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Fluorometric assays for the evaluation of chemokine receptor inhibitors with anti-HIV activity

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Background and objectives

In order to infect a target cell, the HIV envelope glycoprotein gp120 has to interact with both the cellular CD4 receptor and a chemokine receptor, CCR5 or CXCR4, the so-called HIV co-receptors. Several flow cytometric/fluorometric assays are developed in our lab and are very helpful in deciphering the mode of action and interaction site of several classes of HIV entry inhibitors, such as the chemokine receptor antagonists.

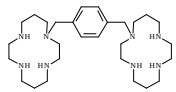

FIGURE 1. Chemical structure of the CXCR4 inhibitor AMD3100 (Mozobil, AnorMED)

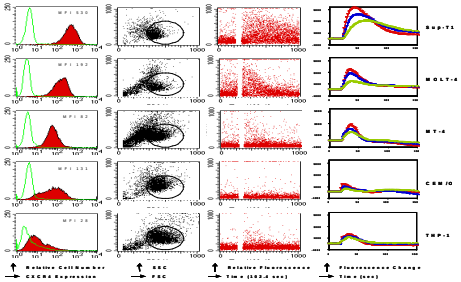

FIGURE 2. Evaluation of chemokine-induced intracellular calcium mobilization in five CD4+CXCR4+ cell lines using the FLIPR system compared with a flow cytometric assay. Correlation of calcium response with CXCR4 cell surface expression and cell viability. **First column**, flow cytometric analysis of the membrane expression of CXCR4 in the different cell lines. The red curves represent staining by the CXCR4-specific 12G5 mAb. The green curves represent aspecific background fluorescence. MFI = mean fluorescence intensity. **Second column**, forward and side angle scatter dot plot of Fluo-3 loaded cells. Viable cells were gated by FSC/SSC profile. **Third column**, SDF-1-induced intracellular calcium mobilization using the flow cytometric method. Fluo-3 loaded cells were stimulated with 100 ng/ml SDF-1 (t = 20 s). The transient increase in intracellular calcium concentration was recorded by monitoring the change in green fluorescence intensity of the cells as a function of time. **Fourth column**, SDF-1-induced intracellular calcium mobilization using the FLIPR system. Fluo-3 loaded cells were stimulated with SDF-1 at 500 ng/ml (red curve), 100 ng/ml (blue curve) and 20 ng/ml (green curve). The transient increase in intracellular calcium concentration was recorded by monitoring the change in green fluorescence intensity of the cells as a function of time.

Conclusion

Flow cytometric/fluorometric assays are very valuable in the search for CCR5 and CXCR4 inhibitors to combat HIV transmission and infection.

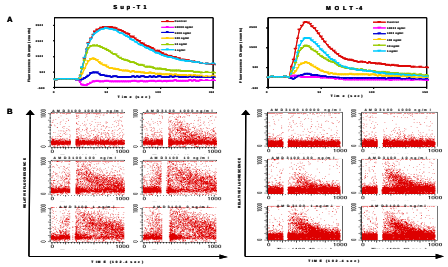

FIGURE 3. Concentration-dependent inhibition of SDF-1-induced intracellular calcium mobilization by AMD3100. Fluo-3 loaded Sup-T1 and MOLT-4 cells were preincubated for 15 min with AMD3100 and then stimulated with 20 and 100 ng/ml SDF-1/CXCL-12 respectively. The transient increase in intracellular calcium concentration was recorded by monitoring the change in green fluorescence intensity of the cells as a function of time using **A.** FLIPR (Molecular Devices) or **B.** flow cytometry (FACSCalibur, Becton-Dickinson).

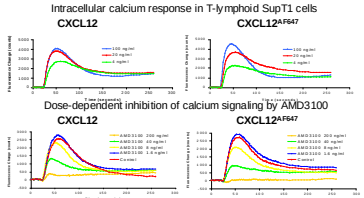

FIGURE 4. Dose-dependent intracellular calcium flux induced by unlabeled *versus* fluorescent CXCL12 and inhibition by the CXCR4 antagonist AMD3100. Sup-T1 cells were loaded with the fluorescent calcium indicator Fluo-3 and then stimulated with the chemokines at the indicated concentrations. The fluorescence change was monitored by the use of the Fluorometric Imaging Plate Reader (FLIPR). To evaluate the inhibitory effect of the CXCR4 antagonist AMD3100 on chemokine-induced intracellular calcium signaling, the cells were preincubated with AMD3100 at the indicated concentrations prior to stimulation with the chemokine (20 ng/ml). The red curves represent the positive control without AMD3100.

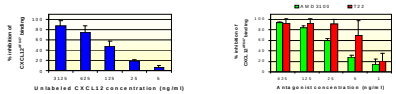

FIGURE 5. Inhibitory effect of unlabeled SDF-1/CXCL12 and CXCR4 inhibitors on the binding of $CXCL12^{AF647}$ in human T-lymphoid Sup-T1 cells. The cells were incubated with 25 ng/ml $CXCL12^{AF647}$ in the presence of increasing concentrations of unlabeled CXCL12 (left diagram) and the bicyclam AMD3100 (green bars) or the oligopeptide T22 (red bars) (right diagram). The bars represent the percentages of inhibition of $CXCL12^{AF647}$ binding in the presence of the inhibitor, relative to the positive control where the cells were exposed to 25 ng/ml $CXCL12^{AF647}$ alone.

References

Evaluation of SDF-1/CXCR4-induced Ca2+ signaling by fluorometric imaging plate reader (FLIPR) and flow cytometry. 2003. – K. Princen, S. Hatse, K. Vermeire, E. De Clercq & D. Schols. Cytometry 51, 35-45.
Fluorescent CXCL12AF647 as a novel probe for non-radioactive CXCL12/CXCR4 cellular interaction studies. 2004. – S. Hatse, K. Princen, S. Liekens, K. Vermeire, E. De Clercq & D. Schols. Cytometry, 61, 178-186.

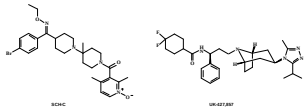

FIGURE 6. Chemical structures of the CCR5 inhibitors SCH-C (Vicriviroc, Schering-Plough) and UK-427,857 (Maraviroc, Pfizer)

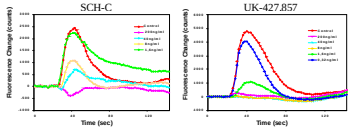

FIGURE 7. Concentration-dependent inhibition of LD78beta-induced intracellular calcium mobilization by SCH-C and UK-427,857 in U87.CD4.CCR5-transfected cells.

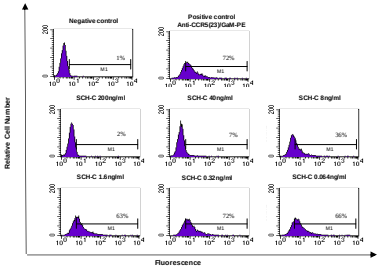

FIGURE 8. Red fluorescence histograms of CEM.CCR5-transfected cells stained with an specific anti-CCR5 mAb (2D7, R&D Systems) in the presence of different concentrations of the CCR5 inhibitor SCH-C. The % of CCR5 positive cells (defined by M1) are indicated in each histogram.

Results

The chemical structures of the specific CXCR4 inhibitor, AMD3100, and two specific CCR5 inhibitors, SCH-C and UK-427,857 are shown in Fig. 1 and Fig. 6 respectively. Flow cytometric and fluorometric calcium mobilization assays in five different cell lines were compared in Fig. 2. The SDF-1/CXCL-12-induced calcium signaling was correlated, although not strictly, with the expression of the CXCR4 receptor. The higher the expression of CXCR4 (MFI values), the better the calcium responses induced by CCXCL-12. In Fig. 3 are the inhibitory potential of the CXCR4 antagonist AMD3100 shown in two different T cell lines (Sup-T1 and MOLT-4) by fluorometric (FLIPR) and flow cytometric assays. Both assays show the inhibition of calcium signaling by the CXCR4 inhibitor, but is better qualified by the FLIPR. Fig. 4 shows the calcium signaling induced by the unlabeled and a fluorescent labeled CXCL12 and its dose-dependent inhibition by AMD3100. Fig. 5 showed that the unlabeled CXCL12 and the CXCR4 inhibitors AMD3100 (AnorMed) and T22 (Bachem, Germany) blocked the binding of the fluorescent SDF-1/CXCL12 to T cells with IC_{50} values of 92, 13 and 8 ng/ml, respectively. Fig. 7 shows the dose-dependent inhibition of the CCR5-induced calcium signaling by a specific CCR5 ligand LD78-beta and the inhibition of two different CCR5 antagonists. The effect of SCH-C on the binding of a specific CCR5 mAb is shown in Fig. 8.