

The Effect of Small Molecule 390 on CXCR4 Receptors

Selam B. Zenebe-Gete, Shruti R. Topudurti, Andrew Shum, and Richard J. Miller

KEYWORDS. CXCR4 receptor, SDF-1, novel molecule 390, stem cells

BRIEF. Small molecule 390 was synthesized and is a possible agonist of the CXCR4 receptor.

ABSTRACT. CXCR4 is the chemokine receptor which aids in chemotaxis of stem cells, such as those in the bone marrow or the brain. SDF-1 is the natural ligand for the CXCR4 receptor. Similarities between novel molecule 390 synthesized by the Miller Lab and SDF-1 make this novel small molecule a possible agonist of the CXCR4 receptor. To determine whether 390 is an agonist to the CXCR4 receptor, we transfected cells with CXCR4 and exposed them to no agonist [vehicle control], SDF-1, or varying concentrations of our agonist drug. Next, we took calcium images using the dye fura-2, which indicates changes in calcium concentration in the cell due to CXCR4 activation. There was an increase in calcium in the cells when 390 was added, indicating that the receptor was being activated. When compared to the natural ligand, SDF-1, the levels were not as high, but they were higher than with the vehicle control. In the internalization assay, both SDF-1 and 390 internalized the receptor. The results suggest 390 is an agonist or partial agonist of the CXCR4 receptor. 390 may be the first small molecule agonist of CXCR4 receptors ever identified and may have a number of uses in medicine.

INTRODUCTION.

CXCR4 is a chemokine receptor, and is found on a variety cells, including cancerous and stem cells [1]. Chemokines are small signaling proteins, or cytokines, that are released by the cell to induce chemotaxis, or cell movement, in nearby cells. For example, in the immune response, damaged tissue releases chemokines that induce chemotaxis of white blood cells to the area of injury. As a CXC-type chemokine receptor, or a receptor that responds to CXC chemokines, CXCR4 aids in cell chemotaxis when it is stimulated by chemokines [2]. The chemotactic function of CXCR4 is demonstrated in the receptor's role of anchoring hematopoietic stem cells in the bone marrow [3]. Once it is activated by its natural ligand and chemokine, Stromal Cell Derived Factor 1 (SDF-1/CXCL12), the CXCR4 ligand stimulates the stem cells to undergo chemotaxis [4]. While an agonist activates a receptor, an antagonist prevents agonist-mediated stimulation of a receptor. Since these stem cells can be moved in the body through agonist interaction with CXCR4, discovering a small molecule receptor stimulant can potentially facilitate organ or tissue transplants [5]. Conversely, CXCR4 antagonists may be used to block the metastasis, or spread, of cancer cells by preventing them from moving into other parts of the body. Many types of cancer cells express the CXCR4 receptor and studies have shown that interference with the activation of the receptor by SDF-1 inhibited the spread of the cancer cells [3]. This receptor is also used by the HIV-1 virus to infect human body cells [6]. Finding an antagonist of the receptor could prevent the entry of HIV-1 into cells and, in turn, the spread of the virus; however, the usage of an agonist could still benefit treatment of HIV [7]. As mentioned earlier, CXCR4 agonists may be crucial in stem cell chemotaxis.

390 is a novel small organic molecule synthesized by the Miller Lab at Northwestern University, which we have now demonstrated may act on the CXCR4 receptor as an agonist. We thought it might be an agonist for CXCR4 because the structure was similar to prior known agonists of CXCR4. Previously, the only molecules that have been made to activate CXCR4 are large peptides; this means that 390 may be a more appealing drug candidate than other CXCR4 agonists. While other similar compounds are peptide based and have large molecular weights, molecule 390 is small and compact. This suggests that 390 may be both easier and cheaper to produce than other CXCR4 ago-

nist compounds, although peptide alternatives mass-produced in *E.coli* may be cheaper to produce.

During this experiment, we conducted two assays: a calcium imaging assay and a receptor internalization assay. These two experiments were chosen to target two of the characteristics of CXCR4 agonists. The calcium imaging assay was chosen because CXCR4 agonists, such as SDF-1, are known to signal via G proteins in the cell, which result in increased cellular calcium levels when the receptor is activated [8]. Hence, increased calcium levels can indicate activation of CXCR4 by an agonist. Also, internalization of the CXCR4 can occur when the receptor is overstimulated or desensitized i.e., during CXCR4 agonist stimulation on CXCR4 expressing cells [9]. Therefore, an increase in calcium as well as internalization of CXCR4 was used to confirm that drug 390 was an agonist of the receptor.

MATERIALS AND METHODS.

Cell Passage.

Cell passage using C8161 melanoma cells (passage 17-21) that already have CXCR4 expressed in them were used in the Calcium Imaging Assay. Human Embryonic Kidney 293 cells (HEK 293-passages 71) were used in the internalization assay. Standard Practices for trypsinization and cell culture were done, using Life Technologies DMEM, DMEM + 10% FBS + Pen/Strep, and poly-L-lysine.

Calcium Imaging Assay.

In the calcium imaging assay, after the cell passage of the C8161 melanoma cells, fura-2 and calcium buffer were added. An Olympus IX71 calcium imaging machine was used. A line well with calcium buffer was set up, providing the ability to add either drug 390 or SDF-1 to cells and wash it off afterwards, while recording the change in the calcium levels. Then, one of the coverslips was taken and placed on the stage of the confocal microscope attached to the calcium imaging machine. Calcium buffer warmed to 37°C was applied on top of the coverslip and then put it in the machine. Compound 390 or SDF-1 was then added at appropriate concentrations (10 µM, 1 µM, 0.1 µM, and 0.01 µM); once the observation of the change in calcium levels occurred, the cells were washed and we added four concentrations four concentrations (10 µM, 1 µM, 0.1 µM, and 0.01 µM) of Molecule 390 or AMD 3100, a known antagonist of CXCR4 [10]. Data from this assay were recorded and saved using MetaFluor Fluorescence Ratio Imaging Software. This data was averaged and graphed with Microsoft Excel. The experiment was conducted eight times.

Receptor Internalization Assay.

For the receptor internalization assay, after passaging the HEK 293 cells, they were transfected with cDNA for CXCR4-YFP. This version of CXCR4 has a yellow fluorescent tag attached to it. The cells were transfected by aspirating old media from cells and replacing it with DMEM + 10% FBS. This was done on cells that were 80-90% confluent. The cells were allowed to equilibrate for one to one and a half hours. Then in six different tubes, DMEM and CXCR4-YFP were mixed. These tubes were allowed to incubate for five minutes and then a Lipofectamine and DMEM mixture (8:1000) were added to the tubes. Afterwards, each tube was triturated for twenty to thirty seconds and incubated for twenty minutes with the caps open. Afterwards, we added the complex to cells and incubated for 48 hours. Then, four concentrations of drug 390 or SDF-1 were added to one well each, leaving one well for the negative control. These cells were incubated for two hours before taking the images of the cells. A confocal microscope was then used image the cells at 10x and 60x. DAPI was

used to stain the nucleus of the cells to differentiate between each cell after taking the images. After the images were taken, the program, "Fiji is just ImageJ," was utilized in order to quantify the data from the images. Regions of interests were then drawn around the cells and their periphery to obtain the integrated density of CXCR4 measured either internalized into the cell or expressed in the cell membrane. Next, the percentages of CXCR4 internalized and on the membrane of the cells were calculated using Microsoft Excel. This assay was conducted five times.

RESULTS.

Calcium Imaging Assay.

Life Technologies Fura-2 is a fluorescent dye that binds to calcium ions and emits a specific fluorescent signal in order to see the increase in the cellular calcium concentration when the experiment was performed. The calcium imaging assay reports a fluorescent ratio which is related to the intracellular calcium concentration using the average of 20 cells. From a baseline ratio of around 50% calcium, the calcium ratio increased to 60% (Fig. 1) following addition of SDF-1. In the second run of the calcium imaging assay (Fig. 1), there was a baseline of about 40% calcium. After the first application of 390, the Calcium levels increased to around about 70%. When AMD 3100 was applied and then 390, there was no increase in Calcium, clearly demonstrating that the agonist like effect of 390 was blocked by a well validated CXCR4 antagonist AMD 3100. Following washout of the AMD 3100 upon the third application of 390, the calcium levels increased from around 45% to around 75%, demonstrating that the block by AMD 3100 was reversible.

Receptor Internalization Assay.

In the receptor internalization assay, using the averages of at least 20 cells per treatment, it was found that in the control, 69% of the CXCR4-YFP was associated with the cell membrane while, 31% was internalized into the cytoplasm (Fig. 2). Following treatment with SDF-1, 10 μ M treatment, 1 μ M treatment, and .01 μ M treatment, the percentage of CXCR4 internalized increased, while the percentage of CXCR4 in the cell membrane decreased (Fig. 2). Thus 390 produced a dose dependent increase in the degree of CXCR4-YFP internalization.

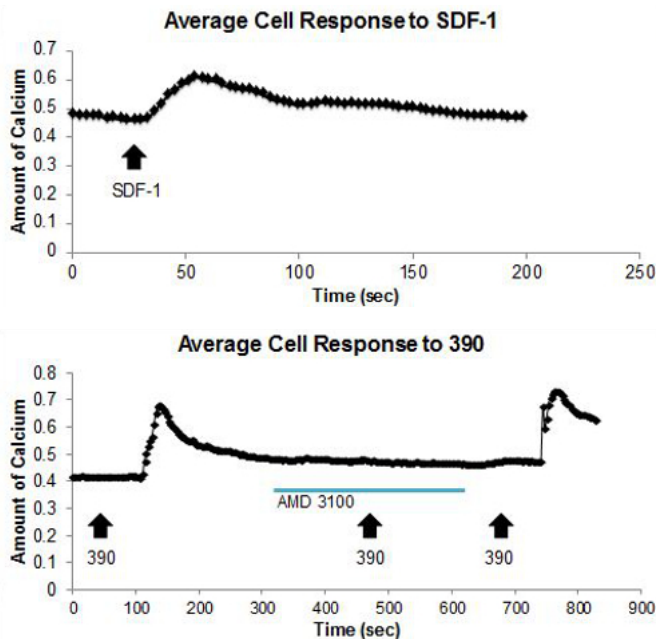


Figure 1. Top graph: Amount of calcium from CXCR4 transfected cells before and after application of SDF-1 at 30 seconds. Bottom graph: Average response of cells responding to application of small molecule 390 at 80 seconds, 525 seconds, and 725 seconds, which is indicated by black arrows. Notice that there is no increase in calcium in the presence of AMD 3100 (blue line).

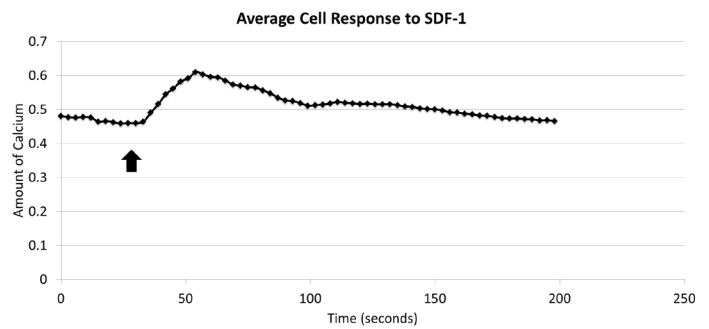


Figure 2. Average expression of CXCR4 in the periphery (dark blue) and internal (light blue) regions of the cell.

DISCUSSION.

Both the calcium imaging and receptor internalization assays that were conducted resulted in a promising future for small molecule drug 390 as an agonist at CXCR4 receptors. First of all, in the calcium images, an increase in the amount of calcium ions after the application of SDF-1 to the C8161 cells was observed (Figure 1). Since this increase in calcium ions is indicative of CXCR4 activation, this result was expected because SDF-1 is the natural ligand to CXCR4. After 390 was applied to the CXCR4 receptor at both 70 and 690 seconds, the concentration of calcium in the cell increased (Figure 2). In addition, drug 390 was applied to the cells in the presence of AMD 3100. AMD 3100 is a known antagonist, or inhibitor, of the CXCR4 receptor. Because it binds to the receptor and prevents anything else from stimulating it, calcium ion levels were expected to be unaffected in the presence of both AMD 3100 and 390. This hypothesis was confirmed by the result in Figure 2 during the time of AMD 3100 application, from 320 to 620 seconds. This confirmed that the increases in calcium that were seen after the application of 390 occurred as a result of the drug's agonist properties instead of as a byproduct of an outside process.

The results of the internalization assay are summarized by Figure 2. In the control trial, use of a confocal microscope allowed us to determine high levels of CXCR4 receptors on the periphery associated the cell membranes of the transfected HEK 293 cells. An image taken by a confocal microscope shows that, in the control trial, the majority of the CXCR4 receptor, labeled in yellow, was on the periphery of the cell. Unlike the control, SDF-1 application resulted in higher concentrations of CXCR4 on the inside of the cells. This indicates that the CXCR4 was internalized in the presence of SDF-1 as a result of desensitization and overstimulation of the receptor. After the addition of 10 μ M of drug 390, high levels of CXCR4 internalization were also observed (Fig. 2). This quantified data is visible in Figure 3, where numerous CXCR4 expressing cells exhibit high concentrations of CXCR4 inside of their cell membranes in vesicles in the cell cytoplasm. Similarly, the application of 1 μ M of molecule 390 led to high levels of CXCR4 internalization (Fig. 2). Images of the 1 μ M trial

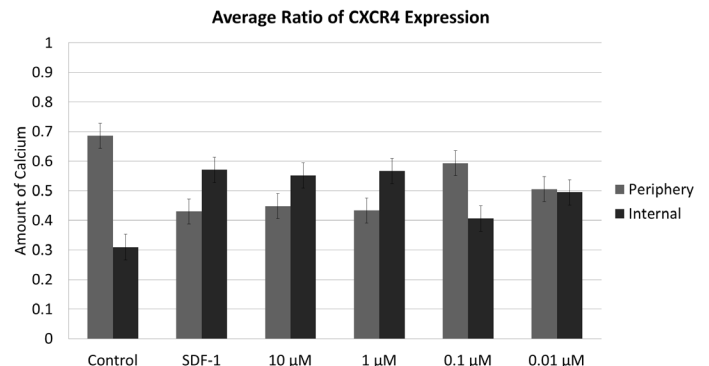


Figure 3. Receptor Internalization images of all treatments showing expression of CXCR4 (yellow) in C8161 melanoma cells with nuclei stained with DAPI.

showed many CXCR4 expressing cells with high levels of yellow inside of the cell, indicating receptor internalization (Fig. 3). While the 0.1 μM and 0.01 μM concentrations of 390 showed some internalization of CXCR4, the 10 μM and 1 μM trials produced images that were significantly more similar to the Figure 2, which showed CXCR4 internalization after the application of CXCR4. This led to the conclusion that small molecule 390 is an agonist or partial agonist of the CXCR4 receptor and is the most effective in higher concentrations and is not effective in the presence of AMD3100.

CONCLUSION.

In conclusion, novel small molecule 390 caused cell calcium ions concentration to increase in a dose dependent manner (Fig. 1). 390 also caused the internalization of CXCR4 from the cell membrane to the cytoplasm (Fig. 2).

FUTURE DIRECTIONS.

Although the results of the calcium imaging assay shows that 390 acts as an agonist of CXCR4 receptors, the fact that it eventually causes the receptor to internalize into cells may mean that it acts as a *de facto* antagonist when used *in vivo* through overall desensitization of receptor signaling. This possible antagonist effect *in vivo* could have several uses. When it is activated, CXCR4 holds stem cells in the bone marrow, where they are made. Anything that inhibits SDF-1 will allow for the removal of hematopoietic stem cells from the bone marrow and the use of these stem cells to help with organ transplants. In addition, the CXCR4 is used by HIV-1 to gain entry into cells. If the receptor is inhibited or internalized by a drug, the virus may be prevented from infecting the cell.

An interesting future study of CXCR4 involves monitoring the effects of the agonist drug *in vivo*. Because certain compounds that are verified as agonist in the lab setting have been shown to be antagonists in the body because they cause receptor internalization, seeing the effects of our small molecule agonist is used *in vivo* could affect in which situations 390 is administered.

ACKNOWLEDGMENTS. We would like to thank Dr. R. J. Miller and the rest of the Miller Lab researchers for their assistance in helping us to complete this project. Special thanks go out to Andrew Shum, Dr. John VanLeeuwen, Caroline Freitag, and Dongjun Ren. We would also like to thank Dr. Judith Scheppler and the Student Inquiry and Research faculty members for the opportunity to conduct research this year. In addition, we would like to recognize the National Institutes of Health for supporting this research through their funding.

SUPPORTING INFORMATION.

Supplemental Methods.

Figure S1. The image on the left is SDF-1, the natural ligand of CXCR4. On the right is the chemical structure of Molecule 390. The molecular weights of both compounds are included in the image.

Figure S2. The image on the right shows cells with a negative control at 10X magnification. The image on the left shows cells with a negative control at 60X magnification.

Figure S3. The image on the right shows cells with SDF-1 at 10X magnification. The image on the left shows cells with SDF-1 at 60X magnification.

Figure S4. The image on the right shows cells with 10 μM 390 at 10X magnification. The image on the left shows cells with 10 μM 390 at 60X magnification.

Figure S5. The image on the right shows cells with 1 μM 390 at 10X magnification. The image on the left shows cells with 1 μM 390 at 60X magnification.

Figure S6. The image on the right shows cells with 0.1 μM 390 at 10X magnification. The image on the left shows cells with 0.1 at 60X magnification.

Figure S7. The image on the right shows cells with 0.01 μM 390 at 10X magnification. The image on the left shows cells with 0.01 at 60X magnification.

REFERENCES.

1. Fricker SP, *et al.* *Biochemical Pharmacology.* 72 (2006).
2. Kucia M, *et al.*, *Journal of Molecular Histology.*35 (2004).
3. Harvey JR, *et al.*, *Clin Canc Res.*13 (2007).
4. Fernandis AZ, *et al.* *Oncogene.* 23, 1 (2004).
5. Xu, J, *et al.*, *J Heart Lung Trans.* 27,12 (2008).
6. Busillo JM and Benovic JL, *Biochim Biophys Acta.* 4 (2007).
7. Baleux F, *et al.*, *Nat Chem Biol.* 5 (2009).
8. Tchernychev B, *et al.*, *PNAS.* 107, 51 (2010).
9. Cheng Z, *et al.*, *J Biol Chem.* 24 (2000).
10. Hatse S, *et al.* *FEBS Letters.* 527 (2002).



Selam Zenebe-Gete and Shruti R. Topudurti are students at Illinois Math and Science Academy in Aurora, Illinois; they participated in research at Northwestern University.