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A study of the anti-invasive properties of *N*-α-phthalimidomethyl-ketomethylene tripeptide-based metalloprotease inhibitors

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Abstract

We have developed matrix metalloprotease (MMP) inhibitors based on synthetic peptides incorporating a non-cleavable peptide-bond isostere at the site of the putative scissile bond. These inhibitors, *N*-α-phthaloyl-Gly-Ψ(CO-CH_2)-Leu-Tyr-Ala-NH_2 (Pht-G-CH_2-LYA-NH_2) and *N*-α-phthaloyl-Gly-Ψ(CO-CH_2)-Leu-Tic-Ala-NH_2 (Pht-G-CH_2-LTcA-NH_2) were kinetically evaluated against the type IV collagenases, gelatinase A (MMP-2) and B (MMP-9), and compared with an exactly analogous chelating-based inhibitor, *N*-α-mercaptoacetyl-Leu-Tyr-Ala-NH_2 ($HSCH_2CO$-LYA-NH_2). The peptide inhibitors were also tested for their anti-invasive effects on breast carcinoma cell lines using a modification of the Boyden chamber assay. Gelatin zymography was utilized to identify gelatinolytic activities present in media removed from cultured breast cancer cells. Of the two *N*-α-phthalimidomethyl-ketomethylene peptide-based inhibitors, Pht-G-CH_2-LYA-NH_2 proved the more effective inhibitor of MMP-2 and MMP-9 (K_i 34.27 and 45.75 μM, respectively). However, when tested against two breast cancer cell lines, T47D and MDA-MB-231, both inhibitors were able to effectively reduce tumour cell invasion through a type IV collagen matrix by up to 91.2 %. Of particular interest was the observation that Pht-G-CH_2-LYA-NH_2 was the most potent inhibitor of invasion by the highly aggressive MDA-MB-231 cells, despite the cells' relative lack of active secreted metalloprotease activity. The results obtained from this kinetic and anti-invasive analysis of the new inhibitors suggest that compounds incorporating the *N*-α-phthalimidomethyl-ketomethylene peptide-bond isostere may have potential for development as new agents with anti-metastatic properties.

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Introduction

Cell invasion and metastasis are critical targets for the therapeutic treatment of malignant carcinomas. One essential mechanism involved in these pathological processes is the degradation of basement membranes and stromal tissues by tumour-cell-associated proteases. In particular, the production of matrix metalloproteases (MMPs) has long been associated with the development of the malignant phenotype (Azzam et al 1993).

During tumour-cell invasion, the basement membrane and interstitial stromal tissue stand as barriers through which an invading tumour cell must pass. The major structural component of the basement membrane is type IV collagen, while the stroma consists of interstitial collagens, fibronectin and proteoglycans (Lochter

Table 1 MMP families and their physiological actions.

MMP family	Examples	Actions
Collagenases	Interstitial collagenase (MMP-1) Neutrophil collagenase (MMP-8) Collagenase-3 (MMP-13)	Degradation of fibrillar collagens
Gelatinases	Gelatinase A (MMP-2) Gelatinase B (MMP-9)	Degradation of type IV collagen and non-helical proteins such as fibronectin and laminin
Stromelysins	Stromelysin-1 (MMP-3) Stromelysin-2 (MMP-10) Stromelysin-3 (MMP-13)	Degradation of proteoglycans, fibronectin and laminin
Membrane-type (MT-MMPs)	MT-MMP-1 (MMP-14) MT-MMP-2 (MMP-15) MT-MMP-3 (MMP-16) MT-MMP-4 (MMP-17)	Activation of other MMPs, including latent MMP-2, degradation of collagens

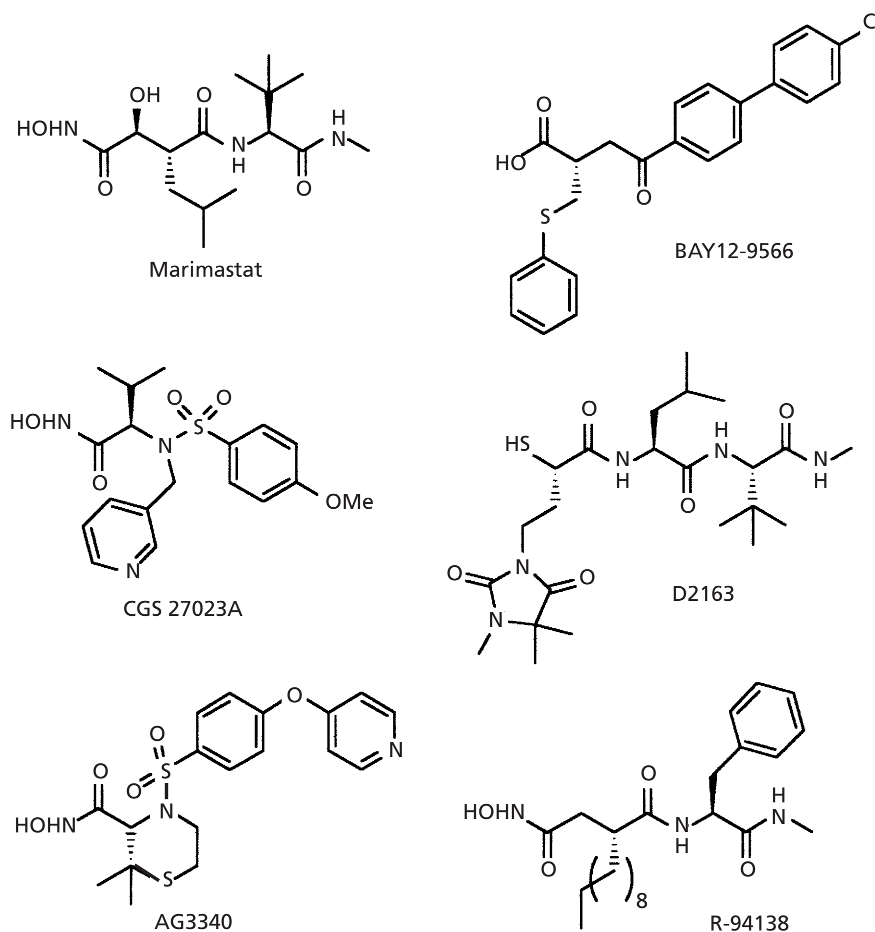

Figure 1 Matrix metalloprotease inhibitors currently in clinical oncology trials (reviewed by Whittaker et al 1999).

& Bissell 1995). MMPs can selectively cleave all these structural proteins, facilitating the progression of tumour cells into the surrounding tissue (Woessner 1991). Additionally, MMPs have been shown to play a critical role in tumour-associated neovascularization (angiogenesis) (Moses 1997), an essential process in the

establishment and spread of solid tumours, enabling endothelial cells to migrate from pre-existing vasculature. As a consequence, substantial research efforts have been aimed at developing new synthetic inhibitors of these enzymes as potential therapeutic agents.

Approximately 20 different MMPs, which can be broadly classified into four main groups, have now been identified in mammalian tissues (Table 1). In practice, a considerable degree of homology exists between such MMP species and other MMPs, such as matrilysin (MMP-7), macrophage metalloelastase (MMP-12) and the recently discovered ADAM (A Disintegrin And Metalloprotease) family of MMP-like enzymes, responsible for the shedding of important cell-surface proteins (Stone et al 1999). The design and development of inhibitors with high selectivity for individual enzyme sub-species has thus been extremely difficult. Despite this problem, a number of synthetic MMP inhibitors are currently in clinical trials for the treatment of cancer (Figure 1).

While some recent reports have described the use of pharmacologically active natural products, such as tetracyclines (Bols et al 1992), betulinic acid (Sun et al 1996) and nicotianamine (Suzuki et al 1996) as moderately effective MMP inhibitors, the principal drug design strategy still remains the development of compounds incorporating a metal-chelating moiety into a synthetic substrate or non-substrate-based targeting group. Such chelating groups bind to the zinc atom present in the active site of all MMPs thereby preventing substrate hydrolysis. Although a number of chelating groups have been reported in the literature (reviewed by Whittaker et al 1999), physicochemical studies by Castelhano's group (Browner et al 1995) have shown that the hydroxamic acid moiety is the optimal zinc-binding group, a fact reflected in the large number of synthetic inhibitors incorporating this particular moiety (Hodgson 1995; Wahl et al 1995). Unfortunately, hydroxamates have a number of pharmacokinetic deficiencies, including poor metabolic stability and bioavailability. For example, the hydroxyl group of these inhibitors is rapidly glucuronidated and may also be reduced and ultimately metabolized to a free acid (Hodgson 1995). Consequently, a number of groups, including ourselves, have attempted to utilize other chelating groups such as the carboxylates (Chapman et al 1993) and thiols (Celltech-Chiroscience) (Baxter et al 1997; Lynas et al 2000).

An alternative strategy in MMP-inhibitor development is the utilization of peptidomimetic compounds incorporating a non-cleavable peptide-bond isostere spanning the putative cleavage site. Previously, we reported the synthesis and kinetic characterization of extended peptide-based compounds bearing a ketomethylene amino isostere as inhibitors of collagenase (Wallace et al 1986). We now wish to describe the anti-invasive properties of two tripeptide-based compounds, incorporating an *N*-α-phthalimidomethyl-ketomethylene grouping, in comparison with an exactly analogous thiol-based inhibitor. The results obtained illustrate that these compounds may provide a useful lead for the development of new compounds with anti-metastatic properties.

Materials and Methods

Synthetic procedures

N-α-(9-Fluorenylmethoxycarbonyl)-1, 2, 4, 6-tetrahydroisoquinoline carboxylic acid (Fmoc-Tic-OH) was obtained from Bachem Ltd (Bubendorf, Switzerland). *N*-Phthaloylglycine was from Senn Chemicals (Basle, Switzerland). All other peptide synthesis reagents were purchased from NovaBiochem Ltd (Nottingham, UK).

The synthetic scheme for solid-phase synthesis of the compounds is outlined in Figure 2. Briefly, -Leu-Tyr-Ala-NH_2 and -Leu-Tic-Ala-NH_2 peptide-targeting groups were synthesized, on Rink Amide AM polystyrene resin (100–200 mesh) (Novabiochem Ltd, Nottingham, UK) using solid-phase synthetic procedures employing standard Fmoc- and acid-labile orthogonal protecting strategies (reviewed by Walker 1995) with (1*H*-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole/*N*, *N*-diisopropylethylamine (HOBt/HBTU/DIPEA) coupling protocols. Removal of the Fmoc-protecting group was afforded in each instance with 20% (v/v) piperidine in dimethylformamide (DMF). Tyrosine was incorporated as the Fmoc-Tyr(OBut)-OH derivative while tetrahydroisoquinoline carboxylic acid was incorporated as Fmoc-Tic-OH. Mercaptoacetic acid was incorporated via the *S*-acetyl-*O*-pentafluorophenyl ester derivative, in the presence of triethylamine (TEA) and a catalytic amount (0.1 equivalents) of HOBt.

N-α-Phthalimidomethyl-diazomethyl ketone (Pht-Gly-CHN_2) was prepared via the acylation of an unsymmetrical anhydride, formed by reaction of *N*-α-phthalimido acetic acid with isobutyl chloroformate, using excess ethereal diazomethane (Ye & McKervey 1992). *N*-α-Phthalimidomethyl-bromomethyl ketone (Pht-Gly-CH_2Br) was generated by a 5-min treatment of Pht-Gly-CH_2Br with 30% (v/v) HBr/AcOH. Deprotected tripeptide resin was alkylated with Pht-Gly-CHN_2 in the presence of TEA for 24 h.

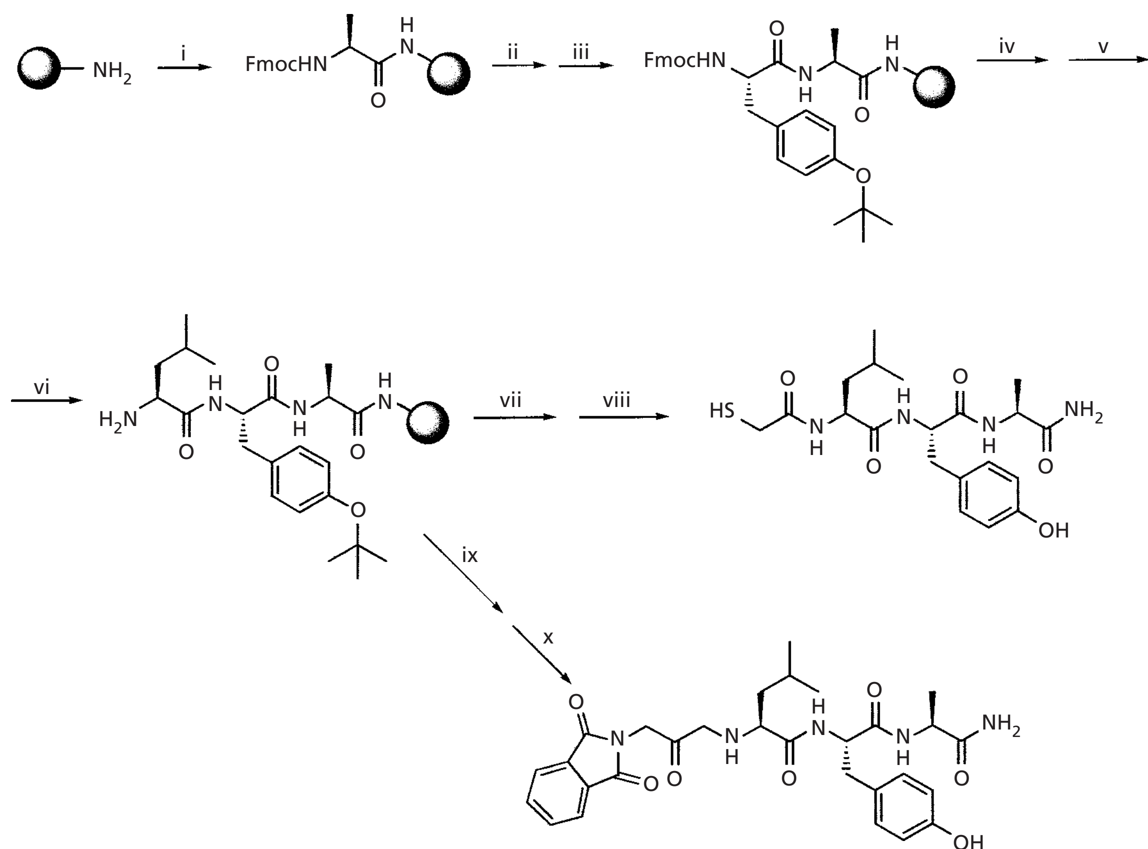

Figure 2 Divergent solid-phase synthesis of N-α-phthalimidomethyl-ketomethylene and N-α-mercaptoacetyl tripeptides. i, Fmoc-Ala-OH (3 eq.)/HBTU (3 eq.)/HOBt (3 eq.), 1 h. ii, 20% (v/v) piperidine/DMF, 45 min. iii, Fmoc-Tyr(OBut)-OH (3 eq.)/HBTU (3 eq.)/HOBt (3 eq.)/DIPEA (6 eq.), 1 h. iv, repeat ii. v, Fmoc-Leu-OH (3 eq.)/HBTU (3 eq.)/HOBt (3 eq.)/DIPEA (6 eq.), 1 h. vi, repeat II. vii, Ax-SAMA-OPfp (3 eq.)/TEA (3 eq.)/HOBt (0.3 eq.), 3 h, then 10% (v/v) MeOH/NH_3 in DMF, 2 h. viii, 95% TFA/anisole. ix, Phthaloyl-Gly-bromomethylketone (5 eq.)/DIPEA (5 eq.), 24 h. x, 95% (v/v) TFA/H_2O.

After the completion of solid-supported synthesis, cleavage of products was afforded by treatment with 95:2.5:2.5 (v/v/v) trifluoroacetic acid–thioanisole–H_2O. Purity and identity of each compound was established by electrospray ionization mass spectrometry (ESI-MS), elemental and HPLC analysis.

The N-α-mercaptoacetyl-based inhibitor ($HSCH_2CO$-LYA-NH_2) was synthesized, on a solid support, as previously described (Lynas et al 2000) with the exception that S-acetylthioglycolic acid was introduced as an O-pentafluorophenyl ester derivative. Purity and identity were established as described above.

Kinetic evaluation of inhibitors

Recombinant human gelatinase A (MMP-2) and gelatinase B (MMP-9) were purchased from Roche Diagnostics Ltd. (Lewes, East Sussex, UK). 4-Aminophenyl mercuric acetate (APMA) was obtained from Sigma-Aldrich (St Louis, MO). The internally quenched substrate, N-α-(D, L)-2-amino-3-(7-methoxy-4-coumaryl) propionyl-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH_2 (where Dnp = 2, 4-dinitrophenyl) (Amp-PLGLK-(Dnp)AR-NH_2) was synthesised by Dr Patrick Harriott (School of Biology and Biochemistry, The Queen's University of Belfast). Spectrofluorimetric studies were performed at 25°C on a Perkin-Elmer MPF-44B spectrofluorimeter set to an excitation wavelength of 328 nm and an emission wavelength of 392 nm.

MMP-2 and MMP-9 (1 μg) were each activated by incubation with 1 mM APMA in 50 mM Tris-HCl buffer, pH 7.6, containing 150 mM NaCl, 5mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.01 % (w/v) Brij-35 (assay buffer), for 45 min and 2 h, respectively, at 37°C. Activated MMP-2 (2.5 μL; 0.1 μg) or MMP-9 (5 μL; 0.1 μg) and Amp-PLGLK(Dnp)AR-NH_2 (5 μL of a 1 mM stock in DMF) were added to assay buffer to give a final volume of 500 μL. The inhibitors, Pht-G-CH_2-LYA-NH_2, Pht-G-

CH_2-LTcA-NH_2 and $HSCH_2CO$-LYA-NH_2 were included in the assay at concentrations of 50–250 μM.

Determination of K_i

The inhibitor constant (K_i) for each inhibitor was calculated using the method of Walker (1982). If the rate of hydrolysis progressively decreases in the presence of increasing concentrations of inhibitor, it can be assumed that the inhibition is competitive, provided the maximum velocity (V_{max}) of the substrate is also found to be unaltered at different substrate concentrations, in the presence of the inhibitor. The K_i can therefore be calculated from equation 1.

$$\frac{V_s - V_i}{V_i} = \frac{K_m . [I]}{K_i . (K_m + [S])} \tag{1}$$

where, V_s = rate of hydrolysis of substrate in the absence of inhibitor, V_i = rate of hydrolysis of substrate in the presence of inhibitor concentration [I], [S] = substrate concentration and K_m = Michaelis constant for the hydrolysis of the substrate.

The above equation can be derived from the respective Michaelis-Menten equations (equations 2 and 3) for the hydrolysis of a substrate in the absence and presence of an inhibitor.

$$V_s = \frac{V_s . [S]}{K_m + [S]} \tag{2}$$

$$V_i = \frac{V \cdot [S]}{K_m . (1 + [1]/K_i) + [S])} \tag{3}$$

Now, from equation 1, it follows that a plot of $(V_s - V_i)/V_i$ against [I] will yield a straight line of gradient $K_m/K_i.(K_m + [S])$. Having determined K_m in a separate experiment (16.11 ± 1.33 μM for MMP-2; 17.32 ± 4.6 μM for MMP-9), it is then possible to evaluate K_i from the gradient.

Cell lines and cell culture

The breast cancer cell lines T47D and MDA-MB-231 were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK).

Cells from the oestrogen-dependent cell line, T47D, were maintained in Dulbecco's Modified Eagles Medium (DMEM; ICN Biomedicals; High Wycombe, UK) supplemented with 20 mM HEPES (Sigma-Aldrich), 0.38% (w/v) $NaHCO_3$ and 10% (v/v) foetal calf serum (FCS; PAA Laboratories, Durham, UK). Cells from the oestrogen-independent cell line, MDA-MB-231, were maintained in Liebowitz-15 medium (L-15; ICN Biomedicals) supplemented with 10% (v/v) FCS. All media for routine culture were supplemented with penicillin (1.0 IU mL^{-1}) and streptomycin (1.0 μg mL^{-1}) (GIBCO Ltd, Paisley, UK). Cells were routinely grown, in a CO_2 incubator, in 75-cm^2 tissue-culture flasks (Falcon, Becton Dickinson Ltd, Oxford, UK) and subcultured when pre-confluent. Cells were examined using an Olympus IMT microscope (Olympus Opital Co. Ltd, UK).

Cytotoxicity assay

Evaluation of inhibitor cytotoxicity was carried out using a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Slater et al 1963). Briefly, cells were inoculated onto 96-well cell-culture plates (Falcon, Becton Dickinson Ltd, Oxford, UK) at a concentration of 1×10^5 cells/well and incubated overnight at 37°C. After 24 h, the media was replaced with fresh media (100 μL) containing the peptide treatments (final concentrations 1–1000 μM). Following a further incubation at 37°C for 24 h, MTT (10 μL of a 10 mg mL^{-1} aqueous solution) was added to each well and the plates incubated for 2 h. The media was then removed and the resultant formazan crystals dissolved in dimethyl sulfoxide (200 μL). The optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 510 nm.

Peptide stock solutions in DMF were diluted with media to give 0.4% (v/v) DMF at the highest concentration and sterilized through a 0.22-μm filter (ICN Biomedicals Ltd) before application to cells. Control experiments were carried out in the absence of inhibitor and in the presence of equivalent dilutions of DMF.

Tumour cell invasion assay

Anti-invasive properties of the compounds were evaluated using an in-vitro cell invasion assay modified from the Boyden Chamber method described by Albini et al (1987).

Polycarbonate transwell cell culture inserts (0.65 cm diameter; 8 μm pore size; Costar, Northumbria Biologicals, Paisley, UK) were coated with type IV collagen (6 μg cm^{-2}; Sigma-Aldrich). Coated transwell filters were then air-dried overnight, under UV light, before insertion into wells containing 600 μL of DMEM (for MDA-MB-231 cells) or L-15 (for T47D cells) supplemented with murine epidermal growth factor (EGF; Biogenesis, Bournemouth, UK) (receptor grade) (100 ng mL^{-1}). Cells (5×10^4 cells/well; 50 μL) were added to the transwells followed by the peptide treatments (50 μL

of 2, 20 or 200 μM solutions in DMEM or L-15) before incubation at 37°C. MDA-MB-231 cells were incubated for 24 h while T47D cells required 72 h. Experiments were performed in triplicate.

After incubation, transwells were transferred to a new plate and the media carefully removed using a cotton bud. Cells on the topside of the membrane were then removed by gently stroking the surface with a fresh cotton bud. This was repeated a further four times to ensure no cells remained. Membranes were then fixed and stained (Diff-Quik; DADE AG, Düdingen, Switzerland) to visualize the cells present on the underside of the filter. Excess stain was removed by washing in double-distilled water before air-drying overnight.

Image analysis was performed using a Leica DMIRB phase contrast microscope and Q500MC system, incorporating a JVC TK-1280E colour camera at × 50 magnification (Leica, Milton Keynes, UK). The total cell area in five non-overlapping views of the filter were calculated and added together to give a total cell surface area.

Scanning electron microscopy

Scanning electron microscopy was performed on a JSM-840A instrument. Membranes were prepared by fixing cells with 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, for 1 h, at room temperature. The transwell was then washed in the same buffer for 5 min before sequential dehydration in a series of ethanol solutions of 50% (v/v), 70% (v/v) and 100% concentration.

Preparation of cell-conditioned media

Growth media was removed from the cell-culture flask and the sub-confluent cells rinsed twice with 0.02 M phosphate buffer, pH 7.2, containing 150 mM NaCl. The cells were then incubated in 10 mL of fresh serum-free medium for 48 h, at 37°C. After this period, serum-free media was removed from the flasks and centrifuged at 1000 rev min^{-1} for 5 min to remove any cells or cell debris. The media was then concentrated approximately 20-fold, at 4°C, on an ultrafiltration apparatus (Amicon, Ltd; Gloucester, UK) equipped with a 10-kD cut-off filter and at a pressure of 30 lb in^{-2}.

Detection of breast-tumour-cell MMP activity by gelatin zymography

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a modification of the method of Laemmli (1970).

SDS-PAGE was performed on 10% polyacrylamide gels (8%T; 2.5%C), containing 0.1% (w/v) gelatin, under non-reducing conditions. Electrophoresis was performed for 1.5 h at 20 mA and 4°C. SDS-PAGE markers were included on all gels for determination of molecular weight. After electrophoresis, gels were washed twice for 15 min in 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 5 mM $CaCl_2$ and 2.5% (w/v) Triton X-100. Fresh buffer was then added and the gel incubated overnight at 37°C to ensure complete renaturation of protease structure and activity. Zones of gelatin lysis were visualized by staining gels with an aqueous solution of 0.25% (w/v) Coomassie Brilliant Blue R-250 containing 5% (v/v) acetic acid and 30% (v/v) methanol.

Results

Kinetic evaluation of inhibitor efficacy

The K_i values of the three peptide inhibitors are shown in Table 2. Pht-G-CH_2-LYA-NH_2 was found to be 10-fold more effective at inhibiting MMP-2 and 3.5-fold more potent for MMP-9 than Pht-G-CH_2-LTcA-NH_2. However, the *N*-α-mercaptoacetyl-containing peptide counterpart, $HSCH_2CO$-LYA-NH_2, was found to be the most potent inhibitor, inactivating MMP-2 and MMP-9, respectively, some 10-fold and 4-fold more efficiently than Pht-G-CH_2-LYA-NH_2. The *N*-α-phthalimidomethyl-ketomethylene peptide-based inhibitors displayed simple, competitive, reversible inhibition whereas the *N*-α-mercaptoacetyl analogue displayed slow-binding characteristics against MMP-2 but not MMP-9. The progress curves for this inhibitor against MMP-2 were analysed according to the models for slow, tight binding described by Morrison (1982) and Duggleby et al (1982).

Cytotoxicity studies

The results are summarized in Figure 3. No cytotoxic effects were observed when the peptides Pht-G-CH_2-

Table 2 Kinetic evaluation of the peptide-based inhibitors.

Peptide inhibitor	MMP-2 K_i (μM)	MMP-9 K_i (μM)
$HSCH_2$-LYA-NH_2	3.16 ± 0.40	11.84 ± 1.30
Pht-G-CH_2-LYA-NH_2	34.27 ± 4.10	45.75 ± 4.40
Pht-G-CH_2-LTcA-NH_2	362.8 ± 38.4	157.0 ± 19.5

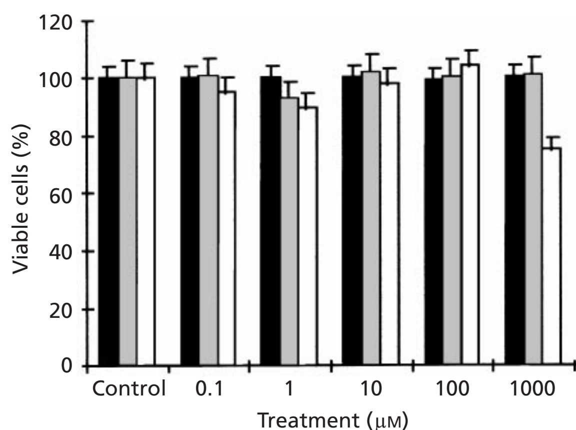

Figure 3 T47D cell viability as determined by the MTT assay. Cells were incubated either in the absence (control) or presence of Pht-G-CH_2-LTcA-NH_2 (■), Pht-G-CH_2-LYA-NH_2 (▨) or $HSCH_2CO$-LYA-NH_2 (□) for 24 h, at a concentration of 0.1–1000 μM.

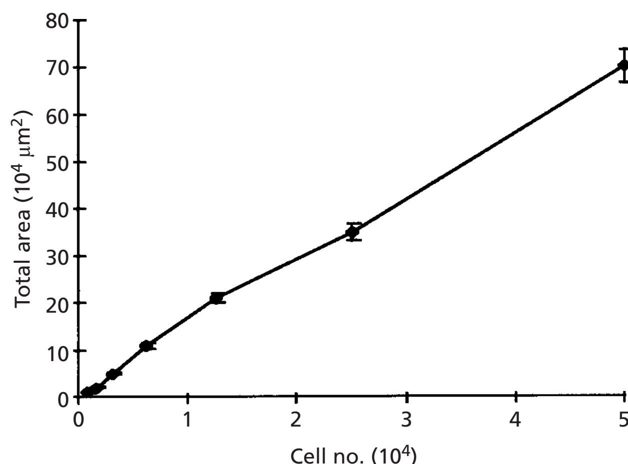

Figure 5 Relationship between total cell surface area, as calculated by image analysis and cell number. T47D cells were seeded onto transwell filters using doubling dilutions from 5×10^4 cells/well. The cells were incubated for 2 h at 37°C to allow adhesion onto the filter surface before fixation and staining.

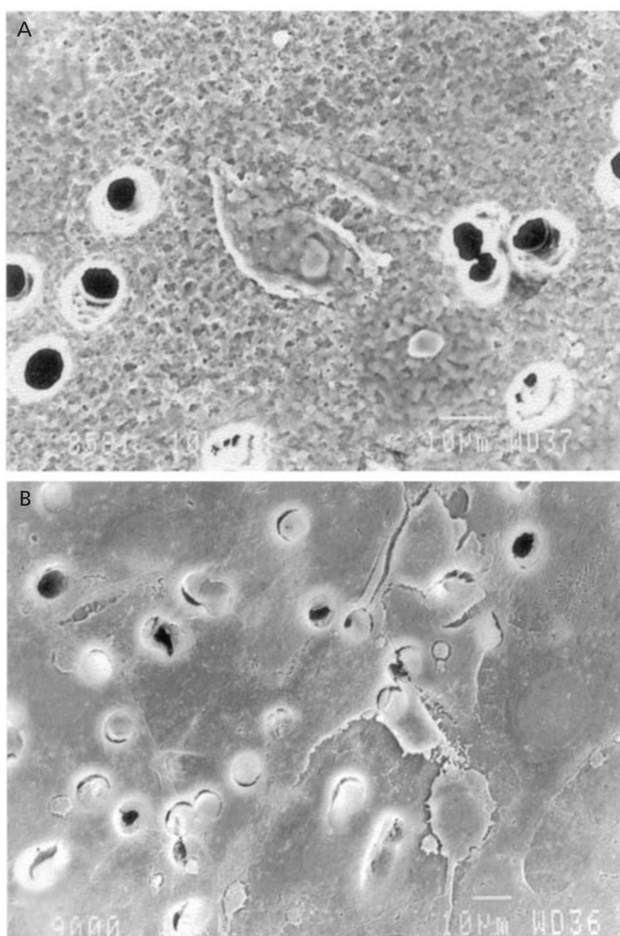

Figure 4 Scanning electron micrographs of T47D (A) and MDA-MB-231 cells (B) after invasion to the lower surface of polycarbonate transwell filters.

LYA-NH_2 and Pht-G-CH_2-LTcA-NH_2 were used within the concentration range 0.1–1000 μM. A diminished cell number (75% of control) was observed in the presence of $HSCH_2CO$-LYA-NH_2, but only when the peptide was tested at the upper concentration limit (1000 μM).

Invasion assay

To investigate the anti-invasive effects of the peptide-based inhibitors, an invasion assay was designed utilizing a type IV collagen-coated porous filter as a barrier to tumour-cell invasion. The chemoattractant EGF was included in the lower chamber to stimulate rapid penetration of the cells. The lower surfaces of the filters were successfully visualized using scanning electron microscopy. T47D and MDA-MB-231 cells are shown in Figure 4A and 4B, respectively.

By utilizing image analysis technology it was possible to solely select the stained tumour cells and subsequently calculate a value of the total cell surface area. To confirm that this measurement was relative to cell number, invasion chambers were seeded with doubling dilutions of cells from 5×10^4 cells/well. The cells were allowed two hours to adhere to the filter before fixation and staining, to diminish any proliferative effect. The total cell surface area on each filter was then calculated and compared with cell number. Figure 5 clearly shows that cell surface area correlates well with cell number, therefore any reduction in the number of invading cells due to the presence of the peptide treatments would be

observed by a comparative reduction in cell surface area on the filter base.

Anti-invasive effects of the peptide treatments

Results are summarized in Figure 6. The *N*-α-phthalimidomethyl-ketomethylene peptide-based inhibitors were tested firstly against the T47D cell line. Pht-G-CH_2-LYA-NH_2 was found to inhibit cell invasion through the collagen coat by 62.87 % at a concentration of 100 μM and 23.3 % at 1 μM. Pht-G-CH_2-LTcA-NH_2 was a much poorer anti-invasive agent, causing only a 34.6 % decrease in the number of invading T47D cells at 100 μM. The thiol-based peptide inhibitor was found to inhibit T47D cell invasion in the concentration range 1–100 μM. At 100 μM, $HSCH_2CO$-LYA-NH_2 inhibited cell invasion by 85.2 %. At lower concentrations the percentage inhibition fell to 62.3 % (10 μM) and 52.5 % (1 μM).

The MDA-MB-231 cells rapidly invaded the collagen-coated transwells which led to these experiments being carried out using a much diminished incubation period (24 h compared with 72 h for the T47D cells). Pht-G-CH_2-LTcA-NH_2 was found to inhibit invasion by 58 % at 100 μM, reducing to 24.7 % at 1 μM. Pht-G-CH_2-LYA-NH_2 however, proved to be an excellent inhibitor of MDA-MB-231 cell invasion, causing a 91.2 % reduction in the number of cells invading the transwell filter at 100 μM. This anti-invasive effect was also observed at 1 and 10 μM, resulting in a 71.6 % and 81.6 % reduction in invading cells, respectively. In contrast, the anti-invasive effect of the mercaptoacetyl peptide was not as pronounced against this cell line compared with the T47D cells and inhibited invasion by only 32.1 % at 100 μM.

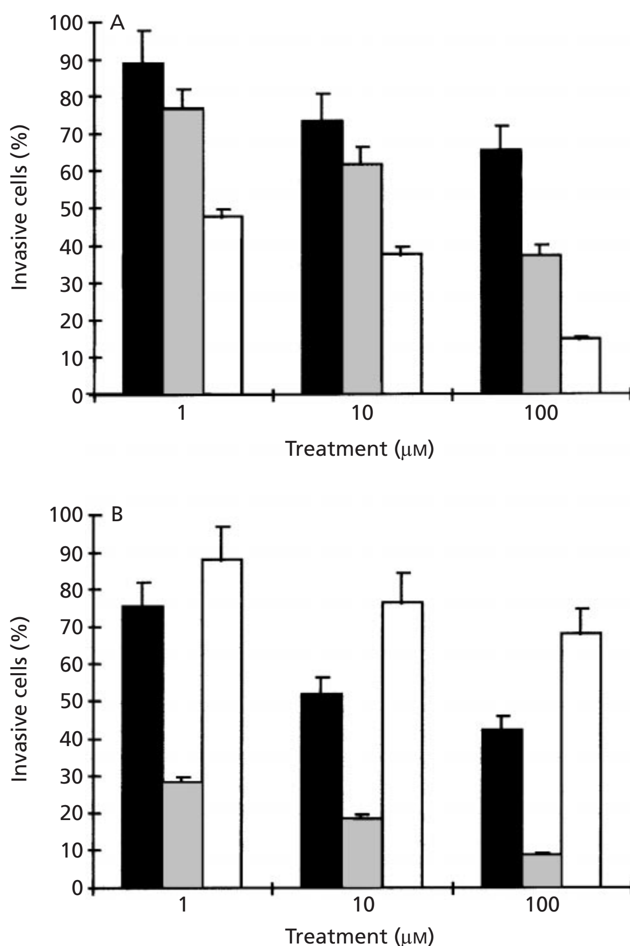

Figure 6 Anti-invasive effects of the peptide-based inhibitors tested against T47D (A) and MDA-MB-231 breast tumour cells (B). Cells were incubated at 37°C in media (100 μL) containing the following peptide treatments: Pht-G-CH_2-LTcA-NH_2 (■); Pht-G-CH_2-LYA-NH_2 (▩) and $HSCH_2CO$-LYA-NH_2(□) at 1, 10 and 100 μM. After 24 h (MDA-MB-231) or 72 h (T47D), the media and any residual cells on the top surface of the transwell filters were removed. The filters were stained with Diff-Quik, and allowed to dry. Image analysis was then used to determine the total area on the lower surface of the transwell covered by invaded cells. These experiments were carried out in triplicate.

Detection of gelatinase activity in breast tumour cell lines

To elucidate the molecular basis of these effects, we decided to analyse the cell-conditioned media of both breast tumour cell lines to profile their gelatinolytic activities. This was performed using the simple procedure of gelatin zymography, which allows a snap-shot of activity from both latent and active MMP species to be examined. Visualization of latent MMP bands is due to a conformational change induced by SDS which results in the exposure of the active site. T47D cells were found to secrete a variety of activities with molecular weights ranging from 92 kDa to 52 kDa (Figure 7A). By altering the pH of the incubation buffer it was observed that these bands were active in a pH range 7.0–8.0, with optimal activity being observed at pH 7.5. The predominant activity was found to have a molecular weight of 63 kDa, consistent with the activated form of MMP-2. Four other activities were observed of molecular weights 92, 86, 55 and 52 kDa. The 92- and 86-kDa bands are consistent with the latent and intermediate forms of MMP-9, respectively. The lower bands could be due to active fragments of the gelatinases.

The cell-conditioned media of MDA-MB-231 cells was found to contain only one gelatinolytic activity (Figure 7B). The single active band, of molecular weight

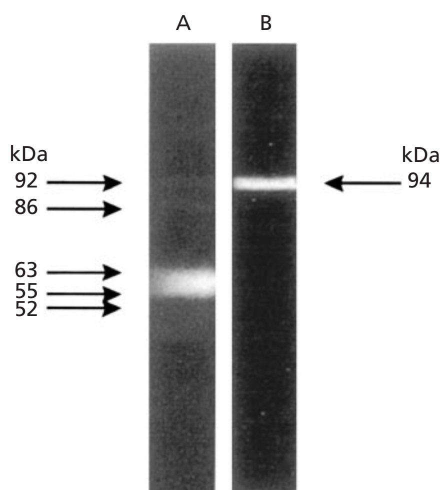

Figure 7 Sodium dodecyl sulfate–polyacrylamide (10%) gelatin zymograms illustrating active and latent gelatinolytic activity in cell-conditioned serum-free media from T47D (A) and MDA-MB-231 cells (B).

94 kDa, was thought to be the latent form of MMP-9. Activity was observed in the pH range 7.5–8.5, with activity being only slightly diminished at pH 7.0. These cell lines provide an excellent contrast as MMP-2 appears to be the predominant activity secreted by T47D cells whereas MMP-9 is the only activity produced in the media of MDA-MB-231 cells.

Discussion

This paper describes the kinetic characterization and anti-invasive properties of new synthetic MMP inhibitors based on peptides incorporating an N-α-phthalimidomethyl-ketomethylene group. Unlike the vast majority of MMP inhibitors reported to date, this group acts as a non-cleavable peptide-bond isostere. Integrating such a group into a peptide-based targeting motif has enabled us to develop MMP inhibitors which may avoid some of the pharmacological problems associated with other chelating group-based MMP inhibitors, such as hydroxamic acids and thiols, reported to date. Kinetic evaluation against MMP-2 and MMP-9, the key gelatinolytic MMPs involved in tumour invasion and metastasis, revealed that the inhibitors had only modest activity (Table 2); however, it was interesting to note that an exactly analogous inhibitor incorporating an N-α-mercaptoacetyl group was only 10-fold and 4-fold more active against MMP-2 ($K_i = 3.16\ \mu M$) and MMP-9 ($K_i = 11.53\ \mu M$), respectively. These results are probably a reflection of the choice of peptide targeting motif, as we and others have previously shown that analogous dipeptides have sub-micromolar activity (Baxter et al 1997; Lynas et al 2000). Extended peptide targeting motifs were chosen in this instance to enhance binding between enzyme and inhibitor, although results suggest that elaboration of pharmacophoric groups around the primary and prime subsite region may have greater potential. We are currently pursuing such an approach with the aim of improving inhibitor potency.

We initially performed cytotoxicity assessments which indicated that neither of the N-α-phthalimidomethyl-ketomethylene-based inhibitors displayed toxic effects at concentrations as high as 100 μM, thus establishing that any inhibition in invasion would be attributable to an anti-invasive effect of the inhibitors as opposed to a decrease in cell numbers due to cytotoxicity.

The compounds were then assessed for their ability to block the invasion in-vitro of two breast cancer cell lines, T47D (oestrogen-receptor positive) and MDA-MB-231 (oestrogen-receptor negative). Figure 6 clearly shows that both N-α-phthalimidomethyl-ketomethylene-based inhibitors and the mercaptoacetyl-based inhibitor, $HSCH_2CO$-LYA-NH_2, display dose-dependent anti-invasive effects on these cell lines. It can be seen that the effects against T47D cells are reflected, to a degree, in the K_i values obtained for each compound, with $HSCH_2CO$-LYA-NH_2 being the most effective gelatinase inhibitor as well as the most effective anti-invasive agent. The most interesting results were, however, obtained with the highly invasive hormone-independent MDA-MB-231 cells against which the most potent anti-invasive compound was found to be Pht-G-CH_2-LYA-NH_2, which prevented invasion by up to 71.2% at a concentration as low as 1 μM. With this particular cell line, the mercaptoacetyl-based inhibitor was almost 4-fold less effective, and indeed, Pht-G-CH_2-LTcA-NH_2, a relatively poor gelatinase inhibitor, was almost 1.5-fold more effective than $HSCH_2CO$-LYA-NH_2. These results strongly suggested that the inhibitors were acting by a mechanism which differed between the two cell types. We considered one possibility to be considerable differences in their expression of gelatinolytic enzymes.

To test this hypothesis we analysed, by zymography, the gelatinolytic activity found in serum-free cell-conditioned media from both cell lines. This experiment provided some interesting results, revealing that T47D cells secreted both MMP-2 and MMP-9 in latent and active forms, while MDA-MB-231 cells, which are much more invasive in-vitro than T47Ds, only secreted the latent form of MMP-9. Thus, the invasive mechanisms

must involve other proteolytic enzymes. We have probed the cell-conditioned media from MDA-MB-231 cells with a panel of commercially available anti-MMP antibodies (anti-MMP-1, -2, -3, -8, -9 and -13) (results not shown) and the results confirmed our finding that the only MMP species secreted into the media appeared to be latent MMP-9. Thus it appears that the highly invasive nature of this cell line is either a consequence of the activity of a non-metalloprotease(s) or, more likely, a result of a membrane-bound MMP species. One family of MMPs which has received increasing attention due to its members' perceived role in invasion is the membrane-type MMPs (MT-MMPs), in particular MT-MMP-1, which has been shown to degrade a number of matrix molecules, including collagen (Ohuchi et al 1997). The *N*-α-phthalimidomethyl-ketomethylene-based inhibitors may have some activity against this enzyme or related family members. This aspect is currently under investigation in these laboratories.

In conclusion, as MDA-MB-231 breast carcinoma cells are both highly invasive and refractory to oestrogen-receptor antagonists such as tamoxifen, our observation that the *N*-α-phthalimidomethyl-ketomethylene-based inhibitor Pht-G-CH_2-LYA-NH_2 strongly abrogates the invasive phenotype in-vitro makes this a promising lead compound for the development of new anti-metastatic agents for the treatment of malignant breast carcinoma.

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