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Screening of Anti-Metastatic Compounds by a Fluorescence-Based Tumor Cell Invasion Assay

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Introduction

Discovery of new cancer therapeutics has been constrained by the lack of reliable and high-throughput primary and secondary biological assays. To address this need BD Biosciences developed a quantitative, high-throughput, automation-compatible, fluorescence-based tumor cell invasion assay system.

Measurement of the inhibition of tumor cell invasion through a microporous membrane coated with BD Matrigel™ Matrix in Boyden-like chambers is a widely accepted *in vitro* assay of anti-metastatic activity. However, conventional methods employing these individual chambers in conjunction with cumbersome methods of cell counting make this procedure impractical for screening. Additionally, the difficulty in reliably coating microporous membranes has resulted in wide variations in assay results. BD Biosciences has coupled a multiwell insert device containing a fluorescence blocking microporous membrane that allows separation of fluorescence readings in the top and bottom compartments of the chamber with a unique BD Matrigel Matrix coating process. By optimizing labeling and assay conditions, a practical, reproducible high-throughput screening assay for anti-metastatic drugs was developed.

Using three cell lines, human fibrosarcoma (HT-1080), human breast carcinoma (MDA-MB-231), and human prostate carcinoma (PC-3), BD Biosciences tested three anti-metastatic compounds and calculated IC₅₀ values of each to establish the relevance of the assay to compound screening.

Materials and Methods

BD Falcon™ FluoroBlok™ 24-Multiwell Insert System

The BD Falcon™ FluoroBlok™ 24-Multiwell Insert System (BD Cat. No. 351161 or 351162) consists of a cell culture insert containing a fluorescence blocking, microporous PET membrane which blocks the passage of light at wave-lengths 490-700 nm at >99% efficiency. The light emitted by fluorescently labeled cells on the upper surface of the membrane is separated from those on the lower. This allows for real-time, non-destructive monitoring and quantitation of invading or migrating cells without further manipulation. The 24 insert wells are contained within a single unit, which facilitates both manual and automated handling.

BD Matrigel™ Matrix

BD Matrigel™ Matrix is a biologically active reconstituted extracellular matrix preparation derived from the Englebreth-Holm-Swarm (EHS) mouse tumor. BD Matrigel Matrix coated on the membrane functions as a barrier to the passage of non-invasive cells analogous to the *in vivo* extracellular basement membrane.

BD BioCoat™ Tumor Invasion System

The BD BioCoat™ Tumor Invasion System was prepared by coating the membrane of a BD Falcon FluoroBlok 24-Multiwell Insert System (8 µm) with BD Matrigel Matrix. The coating was dried under unique conditions. This procedure results in occlusion of the membrane pores.

Reagents

Calcein AM (C-3100) was obtained from Invitrogen. DOXY, Paclitaxel and 1,10-Phenanthroline were obtained from Sigma.

Calcein AM is now available from BD Biosciences.

BD™ Fluorescent Dyes

BD™ Fluorescent Dyes are available for labeling cells when performing tumor cell invasion, angiogenesis assays, and other cell-based assays. For additional information about BD™ Calcein AM Fluorescent Dyes (Cat. Nos. 354216 and 354217) and BD™ DiI_{C₁₂(3)} Fluorescent Dye (Cat. No. 354218), visit our website at: bdbiosciences.com.

Cells

HT-1080 human fibrosarcoma cells, MDA-MB-231 human breast adenocarcinoma, and PC-3 human prostate adenocarcinoma cells were obtained from American Type Culture Collection (ATCC). HT-1080 cells are a commonly used invasive tumor cell.

Preparation of Cell Suspensions

HT-1080 cells were grown to near confluence in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). MDA-MB-231 cells were grown to 70-80% confluence in Leibovitz L-15 medium supplemented with 10% FBS under CO₂-free conditions. PC-3 cells were grown to 70-80% confluence in F-12 Nutrient Mixture (Kaign's Modification) supplemented with 10% FBS. Cell suspensions were prepared by trypsinizing the monolayer and resuspending in DMEM without FBS at 5 x 10⁴ cells/ml for HT-1080 cells, and 1 x 10⁵ cells/ml for MDA-MB-231 and PC-3 cells.

Invasion Assay Procedure

The insert plates were prepared by rehydrating the BD Matrigel™ Matrix coating with phosphate buffered saline for two hours at 37°C. The rehydration solution was carefully removed, 0.75 ml DMEM containing chemo-attractant (5% FBS for HT-1080 cells and 10% FBS for MDA-MB-231 and PC-3 cells) was added to the plate well, and 0.5 ml of cell suspension (2.5×10^4 of HT-1080 cells, 5×10^4 of MDA-MB-231 and PC-3 cells) was added to each insert well. For invasion inhibition assays, the compound of interest was added to the medium in both upper and lower chambers along with cells and chemoattractant solution. Uncoated insert plates, included as migration controls, were used without rehydration. HT-1080 and MDA-MB-231 assay plates were incubated for 20-22 hours at 37°C. PC-3 assays were incubated for 48 hours. Following incubation, the medium was removed from the upper chamber and the entire insert plate was transferred to a second 24-well plate containing 0.5 ml/well of 4 µg/ml Calcein AM in Hanks buffered saline. The plates were incubated for one hour at 37°C and read in a fluorescence plate reader without further manipulation (Figures 1-3).

Fluorescence Reading

Fluorescence data was collected using an Applied Biosystems CytoFluor® 4000 Fluorescence Plate Reader at excitation wavelength (ex) of 485 nm and emission wavelength (em) of 530 nm at a gain of 55 nm. Only those labeled cells that pass through the BD Matrigel Matrix layer and the membrane are detected (Figures 1-3).

Data Analysis

Data was analyzed using the a Graphical User Interface (GUI)-based protocol editor allowing input of compound names, concentrations, and cell lines, to facilitate subsequent data analysis. IC₅₀ values were calculated using either the classical four-parameter logistic model or the non-parametric smoothing spline. (Figures 6-14).

Results and Discussion

Assay Robustness

DOXY, a synthetic tetracycline analog and antimicrobial agent, has been shown to suppress tumor metalloproteinase (MMP) production and function as a non-competitive inhibitor. While other proteases have been shown to be involved in the invasive process, MMPs appear to be responsible for the initiation of matrix degradation. Inhibition of these enzymes has been shown to inhibit invasion and metastasis.

BD BioCoat™ Tumor Invasion Systems were used to quantitate the inhibitory effect of DOXY on both migration (no basement membrane) and invasion (enzymatic degradation of BD Matrigel Matrix). DOXY affected the human fibrosarcoma cell line HT-1080 in a dose dependent manner with the IC₅₀ value for invasion at 80 µM. Migration of these cells was also inhibited (IC₅₀ value of 75 µM) suggesting a more complex mechanism than inhibition of MMPs alone may be at play.

These DOXY studies were used to assess precision (reproducibility and correlation coefficients [CV]), accuracy (standard error), and robustness (long-term performance over repeated assay use). In all cases, the assay performed at a higher level than expected for a cell-based assay. CVs for inter-assay reproducibility were at 6% over a four-week, four-assay period. Lot-to-lot variation gave an IC₅₀ CV of only 2% over three lots of BD BioCoat™ Invasion Systems. Standard deviations were routinely low and are reported on the graphs (Figures 4 and 5).

Assay Relevance

Three cell lines of importance to the discovery of anti-invasive compounds were tested. HT-1080, a human fibrosarcoma cell line frequently used in other *in vitro* invasion models; MDA-MB-231 cells, a human breast adenocarcinoma; and PC-3 cells, a human prostate adenocarcinoma. We have also tested non-invasive cell lines, MCF-7 human breast carcinoma, LNCap FGC human prostate carcinoma, RPMI 7951 human melanoma, WM115 human melanoma, and NIH-3T3 mouse fibroblast, with the expected negative results.

In addition to DOXY, two other compounds were screened with the three invasive cell lines. Paclitaxel, an inhibitor of disassembly of microtubules is an established chemotherapeutic agent. 1,10-Phenanthroline, a zinc chelator and MMP inhibitor has been shown to prevent tumor cell invasiveness as well as endothelial cell invasion, an important event occurring during angiogenesis. All were shown to inhibit both invasion (through a basement membrane) and migration (without a basement membrane) in three cell lines. Compound IC₅₀ values are reported. (Figures 6-14).

Retinoic Acid, TIMP-2, GM6001, and MMP2/MMP9 Inhibitor 1 were tested on HT-1080 cells. Unexpectedly, these compounds had no inhibitory effect on invasion/migration as tested. Modifications of the assay, such as the addition of a carrier or pre-incubation of the cells with the compound, could provide results consistent with the expected activity of these compounds (Table 1).

The development of this assay is a significant advancement toward the routine use of a cell-based invasion assay system in compound screening.

Compound	Concentrations Tested	IC ₅₀
Doxycycline	25 - 250 µM	68.6
1,10-Phenanthroline	0.1 - 50 µg/ml	12.6
GM6001	0.25 - 25 µM	No inhibition
MMP2/MMP9 Inhibitor 1	0.1 - 100 µM	No inhibition
Paclitaxel	1.0 - 1,000 nM	10.4
TIMP 2	12.5 - 200 ng/ml	Inhibitory (IC ₅₀ not determined)
Retinoic Acid	0.1 - 100 µM	No inhibition

Table 1: Compound tested for inhibition of HT-1080 invasion.

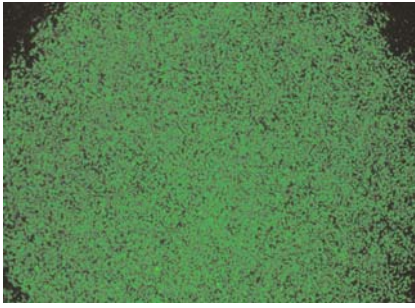


Figure 1: Post-invasion, calcein-stained HT-1080 human fibrosarcoma cells.

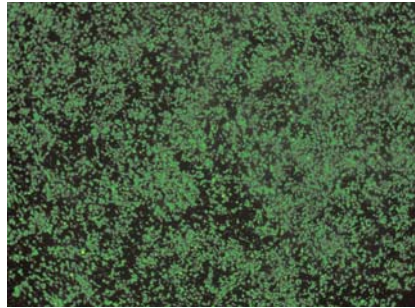


Figure 2: Post-invasion, calcein-stained PC-3 human prostate adenocarcinoma cells.

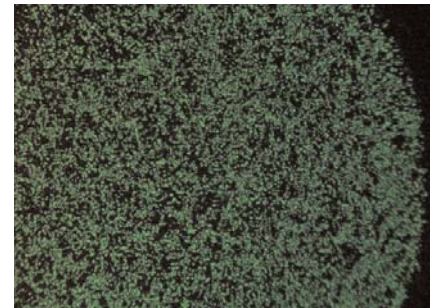


Figure 3: Post-invasion, calcein-stained MDA-MB-231 human breast adenocarcinoma cells.

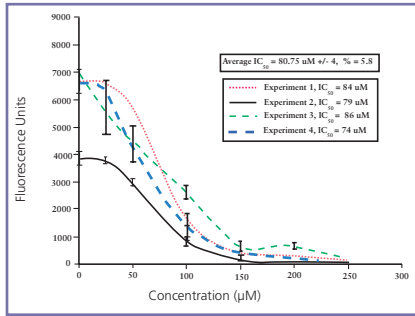


Figure 4: Inter-assay Reproducibility. A single lot of coated inserts was assayed four times over a four-week period. IC_{50} values were calculated using two order polynomial trendlines.

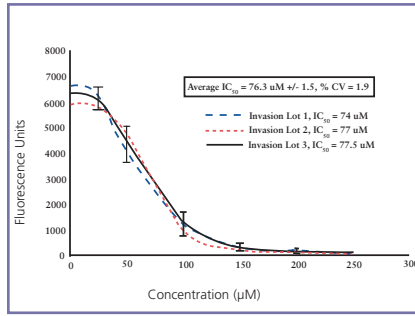
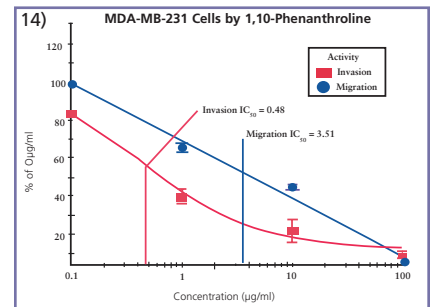
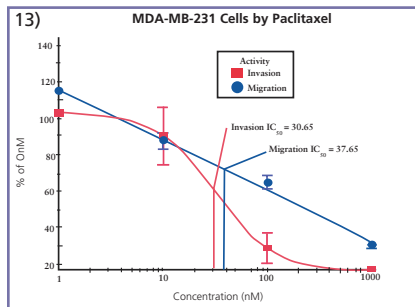
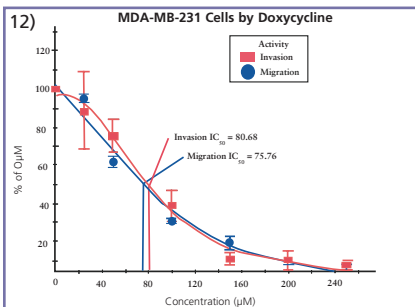
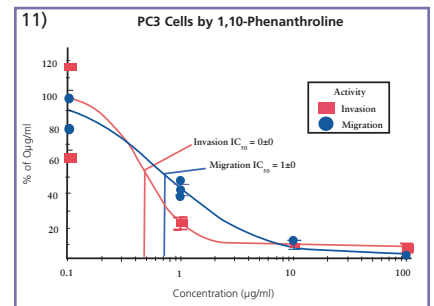
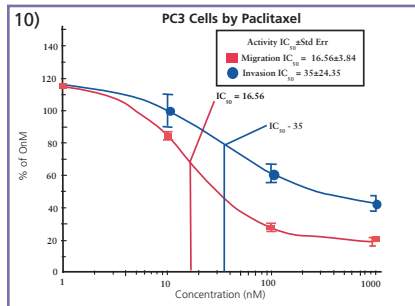
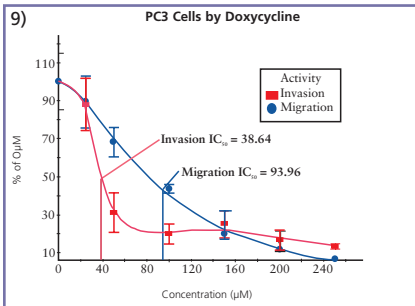
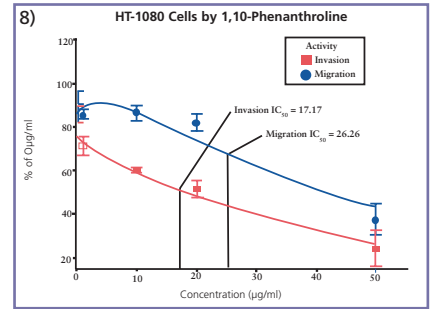
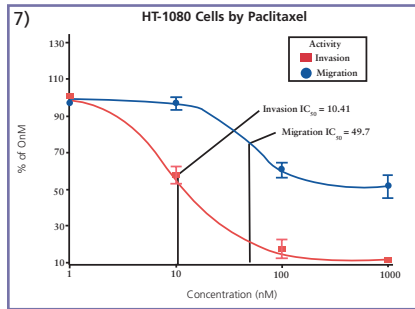
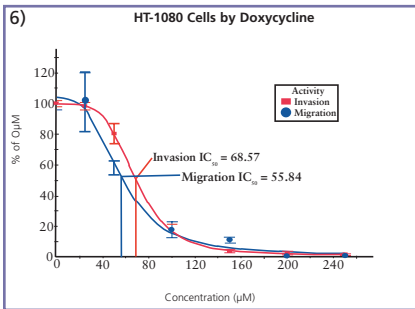


Figure 5: Lot-to-Lot Reproducibility. Three lots of coated inserts were evaluated in a single experiment. IC_{50} values were calculated using two order polynomial trendlines.



Figures 6-14: Inhibition of invasion/migration IC_{50} value results for HT-1080, PC3, and MDA-MB-231 cells.

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