XR5967, a novel modulator of plasminogen activator inhibitor-1 activity, suppresses tumor cell invasion and angiogenesis *in vitro*

Teresa D. Brooks[a], Shouming W. Wang[a], Nils Brünner[b] and Peter A. Charlton[a]

Recent reports suggest that elevated levels of plasminogen activator inhibitor (PAI)-1 may contribute to tumor progression. We have recently shown that antibodies to PAI-1 block the invasive and migratory potential of human fibrosarcoma cells and suppress angiogenesis *in vitro*. Here we report the *in vitro* evaluation of a low-molecular-weight modulator of PAI-1, XR5967, on invasion, migration and angiogenesis. XR5967, a diketopiperazine, dose-dependently inhibited the activity of human and murine PAI-1, towards urokinase plasminogen activator (uPA), with IC_{50} values of 800 nM and 8.3 μM, respectively. This was confirmed by SDS–PAGE, revealing that XR5967 inhibited complex formation between PAI-1 and uPA. This suppression may be caused by XR5967 promoting insertion of the reactive center loop within PAI-1. XR5967 dose-dependently inhibited the invasion of human HT1080 fibrosarcoma cells through Matrigel. Their invasion was reduced by 57% ($p<0.001$) at 5 μM. HT1080 cell migration was inhibited in a similar manner, indicating that PAI-1 may play an additional role in invasion, which is distinct to its role in the regulation of proteolysis. The potential of XR5967 to inhibit the invasion/migration of human endothelial cells was investigated in an *in vitro* model of angiogenesis. In this model XR5967 reduced tubule formation by 77% at 5 μM ($p<0.001$), highlighting a crucial role for PAI-1 in angiogenesis. These data stress the importance of a balanced proteolysis in the processes of invasion, migration and angiogenesis. Our results support the clinical findings and indicate that modulation of PAI-1 activity, with low-molecular-weight inhibitor of PAI-1 activity, may be of therapeutic benefit for the treatment of cancer. *Anti-Cancer Drugs* 15:37–44 © 2004 Lippincott Williams & Wilkins.

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[a]Xenova Ltd, Slough, Berkshire, UK and [b]Institute of Pharmacology and Pathobiology, Royal Veterinary and Agriculture University, Frederiksberg, Denmark.

Correspondence to T. Brooks, The Institute of Cancer Research, Belmont, Surrey SM2 5NG, UK.
Tel: +44 208 643 8901;
e-mail: teresa.brooks@icr.ac.uk

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Introduction

The processes of tumor cell invasion, angiogenesis and metastasis require the coordination and regulation of a series of adhesive, migratory and proteolytic events. Urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI)-1 are implicated in these processes. Over the past two decades elevated levels of uPA, its receptor (uPAR) and its inhibitor (PAI-1) have been identified in many malignant tumors [1,2].

uPA is a serine protease which catalyzes the conversion of the inactive zymogen, plasminogen, into the active enzyme plasmin. Plasmin is able to degrade components of the extracellular matrix either directly or indirectly via activation of selected matrix metalloproteinases (MMPs). PAI-1, a member of the serine protease inhibitor (serpin) family, is the primary inhibitor of uPA activity and hence is a regulator of extracellular proteolysis [3]. PAI-1 can exist in four different conformational forms: active, latent, substrate and cleaved [4]. Active PAI-1 has a reactive bond or bait sequence, in the form of a reactive center loop (RCL) that extends up from the main body of the protein. It can spontaneously convert to a latent, inactive, conformation via insertion of the exposed RCL, with a half-life of 3 h [5]. uPA binds to PAI-1 via the 'bait' sequence on the exposed RCL, which is cleaved, by uPA, during the generation of plasmin [6]. Cleaved and latent PAI-1 can no longer bind to or inhibit uPA activity. PAI-1 can also bind to vitronectin, which extends its active half-life and can localize PAI-1 activity to the cell surface [7]. Elevated levels of uPA and, paradoxically, elevated levels of its inhibitor, PAI-1, are associated with a poor prognosis in a variety of cancers including breast, skin, lung, colon, brain, stomach and ovarian [1].

Recent reports suggest that levels of these proteins are not merely elevated as a consequence of cancer, but may actually contribute to disease progression and, as PAI-1 is known to inhibit uPA activity, it may seem surprising to find that PAI-1 is also implicated in disease progression. Nevertheless, recent data from animal models support the clinical findings and have shown that high PAI-1 levels correlate with the ability of human melanoma cells to metastasize in the nude mouse [8]. It has also been

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demonstrated that PAI-1, added exogenously, increased the number of metastases in mice that were injected with human HT1080-P4 fibrosarcoma cells, by 60% [9]. In the same study a monoclonal antibody to PAI-1 was shown to suppress the metastatic potential of these cells by 39% over an 11-day period. Furthermore, studies with PAI-1 knockout mice have revealed that the hosts are resistant to invasion and neoangiogenesis of implanted malignant cells. When the PAI-1 deficiency in this model was circumvented, however, by i.v. injection of an adenoviral vector expressing human PAI-1, invasion and associated angiogenesis were restored [10]. Thus, it appears that PAI-1 may contribute to disease progression. These data, therefore, support the possibility that modulation of PAI-1 activity may have therapeutic potential in cancer.

We have previously demonstrated the potential of antibodies to PAI-1 to inhibit tumor cell invasion, migration and angiogenesis [11]. Having identified the role of PAI-1 in these processes, we aimed to determine whether a low-molecular-weight modulator of PAI-1 activity could also suppress tumor cell invasion, migration and angiogenesis.

Methods

Materials

Human HT1080 fibrosarcoma cells were kindly provided by Dr S. Eccles (ICR, Sutton, UK). Eagle's minimum essential medium (EMEM), fetal bovine serum, L-glutamine and trypsin, and non-essential amino acids were all from Life Technologies (Paisley, UK). Matrigel invasion chambers were purchased from Stratech Scientific (Luton, UK). Active human, recombinant, PAI-1, active murine PAI-1, NBD-tagged PAI-1 and human vitronectin were purchased from Molecular Innovations (Southfield, MI). SDS–PAGE gradient gels and silver staining kits were obtained from Amersham Pharmacia Biotech (St Albans, UK). Aprotinin, uPA, MTT and BSA were from Sigma (Poole, UK). The S2444 substrate and plasminogen were purchased from Quadratech (Epsom, UK). The *in vitro* angiogenesis kit and PECAM-1 staining kit were from TCS CellWorks (Botolph Claydon, UK).

Cell lines and cell culture

HT1080 fibrosarcoma cells were maintained in EMEM with the addition of 10% fetal bovine serum, 2 mM L-glutamine and 1% non-essential amino acids.

uPA activity assay

uPA activity was determined using the chromogenic substrate S2444 (pyroGlu-Gly-Arg-pNA.HCl), which liberates pNA on cleavage by uPA. Recombinant active PAI-1 was standardized by titration against uPA and a concentration of PAI-1 was chosen that just inhibited uPA activity. The inhibitory activity of XR5967 towards PAI-1 was measured by incubating 45 µl of PAI-1, in 50 mM Tris–NaCl, with various concentrations of XR5967 (in a 5 µl volume) for 10 min at 25°C. An aliquot of 80 µl of uPA (93.8 PU/ml) in Tris–NaCl was added and incubated for a further 10 min at 25°C. An aliquot of 20 µl of 3 mM S2444, in distilled water, was then added. The reaction mixture was incubated at 37°C for 30 min and the absorbance change monitored at 405 nm. Inhibition of PAI-1 activity was measured as the percentage restoration of uPA activity.

SDS–PAGE: PAI-1/uPA complex formation

The ability of XR5967 to modulate PAI-1/uPA complex formation was studied using SDS–PAGE. An aliquot of 10 µl of 50 mM Tris buffer (pH 8.3) was mixed with 5 µl of 18.6 µM PAI-1 (or Tris control) and 5 µl of various concentrations of XR5967 (or Tris control). This was incubated at 25°C for 15 min. An aliquot of 15 µl of 19.8 µM uPA (or Tris control) and 5 µl of Tris was then added, mixed and incubated at 25°C for a further 15 min. An aliquot of 8 µl of a mixture of SDS 350 mM, bromophenol blue 0.1% and EDTA 0.1% was then added to each sample, and incubated for a further 2 min at 25°C. The reaction was stopped by the addition of a 2-fold concentration of ice-cold Tris buffer. An aliquot of 1 µl of each sample was then applied to a 10–15% gradient PhastGel and the samples run on a PhastSystem unit at 250 V, 10 mA and 3 W at 15°C for 60 Vh. The bands were visualized with a standard silver stain. The concentrations of PAI-1 and uPA had been previously titrated in this assay to achieve optimal complex formation, which was 50–60% under these conditions.

RCL insertion assay

The ability of XR5967 to cause insertion of the RCL of PAI-1 (and hence cause latency) was investigated using an NBD fluorescently tagged PAI-1 molecule, whose fluorescence emission is increased 6- to 7-fold when the RCL is inserted into the β-sheet A. An aliquot of 25 µl of the NBD–PAI-1 at 40 µg/ml was added to 50 µl of Tris buffer (pH 8.3). A further 25 µl of either buffer, uPA at 2.5 KU/ml (which gave maximal RCL insertion, data not shown) or XR5967 at increasing concentrations was added. Fluorescence was measured after 0.5, 5, 10 and 15 min (excitation 485, emission 530). The degree of RCL insertion induced by XR5967 was calculated relative to the maximal insertion caused by uPA and expressed as a percentage of maximum RCL insertion.

Matrigel invasion assay—HT1080 human fibrosarcoma cells

Pre-coated Matrigel invasion chambers, containing filters with 8-µm pores, were used for this assay. Briefly, 200 µl of serum-free medium (containing 0.1% BSA) was added to

the Transwell chambers, which were incubated at 37°C to allow gelation. HT1080 cells were pre-incubated overnight in serum-free medium and harvested using 0.05% trypsin/0.02% EDTA. The cells were washed 3 times in PBS and suspended in serum-free medium, containing 0.14 μM plasminogen, at 5×10^5 cells/ml. Medium was then eluted from the upper chambers and 100 μl of cell suspension added. An aliquot of 400 μl of serum-free medium containing 0.14 μM plasminogen, with or without treatment, was also added to the upper well. An aliquot of 750 μl of 3T3 fibroblast-conditioned medium was added to the lower chambers as a chemotractant, with 0.14 μM plasminogen, plus or minus the treatments. After 24 h of culture at 37°C the medium was removed and the upper surfaces of half of the filters, from each treatment group, were swabbed to remove non-invading cells. The other half was used to determine the total number of cells per chamber. An aliquot of 500 μl of medium containing 0.2 mg/ml of the cell stain MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] was added to both the upper and lower chambers. The chambers were incubated for 1 h at 37°C. The medium was then eluted and 500 μl of DMSO added, to both the upper and lower chambers, to solubilize the cell stain. The OD of each chamber was determined at 490 nm. The percentage of tumor cell invasion was determined as the OD of the invaded cells divided by the OD of the total cells. Cytotoxicity of the treatments was determined by comparing their 'total' cell OD with that of the untreated (control) 'total' cell OD.

Chemotactic migration assay—HT1080 cells

Migratory capacities of the cells were investigated by using a Transwell system with 8-μm pore filters. The filters were coated with a 10 μg/ml solution of vitronectin, for 4 h at 37°C, before drying. The experimental conditions were identical to those used in the invasion assay.

Angiogenesis assay

In this *in vitro* model of angiogenesis (TCS CellWorks), human endothelial cells were grown in co-culture with human fibroblast (matrix) cells. An aliquot of 50 μl of either XR5967 or media controls was added to the culture at day 1, with 450 μl of fresh media, and the media and treatments replaced every 2–3 days. Capillary development was visualized after 9 days by staining with an antibody to PECAM-1. The total area (per field) covered by tubules was analyzed using DataCell Image Analysis Software.

Statistics

Significance levels (p values), for the HT1080 invasion, migration and RCL assays, were calculated using Student's unpaired t-test.

Results

The. structure of XR5967 (HCl salt, molecular weight 463 kDa) is shown in Figure 1. XR5967 was synthesized at Xenova and is an analog of a diketopiperazine, originally isolated from the lyophilized biomass of a *Streptomyces* species [12].

uPA activity assay

The effect of XR5967 on PAI-1 activity was studied indirectly by measuring restoration of uPA activity using the S2444 substrate (Fig. 2). XR5967 dose-dependently inhibited the activity of human PAI-1, toward uPA, with an IC_{50} of 800 nM. Additionally, XR5967 demonstrated a dose-dependent inhibition of murine PAI-1, although to a lesser extent, with an IC_{50} of 8.3 μM.

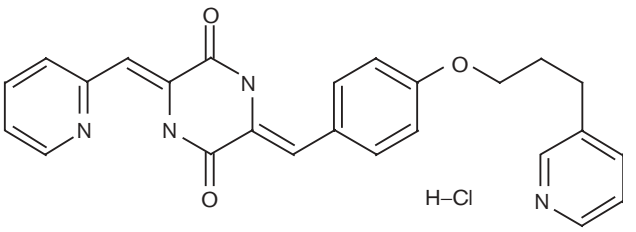
Effect of XR5967 on PAI-1/uPA complex formation by SDS–PAGE

When PAI-1 and uPA are incubated together they form an SDS-stable complex of approximately 90 000 kDa. The effect of XR5967 on PAI-1/uPA complex formation is shown in Figure 3. Complex formation between PAI-1 and uPA is evident in lane 3 and a dose-dependent inhibition of complex formation is clearly shown by XR5967 in lanes 4 –7.

Effect of XR5967 on RCL insertion

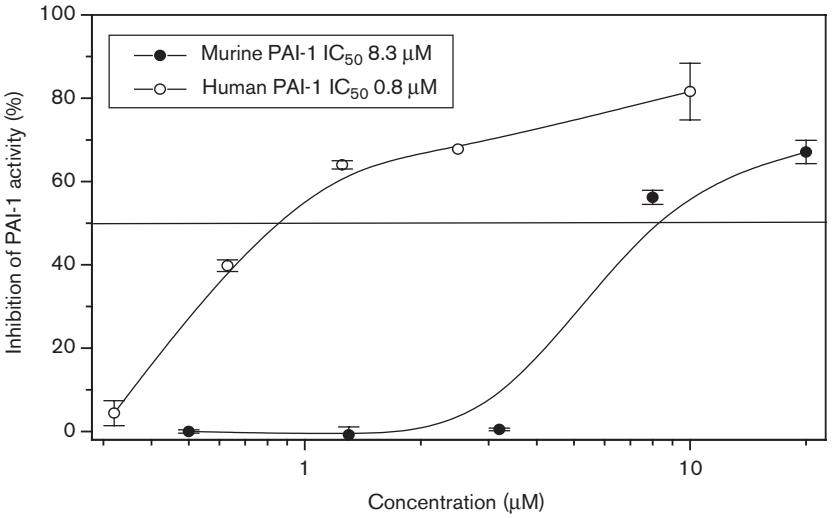
Insertion of the RCL of PAI-1 was monitored using an NBD fluorescently tagged PAI-1 molecule, whose fluorescence emission is increased when the RCL becomes inserted into the main body of the PAI-1 molecule. XR5967 caused a significant dose-dependent increase in the degree of RCL insertion and this effect was seen as early as 30 s after its addition (Fig. 4). Full RCL insertion (compared to the maximum effect seen with uPA) was achieved at 20 times the IC_{50} concentration. All concentrations of XR5967 and all time points gave a highly significant increase in loop insertion compared to controls ($p < 0.001$, Student's unpaired t-test).

Fig. 1



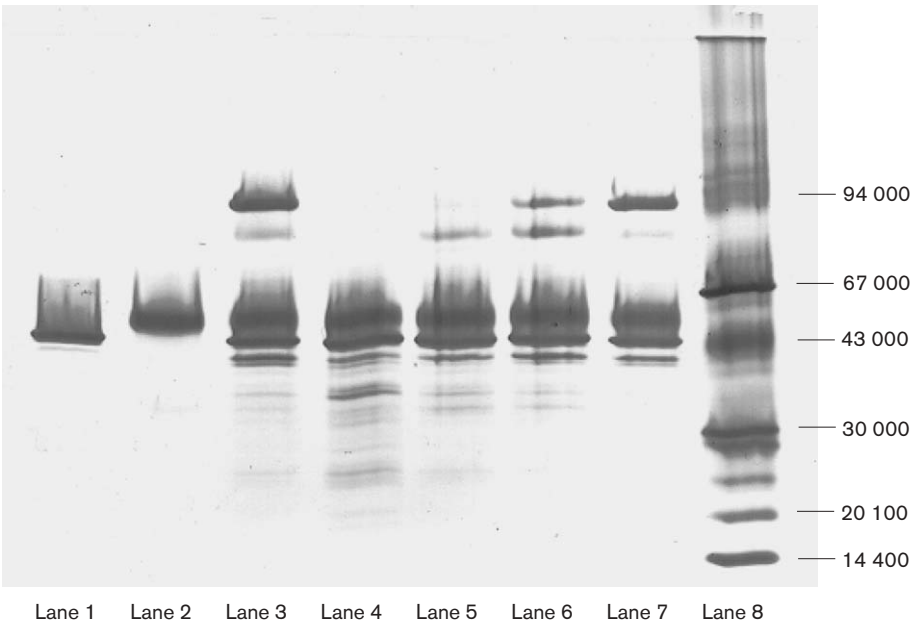
Structure of the diketopiperazine, XR5967 (HCl salt), a synthetic analog of a structure originally isolated from the culture biomass of a *Streptomyces* species.

Fig. 2



S2251 uPA activity assay. The effect of XR5967 on human (open circles) and murine (closed circles) PAI-1 activity. Results are expressed as mean percentage inhibition of PAI-1 activity ($n=2$ experiments $\times$ 3 replicates $\pm$ SEM).

Fig. 3

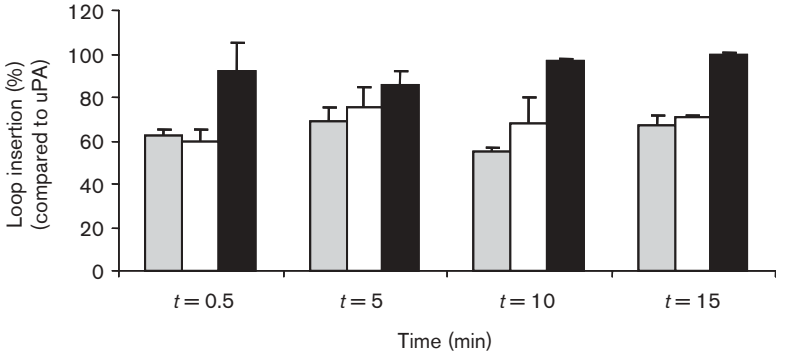


SDS–PAGE. The effect of XR5967 on PAI-1/uPA complex formation. Lane 1, PAI-1; lane 2, uPA; lane 3, PAI -1 + uPA; lanes 4, 5, 6 and 7 PAI-1 + uPA + XR5967 at an equivalent concentration of 10, 2, 0.4 and 0.08 μM, respectively; lane 8, standards. This result is representative of experiments carried out on three separate occasions.

Effect of XR5967 on human HT1080 fibrosarcoma cell invasion

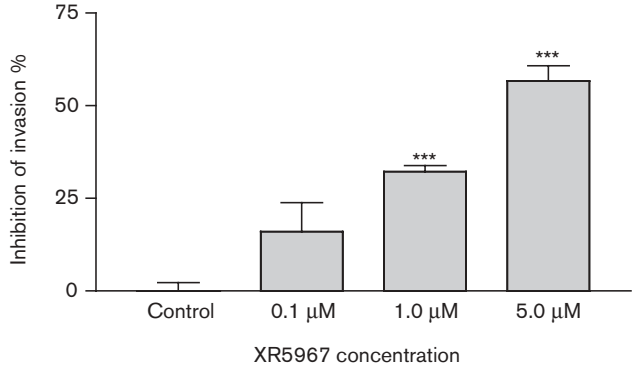
Human HT1080 fibrosarcoma invasion was studied in Matrigel-coated invasion chambers, towards a chemotractant. The HT1080 cells were highly invasive, with approximately 30% of the cells invading through the Matrigel layer in just 24 h (data not shown). The effect of XR5967 on HT1080 cell invasion is shown in Figure 5. A significant and dose-dependent inhibition of invasion was shown by XR5967, reaching 57% at 5 μM ($p < 0.001$).

Fig. 4



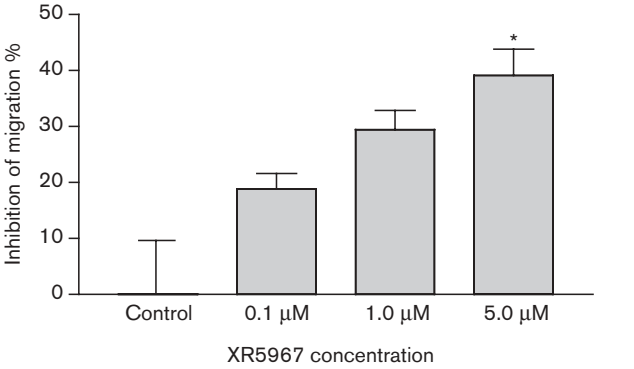
RCL insertion. The effect of XR5967, at $\times 4$ (light grey bars), $\times 8$ (white bars) and $\times 20$ (dark grey bars) its IC_{50} value, on the folding of PAI-1's RCL. Results represent the mean percentage loop insertion ($n=2$ experiments $\times$ 3 replicates $\pm$ SEM) compared to the maximal loop insertion produced by uPA (normalized to 100%). All results gave a highly significant increase in loop insertion compared to controls ($p<0.001$, Student's unpaired *t*-test).

Fig. 5



Matrigel invasion assay. The effect of XR5967 on the invasion of human HT1080 fibrosarcoma cells through Matrigel-coated filters. These results are from representative experiments that were performed on three occasions. Determinations were performed in triplicate and data expressed as the mean + SE with associated significance from control invasion (*t*-test, ***$p<0.001$).

Fig. 6



Tumor cell migration assay. The effect of XR5967 on the migration of human HT1080 fibrosarcoma cells over vitronectin-coated filters. These results are from representative experiments that were performed on three occasions. Determinations were performed in triplicate and data expressed as the mean + SE with associated significance from control invasion (t-test, *$p<0.05$).

The inhibition of invasion was not due to a cytotoxic effect as XR5967 did not affect the number or viability of the HT1080 cells (data not shown).

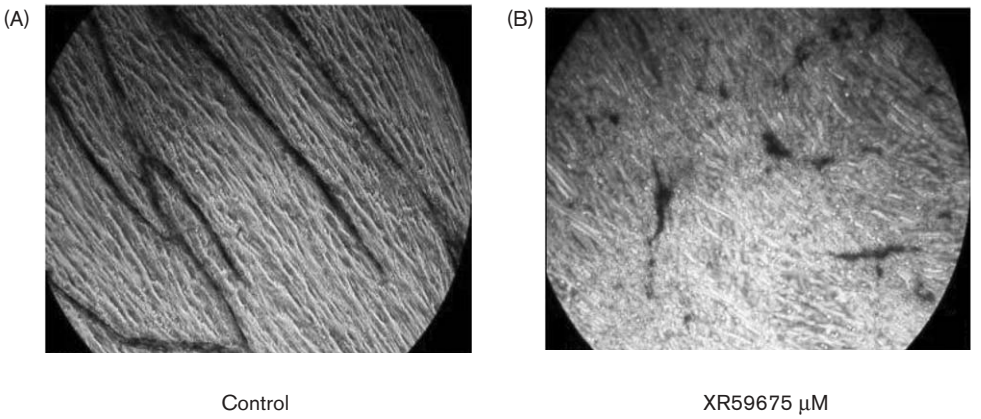
Effect of XR5967 on human HT1080 fibrosarcoma cell migration

Directed migration of the fibrosarcoma cells, through vitronectin-coated filters, was studied over a 24-h period. XR5967 suppressed the migration of the HT1080 cells over the adhesion molecule, vitronectin (Fig. 6). Once again, this effect was dose dependent. At 5 μM, XR5967 conferred 39% inhibition of migration ($p < 0.05$).

Effect of XR5967 on angiogenesis *in vitro*

The effect of XR5967 was studied in a co-culture model of angiogenesis using human endothelial cells seeded in a human cellular matrix. A well-developed network of tubules was visualized in the angiogenesis assay, by staining for PECAM-1, after a 9-day period (Fig. 7A). Inclusion of XR5967 in the media, at 5 μM, stunted tubule formation and prevented the development of a tubular network (Fig. 7B). Image analysis revealed a 77% inhibition, by XR5967, of the total area covered by tubules. This effect was not due to compound toxicity as no apparent cytotoxicity was shown by XR5967 in a separate assay (data not shown).

Fig. 7



The effect of XR5967 on angiogenesis. Photograph of representative wells in the *in vitro* angiogenesis assay (taken at magnification × 100). (A) Control well, (B) with the addition of XR5967 at 5 μM. The percentage reduction of tubule area (77% at 5 μM) represents the mean of four wells × 2 analyzed fields (SE ± 8.2%). These results are from representative experiments that were performed on three separate occasions.

Discussion

Recent studies indicate that PAI-1 contributes to the progression of cancer [8–10]. We have previously shown that antibodies to PAI-1 inhibit the invasive and migratory properties of human tumor cells *in vitro*, and reduce tubule formation in an *in vitro* model of human angiogenesis [11,13]. We have also shown that a low-molecular-weight, diketopiperazine-based, molecule can block the functional activity of PAI-1 both *in vitro* and *in vivo* [14,15]. In this report we have determined whether a low-molecular-weight inhibitor of PAI-1 activity can modulate tumor cell invasion, migration and angiogenesis.

The metastatic process involves a series of tightly coupled events. These include the detachment of tumor cells from the primary site, migration of the cells accompanied by regulated proteolysis of the extracellular matrix/basement membrane, dissemination of the tumor cells through the vasculature, and, finally, adhesion and proliferation of the cells at a secondary site. Evidence is mounting to show that the neutralization or absence of PAI-1 activity can have a dramatic effect on these processes. For example, studies using neutralizing antibodies to PAI-1 have shown reductions in the invasion of human lung cancer cells, significant decreases in human fibrosarcoma invasion/migration and also the suppression of pulmonary metastases in athymic mice [9,11,16]. Additionally, investigations using PAI-1 knockout mice have revealed a suppression of tumor growth and tumor angiogenesis together with a diminished proliferative response and an up-regulation of apoptosis in the tumor tissue [17,18]. Prevention of keratinocyte invasion and associated angiogenesis has also been documented in PAI-1 knockout mice [10].

These data led us to determine whether a low-molecular-weight inhibitor of PAI-1 activity could modulate some of the processes involved in metastasis. XR5967, a synthetic analog of a structure originally isolated from the biomass of a *Streptomyces* species that was shown to inhibit PAI-1 activity, was selected for this purpose [12]. Our results indicate that this diketopiperazine-based compound reversed the inhibitory effect of PAI-1 in a chromogenic assay measuring uPA activity. This result was not due to a direct effect on uPA as uPA activity was not enhanced in the absence of PAI-1 and XR5967 also reversed the inhibitory effect of PAI-1 on tissue PA (data not shown). In addition, the inhibition of PAI-1 functional activity was confirmed mechanistically, by SDS–PAGE, revealing that XR5967 prevented complex formation between PAI-1 and its target protease uPA. XR5967 was also shown to inhibit murine PAI-1 activity, although to a lesser extent (which may represent species selectivity or differences in the activity and purity of the two sources of PAI-1). This diketopiperazine-based molecule caused a dose-dependent increase in the degree of RCL insertion (similar to the maximal loop insertion caused by uPA) and hence may have caused PAI-1 to convert from the active to a latent form. In this conformation PAI-1 would no longer be able to bind to and suppress uPA activity.

Our results demonstrate that the process of tumor cell invasion can be modulated by a low-molecular-weight inhibitor of PAI-1 activity. The inhibition of human fibrosarcoma cell invasion, shown by XR5967, indicates that although PAI-1 potentially inhibits uPA-mediated degradation of the extracellular matrix, its presence is still vital for tumor cell invasion. An essential role of PAI-1 therefore may be to confine and direct areas of invasive

growth by regulating matrix degradation. A direct role for PAI-1 in controlling extracellular matrix degradation, in the human H1080 fibrosarcoma cells, was confirmed by Cajot *et al.* [19]. It is possible therefore that the low-molecular-weight inhibitor of PAI-1, XR5967, may inhibit invasion by disrupting this vital regulatory role of PAI-1. It appears from our previous studies that the maximum contribution of the plasminogen activation system to invasion, in this particular model, is about 60% [11]. At 5 μM, it therefore appears that XR5967 may have achieved maximum inhibition of the plasminogen activation component of invasion (57%) as higher concentrations of XR5967 gave a similar result (data not shown). Hence other components, e.g. MMPs, may also be involved in the invasion of the HT1080 cells through Matrigel in this model. It must be stressed, however, that this model is an isolated system, and may not reflect the true balance and interplay of the *in vivo* environment. In fact, *in vivo* studies have clearly demonstrated that the absence of PAI-1 gives rise to a complete blockade of tumor cell invasion in mice [10].

XR5967 also inhibited the migratory capacity of the fibrosarcoma cells, although to a slightly lesser extent. As there is no proteolytic barrier (Matrigel layer) in this migration assay, it may indicate that active PAI-1 plays an additional role in the migratory process, which is distinct from its regulatory role in proteolysis. We have previously shown this additional role to be in the detachment of tumor cells from the extracellular matrix adhesion molecule, vitronectin [11]. Furthermore, detachment of these cells did not occur in the presence of XR5967 (data not shown). It has been shown that the uPA receptor (uPAR) mediates the adhesion of tumor cells to vitronectin in the extracellular matrix and that uPA increases the strength of this association [20]. The observed effect with PAI-1 may be due to direct competition between active PAI-1 and uPAR for binding to the somatomedin B domain of vitronectin, as has been shown with monoclonal antibodies, thus causing a release of uPA/uPAR-mediated attachment [21]. Inhibition of PAI-1 by XR5967 may therefore prevent the detachment of cells from vitronectin in the extracellular matrix, thereby inhibiting cell migration.

Both tumor cell invasion and migration are essential features of angiogenesis, and as XR5967 was able to modulate these processes we investigated the effect of this molecule in a model of *in vitro* angiogenesis. XR5967 significantly reduced tubule formation, which was probably mediated by the diminished regulation of uPA-mediated proteolysis, and also by the reduced ability of the endothelial cells to detach and migrate, and hence form tubular structures. The inhibition of angiogenesis achieved in our model was consistent with the results reported by Bajou *et al.* which showed a role for PAI-1 in this process, and revealed that absence of host PAI-1 in mice prevented both tumor cell invasion and angiogenesis [10].

XR5967 clearly demonstrates *in vitro* proof of principal that a low-molecular-weight inhibitor of PAI-1 activity can inhibit tumor cell invasion, migration and angiogenesis.

Although the benefits of inhibiting PAI-1 in man have yet to be determined, studies on PAI-1 knockout mice, coupled with the existence of PAI-1-null individuals, indicate that the inhibition of PAI-1 activity may be a safe and viable approach to suppress tumor growth [10,18,22]. Further development is underway to optimize the potency, physicochemical properties and pharmacokinetic profile of XR5967, to enable the evaluation of this series of diketopiperazine-based inhibitors of PAI-1 activity on tumor growth *in vivo*. Ultimately it is therefore anticipated that XR5967 may act as a template for the development of a novel class of chemotherapeutic agents. In conclusion, these data clearly support the proposal that the development of inhibitors of PAI-1 activity may be of benefit for the treatment of a variety of cancers.

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