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RESEARCH PAPER

Novel Integrin-Targeted Binding-Triggered Drug Delivery System for Methotrexate

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ABSTRACT

Purpose To design a binding-induced conformation change drug delivery system for integrin-targeted delivery of methotrexate and prove the feasibility of using hairpin peptide structure for binding triggered drug delivery.

Methods Methotrexate prodrugs were synthesized using solid phase peptide synthesis techniques by conjugating methotrexate to Arg-Gly-Asp (RGD) or a hairpin peptide, RWQYV^{D}PGKFTVQRGD (hairpin-RGD). Levels of integrin $\alpha_V\beta_3$ in HUVEC were up-regulated using adenoviral system and knocked down using siRNA. Stability of prodrugs and methotrexate release from prodrugs were evaluated in plasma, in presence or absence of integrin $\alpha_V\beta_3$-expressing cells. Molecular modeling was performed to support experimental results using MOE.

Results Prodrugs recognized and bound to integrin $\alpha_V\beta_3$-expressing cells in integrin $\alpha_V\beta_3$ expression level-dependent manner. Prodrug with hairpin peptide could resist *Streptomyces griseus*-derived glutamic acid-specific endopeptidase (SGPE) and plasma enzyme hydrolysis. Drug release was triggered in presence of HUVEC cells and SGPE. Analysis of conformation energy supported that conformational change in MTX-hairpin-RGD led to exposure of labile link upon binding to integrin $\alpha_V\beta_3$-expressing cells.

Conclusions Binding-induced conformation change of hairpin peptide can be used to design integrin-targeted drug delivery system.

KEY WORDS hairpin peptide · integrin $\alpha_V\beta_3$ · methotrexate · targeted delivery · triggered release

INTRODUCTION

In cancer therapy, achieving high tumor to non-tumor tissues drug concentration ratio is required to reduce side effects. Targeted drug delivery of anticancer agents can be designed using prodrug strategy to release the active drug in the vicinity of tumor via tumor-targeted enzymatic activation (1). Several ligands and monoclonal antibodies specific to receptors and antigens associated with tumors have been reported to bring anticancer agents specifically to tumors and then release the anticancer agent by enzymatic activation (1–3). Though these strategies have been shown to be promising in achieving high tumor to blood drug ratios, they suffer from serious limitations, such as immunogenicity posed by antibodies and premature prodrug activation by residual enzyme present in the blood (1–3). Due to these drawbacks, refining the reported methods and search for new approaches based on the ligand-specific interaction and enzymatic activation continues.

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Most improvements for tumor-targeted enzyme-based prodrug activation delivery methods have been focused on optimizing specificity (K_{cat}/K_M) and reducing the half-life in order to reduce prodrug activation away from the tumor environment (4, 5). In addition, introducing clearing agents to expedite the removal of residual enzyme antibody conjugate (E-Ab) from blood, restricting the enzyme expression within the tumor cell, and using humanized antibodies have alleviated these problems to some extent (1, 3, 6, 7).

Methotrexate (MTX)-amino acid prodrugs have been designed to be specifically cleaved by carboxypeptidase (CPA) enzyme for tumor targeting. These prodrugs were made by allowing glutamic acid residue, α-COOH of MTX, to react with an additional amino acid. When phenylalanine (MTX-α-Phe) is used as the additional amino acid, it was shown to be a better substrate for CPA among all other single amino acid conjugated prodrugs (4). The bulkier structure of the labile link in MTX-α-Phe prodrug made it insensitive to the endogenous carboxypeptidase enzyme and hence metabolically more stable in the plasma.

Several tumors, such as gliomas and ovarian cancers, have been shown to over-express integrin $\alpha_V\beta_3$ receptor (8), a membrane-bound hetero-dimer associated with angiogenesis and tumor metastasis (9, 10). Arg-Gly-Asp (RGD), a tripeptide sequence commonly conserved in endogenous $\alpha_V\beta_3$ binding ligands such as firbronectin and vitronectin (11), has been used for radioimaging (12), viral transfection (13) and drug delivery (14).

In this study, a two-pronged approach for drug accumulation at tumor site was investigated which could potentially eliminate the immunogenicity problems associated with tumor-targeted enzyme-based prodrug activation. First, prodrugs are designed to target a specific receptor using a small ligand that can negate the necessity for enzyme-antibody (E-Ab) conjugation to eliminate immunogenicity-related problems. Second, a beta-hairpin peptide structure is introduced to the delivery system to reduce the premature activation of prodrug in the blood circulation. A prodrug built on the β-hairpin peptide structure is designed to achieve the above-mentioned characteristics. The prodrug consisted of MTX as a model anticancer agent, RGD as targeting moiety, and β-hairpin peptide to protect the enzymatic labile linkage to MTX. It is hypothesized that prodrug would resist enzymatic hydrolysis prior to reaching the target and reduce the premature activation by residual enzyme in the blood circulation. Upon binding to the target, overexpressed integrin $\alpha_V\beta_3$ on tumor cells, the β-hairpin structure would be perturbed to expose the labile linkage of MTX for enzymatic cleavage. A twelve amino acid peptide, RWQYV^{D}PGKFTVQ, previously demonstrated to fold into an anti-parallel β-hairpin in aqueous environment (15), is used to mask enzymatic hydrolysis. The tumor binding would trigger the prodrug activation by specific foreign enzyme, which eliminates the need for clearing agent. This design provides significant advantages over antibody-mediated enzyme targeting. Absence of antibodies circumvents the immunogenicity of antibody-mediated enzyme targeting. The objectives of this study are to design and synthesize the RGD-targeted MTX prodrug with β-hairpin peptide structure and demonstrate binding-induced targeted release of MTX *in vitro*.

MATERIALS AND METHODS

Materials

All 9-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids and Rink amide resin were obtained from Novabiochem (Darmstadt, Germany). Dimethylformamide (DMF), diisopropylcarbodiimide (DICI), piperidine, hydroxybenzotriazole (HOBT), dimethylchloride (DMC), trifluoroacetic acid (TFA), diisopropylethylamine (DIPEA), 4-amino-4-deoxy-10-methyl pteroic acid HCl (MAPA), 2,4-dinitrophenol, ethylcarbodiimide hydrochloride (EDC), bovine serum albumin, methotrexate, azo-casein, cell lysis RIPA buffer and alkaline phosphatase secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, Milwaukee, WI). Thioanisole and 1, 2 ethanedithiole were obtained from TCI (Portland, OR) and Alfa Aesar (Ward Hill, MA), respectively. *Streptomyces griseus*-derived glutamic acid endopeptidase (SGPE) was purchased from Fluka. GRGDS was purchased from polypeptide laboratories. Human Umbilical Vein Endothelial Cells (HUVEC, pooled), EGM complete medium, and bovine brain extract (BBE) were obtained from Lonza (Walkersville, MD). $VnRC_3$ cells were kindly donated by Dr. Ginsberg (Scripps Research Institute, La Jolla, CA). All other cells were obtained from ATCC. AdEasy™ adenoviral vector system kit was obtained from Stratagene (Stratagene, La Jolla, CA). The lipofectamine and primers were purchased from Invitrogen. The integrin β_3 siRNA (human) was purchased from Santa Cruz biotechnology (Santa Cruz, CA). The $\alpha_V\beta_3$ Integrin Investigator Kit, rabbit polyclonal antibody against integrin β_5 and Immobilon P (polyvinylidene difluoride, PVDF) for electroblotting were purchased from Millipore. Fetal bovine serum (FBS) was purchased from Innovative Research (Novi, MI); Fibrinogen was purchased from Calbiochem (Gibbstown, NJ).

Synthesis of MTX Prodrugs

Two prodrugs were synthesized by conjugating MTX with either RGD (referred as MTX-RGD) or a twelve amino acid peptide-RGD, RWQYV^{D}PGKFTVQ-RGD (referred to as MTX-hairpin-RGD). Peptides were built from C to N terminus on Rink amide resin using standard N-9-fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide syn-

thesis (SPPS) protocols at room temperature. Briefly, coupling of amino acid was carried out in Chemglass® synthesizer for one hour with intermittent shaking by adding 2.5 mol equivalents of amino acid, diisopropylcarbodiimide (DICI) and 1-hydroxy-benzotriazole (HOBt) in dimethylformamide (DMF) to one mol of resin. Before coupling of a new amino acid, amine groups were deprotected by treating with 20% piperidine in DMF. After the coupling, the unreacted groups were blocked by acylation (335 μL acetic anhydride and 530 μL diisopropylethylamine mixture in DMF for each mol. of resin). After each step, the resin was successively washed with DMF and dichloromethane (CH_2Cl_2) three times each to remove residual reactants. Completion of reaction was confirmed by ninhydrin test. Typical coupling cycle involved repetition of coupling step two times with 2.5 equivalents of each amino acid followed by capping. The resin-bound peptide was used for further coupling of dinitrophenyl ester of 4-amino-4-deoxy-10-methyl pteroic acid (MAPA) and 2, 4 dinitrophenol ester (DNPE).

Activation of COOH of MAPA was achieved by forming an active ester of 2, 4 dinitrophenol (DNPE). The DNPE was synthesized by carefully heating reaction mixture consisting of MAPA and 2, 4-diniotrophenol (DNP) in 2:3 mol ratio and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC, 1.5 parts in mol) in DMF at 48 ± 2°C for 72 h. For every 24 h, additional amounts of EDC and DNP were added. After 72 h, solvent was evaporated under vacuum using rotary evaporator, and residue was triturated in isopropanol. Completion of reaction was monitored by TLC. The molecular weight of final product was confirmed by Matrix-Assisted Laser Desorption Ionization—Time of Flight (MALDI-TOF) mass spectrometry. The DNPE (1 part) was condensed with free amine group of N-terminal glutamic acid (E) on the resin (1 part) in presence of diisopropylethylamine (DIPEA, 1.5 parts) in DMF at room temperature with stirring for 72 h. Schematic is shown in Fig. 1.

The prodrug was cleaved from the resin after coupling with DNPE using a cleavage cocktail containing, 2.5% triisopropylsilane (TIS), and 2.5% water containing sufficient quantity of thioanisol and ethanedithiol in trifluoroacetic acid (TFA). Resin was filtered, and filtrate was evaporated carefully using rotary evaporator under low pressure below 30°C to obtain crude concentrated prodrug.

Purification and Characterization

The concentrated prodrug was precipitated by adding it into chilled isopropylether. This procedure was repeated three times to remove ether-soluble impurities. The water-soluble peptide was lyophilized to obtain a dry solid. Prodrug was stored at −20°C until further processing. Possible deletion products and unconjugated peptide without MAPA portion were separated by reverse phase high performance liquid chromatography (RP-HPLC, Thermo separation products, P4000, AS3000, UV2000, ChromQest v2.51, Agilent C18, 5 μ, 4.6×250 mm, detection at 305 and 214 nm). Separation was achieved by using a mobile phase gradient method. Peaks were characterized by either MALDI-TOF (Shimadzu-Kratos PC Axima CFR V2.2.1) or Electro Spray Ionization Mass Spectrometry (ESI-MS, Varian 500-MS Ion Trap Mass Spectrometer equipped with an ESI source, MS Workstation Version 6.9.1) to confirm the molecular weight. Elutions containing the prodrugs were pooled, evaporated and finally lyophilized to obtain prodrug with a purity of 95% or higher. They were stored at −20°C till use.

Cell Culture

All cancerous cell lines (VnRC3, A2058, MCF7, A375 and HTB 129) were grown in 75 cm^2 cell culture flasks obtained from TPP in Dulbecco's Modified Eagles Complete Growth Medium (DMEM) supplemented with 10% FBS, glutamine (1%v/v, 200 mM) and antibiotics (1%v/v, 5,000 I.U/mL of penicillin and 5,000 μg/mL of streptomycin) in 5% CO_2 incubator at 37°C. HUVEC cells were grown under similar conditions using the EGM complete growth medium supplemented with BBE.

Characterization of Integrin Expression

Western blot analysis was carried out on the cell lysates of $VnRC_3$, HUVEC, A2058, MCF7, A375 and HTB129 to determine the expression pattern of α_V, β_3, and β_5. Cell lysate was prepared using RIPA buffer from the centrifuged cell pellet of confluent cells by subjecting the pellet to four freeze-thaw cycles (5 min in −80°C and 1 min at 37°C). The protein content was determined by using BCA assay kit. Proteins were analyzed using 8% SDS-PAGE gel at 200 mV for 30 min. PVDF membranes were used to transfer the proteins, and they were blocked with 15 mL of SEA BLOCK blocking buffer in TBST (SB-TBST) for 1 h. The PVDF membranes were then incubated with primary antibodies (α_V, β_3 or β_5 diluted in SB-TBST, 1:1000 times) overnight at 4°C, followed by incubation for 1 hr with alkaline phosphatase-coupled rabbit anti-goat antibody. Antibody-protein complexes were then detected using the Lumiphos-based chemiluminescence technique to determine integrin protein levels.

Modulation of β_3 Expression

The cDNA for β_3 was cloned into adenovirus using AdEasy™ adenoviral vector system according to the manufacturer's recommendations. Briefly, 2.5 μL of 10^5 times diluted pc-DNA3 stock containing β_3 cDNA (a gift from Dr. David Wilcox, Medical College of Wisconsin

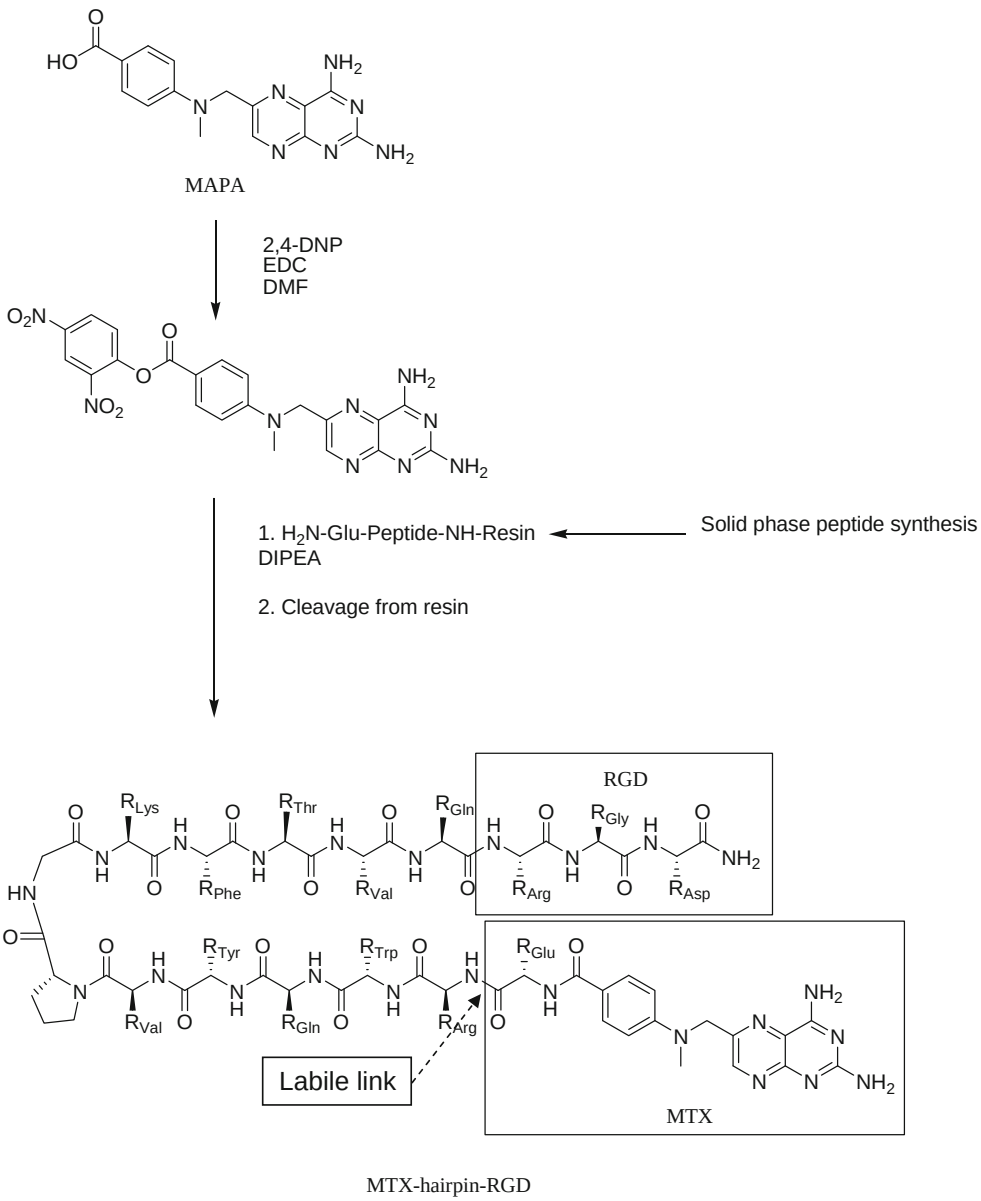

Fig. 1 Synthetic scheme for MTX-hairpin-RGD.

Cancer Center) was electroporated (Eppendorf, Electroporator 2510) into 40 μL of electrocompetent JM109 bacteria to produce the plasmid for further use. The β_3 cDNA was amplified from pc-DNA3 by the forward and reverse primers (I-F and I-R, Table I) with EcoRV and XhoI sites at 5′ and 3′ ends, respectively. The cDNA collected from pGEM-T-β_3 was then ligated between EcoRV and XhoI sites with T4 DNA ligase at the multiple cloning site of pCMV-Tag 2A with the FLAG tag sequence at the 5′ end of the insert. The resultant pCMV-Tag 2A-β_3 construct was used to amplify the FLAG-β_3 cDNA with BglII and XhoI sites on forward and reverse primers (II-F and 1-R, Table I). The FLAG-β_3 cDNA was harvested from pGEMT-FLAG-β_3 by restriction digestion with XhoI and BglII. The cDNA was then ligated to the linearized pShuttle between BglII and XhoI sites in the multiple cloning sites. The product (pShuttle-FLAG-β_3) was confirmed by EcoRV digestion (positions 1007 and 3404) after selecting the transformants from the electroporated JM109 cells grown on 0.03% Kanamycin plates. BJ5183-AD1 cells containing pAdEasy-1 vector were electroporated with linearized pShuttle-CMV-flag-β_3. The pAdEasy-FLAG-β_3 constructs were purified using large prep, and AD-293 cells were transfected with the purified constructs using Lipofectamine PLUS. After four days of incubation, the AD-293 cells were harvested and lysed. To overexpress β_3, 50 μL of the lysate was added to T-25 flask, and the expression of β_3 was confirmed by western blot after 48 h.

Downregulation of β_3 was achieved by treating the cells in T-25 flask with 16 μL of 10 μM siRNA specific for β_3 and lipofectamine® in Optimem medium (2 mL) for 5 h.

Table I Primers for PCR

Primer	Sequence	T_m (°C)
I – F	CG ATA TCA ATG CGA GCG CGG CCG CGG CC	96
I – R	CG CTC GAG TTA AGT GCC CCG GTA CGT GAT ATT GG	106
II – F	CG AGA TCT GCC ACC ATG GAT TAC AAG GAT GAC G	100

A Adenine; *C* Cytosine; *G* Guanine; *T* Thiamine; *F* Forward primer; *R* Reverse primer; T_m Melting temperature (approx.)

Then, the medium was replaced with complete growth medium, and the expression was examined by western blot after 48 hrs. The suppression of β_3 expression was determined by western blot analysis.

Image J (Version 1.44p, Wayne Rasband, NIH, USA) was used to quantify western blots. Image densities were corrected for background, and the relative density compared to the siRNA-treated sample was reported.

Binding Specificity of Prodrugs

Binding specificity of the prodrugs to $\alpha_V\beta_3$ integrins was demonstrated by endothelial adhesion assay as described previously (17) with modifications. Untreated 96-well plate wells were coated with 50 μL each of fibrinogen (FG, 1 mg/mL), bovine serum albumin (BSA, 3% w/v), MTX-RGD (10 μmolar) and MTX-hairpin-RGD (10 μmolar) dissolved in Hank's balanced buffer saline (HBSS) and incubated at 4°C overnight. Before the experiment, wells were briefly blocked by 0.5% w/v of BSA. Each well was incubated with 20,000 trypsinized cells expressing different levels of $\alpha_V\beta_3$ (overexpressed, wild or knockdown) in complete growth medium for 90 min with or without a pre-incubation step (1 h at 4°C) with RGD peptide. Non-adherent cells were washed with HBSS. Adhered cells were fixed in methanol for 30 min at 4°C and were stained with crystal violet for 15 min after HBSS washings. Absorbance was read at 565 nm after dissolving in 200 μL of 1% sodium dodecyl sulfate (SDS).

SGPE Enzyme Hydrolysis Studies

One mL of 100 μM MTX-hairpin-RGD was incubated with 8 mU of SGPE in presence or absence of $\alpha_V\beta_3$-expressing HUVEC in a 12-well culture plate. At predetermined time points, 25 μL of samples were drawn and immediately added to 100 μL of cold acetonitrile to inactivate enzyme. These samples were frozen until the analysis. MTX-RGD was used as control to estimate the hydrolysis rate. Samples were thawed, appropriately diluted and analyzed for MTX by HPLC (Thermo Separation Products, P4000 pump, AS3000 autosampler and UV2000 detector; SN4000 Spectra System; ChromQuest v.2.51) using Agilent (C-18, 4.6×250 mm) column at 305 nm. Initial hydrolysis rates of prodrugs were estimated by measuring the amount of MTX released at different time points and compared with those obtained in the growth medium without cells.

Plasma Stability Studies

Blood was collected into heparinized vacutainer (Becton Dickinson) from anesthetized rabbits, and plasma was collected by centrifugation. Prodrugs were incubated in plasma at 37°C for 24 h at 100 μM final concentrations. Amounts of released MTX at different time points were estimated by HPLC following a reported sample preparation method (16). Briefly, at predetermined time points, 25 μL of sample and 50 μL of chilled acetonitrile (ACN) were collected. The samples were centrifuged, and supernatant was added to chloroform (four times volume of supernatant). Aqueous supernatant was analyzed by HPLC for MTX. Stability was expressed as the amount of MTX released at different time points. Free MTX was also incubated as a control to observe the degradation of MTX in the plasma. The proteolytic enzyme activity of the plasma was confirmed using standard azo-casein method. MTX recovery for the extraction procedure was determined to be not less than 97%.

Molecular Modeling

Molecular Operating Environment (MOE-2006.08, Chemical Computing Group, Inc.) software was used for building molecules, energy minimization, peptide-integrin docking and dynamics. A twelve amino acid peptide with sequence of RWQYV^{D}PGKFTVQ was built. The anti-parallel β-hairpin conformation was induced by applying restrains for distances between alpha carbon atoms on opposite strands based on the NMR NOEs reported for this sequence in the literature (15). Arg-Gly-Asp (RGD-NH_2) and MTX were attached at the C and N terminus, respectively. Energy was minimized using AMBER99 forcefield without restrains for distances in aqueous environment to observe the propensity to form hairpin. Local energy minimum was determined by repeated *in silico* heating and cooling with concomitant search for low energy conformations followed by energy minimization of all molecules in the database. Binding simulation studies were carried out with integrin $\alpha_V\beta_3$ X-ray crystal structure (Protein Data Base file, 1L5G) using cyclic-RGD as template for bound RGD conformation. β-hairpin

peptide with MTX was coupled to bound RGD conformation for the simulation. Energy minimization was carried on selected atoms with the entire receptor and RGDs kept fixed. The preferred conformation upon ligand binding was determined by comparing the potential energy values in open and folded conformations in the bound state.

RESULTS AND DISCUSSION

Synthesis of Prodrugs

The addition of amino acid residues to the MTX-α-carboxylate has been shown to generate prodrugs that can be easily activated by selective enzymatic hydrolysis (4). Many synthetic schemes have been reported to selectively obtain MTX-α-amino acid derivatives in high yield (17, 18). Using the method reported by Coughlin *et al.* (19), α-amino acid-conjugated prodrugs were synthesized (Fig. 1). The MTX was synthesized *in situ* by conjugating Glu and DNPE. The Fmoc-based peptide synthesis method involved synthesis from C to N terminus. Accordingly, the synthesis was started with coupling α-COOH of Asp of the RGD onto the Rink amide resin. In every step of coupling, the free alpha-COOH was linked to deprotected NH_2 of the previously coupled amino acid. All other reactive groups, such as γ-COOH in the case of Glu, do not interfere in the synthesis since they are protected orthogonally. Once the coupling of RGD and 12 amino acids of the hairpin peptide completed, Glu was added where only the α-COOH is reacted with NH_2 of Arg of the hairpin peptide. Thus, when the pteroic acid portion was reacted with NH_2 of Glu, it formed MTX-α-hairpin-RGD. The MTX-RGD, without bearing a hairpin structure, was used as control for MTX-hairpin-RGD peptide. Characterization data of these synthesized MTX prodrugs are summarized in Table II.

Specific Binding of RGD to Integrin $\alpha_V\beta_3$

After screening the expression pattern for integrin α_V, β_3 and β_5 in several cell lines, HUVEC was found to be the most suitable for binding experiments, as the expression of integrins is prone to fewer fluctuations in normal cells. Also, HUVEC cell line has been widely used to test the RGD-specific recognition and binding (14, 20). Wild-type HUVEC expresses both $\alpha_V\beta_3$ and $\alpha_V\beta_5$. Cheresh *et al.* carried out experiments to modify the integrin $\alpha_V\beta_3$ expression by altering the α_V subunit. LM609, an antibody specific to integrin $\alpha_V\beta_3$, bound to M21 cells proportional to the receptor expression level (21). From the study using AP3 antibody directed to β subunit, it was found that the cells with altered level of α subunit also exhibited similar altered β expression. The 22C4 antibody, specific to uncomplexed β chain failed to bind to any of the cells when α subunit was knocked down. This suggested that β subunit cannot be expressed on the membrane alone without its complementary α subunit (22). These studies suggest that by altering one of the subunits of heterodimeric receptor, it is possible to control the membrane expression of a specific integrin dimer. Similarly, in our case, increase in β subunit expression is expected to translate into the overexpression of the whole integrin. While siRNA could almost completely knock down this subunit expression, the overexpression of β_3 was increased with increasing $Ad\beta_3$ viral volume to transfect the cells. The results of knockdown and overexpression of β_3 are shown in Fig. 2. The integrin β_3 expression was modulated to create three levels of integrin $\alpha_V\beta_3$ expression (knockdown, wild-type, and overexpressed) for testing the responses of RGD-specific binding on the amount of expressed integrin $\alpha_V\beta_3$. As shown in Fig. 3, at any given expression level, it is evident that significantly higher (*t*-test $P<0.05$) numbers of cells expressing $\alpha_V\beta_3$ bound to RGD-containing peptides, such as fibrinogen, MTX-RGD, and MTX-hairpin-RGD compared to a non-specific substrate, BSA. Within each substrate group (coating), the number of bound cells decreased significantly (ANOVA $P<0.05$) with decreased expression of $\alpha_V\beta_3$. Within each level of expression, there was no binding difference between prodrugs. Apart from RGD, other portions and higher order of conformations of fibrinogen are attributed to higher cell binding compared to RGD prodrugs. The specificity of RGD binding dependence was demonstrated by preincubating wild-type HUVEC with RGD peptide prior to binding to prodrugs.

Table II Synthesis of Methotrexate Prodrugs

Molecule Sequence	Yield (%)	$[M+H]^{+a}$	Purity (%)
MTX-RGD	47	782.6	96
MTX-RWQYV-^{D}P-GKFTVQ-RGD	31	2273.3	93

D Asp; *E* Glu; *F* Phe; *G* Gly; *K* Lys; ^{D}*P d*-Pro; *Q* Gln; *R* Arg; *T* Thr; *V* Val; *W* Trp; *Y* Tyr

[a] MALDI-TOF molecular ion peak

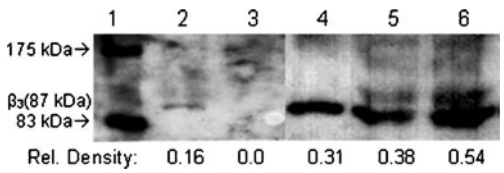

Fig. 2 Modulation of integrin β_3 in HUVEC. 1, Molecular weight standards; 2, HUVEC wild; 3, SiRNA treated; 4, 20 μL $Ad\beta_3$; 5, 40 μL $Ad\beta_3$; 6, 80 μL $Ad\beta_3$.

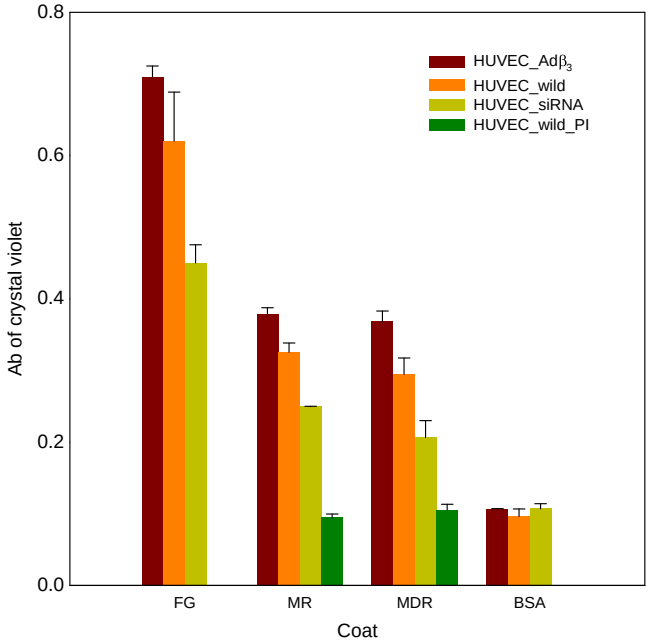

Fig. 3 Cell adhesion assay with HUVEC. Y-axis represents the absorbance of crystal violet proportional to the number of cells bound. The bars represent means ($n=6$), and error bars represent standard deviation. Ab = Absorbance; HUVEC_Adβ_3 = HUVEC transfected with Adβ_3 virus; HUVEC_siRNA = HUVEC treated with siRNA; HUVEC_wild = HUVEC wild type; HUVEC_wild_PI = HUVEC wild type preincubated with RGD containing peptides.

The cell binding to prodrug-coated surface abolished completely for pre-incubated cells. Therefore, it can be concluded that the prodrugs can specifically recognize and bind to $\alpha_V\beta_3$-expressing cells in an RGD-dependent manner.

The prodrug was designed to exist in blood circulation with labile linkage between MTX and β-hairpin (Bond between MTX's Glu and hairpin's Arg) protected by steric hindrance, and the labile linkage will be exposed after conformation change upon binding to integrin $\alpha_V\beta_3$. These two conformations of prodrug were studied experimentally and supported by the molecular modeling results.

The β-Hairpin-Based Steric Hindrance to Labile Link

The MTX-α amino acid prodrugs reported in literature utilized CPA as the specific enzyme for activation, since it can cleave the terminal amino acid with a free α-carboxylic acid group (COOH) (4). Prodrugs of the current study differ from MTX- α amino acid prodrugs in two ways and hence cannot use CPA enzyme to activate the prodrug. First, MTX is attached to a β-hairpin-RGD peptide consisting of 15 amino acids instead of a single amino acid. Second, the bond between MTX and Arg of hairpin peptide does not have a free α-carboxylic group. Therefore, *Streptomyces griseus*-derived glutamic acid-specific endopeptidase (SGPE) capable of preferentially cleaving the bond between Arg (R) and Glu (E) (23) was used. It is evident from the structure of prodrugs that both (MTX-RGD and MTX-hairpin-RGD) of them have MTX (with a Glu) attached to an Arg moiety and hence are cleavable by SGPE. The protection of labile linkage between MTX and β-hairpin was demonstrated by measuring the initial MTX hydrolysis rates in presence of SGPE. In the absence of integrin binding (absence of HUVEC cells), the rate of MTX release was significantly ($P<0.05$) slower from MTX-hairpin-RGD (0.7 ± 0.1 ng/hr) than from MTX-RGD (1.0 ± 0.1 ng/hr) (Fig. 4). The bare hairpin structure was shown not to self-associate at 1.6 mM concentration (15). Therefore, self-association can be ruled out as a possible reason for slower hydrolysis rate in the present study, since all experiments were conducted at 15 times diluted concentrations compared to the concentration-reported result in association. The prodrug can also protect the labile linkage from non-specific enzymes usually present in plasma. The appearance rate of free MTX from different prodrugs in plasma was used as an indicator for the stability of prodrug in plasma. The rate of MTX appearance was significantly ($P<0.05$ at each time point and for the slope) lower for MTX-hairpin-RGD than that of MTX-RGD (Fig. 5). The higher stability or resistance to non-specific hydrolysis of MTX-hairpin-RGD can be attributed to several reasons. The masking effect of hairpin conformation plays an important role in preventing the labile linkage from endopeptidase hydrolysis. Though it is challenging to avoid endopeptidase-mediated hydrolysis, literature evidence showed that creating a steric hindrance to mask the labile linkage can improve the stability. Rozek and co-workers designed an anti-microbial cyclic peptide by joining two ends with a disulfide bond to stabilize lipid-bound structure as well as improve the stability of peptides in presence of protease. The stability of the cyclic peptide was improved 4 to 5 times in presence of trypsin. From structural analysis of cyclic peptide it was revealed that the lysine and tryptophan

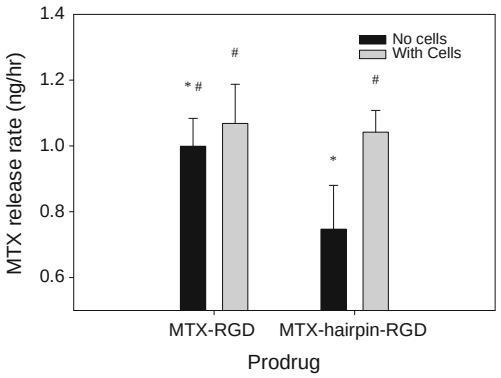

Fig. 4 SGPE mediated specific hydrolysis of prodrugs. The MTX (in ng) initial release rate was determined and compared in presence and absence of cells. Statistics: *: *t*-test $P<0.05$; #: ANOVA, No significant difference.

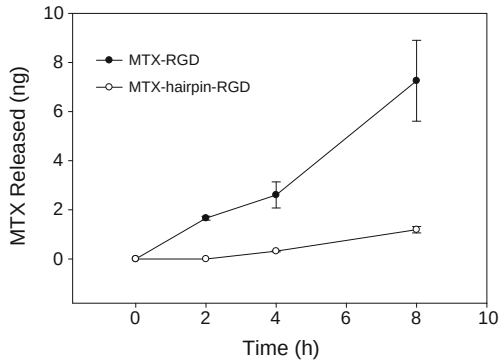

Fig. 5 MTX release in plasma.

sidechains are compactly packed and might contribute to the higher protease resistance (24). A simple way to avoid hydrolysis by exopeptidases such as carboxypeptidases and aminopeptidases is to block the C and N termini. Making the C-terminus an amide is one of such methods to block the C-terminus (25). After MTX-hairpin-RGD was cleaved from Rink amide resin, MTX-hairpin-RGD was left with an amide at its C-terminus. On the N-terminus, Glu and pteroic acid were attached to form MTX. These structures in MTX-hairpin-RGD contribute to the exopeptidase hydrolysis masking. Furthermore, the introduction of an un-natural amino acid isomer, D-Pro, in the sequence was also playing a role in stabilizing the prodrug. It has been shown that substitution of L-amino acids with their D-isomers improved the plasma stability of Cetrorelix, a decapeptide (25). Radiolabelled compounds conjugated to RGD peptides were shown to accumulate near the tumor within 2 h of IV administration (12, 26, 27). Hence, it is desirable for RGD-conjugated prodrugs to be stable for at least 2 h so that they can reach the target tumor. Both MTX-hairpin-RGD and MTX-RGD retained at least 80% prodrug form in plasma for 2 h; however, MTX-hairpin-RGD had significantly higher stability (Fig. 5). This suggested that the introduction of hairpin peptide-based stearic hindrance in the prodrug was beneficial in improving the plasma stability Non-specific plasma enzyme hydrolysis of unfolded (straight chain) conformer that exists in equilibrium with hairpin conformer when prodrug is not bound to cells may cause the release of MTX from MTX-hairpinm-RGD. Compared to similar targeting strategies, this approach can curb the non-specific hydrolysis to a greater extent, leading to reduced availability of drug non-specifically.

Binding-Induced Conformation Change

In addition to imparting masking capability (stability in plasma) to the prodrug, the hairpin structure was also introduced with an intent to expose the labile link (bond between MTX's Glu and hairpin's Arg) by an integrin binding-induced conformational change. The increase in enzymatic hydrolysis and MTX release in such scenario was demonstrated by incubation of MTX-hairpin-RGD peptide and MTX-RGD with SGPE in presence of $\alpha_V\beta_3$-expressing HUVEC as shown in Fig. 4. Initial MTX release rates suggested that the hairpin peptide did not pose any steric hindrance when bound to $\alpha_V\beta_3$, and the release rate (1.0 ± 0.1 ng/hr) was not significantly different from the MTX-RGD (1.1 ± 0.1 ng/hr) that does not exhibit any hairpin-based steric hindrance (Fig. 4). Compared to the MTX release rate from MTX-hairpin-RGD in the absence of cells (0.7 ± 0.1 ng/hr), there was 40% increase in the release rate in presence of cells. This result is comparable to previously reported model system (28). These results support exposure of labile link due to unfolding upon binding of RGD to integrin $\alpha_V\beta_3$. Previously, it has been shown that equilibrium is established between the hairpin (folded) and straight chain (unfolded) conformations of hairpin structure peptide (15). In presence of $\alpha_V\beta_3$ integrin, straight chain conformation preferentially binds with integrin $\alpha_V\beta_3$, which triggers the equilibrium shift towards the unfolded species leading to more folded forms to unfold. A mechanism for inducing drug release from MTX-hairpin-RGD via this route is proposed in Fig. 6 (Mechanism A). It is also possible that binding induces unfolding of MTX-hairpin-RGD to expose the labile linkage and resulted in enzymatic hydrolysis; therefore, an alternative mechanism was also proposed (Fig. 6, Mechanism B). Binding of folded form brings the RGD end of MTX-hairpin-RGD very close to the receptor, which may result in an unfavorable conformation that leads to unfolding of the hairpin. It may also be possible that these two mechanisms operate simultaneously. The preferred mechanism is not clear, and further studies will be needed to understand the exact mechanism. Nevertheless, the experiment results support the design concept of binding-triggered drug release for integrin-targeted delivery.

Since the drug release occurs upon binding to integrin $\alpha_V\beta_3$-overexpressing cells, drug will be released in the vicinity of the target cells. MTX is a folate mimic and is known to be taken up by cells via active (folate receptor) and passive transport mechanisms. Preferential binding of MTX prodrugs followed by a specific enzyme mediate hydrolysis results in accumulation of MTX in the cells overexpressing the integrin $\alpha_V\beta_3$. Apart from achieving targeted delivery of anti-cancer drug, due to small size, hairpin peptide is unlikely to elicit immunological reactions. Furthermore, unlike in ADEPT (antibody directed enzyme prodrug therapy), the current strategy does not utilize any antibody or anti-antibodies (clearing agents) to either target the enzyme to tumor or clear the residual enzyme in the blood. This may lead to significant reduction of immunological complications associated with ADEPT.

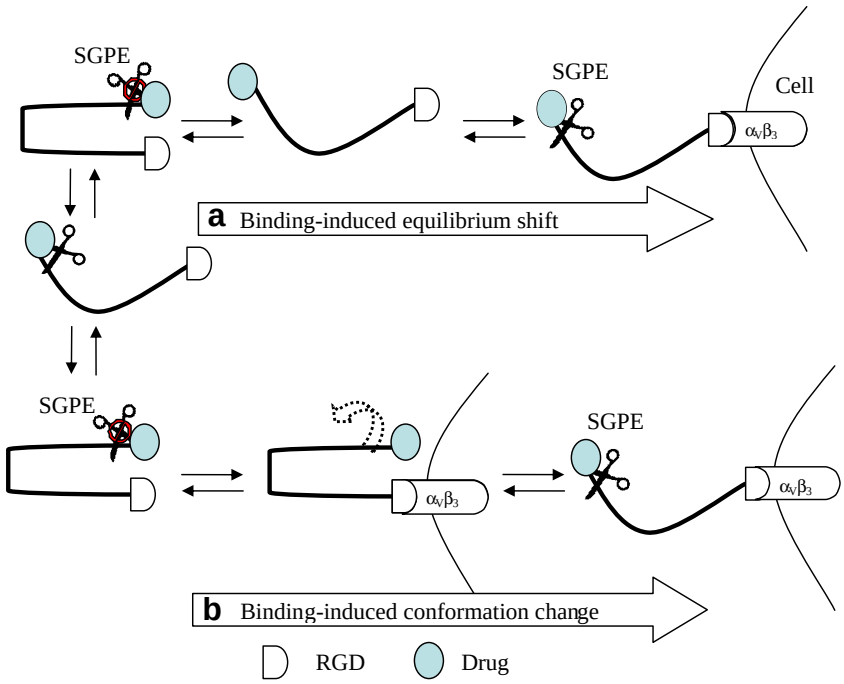

Fig. 6 Proposed mechanism of binding induced unfolding and exposure of labile link. (**a**) Equilibrium shift towards the unfolded species is triggered by preferential binding of unfolded structure. (**b**) Hairpin unfolds due to high free energy of bound hairpin conformation. RGD = Arg-Gly-Asp; SGPE = *Streptomyces griseus*-derived Glu specific endopeptidase.

Molecular Modeling Studies

The molecular modeling was used as secondary supporting evidence to show the propensity of hairpin formation and the binding-induced unfolding of MTX-hairpin-RGD. The 12-amino acid peptide was built using Arg-Trp-Gln-Tyr-Val-^{D}Pro-Gly-Lys-Phe-Thr-Val-Gln (referred as "peptide" from now on) sequence using MOE to analyze the energetic state for preferentially forming a hairpin conformation in aqueous medium after conjugating MTX and RGD. A 6Å distance restraint was applied between the alpha C atoms of Trp-Val and Tyr-Phe to bring the two strands sufficiently close to form a hairpin conformation. The energy was minimized using MOE after placing the molecule in water using "WATER SOAK" function without distance restraints to verify its propensity to remain in a hairpin conformation. Previous studies suggested that the hairpin conformation is facilitated by hydrophobic side chain interactions and hydrogen bonding between the strands (15). These studies suggested that the entropic penalty of more compact structure is compensated by hydrophobic core formation comprising side chains away from the external aqueous environment. The NOEs observed in previous proton NMR studies of the core peptide in aqueous environment support the distance restraints between these amino acids (15). In aqueous environment, the energy of MTX-hairpin-RGD in hairpin conformation was 31 kcal/mol less than its straight chain counterpart (Table III and Fig. 7). Due to the strong beta turn propensity of ^{D}Pro-Gly sequence, the straight chain conformer also showed a U-shaped structure. However, larger distance between two strands, absence of both H-bonds and alternatively arranged 10 and 14 member rings suggest that it is not a β-hairpin conformation. Therefore, this conformation was referred to as "straight chain" conformer in the subsequent paragraphs. In contrast, the hairpin conformer (Fig. 7b) showed H-bonds forming typical 10-and 14-member rings. The predicted lower energy of hairpin structure in aqueous environment compared to its straight chain conformation suggested that this peptide preferentially adopts a hairpin structure in aqueous environment.

Table III Potential Energy Values of Different Conformers

Molecule	Energy (kcal/mol)[a]
MTX-hairpin-RGD straight chain conformer in water	−4606
MTX-hairpin-RGD hairpin conformer in water	−4637
Integrin $\alpha_V\beta_3$ bound RGD (from Protein data bank file, IL5G)	20872
Integrin $\alpha_V\beta_3$ bound MTX-hairpin-RGD	5×10^6
Integrin $\alpha_V\beta_3$ bound MTX-straight chain-RGD	21104

MTX Methotrexate; *RGD* Arg-Gly-Asp

[a] Local minimum

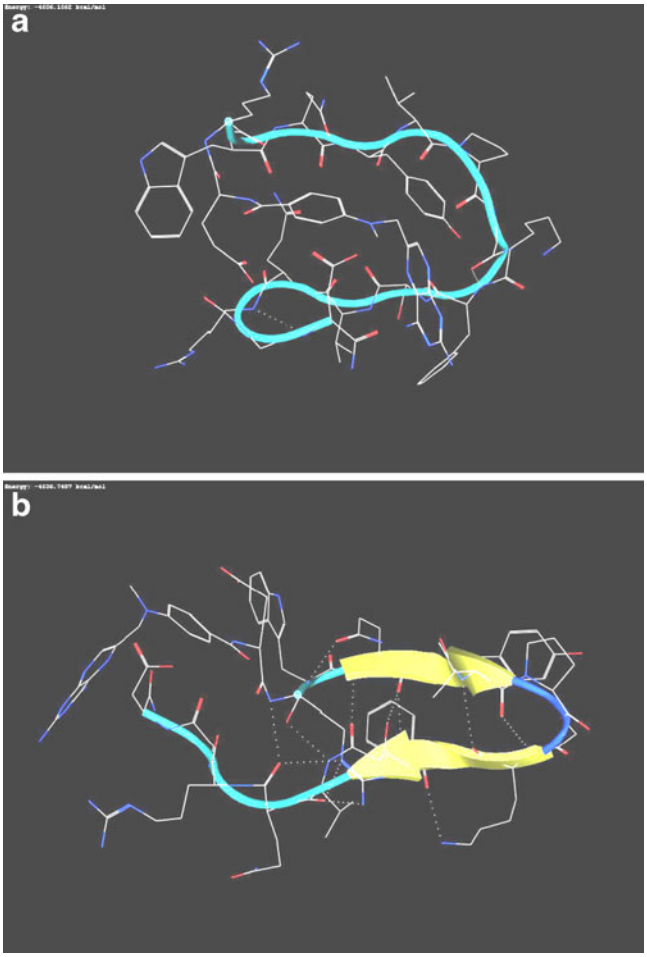

Fig. 7 Minimum energy conformations of MTX-peptide-RGD in aqueous environment. (**a**) No restraints were applied for distances before energy minimization, and it represents straight chain conformer (non-hairpin structure). (**b**) Restraints were applied for distances between two strands to bring them close, and energy was minimized without any restrains. This represents anti-parallel beta hairpin conformation (Note: Rings formed by H-bonds are characteristic for an anti-parallel beta hairpin.)

Data for integrin $\alpha_V\beta_3$ receptor crystal structure bound with cyclic RGD are available from protein databank. Cyclic RGD chemical structure was modified to obtain RGD by deleting the linking molecule between R and D amino acids without disturbing the conformation. This resulted in RGD tripeptide in its bound conformation to $\alpha_V\beta_3$ receptor. The α-COOH of Asp is not essential for integrin binding. In cyclic RGD the alpha COOH of Asp is involved in cyclization and thus not free. Additionally, guanidine group of Arg and β-COOH of Asp, essential for prominent interaction, are intact in RGD. Therefore, amide modification may not adversely affect the binding specificity of RGD ligand, and it can be safely assumed that both cyclic RGD and RGD-NH_2 would bind to integrin receptor in a similar fashion though with different affinities. The free energy of RGD bound to $\alpha_V\beta_3$ receptor was determined by MOE without any further modification and was used as reference for the minimum possible energy. The MTX-peptide was coupled to the bound N terminus of RGD either in its hairpin or straight chain conformation. The energy of these two structures was minimized while

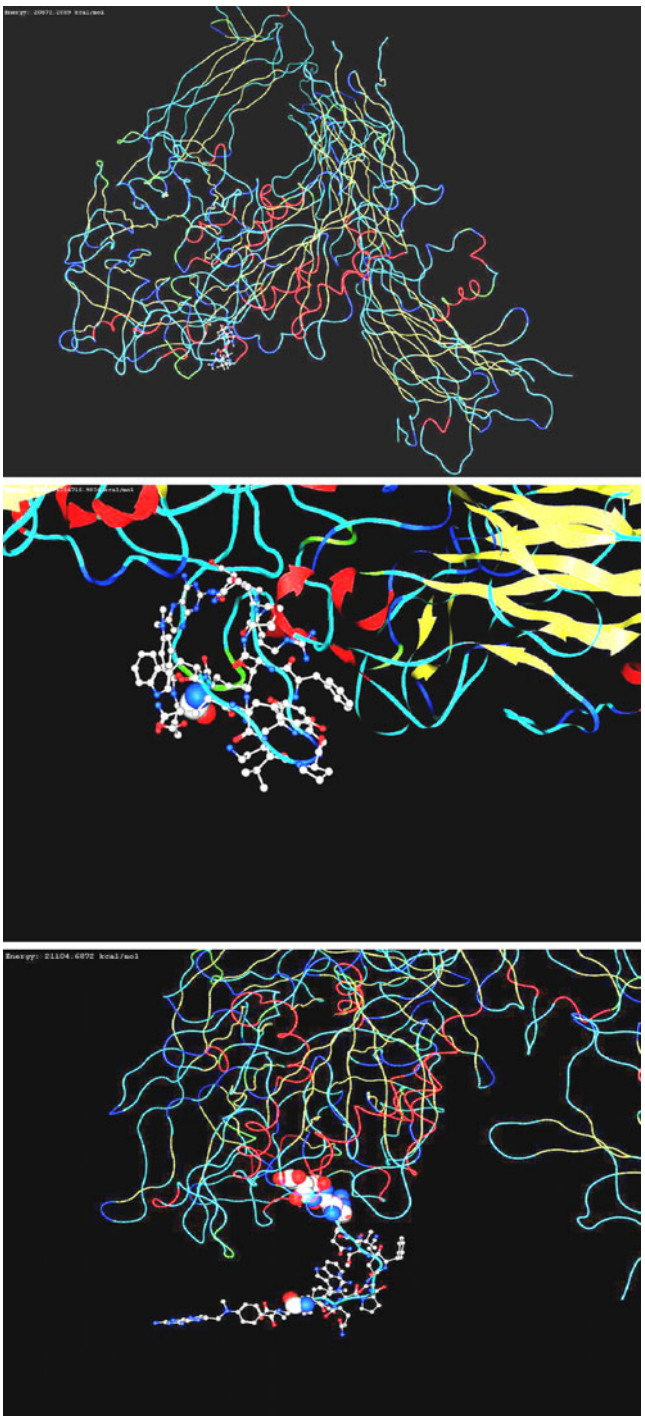

Fig. 8 Molecular modeling of binding induced unfolding. Top: Integrin $\alpha_V\beta_3$ bound RGD; Middle: Integrin $\alpha_V\beta_3$ bound hairpin conformer (folded); Bottom: Integrin $\alpha_V\beta_3$ bound straight chain conformer.

keeping the RGD and $\alpha_V\beta_3$ receptor fixed. Keeping the RGD and $\alpha_V\beta_3$ receptor unchanged (fixed) allowed the distal end (MTX end) of the peptide to adopt minimum energy conformation in bound state. The straight chain conformation could re-arrange itself and reach an energy value close to the reference, whereas hairpin structure coupled to bound RGD had higher energy compared to straight chain conformer after minimization. Probably, hairpin conformation could not rearrange itself to straight chain conformation, as it was unable to overcome the energy barrier of its local minimum under the MOE experimental conditions (Table III, Fig. 8). In reality, the same energy barrier may prevent it from assuming hairpin conformation in bound state. These results suggest that the hairpin peptide prefers to be in straight chain conformation (unfolded) when it is bound to integrin $\alpha_V\beta_3$ receptor, since staying close to the receptor in its hairpin form was not energetically favorable. Therefore, hairpin structure adopts straight chain conformation, which leads to disruption of hairpin conformation. The molecular modeling results support the experimental observations and are consistent with design concept to create a binding-triggered targeted delivery of anticancer agent to integrin $\alpha_V\beta_3$ overexpressed cells.

In summary, methotrexate prodrugs conjugated to bare RGD or RGD with a hairpin peptide were synthesized, and these prodrugs recognize and bind to integrin $\alpha_V\beta_3$-expressing cells. The MTX-hairpin-RGD prodrug can resist non-specific enzymatic hydrolysis in the plasma as well as specific hydrolysis mediated by SGPE when compared with MTX-RGD prodrug. Enzymatic hydrolysis studies and molecular modeling supported that the MTX-hairpin-RGD prodrug prefers to assume a straight chain conformation when bound to integrin, leading to the SGPE hydrolysis-mediated drug release. These results suggested that the design of an integrin binding-triggered conformation change is feasible and may pave the way to a new group of stimuli-sensitive drug-release-based carrier systems.

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