Combining Kinetic Ligand Binding and 3D Tumor Invasion Technologies to Assess Drug Residence Time and anti-Metastatic Effects of CXCR4 Inhibitors

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Introduction

Metastasis, the spread of cancer cells from the original tumor to secondary locations within the body, is linked to approximately 90% of all cancer deaths (Muller et al., 2001). The ability of cancer cells to detach from the primary tumor and to invade and metastasize has been associated with expression of specific chemokine receptors and their ligands, such as CXCR4 and CCR7, highly tightly correlated with the metastatic properties of breast cancer cell lines. In vivo, neutralizing the interaction of CXCR4 and its ligands, SDF-1α and SDF-1β, significantly impairs the metastasis of breast cancer cells and cell migration (Muller et al., 2001). Therefore, a phenotypic analysis of the CXCR4 inhibitor panel was then performed to determine whether changes in the level of tumor migration could be detected and if so, if compounds exhibiting longer residence times compared to SDF-1α exhibited a higher inhibitory effect on migration through the 3D matrix.

Materials

AM30100, AM30455, FT14016, CTCX-9508, FC-131, ITI and WE111 were purchased from Tocris Bioscience. Tag-lite® CXCR4 Stable Cell Line, SDF1-α-d2 and all labeling reagents used for the characterization on the inhibitors’ kinetic properties were from Cisbio Assays.

Methods

1- Kinetic Binding

All binding experiments were performed in 384-well loss volume, white bottom, round bottom, non-treated microplates (Corning Catalog No. 36196), using Gen5 and kinetic reading capabilities of the Synergy Neo. SDF1-α-d2 Association rate constant

The association and dissociation rate constant of labeled SDF-1α must be determined with reasonable precision. The values are later used in the equations developed by Motulsky & Mahan for the characterization of the unlabeled inhibitor panel.

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<th>Table 1. SDF1-α kinetic Characterization</th>
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2- Three-Dimensional (2D) Tumor Invasion

All tumor invasion experiments were performed in 16-well black, clear-bottom spheroid microplates, coated with the original Attachment surface (Corning Catalog No. 4322), using the live cell imaging capabilities of the Cytation 5.

Cell Preparation, Spheroid Formation and Mounding

Madin-Darby Bovine Kidney (MDBK) cells expressing CXCR4 and human normal dermal (Fibroblast expressing RFP) were combined to create final concentrations of 2.5x10⁵ cells/ml for each cell type. 100 µl of cell suspension was then pipetted into appropriate wells, and the plate placed in an incubator at 37 °C/CO₂. Spheroid formation was monitored for 24 hours. The plate was then transferred to the Cytation 5, pre-disabled at 37 °C/CO₂ using Gen5 as a gas control module. Imaging was performed using the brightfield channel. The typical spheroid generation period was 48 hours.

Invasion Matrix Preparation

Upon spheroid formation completion, 70 µl of complete medium was removed from each well, washed with an equal volume of invasion medium (serum-free, phenol red-free medium), and the spheroid placed on ice for 5 minutes to cool the cells. Cytoskeleton, Inc. (Permea Red-Free (Corning Catalog No. 3562371), was then placed on ice and diluted 1:2 in invasion medium containing unlabeled SDF1-α and CXCR4 inhibitors. With the plate still on ice, 70 µl of invasion medium with unlabeled SDF1-α and inhibitors was added to each well. Diluted Matrigel, 100 µl was then added as an overlay to each well. The plate was then centrifuged at 300 g for 5 minutes in a swinging bucket centrifuge that had been previously set to 4 °C for 5 minutes in a swinging bucket centrifuge that had been previously set to 4 °C for 5 minutes.

Tumor Invasion Assay

Using a 4x objective, exposure settings were optimized for the brightfield and fluorescent imaging channels. Following the optimization process, automated day 0 imaging was performed, and continued every 24 hours thereafter, to track tumor invasion. Cytation 5 analysis was performed with captured 4x images to track invasion in the presence or absence of CXCR4 inhibitors.

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<th>Table 2. CXCR4 Inhibitor Disinhibition Rate and Residence Time Values</th>
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Inhibitor Panel Kinetic Characterization

In the theory developed by Motulsky and Mahan, an unlabeled competitor is co-incubated with a labeled ligand during a kinetic association experiment. From the curve fitting, the kinetic constant of the unlabeled competitor may be derived. From the curve shape, qualitative assumptions about the binding constant may be found. If the competitor dissociates faster from its target than the ligand, the specific binding of the ligand will slowly and monotonically approach its equilibrium in time. However, when the competitor dissociates slower, the association curve of the ligand will consist of two phases, starting with a typical “overshoot” and then a decline until a new equilibrium is reached. The results of testing several known CXCR4 inhibitors are presented below.

Three-Dimensional Spheroid Model

As stated previously, interruption of the interaction between CXCR4 and its known ligand, SDF1-α, impairs metastasis of breast cancer cell migration (Muller et al., 2001). Therefore, a phenotypic analysis of the CXCR4 inhibitor panel was then performed to determine whether changes in the level of tumor migration could be detected and if so, if compounds exhibiting longer residence times compared to SDF-1α exhibited a higher inhibitory effect on migration through the 3D matrix.

Figure 1. Association binding graph of SDF1-α-d2. Binding increases over time until it plateaus after several minutes. The plateaus in an association experiment depends on the concentration of labeled SDF1-α used. Higher plateaus will be obtained with higher concentrations of SDF1-α, which was found to be true. Graph Pad Prism yields the observed association rate values for all concentrations tested or follow.

Figure 2. Saturation Binding & Law of Mass Action. In a saturation binding experiment, increasing concentrations of labeled SDF1-α result in increased binding. Saturation is obtained when no further change in binding is observed. In this case, if the system follows the Law of Mass Action then Koff increases linearly with increasing concentrations of SDF1-α. Non-linear association plots were used to derive association and dissociation rate constants from the linear regression line.

Figure 3. Kinetics of Competitive Binding. Examples of competitive binding graphs for CXCR4 inhibitors

Koff (µl/h) Kd (nM) StDev

SDF1-α-d2 1.342 3.125 0.25 12.5 20 50 100

Kon (nM⁻¹.s⁻¹) calculated from Kd=Koff/Kon

AM30100 2.09 0.12 3.51E-06

FT14016 2.28 0.44 2.40E-08

AM30455 2.38 0.09 1.00E-08

CTCE9908 0.933 0.025 0.0003

CCX20146 0.917 0.046 0.0005

AM3465 1.1 0.12 3.84E-06

Table 3. SDF1-α kinetic Characterization

Figure 4. Image-Based Monitoring of MDA-MB-231 Fibroblast Tumor Invasion. Overlaid brightfield and fluorescent images captured using a 4x objective and injection capabilities of the Synergy Neo allow accurate assessment: Brightfield – Total cells and invadopodia; GFP – Fibroblasts

Figure 5. Tumor Invasion Inhibition Quantification. (A) 4x overlaid images captured following 5 day 100 and 0 µM ITI incubation with tumoroids. Object masks automatically drawn by Gen5 using the following criteria. Threshold: 10000 RFP Min. Object Size: 400 µm Max. Object Size: 1500 µm; Image Smoothing Strength: 5 Background Flattening Box. (B) Graphs of individual tumoroid areas over day 0 and subsequent to five day incubation period with AM3465, MDA-MB-231 cells, RFP – Fibroblasts

1. The Tag-lite® CXCR4 ligand binding assay offers a simple, yet robust cell-based approach to determine kinetic binding of known receptor ligands, as well as competitive binding of test molecules

2. The simultaneous dual emission capture and injection capabilities of the Synergy Neo allow accurate calculation of kinetic association and dissociation rates.

3. Cytoskeleton Microplates provide an easy-to-use, consistent method to perform spheroid aggregation and 3D tumor invasion assays

4. Imaging of spheroid formation, as well as invading structures can be easily performed by the Cytation 5 using brightfield or fluorescent channels

5. The flexible cellular analysis capacity of the Gen5 Data Analysis Software allows for accurate assessment of 3D tumor invasion during the entire incubation period

6. The combination of assay chemistry, microscope, image-based monitoring, and cellular analysis provide an ideal method to better understand the target-based and phenotypic effects of potential inhibitors of tumor invasion and metastasis.