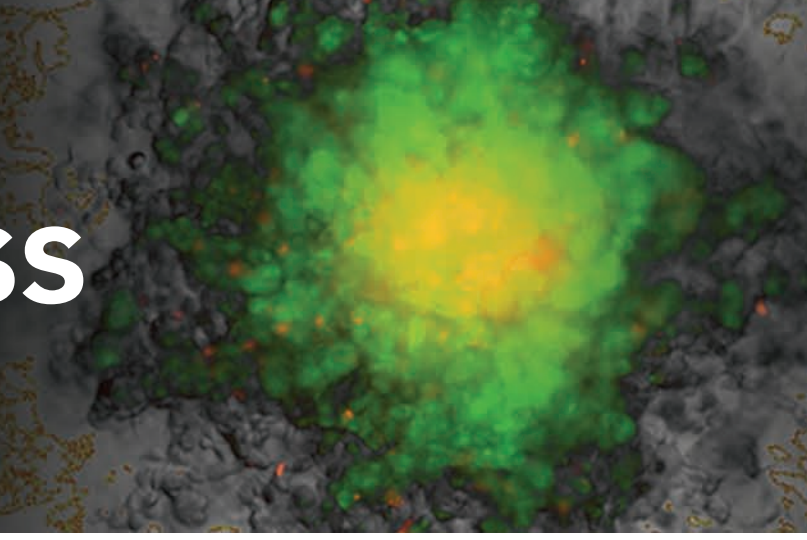


# Combining Kinetic Ligand Binding and 3D Tumor Invasion Technologies to Assess Drug Residence Time and anti-Metastatic Effects of CXCR4 Inhibitors



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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 deaths (Saxe, 2013). The expression of chemokine receptors, such as CXCR4 and CCR7, is tightly correlated with the metastatic properties of breast cancer cells. *In vivo*, neutralizing the interaction of CXCR4 and its known ligand, SDF1- $\alpha$  (CXCL12), significantly impaired the metastasis of breast cancer cells and cell migration (Muller *et al.*, 2001). Traditionally, the discovery of novel agents has been guided by the affinity of the ligand for the receptor under equilibrium conditions, largely ignoring the kinetic aspects of the ligand-receptor interaction. However, awareness of the importance of binding kinetics has started to increase due to accumulating evidence (Swinney, 2004; Copeland *et al.*, 2006; Tummino and Copeland, 2008; Zhang and Monsma, 2009) suggesting that the *in vivo* effectiveness of ligands may be attributed to the time a particular ligand resides at its receptor (Drug-Target Residence Time).

Similarly, appropriate *in vitro* cell models have also been lacking to accurately assess the ability of novel therapies to inhibit tumor invasion. Tumors *in vivo* exist as a three-dimensional (3D) mass of multiple cell types, including cancer and stromal cells (Mao *et al.*, 2013). Therefore, incorporating a 3D spheroid-type cellular structure that includes co-cultured cell types forming a tumoroid, provides a more predictive model than the use of individual cancer cells cultured on the bottom of a well in traditional two-dimensional (2D) format.

Here we examine the drug-target residence time of various CXCR4 inhibitors using a direct, homogeneous ligand binding assay and CXCR4 expressing cell line in a kinetic format. This inhibitor 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 phenotypic effect of tumor invasion. MDA-MB-231 breast adenocarcinoma cells, known to be invasive, and metastasize to lung from primary mammary fat pad tumors (Kamath *et al.*, 2001), were included, in addition to primary human dermal fibroblasts. The cells were aggregated into 3D structures using Corning Spheroid Microplates containing an Ultra Low Attachment surface. A novel cell imaging multi-mode reader was incorporated to provide PMT-based assessment of drug-target residence time, as well as automated image-based monitoring of tumor invasion through a basement membrane matrix. Cellular analysis algorithms provided accurate quantification of changes to the original tumoroid structure, as well as invadopodia development. The combination presents an accurate, yet easy-to-use method to assess target-based and phenotypic effects of new, potential anti-metastatic drugs.

## BioTek Instrumentation

**Synergy™ Neo Multi-Mode Reader:** Synergy Neo combines a filter- and monochromator-based detection system in one compact unit. The HTRF® certified reader uses a high performance xenon flash lamp and dual PMTs in the filter-based optics to simultaneously detect the assay's 665 nm and 620 nm fluorescent emissions when the excitation was set to 340 nm. The dual reagent injection capabilities enables kinetic analyses of known ligand and competitive binding.

**Cytation™ 5 Cell Imaging Multi-Mode Reader:** Cytation 5 is a modular multi-mode microplate reader that combines automated digital microscopy and microplate detection. Cytation 5 includes filter- and monochromator-based microplate reading; the microscopy module provides up to 60x magnification in fluorescence, brightfield, H&E and phase contrast. With special emphasis on live-cell assays, Cytation 5 features temperature control to 65 °C (37  $\pm$  0.2°C), CO<sub>2</sub>/O<sub>2</sub> gas control and dual injectors for kinetic assays. Shaking and Gen5 software are also standard. The instrument was used to image spheroids, as well as individual cell invasion through the Matrigel matrix.

## Materials

AMD3100, AMD3465, TF14016, CTCE-9908, FC-131, iT1t and WZ811 were purchased from Tocris Biosciences. Tag-lite® CXCR4 Stable Cell Line, SDF1- $\alpha$ -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 low volume, white, round bottom, non-treated microplates (Corning Catalog No. 3674), using the injection and kinetic reading capabilities of the Synergy Neo.

#### SDF1- $\alpha$ -d2 Association rate constant

The association rate constant of SDF1- $\alpha$ -d2 (Kon<sub>SDF1- $\alpha$ -d2</sub>) was determined by placing the CXCR4 expressing cells (5000 cells/well) in the presence of SDF1- $\alpha$ -d2 and measuring specific binding at various times thereafter. A total of seven [SDF1- $\alpha$ -d2] were tested, thus also allowing for calculation of the half saturation binding constant at equilibrium (Kd<sub>SDF1- $\alpha$ -d2</sub>)

#### SDF1- $\alpha$ -d2 Dissociation rate constant

The dissociation rate constant of SDF1- $\alpha$ -d2 (Koff<sub>SDF1- $\alpha$ -d2</sub>) was determined by adding to the previous mix a 100 fold molar excess of AMD3100, effectively blocking further binding of SDF1- $\alpha$ -d2 to its receptor.

#### Kinetic characterization of inhibitor panel

The association and dissociation rate constant of the different CXCR4 inhibitors were tested following the kinetic competitive binding protocols described elsewhere. All experimental data were analyzed by using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

### 2- Three Dimensional (3D) Tumor Invasion

All tumor invasion experiments were performed in 96-well black, clear-bottom spheroid microplates, coated with the Ultra Low Attachment surface (Corning Catalog No. 4520) , using the live cell imaging capabilities of the Cytation 5.

#### Cell Preparation, Spheroid Formation and Monitoring

MDA-MB-231 cells expressing GFP and human neonatal dermal fibroblasts expressing RFP were combined to create final concentrations of 2.5x10<sup>4</sup> cells/mL for each cell type. 100  $\mu$ L of cell suspension was then pipetted to appropriate wells, and the plate placed at 37 °C/5% CO<sub>2</sub>. Spheroid formation was monitored every 24 hours. The plate was placed into the Cytation 5, previously set to 37 °C/5% CO<sub>2</sub> using Gen5 as well as a gas control module. Focusing was performed using the brightfield channel. The typical cell aggregation period was 48 hours.

#### Invasion Matrix Preparation

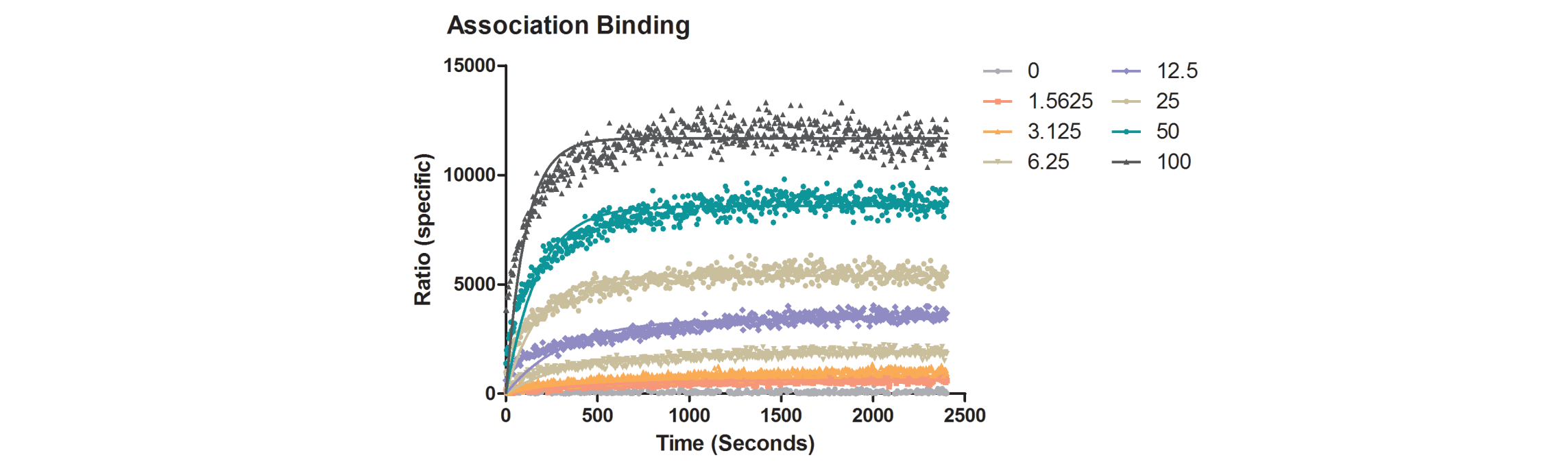
Upon spheroid formation completion, 70  $\mu$ L of complete medium was removed from each well, washed with an equal volume of invasion medium (serum-free, phenol red-free medium), and the spheroid plate placed on ice in a refrigerator for 5 minutes to cool the wells. Corning® Matrigel® Matrix, Phenol Red-Free (Corning Catalog No. 356237) was then thawed on ice and diluted 1:2 in invasion medium containing unlabeled SDF1- $\alpha$  ligand and CXCR4 inhibitors. With the plate still on ice, 70  $\mu$ L of invasion medium with unlabeled SDF1- $\alpha$  ligand and inhibitors was added to each well. Diluted Matrigel, 100  $\mu$ L, was then added as an overlay to each well. The plate was centrifuged at 300 x g for 5 minutes in a swinging bucket centrifuge that had been previously set to 4 °C for spheroid positioning, and then transferred to a 37 °C/5% CO<sub>2</sub> incubator for one hour to initiate gel formation.

#### Tumor Invasion Assay Performance

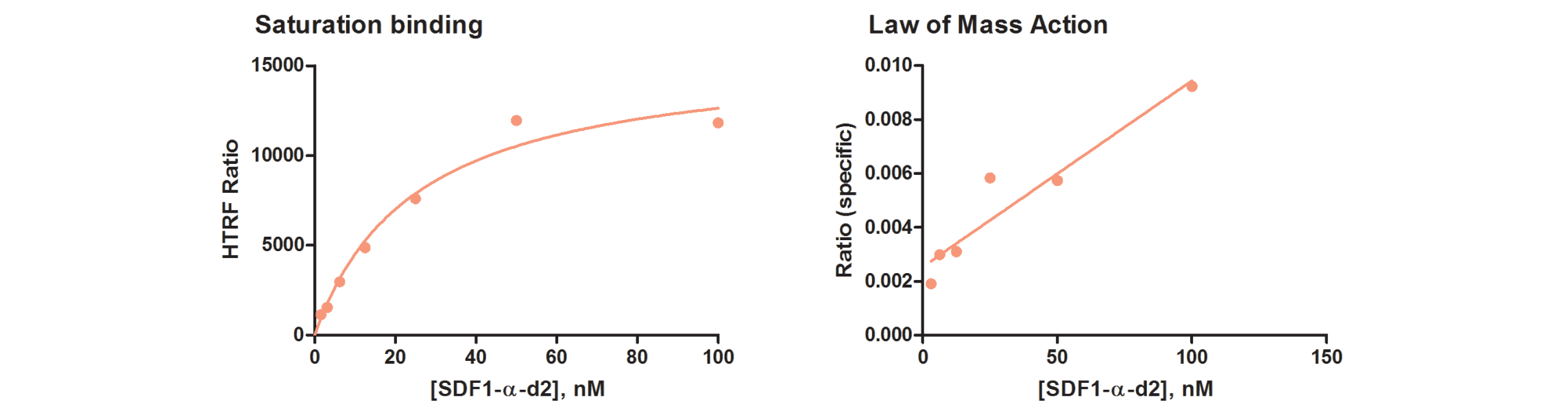
Using a 4x objective, exposure settings were optimized for the brightfield and fluorescent imaging channels. Following the optimization process, automated day 0 imaging was performed, and continued every 24 hours pursuant, to track tumor invasion. Cellular analysis was performed with captured 4x images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

## SDF1- $\alpha$ -d2 Kinetic Characterization

The association and dissociation rate constant of labeled SDF1- $\alpha$  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.



**Figure 1. Association binding graph of SDF1- $\alpha$ -d2.** Binding increases over time until it plateaus after several minutes. The plateau in an association experiment depends on the concentration of labeled SDF1- $\alpha$  used. Higher plateaus will be obtained with higher concentrations. Fitting of the curves with Graph Pad Prism yields the observed association rate values for all concentrations tested or Kobs.



**Figure 2. Saturation Binding & Law of Mass Action.** In a saturation binding experiment, increasing concentrations of labeled SDF1- $\alpha$  result in increased binding. Saturation is obtained when no further binding can be recorded. The ligand concentration that binds to half the receptor sites at equilibrium or Kd was 29nM. If the system follows the Law of Mass action then Kobs increases linearly with increasing concentrations of SDF1- $\alpha$ , which was found to be true. Graph Pad Prism software was used to derive association and dissociation rate constants from the linear regression line.

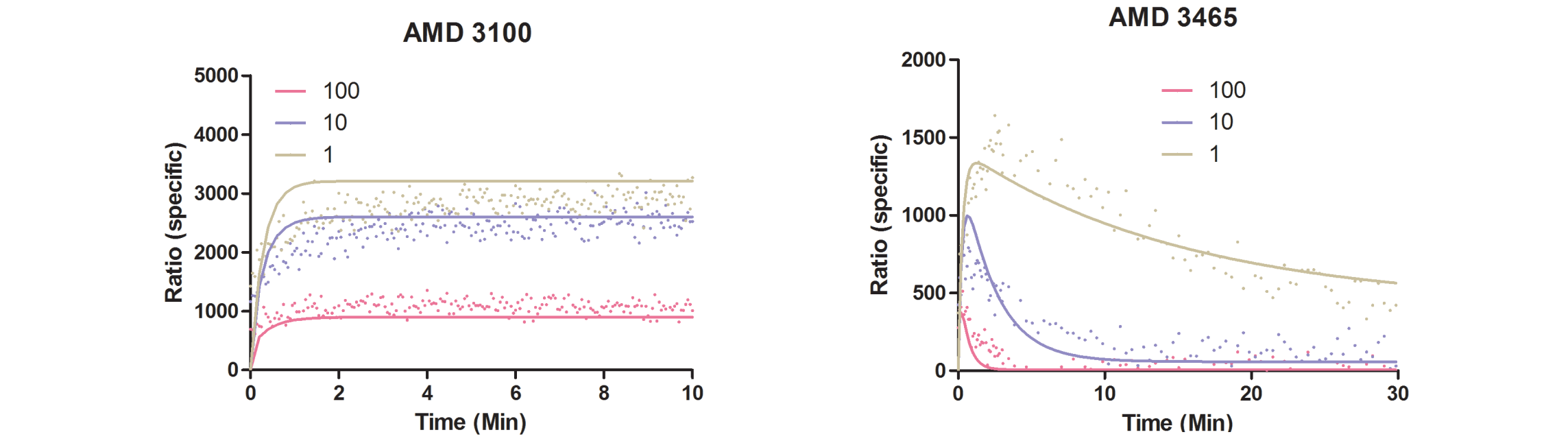
The Rate constant values experimentally found or mathematically derived are summarized in Table 1. Kon<sub>SDF1- $\alpha$ -d2</sub> and Koff<sub>SDF1- $\alpha$ -d2</sub> were 0.001 nM-1.S-1 and 0.04 S-1 respectively.

SDF1- $\alpha$ -d2 Kinetic Characterization							
[SDF1- $\alpha$ -d2] (nM)	1.562	3.125	6.25	12.5	25	50	100
Koff (S-1) Experience	0.045	0.031	0.034	0.041	0.042	0.043	0.034
Kobs (nM-1.S-1) Experience	0.003	0.006	0.007	0.11	0.012	0.015	0.019
Kon (nM-1.S-1) calculated from kobs=*[L]+Koff	0.03033	0.01201	0.00647	0.00415	0.00217	0.00115	0.00054
Kon (nM-1.S-1) calculated from Kd=Koff/Kon	0.0010						
Kd (nM) Experimental	29.3						

Table 1. SDF1- $\alpha$  Kinetic Binding Characterization

## 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 assumption regarding the constant may be found. In particular, 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.



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

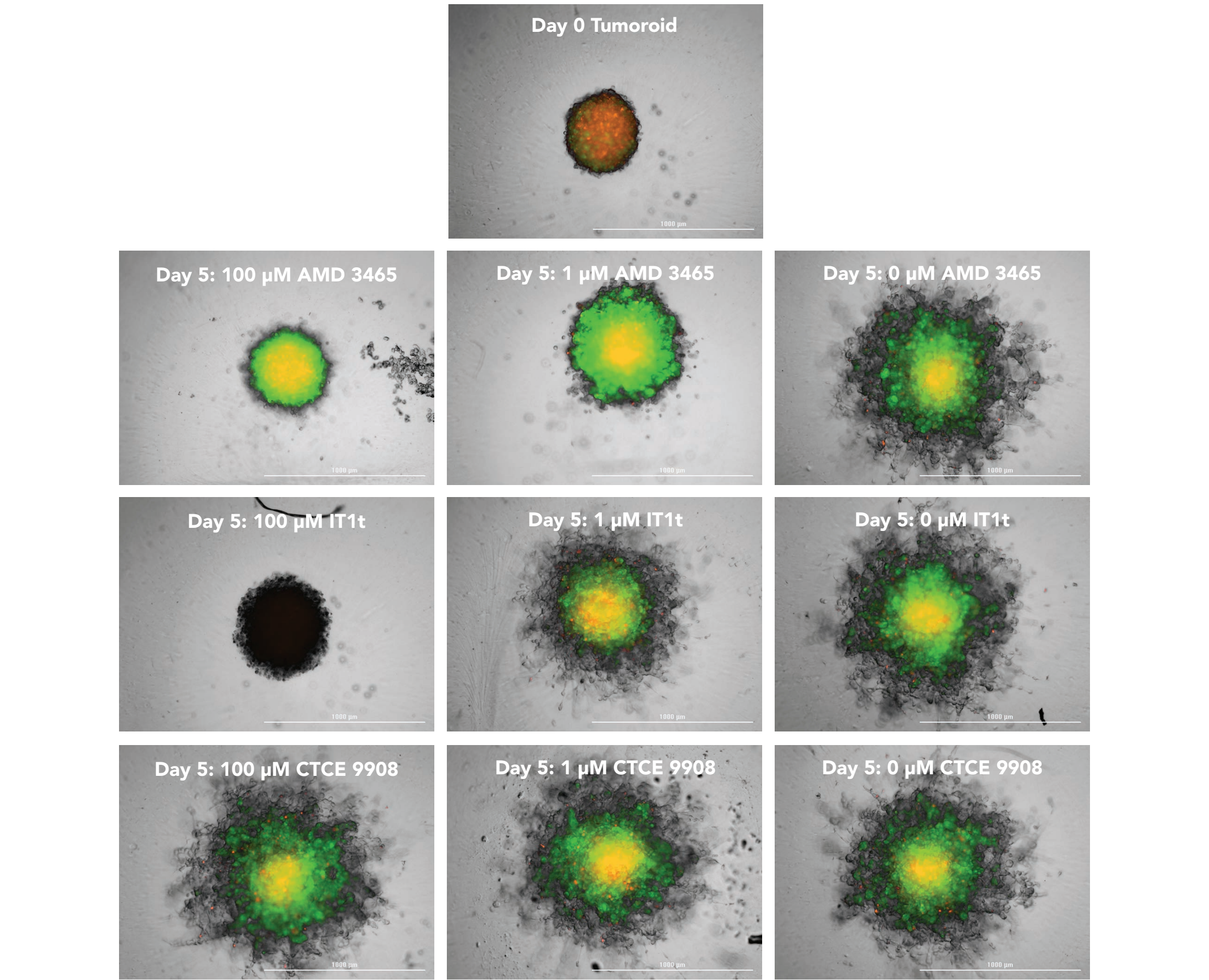
	Koff (Min-1)	R (Min)	Ki (M)
SDF1- $\alpha$ -d2	2.28	0.44	2.40E-08
AMD3465	0.003044	328.52	1.44E-10
IT1t	0.02844	35.16	1.93E-10
AMD3100	5.496	0.18	1.56E-08
CTCE9908	8.692	0.12	3.51E-06
FC131	ND	ND	1.52E-09

Table 2. CXCR4 Inhibitor Dissociation Rate and Residence Time Values

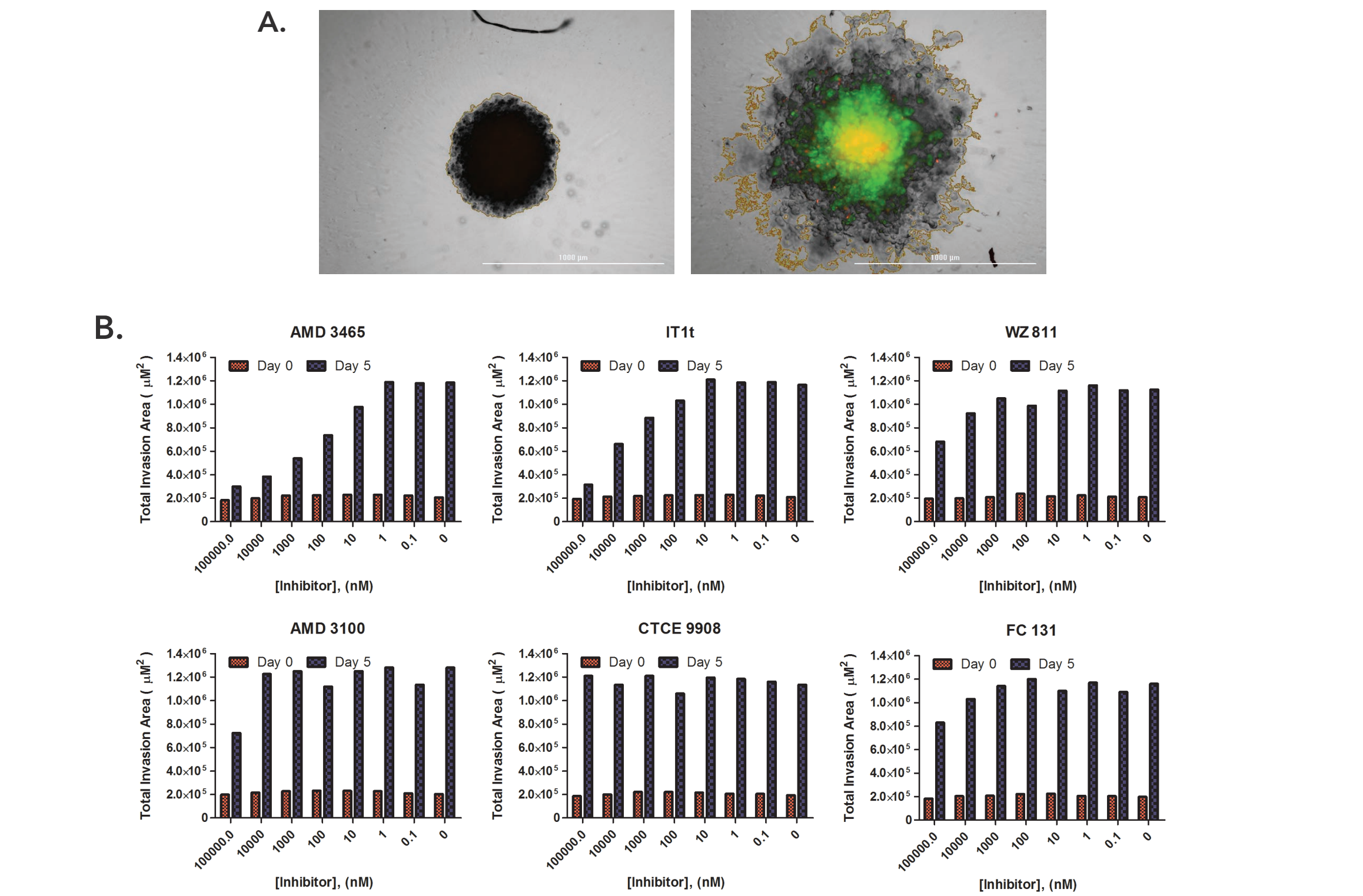
From the shape of the curves in Figure 3, and Koff results listed in Table 2, compounds such as AMD3100 dissociate faster than labeled SDF1- $\alpha$ . Others, such as AMD3465 dissociate slower. Lower Koff rates calculate to higher residence times of the molecule on the target receptor. AMD3465 and IT1t, whose residence times are greater than that of the SDF1- $\alpha$  ligand, may exhibit a stronger inhibitory response when used in a confirmatory phenotypic assay.

## Three-Dimensional Spheroid Model

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



**Figure 4. Image-based Monitoring of MDA-MB-231/Fibroblast Tumor Invasion.** Overlaid brightfield and fluorescent images captured using a 4x objective, after a 0 and 5 day incubation period with AMD 3465, IT1t, and CTCE 9908. Imaging channel representation: Brightfield – Total cells and invadopodia; GFP – MDA-MB-231 cells; RFP – Fibroblasts.



**Figure 5. Tumor Invasion Inhibition Quantification.** (A) 4x overlaid images captured following 5 day 100 and 0  $\mu$ M IT1t incubation with tumoroids. Object masks automatically drawn by Gen5 using the following criteria: Threshold: 5000 RFU; Min. Object Size: 400  $\mu$ m; Max. Object Size: 1500  $\mu$ m; Image Smoothing Strength: 0; Background Flattening Size: Auto. (B) Graphs of individual tumoroid areas on day 0, and subsequent to five day invasion period in the presence of inhibitor concentrations.

The 4x images displayed in Figure 4, as well as the graphs demonstrating total tumoroid area coverage before and after the incubation period illustrate the ability of CXCR4 inhibitors to interrupt tumor invasion consistent with the previously determined residence time. AMD 3465 and IT1t, which exhibit a residence time longer than SDF1- $\alpha$ , effectively minimize tumor invasion in a dose dependent manner. All other compounds show little to no effect on the ability of the tumoroid to migrate through the 3D matrix. While AMD 3465 and IT1t display the same sub-nanomolar potency, AMD3465 prevails as a CXCR4 inhibitor due to its greater residence time.

## Conclusions

- The Tag-lite CXCR4 ligand binding assay affords a simple, yet robust cell-based approach to determine kinetic binding of known receptor ligands, as well as competitive binding of test molecules
- The simultaneous dual emission capture and injection capabilities of the Synergy Neo allow accurate calculations of kinetic association and dissociation rates
- Corning Spheroid Microplates provide an easy-to-use, consistent method to perform spheroid aggregation and 3D tumor invasion assays
- Imaging of spheroid formation, as well as invading structures can be easily performed by the Cytation 5 using brightfield or fluorescent channels
- The flexible cellular analysis capacity of the Gen5 Data Analysis Software allows for accurate assessment of 3D tumor invasion during the entire incubation period
- The combination of assay chemistry, microplate, 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.