Design, Synthesis, and Biological Evaluation of a Dual Tumor-specific Motive Containing Integrin-targeted Plasmin-cleavable Doxorubicin Prodrug


Department of Organic Chemistry, NSR-Center for Molecular Structure, Design and Synthesis, University of Nijmegen, 6525 ED Nijmegen [F. M. H. d. G., H. P. H. M. A., G. I. T., H. W. S.]; Department of Medical Oncology, Academic Hospital Vrije Universiteit, 1007 MB Amsterdam [H. J. B., Y. W. E., H. M. P.]; Ansynth Service B.V., 4703 LE Roosendaal [A. v. V.]; Department of Pathology and Laboratory Medicine, Medical Biology Section, Tumor Immunology Laboratory, 9713 GZ Groningen [A. J. S., G. M.]; and Department of Pharmacokinetics and Drug Delivery, University Centre for Pharmacy, 9713 AV Groningen, [R. J. K.], the Netherlands

Abstract
The design, synthesis, and initial biological evaluation of a doxorubicin prodrug that contains a dual tumor specific moiety, which allows enhanced tumor recognition potential, is reported. Both a tumor-specific recognition site and a tumor selective enzymatic activation sequence are incorporated in the prodrug. The first tumor-specific sequence is the bicyclic CDCRGDCFC (RGD-4C) peptide that selectively binds \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins. These integrins are highly overexpressed on invading tumor endothelial cells. The second tumor-specific sequence is a \( \alpha \)-Ala-Phe-Lys tripeptide that is selectively recognized by the tumor-associated protease plasmin, which is involved in tumor invasion and metastasis. An aminocaproyl residue was incorporated as a spacer between the two peptide sequences, whereas a self-eliminating 4-aminobenzyl alcohol spacer was inserted between the plasmin substrate and doxorubicin. Although the prodrug showed a decreased binding affinity as compared with the unconjugated reference peptide, it was still a potent ligand for \( \alpha_v \beta_3 \), \( \alpha_v \beta_5 \), and \( \alpha_v \beta_3 \) integrin receptors. The synthesized construct also possessed plasmin substrate properties as demonstrated by doxorubicin release from 1 upon incubation with plasmin. The release of doxorubicin from 1 was not complete, possibly related to low prodrug solubility. In vitro prodruk 1 showed plasmin-dependent cytotoxicity for endothelial cells and HT1080 fibrosarcoma cells. On the basis of these in vitro results, derivatives of 1 with improved water solubility are considered good candidates for additional development and in vivo evaluation of this dual targeting concept.

Introduction
Cancer chemotherapy is in many cases the only treatment option for disseminated cancer. Because of the inherent toxicity of currently available chemotherapeutic agents for normal cells and tissues, limited doses of agents that can be administered are often not high enough to eradicate the cancer cells. One approach to improve the effectiveness and decrease toxicity of anticancer chemotherapy is to administer an inactive prodrug that specifically releases the active parent drug at the tumor site (1–3). Whereas site-specific recognition can result from incorporation of a specific receptor binding domain, drug release can be induced by incorporation of a selective substrate for an enzyme that is present in elevated levels at the tumor target site. In this study, a prodrug that contains both a receptor binding domain and an enzyme substrate moiety is reported.

A relatively new approach is targeting drugs to receptors involved in tumor angiogenesis, the formation of new blood vessels from existing ones. Specific integrin receptors are required for tumor angiogenesis (4). Integrins are important in cancer as they mediate contact between cells and between cells and the extracellular matrix (5). In particular, the \( \alpha_v \beta_3 \) integrins \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) are highly expressed by endothelial cells in human tumor microvessels.

Tumor vasculature has been shown to be an interesting target for anticancer therapy because tumor cells depend on the tumor microcirculation for oxygen and metabolic supply. The effect of the elimination of one endothelial cell may result in the death of many tumor cells (6). Furthermore, tumor endothelial cells are in direct contact with the bloodstream and are therefore easily accessible for systemically applied therapeutics. Finally, endothelial cells are genetically stable, making the occurrence of the development of drug resistance less likely.

The tumor-homing bis-disulfide containing RGD-4C peptide (CDCRGDCFC; Ref. 7) has been shown to have high selectivity for binding to the \( \alpha_v \beta_3 \) integrin receptor. Linear RGD peptides bind to many integrins, whereas it is believed that the presence of two disulfide bridges is responsible for selective binding to the \( \alpha_v \beta_3 \) member of the integrin receptor family. The most potent isomer of RGD-4C for binding to \( \alpha_v \beta_3 \) integrin is obtained when the exact configuration is such that the disulfide bridges span the first and fourth and the second and third cysteine residues (1–4;2–3, Fig. 2; Ref. 8). When an RGD-4C peptide was conjugated to doxorubicin...
and given to nude mice bearing human breast carcinoma xenografts, the conjugate appeared much more toxic and demonstrated an increased anticancer effect in comparison with free doxorubicin (9). Also a conjugate of the RGDS-4C sequence with the proapoptotic peptide D(KLAKLAK)2 showed selective cytotoxicity to angiogenic endothelial cells and showed in vivo anticancer activity in nude mice (10).

In another approach to selectively generate active parent drug at the target site, tumor-associated enzymes can be exploited as a trigger for specific prodrug activation. Particularly, tumor-associated proteases have served as target enzymes for selectively activated prodrugs in recent studies. Anticancer prodrugs that are activated by prostate-specific enzymes for selectively activated prodrugs in recent studies.


target enzyme, for example, a,6-antiplasmin (15).

Both tumor-associated proteins a,6 integrin and plasmin are often present in tumor tissue in cellular plasma membranes that allow physical association in multiprotein complexes (22). Interaction of integrins and components of the urokinase-type plasminogen activator system is essential for the processes of extracellular matrix breakdown and cell migration (23, 24). This provides a basis for combination of two peptide sequences in one prodrug that can be recognized by either a member of the integrins or by a component of the urokinase-type plasminogen activator system.

Here, the design, synthesis, and initial biological evaluation of a tumor vasculature-directed doxorubicin prodrug that contains both an a,6 integrin binding RGDS-4C sequence and a specific tripeptide sequence that is a substrate for the tumor-associated protease plasmin is presented. This approach may lead to a synergistic mode of action in which both proteins act effectively together to release active drug at the tumor site.

Materials and Methods

Chemistry

Linear Undecapeptide 2. The undecapeptide was synthesized applying the Fmoc4 protocol on a Barlos resin (Bachem, Bubendorf, Switzerland) (2 * 2g) with a load of 0.7 mmol/g. Deprotection of Fmoc groups was performed with 20% piperidine in DMF. Both the removal of the Fmoc group and the efficacy of each new acylation were controlled using the Kaiser test (25). When the Kaiser test indicated incomplete coupling, acylation was repeated with a Pfp ester. After deprotection of the final Fmoc group from the Ala residue, the NH2 terminus was acetylated using acetic anhydride/pyridine. The peptide was subjected to acidolysis in trifluoro ethanol/acetic acid/DCM (2/2/6) for 30 min. The resulting mixture was filtered, the filtrate was concentrated in vacuo, and freeze-dried from dioxane; ESI-MS: (M + H)+ = 2252.

Monocyclic RGD-Peptide 3. To a stirred solution of iodine (930 mg, 7 eq) in hexafluoroisopropanol/DCM (12ml/120 ml), the linear peptide 2 (1.12 g, 0.497 mmol) was added dropwise. After 15 min, the reaction was quenched by pouring the mixture into an ice-cold (100 ml, 0°C) aqueous solution containing a large excess of L-ascorbic acid (3.2 g, 18 mmol) and ammonium acetate (2.3 g, 30 mmol). The resulting mixture was concentrated in vacuo, and the suspension was extracted with CHCl3/methanol (9/1) in three portions. The organic layer was washed with water, dried over anhydrous sodium sulfate, and concentrated to dryness. The residue was dissolved in dioxane and freeze-dried to yield monocyclic 3; ESI-MS: (M + H)+ = 1766.

Bicyclic RGD-Peptide 4. The monocyclic RGD-peptide 3 was dissolved in 30 ml of DMF and added to a stirred solution of iodine (1.62 g, 12 eq) in 65 ml of DMF. After 15 min, the reaction was quenched by adding an aqueous solution containing L-ascorbic acid (1.289 g, 7.32 mmol) and ammonium acetate (0.894 g, 11.6 mmol). The mixture was added dropwise to stirring water to precipitate the product. After centrifugation and several washings with water and diethyl ether, the residue was dissolved in dioxane and freeze-dried, affording 610 mg (two steps: 76%) of white and fluffy bicyclic 4; ESI-MS: (M + H)+ = 1622.

H-D-Ala-NH2-Boc. To a stirred solution of Z-D-Ala-OH (2.00 g, 9.96 mmol) and H2N-NH-Boc (1.30 g, 1.1 eq) in EtOAc at 0°C, was added 2.22 g (1.2 eq) dicyclohexyl carbodiimide. After 1 h, precipitated dicyclohexylurea was filtered, and the filtrate was washed with 10% citric acid, saturated sodium bicarbonate, and saturated sodium chloride (brine), and the organic layer was dried over anhydrous sodium sulfate. The solvent was evaporated, cold EtOAc was added, and the residual dicyclohexylurea was filtered. The filtrate was concentrated in vacuo to yield Z-D-Ala-NH2-Boc as a white foam. The compound was dissolved in methanol and subjected to catalytic hydrogenolysis using 10% Palladium on activated carbon (2 h, 60 bar). The mixture was filtered over hyflo, and the filtrate was concentrated in vacuo. The residue was dissolved in EtOAc, filtered over hyflo, and the filtrate was concentrated in vacuo to yield 1.34 g of H-D-Ala-NH2-Boc (two steps: 66%). 1H-NMR (300 MHz, high-performance liquid chromatography; HPLC, human umbilical vein endothelial cell; MT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Aca, aminocaproic acid; Aloc, allyl oxycarbonyl; Trt, trityl; Acm, aceticamidomethyl; Pfp, pentafluorophenyl.)
After two additional washings with ether, the residue was added dropwise to cold diethyl ether, and the solution was added to 5 h. Then 10% 2-propanol in EtOAc (200 ml) was added, the organic layer was washed with water (three times), 10% citric acid solution (three times), and brine. The organic layer was concentrated in vacuo, dioxane was added, and the solution was freeze-dried to yield Boc-hydrazide 5; ESI-MS: (M + H)⁺ = 1808.

Deprotected Hydrazide 6. A solution of 330 mg of 5 in 7 ml trifluoroacetic acid/water (95/5) was stirred for 5 h. The solution was added dropwise to cold diethyl ether, and the suspension was centrifuged; the supernatant was discarded. After two additional washings with ether, the residue was dissolved in dioxane and freeze-dried to yield 100 mg (0.064 mmol) of hydrazide 6.

To a solution of 2.5 g of 10 (300 MHz, CDCl₃/CD₂OD, δ): 1.28 (d, 3H, J = 6.7 Hz, dox sugar 1), 1.80 (d, 3H, J = 4.4 Hz, dox 8), 2.15–3.35 (m, 6H, Bn Phe, N-CH₂ Lys and dox 10), 3.61 (bs, 1H, dox sugar 4'), 3.85 (m, 1H, dox sugar 3'), 4.07 (s, 3H, dox OMe), 4.15 (m, 2H, Hα), 4.30–4.60 (m, 5H, dox sugar 5', 2 Fmoc and 2 Aloc), 4.76 (m, 1H, dox 7), 5.35 (m, 2H, Bn Phe and dox 14), 5.65 (m, 1H, dox OMe), 5.80 (m, 1H, dox sugar 3), 6.80 (m, 1H, dox 1), 7.05–7.90 (m, 19H, aromatic), 8.08 (d, 1H, J = 7.6 Hz, dox 1) ppm. 4-Nitrophenylcarbonate 11 (1.349 g, 1.55 mmol) and doxorubicin-HCl (989 mg, 1.1 eq) were dissolved in N-methylpyrrolidinone, and triethyl amine (322 µL, 1.5 eq) was added at room temperature. The reaction mixture was stirred in the dark for 16 h and subsequently diluted with 10% 2-propanol in EtOAc. The organic layer was washed with water and brine and dried over anhydrous sodium sulfate. After evaporation of the solvents, compound 12 was chromatographically pure. 1H-NMR (300 MHz, CDCl₃/CD₂OD, δ): 1.28 (d, 3H, J = 6.7 Hz, dox sugar 1), 1.20–1.95 (m, 8H, CH₂ Phe and dox 2'), 2.16 (dd, 1H, J = 4.4 Hz, dox 8), 2.37 (m, 1H, dox 8), 2.95–3.35 (m, 6H, Bn Phe, N-CH₂ Lys and dox 10), 3.61 (bs, 1H, dox sugar 4'), 3.85 (m, 1H, dox sugar 3'), 4.07 (s, 3H, dox OMe), 4.15 (m, 2H, Hα), 4.30–4.60 (m, 5H, dox sugar 5', 2 Fmoc and 2 Aloc), 4.76 (m, 1H, dox 7), 5.35 (m, 2H, Bn Phe and dox 14), 4.98 (s, 2H, Bn spacer), 5.10–5.35 (m, 2H, Aloc), 5.49 (s, 1H, '1'), 5.65–5.95 (m, 2H, dox 7 and Aloc), 7.05–7.90 (m, 19H, aromatic), 8.03 (d, 1H, J = 7.6 Hz, dox 1) ppm.

H-Phe-Lys(Aloc)-PABC-Doxorubicin 13. To a solution of 12 (300 mg, 0.235 mmol) in N-methylpyrrolidinone (1 ml) and THF (10 ml) was added 12.5 ml of 2% 1,8-diazabicyclo[5.4.0]undec-7-ene in THF, and the mixture was stirred for 1 min. Diethyl ether was added, and the mixture was centrifuged. The supernatant was removed, fresh diethyl ether was added again, the mixture was centrifuged, and the remaining pellet was dissolved in dioxane. HCl in EtOAc was added and the mixture was freeze-dried yielding 250 mg (98%) of hydrochloride 13. 1H-NMR (300 MHz, CDCl₃/CD₂OD, δ): 1.28 (d, 3H, J = 6.7 Hz, dox sugar 1), 1.20–1.95 (m, 8H, CH₂ Phe and dox 2'), 2.16 (m, 1H, dox 8), 2.37 (m, 1H, dox 8), 2.95–3.35 (m, 6H, Bn Phe, N-CH₂ Lys and dox 10), 3.61 (bs, 1H, dox sugar 4'), 3.85 (m, 1H, dox sugar 3'), 4.07 (s, 3H, dox OMe), 4.09 (m, 1H, dox sugar 5'), 4.25 (m, 1H, Hα), 4.40 (m, 1H, Hα), 4.50 (m, 2H, Aloc), 4.76 (s, 2H, dox 14), 4.98 (m, 2H, Bn spacer), 5.15–5.30 (m, 2H, Aloc), 5.49 (s, 1H, dox sugar 1'), 5.65 (m, 1H, dox 7), 5.80–5.95 (m, 1H, Aloc), 7.05–7.85 (m, 19H, aromatic), 8.04 (d, 1H, J = 7.6 Hz, dox 1) ppm.

Azide Coupling to Give Protected Prodrug 15. To a solution of 100 mg (0.064 mmol) of hydrazide 6 in dry DMF at −30°C were added dropwise 127 µL (4.1 eq) of a 2.1 M HCl/EtOAc solution and 10 µL of t-butylnitrite (1.2 eq). The diazotation was followed using the Barton test (26), which indicated disappearance of hydrazide. The reaction mixture...
was stirred for 1 h and neutralized with diisopropylethyl amine (45 µl, 4.1 eq). Subsequently, a solution of amine hydrochloride 13 (77 mg, 1.1 eq) and diisopropylethyl amine (12 µl, 1.1 eq) in DMF was added at −15°C. The reaction mixture was stirred at −4°C for 16 h and subsequently added dropwise to cold diethyl ether. After centrifugation, the supernatant was discarded and after two additional washings with diethyl ether, a red pellet was obtained upon centrifugation. The residue was dissolved in dioxane and freeze-dried to obtain 17 in a yield of 83%; ESI-MS: (M + H)⁺ = 2348; (M + Na)⁺ = 2370.

Bifunctional Prodrug 1. Argon was bubbled through a solution of 68 mg (0.029 mmol) of protected prodrug 15 in dry DMF for 20 min. Morpholine (25 µl, 10 eq) and a catalytic amount of Pd(PPh₃)₄ were added. After 30 min, again a catalytic amount of Pd(PPh₃)₄ was added. After an additional 30 min, the reaction was quenched with AcOH (25 µl, 15 eq). The reaction mixture was added dropwise to cold diethyl ether. After centrifugation, the supernatant was removed and after two additional washings with diethyl ether, a red pellet was obtained. The product was dissolved in dioxane/water and freeze-dried. Mass spectroscopy indicated presence of Aloc-deprotected 13 [(M + H)⁺ = 968]. The crude product was washed with acetonitrile/0.05 M ammonium acetate (50/50), the mixture was centrifuged, and the supernatant was removed. This procedure was repeated several times until the supernatant remained colorless and was repeated once using acetonitrile/water (50/50). The remaining solid was suspended in dioxane/water and freeze-dried to obtain 17 mg (26%) of the desired bifunctional prodrug 1. Both matrix-assisted desorption ionization-time of flight [(M + H)⁺ = 2265; (M + Na)⁺ = 2287] and ESI-time of flight [(M + H)⁺ = 2265; (M + 2H)⁺ = 1133] showed only signals from the desired compound or fragments thereof.

Biological Evaluation

Dissolution of Prodrug 1. The chemically pure prodrug 1 poorly dissolved in a range of solvents. For the in vitro experiments reported herein, prodrug 1 was dissolved in DMSO, and undissolved material was removed by centrifugation. The concentration was then determined by using a molar extinction coefficient at 480 nm of 11.5 × 10⁵. By HPLC analysis (conditions see next paragraph), the percentage of free doxorubicin was determined to be 0.14%. The percentage of dissolved prodrug was 25% in three independently dissolved samples. Stock solutions of 250 µM were stored at −20°C.

HPLC Analysis. Prodrug 1 was analyzed using separation by HPLC and spectrofluorometric detection. The separation was performed by isocratic elution [25% acetonitrile, 75% HPLC buffer consisting of 10 mM triethylamine, and 10 mM NaH₂PO₄ (pH 3.5)], with a flow rate of 1 ml/min on an Absorbosphere HS C18, 3-µm particle column (Alltech) with a LiChroCART4–4/Lichrophase 100 RP-18, 5-µm guard column (Merck). The detection of fluorescent peaks was at λₑₓᶜₑ 475 nm and λₑₓₘ 551 nm. Rₘ prodrug = 26.5 min; Rₘ doxorubicin = 8.6 min. For the determination of the effect of plasmin on doxorubicin formation, 4 µM prodrug 1 in Tris buffer (pH 7.5) was incubated with concentrations between 3–45 µg/ml plasmin with or without 250 kIU/ml aprotinin at 37°C. Plasmin (15 µg/ml) was shown to be an optimal concentration because at higher plasmin concentration no higher concentration of free doxorubicin was formed.

Cells. HUVECs were cultured in 1% gelatin-precoated 25 cm² tissue culture flasks (Costar, Cambridge, MA) at 37°C under 5% CO₂/95% air. The culture medium, hereafter referred to as EC medium, consisted of RPMI 1640 (BioWittaker, Verviers, Belgium) supplemented with 20% heat-inactivated FCS (Integro B.V., Zaandam, the Netherlands), 2 mM L-glutamine (Life Technologies, Inc., Paisley, Scotland), 5 units/ml heparin (Leo Pharmaceutical Products B.V., Weesp, the Netherlands), 100 units/ml penicillin (Yamanouchi Pharma B.V., Leiderdorp, the Netherlands), 100 µg/ml streptomycin (Radiumfarma-Fisipharma, Italy), and 50 µg/ml EC growth factor supplement extracted from bovine brain. After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml in PBS) treatment and split at a 1:3 ratio. Cells were used for experiments up to passage four. For the experiments described in Fig. 6 and Table 1, HUVECs were isolated in a slightly different way, according to previously described methods (27, 28, and HUVECs were used in passage 2 or 3. HT1080 fibrosarcoma cells were cultured in DMEM with 10% FCS.

The H5V mouse endothelioma cell line was kindly provided by Dr. A. Vecchi (Milan, Italy) and were grown in tissue culture flasks or plates (Costar) at 37°C under 5% CO₂/95% air. The culture medium consisted of DMEM (BioWittaker) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 300 µg/ml gentamicin (Life Technologies, Inc., Paisley, Scotland). After attaining confluence, cells were detached from the surface by trypsin/EDTA (0.5/0.2 mg/ml in PBS) treatment and split at a 1:3 ratio.

Endothelial Cell Adhesion Assay. The endothelial cell adhesion assay was performed as previously described (29) with minor modifications. Briefly, flat-bottomed 96-well culture plates (Costar) were coated with 0.5 µg/well vitronectin (Sigma, St. Louis, MO) overnight at 4°C in PBS. Wells were blocked with 1% BSA for 2 h at 37°C and washed two times with PBS. HUVECs were trypsinized, resuspended in serum-free medium, and plated at 2 × 10⁴ cells/well. For inhibition experiments, cells were preincubated with peptides for 15 min on ice before plating. Cell numbers were determined by crystal violet staining or by the MTT assay. Coefficient of variation: after incubation at 37°C for 24 h, unattached cells were removed by rinsing the wells with PBS. Attached cells

<table>
<thead>
<tr>
<th>IC₅₀ doxorubicin (µM)</th>
<th>IC₅₀ prodrug 1 (µM)</th>
<th>IC₅₀ prodrug 1 (µM) + plasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-1080</td>
<td>0.18</td>
<td>2.4</td>
</tr>
<tr>
<td>HUVEC</td>
<td>0.45</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 1 Effect of plasmin on prodrug 1 cytotoxicity
were fixed in 2% paraformaldehyde, stained with 0.5% crystal violet (Sigma), and quantified by absorbance reading at 575 nm on an Emax microplate reader (Molecular Devices, Sunnyvale, CA). MTT: cells were incubated with MTT for 3 h, and color formation was determined by absorbance reading at 450 nm by a standard MTT assay (Fig. 6 and Table 1; Ref. 30).

Radioactive Binding Assay. The binding specificity of prodrug 1 to H5V cells was studied using a competition assay exploiting a radioiodinated RGD-based protein conjugate that selectively binds to αvβ3/αvβ5. This conjugate consisted of RGD peptides (cRGDFK) chemically coupled to a protein backbone, which was shown to have a high affinity for αvβ3 (31). Confluent H5V monolayers in 24-well culture plates (Costar) were preincubated with binding buffer [50 mM Tris-HCl (pH 7.4), supplemented with 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2, and 1% BSA]. The cells were subsequently incubated with a radiolabeled RGD-based protein conjugate in the presence of different concentrations of prodrug 1 in binding buffer at 4°C for 4 h. After incubation, the supernatant was harvested, and cells were washed three times with binding buffer and lysed with 1 M NaOH. Cell-associated radioactivity was counted in a LKB multichannel counter (LKB, Bromma, Sweden). Control experiments included competition experiments with RGD-4C peptides and Phe-Lys-PABC-Doxorubicin.

Results

Synthesis

When the receptor recognizing peptide is incorporated into the prodrug at one end of the molecule and the tripeptide is built between the RGD sequence and the drug (doxorubicin), a multifunctional prodrug containing a dual specificity motive is obtained. It was hypothesized that drug release can occur after receptor binding or specific enzymatic activation. In this approach, integrin recognition must not be hindered by the presence of the tripeptide-drug part of the prodrug. On the other hand, plasmin activation of the prodrug must not be hindered by the presence of the bulky RGD-4C peptide on one side of the tripeptide substrate and the relatively bulky doxorubicin molecule on the other side.

The target prodrug to be synthesized contains a RGD-4C sequence, a plasmin substrate sequence, the parent drug doxorubicin, and two spacers (Fig. 1). The 1,6-Aca spacer (spacer 1), positioned between the RGD-4C and the d-Ala-Phe-Lys, should maintain appropriate spacing between the receptor binding unit and the enzyme substrate unit. Spacer 2 is a self-immolative connector (32) placed between the tripeptide and doxorubicin to enable plasmin to cleave the Lys-spacer bond. Without a spacer, plasmin is not able to efficiently cleave the tripeptide-doxorubicin bond (20). After subsequent spontaneous spacer-elimination, doxorubicin is released.

For the synthesis of this bifunctional molecule, a synthetic strategy had to be followed that was compatible with the presence of both two base-labile disulfide bridges of the bicyclic RGD sequence and the acid-labile acetal function in the doxorubicin molecule. Therefore, a convergent synthesis was chosen in which a base-labile disulfide fragment was coupled with an acid-labile doxorubicin fragment in the final stage of the synthesis. The final step of the synthesis should comprise removal of the protecting group from the side chain of the lysyl residue of compound 15 (Fig. 4). This protective group is indispensable during synthesis and should prefer-
ably be removable in neutral medium to respect the sensitivity of the end product. This condition was realized by choosing the Aloc group for protection of this side chain. The Aloc protecting group has already been successfully applied in combination with doxorubicin.

The final coupling in the synthesis was accomplished by connection of the COOH-terminus of Ac-Ala-RGD-4C-Aca-D-Ala (14) with the NH₂ terminus of Phe-Lys-PABC-Doxorubicin (13, PABC; Fig. 4). In this approach, the NH₂-terminal RGD-4C-containing undecapeptide sequence (2, Fig. 2) was designed to be prepared as a protected peptide derivative using the solid phase method. This can be realized using the Fmoc protocol on the 2-chlorotrityl polymer support of Barchos et al. (33). This technique ensures the preservation of all protecting groups upon peptidolysis, some of them (Trt) being rather sensitive toward acids. Because the product of a solid phase synthesis is a carboxylic acid, which should in this case be condensed to an amino component, the possibility of racemization is present if a chiral amino acid terminates the sequence. Therefore, an achiral COOH-terminus was preferred. To space the RGD sequence from the plasmin substrate sequence, we chose to incorporate /H9280-Aca as the COOH-terminal amino acid (2, Fig. 2).

To complete the synthesis of the NH₂-terminal undecapeptide derivative 6, the disulfide bridges were selectively introduced using the method of Kamber (34, 35), giving 3 and 4. Compound 4, the bicyclic disulfide, arose upon two iodolysis reactions of the selectively protected peptide 2. Both cyclizations were performed in highly diluted solution after peptidolysis of the linear peptide from the resin to prevent intermolecular disulfide formation. Although in another synthetic approach to selectively introduce the 1-4;2-3 disulfide into the RGD-4C molecule, only two cysteine residues were protected (8), we were interested to investigate the possibility to selectively introduce two disulfides through protection of all four cysteine residues. This strategy might allow additional modification of the linear peptide before the disulfides are introduced, if desired. Kamber et al. (34, 35) reported that combination of two Trt- and two Acm-protecting groups allows for selective conversion of two Trt-protected thiol groups to one disulfide in the presence of the two Acm-protected thiol groups. This selective conversion was performed with iodine and depends on a proper choice of the solvent. Because the compatibility of the indispensable allyl protection during iodolysis of Trt and Acm groups has not been proven, we chose to introduce the disulfide bridges at the undecapeptide stage.

ESI-MS indicated exclusive formation of both the desired monodisulfide product 3 after the first iodine oxidation and the bis-disulfide product 4 after a second iodine oxidation.

The hydrazide was introduced in protected form by acylation of H-D-Ala-N₂H₂-Boc with the Nα-acetylated undecapeptide derivative 4, giving 5. Because the COOH terminus of compound 4 is achiral, coupling to the amine H-D-Ala-N₂H₂-Boc was performed via a mixed anhydride method without risking racemization. Because a protonated NH₂ terminus of the RGD-4C peptide appeared as not essential for biological activity, it was decided to permanently cap this NH₂ terminus with an acetyl group. H-D-Ala-N₂H₂-Boc was synthesized by coupling Z-D-Ala-OH with H₂N-NH-Boc and subsequent hydrogenolysis of the Z protecting group. This latent activation had to be introduced after formation of the disulfide bonds because it is incompatible with iodine-mediated oxidation. The D-alanyl residue containing a latent activation in the form of a protected hydrazide was introduced at this stage to allow selective condensation with the COOH-terminal fragment 13 in the presence of the deprotected carboxylic acid functions of Asp (3, 7). Acidolysis of all acid-labile groups of 5 afforded compound 6, which can be selectively converted into an acid azide (14) upon diazotation using Honzl and Rudinger’s in situ procedure (36). Compound 14 was activated as the acid azide to prevent racemization during the assembly of fragments 13 and 14 in this stage of the synthesis.

---

**Fig. 2.** Synthesis of the bicyclic RGD-4C containing NH₂-terminal dodecapeptide 6 and the deprotected bicyclic RGD-4C reference undecapeptide 7.
A small portion of bicyclic 4 was deprotected using 95% trifluoroacetic acid to obtain an unconjugated RGD-4C derivative (7, Fig. 2) that could be tested for its functionality as $\alpha_v\beta_3$ integrin ligand.

The amino component to be condensed with the acid azide derivative of 6 is the fragment that involves the acid-sensitive parent drug doxorubicin [H-Phe-Lys(Aloc)-PABC-Doxorubicin, 13]. The synthesis of compound 13 is depicted in Fig. 3; it represents the partially protected version of this COOH-terminal. Compound 13 is the hydrochloride of L-phenylalanyl-$\alpha_2$2-L-lysyl-4-aminobenzyl-oxycarbonyl-doxorubicin. It was obtained from 4-amino-benzyl alcohol after acylation with Fmoc-Lys(Aloc)-OH through a mixed anhydride acylation (to give 9), deprotection with a base and subsequent acylation with Fmoc-Phe-OPfp giving 10. To avoid racemization of the lysyl residue, the dipeptide amide was prepared by stepwise acylation. The benzylic alcohol function was activated with $p$-nitrophenyl chloroformate, and the resulting mixed carbonate 11 was used to establish the carbamate linkage by acylation of the sole amino group of doxorubicin, which afforded compound 12. Base-catalyzed deprotection of the Fmoc group using the required strong base 1,8-diazabicyclo[5.4.0]undec-7-ene afforded the doxorubicin-containing amine fragment 13.

Fig. 3. Synthesis of the COOH-terminal doxorubicin fragment 13, which contains the plasmin-cleavable amide bond.

Fig. 4. Coupling of the NH$_2$-terminal RGD-4C and COOH-terminal plasmin-cleavable doxorubicin derivatives and final deprotection to give prodrug 1.

A small portion of bicyclic 4 was deprotected using 95% trifluoroacetic acid to obtain an unconjugated RGD-4C derivative (7, Fig. 2) that could be tested for its functionality as $\alpha_v\beta_3$ integrin ligand.

The amino component to be condensed with the acid azide derivative of 6 is the fragment that involves the acid-sensitive parent drug doxorubicin [H-Phe-Lys(Aloc)-PABC-Doxorubicin, 13]. The synthesis of compound 13 is depicted in Fig. 3; it represents the partially protected version of this COOH-terminal. Compound 13 is the hydrochloride of L-phenylalanyl-$\alpha_2$2-L-lysyl-4-aminobenzyl-oxycarbonyl-doxorubicin. It was obtained from 4-amino-benzyl alcohol after acylation with Fmoc-Lys(Aloc)-OH through a mixed anhydride acylation (to give 9), deprotection with a base and subsequent acylation with Fmoc-Phe-OPfp giving 10. To avoid racemization of the lysyl residue, the dipeptide amide was prepared by stepwise acylation. The benzylic alcohol function was activated with $p$-nitrophenyl chloroformate, and the resulting mixed carbonate 11 was used to establish the carbamate linkage by acylation of the sole amino group of doxorubicin, which afforded compound 12. Base-catalyzed deprotection of the Fmoc group using the required strong base 1,8-diazabicyclo[5.4.0]undec-7-ene afforded the doxorubicin-containing amine fragment 13.

Fig. 4 summarizes the final stages of the synthesis, which involved formation of compound 15 by acylation of the sole amino function in 13 with 14 and a subsequent final deprotection of the Aloc group of 15 in neutral medium to afford target compound 1.
Integrin Targeted Plasmin-cleavable Doxorubicin Prodrug

Fig. 5. Peptide 7 inhibited $\alpha_\beta_3$-mediated adhesion of HUVECs to vitronectin. Cells were added to vitronectin-coated wells in the presence of 10 $\mu$g/ml of the reference RGD-4C peptide (ACDCRGDCFCG; 8.7 $\mu$m) or unconjugated RGD-4C peptide 7 prepared via the herein described route (8.3 $\mu$m). The number of adherent cells was determined after 24 h of incubation at 37°C. Data were corrected for nonspecific adhesion to BSA-coated wells and expressed as percentage of control [HUVEC adhesion in the absence of inhibitor (−)]. Data are presented as the mean of a triplicate ± SD. *P < 0.05 compared with control.

Extensive efforts to purify the product on a Sephadex column or with HPLC (0.05 $\mu$m aqueous ammonium acetate/acetoniitrite; Vydac TP218 C18 column) failed because of poor solubility of the product. The product was isolated in pure form by selective precipitation in several steps from dioxane/0.05 $\mu$m ammonium acetate (50/50), thereby removing all undesired components. The residual product unambiguously proved to be the desired bifunctional prodrug. Both a correct matrix-assisted desorption ionization-time of flight measurement and an electrospray measurement in which all peaks could be assigned to the desired product or fragments thereof, both single and multiple charged compounds, combined with the fact that the purified product showed only one major HPLC peak (>99%; free doxorubicin contamination: 0.14%), indicate isolation of pure and desired material.

Biological Evaluation

Endothelial Cell Adhesion Assay. Inhibition experiments with anti-$\alpha_\beta_3$, anti-$\alpha_\mu$, and anti-$\beta_3$ antibodies demonstrated that adhesion of HUVECs to vitronectin was dependent on both $\alpha_\beta_3$ and $\alpha_\beta_5$, but not on $\beta_3$ integrins (data not shown). As shown in Fig. 5, unconjugated RGD-4C containing peptide 7 was able to completely inhibit the adhesion of HUVECs to $\alpha_\beta_3$/$\alpha_\beta_5$ ligand vitronectin and was more potent than the reference bicyclic RGD-4C peptide ACDCRGDCFCG. Also conjugated RGD-4C-containing prodrug 1 was able to completely inhibit the adhesion of HUVECs to vitronectin (Fig. 6). In contrast, the unconjugated doxorubicin did not affect the adhesion of HUVECs to vitronectin. Furthermore, RGD-4C-containing prodrug 1 inhibited adhesion of HUVECs to vitronectin in a concentration-dependent fashion with an IC50 of ~150 nM.

Competitive Displacement Study. To compare the affinity of RGD-4C containing peptide 7 and the RGD-4C conjugated prodrug 1 for $\alpha_\beta_3$ and $\alpha_\beta_5$-integrins, a competitive displacement study was performed. We studied whether peptide 7 and prodrug 1 selectively interacted with $\alpha_\beta_3$ by interfering with binding of a radiolabeled $\alpha_\beta_3$-specific RGD-modified protein ligand to H5V cells. This RGD-modified protein consists of monocyclic RGDfK peptides coupled to a protein backbone and binds specifically with high affinity to $\alpha_\beta_3$ on H5V cells (31). Both unconjugated peptide 7 and prodrug 1 dose dependently inhibited the binding of $^{125}$I-RGD-modified protein to H5V cells (Fig. 7) with an IC50 of ~0.4 and 25 nM, respectively. The control compound Phe-Lys-PABC-Doxorubicin (16), which does not have the RGD-4C sequence, did not affect the binding of the RGD-modified protein to H5V cells.

Susceptibility of Prodrug 1 to Plasmin Cleavage. In the HPLC chromatograms of Fig. 8, it is clearly shown that incubation of the prodrug with plasmin leads to the formation of free doxorubicin (B), which is completely blocked by addition of the specific plasmin inhibitor aprotinin (C). This experiment showed that prodrug 1 is susceptible to enzymatic activation by plasmin. After 1 h, 30% of prodrug 1 is...
cleaved, a percentage that does not increase largely upon extended duration of incubation. Obviously, approximately one-third of prodrug is rapidly converted to yield free doxorubicin, after which the enzymatic cleavage rate is substantially reduced.

Cytotoxicity of Prodrug 1 for HUVECs and HT1080 Cells in the Presence or Absence of Plasmin. As a final proof of principle that the prodrug was susceptible to plasmin activation to generate doxorubicin, it was demonstrated that plasmin was capable of increasing the cytotoxicity of 1 for both HUVECs and HT1080 cells. In the presence of 15 μg/ml plasmin, prodrug 1 was almost as toxic as free doxorubicin, whereas in the absence of plasmin, prodrug 1 was much less toxic (Table 1). Similar short-time incubations with control cyclic RGD peptides were not cytotoxic to either HUVECs or HT1080 cells (data not shown).

Discussion

A doxorubicin prodrug (1) containing a dual specificity peptide sequence was designed and synthesized. We hypothesized that this prodrug with its double specificity motive could show enhanced tumor recognition potential. Tumor-specific recognition can occur through integrin receptor binding of the RGD-4C peptide and/or enzymatic activation by the protease plasmin.

Surprisingly, compound 1 showed poor solubility, which ruled out purification by gel-filtration techniques or by HPLC. Compound 1 could be isolated in a pure state by fractional precipitation, as demonstrated by mass spectrometry and analytical HPLC. It remains unclear how the poor dissolution behavior of 1 in a range of solvents such as water, ethanol, DMSO, and DMF can be explained. The prodrug possesses several charged functional groups such as the lysine, arginine, and aspartic acid side chains, which should facilitate dissolution in aqueous media. Possibly, a part of the molecules is locked in a particular conformation that decreases their solubility.

The unconjugated RGD-4C peptide 7 synthesized via the herein presented route appeared to be a potent inhibitor of adhesion of HUVECs to vitronectin. The results obtained with the endothelial cell adhesion assay show that also the RGD-4C peptide conjugated to doxorubicin in prodrug 1 was still able to bind to α5β3/α6β4 receptors on HUVECs and was functional in preventing binding of the cells to vitronectin. Prodrug 1 showed an approximate 60-fold decreased binding affinity as compared with unconjugated peptide 7 (Fig. 7), which might be explained by the presence of the tripeptide-drug portion of the molecule that sterically interferes with receptor binding. However, prodrug 1 was still a potent integrin ligand with IC50 of 25 (radioactive binding assay) and 150 nM (endothelial cell adhesion assay). Doxorubicin was unable to inhibit binding of HUVECs to vitronectin (Fig. 6), and Phe-Lys-PABC-Doxorubicin (16) was unable to inhibit binding of RGD-based protein conjugates to HSV cells (Fig. 7), indicating that the effect of prodrug 1 was RGD-4C dependent.

Moreover, the fact that RGD-4C peptide 7 and the RGD-4C-containing prodrug 1, synthesized according to the route
described herein, are both capable of completely blocking adhesion of HUVECs to vitronectin (Figs. 5 and 6) indicates that the RGD-4C peptides in these compounds can bind to both $\alpha_5\beta_3$ and $\alpha_v\beta_3$ integrin receptors, both reported to be involved in tumor angiogenesis (4).

The synthesized construct 1 possessed plasmin substrate properties as investigated by in vitro incubation with plasmin, followed by HPLC analysis. These experiments (Fig. 6) showed that the plasmin-sensitive tripeptide has access to the enzyme active site. However, only 30% of prodrug were easily converted to parent drug. The poor solubility of prodrug 1 may explain incomplete activation of prodrug 1 by plasmin. It can be questioned whether the prodrug remains completely dissolved upon mixing of the DMSO solution containing prodrug 1 with aqueous buffer. Partly undissolved prodrug 1 would not be available for plasmin activation. The rate of plasmin cleavage of prodrug 1 (30% after 60 min incubation with 15 $\mu$g/ml plasmin; 4 $\mu$M prodrug) seems to be slightly lower than the rate of plasmin activation of the previously reported spacer-containing plasmin-activated prodrug lacking the RGD-4C-Aca peptide (Ref. 18; 50% after 19-min incubation with 50 $\mu$g/ml plasmin; 100 $\mu$M prodrug). This may indicate that incorporation of the Aca spacer (partly) prevented steric interference of the RGD-4C portion of the molecule with plasmin cleavage.

Additional proof of principle for susceptibility of 1 for plasmim cleavage was delivered in an in vitro cytotoxicity experiment. The IC50s reported in Table 1 show that in the presence of plasmin, prodrug 1 exerts cytotoxicity to HT1080 cells as well as HUVECs, approaching that of free doxorubicin. These data indicate that plasmin mediation is involved in generation of cytotoxicity.

Having established proof of principle for both integrin binding and plasmin susceptibility, a number of pathways leading to drug accessibility into the target cells under pathological conditions could be considered to be operative: (a) binding of the bicyclic RGD sequence to the $\alpha_5\beta_3$ integrin receptor on either tumor vasculature or tumor cells, followed by extracellular drug release by plasmin cleavage; (b) integrin binding and subsequent internalization of the entire prodrug, leading to degradation by intracellular proteases such as lysosomal enzymes (for example cathepsins); and (c) extracellular plasmin activation of the prodrug without integrin recognition. It should also be noted that a proper choice of the chemical linkage between promoiety (i.e., part of prodrug that is attached to the parent drug) and drug is essential for drug release. This linkage should possess tumor-selective lability. The tumor-selective lability in the conceptually novel prodrug 1 is incorporated in its tripeptide sequence that is designed to be extracellularly cleavable by tumor-associated plasmin at the COOH-terminal side of the lysyl residue. If the prodrug is localized via its RGD peptide and internalized (release mechanism ii), the presence of the tripeptide sequence may make drug release more efficient by facilitating degradation of the prodrug by intracellular proteases. The D-configuration of the alanyl residue must assure stability against unspecific proteolysis by ubiquitous endoproteases (37). If free drug is generated on the surface of target cells through mechanism i, the RGD sequence may retain the prodrug in tumor-angiogenic tissue and may give plasmin more time to actively liberate doxorubicin. If drug release occurs according to mechanisms i and/or iii, a bystander effect can be expected, as the extracellularly released drug can kill also tumor or endothelial cells in proximity that do not possess the $\alpha_5\beta_3$ and/or $\alpha_v\beta_3$ receptor. The possibility of partial redistribution of free drug generated through mechanisms i and/or iii cannot be completely ruled out. However, an improved therapeutic index could also be achieved by tumor-specific delivery of a portion of targeted doxorubicin. This may result in lower and therefore less toxic systemic doses that are necessary to obtain antitumor efficacy.

Proof of principle for the dual specific mode of action of prodrug 1 has been delivered. In vitro, both the RGD-4C peptide binds to integrin receptors and the tripeptide sequence is susceptible to plasmin cleavage. On the basis of the obtained results, development of derivatives of prodrug 1, which possess improved solubility, may yield dual tumor-specific prodrugs that act according to this newly proposed approach. Such derivatives are considered as promising candidates for future in vivo evaluation of antitumor efficacy and toxicity.

Acknowledgments
We thank Dr. Dirk T. S. Rijkers, Dr. Wil J. G. M. Cuppen, and Dr. Ivo F. Eggen for their technical contributions.

References


Molecular Cancer Therapeutics

Design, Synthesis, and Biological Evaluation of a Dual Tumor-specific Motive Containing Integrin-targeted Plasmin-cleavable Doxorubicin Prodrug 1 This work was partly supported by the Spinoza Award (to H. M. P.).


*Mol Cancer Ther* 2002;1:901-911.

Updated version  Access the most recent version of this article at:
http://mct.aacrjournals.org/content/1/11/901

## Cited articles
This article cites 34 articles, 7 of which you can access for free at:
http://mct.aacrjournals.org/content/1/11/901.full#ref-list-1

## Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/1/11/901.full#related-urls

## E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

## Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

## Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.