Differential Effects of Protease Inhibitors in 2-Dimensional and 3-Dimensional Cell Invasion Assays

KI Hulkower[1], SR Soltaninassab[1], KM Anhalt[1], RL Herber[1], K Moin[2], BF Sloane[2]

[1]*Platypus Technologies LLC, Madison, WI,* [2]*Department of Pharmacology & Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI*

Program/Board: 1816/B278

Abstract

Tumor cell invasion through an extracellular matrix has long been established as a key component in the metastasis of cancer. Previous studies have shown that inhibitors of proteases, such as matrix metalloproteinases (MMP's), urokinase, and cathepsins B and L, are effective in reducing the invasion of tumor cell lines in 2-D assays, that employ matrix coated porous transmembrane filters. We have recently developed a 3-D invasion assay in a 96-well plate format wherein cells were seeded onto a layer of basement membrane extract (BME) in an annular fashion around a centrally placed silicone stopper within the plate well. Adherent cells were covered with a second layer of BME once the stoppers were removed. Cells were then permitted to invade three dimensionally into the analytic zone formed upon the removal of the stopper. We confirmed the presence of proteases in HT-1080 cells and in HT-1080DM cells, a cell line that was selected for growth in serum free, defined medium and studied them in this 3-D invasion assay. The presence of proteases was detected via immunostaining and by a whole cell enzymatic activity assay. While we demonstrated that the enzymatic activity of the HT-1080 and HT-1080DM cells could be abolished by treatment of the cells with E-64 and leupeptin in whole cell assays of cathepsin B, we found that these inhibitors, as well as inhibitors against urokinase and MMPs, were ineffective in blocking invasion and invadopodia formation by these cells in our 3-D invasion assay. However, inhibitors of actin-myosin components of the cytoskeleton were effective in blocking invasion in our assay. We conclude that cells can utilize different mechanisms to maneuver and invade through BME when subjected to 2-D versus 3-D conditions. Our findings that cell invasion in a 3-D assay can occur independently of protease activity may be more physiologiacally relevent for drug discovery efforts as these results substantiate the lack of efficacy of protease inhibitors observed in *in vivo* settings.

Introduction

Previous studies have shown that inhibitors of a broad range of proteases, including MMPs, the cysteine proteases cathepsins B and L, and the serine protease urokinase (uPA), are effective in blocking invasion by HT-1080 cells utilizing BME coated transmembrane filters or Boyden chambers in the absence of a 3-Dimensional overlay:

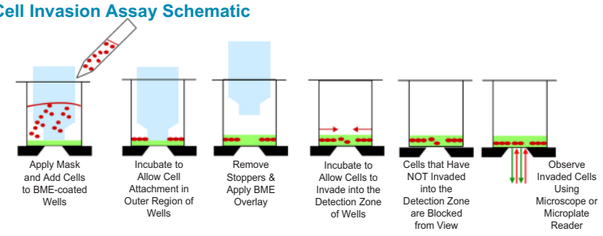
- Boyden chamber assay using leupeptin analogues to inhibit collagenase, uPA.[1]
- Transwell™ assay using the MMP inhibitors Ro-28-2653 and BB2516 (Marimastat).[2]
- Matrigel™ coated filter assay using the broad spectrum protease inhibitor hyperforin-DCHA.[3]
- Boyden chamber assay using Tetrahydrocurcumin to inhibit MMP-2, MMP-9 and uPA.[4]

However, preclinical studies with MMP inhibitors did not translate into efficacy as cancer therapy in human clinical trials. [5,6] It was subsequently found that protease independent mechanisms of invasion are possible in 3-D environments that may be more representative of physiological conditions *in vivo*:

- Cells in 2-D models depend more upon adhesion while cells in 3-D scaffolds compensate through diverse low adhesion or adhesion independent mechanisms. [7]
- Proteolytically potent HT-1080 and MDA-MB-231 cells embedded in 3-D collagen matrices, under conditions of near-total inhibition of a broad range of proteases, convert to spherical morphology and continue to migrate at undiminished rates. [8]
- Distinct forms of cell invasion differ in requirements for Rho/ROCK signaling and proteolysis [9] with ROCK- and myosin-dependent ECM deformation enabling protease independent tumor cell invasion in vivo. [10]

We therefore sought to examine the effects of protease inhibitors in a novel 3-D cell invasion assay as well as in a cell line with reduced protease activity that we established to grown in a serum-free, defined medium.

Oris™ Cell Invasion Assay Schematic



Materials and Methods

Reagents and Sources: Calcein AM (C3099), Invitrogen; Calbiochem; Amelioride (CA-200), Actinonin (P-8702), E64 (PI-105), CTT (PL-136), Leupeptin (PI-118),, Z-RR-AMC (P-137) BIOMOL; Blebbistatin (203391) Calbiochem; FBS, Atlanta Biological; Growth Factor Reduced Cultrex™ BME (3433-005), Trevigen; cellgro COMPLETE™ (40-101-CV) and Cellstripper™ (25-056-CI), Mediatech; 10X HBSS (14065-65) GIBCO; PIPES (P1851),Sigma; L-cysteine (168149) Aldrich; Microfluor 2 96-well plates (7905) Thermo.

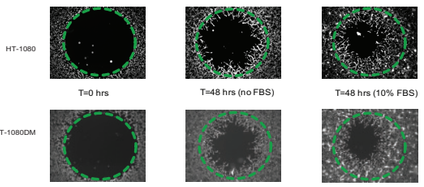
Antibodies and Sources: Polyclonal anti-MMP-9 (1:50 dilution, sc-10737), Santa Cruz Biotechnology; polyclonal anti-Cathepsin B (1:50 dilution, SA-361), BIOMOL; Alexa Fluor® 488 SFX kit containing highly cross-adsorbed conjugated goat anti-rabbit antibodies (A31628) with Image-iTTM FX signal enhancer, Invitrogen.

Cell Lines and Sources: HT-1080 fibrosarcoma cell line, ATCC; HT-1080DM cells were established from the parental HT-1080 cell line by gradual weaning from growth medium containing 10% FBS to select for growth in a defined serum-free, low protein medium (cellgro COMPLETE™). Upon reaching confluency in growth flasks, an aliquot of the conditioned medium was removed and clarified by centrifugation. The HT-1080DM cells were harvested non-enzymatically using Cellstripper™ and split into fresh flasks containing 75% fresh defined medium and 25% of clarified conditioned medium (V/V).

Funded by NIH/NIGMS, Grant # 2R44GM069026-03

* Patent Pending

3-Dimensional Cell Invasion of HT-1080 & HT-1080DM Cell Lines



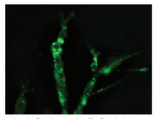
Serum starved HT-1080 cells and HT-1080DM cells were seeded (50,000 cells/well) onto BME coated plates, and allowed to adhere for 4 hours. Stoppers were removed and the Oris™ BME (+/- 10% FBS) was overlayed on the cells. Following a 48 hour incubation, the cells were labeled with Calcein AM and imaged by using a Zeiss Axiovert microscope (5X magnification). The images, captured without a detection mask, represent pre-invasion (t=0 hrs) and post-invasion (t=48 hrs) wells.

Results: Serum starved HT-1080 cells form invadopodia-like projections within the 3-dimensional overlay of ECM. This invasive movement is potentiated by 25 - 30% when 10% FBS is present in the ECM overlay. Similar results were obtained from the HT-1080DM cells.

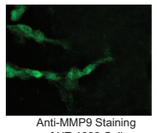
Detection of Proteases in HT-1080 Cell Invadopodia in the Oris™ 3D-Invasion Assay

Cathepsin B is a lysosomal protease that facilitates cell invasion by degrading the surrounding extracellular matrix. MMP-9 is a protease that is involved in matrix degradation in cancer cell invasion.

Cells from the Oris™ Cell Invasion Assay were fixed, permeabilized, and pre-treated by sequential incubations in 3.7% formaldehyde, PBS, 0.5% Triton X-100, PBS, Image-iTTM FX signal enhancer (30 minutes), and PBS. Immunostaining was performed by incubating with primary antibodies (2 hours, 37°C) and Alexa Fluor® 488 conjugated secondary antibodies at 1:200 dilution (1 hour, 37°C). Images were collected using a Nikon TE300 inverted microscope.



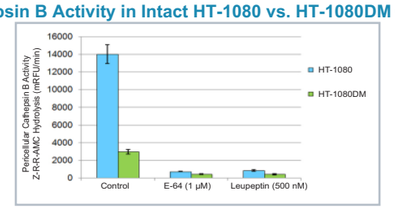
Anti-Cathepsin B Staining of HT-1080 Cells



Anti-MMP9 Staining of HT-1080 Cells

Results: Distinct areas of lysosomal and peripheral cellular expression of Cathepsin B are visible in invading HT-1080 cells, characteristic of protease expression observed in cancer cell invasion[11]. There is also visible evidence of MMP-9 expression and secretion into surrounding areas of ECM by invading HT-1080 cells, consistent with known protease characteristics observed at invadopodia sites[12].

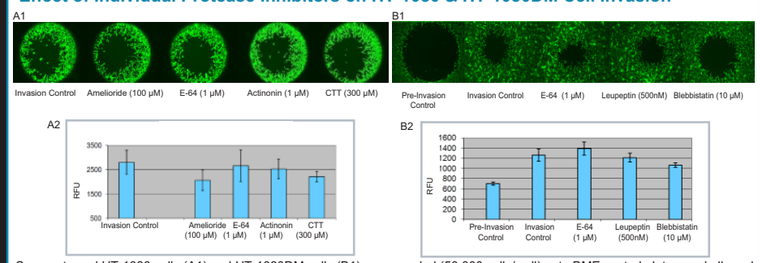
Differential Cathepsin B Activity in Intact HT-1080 vs. HT-1080DM Cells



The cells, pericellular assay buffer (PAB), and substrate were prepared and the assay run as described by Hulkower et al[11]. Cathepsin B activity was assessed in Thermo Microfluor 2 96-well plates in a final volume of 200 µL consisting of: 150 µLcell suspension in PAB (150,000 cells), 20 µL inhibitor or 0.1% DMSO vehicle control, 10 µL PAB; 20 µL of 2 mM Z-RR-AMC was added to begin the assay. The assay plates were read at 37 °C in a BioTek Synergy™ 2 microplate reader in kinetic mode with readings taken every 30 seconds for 10 minutes using a 355 nm excitation filter and a 460 nm emission filter. Each column represents the mean value +/- S.D. of at least 4 wells.

Results: The native Cathepsin B activity of HT-1080DM cells, established and propagated in serum-free, defined medum, is only about 20% that as compared to HT-1080 cells. Cathepsin B activity from both cell lines is abolished by the cysteine protease inhibitors E-64 and Leupeptin, thus indicating the efficacy of these inhibitors on intact cells.

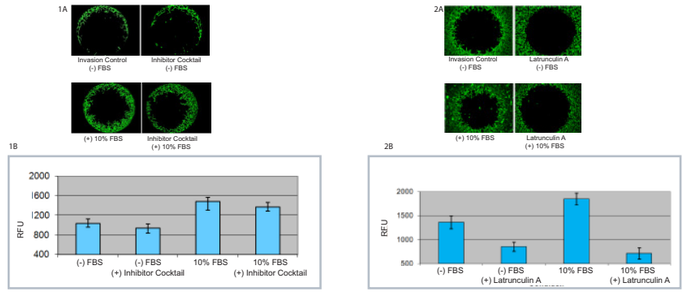
Effect of Individual Protease Inhibitors on HT-1080 & HT-1080DM Cell Invasion



Serum starved HT-1080 cells (A1) and HT-1080DM cells (B1) were seeded (50,000 cells/well) onto BME coated plates, and allowed to adhere for 4 hours. Stoppers were removed and the Oris™ BME (with 10% FBS) was overlayed on the cells. A medium overlay containing the protease inhibitors (in 0.1% DMSO) was then added to the wells. Following a 48 hour incubation, cells were labeled with Calcein AM and images were obtained from plate wells via fluorescence microscope (A1, B1). The Oris™ Detection Mask was attached to the bottom of the plate and fluorescence was quantitated using a BioTek Synergy™ 2 microplate reader (B1, B2). Each column represents the mean value +/- S.D. of at least 4 wells.

Results: The uPA inhibitor (Amelioride), the cysteine protease inhibitor (E-64), the elastase/MMP inhibitor (Actinonin), and the MMP-2/MMP-9 inhibitor (CTT) demonstrated no significant inhibitory effects on HT-1080 cell invasion into the BME. Similarly, the cysteine protease inhibitors, E-64 and Leupeptin, did not inhibit HT-1080DM cell invasion. However, the myosin inhibitor, Blebbistatin, did reduce HT-1080DM cell invasion.

Effects of Protease Inhibitor Cocktail & Latrunculin A on HT-1080DM Cell Invasion



HT-1080DM cells were seeded (50,000 cells/well) onto BME coated plates and allowed to adhere for 4 hours. Stoppers were removed and the Oris™ BME (+/-10% FBS) was overlayed on the cells. A medium overlay containing a mixture of protease inhibitors (30 nM Amelioride, 30 nM E-64, 30 nM Actinonin) or Latrunculin A (200 nM) was then added to the wells. Following a 48 hour incubation, cells were labeled with Calcein AM, images were obtained from plate wells via fluorescence microscope (1A & 2A), and fluorescence was quantitated using a BioTek Synergy™ 2 microplate reader (1B and 2B). Each column represents the mean value +/- S.D. of at least 4 wells.

Results: As observed previously, cell invasion by HT-1080DM cells was more robust when 10% FBS was present in the BME overlay. A cocktail consisting of uPA, cysteine protease, and elastase/MMP inhibitors (Amelioride, E-64, and Actinonin) demonstrated no significant inhibitory effects on HT-1080DM cell invasion. In contrast, the actin polymerization inhibitor, Latrunculin A, effectively abolished HT-1080DM cell invasion. Similar observations were made with HT-1080 cells (not shown).

Comparison of Protease Inhibitor Effects in Boyden Chamber / Transmembrane vs. 3-D Cell Invasion Assays

	Cathepsin B/L	uPA	MMP	Elastase	Myosin or Actin Polymerization	Inhibitor Cocktail Cat B/L, uPA, MMP
Boyden Chamber / Transmembrane	Yes	Yes	Yes	N/A	N/A	Yes
3-D	No	No	No	No	Yes	No

Conclusions

1. **HT-1080 cells moving within the 3D ECM express several classes of proteases within invadopodial structures, such as Cathepsin B and MMP-9, as evidenced by enzymatic activity assays and immunocytochemistry.**
2. **Serum stimulation enhances invasion through protease-independent mechanisms.**
3. **Serum deprived HT-1080DM cells have significantly less pericellular Cathepsin B activity than the parental HT-1080 cells, but still retain the capacity to invade into 3D BME matrix.**
4. **Inhibitors, including those for serine and cysteine proteases, and MMPs, have little effect alone or in combination on cell invasion into 3D BME matrix.**
5. **Inhibitors of myosin and actin polymerization are effective in blocking cell invasion into 3D BME matrix.**

References

1. Kawada and Umezawa, Biochem. Biophys. Res. Commun. 209:25 (1995)
2. Maquoi et al.,Clin. Cancer Res. 10:4038 (2004)
3. Dona et al., Cancer Res. 64:6225 (2004)
4. Yodkeeree et al., Acta Pharmacologica Sinica 29:853 (2008)
5. Coussens et al., Science 295:2387 (2002)
6. Fingleton, Expert Opin. Ther. Targets 7:385 (2003)
7. Friedl and Wolf, Nature Reviews Cancer 3:362 (2003)
8. Wolf et al., J. Cell Bio., 160:267 (2003)
9. Sahai and Marshall, Nature Cell Biology 5:711 (2003)
10. Wyckoff et al., Current Biology 16:1515 (2006)
11. Hulkower et al., Eur. J. Biochem. 267:4165 (2000)
12. Weaver, Clin. Exp. Metastasis 23:97 (2006)
13. Fumaniak-Kazmierczak et al., Circulation Res. 100:1328 (2007)