Original Article

Doxorubicin prodrug for cytoplasmic and nuclear delivery in breast cancer cells

Abstract

Purpose: The objective of this study was to develop a novel peptide prodrug of doxorubicin which can evade over-expressed efflux pumps in breast cancer cells. This approach may lead to increased uptake and higher drug accumulation in nuclei of cancer cells. **Materials and Methods:** L-val-L-val doxorubicin prodrug was synthesized following standard f-moc chemistry. The prodrug was analyzed for stability, cellular and nuclear uptake and interaction with efflux and peptide transporters. Breast cancer cells (T47D) were grown on polystyrene 12-well plates. **Result:** The prodrug Val-Val-doxorubicin was found to be very stable in breast cancer cell homogenate. It was able to evade efflux pumps. The prodrug penetrated cytoplasm and nucleus of cancer cells by interacting with peptide transporters.These transporters (pepT1 and pepT2) are expressed both on plasma and nuclear membrane of breast cancer cells. Uptake of prodrug was found to be 10 times more than parent drug. **Conclusion:** Peptide prodrug derivatization of doxorubicin has potential to evade efflux pumps and increase availability and nuclear accumulation of doxorubcin in breast cancer cells.

Key words:
Breast cancer, drug delivery, efflux pumps, peptide transporter, prodrug

Introduction

Breast cancer (BC) is the most common form of cancer in women.[1] Tumor spreads early during disease development. En block resection of tumor is usually not possible. BC accounts for approximately 39,620 female deaths per year according to statistics released by the American Cancer Society in 2013.[2] Anthracyclines such as doxorubicin (DOX) have been widely used in BC chemotherapy.[3] However, multidrug resistance (MDR) caused by efflux pumps localized on plasma as well as nuclear membrane of BC cells (BCCs) is a major barrier to delivery of these agents. MDR significantly compromises clinical outcome.[4]

MDR in breast cancer cells (BCCs is associated with overexpression of efflux pumps. When MDR genes are overexpressed, tumor cells develop resistance to a wide range of structurally and functionally unrelated therapeutic agents.[5] MDR genes produce three main types of efflux proteins: P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and BC resistance protein (BCRP). Such efflux proteins play a key role in protecting cells against xenobiotics.[6-15] However, in diseased condition, they may identify drugs as foreign agents and efflux them out of cells. Multidrug efflux pumps are localized on both plasma and nuclear membranes, which may pump cytotoxic agents out of cytoplasm and nucleus, respectively.[16-18] Development

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of resistance to chemotherapeutics is one of the major factors responsible for low efficacy of anticancer chemotherapeutic drugs. Several mechanisms can lead to the development of drug resistance. However, MDR due to overexpression of efflux pumps such as P-gp is a major reason of drug resistance.[19] MDR1 gene encodes P-gp which is a transmembrane protein. It is a member of the ATP-binding cassette family of drug transporters. P-gp is responsible for efflux of many of hydrophobic, neutral, and positively charged drugs out of the cell. Expression of P-gp is a function of normal cellular defense system against xenobiotics.[20] Unfortunately, in majority of human cancers, enhanced expression of P-gp causes reduced survival and poor prognosis.[21,22]

Drug accumulation inside the cell is a complicated process.[23] It involves cellular drug uptake, retention, and distribution. Generally, most hydrophobic chemotherapeutic agents enter the cell by passive diffusion. However, efflux transporters bind drug molecule and pump it outside. At any instance, accumulation of a drug inside cells is the difference between amount of drug uptake and effluxed amount. Drug efflux caused by P-gp lowers intracellular net drug uptake or accumulation.

Therapeutic efficacy of DOX in cancer treatment is restricted primarily due to the emergence of drug resistance. Reversal of this process may lower dose necessary to eradicate tumor which will reduce drug toxicity. DOX efficacy is limited because of cardiotoxicity, a dose-dependent phenomenon and an important adverse effect that impairs treatment, outcome, and survival.[24] Chintamani *et al.*[25] reported that P-gp positive patients are clinically less responsive to DOX compared to P-gp negative group.

Due to drug exposure, tumor cells produce elevated levels of MDR proteins, resulting in enhanced efflux and diminished accumulation of anticancer agents.[26,27] Thus, progressively higher doses are needed to achieve cytotoxic concentrations in cancer cells. However, such doses may cause toxicity to nonmalignant cells such as bone marrow and cardiac and renal cells. Therefore, primary objective of this work is to design a prodrug (PD) of anticancer agent (DOX), which can evade efflux pump, recognized and taken up by influx transporters and lower drug resistance. Influx transporter-targeted PD design may be a promising strategy which can bypass efflux pumps and enhance intracellular drug accumulation in BCCs.[28,29] It involves conjugating small peptide to anticancer agents which may serve as substrates to the membrane influx transporters, such as human peptide transporter (hPEPT), highly expressed on BCCs [Figure 1].

PEPT1 and 2 belong to the peptide transporter (PEPT) family.[30] These proteins are expressed as integral membrane proteins and are responsible for uptake of di- and tri-peptides across membranes. These transmembrane transporters are able to transport peptidomimetics and peptide-conjugated therapeutic agents.

In this study, we have focused on two isoforms of PEPT-PEPT1 and PEPT2. PEPT1 is a low-affinity and high-capacity transporter whereas PEPT2 is a high-affinity and low-capacity transporter.[24] The PD developed in this work is L-Val-L-Val peptide conjugated to DOX. L-Val-L-Val is an excellent substrate of both PEPT1 and PEPT2, which are overexpressed in plasma and nuclear membranes of BCC.

Hence, the objective of this work is to design peptide transporter-mediated delivery of DOX which can accumulate in the nuclei of BCC. This article provides proof-of-concept strategy to establish that PD modification of DOX can enhance DOX exposure to tumor cells and reduce dose-related toxicity. This approach may (i) evade efflux transporters since the parent drug is a substrate of efflux pumps but not the PD, (ii) the peptide PD (Val-Val-DOX) will be recognized by peptide transporters highly expressed on both plasma and nuclear membranes of tumor cells. Once inside the nucleus, the PD will be cleaved to its parent drug DOX. This dual approach may significantly enhance drug efficacy and

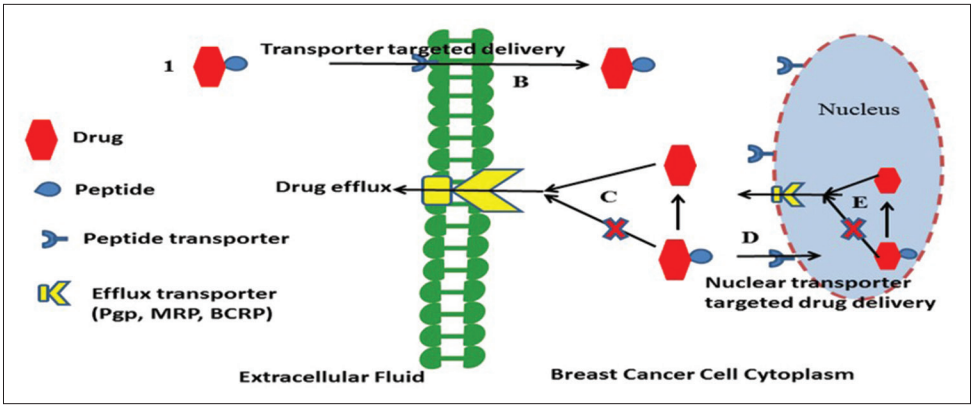

Figure 1: Translocation of prodrug across plasma and nuclear membrane of breast cancer cells

reduce the dose required to achieve pharmacological action.

Materials and Methods

Materials

T-47D (breast cancer cell line) was obtained from ATCC. Glysar, cefadoxil, cephradine, PGP-4008, MK-571, Diisopropylethylamine (DIEA), DMF, dichloromethane, diethyl ether, o-benzotriazol-N,N,N^1,N^1 –tetramethyl –uronium hexafluoro-phosphate (HBTU) and doxorubicin were purchased from Sigma-Aldrich, USA. GF-120918 was obtaines from glaxoSmithKline, USA.

FMoc-Val-Val and Piperidine was purchased from Fisher-scientific.

Methods

Cell culture method

BCC line, T47D cells, of passage numbers (20–25) was selected for studies. Cells were cultured in 75 mL flasks (T-75). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. Nutrient medium to maintain cells consisted of Dulbecco's modified minimal essential medium at pH 7.4 supplemented with 10% fetal bovine serum (heat inactivated), 1% nonessential amino acids, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 29 mM sodium bicarbonate, and 100 μg/mL of penicillin and streptomycin each. The pH of the medium was adjusted to 7.4. The medium was replaced every alternate day to ensure proper growth. Cells were allowed to reach 80–90% confluency and then were passaged with TrypLE™ Express solution. Cells were seeded at a density of 250,000 cells/well in 12-well tissue culture-treated plastic plates and then were allowed to grow for 5–7 days. These cells were subsequently utilized for further studies.

Uptake studies

Uptake studies were performed using 12-well plates according to the established protocol[31] with minor modifications. After 6–8 days of seeding, confluent T47 D cell monolayers were rinsed 3 times with Dulbecco's phosphate-buffered saline (DPBS) (pH 7.4). Uptake was initiated by the addition of 1 mL drug solution containing 0.5 μCi/mL [^{3}H] Gly-Sar on apical side. Experiments were performed in the presence or absence of competing substrates and inhibitors. Incubation was carried out over a period of 30 min. Following incubation, cell monolayers were rinsed 3 times with ice-cold solution (200 mM KCl and 2 mM HEPES) to terminate drug uptake. Cells were lysed overnight using 1 mL 0.1% (wt/vol) Triton X-100 in 0.3 N sodium hydroxide at room temperature. Aliquots (500 μL) were withdrawn from each well and transferred to scintillation vials containing 5 mL scintillation cocktail. Samples were then analyzed by liquid scintillation spectrophotometry with a Beckman scintillation counter (Model LS-6500, Beckman Instruments, Inc.) (Minnesota, USA). Uptake data was normalized to the protein content of each well. The amount of protein in the cell lysate was quantified by the Bradford method utilizing BioRad protein estimation kit (BioRad, Hercules, CA, USA).

Nuclear isolation

Isolation of nucleus from T47D cells was performed based on the principle of cell fractionation and differential centrifugation. Briefly, confluent T47D cells grown in 150 cm^2 flask were harvested by trypsinization and washed 2 times with ice-cold PBS. The pellet was generated at 4°C and centrifuged at 8000 rpm for 10 min. The resulting pellet was resuspended in 500 mL of ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES; pH 7.4) and incubated on ice for 10 min. Following incubation, cells were homogenized with prechilled Dounce homogenizer (Thomas Scientific,Swedesboro, NJ, USA) (40–50 strokes) and cell lysis was ensured by lactate dehydrogenase assay. The resulting homogenate contained the nuclear fraction, which was transferred into 10 mL centrifuge tube by making the volume up to 5 mL with homogenization buffer and centrifuged at low speed (1000 g, 10 min, 4°C) to remove unlysed cells.

Synthesis of doxorubicin prodrug

Val-Val-DOX PD was synthesized by solution phase method. DIEA (*N*, *N*-Diisopropyl ethylamine) in DMF was selected as a coupling reagent. A mixture of DOX and DIEA (1:2 equivalents) in dry DMF was stirred for 10 min under nitrogen atmosphere at room temperature in dark atmosphere. Following stirring of DOX and DIEA, FMoc-Val-Val and HBTU (1:1 equivalent) in DMF were added to the solution and stirred for 4.5 h at room temperature. The solvent was evaporated overnight under high vacuum. Fmoc-Val-Val DOX was treated with 20% piperidine in DMF for 20 min at 0°C. The obtained amide bond DOX peptide PD residue was coevaporated with DCM. The final product, dipeptide – DOX dark red flakes, was purified by crystallization with cold diethyl ether [Figure 2].

Prodrug characterization

Mass spectrometer

The mass of the final purified product was identified by a mass spectrometer (MDS Sciex API 2000 Triple Quadrupole linear

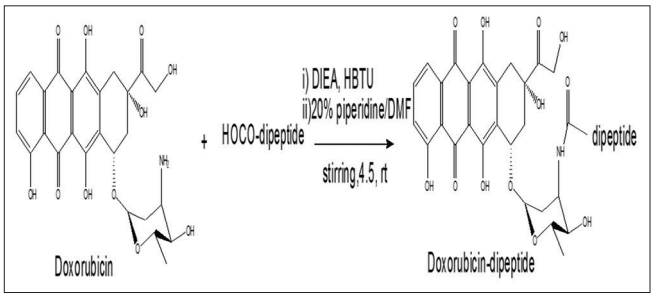

Figure 2: Synthesis scheme for valine dipeptide doxorubicin prodrug

QTrap mass spectrometry, Applied Biosystems/MDS Sciex, Faster City, CA, USA). The system was interfaced with turbo ion spray in positive ion source for detection. A constant flow rate of 15 µl/min was infused into the mass spectrometer.

High-performance liquid chromatography method

In vitro analyses of DOX and its peptide PD were performed by a reversed phase-high-performance liquid chromatography (HPLC) method with a Waters 515 HPLC pump (Waters corporation, Milford, MA, USA), Alcott autosampler (model 718 AL), Agilent 1100 series fluorescence detector, Zorbax SB-phenyl column (5 µm, 25mm×4.6mm) (Agilent technologies, Santa Clara, CA, USA), and Hewlett Packard HPLC integrator (Hewlett–Packard, Palo Alto, CA, USA). The mobile phase was comprised of 65% of 10 mM phosphate buffer as aqueous phase and 35% acetonitrile as an organic phase which was pumped at a flow rate of 1.2 mL/min. The detection wavelength was set at emission and excitation of 480 and 560 nm, respectively. Calibration curve (0.5–5.0 µg/mL) for DOX was prepared by making appropriate dilution from the stock solution. An injection volume of 50 µl was injected into the HPLC column for analysis.

A calibration curve with a regression coefficient of 0.995 was prepared. The other HPLC parameters are provided in Table 1.

Stability studies

Preparation of T47D cell homogenate

Confluent T47D cells grown in tissue culture flasks were isolated with the aid of mechanical scraper and washed 3 times with DPBS. Cells were homogenized in 2 mL of chilled (4°C) DPBS for about 10 min, with a mechanical homogenizer in an ice bath. Subsequently, the homogenates were centrifuged at 12,500 rpm for 25 min at 4°C to remove cell debris, and the supernatant was subjected to stability studies.

Stability studies

Supernatant obtained from T47D cells as described previously was equilibrated at 37°C for about 15 min prior to an experiment. Supernatant (0.8 mL) was incubated with 0.2 mL of 1 mM PD solution at 37°C in a shaking water bath for the entire study period. Positive control consisted of 0.8 mL of DPBS instead of supernatant. Aliquots (50 µL) were withdrawn at appropriate time intervals over 24 h. The samples were immediately diluted with 50 µL of chilled acetonitrile/methanol (4:5 mixture) to precipitate the proteins, and the supernatant was stored at −80°C until further analysis. The protein content of the supernatant was determined by the method of Bradford (1976), using bovine serum albumin as the standard (protein estimation kit; Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± standard deviation. Student's *t*-test was applied to detect statistical significance between the parameters, and $P < 0.05$ was considered to be statistically significant.

Results and Discussion

The purified product resulted in 56% yield with a purity of >95% as determined by HPLC. Val-Val-DOX PD was characterized with the mass spectroscopy. The mass spectrometer indicated the peptide DOX PD peak at 742.7 amu.

Stability studies

The stability data show that peptide conjugate of DOX is metabolically more stable in T47D cell homogenate (≈5% degradation of L-Val-L-Val DXR in 12 h). This will allow sufficient time for the PD to be recognized by peptide transporter present on nuclear membrane. Hence, nuclear targeting becomes a possibility. It is a promising strategy since the PD can provide sustained and targeted delivery of DOX [Figure 3]. The stability of PD is due to amide bond conjugating valine and DOX. Therefore, it prevents the peptide moiety (Val-Val) cleavage outside the cell membrane.

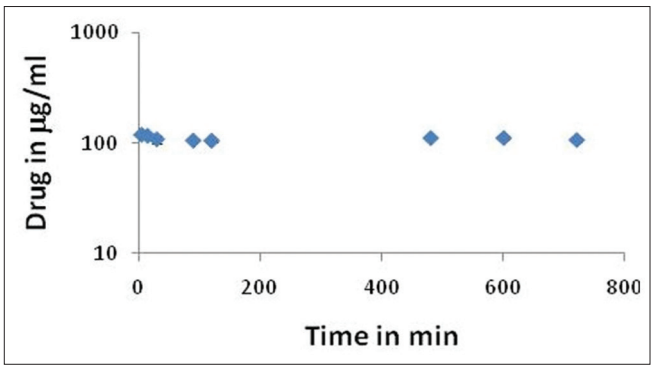

Figure 3: Stability data of doxorubicin prodrug in T47D cell homogenate

Table 1: HPLC parameters for analysis of prodrug stability

Drug/prodrug	Aqeuous phase	Organic phase	Mobile phase Aq : Org	Retention time (mins)	R^2	*r*
Doxorubicin	10mM phosphate buffer	ACN	65:35	9.8	0.995	0.997
L-val-L-val-doxorubicin	10mM phosphate buffer	ACN	65:35	12.5	0.997	0.998

ACN – Acetonitrile; HPLC – High-performance liquid chromatography

Expression of efflux pumps and interaction with peptide prodrug

Expression of efflux pumps in BCCs was evaluated utilizing various inhibitors which suppress the activity of efflux transporters. Uptake of [^{14}C] erythromycin was performed in the presence of various inhibitors of P-gp and MRP. PGP-4008, a specific inhibitor for P-gp, GF-120918, a dual inhibitor for both P-gp and BCRP and MK-571 and inhibitor of MRP are selected to determine the specificity of the PD. Moreover, excess DOX was also included as a competitive substrate inhibitor for the transporters. Erythromycin is a substrate for both P-gp and MRP. Uptake of ^{14}C-Erythromycin was elevated 2.5 fold in response to PGP-4008 in BCC monolayers. This result suggests expression of P-gp in these cells. Similarly, ^{14}C-Erythromycin uptake was enhanced by almost five-fold in the presence of MK-571. This data indicates inhibition of MRP in this process. The result indicates high expression of MRP in T47D cells. Surprisingly, GF-120918 caused insignificant enhancement of erythromycin uptake compared to positive control (^{14}C-Erythromycin in the absence of any inhibitor). GF-120918 is a strong inhibitor of BCRP and a weak inhibitor of P-gp. Hence, it is evident from this result that erythromycin is not a substrate of BCRP. Furthermore, the addition of unlabeled DOX elevated uptake of ^{14}C-Erythromycin by almost 4.5 times. It may be due to competitive substrate inhibition of both P-gp and MRP. From these experiments, it becomes clear that DOX is a substrate of efflux transporters whereas the PD L-Val-L-Val-DOX is not a substrate. One of the reasons for low bioavailability of DOX in cancer patients is possibly due to drug efflux. Hence, higher doses are required for therapeutic efficacy which leads to dose-related toxicity. If this form of resistance development can be prevented, it will lead to a much better therapeutic outcome. From Figure 4, it is apparent that the PD derivatization of DOX may achieve that goal. The peptide PD can bypass the efflux pumps and therefore may be able to reduce dose-related toxicity.

Peptide transporter on plasma membrane of breast cancer cell

Gly-Sar is a model substrate of hPEPT. Cephradine and cefadroxil are inhibitors of PEPT1 and PEPT2, respectively. Uptake of [^{3}H] Gly-Sar was measured in the presence of 1 mM Gly-Sar, 1 mM cephradine, 1 mM cefadroxil. This study suggests that there is indeed an inhibition of [^{3}H] Gly-Sar uptake in the presence of unlabeled Gly-Sar and PEPT inhibitors. Unlabeled Gly-Sar is a substrate of both PEPT1 and PEPT2. Hence, the peptide PD competes with [^{3}H] Gly-Sar resulting in almost 50% decrease in uptake of [^{3}H] Gly-Sar. In the presence of both cephradine and cefadroxil, there is approximately 25% inhibition in the uptake of [^{3}H] Gly-Sar. These results clearly indicate the expression of both PEPT1 and PEPT2 on the plasma membrane of BCC [Figure 5]. Hence, these transporters may offer an attractive target for the development of PDs which may enhance accumulation of DOX in cancer cells.

Peptide transporter on nuclear membrane

We have also performed experiments to show the presence of peptide transporter on nuclear membrane of T47D. Our studies indicate that in the presence of unlabeled Gly-Sar, nuclear uptake of [^{3}H] Gly-Sar is competitively inhibited [Figure 6]. In the presence of unlabeled Gly-Sar (2.5 mM), uptake of [^{3}H] Gly-Sar decreases approximately 1.8 times. This study suggests that nuclear membrane of T47D cells express peptide transporters. This may be exploited for targeted drug delivery. Peptide transporters might be able to translocate our peptide PD into the nucleus. This has the potential to enhance bioavailability and reduce dose-related toxicity.

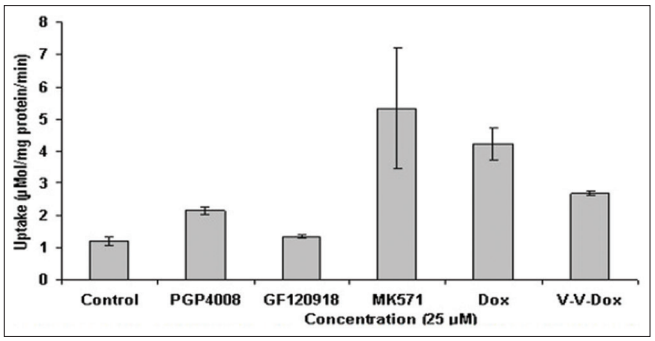

Figure 4: Uptake of [^{14}C] erythromycin in the presence of various efflux pump inhibitors, doxorubicin, and L-Val-L-Val-doxorubicin

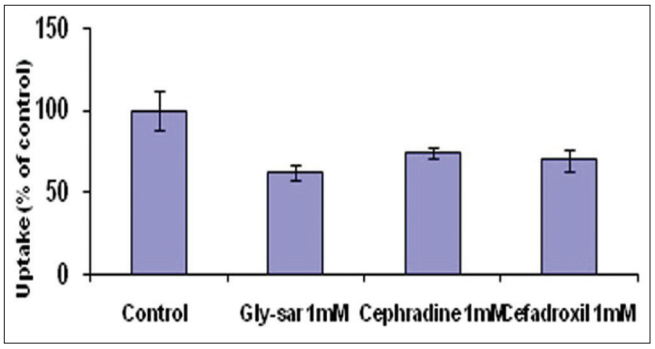

Figure 5: Uptake of [^{3}H] Gly-Sar in the presence of unlabeled Gly-Sar, cephalosporins (cephradine and cefadroxil) in T47D

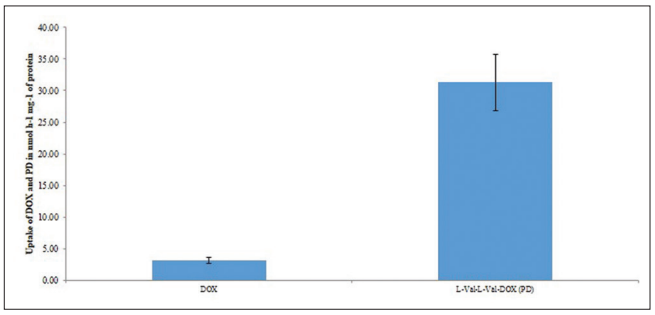

Figure 6: Uptake of doxorubicin and L-Val-L-Val-doxorubicin in T47D cells

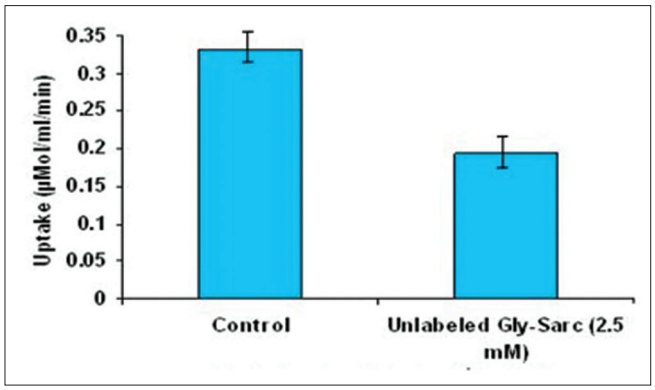

Figure 7: Nuclear uptake of [^{3}H] Gly-Sar in the presence of unlabeled Gly-Sar

In-vitro efficacy of prodrug

Figure 7 depicts approximately ten-fold elevated accumulation of peptide-PD of DOX (L-Val-L-Val-DOX) compared to parent drug DOX in T47D cells. Therefore, PD of anticancer agents targeted to hPEPT may undergo facilitated transport into the cytoplasm as well as the nucleus of BCC. This result indicates that the PD may exert more potent anticancer activity than the parent drug DOX.

Discussion

Chemotherapeutic agents such as DOX which are mostly administered systemically exhibit limited permeability into BCC. Efflux pumps such as P-gp and MRP are responsible for poor uptake of various chemotherapeutic agents. The inability of drugs to evade efflux may be one of the major impediments to cancer chemotherapy. Cancer cells exhibit higher expression of efflux pumps relative to normal nonmalignant cells. Most cancer drugs such as DOX are substrates of efflux transporters such as P-gp, MRP, and BCRP.

Cyclosporine has been included in cancer treatment regimen as a P-gp inhibitor by a few studies.[32] It was anticipated that anticancer agents would show better efficacy. However, this approach has not been very successful.[21] For therapy to be effective, cancer cells should be exposed to chemotherapeutic agents in therapeutic concentrations. Usually, intravenous route is preferred for the administration of chemotherapeutic agents. As a result, normal cells are exposed to a high concentration of anticancer agents resulting in cytotoxicity.[22] Furthermore, exposure to chemotherapeutics causes up-regulation of MDR genes. It may enhance expression of efflux pumps (P-gp, MRP, and BCRP), leading to elevated drug efflux out of cancer cells.[23] Most chemotherapeutics are excellent substrates of efflux pumps.

Failure of chemotherapeutics to elicit pharmacological response in cancer patients may be caused due to the presence and/or development of drug resistance. The present study shows this issue can possibly be resolved by PD derivatization.

The peptide PD synthesized is not a substrate of the efflux transporters as evident from Figure 4. However, it is a substrate of the influx peptide transporters expressed both on the cell membrane and the nuclear membrane [Figures 6 and 7]. Hence, there is a higher probability of accumulation of the chemotherapeutic agent inside the cancer cell nuclei. Our data provide evidence of hPEPT expression on plasma and nuclear membranes of T47D BCCs [Figures 6 and 7]. Inhibition of [^{3}H] Gly-Sar (preferred substrate for hPEPT) uptake by unlabeled Gly-Sar and cephalosporins indicates expression of specific hPEPT on BCCs. Enzymatic hydrolysis will follow to generate parent drug. Of importance is the ability of drug peptide conjugate to overcome drug efflux pumps at both cell and nuclear membranes [Figures 6 and 7]. The data clearly provides evidence that peptide conjugate of DOX can bypass efflux pumps. Since nucleus also express efflux pumps, PD of DOX may also bypass efflux at the nuclear membrane. This strategy may be suitable for targeting both plasma and nuclear membranes of BCCs, thereby enhancing anticancer efficacy at target organ.

Another interesting property of the synthesized PD is its stability. As Figure 3 shows this, PD is highly stable. Hence, the PD may not readily revert back to the parent drug in the cytoplasm. It would allow the PD sufficient time to enter the nucleus. If the drug is not stable and conversion of the PD to drug occurs even in cytoplasm, there is a possibility that the parent drug will be effluxed out. Hence, a stable PD-like Val-Val-DOX has the potential to enhance availability of the chemotherapeutic agent in tumor cells.

Conclusions

Peptide PD derivatization has the potential to deliver anticancer drugs effectively into nucleus of cancer cells. As a result, cytotoxic activity is enhanced. Hence, this approach needs to be developed as an effective cancer treatment.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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