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Rationalized Computer-Aided design of Matrix Metalloprotease-Selective Prodrugs

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Protease, *in silico* modelling, Matrix Metalloproteinase

ABSTRACT: Matrix metalloproteinases (MMPs) are central to cancer development and metastasis. They are highly active in the tumour environment and absent or inactive in normal tissues; therefore they represent viable targets for cancer drug discovery. In this study we evaluated *in silico* docking to develop MMP-subtype-selective tumour-activated prodrugs. Proof of principle for this therapeutic approach was demonstrated *in vitro* against an aggressive human glioma model, with involvement of MMPs confirmed using pharmacological inhibition.

INTRODUCTION

Matrix Metalloproteinases (MMPs) are zinc-dependent endoproteases central to digestion of extracellular matrix (ECM) and pericellular proteins involved in regulation of many normal physiological processes including tissue growth and embryogenesis.^{1,5} Their activity is regulated by both post-secretion zymogenic activation and inhibition by endogenous inhibitors termed TIMPs (tissue inhibitor of metalloproteinase).⁶ However, dysregulation of MMP expression and unbalanced endoproteolytic activity of specific MMPs is a major contributor to many degradative diseases including arthritis, cancer, cardiovascular disease, inflammatory disorders, and neurodegeneration,^{5,7-9} thus making them attractive drug targets.^{2, 10, 11}

In the last two decades significant drug discovery effort was focused on inhibition of MMPs as a strategy to prevent tumour invasion and subsequent tumour metastases.¹² As a result the pharmaceutical industry produced a number of well tolerated orally active MMP inhibitors (MMPi).¹³ These agents were largely peptidomimetic zinc-binding hydroxamates, based upon an MMP peptide substrate.^{2,14,15} Although many of these inhibitors progressed to late stage clinical trials against metastatic cancer, limited clinical success was seen due to a lack of inhibitor MMP-subtype specificity and insufficient knowledge about the complexity of the disease biology.^{2,16,17} Several additional strategies have been evaluated over recent years, including development of inhibitors exploiting the enzymatic transition state,^{2,18} inhibitors binding enzyme cavity subsites,¹⁹ or alternative zinc chelation groups.²

Generation of MMP subfamily-selective inhibitors still proves elusive however, because of the broad structural similarity of their active site, substrate complexity, and identification of specific MMPs as anti-targets.^{2,17}

In contrast to inhibition of MMP function, exploitation of the MMP-mediated proteolysis within diseased tissues has also been investigated as a diagnostic and prognostic approach. These studies used activity-based probes comprising a 'broad-spectrum' or selective MMP-cleavable peptide labelled with a quenched fluorophore or imaging moiety.^{20,21} In this approach, elevated MMP activity in the diseased tissue results in activation of the probe via selective cleavage of the peptide and release of the contrast agent, facilitating imaging and quantification of MMP activity.^{20,21}

In line with the MMP-activated probe-based approaches, elevated activity of MMPs within diseased tissue has also been explored as a strategy for conversion of a non-toxic peptide-conjugated prodrug into a potent therapeutic entity within the disease site. The advantage of this approach is dose intensification and reduced systemic drug exposure.^{1,22-26} A requirement for success in activity-probes, prodrugs, or theranostic approaches is MMP-selectivity through incorporation of MMP-subtype unique (e.g. MMP-2 or MMP-9) peptide sequences and subsequent disease-selective activation.

The rationale for this study is to exploit the MMP binding subsites and modify the substrate residues to produce a prodrug selective for MMP-2 over MMP-9, and create a robust approach which could be exploited for develop-

ment of endoprotease-activated diagnostic probes and therapeutics. Visualising and quantifying binding preferences and motifs can provide valuable insight into the structural determinants of substrate selectivity and enable MMP-targeted drug development.²⁷ In order to achieve this the following steps were undertaken; definition of the catalytic domains within the relevant MMPs through *in silico* study, docking of known MMP-selective sequences to highlight key catalytic binding determinants, subsequent rational design of novel MMP-selective prodrugs, and *in vitro* confirmation of MMP-selectivity and therapeutic proof-of-concept. In this work a reiterative approach using *in silico* proteolytic docking coupled to *in vitro* biochemical assessment have been applied to enable the development of prodrugs that are selectively activated by MMP-2 over MMP-9, the closely related gelatinase family members. The availability of three-dimensional crystal structures of MMPs allowed us to critically examine the differences existing between the catalytic domains of the MMP-2 (PDB ID: 1QIB) and MMP-9 (PDB ID: 1GKC).²⁸ This allowed for successful development of *in silico* models of MMP-2 and MMP-9. The *in silico* model was able to accurately predict known cleavage sites on substrates and prodrugs by MMPs, thus enabling rationalized design of an MMP-2 selective peptide.

RESULTS AND DISCUSSION

The catalytic domain of MMPs consists of five β -sheet strands and three α -helices. The catalytic centre comprises of a catalytic zinc ion coordinated by three histidine residues and a glutamic acid.²⁸ The specificity loop within the catalytic site of MMPs shows the largest structural differences, as visualised for MMP-2 and MMP-9 in Figure S1. The overall folding of MMP-2 and MMP-9 resemble those of other MMPs, which is expected based on their structural similarity (Figure S1).²⁹ The cavity of S1' pockets in MMPs are well-suited to accommodate a wide-range of hydrophobic residues, with the main functional difference between MMP subtypes lying in this region. In MMP-9, residues 421 – 423 form the wall of the binding pocket and the specificity loop is formed by the residues 424 – 430. Arg424 is present at the bottom of S1' pocket and closes off the end. Arg424 is therefore responsible for making the pocket cavity smaller in MMP-9 than in MMP2 (Figure S1). Whereas in MMP-2, the external wall of the S1' pocket is largely formed by Thr227 – Phe232 specificity residues, creating a deeper pocket. These differences can potentially be exploited for rational design of MMP-selective substrates/conjugates.

To probe the selective binding of potential substrates the peptide sequence of the non-specific gelatinase substrate **1** (M-2055)³⁰ was minimised with respect to its geometry, and then docked into the MMPs. In order to validate modelling work, attempts at crystallisation of this and other substrates were undertaken in order to determine the X-ray crystal structure. Crystallisation experiments failed to yield suitable crystals for structure determination, therefore only CHARMM minimised geometries

of the substrates were employed throughout this study.

Figure 1 shows the interaction of **1**'s peptide sequence with human MMP-2 and MMP-9. In both MMPs the zinc ion interacts with the Gly and Cys(Me) bond, the known cleavage site according to Bickett et al.³⁰ MMP-9 is able to bind tightly with the substrate residues compared to MMP-2, as determined by differences in their predicted inter-atomic zinc distances and overall binding energies. The substrate bound MMP-complexes provided crucial insight into the differences in their subsite interactions, as S1 and S3 subsites in MMP-2 demonstrated affinity to accommodate longer side-chains than MMP-9. The charged nature of the S2 subsite (presence of His205) in MMP-2 lends affinity for acidic residues, whereas this feature is not observed in MMP-9. In the MMP-9 structure, the carboxylic acid between Gly and Cys(Me) chelates the zinc ion (2.1 Å) and is involved in a strong H-bond to the carboxylate O of Glu402. The zinc ion is further coordinated by three Histidine residues namely His 401, His 405 and His 411 present in Helix $\alpha\beta$ segment of the protein. Only the P1' amino acid is involved in strong H-bonds with Arg424 (2.2 Å), which creates the wall-forming segment. The strong binding of Arg424 with the P1' residue is an important determinant of the specificity pocket. Remaining substrate residues are involved in strong interactions with the bulge-edge segment molecules (Gly86 to His190) with interatomic distances ranging from 2.5 to 3.1 Å. The docked complex of **1** and MMP-9 has an overall binding energy of 706 kcal/mol (Figures 1 and S2).

Consideration of the interaction of **1** with the active site of MMP-2 shows a marked reduction in affinity in energetic terms, the predicted interaction is seven times weaker

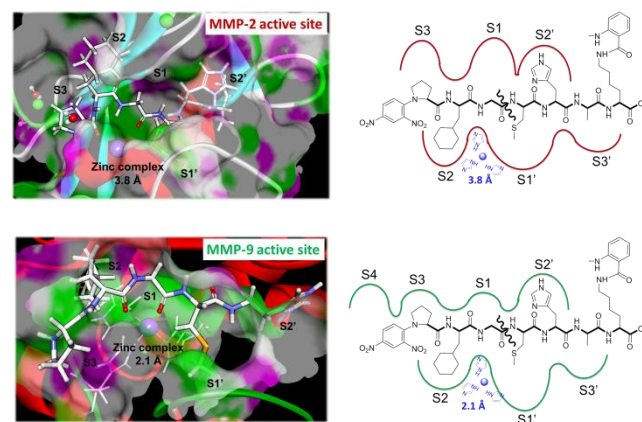


Figure 1. (Left) Stereo view of the docked complexes of **1** substrate (white sticks) and the catalytic domain of human MMP-2 (PDB ID: 1QIB) and MMP-9 (PDB ID: 1GKC). Catalytic and structural zinc ions are shown as purple spheres. Active-site cleft residues (the $\alpha\beta$ -helix loop and the specificity loop) are shown in green. (Right) Schematic representation of **1**: active site binding interaction in human MMP-2 and MMP-9. MMP-2 and MMP-9 enzyme binding pockets are shown in red and green respectively. Substrate chemical structure and its scissile bond is shown in black. The zinc ion coordinated by histidine is indicated in blue.

than that of **1** and MMP-9 (binding energy of 101 kcal/mol). Gly forms the P₁ subsite and Cys(Me) forms the P₁' subsite and the presence of a zinc ion, chelated by the carboxylic acid between P₁-P₁' residues (3.8 Å), further confirms this. The P₁' residue, although favourable for the MMP-2 specificity pocket, is not involved in any significant interaction with MMP-2 residues. The remaining substrate residues have weak H-bond interactions with wall-forming and bulge-edge segments of MMP-2 with interatomic distances ranging from 3.1 to 5.1 Å. This is expected as **1** residues are oriented away from further MMP-2 binding pockets (Figure 1 and S₂). Key observed differences between the binding affinity of MMP-2 and MMP-9 with **1** are: S₁ and S₃ subsites in MMP-2 can accommodate longer side-chains than MMP-9. Charged nature of S₂ subsite in MMP-2 has affinity for acidic residues, whereas this feature is not observed in MMP-9.

To experimentally validate the predictability of this model, and confirm the *in vitro* cleavage position of **1**, hydrolysis of the substrate by recombinant MMP-2 and MMP-9 was assessed over a 12 hour period. The resultant products were analysed by LCMS using a reverse phase gradient system to separate the substrate **1** and proteolytic products. The identification of these species was confirmed by retention time and mass spectrometry (MS) data. **1** demonstrated a retention time (tR) of 2.8 minutes (Figure S₃) and rapid cleavage by MMP-9 at Gly-Cys(Me) bond was confirmed by LCMS, two peaks corresponding to Dnp-Pro-β-Cyclohexyl-Ala-Gly at tR of 2.3 minutes (m/z 491.5Da, [M+H]⁺) and Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH₂ at tR of 2.47 minutes (m/z 604.7Da, [M+H]⁺) (Figures 2 and S₄). Slow hydrolysis of **1** by MMP-2 (compared to MMP 9) at Gly-Cys(Me) bond was confirmed by two peaks at tR of 2.3 minutes and tR of 2.47 minutes. MMP-2 cleavage experiments displayed a parent peak of **1**, detected at tR of 2.8 minutes (m/z 1077.5Da, [M+H]⁺), suggesting that MMP-2 metabolised **1** at a slower rate than MMP-9 (Figures 2 and S₅). This *in vitro* assessment supports the validity of the predicted *in silico* model of substrate and MMP interactions and was subsequently used for further design of MMP-targeted therapeutics.

The next phase of the study was to design a prodrug (substrate and warhead) which would be selectively-

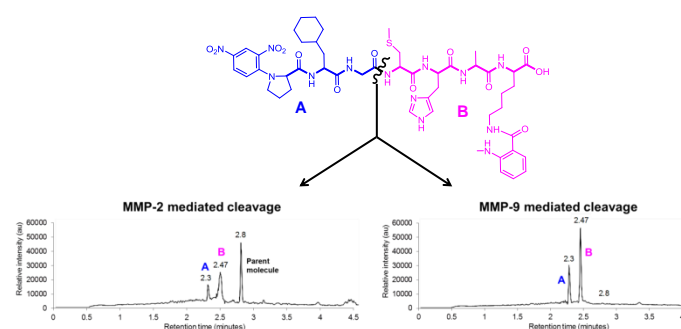


Figure 2. Schematic representation of the cleavage of **1** substrate by recombinant MMP-2 and MMP-9 enzymes at Gly-Cys(Me).

activated by a specific MMP over a close family homologue i.e. MMP-2 not MMP-9. A known MMP-targeted peptide-conjugated doxorubicin prodrug^{31,32} was evaluated as it is cleaved by both MMP-2 and MMP-9, presenting an excellent model for further modification. MMP-targeted peptide-conjugates were synthesised via solid phase chemistry and purified by reverse phase HPLC, the chemotherapeutic drug doxorubicin conjugated to the C-terminus (Scheme S6). Predicted *in silico* interaction of the **2** (reference compound) with MMP-2 and MMP-9 shows the zinc ions are chelated by the carboxylate between Gly-Ser(O-Benzyl) bond (2.6 Å and 2.8 Å respectively), the known cleavage site.^{31,32} Figure S₇ shows the binding pockets of MMP-2 are larger and deeper than MMP-9, S₁ subsite allowing for larger aromatic residues. The compound **2** aligns tightly into the active site of both MMPs as shown by their predicted interatomic zinc distances and binding energies (555 kcal/mol and 492 kcal/mol) in MMP-2 and MMP-9 docked complexes respectively. Active site residues interact with the compound in a similar way to that previously explained. His205 in MMP-2 αβ-Helix loop makes the S₂ pocket charged in nature and could potentially accommodate acidic residues (Figures S₇ and S₈). To experimentally validate the *in silico* docking of **2**^{31,32} and confirm the *in vitro* cleavage site, the lysis of this prodrug by recombinant MMP-2 and MMP-9 was assessed over a 12 hour period; with the resultant products being assessed by LCMS. HPLC (reverse phase gradient) was used to separate **2** (Cbz-Glu-Pro-Leu-Gly-Ser(O-Benzyl)-Tyr-Leu-Doxorubicin),^{31,32} identification confirmed by mass spectrometry (MS) with a retention time (tR) of 3.63 minutes (Figure S₉). Cleavage of **2** by both MMP-2 and MMP-9 at Gly-Cys(Me) bond was confirmed by LCMS, two peaks corresponding to Cbz-Glu-Pro-Leu-Gly at tR of 2.87 minutes (m/z 547.2Da, [M+H]⁺) and Ser(O-Benzyl)-Tyr-Leu-Doxorubicin at tR of 3.23 minutes (m/z 997.4, [M+H]⁺). (Figures 3, S₁₀ and S₁₁). A parent peak was also detected at tR of 3.63 minutes (m/z 1525.8Da, [M+H]⁺). This *in vitro* assessment supports the predictability of *in silico* model of anticancer therapeutics with MMPs, for further design of MMP-2 selective prodrugs.

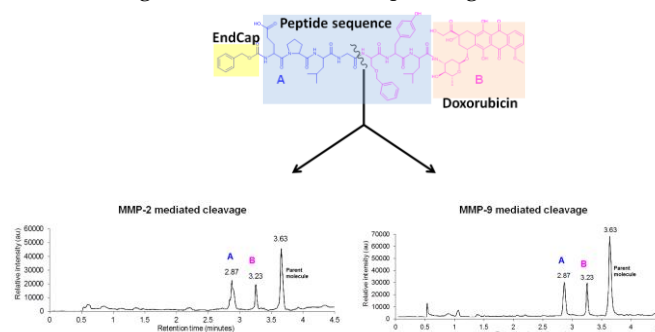


Figure 3. Schematic representation of the cleavage of **2** by recombinant MMP-2 and MMP-9 enzymes at the Gly-Ser(O-Bn) bond.

Rational design of a peptide conjugate selective for MMP-2 over MMP-9 was achieved by incorporating residues into the peptide chain to fit S₁, S₂, S₃ and S₁' pockets of MMP-2 which differ in size and polar affinity compared to MMP-9. The following modifications were incorporated: Aromatic residues in S₁ subsite; acidic side-chain in S₂ subsite and a polar side-chain at the S₃ subsite. Small non-polar residues were included at the S₁' subsite despite S₁'s potential to accommodate longer hydrophobic residues. This was due to longer residues leading to a negative effect on the predicted binding affinity, due to conformational alteration.

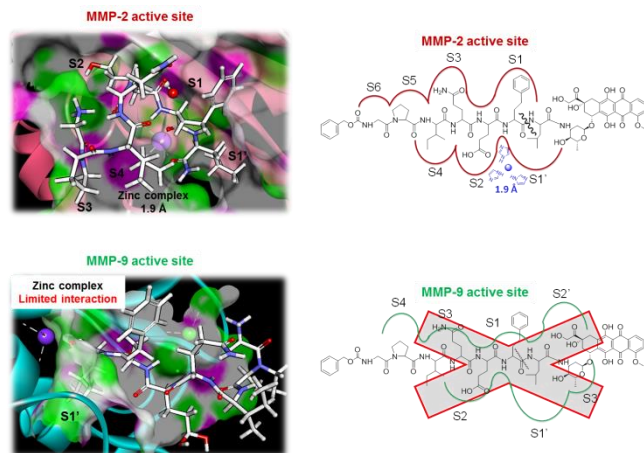


Figure 4. (Left) Stereo view of docked complexes of **3** (white sticks), catalytic domain of human MMP-2 (PDB ID: 1GKC) (Red) and MMP-9 (PDB ID: 1GKC) (Green). Catalytic and structural zinc ions are shown as purple spheres and active-site cleft residues (α -helix loop and the specificity loop) are shown as green. (Right) Schematic representation of **3** substrate: active site binding interaction in MMP-2 and MMP-9, are shown in red and green respectively. Substrate chemical structure and its scissile bond is shown in black and zinc ion coordinated by histidine is indicated in blue.

The zinc ion in MMP-2 demonstrated interaction with **3** at carboxylate between Homophenylalanine (Hof)-Leu bond (1.9 Å), indicating the predicted cleavage site. Residues of MMP-2 tightly bind with **3** as demonstrated by strong interactions with the bulge-edge segment molecules (Gly162 to His166) and the wall-forming segment molecules (Tyr223 to Thr229) with interatomic distances ranging from 1.8 to 2.8 Å. The docked complex of **2** and MMP-2 has an overall binding energy of 805 kcal/mol. In MMP-9 the zinc interaction is not detectable and the predicted binding energy is negative (-107 kcal/mol) suggesting the modified peptide residues should give selectivity of MMP-2 over MMP-9 (Figures 4 and S12). The hydrolysis of **3** by recombinant MMP-2 and MMP-9 was assessed over a 12 hour period and analysed by LCMS. Reverse phase HPLC identified **3** at tR of 3.58 minutes (Figure S13). **3** was preferentially cleaved by MMP-2 at Hof-Leu two peaks identified; corresponding to Leu-Doxorubicin at tR of 2.078 minutes (m/z 657.2Da, [M+H]⁺) and Cbz-Gly-Pro-Ile-Gln-Glu-Hof at tR of 2.826 minutes (m/z 821.4Da, [M+H]⁺) (Figures S14 and S15).

Conversely MMP-9 did not cleave **3** in the same timeframe, indicating that **3** is MMP-2 selective supporting the in silico prediction (Figure S14).

In order to assess efficacy and demonstrate proof-of-concept for the developing approach, the effects of **3** were assessed against the U87-MG malignant human glioma cell line. This cell line is derived from a highly aggressive glioma tumour and expresses both MMP-2 and MMP-9 (Figure 5). Cytotoxicity was observed in this cell line with doxorubicin, Leucine-doxorubicin (Leu-Dox) and **3**, with IC₅₀ values of 0.3±0.2 μM, 0.6±0.2 μM and 5.0±1.2 μM, respectively. The differential cytotoxicity between doxorubicin and **3** supports the requirement for **3** to be activated prior to inducing its effects. Furthermore, **3** remained inactive in the presence of a pan-MMP inhibitor (GM6001; Ilomastat), demonstrating MMP-selective chemotherapeutic action of this prodrug.

In order to further determine the tumour-selective activation of **3**, its metabolism was studied *ex vivo* using MMP-expressing HT1080 human tumour xenograft³³ and homogenized murine liver and kidney tissues (Figure S16). Rapid metabolism of **3** was observed in the HT1080 xenograft homogenate (t_{1/2} = 9.8 minutes). In comparison, **3** was relatively stable in murine liver (t_{1/2} = 17 minutes) and murine kidney (t_{1/2} = 38.5 minutes). Metabolism of **3** in HT1080 homogenates resulted in rapid production of the chemotherapeutic agent.

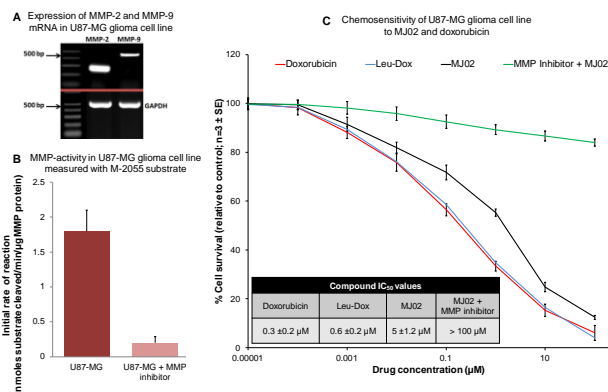


Figure 5. Therapeutic efficacy of **3** against human cancer. A) Expression of MMP-2 and MMP-9 mRNA in the U87-MG glioma cell line; B) MMP-activity in the U87-MG cell line, as demonstrated by activation of the M2055 substrate; C) Cytotoxicity of doxorubicin, Leu-Dox and **3** against U87-MG cell line. MMP-selective activation of **3** in the presence of a pan-MMP inhibitor (Ilomastat).

CONCLUSIONS

Targeted cancer therapies offer the potential of reduced side effects along with benefits of prolonging drug exposure to cancerous tissues, enabling improved tumour response and survival rates.³⁴⁻³⁵ Harnessing the elevated enzymatic activity of MMPs within the tumour microenvironment to selectively convert a non-toxic prodrug into a potent chemotherapeutic agent is one such approach with significant potential therapeutic scope.^{36,37} In this study a reiterative approach using in silico docking cou-

pled to *in vitro* biochemical proteolytic assessment have been applied to enable the development of anticancer prodrugs selectively activated by MMP-2, but not the close family homologue MMP-9. Proof-of-concept for this therapeutic approach was demonstrated against a glioma cell line *in vitro*, with the involvement of MMPs confirmed using pharmacological inhibition and by tumour-selective activation with *ex vivo* tumour xenografts. This study has shown that it is feasible to utilise *in silico* predictive approaches to rationally design MMP-selective prodrugs with possible utility in the treatment of cancer.

EXPERIMENTAL

3D-Molecular Modelling

All the molecular modelling and calculations were performed using the BIOVIA Discovery Studio 4.0 molecular modelling package, developed by Accelrys. The X-ray crystal structure of proteins MMP-2 (PDB ID: 1QIB) and MMP-9 (PDB ID: 1GKC) were acquired from the Protein Data Bank (PDB) and prepared by omitting the ligands associated with the co-crystal structure. Substrate ligand structures were prepared in Avogadro and converted to Discovery studio in MOL format. Using the 'Receptor-Ligand Interactions' tool, the substrate 3D-structures were optimised by complete energy minimisations using the CHARMM (Chemistry at Harvard Macromolecular Mechanics) force field (using 10000 minimisation cycles) and prepared for flexible receptor-ligand docking (refer to Figure S17 for 3D structure of MMP-9 demonstrating the adaptation of Zn²⁺ before and after flexible docking). The substrate ligands were docked into the active site clefts of MMPs via flexible docking, wherein the protein structure was selected as the 'receptor molecule' and the substrate structures as the 'ligand molecule'. Docking results were analysed in Discovery Studio by viewing the ligand interactions, hydrophobic receptor surfaces and the location of zinc ion in the receptor with respect to the ligand. The binding free energies between a receptor and a ligand were also calculated using the CHARMM implicit solvation models. The binding energy was calculated using the following equation:

$$\text{Energy binding} = \text{Energy complex} - \text{Energy ligand} - \text{Energy receptor (in kcal/mol)}$$

Synthesis of MMP-targeted peptide conjugates

Custom designed peptide sequences with Cbz (Benzyloxycarbonyl) as the chemical endcap were supplied (Bachem, Switzerland)/ synthesised using solid phase strategy. Activation of the pre-loaded 2-chlorotrityl resin was carried out in a fritted polypropylene reaction chamber. 0.1mmol of resin was weighed into the reaction chamber and 2 ml of dry DCM added. The reaction vessel was shaken for 45 minutes. After this time, DCM removed and the resin washed further with DCM. Single couplings were carried out using 5 equivalents of peptide (compared to resin), 5 equivalents of benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP[®]), 10 equivalents of N,N-Diisopropylethylamine

(DIPEA) and 2 ml of DMF under agitation for 45 minutes. Double peptide couplings were carried out- 2 x 45 minute couplings for each residue addition- the reaction drained after each coupling and fresh reagents added. After each set of coupling reactions, the reaction solution was drained and resin washed with 5 portions of 2 ml DMF. Removal of the Fmoc group was carried out using 5 ml of a solution of 20% piperidine in DMF for 5 under agitation. Piperidine solution was drained and fresh solution added for a further 10 minutes under agitation. Piperidine solution was drained and the resin rinsed using 5 portions of 2 ml DMF. Peptide-resin was treated with a solution of 20% hexafluoroisopropanol in DCM for 1 hour. The resin was removed by filtration and the solvent removed from the filtrate under reduced pressure before precipitation using ether and decanting of the liquid (followed by subsequent ether washes). The resulting solid peptide (Cbz-GPIQ(Trt)-E(tBu)-hPhe-L-OH) was dissolved in deionized water and acetonitrile mix and lyophilized. Purification of peptides was carried out using Perking Elmer HPLC. Samples were injected into a column and a gradient of 0-100% solvent B (solvent A= 95% H₂O, 5% MeCN, 0.01% TFA, solvent B = 95% MeCN, 5% H₂O, 0.01% TFA) over 95 minutes with a flow rate of 2.0 ml/min. Doxorubicin was conjugated to the peptide C-terminus as follows: Doxorubicin.HCl (0.0012 g, 0.002 mol, 1 equiv.), peptide (0.0022 g, 0.002 mol, 1 equiv.), PyBOP[®] (0.0015 g, 0.003 mol, 1.3 equiv.), and hydroxybenzotriazole hydrate (0.0073 g, 0.0054 mol, 2.6 equiv.) were added together under nitrogen in anhydrous DMF (2 mL). DIPEA (8 equiv., 0.016 mol) was added and the reaction mixture was stirred overnight in the absence of light. Solvent was removed *in vacuo* and the mixture triturated with cold Et₂O (5 mL) to precipitate the crude peptide which was then obtained through centrifugation to obtain the crude solid peptide conjugate. The product was then purified using a C₁₈ column and reverse phase HPLC (H₂O/MeCN) gradient system using mass spectrometry as confirmation of molecular mass to give a pale red solid (0.0021 g, 70 % yield).

Liquid Chromatography and Mass Spectrometry (LCMS) detection of substrates

LC conditions: High-purity HPLC-grade solvents (Sigma-Aldrich), analytical grade chemicals (Sigma-Aldrich) and triple distilled water were used throughout. Reverse-phase chromatographic separation of substrates was performed using an Acquity UPLC comprising a BEH C₁₈ 1.7μM column (2.1mm x 100mm) (Waters, UK). Mobile phases were as follows: Mobile Phase A consisted of 90% HPLC grade water, 10% HPLC grade MeCN and 0.1% HCO₂H. Mobile phase B consisted of 40% HPLC grade water, 60% MeCN and 0.1% HCO₂H.

MS conditions: A Micromass ZMD single quadrupole electrospray MS was used in positive mode (Micromass, Manchester, UK) and MassLynx software was used to identify substrates and anticipated metabolites. MS source parameters were optimised to: desolvation gas 375 L/hr, cone gas 33 L/hr, capillary 2.9 kV, sample cone 16V,

extraction cone 5V, fR lens 0.1V, source block temperature 150°C and desolvation temperature 200°C. Parent compounds and metabolites were detected as singularly charged ions using selected ion readings (SIR).

Cleavage of substrates by recombinant MMPs

Recombinant human MMP proteins (MMP-2 and MMP-9, R&D Systems, UK) were assayed for their ability to cleave MMP-selective substrate **1**, and peptide conjugates. Reactions (100 µL) contained 20 ng recombinant protein and 10 µM substrate in MMP activity buffer (100 mM Tris-HCl pH 7.6, 10 mM CaCl₂, 100 mM NaCl, 0.16% v/v Brij-35), and incubated at 37°C for 12h. At T₁₂, The sample was removed and diluted 1:3 with MeCN to precipitate proteins. The sample was then centrifuged at 10,000 g for 3 minutes and supernatant assayed for substrate cleavage by LCMS.

Determination of MMP mRNA expression by semi-quantitative RT-PCR analysis

Refer to section S18.

MTT assay

Drug cytotoxicity was evaluated against the human U87-MG glioma cell line. Cells were seeded into 96-well plates at 2000 cells/well. After 24 h incubation, cells were treated with various concentrations of compound or relevant vehicle control. Following a further 96 h drug exposure, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) (500 µg/well) was added and incubated for 4 h at 37°C. MTT was carefully removed, the blue formazan crystals dissolved in DMSO, and absorbance measured at 550 nm (GO Multiskan, Thermo Life Sciences). Cell survival was normalised to vehicle treated control wells, which represented 100% viability (data representative of three independent experiments).

Metabolism of **3** in tissues *ex vivo*

Refer to section S19.

ASSOCIATED CONTENT

Supporting Information Availability: Structural analysis of MMP 2 and MMP 9, Fig. S1., Electrospray Ionisation-Liquid Chromatography Mass Spectrometry (ESI-LCMS) identification of substrates and their metabolites Fig. S2-S4., Synthetic scheme for the production of the peptide doxorubicin conjugates Fig. S5., Docking analysis and LCMS analysis of known prodrug cleavage, Fig. S6-S9., Cleavage of **3** by MMP 2 and MMP 9, fig. S10-S12. Embedded Mol2 files. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions\

‡The manuscript was written through contributions of all authors.

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ABBREVIATIONS AND ACRONYMS

DCM	Dichloromethane
DIPEA	N,N-Diisopropylethylamine
DMF	Dimethylformamide
Et ₂ O	Diethyl Ether
HCO ₂ H	Formic Acid
HPLC	High Performance Liquid Chromatography
HT1080	Human Fibrosarcoma
LCMS	Liquid Chromatography - Mass Spectrometry
MeCN	Acetonitrile
MMPs	Matrix Metalloproteases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
PyBOP	benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
TFA	Trifluoroacetic Acid
U87-MG	Human Malignant Glioma

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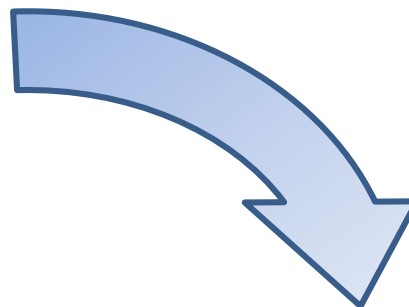
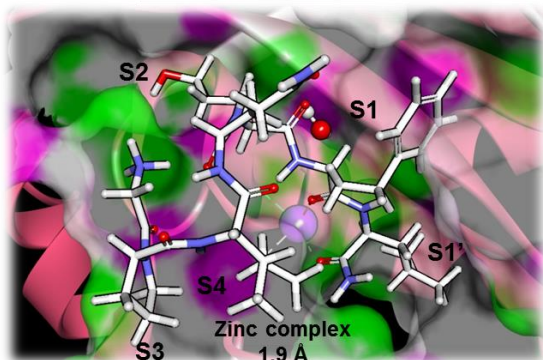
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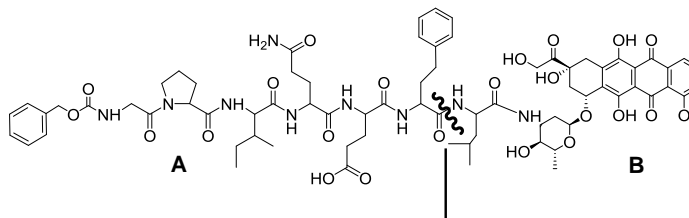
Development of MMP-2 selective peptide prodrug

In silico prediction

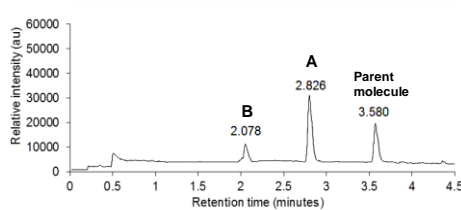
MMP-2 active site



In vitro confirmation



MMP-2 mediated cleavage



No cleavage by MMP-9

