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Design of a doxorubicin-peptidomimetic conjugate that targets HER2-positive cancer cells

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Abstract

Doxorubicin (DOX) belongs to the anthracycline class of drugs that are used in the treatment of various cancers. It has limited cystostatic effects in therapeutic doses, but higher doses can cause cardiotoxicity. In the current approach, we conjugated a peptidomimetic (Arg-aminonaphthylpropionic acid-Phe, compound **5**) known to bind to HER2 protein to DOX via a glutaric anhydride linker. Antiproliferative assays suggest that the DOX-peptidomimetic conjugate has activity in the lower micromolar range. The conjugate exhibited higher toxicity in HER2-overexpressed cells than in MCF-7 and MCF-10A cells that do not overexpress HER2 protein. Cellular uptake studies using confocal microscope experiments showed that the conjugate binds to HER2-overexpressed cells and DOX is taken up into the cells in 4 h compared to conjugate in MCF-7 cells. Binding studies using surface plasmon resonance indicated that the conjugate binds to the HER2 extracellular domain with high affinity compared to compound **5** or DOX alone. The conjugate was stable in the presence of cells with a half-life of nearly 4 h and 1 h in human serum. DOX is released from the conjugate and internalized into the cells in 4 h, causing cellular toxicity. These results suggest that this conjugate can be used to target DOX to HER2-overexpressing cells and can improve the therapeutic index of DOX for HER2-positive cancer.

Graphical Abstract

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Supplementary data related to this article can be found at

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Keywords

HER2; Lung cancer; Breast cancer; Doxorubicin; Peptidomimetic; Conjugate

1. Introduction

Doxorubicin (DOX), an anthracycline class antibiotic, is a well-known anticancer drug used in treating different types of cancers such as lung cancer, colon cancer, breast cancer, leukemia, etc [1, 2]. DOX kills cells via mechanisms such as inhibiting topoisomerase II-DNA complex, resulting in DNA damage; generating free radicals, leading to oxidative stress; and acting on mitochondria by release of cytochrome c [3]. DOX lacks tumor-specific activity because it targets all dividing cells and, at higher doses, DOX treatment causes irreversible cardiotoxicity[4]. The other major limitation when using DOX is the development of resistance due to a decrease in membrane permeability, an increase in efflux pumping, and alteration of target proteins [5–7]. To improve its selectivity and anticancer activity, several studies were carried out in the last decade in which the drug was conjugated with tumor-specific agents that bind to overexpressed cell surface receptors on the tumor cells. This strategy increases the specificity of DOX toward tumor cells that overexpress cell surface receptors. Many researchers conjugated DOX with different tumor-specific agents such as peptides, hormones, and antibodies for tumor-targeted delivery [8–11]. Small peptides and peptidomimetics offer wide advantages as drug carriers because they can be easily modified and the synthesis procedures are relatively simple. Furthermore, many peptidomimetics exhibit lower toxicity and a lack of immunogenicity[12].

Epidermal growth factor receptors (EGFR), which belong to the tyrosine kinase family of receptors, consist of different domains with an extracellular domain that is made up of 620 amino acids, a short and single transmembrane domain, and an intracellular tyrosine kinase domain[13–16]. Among these, human epidermal growth factor receptor-1, -3 and -4 (HER1, HER3, and HER4) have ligands and, upon binding of the ligand to the extracellular domain, these receptors change their conformation and dimerize with other receptors. When receptors dimerize, autophosphorylation occurs, leading to subsequent signal transduction, which ultimately leads to cell proliferation, apoptosis, angiogenesis, and metastasis [14, 17]. However, no ligands were reported for HER2 receptors, which activate by interacting with other EGFR [18]. HER2 receptors are overexpressed in 30% of breast [17, 19], lung[20, 21], and ovarian cancers. Discovery of the role of HER2 receptor in disease pathogenesis led to

the development of different targeted therapies[22] using, for example, trastuzumab, an antibody that binds specifically to HER2 receptors[17, 23] and pertuzumab, which binds to HER2 and inhibits the dimerization of HER2 receptors with other receptors[24, 25].

We have designed several peptides/peptidomimetics that inhibit the dimerization of HER2 with other EGFR, which leads to blockage of HER2-mediated signaling. Among several peptidomimetics we designed, compound **5** (Fig. 1) binds to the extracellular domain IV of HER2 and inhibits HER2 dimerization with other EGFR [26–29]. Obstruction of domain IV for dimerization leads to the interruption of HER2-based signaling that stimulates tumor growth. In our previous studies we have shown that compound **5** has selectivity toward HER2-overexpressed cancer cells; hence, it is advantageous to conjugate compound **5** via a linker to DOX that target HER2 overexpressed cancer cells with reduced delivery to normal cells. Previous reports focus more on using the peptide to target particular type of cancer cells or for cell penetration purposes. However, the method used here is slightly different from the previous approach in which we have used a peptidomimetic compound that targets HER2 and also inhibits cell signaling for cancer. In this sense, our approach is not a prodrug method where the vehicle has no pharmacological effect. Herein, we report the design, synthesis, and in vitro activity of a DOX-compound **5** conjugate (Fig. 1) that specifically targets HER2-positive cancer cells. We attempted the synthesis of this conjugate through different linkers and different methods using the C- and N-termini of the peptide. Synthesis was successful when the N-terminal of the peptide was linked to DOX via 5-carbon linker. Antiproliferative assays were performed on various cancerous cell lines (BT-474, SKBR-3 and Calu-3 and MCF-7) and normal (non-cancerous) breast cell lines (MCF-10A) to evaluate the effectiveness of the conjugate. Results showed that the conjugate has antiproliferative activity with an IC₅₀ value in the low micromolar concentration range. Time-dependent uptake studies were performed to find the localization of conjugates using confocal microscopy experiments on the SKBR-3 cell line. SPR analysis was performed to show that the conjugate binds to HER2 extracellular domain IV.

2. Results and discussion

2.1. Synthesis

Synthesis of the conjugate was attempted by directly attaching (without using a linker) DOX to the C-terminal of the peptidomimetic with the N-terminal protected by the fluorenylmethyloxycarbonyl chloride (Fmoc) group. Although the final compound was obtained, purification was difficult because of its very low yield. Later, by adopting the procedure described by Nasrolahi Shirazi et al. [30], we tried to synthesize the conjugate. However, in the preliminary step, attaching t-butyl anhydride to the C-terminal of compound **5** posed a problem. Finally, the synthesis of the DOX-peptidomimetic conjugate was completed using glutaric anhydride as the linker. In this method, compound **5** was first synthesized using Wang resin and, after Arg-Anapa-Phe, where Anapa is aminonaphthylpropionic acid was synthesized, the resin was not cleaved. The N-terminal of Arg in the peptidomimetic was linked to glutaric anhydride via an amide bond. DOX was linked to compound **5** via 5-carbon linker (Scheme 1). The final product was purified by

high performance liquid chromatography (HPLC) and analyzed by analytical HPLC, mass spectrometry (Fig. 2), and 1D and 2D ^1H NMR (Figs. S1–S6 see Supplementary material).

2.2. Antiproliferative activity

Cell Titer-Glo[®] assay[31] was performed to compare the cytotoxicity of DOX-conjugate with that of DOX and compound **5**. We selected different cancer cell lines that overexpress HER2 receptors (BT-474, SKBR-3, Calu-3) and one that does not overexpress HER2 receptors (MCF-7). Using the cell Titer-Glo (CTG) reagent, cell viability was evaluated and a dose-response curve was used to calculate IC_{50} values. Antiproliferative activity was evaluated for two time points—24 and 48 h. For MCF-7 and MCF-10A, the assay was also performed after 72 h. From Table 1, we can infer that the conjugate IC_{50} value on MCF-7 (which does not overexpress HER2) is many times higher than that of DOX, indicating that the conjugate has achieved selectivity. In MCF-10A, which are normal breast cell lines, compound **5** exhibits IC_{50} values $> 50\ \mu\text{M}$, whereas the conjugate and DOX exhibit IC_{50} values of 0.85 ± 0.07 and $0.15 \pm 0.03\ \mu\text{M}$, respectively.

2.3. Confocal microscopy

Binding to HER2-overexpressed cells and cellular distribution of the conjugate were evaluated by confocal microscopy. In this experiment, we incubated the SKBR-3 cells at $37\ ^\circ\text{C}$ with $5\ \mu\text{M}$ DOX or conjugate for different time periods (30 min, 4 h, 24 h). The cells were imaged using a confocal microscope. The nuclei were observed by staining with 4',6-diamidino-2-phenylindole (DAPI) while the distribution of DOX and the conjugate was studied by the autofluorescence of the DOX. DOX is known to accumulate in the nucleus and, hence, when only DOX is used we expect to see nuclear accumulation of DOX fluorescence. In the 30-min image (Fig. 3A), we see that the cells incubated with free DOX showed strong DOX fluorescence in both the nucleus and cytoplasm whereas the fluorescence in the cells incubated with conjugate was present in the cytoplasm and relatively less in the nucleus. After 4 h (Fig. 3B), there was a decrease in DOX fluorescence compared to that observed after 30 min, but most of the fluorescence was in the nuclear region. However, the conjugate exhibited some fluorescence in the cytoplasm and nucleus. From this it can be inferred that the conjugate was more confined to perinuclear regions. After 24-h incubation (Fig. 3C) with free DOX, the autofluorescence of DOX was weak, which may be due to the degradation of DOX. The DOX fluorescence in the cells incubated with conjugate was weaker than in the 4-h incubation, but was confined to perinuclear regions. These results suggest that the conjugate binds to surface proteins and then internalizes slowly, while DOX is internalized to the nucleus in a short time. Overall, the conjugate is able to bind to the HER2 receptors and DOX is able to enter the cytoplasm and nucleus. Similar studies were carried out on MCF-7 cells to evaluate the specificity of the conjugate. Microscopic images suggest that in the cells that were incubated with conjugate, there was no DOX fluorescence in MCF-7 cells during 30-min or 4-h incubation. DOX fluorescence was observed only in 24-h incubation. In the cells that were incubated with DOX only, fluorescence was seen in 30 min, suggesting that the DOX internalizes in MCF-7 cells and the conjugate does not specifically bind to MCF-7; hence, DOX enters the cells after 24 h (Fig. S7 A,B & C see Supplementary material).

2.4. Stability studies

The stability of conjugate was evaluated in the presence of SKBR-3 cells as well as in human serum. DOX-compound **5** conjugate was incubated in SKBR-3 cells (50 μ M). At different time points (0, 15, 30 min, 1, 2, 4, 6, 8, 24, and 48 h) the cells were lysed and the conjugate was extracted using acetonitrile and analyzed by HPLC. The area under the curve (AUC) for the peak corresponding to the intact conjugate was monitored. Fig. 4A displays the time profile graph of stability of DOX-compound **5** conjugate. There was a decrease in the relative AUC compared to the zero time point up to 4 h after which the relative peak area was stabilized to 50% of the original peak up to 48 h; this suggests that in nearly 4 h 50% of the conjugate is degraded. The remaining part of the DOX-compound **5** conjugate might bind to the proteins non-specifically and remain stable. This result is consistent with microscopy studies in which DOX fluorescence was observed up to 4 h and after that the intensity was decreased.

The proteolytic stability of DOX-compound **5** conjugate was also evaluated in human serum, and the samples were analyzed after incubation of the conjugate in serum for different time points (0, 15, 30 min, 1, 2, 4, 6, 8, 24, and 48 h) using HPLC and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). The stability profile for conjugate using AUC (Fig. 4B) suggests that the conjugate was stable with an estimated half-life of 1 h. Taken together, these stability data suggest that DOX-conjugate **5** will be stable in the presence of cellular enzymes and in serum that allows sufficient time to reach the target cancer cells. Once the conjugate reaches the site, the conjugate binds to cell surface proteins and releases the DOX to internalize into the cells. However, detailed pharmacokinetics studies in an animal model are needed to confirm this.

2.5. Flow cytometry and cell cycle analysis

DOX is known to kill cells by inhibiting the cell cycle [32, 33]. The aim of this experiment is to determine whether the conjugate will inhibit the cell cycle in BT-474 cells. BT-474 cells were treated with either DOX or conjugate or were untreated for 1 h, then after 24-h incubation they were analyzed using a flow-cytometer. The cells in the untreated sample (Fig. 5) were distributed in the G0/G1 phase ($57 \pm 0.73\%$), S phase ($13.6 \pm 0.56\%$), and G2/M phase ($29.7 \pm 1.0\%$). The distribution of DOX-treated cell samples in different phases was G0/G1 phase ($75.6 \pm 0.4\%$), S phase ($3.7 \pm 0.4\%$), and G2/M phase ($20.6 \pm 0.13\%$) whereas the distribution pattern for the cells treated with conjugate was G0/G1 phase ($65.1 \pm 0.5\%$), S phase ($8.7 \pm 0.8\%$), and G2/M phase ($25.9 \pm 0.32\%$). Cell numbers were significantly lower in untreated cells than in treated groups in the G0/G1 phase while in the S phase and G₂ phase the DOX- and conjugate-treated cell numbers were lower compared to untreated cells. The conjugate showed cell cycle inhibition comparable to that of DOX, suggesting that it is inhibiting the cell cycle in the BT-474 cell line. Similar studies were carried out on compound **5**. The cells in the untreated sample (Fig. S8 see Supplementary material) were distributed in the G0/G1 phase ($63.5 \pm 1.2\%$), S phase ($8.4 \pm 1.3\%$), and G2/M phase ($27.5 \pm 0.3\%$). Cells treated with compound **5** showed slightly different pattern than control cells for the G0/G1 phase ($67 \pm 2\%$), S phase ($5.7 \pm 1.1\%$), and G2/M phase ($23.6 \pm 1.8\%$), respectively. From the above, it can be concluded that there is no significant difference in the cell numbers among different phases between the untreated and compound

5-treated cells, suggesting that compound **5** has no significant effect on cell cycle inhibition. Since compound **5** is known to bind to the extracellular domain and inhibit HER2:HER3 signaling and phosphorylation of HER2, compound **5** does not seem to affect the cell cycle directly.

2.6. 3D antiproliferative assay

The 3D cell-based experiments mimic tumors in some aspects and hence can be used as preliminary investigations before in vivo studies[34–36]. The hanging drop method of obtaining spheroids is advantageous because of its simplicity and reproducibility; moreover, these spheroids resemble tissue[37] unlike in other methods such as extracellular matrix (ECM) scaffolds and hydrogel systems. Spheroids of BT-474 were obtained after 4–5 days of incubation, and they were then treated with different concentrations of conjugate and DOX for 3 days. Cell viability was evaluated using 3D Cell Titer-Glo® reagent. Dose-response curves indicated that conjugate and DOX inhibit the 3D spheroids with IC₅₀ values of 0.8 μM and 0.18 μM, respectively. The IC₅₀ value of the conjugate is higher in 3D plates than in 2D plates, which may be due to the fact that the inner layer of cells in the spheroid is not exposed to the drug. The higher IC₅₀ values in 3D antiproliferative assay compared to 2D antiproliferative assay is in agreement with previous reports[38–40]. Compound **5** inhibited 3D spheroid formation with an IC₅₀ value of 0.94 μM. These results suggest that the conjugate and compound **5** have the same potency. Thus, the designed conjugate may serve to deliver DOX selectively to the HER2-positive cells.

2.7. Surface plasmon resonance (SPR) analysis

This experiment was performed with the aim of showing that the conjugate is binding to the HER2 DIV protein. The HER2 DIV was immobilized on a carboxymethylated (CM5) dextran sensor chip[41] using 10 mM acetate buffer at pH 4, at which the protein showed the highest binding to the sensor chip. The stable surface of immobilized protein was obtained at relative response units (RU) of 10,018. After immobilization of the protein, different concentrations of conjugate were injected (0, 10, 25, 50, and 100 μM) onto the protein. As the concentration of conjugate increased, the RU also increased (Fig. 6). This suggests that the conjugate is binding to the HER2 DIV protein. As a control, a compound that exhibits antiproliferative activity against BT-474 cell lines at concentrations >50 μM was used. The control compound did not show any binding to HER2 domain IV protein and DOX was also used as control (Figs. S9, 10 & 11 see Supplementary material). Kinetics of association and dissociation were analyzed by curve fitting 1:1 Langmuir binding, and the calculated K_d value for conjugate was 0.172 μM and for DOX only 11 mM, suggesting that the conjugate binds to HER2 with high affinity and that DOX has very weak binding to the HER2 protein extracellular domain IV. In previous studies, we have shown that compound **5** binds to the HER2 extracellular domain (ECD) with a K_d value around 1 μM[27].

2.8. Molecular dynamics (MD) simulations and docking

The structures of the conjugate were generated using molecular modeling software InsightII (BIOVIA, San Diego, CA). To determine how the DOX portion of the molecule is oriented with respect to the peptide structure, MD simulations were carried out. The peptidomimetic

portion was folded like a beta-turn-type of structure (pseudo turn)[42, 43] from linker to Phe with Arg and Anapa at the corners of the turn structure (Fig. 7A). The naphthyl group of the peptidomimetic was either parallel or perpendicular to the DOX anthracycline rings during the dynamics (Figs. 7 B&C). Arg formed cation-pi interactions with DOX anthracycline rings, while the Phe of the peptidomimetic was near the sugar moiety. During the dynamics, the entire peptidomimetic portion moved away from DOX rings, but the peptidomimetic part was still folded like a beta-turn (Fig. 7D). Thus, it is clear that, in the absence of a receptor, there is a possible interaction between the naphthyl group and Arg of the peptidomimetic with DOX to form a compact structure. The conformation of the conjugate that has orientation of the peptidomimetic that moves away from the DOX ring may be suitable for binding to the receptor.

SPR studies indicated that the conjugate binds to HER2 protein, specifically to domain IV. To gain insight into how the peptidomimetic portion binds to HER2 receptor, docking studies were performed[44, 45]. A cluster of low-energy docked structures of the conjugate bound to HER2 protein domain IV are shown in Fig. 8A. In the lowest energy docked structure, the peptidomimetic portion of the structure was folded in a beta-turn-type of structure. However, the DOX part of the molecule was away from the peptidomimetic portion (Fig. 8B). There was no interaction of the anthracycline ring of DOX with the Arg or naphthyl group of the peptidomimetic. The peptidomimetic was bound near Val 573, Trp583 of HER2 with the C-terminal of the peptidomimetic forming a hydrogen-bond interaction with the backbone of Lys574 of HER2. The linker carbonyl carbon formed a hydrogen bond with the NH of Ser571 of HER2. The naphthyl group of the peptidomimetic formed a hydrophobic interaction with Trp583. This region is the protein-protein interaction site of domain IV in the HER1 homodimer[46]. The sugar part of DOX formed a hydrogen bond with Asp561 of HER2 protein. The anthracycline ring of DOX formed hydrophobic interactions with Val566, Phe564, Pro534, His533, and Pro563.

Our ultimate aim is to make the conjugate specific for HER2 cells under in vivo conditions. It is clear from these studies that DOX alone binds to HER2 ECD at very high concentrations with a K_d value of 11 mM, whereas the conjugate binds with a K_d value of 170 nM. Docking studies support the high affinity of the conjugate binding to HER2 ECD. When compound **5** binds to ECD of HER2, the DOX part of the conjugate binds to a hydrophobic cavity on ECD (Val566, Phe564, Pro534, His533, and Pro563) and, hence, the K_d value of the conjugate was much lower than that of DOX alone or compound **5** alone. Since we add the conjugate directly to cells in suspension, DOX will be internalized into the cells and cause cellular toxicity depending on the stability of the conjugate. Based on the stability of the conjugate in the presence of cells, we can conclude that in 4 h DOX is released from the conjugate and is internalized into the cells. Since DOX does not have any specificity for cells, the IC_{50} value for MCF10-A was 0.85 μ M after 72 h. DOX is also known to enter the cell directly by a diffusion mechanism because of its small molecular weight and lipophilicity, whereas the conjugate may need a receptor-mediated mechanism. Compound **5** is known to bind specifically to the HER2 protein extracellular domain. From these studies, it is not clear whether DOX alone internalizes into the cells or the conjugate internalizes into the cells and then DOX dissociates. Internalization of compound **5** depends

on several factors. Compound **5** itself may be internalized in 24 h or the bound compound may internalize along with HER2 depending on HER2 trafficking[47, 48]. Based on our stability data of the conjugate in the presence of cells, we can conclude that the conjugate is unstable in the presence of cells after 4 h and, thus, there is a possibility that DOX is internalized. Since the fluorescence observed is due to DOX, we believe that only DOX is internalized in around 4 h. This also explains the toxicity of the conjugate in 24 and 48 h in MCF-7 and MCF-10A cells (Table 1). Compared to previously reported methods, our approach differs in the sense that the peptidomimetic carrier is not a prodrug moiety, rather the carrier also has antiproliferative activity against HER2-positive cancer cells. Serum stability studies indicate that the DOX-compound **5** conjugate is stable for a short duration in serum to possibly reach the target cells and release DOX. Such conjugates can be used to target DOX to HER2-overexpressing cells and can improve the therapeutic index of DOX for HER2-positive cancer.

3. Conclusions

A conjugate of DOX with a peptidomimetic that is highly specific for HER2-overexpressed cancer cell lines was designed and synthesized. Antiproliferative activity data suggested that the conjugate was able to inhibit cell growth of HER2-positive cancer cells such as BT-474, SKBR-3, and Calu-3. The binding of conjugate to the extracellular domain of HER2 protein, in particular to domain IV, was evident from SPR analysis. Cellular assays indicated that the conjugate arrests the cell cycle and DOX enters the cytoplasm of the cell after 4 h. Evaluation of the stability of the conjugate in the presence of cells and in serum indicates that the conjugate is stable in serum for around 1 h, and DOX may be released from the conjugate after 4 h in cells. Based on docking studies, a model was proposed for conjugate binding to HER2 protein domain IV. The proposed model suggests that the peptidomimetic portion of the conjugate binds to the protein-protein interaction site of HER2 domain IV and DOX forms hydrophobic interactions with amino acid residues in domain IV near the C-terminal. The data suggest that the conjugate may be useful for delivering DOX to HER2-overexpressed cancer tumors. Further studies related to the stability of the conjugate and pharmacodynamics of the conjugate in vivo are in progress.

4. Experimental section

4.1. General information

All the chemicals, reagents, solvents, cell lines, etc. were from commercial sources. Fmoc protected amino acids were obtained from AAPPTEC (Louisville, KY) and EMD Biosciences (San Diego, CA). DOX was obtained from Astatech (Bristol, PA). Resin was bought from Chem-Impex (Wood Dale, IL). All cell lines studied were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell Titer-Glo[®] reagent was purchased from Promega (Madison, WI). Propidium iodide staining solution was obtained from BD Biosciences (San Jose, CA). 96-well hanging drop plates for obtaining 3D spheroids were ordered from 3D Biomatrix (Ann Arbor, MI).

4.2. Compound 5 synthesis with glutaric anhydride linker

Compound **5** was synthesized according to the procedure reported in our previous publications[26, 28, 29]. In brief, compound **5** was synthesized using standard solid-phase synthesis peptide chemistry. The synthesis was performed in a column fitted with a polypropylene frit using Fmoc-Phe-Wang resin (128 mg, 0.39 mmol/g) which, after swelling with dimethylformamide (DMF) (30 min), was deprotected using 20% piperidine in DMF (3 × 5 min). The resin was washed with DMF (5 × 30 s), after which Fmoc-S-anapa (109 mg, 5 eq), 2-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium-hexafluorophosphate (HCTU) (103 mg, 5 eq) and N-methylmorpholine (NMM) (2 mL, 0.4 M solution in DMF) were added to the column. After mixing for 30 min, Fmoc deprotection and washes were repeated followed by addition of the next amino acid, Fmoc-Arg (Pbf)-OH (162 mg, 5 eq), HCTU (5 eq), and NMM. After mixing for 30 min, Fmoc deprotection and washes were repeated followed by addition of glutaric anhydride (0.57g, 100 eq, in 1.5 mL of NMP) and 0.5 mL NMM. After mixing, the resin was washed with DMF (5 × 30 s). Small amount of the resin was used to cleave the compound from resin and the product was analyzed by HPLC and mass spectrometry. Molecular formula, C₃₃H₄₀N₆O₇, calculated monoisotopic mass, 632.295, experimental m/z [M+H]⁺ 633.337.

4.3. Conjugation of DOX to compound 5 with glutaric anhydride

DOX (116 mg, 4 eq) and HCTU were dissolved in 2 mL of 0.4 M NMM in NMP and activated by mixing for 2 min. This solution was added to the resin (containing compound **5** linked to glutaric anhydride) and shaken overnight. The resin was drained, followed by washing with DMF (5 × 30 s). The resin was then washed with DCM (5 × 30 s), and the peptide was cleaved from the resin by shaking it in a cleavage cocktail (trifluoroacetic acid (TFA):water:triisopropylsilane (TIPS); 95:2.5:2.5) for 2 h. The solution containing the peptide conjugate was drained and transferred into a 50 mL centrifugation tube. 20 mL of cold diethyl ether was added to the centrifugation tube to precipitate the conjugate, which was centrifuged for 10 min. The ether wash was decanted from the peptide pellet. Fresh cold ether was added to the centrifuge tube and the peptide was resuspended. This procedure was repeated a total of 5 times. The precipitate was dissolved in 4 mL of 0.1% TFA in water and freeze-dried to yield a white powder. The conjugate was purified by HPLC and analyzed by mass spectroscopy.

4.4. DOX-compound 5 conjugate

¹H NMR (DMSO-d₆, 500 MHz, δ ppm): 1.25 (s, 3H, CH₃, 11DOX), 1.30 (m, 2H, C^γH₂ Arg), 1.45 (m, 1H, 12DOX), 1.73 (m, 2H, C^βH₂ Arg), 1.8 (d, 2H, CH₂, 18DOX), 1.97–2.0 (m, 4H, 13, 17DOX), 2.5 (s, DMSO solvent), 2.60 (m, 16DOX), 2.75 (d, 1H, 7DOX), 2.80 (s, 2H, 4DOX), 3.30 (m, 2H, C^δH₂ Arg), 3.39 (s, HDO solvent), 3.85 (s, 10DOX), 3.99 (s, 3H, 5DOX), 4.60 (d, 2H, 6DOX), 5.06 (m, 8DOX), 5.44 (m, 9DOX), 7.65 (m, 3DOX), 7.85 (m, 2DOX), 7.90 (m, 1DOX). Other resonances related to peptidomimetic and DOX are analyzed in 2D NMR (Supporting data). Molecular formula, C₆₀H₆₇N₇O₁₇; calculated monoisotopic mass, 1157.459; experimental m/z [M+H]⁺ 1158.495. Analytical HPLC (acetonitrile/water/TFA) 29.98 min, 95% purity.

4.5. Cell lines

Human breast cancer cell lines (BT-474, SKBR-3, and MCF-7), a normal breast cell line (MCF-10A), and lung cancer cell lines (Calu-3) were purchased from ATCC. All the cells were cultured in 75 cm² cell culture flasks in an incubator at 37 °C and 5% CO₂. Basal medium RPMI-1640 was used for the cell lines BT-474 and MCF-7. Calu-3 and SKBR-3 were grown in the basal medium Eagle's minimum essential medium (EMEM) and McCoy's medium, respectively. MCF-10A was grown in the DMEM basal medium. Media used for cell culture were supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% insulin.

4.6. Antiproliferative assay

The antiproliferative activity of the conjugate, compound **5** and DOX was evaluated on different cell lines such as BT-474, SKBR-3, Calu-3, MCF-7, and MCF-10A using a Cell Titer-Glo[®] luminescent cell viability assay[31] (Promega, Fitchburg, WI). In this assay, each well of the 96-well plates was coated with 10,000 cells; on the following day, the wells were treated in triplicate with different concentrations of conjugate (0.001 to 100 µM) in serum-free medium. 1% sodium dodecyl sulfate (SDS) and 1% dimethyl sulfoxide (DMSO) were used as positive and negative controls. After 24/48 h of incubation at 37 °C, the medium was removed and washed with phosphate-buffered saline (PBS). Later, 100 µL of CellTiter-Glo[®] reagent was added to each well and incubated for about 20 min; then, using a microplate reader, luminiscence readings were taken. Using Prism[®] (GraphPad Software, La Jolla, CA), a dose-response graph was plotted from which IC₅₀ values were calculated. IC₅₀ values of DOX alone were also calculated on all the different cell lines and compared with those of the conjugate. All the experiments were repeated thrice, and standard deviations were calculated.

4.7. Flow cytometry for cell cycle analysis

Approximately 2×10^5 BT-474 cells were treated with either 1 µM DOX or 1 µM conjugate or left untreated for 1 h followed by 24-h incubation in drug-free and serum-free medium. The cells were washed with PBS and fixed with 70% ethanol in water for 2 h at -20 °C with intermittent vortexing. After centrifuging at high speed for 5 min, the supernatant was aspirated and the cells were then washed twice with PBS. Washed cells were resuspended in distilled water with 100 µg/mL RNase and 40 µg/mL propidium iodide, followed by incubation at 4 °C for 30 min. After washing with PBS, the cells were analyzed for DNA content (10,000) using a fluorescence-activated cell-sorting (FACS) calibur instrument. The experiments were repeated in triplicate. Similar studies were carried out with BT-474 in the presence and absence of compound **5** alone.

4.8. Confocal microscopy

SKBR-3 cells were coated in an 8-well plate; after 24 h, the wells were treated with 5 µM DOX or 5 µM conjugate or left untreated. The cells were then incubated at 37 °C for stipulated time points (30 min, 4 and 24 h). After the respective time points, cells were washed twice with PBS. The cells were fixed using 500 µL methanol for 5 min in -20 °C. Later, the cells were centrifuged at high speed for 5 min and the methanol was aspirated.

Cells were then washed twice with PBS; this washing removes traces of methanol and also prevents cells from dehydration. Later, the cells were mounted with DAPI as mounting medium. Slides were viewed under a laser scanning confocal microscope (Olympus Flouview) with a 40 × oil immersion lens at $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 580 \text{ nm}$. Similar experiments were performed with conjugate and DOX for MCF-7 cells.

4.9. Binding of conjugate to HER2 protein using surface plasmon resonance

SPR analyses were performed on a Biacore X100 unit (GE Healthcare Biosciences, Piscataway, NJ) at 25 °C. The protein HER2 domain IV (DIV) (obtained from Leinco Technologies, St. Louis, MO) was immobilized on the CM5 sensor chip (GE Healthcare Biosciences) at a rate of 5 $\mu\text{L}/\text{min}$ using a standard amine coupling procedure. The carboxyl groups on the sensor chip were activated using a solution containing 0.2 M N-ethyl N-(dimethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) (35 μL solution, flow rate 5 $\mu\text{L}/\text{min}$). The running buffer was 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween at pH 7.5. The immobilization of HER2 was performed onto the chip surface until about 10,018 response units (RU) was reached. The unreacted activated groups were blocked with 1 M ethanolamine. Later, using different concentrations of conjugate (flow rate 30 $\mu\text{L}/\text{min}$), we tested for binding to HER2 DIV. Compound **36**, which exhibits antiproliferative activity with an IC_{50} value of $> 50 \mu\text{M}$ in HER2-overexpressed cells, was used as a negative control. K_d values were obtained by assuming 1:1 Langmuir binding and SPR sensograms were curve fitted to the Langmuir equation. Experiments were repeated for by using only DOX as an analyte.

4.10. 3D cell culture

96-well hanging drop plates were used for obtaining spheroids. 40 μL of medium containing about 200 BT-474 cells/ μL were dispensed into the access holes provided in the hanging drop plate wells. The spheroids were formed in 4–5 days by incubating at 37 °C. After confirming the formation of spheroids with a microscope, the spheroids in the medium were transferred to round-bottom 96-well plates by placing the hanging drop plates on top of the plates. Later, the spheroids were treated with different concentrations (0.001 to 10 μM) of conjugate or DOX or compound **5**. 1% sodium dodecyl sulfate (SDS) and 1% dimethyl sulfoxide (DMSO) were used as positive and negative controls. After treatment, the 96-well plates were incubated for 3 days in the incubator at 37 °C. The 3D Cell Titer-Glo[®] reagent was then added and luminescence readings were obtained using a plate reader. The IC_{50} values were obtained from the logarithmic plot of percentage concentration vs. luminescence, which was generated using GraphPad Prism (San Diego, CA). All experiments were repeated thrice and standard deviations were calculated.

4.11. Stability studies in the presence of cells

SKBR-3 cells were grown to confluency and cells were collected by trypsinization. The stock solution of DOX-conjugate (1 mg/175 μL) was prepared by dissolving the powder in 25 μL of DMSO and diluting up to 175 μL with serum-free medium. This solution then further diluted with serum-free medium to obtain the final 50 μM concentration of the conjugate. Around 15,000 cells were resuspended with DOX-conjugate solution prepared in

serum-free SKBR-3 cell medium and incubated at 37°C (for each time point of the stability study, a separate sample was prepared). At different time points (0, 15, 30 min, 1, 2, 4, 6, 8, 24, and 48 h) the cells were lysed with 500 µL of chilled acetonitrile and ultra-sonicated for 15 s. The contents then centrifuged to separate the cell debris from the supernatant. The supernatant solution was then passed through a Sep-pak column to separate the conjugate from other proteins. For analysis, a Shimadzu LC-20AP pump, a Shimadzu FCV-20AH2 manual injector, and a Shimadzu SPD-20A UV/Vis detector were used along with a C18 (5 µm, 250 × 4.6 mm) reversed-phase column from Restek Ultra (Bellefonte, PA). An isocratic mobile phase containing of water (containing 0.1% TFA) 20% and acetonitrile (containing 0.1% TFA) 80% was used while keeping the flow rate at 0.7 mL/min. Two different wavelengths—215 and 254 nm—were used for detection. The area under the curve at the zero-minute time point was considered as 100% and relative areas under the curves were plotted in percentages. Each time point was repeated 3 times to obtain mean and standard deviations.

4.12. Serum stability studies

A stock solution of DOX-conjugate (2 mg/200 µL) was prepared in acetonitrile. One portion of this stock solution was incubated with 9 parts of human serum (Innovative Research, Court Novi, MI, used according to approved guidelines, in a biosafety II cabinet with IBC certification) at 37 °C. Different time points were chosen from 0 min to 24 h. At each time point, 100 µL of serum-conjugate mixture was sampled and treated with 500 µL of cold acetonitrile for DOX-conjugate extraction. After sampling, it was vortexed for 10–150 s and then centrifuged at 1500 rpm for 10 min. The supernatant was passed through a SEP-PAK C18 cartridge (Waters, Milford MA). An excess of acetonitrile (500 µL) was added to wash the SEP-PAK column once more. The filtered supernatant was then lyophilized using Centrivap (Labconco, Kansas City MO). These lyophilized samples were then analyzed by HPLC. The HPLC system consisted of a Shimadzu LC-20AP pump, a Shimadzu FCV-20AH2 manual injector, and a Shimadzu SPD-20A UV/Vis detector, all of which were controlled by Lab Solutions software. Each sample (50 µL) was injected into a Restek Ultra C18 (5 µm, 250 × 4.6 mm) column and detected using a Shimadzu SPD-20A UV/Vis detector at wavelengths 215 and 254 nm. An isocratic system of mobile phase was used for this method in which 80% acetonitrile containing 0.1% TFA was mixed with 20% water containing 0.1% TFA. The flow rate for this method was 0.7 mL/min.

4.13. Molecular modeling, dynamics and docking

Conjugates of DOX-compound **5** were generated using InsightII (BIOVIA, San Diego, CA) and the structure was saved in protein data bank (PDB) and mol2 formats. The generated conjugate structure was optimized by 100 steps of energy minimization using the steepest descent methods followed by the conjugate gradient method until the root mean square (rms) derivative was 0.3 Å. The resulting structure was subjected to MD simulation in vacuum for 200 ps at 300 K. Structures with different conformations from the history file of 200 ps dynamics were analyzed. The final dynamics structures were represented using PyMol software. The crystal structure of HER2 ECD was downloaded from the Protein Data Bank (PDB ID 3N85)[13, 49]. Polar hydrogens were added. Autodock [44, 45] version 4.2 was used for docking studies. A 126 × 126 × 126 Å³ grid box was created around Phe273 of

domain IV of the HER2 protein structure covering most of domain IV. Conjugates were made flexible using the autotorsion option in Autodock software. Docking calculations were made using a Lamarckian genetic algorithm. 10 million energy evaluations were carried out. A Linux cluster (a high performance computational facility) at LSU Baton Rouge was used to perform calculations via the Louisiana Optical Network Initiative (LONI). After all the calculations were performed, the docked structures were ranked according to their energies with the lowest energy structure representing the most probable binding model. The lowest energy structure was saved as a PDB file and viewed using PyMOL software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

A conjugate of doxorubicin-peptidomimetic was synthesized and was shown to exhibit antiproliferative activity against HER2 positive cancer cells.

The peptidomimetic is highly specific for HER2 protein overexpressed cancer cell lines.

The conjugate was shown to bind to HER2 protein domain IV by surface plasmon resonance.

The conjugate binds to HER2 positive cancer cells and doxorubicin internalizes after 4 h as shown by confocal microscopy.

Compound 5 **DOX-Compound 5 conjugate**

Fig. 1.
Structures of compound **5** and DOX-compound **5** conjugate.

Fig. 2.
Mass spectrum of DOX-compound **5** conjugate showing molecular ion corresponding to $[M + H]^+$.

Fig. 3.

Intracellular distribution of free DOX and conjugate. SKBR3 cells were incubated with 5 μ M of DOX or 5 μ M of conjugate or left untreated for different time intervals, i.e., (A) 30 min, (B) 4 h, (C) 24 h. Cells were imaged using a laser scanning confocal microscope. Nucleus was stained using DAPI, and DOX can be identified by its autofluorescence. In the merged image, DOX in the nucleus could be seen. Magnification 40X.

Fig. 4.

A) Stability of DOX-compound **5** conjugate in the presence of SKBR-3 cells and B) in human serum analyzed by HPLC. Relative area under the curve (AUC) was plotted with respect to time. AUC for zero time point chromatogram was considered as 100% and data are from triplicate experiments.

Fig. 5. Effect of DOX and DOX-compound **5** conjugate on cell cycle arrest in HER2-overexpressed cancer cells BT-474. About 2×10^5 BT-474 cells were treated with either 1 μ M DOX or 1 μ M conjugate or left untreated for 1 h followed by 24-h incubation in drug-free and serum-free medium. Fixed cells were incubated with propidium iodide, and cells were analyzed by flow-cytometry.

Fig. 6. SPR analysis of binding of DOX-compound **5** conjugate to HER2 extracellular domain IV. Domain IV of HER2 was immobilized on a CM5 chip, and different concentrations of conjugate were used as analyte. Kinetics of association and dissociation were measured. The sensor chip surface with only protein was used as a reference, and sensograms were represented after subtracting the values from reference surface.

Fig. 7. Structures of DOX-compound **5** conjugate representing different possible conformations of the conjugate during MD simulations. (A) Compound **5** acquires a pseudo β -turn type of structure and the naphthyl group and Arg of compound **5** interact with the anthracycline ring of DOX. The naphthyl ring is either (B) perpendicular to the plane of the anthracycline ring or (C) parallel to the anthracycline ring. D) A conformation with the peptide chain away from the DOX anthracycline ring.

Fig. 8. Model of DOX-compound **5** conjugate binding to HER2 domain IV. A) Ten low-energy structures of the conjugate are shown binding to C-terminal domain IV of HER2. B) Lowest docked energy structure (docking energy -7.09 kcal/mol) of the conjugate bound to HER2 protein. Notice that the anthracycline ring is away from the peptide chain. Amino acids of the HER2 protein are shown with single letter codes and amino acids from compound **5** are shown with three-letter codes for amino acids. Compound **5** binds to HER2 surface at the protein-protein interaction site of EGFR heterodimer in domain IV.

Scheme 1.

Synthesis scheme for DOX-compound **5** conjugate. (A) Reagents used were *N*-methylmorpholine (NMM), *N*-methyl-2-pyrrolidone (NMP); (B) (*O*-(6-chlorobenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate), NMM, NMP, dichloro methane (DCM), trifluoroacetic acid (TFA), triisopropylsilyl ether (TIPS). R-resin.

Table 1

Antiproliferative activity of conjugate, DOX and compound **5** were calculated on both HER2 overexpressing cancer cells (BT-474, SKBR-3, Calu-3) and cancer cells which do not overexpress HER2 receptors (MCF-7) and non-cancerous cells (MCF-10A) to prove that conjugate has potency and selectivity. Activity is represented as IC₅₀ values in μ M.

Compound	IC ₅₀ in μ M					
		BT-474	SKBR-3	Calu-3	MCF-7	MCF-10A
DOX - Compound 5 conjugate	24 h	0.55±0.08	0.053±0.05	0.39±0.01	12.5±1.1	N.D
	48 h	0.07±0.01	0.039±0.02	0.15±0.05	5.9±0.2	N.D
	72h	N.D	N.D	N.D	0.8±0.1	0.85±0.07
DOX	24 h	0.053±0.004	0.034±0.01	0.27±0.09	0.3±0.06	N.D
	48 h	0.041±0.003	0.05±0.02	0.11±0.02	0.16±0.03	
	72h	N.D	N.D	N.D	N.D	0.15±0.03
Compound 5	24 h	1.26± 0.29	0.64±0.03	1.1± 0.3	19.2± 0.2	N.D
	48 h	1.06± 0.15	0.53±0.05	0.72± 0.13	17.8 ±3	N.D
	72h	0.895±0.029	0.39±0.022	0.601±0.02	16.9±1.0	>50

N.D not determined