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Journal of Drug Design and Research

Research Article

Synthesis and Cytoxicity of a TAT Peptide-Doxorubicin Conjugate for Breast Cancer Treatment

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Submitted: 15 January 2015

Accepted: 10 February 2015

Published: 12 February 2015

Keywords
- TAT Peptide
- Doxorubicin
- Doxorubicin resistance
- MCF-7 cells

Abstract

The TAT peptide is a drug delivery tools which has been efficiently proven to deliver drugs, peptides and nucleic acids. A specific sequence of HIV1 protein transduction domain TAT was evaluated for its ability to carry Doxorubicin (Dox) into drug resistant MCF-7 tumor cells. TAT was conjugated to Dox via the formation of a disulfide bond. In this study, we developed an optimal formulation for the conjugation of Dox by this delivery system for tumor treatment. The in vitro study showed that Dox-TAT peptide conjugate was only more potent than free drug at higher concentration on Dox resistance cells. The concentration of drug in resistant cancer cells was increased indicating a partial reversal of drug resistance.

ABBREVIATIONS

Dox: Doxorubicin; SMCC: (Succinimidyl 4-[N-Maleimidomethyl] Cyclohexane-1-Carboxylate); CPPS: Cell-Penetrating Peptides; PTDS: Protein Transduction Domains; TLC: Thin Layer Choromatography

INTRODUCTION

The principle obstacle to the clinical efficiency of chemotherapy has been toxicity to the normal tissues and the development of cellular drug resistance. Tumor resistance is mainly associated with over expression of P-gp in breast cancer [1]. Therefore, in order to achieve better clinical outcome and increase the success of chemotherapy in breast cancer tumors, chemotherapeutic drugs was carried with drug carrier systems to improve the drug influx by escaping P-gp pumps and overcome P-gp transporter mediated multidrug resistance (MDR). Resistance to anticancer agents, including doxorubicin, is a serious limitation to the effective chemotherapeutic treatment of the breast cancer. Doxorubicin is an antracycline antibiotic. It is a commonly used anticancer agent in many cancer types and its most serious side effect is damaging the heart cells [2]. Therefore targeting of this chemotherapeutic agent with a suitable carrier mediated drug delivery system is important. Doxorubicin that is a resistance is generally associated with MDR1/MRP over expression, altered levels of topoisomerase II expression, expression of mutated forms of topoisomerase II, increased glutathione or glutathione peroxidase levels, DNA mismatch repair deficits, cellular resistance to apoptosis and finally changes in the membrane lipid composition [3,4].

Cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) have the ability to transduce or pass through biological membranes independent of classical receptor or endocytosis-mediated pathways. One such peptide is TAT peptide human immunodeficiency virus type 1 (HIV-1) [5-8] and it is able to transport different molecules across biological barriers to be taken up by various cell lines [9,10]. Therefore, the objective of this research was to describe the synthesis of TAT peptide-Doxorubicin conjugates, and the antitumor activity on Doxorubicin resistant MCF-7 cells.

MATERIALS AND METHODS

Dox-TAT Conjugation Reaction with SMCC (Succinimidyl 4-[n-maleimidomethyl] cyclohexane-1-carboxylate)

SMCC was used to conjugate amine containing doxorubicin molecules to TAT peptides with C-terminal cycteine residues. The reaction was performed in several steps. Reaction scheme is shown below in (Figure 1).

Quantification of Sulfhydryl Groups on TAT Peptide

Free -SH groups were quantified by the DTNB method [11]. The molar extinction coefficient of TNB is reported to be 14,150 Mcm at 412 nm [11,12] in 0.1 M phosphate buffer containing

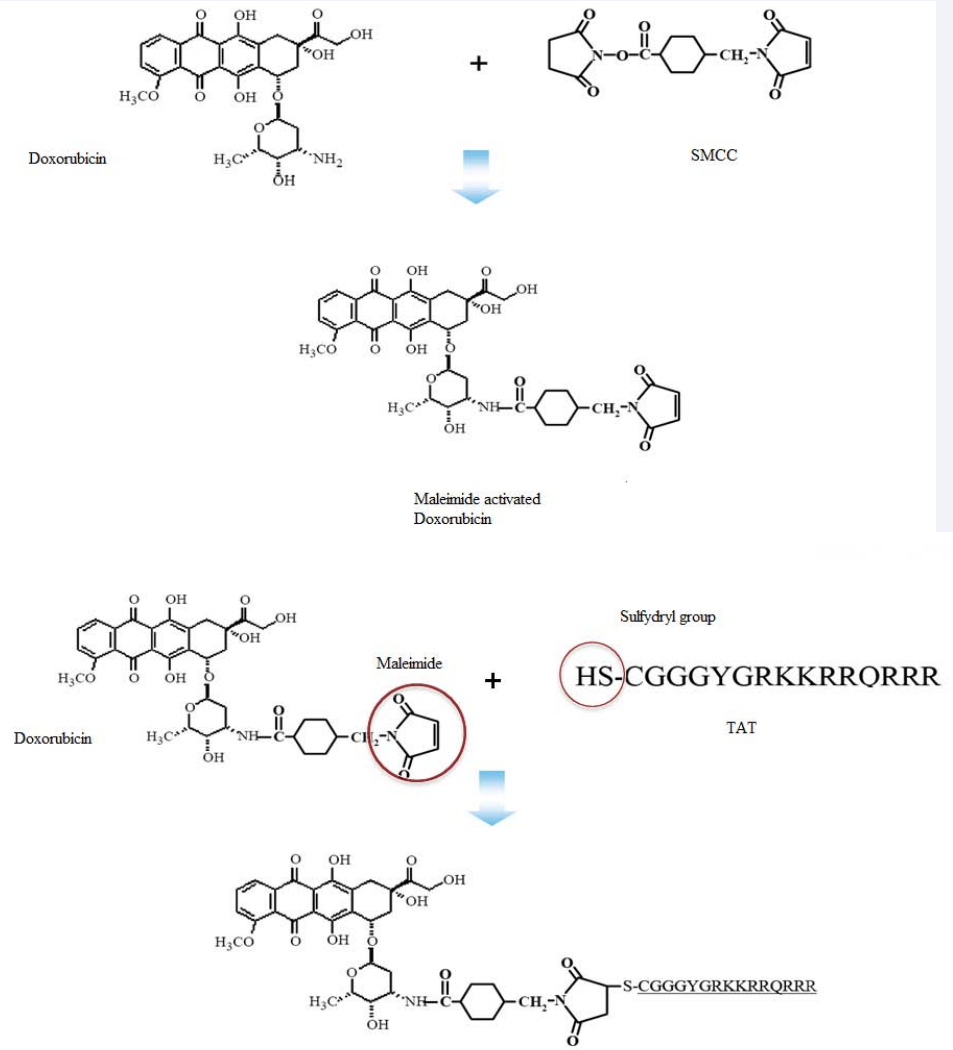

Figure 1 Scheme for doxorubicin–TAT conjugate synthesis.

1 mM EDTA at pH 8.0. The extinction coefficient was used to calculate the number of sulfhydryl groups must be matched to the reaction conditions. Solutions below were prepared and used;

– Reaction buffer: 0.1 M sodium phosphate, 1 mM EDTA at pH 8.0

– Ellman's Reagent Working Solution: 4 mg Ellman's Reagent dissolved in 1 ml of reaction buffer. The unused portion was stored at 4°C for a maximum of two weeks for future use.

– Peptide sample 2: 2 µl TAT stock solution (19 mg/ml) was added to 2.748 ml reaction buffer + 50 µl Ellman's Reagent solution

– Blank: 2.750 ml Reaction buffer + 50 µl Ellman's Reagent solution

Solutions were incubated at RT for 15 minutes. At the end of the incubation spectrophotometer was set to 412 nm and zero on the blank. The absorbance of the peptide sample was measured. The amount and concentration of sulfhydryls in the samples were calculated from the molar extinction coefficient of TNB by using the equation 3:

Molar absorptivity, E, is defined as follows:

$$E = A / (bc)$$

where A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

The concentration of the assay solution (2.8 ml) was calculated by multiplying with the dilution factor. From the molarity values, moles of sulfhydryl in the assay solution were calculated. These moles of sulfhydryl in the assay solution were contributed by the original 2 µl sample. Therefore, the concentration of free sulfhydryl in the original unknown sample was calculated with linear proportion.

Peptide Reduction

Cysteine residues on TAT peptide will invariably polymerize upon reconstitution from their lyophilized form. A detailed protocol on reduction with sodium borohydride is given by James Gailit [13]. A modified form of that procedure was followed in this study.

Reaction buffer for DTNB assay was adjusted to pH 9.8

with NaOH. Peptides were dissolved in this solution to the final concentration 1 mg/ml. At this time point an aliquot was taken and analyzed with Ellman's Reagent. Dry $NaBH_4$ was added to final concentration of 0.1M $NaBH_4$ and solution was mixed slowly to avoid foaming. Cap was left loose. A stream of argon gas was passed over the reaction vessel slowly and constantly. At t=15, 30, 60, 90, 120 minutes aliquots were taken. Borohydride will reduce DTNB so every sample that contains $NaBH_4$ was acidified with 0.5 M HCl for 10 min before analysis. Since DTNB color should be developed in a mild alkaline environment, the pH of the reaction mixture was brought back to pH 8.0. Aliquots were analyzed by DTNB method. When absorbance stopped to increase the solution was acidified back to pH 4.0 using dilute HCl and incubated at RT for 10 min to stop the reaction. Quantization of sulfhydryl groups in the final mixture was calculated.

Activation of Doxorubicin

The amine reactive N-hyroxysuccinimide (NSH-ester) undergoes a nucleophilic substitution to form an amide bond with the primary amines of doxorubicin molecules. In the first step 0,9 mg doxorubicin was dissolved in 500 cL DMSO and dispersed in 2 ml conjugation buffer (0.1 M sodium phosphate, 0.150 M sodium chloride, pH 7.2) 20 cL TEA was added and pH was adjusted back to 8.0. Then 0.5 mg SMCC was added to the solution. Final volume was filled up to 3 ml with conjugation buffer. Reaction proceeded for 2 hours at gentle mixing (200 rpm) at RT, under argon atmosphere and protected from light.

TAT Conjugation to Activated Doxorubicin

225 µl sulfhydryl- (cysteine residue) containing TAT (19 mg/ml) was added to the reaction mixture. The addition of thiols to maleimides occurs via a Michael addition and is usually performed at a low pH due to the base-catalyzed ring opening of the maleimide [14]. Sulfhydryl containing peptide can be added in excess to the amount of maleimide activity present on the drug. 1.5 fold molar excess was used. This second step of the reaction must be performed below pH 7.5 to prevent hydrolization of the maleimide end. Reaction was carried under argon atmosphere and protected from light.

Following the Reaction with Thin Layer Chromatography

To analyze maleimide activation of doxorubicin after the first reaction and TAT conjugation to the activated doxorubicin after the second reaction very small samples of reaction mixtures were taken with a capillary before beginning to the reaction and after each reaction. Solvent systems 1:1 ethanol: chloroform was found to be appropriate from thin layer chromatography (TLC). As stationary phase, a silica coated metal film as a thin layer (~0.25 mm) was used. A start line 1 cm from the bottom of the plate and a stop line 1 cm below the top of the plate were drawn with a pencil. Free doxorubicin, doxorubicin activation reaction mixture and TAT conjugation reaction mixture were spotted at three far apart dots on the start line. A small amount of 1:1 ethanol: chloroform solvent mixture (mobile phase) was placed in a closed container as it was below the start line. A filter paper was placed inside the container to have a concentrated solvent mixture. The lower edge of the plate was then dipped in the solvent. Solvent travels up the matrix by capillarity, moving the components of the samples at various rates because of their different degrees of interaction with the stationary phase and solubility in the developing solvent. When solvent reached the stop line, plate was removed and air dried. Since doxorubicin is a colored substance no other visualization technique was required. Doxorubicin and the reaction products were seen as red spots on the TLC plate. The spots were circled to have a permanent record how far the compound travelled on the plate. The products from two subsequent reactions which were visible as separated spots were identified by comparing the distances they have travelled with those of doxorubicin.

The distance of the start line to the solvent front (stop line) was measured (b) and divided by the distance of centre of the spot to the start line (a). The resulting ratio is called retardation factor (Rf-value).

Purification of Dox-TAT Conjugate

To remove small molecules; unreacted Dox, SMCC and TEA from Dox-TAT conjugate reaction mixture was dialyzed by using a tubing with molecular cut of 1000. To prepare the dialysis tubing 0.9306 gram of EDTA was dissolved in 250 ml water (10 mM) at pH 5.5. Than pH was adjusted back to 7.5 with NaOH. Tubing was boiled in this solution for 30 minutes. Than 10 mM EDTA solution was diluted to 1 mM to have the storage solution. Dox-TAT reaction mixture (3ml) was put into tubing and both ends were sealed. Dialysis was done against 100 ml PBS over night at 4^0C, making three buffer changes.

Visualization of TAT Peptide on Tris Tricine SDS-PAGE

Free TAT peptide and Dox conjugated TAT peptide are run on the tristricine SDS-PAGE then the gel was silver stained. Tris-SDS-PAGE is commonly preferred to separate proteins and peptides in the mass range 1-15 kDa.

RESULTS AND DISCUSSION

Dox-TAT Conjugation Reaction with SMCC

Doxorubicin was conjugated to TAT peptides by using SMCC as the cross-linker. SMCC contains amine reactive N-hyroxysuccinimide (NSH-ester) end and sulfhydryl reactive maleimide group. Maleimide has greater stability in aqueous environments than the NSH ester end. So first NSH-ester was reacted with the amine group on doxorubicin molecule. Second reaction was the conjugation of TAT to activated Dox.

Activation of Doxorubicin

This reaction contains 3 M equivalent of SMCC with respect to doxorubicin. Normally 40-80 fold molar excess is appropriate. Usually after the first reaction excess reagents are removed. Because crosslinker molecules which were not entered into the reaction will be competing for the thiols once there were added. However, the excess SMCC cannot be removed after the first reaction. Due to the very small and similar sizes of products and reactants a purification step was not possible. So the maleimide content contributed from the contaminating reacted cross linker may prevent subsequent conjugate formation. To overcome this SMCC concentration kept at minimum. Some of the TAT peptides

were compromised. In order to compensate for this loss 3 times more TAT was added into the reaction in the second step. This reaction resulted in the formation of an amide bond between the drug and the cross linker and formation of a reactive maleimide group. First dot appeared where the initial reaction mixture was applied. Since Dox is colored on its own no special technique was needed for visualization. The first dot belongs to Dox. 2 hours after the reaction a new sample was applied next to the first. Another dot was appeared further away than the first one. This proves the reaction was successful. These introduced maleimide groups are stable for several days at 4°C. It is reported that only 4% of them will decompose at neutral pH within 2 h at 30°C [15].

Quantification of Sulfhydryl Groups on TAT peptide

Free -SH groups on both of the peptides were quantified by the DTNB method. TAT was dissolved in water to have 19 mg/ml final concentration. 2 µl from this stock solution was analyzed and moles of sulfhydryl in the assay solution were calculated. Calculations were performed as shown below;

A1(412 nm)=0.044

A2(412 nm)=0.045

A3(412 nm)=0.043

Aavr(412 nm)= 0.044

c = A / (bE)

where A(absorbance)=0.044, b(path length in centimeters)=1, c(concentration in M) E(Molar absorptivity)= 14,150 $M\text{-}1cm^{-1}$. Assay solution was 2.8 ml in all experiments.

The concentration in the spectrophotometric cuvette was calculated from the formula:

C = A / (bE)

$$C = \frac{0.004}{1(14.150)} = 3.1*10^{-6} M$$

$$2.8mL \frac{1L}{1000mL} 3.1* \frac{10^{-6} moles}{L} = 8.68*10^{-9} moles$$

$$\frac{8.68*10^{-9} moles}{2\mu L} * \frac{1000000\mu L}{L} = 4.34*10^{-3} M = 0.0043M$$

19 mg/ml TAT stock solution contains 0.0043 M –SH groups. However, theoretically this stock solution is 0.0103 M. There is one cysteine per one molecule of peptide. So –SH concentration should be 0.0103 M as well. Experimental value was 2.4 times less than the theoretical value. Results indicate that 45% of all SH groups of TAT initially present were oxidized to form S-S bonds. In conjugation reaction maleimide end of the cross linker only reacts with reduced cysteine residues (free thiols) on TAT peptides. If they are not in their free form this reaction will not occur and most of the peptide will be compromised. The possibility of degradation of peptide was also considered but the absorptions of stock peptide solutions at 215 nm were not significantly different that working solutions of peptides, which excluded this possibility. In order to restore the SH groups reduction with borohydride was performed.

Reduction of Sulfhydryl Groups on TAT Peptide

In order to determine optimum conditions for TAT peptide reduction, reaction was performed for 90 minutes and aliquots were taken at predetermined time points to analyze with DNTB method. (Figure 2) the chance in absorption as the reaction proceeds.

Absorbance increase corresponds to the increase in free –SH groups. As it is seen from the graph 30 minutes was enough for -SH reduction. Reduction at longer times is not effective as re-oxidation occurred. Before all of the conjugation reactions performed TAT peptides were reduced for 30 minutes then used immediately for the conjugation reaction. Sulfhydryl groups of terminal cysteine residues are readily oxidized and form disulfide bonds when the lyophilized peptides are reconstructed. When the free sulfhydryl groups are oxidized, SMCC conjugation reaction involving free thiols will be impaired. Since oxidation occurs any time during storage and handling it is impossible to fully prevent and it is very important to restore the free sulfhydryl groups before starting any reaction even if the cysteine residues are supposedly free. Reduction with sodium borohydride ($NaBH_4$) is the most suitable method, for reducing the oxidized sulfhydryls for future use in cross-linking reactions. The biggest advantage it that it does not require an additional purification step. The excessive $NaBH_4$ can be eliminated by acidification. The $NaBH_4$ is hydrolyzed to give NaH_2BO_3 and H_2 gas. Afterwards the reduced peptide can be used directly for the coupling reactions.

TAT Conjugation to Activated Doxorubicin

After 30 minutes of reduction TAT peptides were added to activated doxorubicin**.** Conjugation reaction was followed by TLC. Free doxorubicin, doxorubicin activation reaction mixture and TAT conjugation reaction mixture were spotted respectively at first second and third dots apart the start line. TLC paper is shown in (Figure 3).

First spot on TLC paper belongs to doxorubicin before SMCC was added to the solution. Second spot belongs to the sample taken just before TAT addition to reaction mixture. Third spot is the final reaction product. As it is seen from the Figures free doxorubicin before the reaction is compared with the product (Dox-maleimide) at the end of the first reaction and with the final

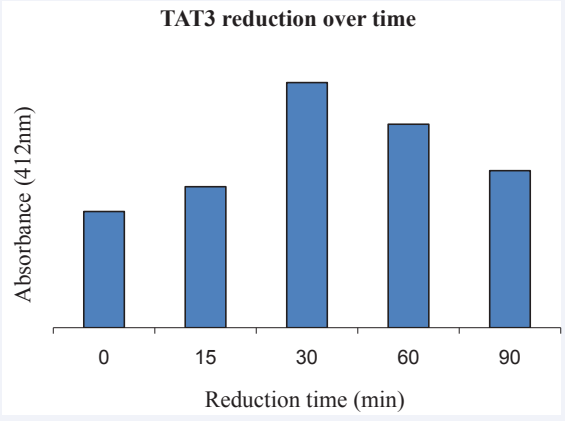

Figure 2 Re-formation of sulfhydryl groups on TAT.

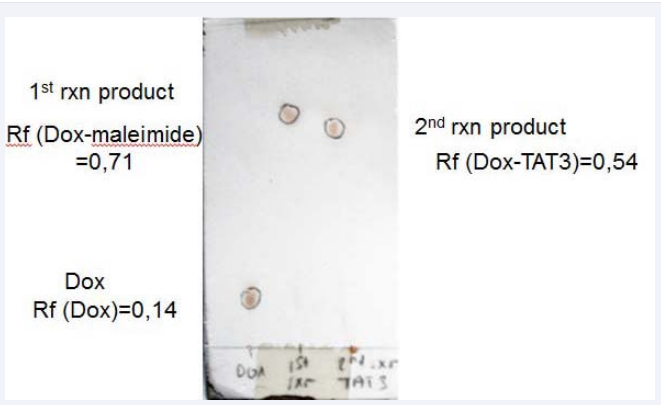

Figure 3 Thin layer chromatography of conjugation reaction products.

product at the end of the second reaction. Retention Factor (Rf) calculations are shown below:

TAT-Dox Plate:

Rf (Dox) =0,14

Rf (Dox-maleimide) =0,71

Rf (Dox-TAT) =0,54

The significant change in Rf values indicates that the activation and conjugation reactions were successful. Another important point is that, in the subsequent steps of the reaction no more free drug was observed on TLC. Also after the second step no more maleimide conjugated Dox was visible. This proves that most of the free doxorubicin was maleimide conjugated and most of the activated Dox was conjugated to TAT.

Thin-layer chromatography is a commonly used technique in synthetic chemistry for identifying compounds, and following the progress of a reaction. It is a rapid, simple, and inexpensive method. The Rf depends on solvent system, absorbent (grain size, water content, and thickness), amount of material spotted and temperature. Hence it will be different for same compounds on different plates. So these were relative values and must be compared with the reference material (Doxorubicin) on the same plate under the same conditions. If Rf values of two compounds are the same they might be the same. If Rf values are different the two compounds are definitely different.

Visualization of Dox-Tat Conjugate on Tris-Tricine SDS-PAGE

The molecular weight of TAT was around 1700Da and doxorubicin was 580Da. Dox-TAT conjugation will be slightly higher than TAT peptide, which would be very difficult, if not impossible, to separate from each other by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) assay. Since doxorubicin is a fluorescent compound, the ultraviolet image of the same SDS–PAGE gel was generated. Figure 4 shows Dox and TAT on SDS-PAGE. After silver staining both of the molecules became visible. The molecular weight of one band was around 600Da and the other was around 1700Da. First band belongs to Dox and the second one belongs to TAT. Dox was illuminated under UV light whereas TAT was not visible. With the help of this property Dox-TAT conjugate can be verified once it was subjected to SDS-PAGE assay.

Figure 4 also shows that Dox moves just above the Coo massive dye which is around 800 g/mol. TAT was larger than both so it's band was observed at a higher level.

Purification of Dox-TAT Conjugate

To remove small molecules; unreacted Dox, SMCC and TEA from Dox-TAT conjugate were dialyzed. Samples were removed inside the tubing at predetermined time intervals (0, 6, 12, and 24 hours) and saved for later use in SDS-PAGE assay. After overnight purification all the samples were run at Tris-tricine SDS-PAGE gel and after silver staining below image was generated.

Samples inside the dialysis bag were removed at t=0, t=6, 6=12, t=24 hours. Bands on the gel correspond to these samples respectively. First band is the marker having 1.5 kDa band at the bottom and 3 kDa band on top of it. Image at the left shows actual gel after silver staining. Image at the right shows UV image of the gel. Bands respectively belong to reaction product after before dialysis, product after 6 hours of dialysis, product after 12 hours of dialysis, product after 24 hours of dialysis. The smear belongs to free drug that was not conjugated. As dialysis continues free drug increases, however it does not go out of the tubing. This result shows that initially there was some free drug but it was not that much. As dialysis continues bonds between Dox and TAT were cleaved. So conjugate was not stable over long periods of time in PBS, pH 7.0. It has to be stored in its lyophilized form (Figure 5).

Cell Growth Inhibition of Dox-TAT Conjugate

Figure 6 shows 600 nM resistant MCF-7 cell viability after 72h of incubation with Dox-TAT chemical conjugation and free Dox.

Cytotoxicity profiles of Dox-TAT conjugate and free Dox was similar at low drug concentrations. Only at higher concentrations (25-50 μM) Dox-TAT seems to be more potent. It was postulated that the thioether linkage is stable to reducing conditions of human serum for several hours [16]. That time might not been enough for the conjugate to enter into the cells and show its effect. The bond between Dox and TAT might have degraded during sample preparation and storage as well. Underlying mechanism needs further evaluation. Either TAT carried Dox inside the cell by its internalization ability or it facilitated drugs internalization into nucleolus with the help of its nuclear localization signal similar

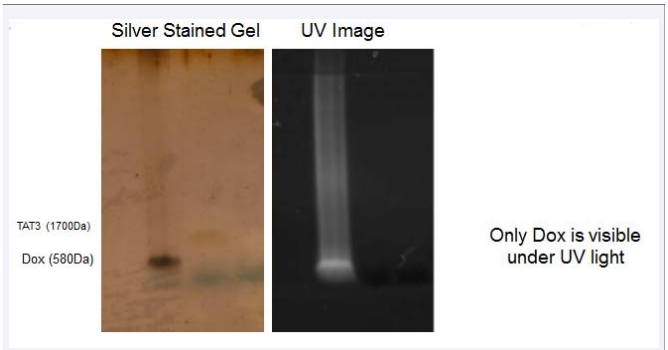

Figure 4 SDS-PAGE image of Dox and TAT.

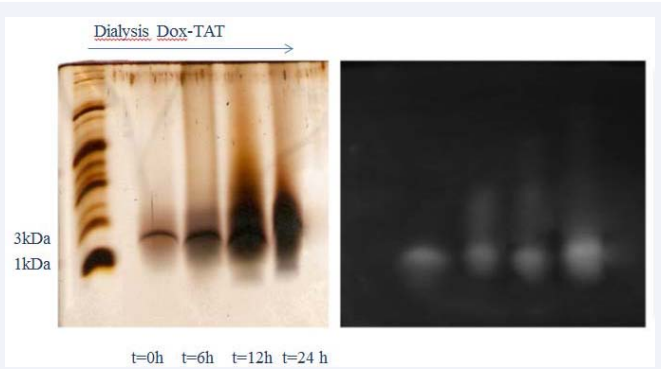

Figure 5 Reaction products on SDS-PAGE gel.

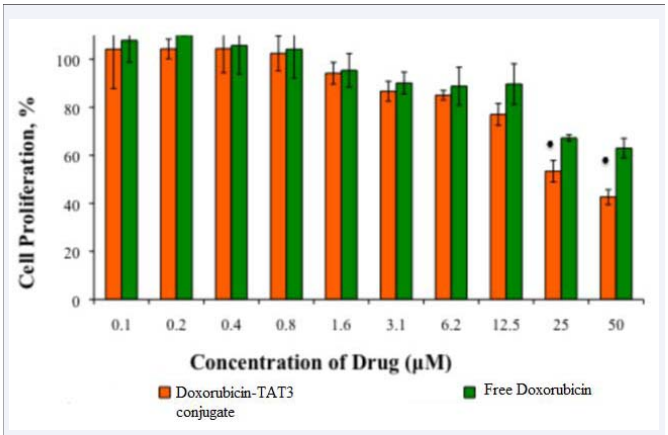

Figure 6 Cell proliferation profiles of 600nm Dox resistant MCF-7 cell line after 72h incubation with Dox-TAT conjugate and free Dox.

sequences. Some drawback on the system is lack of specificity and concern about causing the drug to lose its activity. The bond is not readily degradable in the endosomes so cytotoxicity of some conjugates might be reduced [16].

TAT-peptides have shown a capability for delivery of large variety of biologically active cargoes such as proteins, DNA, antibodies, imaging agents, and nanoparticles [17,18].

Zhao et al., an shown that HIV-1 Tat protein was conjugated with noncovalent bonds to sulfonated aluminum phthalocyanine (AlPcS), doxorubicin or quantum dots in the human nasopharyngeal carcinoma KB cells and cervical carcinoma Hela cells. They found that the Tat peptide-mediated intracellular delivery of anticancer drugs may have the potential for improving efficacy of cancer therapy [19].

In several previous studies, TAT peptide was conjugated with siRNA .TAT-peptide has a great potential due to the formation of stable complexes with desirable physical characteristics, low toxicity and able to carry high amount of siRNA [20].

CONCLUSION

In this study a synthetic peptide with similarities to protein transduction domains are evaluated for its ability to carry doxorubicin, an important P-gp substrate, into resistant cells. Promising results were obtained in delivery of Dox into resistance cancer cells as well. When doxorubicin was chemically conjugated to a cell penetrating peptide, its effect was more obvious on resistant cancer cells when compared to that of free drug. In the future, once mechanism underlying this observation studied further, peptides might be alternative ways of overcoming MDR.

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Cite this article

Şen-Çağlar GP, Yalcin S, Gunduz U (2015) Synthesis and Cytoxicity of a TAT Peptide-Doxorubicin Conjugate for Breast Cancer Treatment. J Drug Des Res 2(1): 1007.