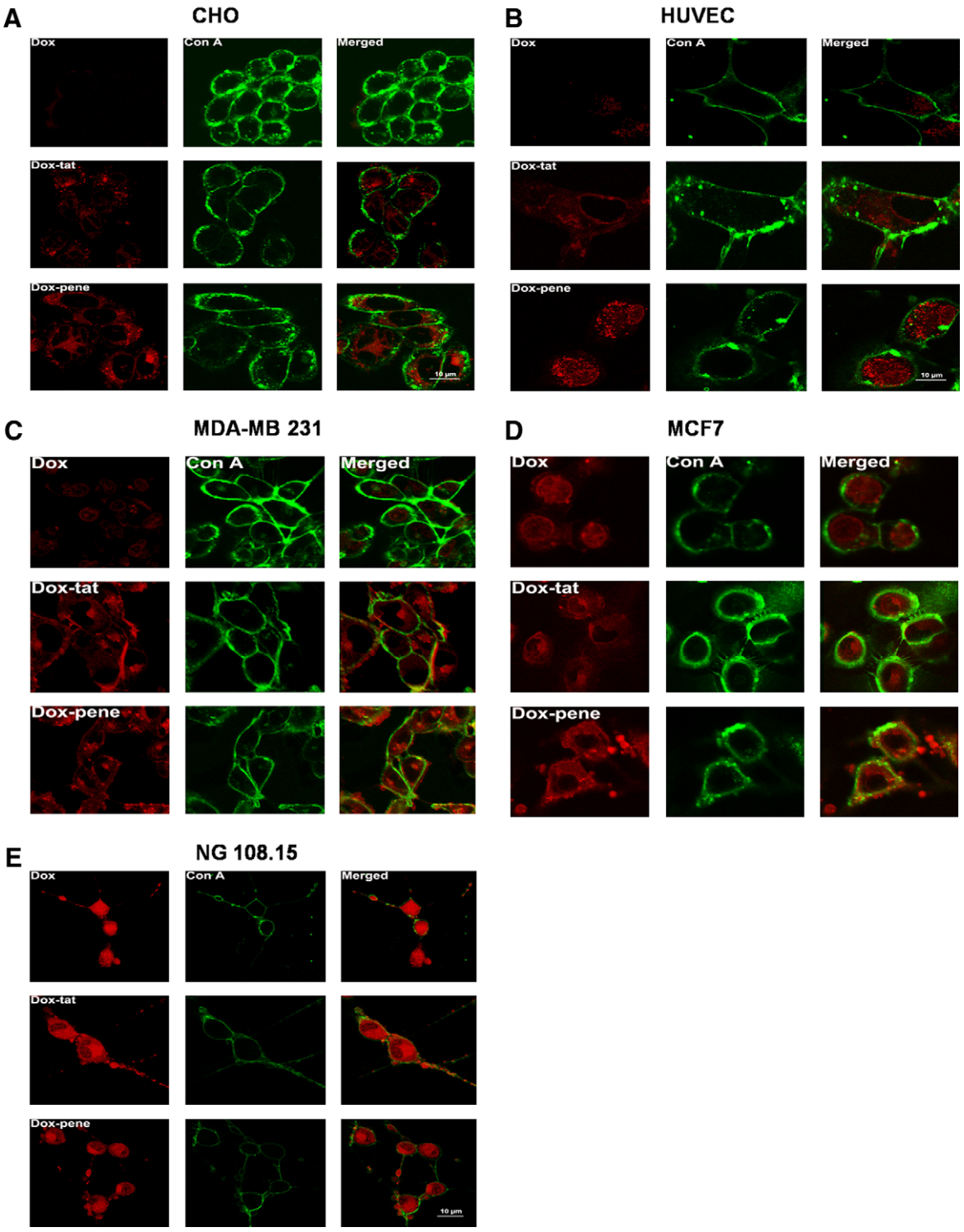

Fig. 3. Subcellular localization of free or conjugated Dox in the five cells after appropriate time treatments. (A) Confocal images of living CHO cells comparing the distribution of Dox fluorescence for free Dox (upper panels) or CPP-conjugated Dox (three lower panels). Cells were incubated with a drug concentration of 3 µM. (B, C, D and E) Same as in (A) but for HUVEC, MDA-MB 231, MCF7 and NG108.15 cells. Dox (red) and Con A (green) labeling, as well as merge pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

of the tumor vasculature and poor lymphatic drainage of the tumor allows drugs delivered by CPPs to accumulate in tumor tissue, resulting in improved therapeutic efficacy when compared to those of low- molecular weight drugs [20,21].

In this study, two CPPs–Dox conjugates were selected for synthesis and evaluation as potential candidate in enhancing Dox cytotoxicity and translocation in CHO, HUVEC and MDA-MB 231 cells but not in NG 108.15 and MCF7 cells. The data indicate that the two CPPs are different in terms of concentration-dependence and efficacy to induce cell death in CHO, HUVEC and NG 108.15 cells but equipotent in MDA-MB 231 and MCF7 cells. Various aspects of the data gathered within this report are discussed hereunder.

We demonstrate that CPPs-bound Dox overcome resistance of CHO or HUVEC or MDA-MB 231 cells to the drug and obviously

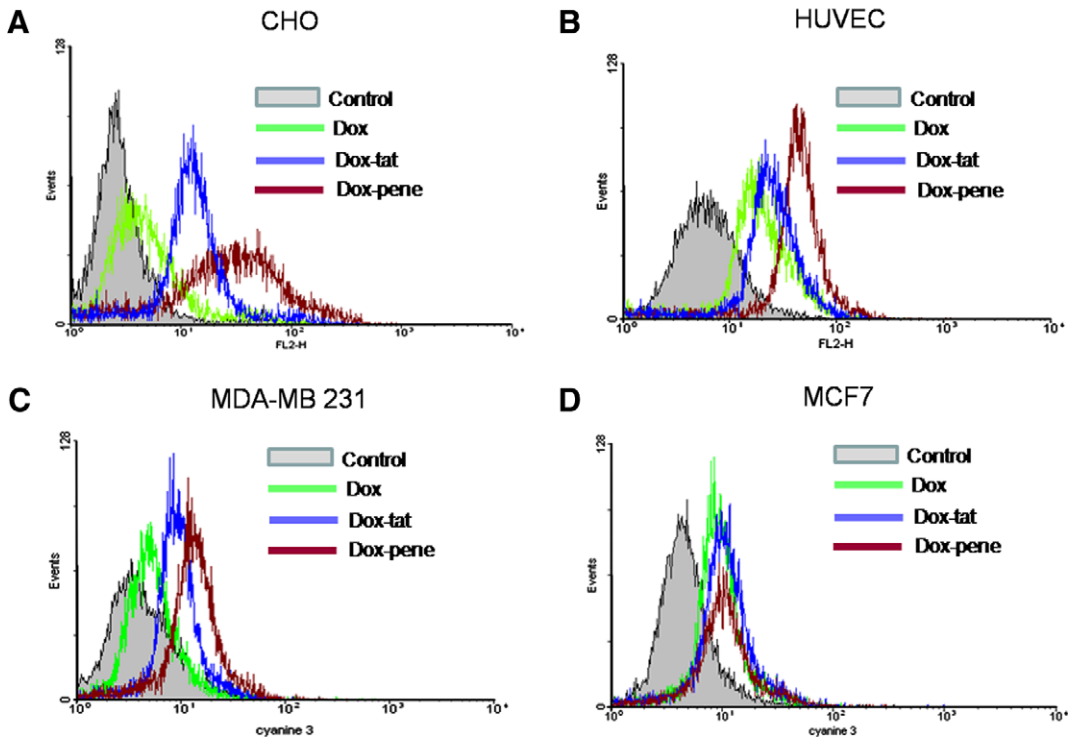

Fig. 4. Cellular uptake of free or conjugated Dox is analyzed by flow cytometry. (A) Histograms show relative fluorescence of CHO cells treated or not with Dox or Dox–CPPs for indicated time at 3 μM. Same think for (B, C, D and E).

enhance cytotoxicity. The conjugates were equally cytotoxic to NG108.15 and MCF7 sensitive cells. In our experiments, CHO, HUVEC and MDA-MB 231 cells had a lower tendency to accumulate free Dox than NG 108.15 or MCF7 cells suggesting that greater efflux might be at the basis of the differences in Dox sensitivity of five cell lines. In any case, this difference can be sustained by P-gp efflux pump. Even though free Dox diffused into the cell more easily than CPPs–Dox, free Dox was rapidly pumped from the resistant cell lines. In contrast, the CPPs-delivered Dox was not pumped from the resistant cells, leading to equal toxicity in both the sensitive and resistant cell lines. The ability of CPP–Dox conjugate to evade the P-gp efflux pump was confirmed using several assays. First, Confocal microscopy analysis showed a lower accumulation of free Dox in the resistant CHO, HUVEC and MDA-MB 231 cells over a high accumulation of drug in sensitive NG108.15 and MCF7 cells even after the drug was removed. This is in contrast to the conjugated-Dox which still remain in the cytoplasm of the cells. The flow cytometry assay revealed that, 24 h after removal of the drug, all the cells treated with conjugate Dox accumulate high level of drug, respectively 97%, 87% and 61% of CHO, HUVEC and MDA-MB 231 cells were positives. As free Dox was pumped out of the cells, the percentage of cells containing high levels of drug decreased to 11%, 20% and 30% in CHO, HUVEC and MDA-MB 231 cells.

The data presented here demonstrate that the CPP–Dox conjugates does not localize to the nucleus but displayed in most case cytoplasmic distribution. This is in contrast to the free drug, which localise mainly in the nucleus where it serves as an intercalator and topoisomerase II poison. The cause of this altered distribution might be the endocytic mode of cellular entry used by CPPs [12] or the modification made to the Dox structure. Interestingly, the CPPs-delivered Dox overcome resistant cells toxicity regardless of its lack of nuclear localization and that pene is powerful in such activity in CHO and HUVEC cells, suggesting an alternate mechanism of toxicity for conjugated Dox. By contrast such distribution allowing a less sensitivity of NG108.15 and MCF7 cells to Dox. These data indicate also that cell distribution of Dox is not a reliable indicator of its toxicity effect and that there are different mechanisms involved in toxicity pathways. Other possible reasons for the greater efficacy of Dox coupled to CPPs in CHO, HUVEC and MDA-MB 231 cells include alterations in efflux pathways or in detoxification reactions. CPPs are cell penetration peptides that tend to accumulate into cells because of their basic properties. They should thus be useful to counteract efflux mechanisms. Besides they possess DNA binding abilities [22] that may come as a synergistic factor for DNA targeting.

The results obtained herein underline the value of CPPs as delivery vectors for bioactive molecules and are in accordance with other studies by Temsamani and co-workers [23,24] showing that Dox and its potent analogue 2-pyrrolinodoxorubicin overcome multidrug resistance by peptide conjugation. They demonstrate that the peptide conjugation of the small drugs made them unrecognisable by the efficient efflux pump P-glycoprotein, thereby increasing the accumulation and cytotoxicity of these drugs.

Generally, CPPs are used for their ability to carry across the cell membrane an important number of impermeable compounds including drugs, peptides, proteins such as antibodies, oligonucleotides, peptide nucleic acids, DNA or inorganic compounds such as nanoparticles [25,26]. For these compounds, the use of CPPs has evident advantages since they overcome the limited availability of these products inside cells. This technological advantage has permitted the development of numerous applications of basic, therapeutic, diagnostic, imaging or technical importance [22,25]. The use of CPPs to promote the cell entry of compounds that are already membrane permeable (such as Dox) is obviously less evident. As a matter of fact, their use in this case is seldom reported. However, our data illustrate the benefit of using CPPs for altering the stability, efficacy and cell compartment targeting of the drug.

Improvements could be brought to this strategy by grafting additional signal sequences to CPPs for their targeting to defined

cell compartments. Enhanced nuclear targeting and enhanced efficacy of a Dox–CPP complex may be obtained by adding a Nuclear Localization Sequence (NLS) to the peptide sequence. This chimera strategy may be extended to cell targeting sequence for *in vivo* application where Dox–CPP delivery could be directed to tumors rather than being diffusely taken up by the entire organism. Such an application has successfully been designed for Dox alone coupled to a cyclic pentapeptide [22].

In conclusion, we have demonstrated that pene is as effective as tat for the efficient delivery of Dox into cells. This vector based delivery can overcome the reduced Dox sensitivity observed in CHO, HUVEC and MDA-MB 231 cells over NG 108.15 and MCF7 cells. Future research avenues will be developed by designing tumor-targeting Dox–CPPs and/or specific cell compartmentalized Dox–CPP analogues with improved cell toxicities. The efficacy of Dox–CPP will be evaluated in *in vivo* tumor models with a special emphasis to low concentrations of Dox–CPPs which were shown to be efficient *in vitro* during long term exposures. Other applications are envisioned such as evaluating *in vitro* and *in vivo* the efficacy of drugs grafted to pene and that possess different cellular targets [26–28].

Acknowledgments

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