Chemotaxis and Angiogenesis Assays in a 96-well Format for Use in High-Throughput Drug Discovery.

MILLIPORE

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Abstract

Purpose. To develop cellular chemotaxis and angiogenesis assays using cancer and endothelial cells in a 96-well format for use in high-throughput cell-based drug discovery applications.

Methods. Chemotaxis assays were performed using Millipore MultiScreen™-MIC (Migration, Invasion and Chemotaxis) 96-well plates using HB124 and K562 (hybridoma and lymphoblastic suspension cell lines) and MDAMB231 and MCF7 (migratory and non-migratory adherent breast cancer cell lines). Chemotaxis, in response to NIH3T3 fibroblast conditioned medium or serum-containing medium as chemoattractants, was assessed for 4-5 h on 3 and 5 µm membrane pore size MIC plates. Suspension cell chemotaxis was quantified using Calcein AM fluorescent label. Adherent cell chemotaxis was evaluated by staining cells migrated to membrane underside with a Hema-3® stain kit. The effects of chemotaxis inhibitors, Tamoxifen and Cytochalasin D, were also evaluated in adherent cell assays. Migrated cells were scanned and quantified with KS300 cell-counting software on a Zeiss Axioplan 2 microscope with an automated stage. Angiogenesis (tube formation) experiments were performed using HUVEC (human umbilical vein endothelial cells) on 5 µm pore size membrane plates pre-coated with extracellular matrix. Angiogenesis in response to EGM-2 (Endothelial cell growth medium) over 24 h was imaged with Zeiss Axiovision software.

Results. An average percent chemotaxis of 36.1 for HB124 and 8.7 for K562 cells was observed. Chemotaxis index of MDAMB231, calculated relative to non-invasive MCF7 cells, was atleast two fold or higher. Greater than 50 percent inhibition of chemotaxis was observed with 10-50 µM Tamoxifen and 5-20 µM Cytochalasin D. Robust tube formation by HUVEC cells was observed in response to EGM-2.

Conclusions. Our results demonstrate the applicability and versatility of a 96-well format for high throughput screening (HTS) of drugs in cell-based assays such as chemotaxis, inhibition and functional differentiation.

Introduction

Cell-based assays are gaining increasing importance in pre-screening of compounds to qualify target leads for drug discovery. These programs are increasingly focused on incorporating functional cell-based assays in the pre-screening stages of lead compounds. Many drugs under development are directed at altering the functional properties of cancer cells such as migration, chemotaxis and invasion. Cells that interact with these cancer cells, such as the Human Umbilical Vein Endothelial Cells (HUVEC), in invasive processes such as angiogenesis are also of interest as drug targets. Development of HTS cell-based assays that are designed to be able to measure effects of lead compounds on such functional processes pose a challenge. We have developed a 96 well MultiScreen-MIC plate to support these HTS cell-based assays. The data presented in this poster indicate that MultiScreen-MIC plates are useful for anti-cancer drug HTS and can serve as an alternative to existing, lower throughput methods.

Materials and Methods

Cell culture

HB124 cells were routinely cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). K562, MDAMB231 and MCF7 cells were cultured in RPMI with 10 % FBS. Sodium pyruvate and insulin were added as additional media supplements for MCF7 cell growth. HUVECs were propagated in EGM-2 complete medium.

Chemotaxis Assays

Cells were grown to 90 % confluency and starved overnight in serum-free media containing 0.2 % bovine serum albumin (BSA) prior to setting up chemotaxis experiments. Chemotaxis assays with HB124 and K562 cells (suspension cells) were set up on 3 µm pore size Millipore MultiScreen-MIC plates (Catalog # MAMIC3S10). 50,000 cells per well (in 50 µl of serum free medium) were added to upper wells and chemotaxis was measured in response to serum free-medium (to assess background migration) and serum-containing medium (to assess stimulated migration) added to bottom wells (150 µl volume). Chemotaxis assays were carried out over a period of 4 hrs at 37ºC. Cells that had migrated to lower wells were labeled with 1mM Calcein AM and quantified against a cell standard curve.

Chemotaxis assays with MDAMB231 and MCF7 cells (adherent cells, passage numbers 18-29 for MDAMB231 and 156-168 for MCF7) were set up on 5 µm pore size Millipore MultiScreen-MIC plates (Catalog # MAMIC5S10). 50,000 cells per well (in 50 µl of serum free medium) were added to the top wells and chemotaxis was measured in response to serum free-medium (to assess background chemotaxis) and serum-containing medium (to assess stimulated chemotaxis) added to bottom wells (150 µl volume). For inhibition experiments, inhibitors were added at various concentrations to cells in upper wells at the start of assay. Chemotaxis assays were carried out over a period of 4 hrs at 37ºC. The non-invaded cells were removed from the upper wells by swiping with cotton swabs followed by rinsing twice with phosphate buffered saline (PBS). Following the removal of cells from the upper wells, cells that had invaded to the membrane underside were enumerated by microscopy. For microscopic enumeration, membranes were stained with Hema-3 stain kit (Fisher Scientific). Stained cells were imaged and quantified with KS300 cell-counting software on a Zeiss Axioplan 2 microscope with an automated stage. Percent chemotaxis was calculated relative to the number of cells seeded per well. Background chemotaxis values were then subtracted to obtain stimulated chemotaxis values.

Angiogenesis Assays

For angiogenesis experiments, wells were coated with 400 µg of extracellular matrix and the matrix was polymerized at 37ºC. 10,000 cells (in PBS, 100 µl volume) were added to coated wells. Cells were allowed to adhere for 30 min at 37ºC. PBS was replaced with EGM-2 complete medium. Plates were incubated for 24 hrs at 37ºC. Tube formation was imaged using a Zeiss Axiovision software after staining wells with Hema-3 stain kit.

Figure 1. MultiScreen-MIC plates

Chemotaxis profiles of suspension cell lines on 3 µm MultiScreen-MIC plates

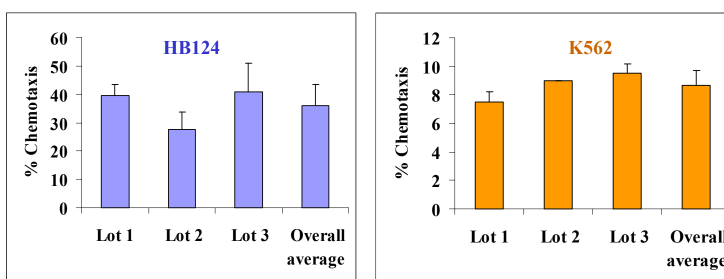

Figure 2. Chemotaxis in response to 10% serum-containing medium as a chemoattractant. Percent chemotaxis (stimulated chemotaxis) is calculated relative to number of cells seeded per well after subtracting out background chemotaxis value in response to 0.2% BSA containing medium. Migrated cells were evaluated using Calcein AM fluorescent label. Percent chemotaxis was calculated using a Calcein AM fluorescent cell reference curve for each cell line (per lot: n=5, r=24)

Inter-plate and Inter-lot variation on 3 µm MultiScreen-MIC plates

	Plate to Plate	Lot to Lot
Percent chemotaxis (HB124)	36	36
sd	9.1	7.4
Number of plates tested	15	5 plates per lot for lots 1, 2, 3
Number of replicates per plate	24	24

Table 1. Chemotaxis in response to 10% serum-containing medium as a chemoattractant. Data shows variability across plates and across lots for experiments performed on two consecutive days.

Chemotaxis of adherent cell lines on 5 µm MultiScreen-MIC membrane

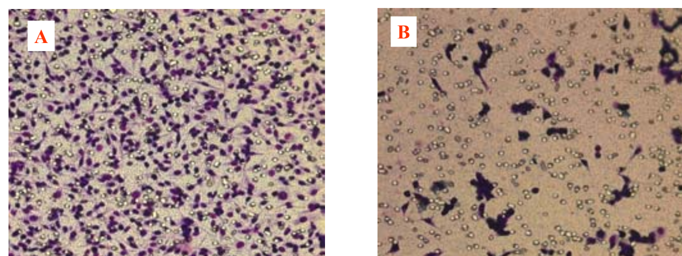

Figure 3 . Chemotaxis of (A) highly migratory MDAMB231 cells and (B) non-migratory MCF7 cells in response to 10% serum-containing medium as a chemoattractant. Migrated cells were visualized after staining as described in Materials and Methods.

Chemotaxis profiles of adherent cell lines on 5 µm MultiScreen-MIC plates

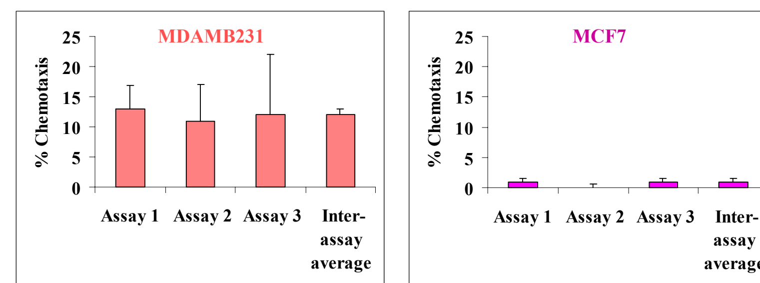

Figure 4. Chemotaxis in response to 10% serum-containing medium as a chemoattractant. Percent chemotaxis (stimulated chemotaxis) is calculated relative to number of cells seeded per well after subtracting out background chemotaxis value in response to 0.2% BSA containing medium. Data represents average percent chemotaxis obtained across two lots tested in three assays (per lot: n=5, per assay condition: r=6).

Chemotaxis profiles of adherent cell lines on alternative device inserts (5 µm)

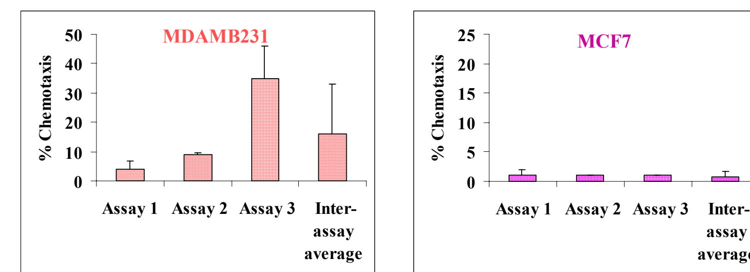

Figure 5. Chemotaxis in response to 10% serum-containing medium as a chemoattractant. Assay conditions were similar to the set-up on MultiScreen-MIC plates. Volume recommendations were as per manufacturer's instructions. Percent chemotaxis (stimulated chemotaxis) is calculated relative to number of cells seeded per well after subtracting out background chemotaxis value in response to 0.2% BSA containing medium. Data represents average percent chemotaxis obtained across three assays (per assay condition: n=2 to 4).

Chemotaxis inhibition of MDAMB231 in response to Tamoxifen and Cytochalasin D

Inhibitor	Concentration of Inhibitor	% Chemotaxis Inhibition of MDAMB231
Tamoxifen	20 µM	58 ± 35
Tamoxifen	50 µM	61 ± 36
Cytochalasin D	5 µM	67 ± 5
Cytochalasin D	10 µM	71 ± 30

Table 2. Chemotaxis in response to 10% serum-containing medium as a chemoattractant in the presence of inhibitors at indicated concentrations. Percent inhibition of chemotaxis is calculated relative to chemotaxis in absence of inhibitors (equated to 100 %) (n=2, r ≥ 3).

Inter-plate and Inter-lot variation on 5 µm MultiScreen-MIC plates

	Plate to Plate	Lot to Lot
Percent chemotaxis (MDAMB231)	5.7	5.2
sd	2.3	1.7
Number of plates tested	7	5 plates, lot 1 2 plates, lot 2
Number of replicates per plate	64	64

Table 3. Chemotaxis in response to 10% serum-containing medium as a chemoattractant. Data shows variability across plates and across lots for experiment performed on the same day. Percent chemotaxis (stimulated chemotaxis) is calculated relative to number of cells seeded per well after subtracting out background chemotaxis value in response to 0.2% BSA containing medium.

Angiogenesis (Tube formation) of HUVEC on 5 µm MultiScreen-MIC membrane

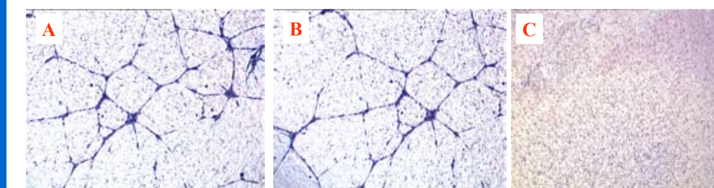

Figure 6. Angiogenesis (tube formation) experiments were performed using HUVEC on 5 µm pore size membrane MIC plates pre-coated with extracellular matrix (400 µg/well). Figure (A, B) demonstrates angiogenesis in response to EGM-2 over 24 h. This tube formation was imaged with Zeiss Axiovision software. No tube formation was observed in control wells (C).

Conclusions

Consistent results were obtained in chemotaxis assays with HB124 and K562 suspension cells and with MDAMB231 and MCF7 adherent cells across plates, lots and assays.

Comparison of adherent cell chemotaxis between the 96-well MultiScreen-MIC plates and alternative devices demonstrated that MultiScreen-MIC plates exhibited superior results, with less variation across assays performed on different days.

Chemotaxis inhibition was successfully performed on MultiScreen-MIC plates demonstrating the usefulness of the 96 well format for high throughput drug discovery screening which target such functional responses.

Excellent angiogenesis formation with HUVEC cells was achieved using MultiScreen-MIC plates demonstrating its utility for application in cell-cell interaction and co-culture assays.

Our results demonstrate the applicability and versatility of a 96-well format for high throughput screening (HTS) of drugs in cell-based assays such as chemotaxis, chemotaxis inhibition and functional differentiation and support the use of MultiScreen-MIC plates in such assays.

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