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Original Research

A new approach towards developing a smart and multifunctional peptide-based drug delivery system for selective targeting and treatment of invasive/metastatic breast cancer.

Walhan Alshaer , Hamdi Nsairat, Malek Zihlif

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Abstract

Cancer remains one of the leading causes of morbidity and mortality worldwide. Breast cancer is the most frequently diagnosed cancer and causes cancer-related deaths in women. In cancer, targeted drugs are often divided into two strategies: "passive targeting" and "active targeting". Passive targeting suffers from low selectivity and poor retention in tumors. Such limitations lead to the development of an active targeting strategy. Active targeting describes the specific interaction between drugs or drug carriers and target cells, which usually occurs through receptor-ligand interactions. Here, we propose to develop a peptide-based drug conjugate as a novel targeted drug delivery system that enhances selectivity, localization, and activity of antitumor therapeutics on metastatic and invasive breast cancer cells by using endoxefin (END) as a targeting ligand for estrogen receptor, Metalloproteinase peptide-substrate (MMP2) for trigger release of drug, and doxorubicin (DOX) as an antitumor therapeutic.

Keywords: Estrogen receptor; Metastasis; Metalloproteinases; Endoxifen; Targeted drug delivery

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INTRODUCTION

A better understanding of tumor biology has supported the development of effective therapeutic and diagnostic strategies[1]. Although conventional therapeutics, including chemotherapy and radiotherapy, are still the main therapeutic choice for treating tumors, they lack specificity and are associated with significant side effects. Therefore, developing therapeutic systems that reduce unwanted side effects while improving therapeutic outcomes is a major focus in tumor therapeutics[2,3]. To achieve this goal, "targeted" therapies have emerged. However, this term may refer to two different strategies: (i) targeting a specific molecular-pharmacological target or a pathway involved in tumor progression, or (ii) targeting specific biomarkers of cancer cells, through specific ligands that are able to guide drugs/drug-loaded carriers to these cells[4,5].

Many metastatic and invasive breast cancer cells show higher levels of estrogen receptors and matrix metalloproteinases compared with normal breast cells. Tamoxifen, a selective estrogen receptor modulator, is one of the most widely used therapies for the treatment of breast cancer in women. Tamoxifen is a prodrug and is metabolized by the cytochrome P450 enzymes in the liver to produce the active metabolites afimoxifene and endoxifen[6,7]. Endoxifen (END) has been shown to bind the estrogen receptor (ER) with around 100-fold greater affinity than tamoxifen[8].

Matrix metalloproteinases (MMPs) can degrade components of the extracellular matrix and cellular membrane[9]. The expression of MMPs is abnormally increased in tumor cells, which increases the degradation of extracellular matrix, leading to increased metastasis and then invasion of distant sites of the primary tumor in the body[10]. Moreover, metalloproteinases have been found to be overexpressed in metastatic tumors compared to nonmetastatic tumors, and such abnormal expression plays an important role in angiogenesis by increasing endothelial cell migration and remodeling tissues necessary for the formation of new blood vessels [11,12]. MMPs are also produced by nonmalignant cells surrounding tumors, such as macrophages and mesenchymal cells[13]. MMP-2 (72 kDa) and MMP-9 (92 kDa) play a critical role in tumor progression, angiogenesis, and metastasis. Therefore, they are considered potential targets in the development of tumor therapeutics [14].

Peptide-based drug conjugates are easy to synthesize, structurally simple, and nonimmunogenic (Figure 1)[15]. One promising approach is the specific protease activity of MMPs towards substrate peptides, which can provide a strategy for controlled drug release. The MMP-activated drug targeting system is designed for enzymatically metabolized prodrugs, in which therapeutic drugs are covalently bound to MMP substrate peptides; the prodrug is then activated upon cleavage of the peptide substrate by MMP[16-21]. Here, we propose to exploit the enhanced activity and localization of MMPs on metastatic and invasive transformed cells by using MMP activity as the trigger for the release of DNA-intercalating topoisomerase II poisons such as doxorubicin (DOX)[22,23]. We build specificity for breast

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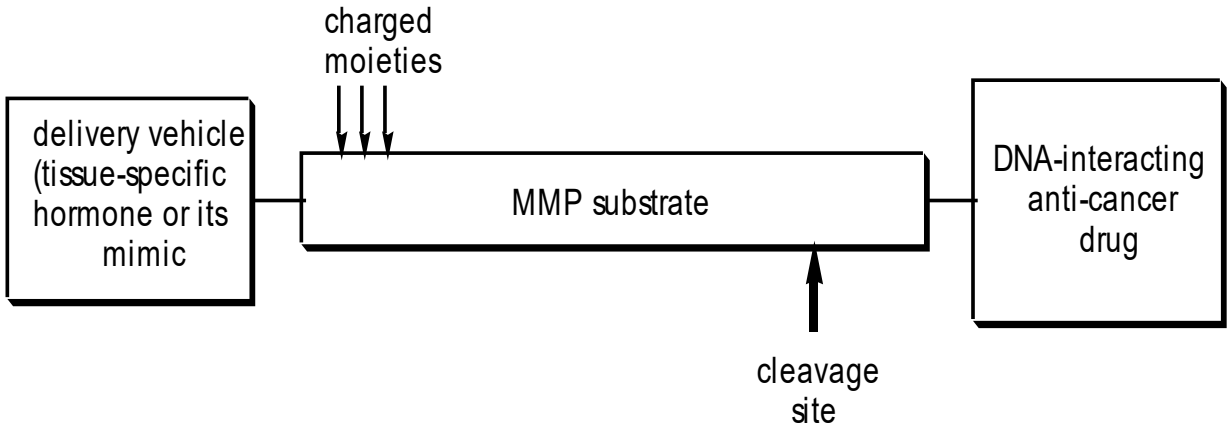

Figure 1. Scheme representing the peptide-based drug conjugate.

cancer cells by further endowing the drug with the capacity to bind to the estrogen receptor. The general structure of the proposed conjugate of DOX-END-MMP2 may be selectively localized in breast carcinoma cells by virtue of endoxifen-estrogen receptor binding and in metastatic and invasive cells; MMPs will cleave the MMP substrate, releasing a cytotoxic DOX within the target cell, or at its surface. By these means, we believe the proposed targeted drug delivery system would lead to the selective generation of a DNA-binding cytotoxin on the surface of invasive and metastatic breast cancer cells: a circumstance that may have beneficial outcomes in the treatment of invasive, metastatic, and minimal residual disease.

MATERIALS AND METHODS

Chemicals

Doxorubicin and (Z)-Endoxifen were purchased from Sigma (St. Louis, MO, USA). Human MMP-2 (Matrix Metalloproteinase 2) and MMP-2-maleimide were purchased from Elabscience (Houston, Texas, USA). Phosphate-buffered saline (PBS) and [4-(2-hydroxyethyl)-piperazino]-ethanesulfonic acid (HEPES) were obtained from LONZA® (USA). Acetone, methanol, ethanol, acetonitrile, and 1-propanol were obtained from the Carbon Group (England). All other chemicals and solvents were of analytical grade. All reagents and chemicals were used without further treatment.

Mass spectrometry

High-resolution mass spectra (HRMS) were acquired (in positive and/or negative mode) using electrospray ionization (ESI) in an ion trap technique by collision-induced dissociation on a Bruker APEX-4 (7 Tesla) instrument. The samples were infused using a syringe pump at a flow rate of 2 µL min^{-1}.

Bio-conjugation of Endoxifen with MMp-2 peptide (END-MMP2 conjugate)

A direct conjugation of Endoxifen (END) to the MMP-2 peptide (MMP2) via secondary amine –maleimide coupling was performed as shown in Scheme 1. The reaction was carried out under ambient atmosphere in air-dried glassware with magnetic stirring.

Preparation of Stock Solution A:

A 1.5 mL HPLC vial was charged with THF (227 µL), followed by LiCl (4.8 mg, 0.045 mmol) and triethylamine (15.8 µL, 0.045 mmol). The resulting mixture was stirred until all LiCl crystals were fully dissolved and the stock solution became homogenous.

Preparation of Stock Solution B:

In a 1.5 mL HPLC vial, MMP-2 -maleimide (25.5 mg, 0.05 mmol) was dissolved in methanol (250 µL). The stock solution was stirred until a homogenous mixture was obtained. To a 1.5 mL HPLC screw-cap vial equipped with a magnetic stir bar, 113.6 µLof stock solution A was added, followed by 0.025 mmol (1.1 equivalents) of END (9.34 mg) at 23 °C. 113.6 µL of stock solution B was added to the reaction mixture, and the resulting solution was stirred at ambient temperature for 72 hours days, followed by reflux at 55 °C for 48 hours.

Bioconjugation of doxorubicin with MMP-2 peptide (DOX-MMP2)

DOX-MMP2 conjugation was performed using the carbodiimide chemistry to form an amide bond (Scheme 2). 2.63 mg of MMP-2 was dissolved in 200 µL DMSO and stirred under nitrogen at room temperature. Further, 1.44mg of EDC, followed by 0.86 mg of NHS, were added to the MMp-2 solution under stirring at room temperature for 1hour. Then, 0.5 µL triethylamine (TEA) was added to a solution of 5.4mg DOX dissolved in 200 µL DMSO. The DOX solution was then added to the MMP-2 solution, and the final solution was stirred at 400 rpm using a magnetic stirrer for at least 24 hours at room temperature.

Bioconjugation of END to DOX-MMP to form the final conjugate END-MMP2-DOX

END-MMP2-DOX final conjugate was performed via secondary amine –maleimide coupling between the already prepared DOX-MMP2 and the END, as shown in Scheme 3.

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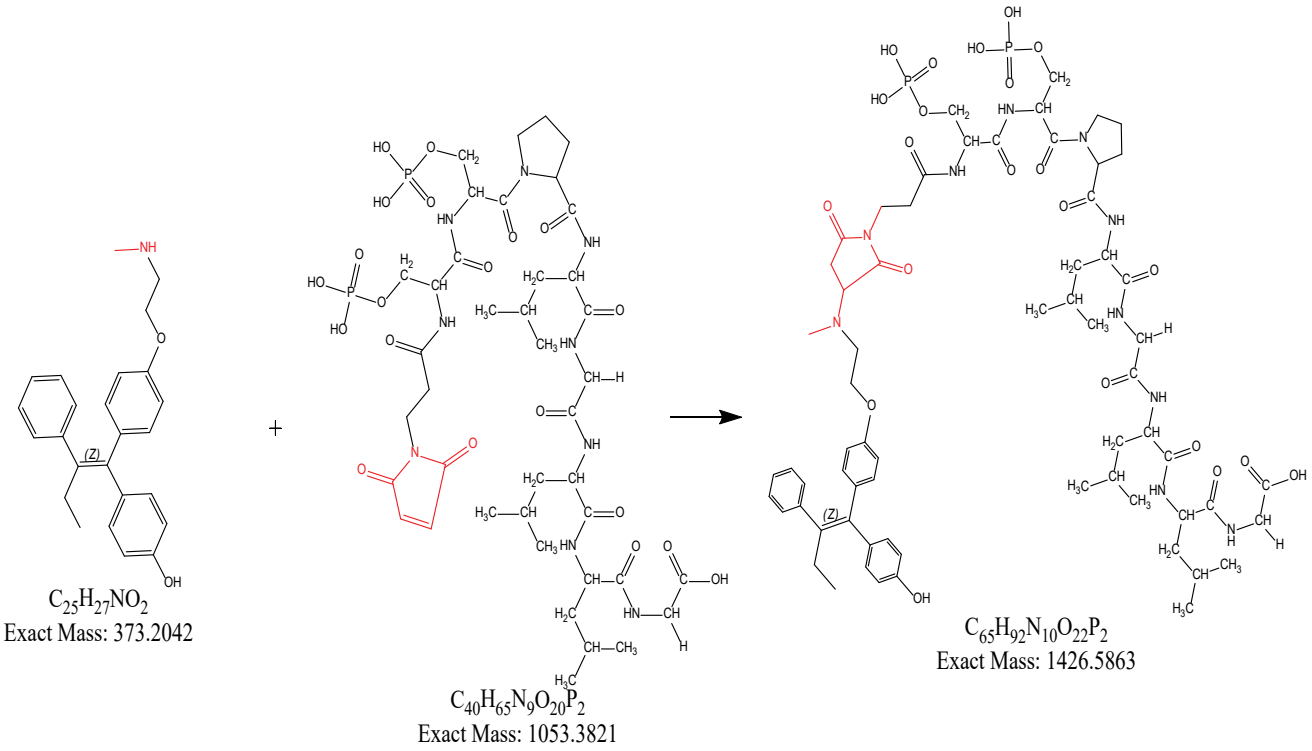
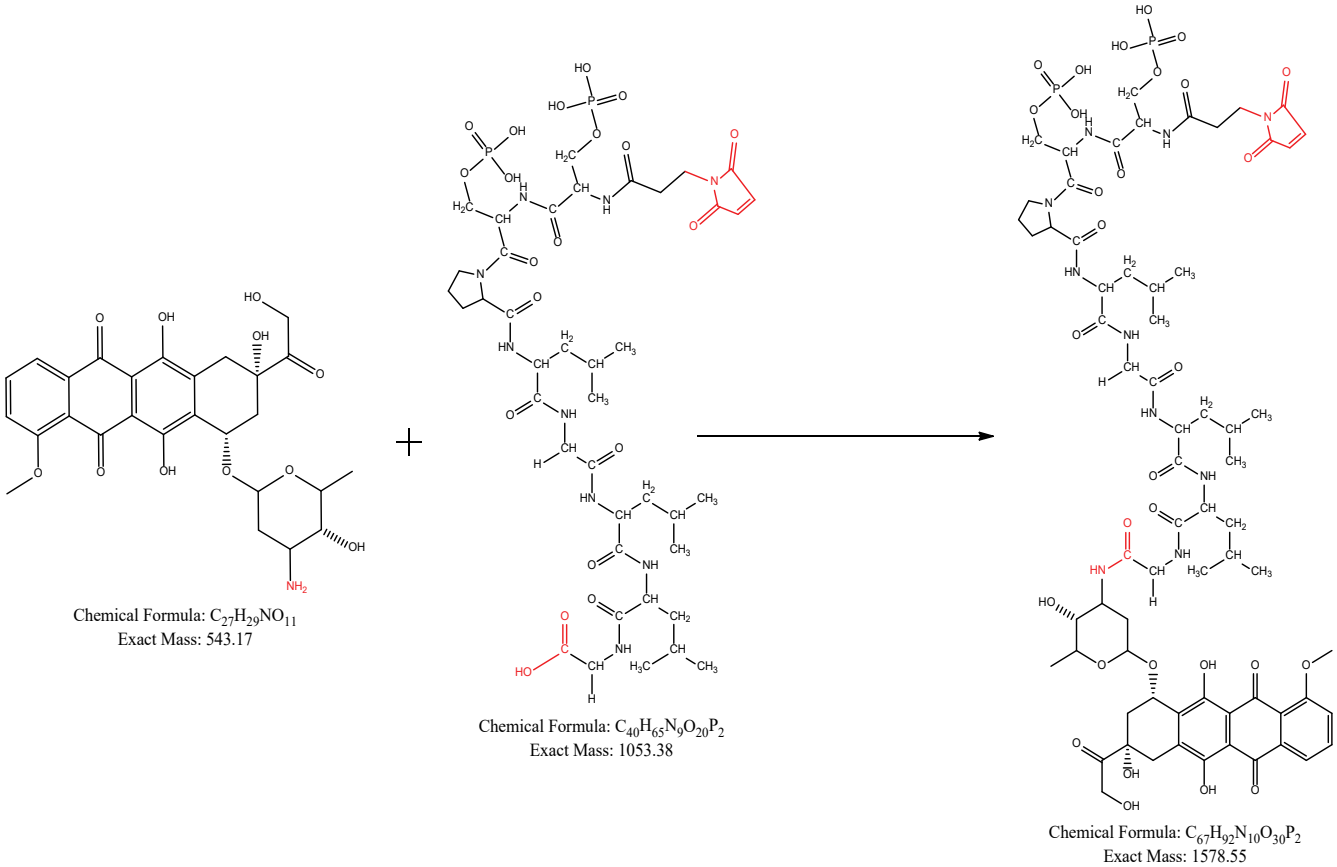


Scheme 1. Schematic representation of END-MMP2 conjugation



Scheme 2. Schematic representation of DOX-MMP2 conjugation

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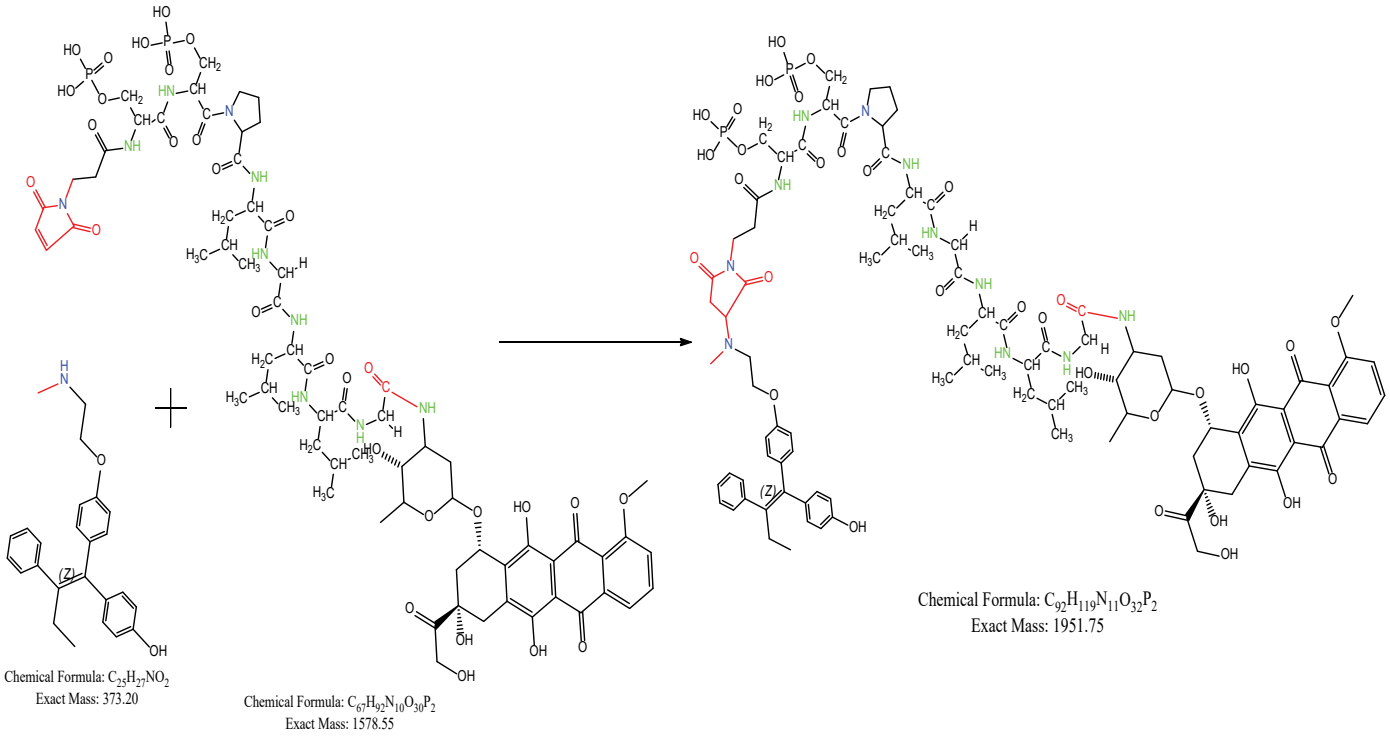

Scheme 3. Schematic representation of END-MMP2-DOX conjugation

Preparation of Stock Solution A:

To prepare a stock solution of co-catalysts, a 10 mL glass vial was charged with THF (3 mL), followed by LiCl (4.8 mg, 0.050 mmol) and triethylamine (15.8 µL, 0.050 mmol). The resulting mixture was stirred until all LiCl crystals were fully dissolved and the stock solution became homogenous.

Preparation of Stock Solution B:

DOX-MMP2 (52.7 mg, 0.05 mmol) was dissolved in 250 µL of methanol in a 1 mL vial to prepare a stock solution of maleimide. The solution was stirred until it became homogeneous.

Procedure for the Synthesis of END-MMP2-DOX :

114 µL of stock solution A was poured into a 1-2 mL screw-cap vial equipped with a magnetic stir, followed by the addition of the secondary amine nucleophile (END) (9.34 mg, 0.025 mmol) at 25 °C. 114 µL of stock solution B was added to the reaction mixture, and the resulting solution was stirred at ambient temperature for 36-48 hr. The crude reaction mixture was subsequently filtered and purified.

Enzyme cleavage protocol for conjugate END-MMP2 hydrolysis

0.350 mg of END-MMP2 (1426.6 g/mol) was dissolved in 100 µL DMSO to form a 2.45 mM stock A. Since not less than 20 µL of stock A can give a TLC signal, 20 µL of stock A was incubated with 100 µL of stock B (0.2 mg/mL MMP2 enzyme solution) for 1 hour at 37 °C to give a ratio of 408 µM to 2.25 µM of End-MMP2 and MMP2 cleavage enzyme, respectively. After incubation, a preparatory thin-layer chromatography (TLC) using a mobile phase of dichloromethane: ethyl acetate: methanol (DCM:EtAc:MeOH) (2:2:1.5) was performed to confirm the END-MMP2 cleavage using MMP2 recombinant protein.

RESULTS AND DISCUSSION

END-MMP2 conjugate purification

The crude reaction mixture was subsequently filtered through a G5-centered glass filter funnel. The filter cake was flushed with EtOAc (3 x 1 mL). The combined organic filtrates were concentrated in vacuo on a rotary evaporator. The concentrated material was separated using thin-layer chromatography (TLC) with different mobile phases that end with (DCM :EtOAc:MeOH:formic acid, 2:3:1:0.05) for three runs, as shown in the following TLC Figure S1. Despite the new intended conjugate (END-MMP2) being more polar than END, and it being difficult to separate them by Silica TLC from the highly water-soluble MMP-2 peptide, it was clearly confirmed by mass spectrometry, as shown in Figure 2.

Mass characterization of END-MMP2 conjugate

The formation of END-MMP2 conjugate was confirmed using high-resolution mass spectrometry. HRMS (ESI) on the negative-ion mode ($[M–H]^-$) and ($[M–2H]^{2-}$) confirmed END-MMP2 ($C_{65}H_{92}N_{10}O_{22}P_2$) synthesis, where the calculated value was 1426.58 and the found values were 712.35 m/z and 1425.41 m/z , for the ($[M–2H]^{2-}$) and ($[M–H]^-$), respectively, (Figure 2).

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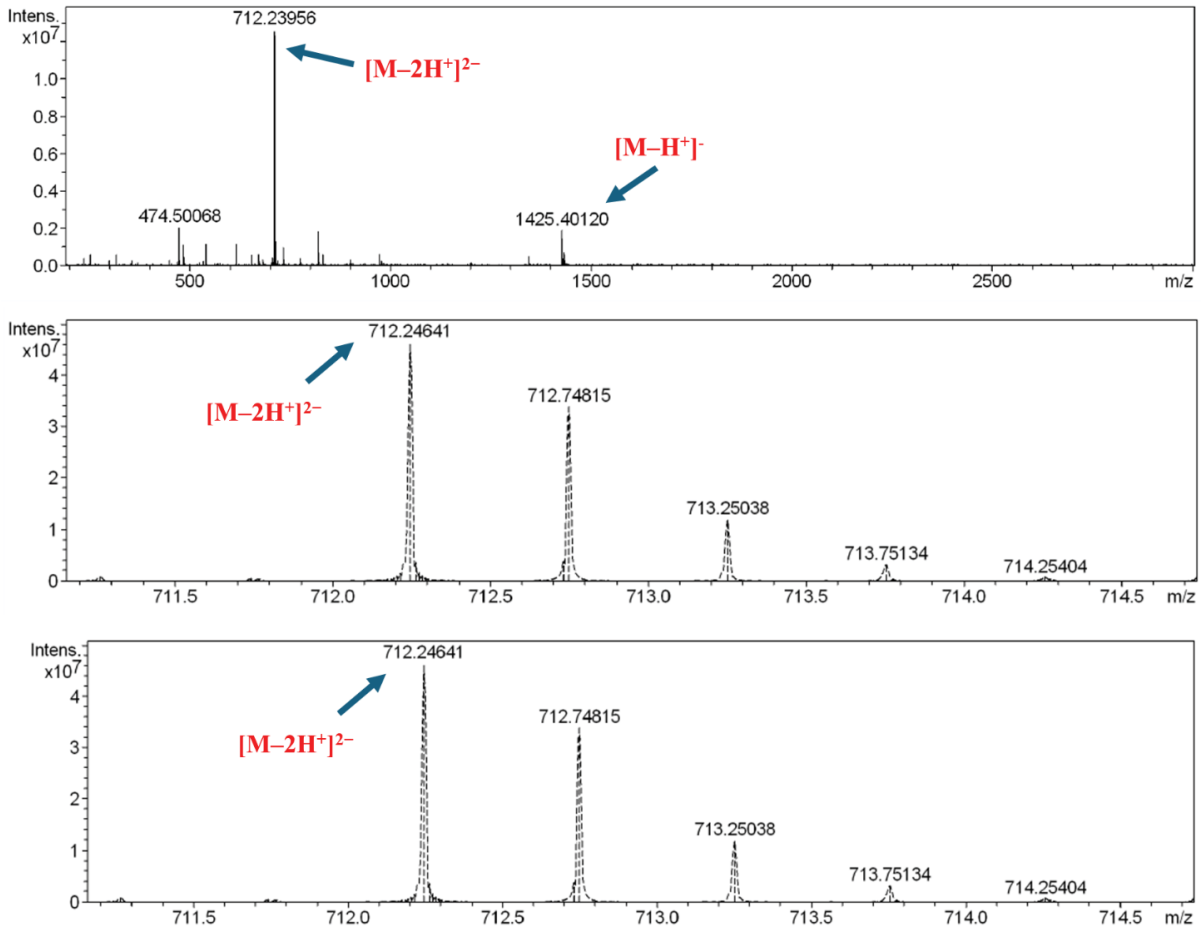

Figure 2. Mass spectrums for END-MMP2 at negative mode (M/1 and M/2).

DOX-MMP2 conjugate purification

Purification was done using preparatory thin-layer chromatography (TLC) using a mobile phase of dichloromethane: ethyl acetate: methanol (DCM:EtAc:MeOH)(2:2.5:0.5), followed by extracting of the product from the silica plate using tetrahydrofuran: H_2O (THF:H_2O)(9:1).

Mass characterization of DOX-MMP2 conjugate

The formation of DOX-MMP2 conjugate was confirmed using high-resolution mass spectrometry. HRMS (ESI) on the positive mode ($[M+2Na+K]^{3+}$) confirmed DOX-MMP2 ($C_{67}H_{92}N_{10}O_{30}P_2$) synthesis, where the calculated value was 1578.55, and the found value was 554.55 g/mol, for ($[M+2Na+K]^{3+}$), (Figure 3).

END-MMP2-DOX Conjugate purification

Purification was done using preparatory thin layer chromatography (TLC) using a mobile phase of (DCM:EtAc:MeOH: formic acid) (2:2:1.5:0.05) for END-MMP2-DOX, followed by extraction the product from the silica plate using tetrahydrofuran: H_2O (THF:H_2O)(9:1).

Mass characterization of END-MMP2-DOX final conjugate

The formation of END-MMP2-DOX conjugate was confirmed using high-resolution mass spectrometry. HRMS (ESI) on the positive mode ($[M+H+Li]^{2+}$) confirmed END-MMP2-DOX ($C_{92}H_{119}N_{11}O_{32}P_2$) synthesis, where the calculated value was 1951.75, and the found value was 977.87 g/mol, for ($[M+H+Li]^{2+}$), (Figure 4).

Enzyme cleavage protocol for conjugate END-MMP2 hydrolysis

Figure S2 represents the migration of spots on TLC plates for END-MMP2 before and after incubation with the MMP2 recombinant enzyme. The TLC showed clear, full cleavage of END-MMP2 by the enzyme, as evidenced by the formation of two new spots in two distinct regions, reflecting the different polarities of the non-polar END and the polar phosphate-modified MMP2. The END-MMP2, before enzymatic incubation, displayed only one spot at the baseline for the large-size END-MMP2, with a high degree of polarity.

CONCLUSION

In the present study, we developed tri-functional peptide–

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drug conjugates, END-MMP2-DOX, which consist of an endoxifen, an estrogen receptor targeting, doxorubicin, a DNA intercalating cytotoxic drug, and a MMP-2–sensitive peptide linker. In addition, we also synthesized the intermediates of END-MMP2 and DOX-MMP2 via maleimide–amine and carbodiimide coupling, followed by ion-exchange and chromatographic separation of the products to obtain their high-resolution mass spectrometry structures. In addition, we confirmed that recombinant MMP-2 cleaves END-MMP2 to detect the protease activity of matrix proteases often found at

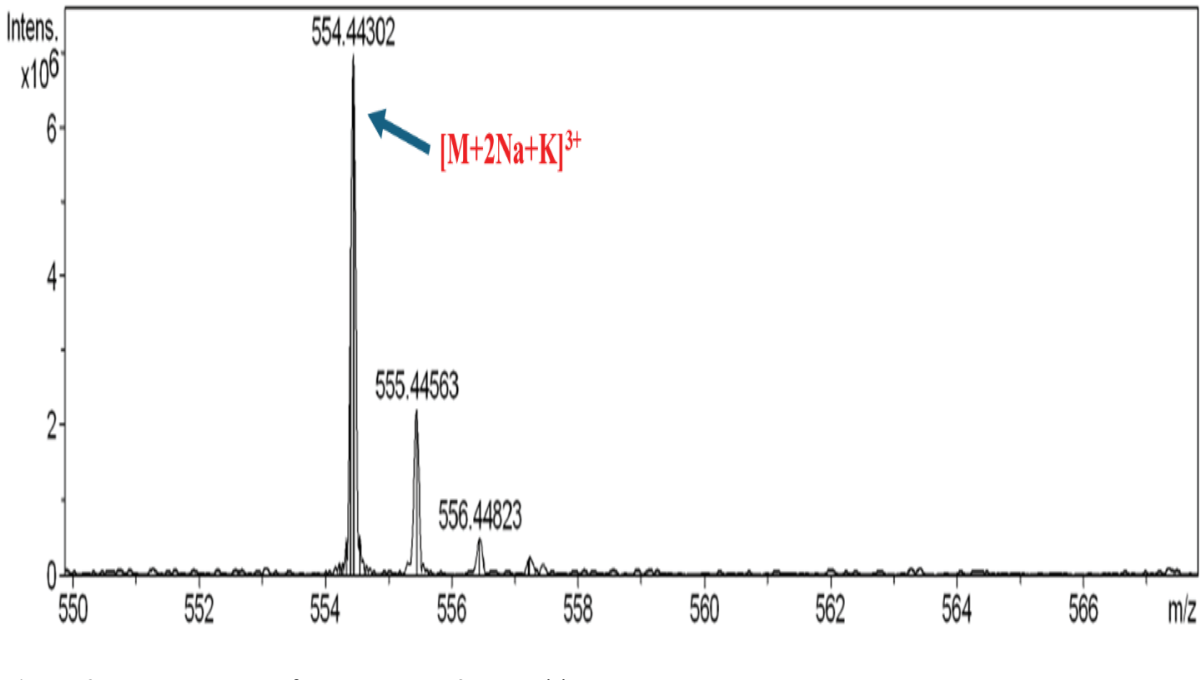

Figure 3. Mass spectrums for DOX-MMP2 at positive mode (M/3)

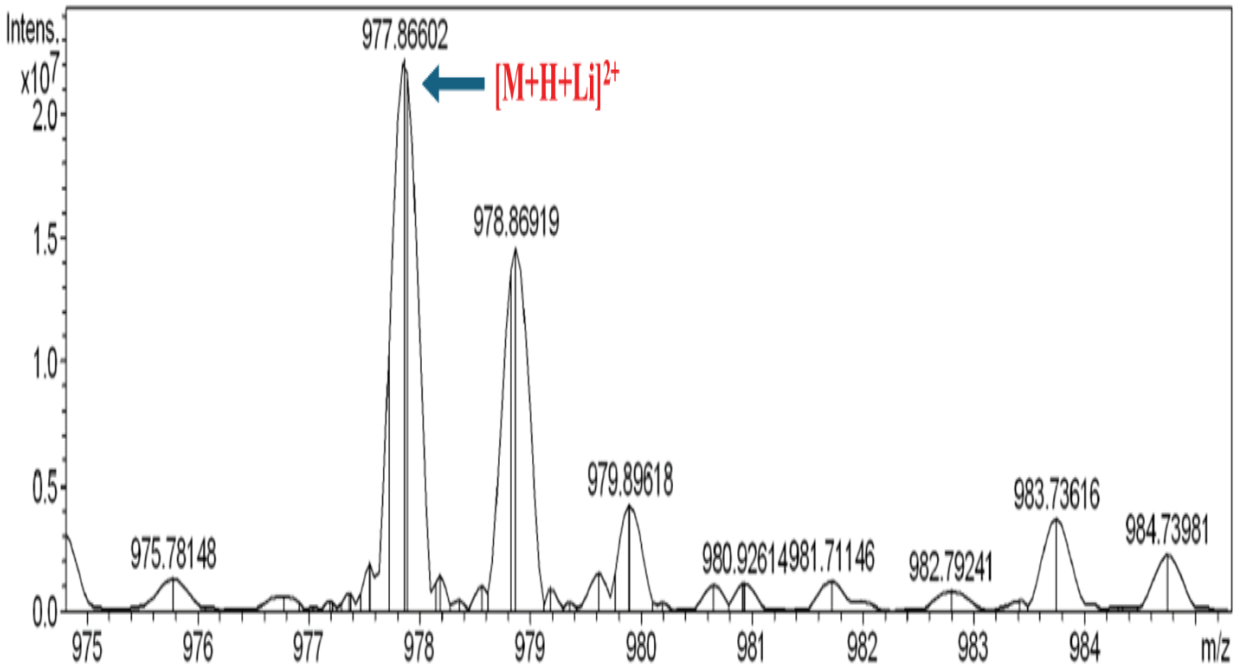

Figure 4. Mass spectrums for DOX-MMP2 at positive mode (M/2).

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tumor sites, thereby enabling site-directed drug release. All of these results represent an initial step towards demonstrating an MMP-activated, ER-targeted topoisomerase II-targeting prodrug for selective treatment of metastatic, invasive, and treatment-resistant breast cancer. Future work to evaluate, via *in vitro* and *in vivo* studies, the selective antitumor activity, pharmacokinetics, safety, and the potential positive on-target activity of the drug compared to the free-dispensed drug in traditional chemotherapy is highly recommended and can provide a comprehensive understanding of the properties of the developed END-MMP2-DOX conjugates.

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