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 cancer-related deaths (Saxe, 2013). The CX chemokine system, including CXCR4 and CCR7, is highly correlated with the metastatic properties of breast cancer cells. In vivo, reevaluating the expression of CXCR4 and its known ligands, SDF-1α and SDF-1β, has provided new insights into metastasis (Ball, 2013). Traditionally, the discovery of novel agents has been guided by the affinity of the ligand for a specific receptor subtype, ignoring the broader aspects of the ligand-receptor interaction. However, awareness of the importance of binding kinetics has started to increase due to accumulating evidence (Jacquet, 2004; Capetulo et al., 2005; Tummino and Copeland, 2008; Zhang and Monsma, 2009). The focus on the temporal aspects of ligand-receptor binding may be attributed to the increasing importance of anti-metastatic drug discovery.

Similarly, appropriate in vitro cell models have also been lacking to accurately assess the ability of novel therapeutics to inhibit tumor invasion. Tumors in vivo are a three-dimensional (3D) mix of cell types, including cancer and stromal cells. As such, a valid in vitro model will include co-cultured cell types forming a tumor, providing a more predictive model than 2D monolayer cultures. Therefore, the development of well-established 3D models is crucial.

Here we present the ligand residence time of various CXCR4 inhibitors using a direct, homogeneous ligand binding assay and Cytation 5 expressing cell line in a kinetic format. This in vitro panel was further tested in a 3D tumor invasion assay to determine whether there is a correlation between the molecule's CXCR4 residence time and inhibition of the pharmacologic effect of tumor invasion. Cytation 5 is a modular multi-mode reader from BioTek that provides reagent-free fluorescence, allowing the assay to be optimized around fluorescent emissions when the excitation was set to 340 nm. The dual reagent injection capabilities enables optics to simultaneously detect the assay's 665 nm and 620 nm fluorescent emissions (Synergy® Neo Multi-Mode Reader).

Materials

Inhibitor Panel Kinetic Characterization

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 Motsiak & Mahan for the characterization of the unlabeled inhibitor panel.

Methods

BioTek Instrumentation

Synergy® Neo Multi-Mode Reader: Synergy Neo combines a filter- and microplate-based Assay Technology (MAT). The LIFERF software reader uses a high performance xenon arc lamp and dual PMTs in the filter-based optics. This dual-channel setup allows for simultaneous detection of excitation and emission from fluorescent emissions when the excitation was set to 340 nm. The dual reagent injection capabilities enables optics to simultaneously detect the assay's 665 nm and 620 nm fluorescent emissions (Synergy® Neo Multi-Mode Reader).

CytoSoft® 5 Cell Imaging Multi-Mode Reader: CytoSoft® 5 is a modular multimode microplate reader that provides a novel approach to homogeneous ligand microplate and microprobe detection. CytoSoft® 5 includes filter and microplate-based fluorescent reading, providing a 1:1:1 signal ratio for the labeled ligand to assay. The assay is performed at a standard temperature control to 65 °C (a 0.2 °C) CO2, constant temperature, and dual PMTs for detection in a standard, 384-well plate format. The instrument was used to image spheroids, as well as individual cell invasion through the Matrigel matrix.

Materials

To assess the ability of novel therapies to inhibit tumor invasion consistent with the previously determined residence time, AMD 3465 and IT1t, which exhibit a residence time longer than SDF-1β, effectively minimize tumor invasion in a dose dependent manner. In vitro compounds show little to no effect on the ability of the tumor to migrate through the 3D microenvironment. AMD 3465 treated tumors exhibit a higher inhibitory effect on tumor invasion compared to AMD 3465 treated tumors, indicating that the 3D spheroid invasion rate is correlated with the residence time of the ligand.

Conclusions

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 provide a novel assay technology to perform phenotypic screening of drug discovery.

2. The simultaneous dual emission capture and injection capacities of Cytation 5 provide accurate calculations of kinetic association and dissociation rates.

3. Cytosoft® 5 Image Microplate provides an easy-to-use, consistent method to perform spherical aggregation and 3D tumor invasion assays.

4. Imaging of spherical formation, as well as invading structure 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 the metastatic phenotype during the entire incubation period.

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

Figure 1: Association binding graph of SDF-1β. Binding increases over time until plateau after second minute. The plateau is an association experiment dependent on the presence of labeled SDF-1β. Higher concentrations will be obtained with higher concentrations. Fitting of the curves with Graph Pad Prism yields the observed association rate curves for all concentrations tested or fold.

Figure 2: Saturation Binding & Loss of Mass Assays. A saturation binding graph is presented for labeled SDF-1β to assess the binding of unlabeled SDF-1β. Saturation is achieved when no further binding can be observed. The kinetic constants for the receptor rate at saturation is reported in Table 2. If the system follows the Law of Mass action then Rik increases linearly with increasing concentrations of SDF-1β which is found to true. Graph Pad Prism software was used to derive association and dissociation rate constants from the assay.

Table 1: SDF-1α Kinetic Characterization

Table 2: CXCR4 Inhibitor Residence Rate and Residence Time Values

The 4x images displayed in Figure 4, as well as the graphs demonstrating total spheroid invasion coverage before and after the incubation period illustrate the ability of CXC4 inhibitors to inhibit tumor invasion consistent with the previously determined residence time. AMD 3465 and IT1t, which exhibit a residence time longer than SDF-1β, effectively minimize tumor invasion in a dose dependent manner. In vitro compounds show little to no effect on the ability of the tumor to migrate through the 3D microenvironment. AMD 3465 treated tumors exhibit a higher inhibitory effect on tumor invasion compared to AMD 3465 treated tumors, indicating that the 3D spheroid invasion rate is correlated with the residence time of the ligand.

References