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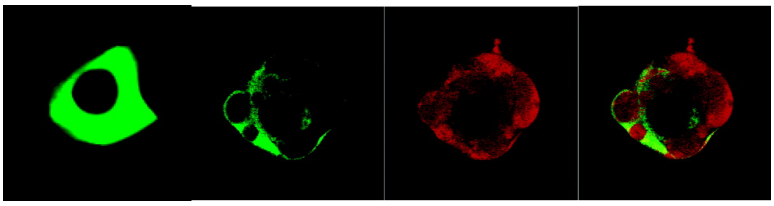

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Real Time Differentiation of G-Protein Coupled Receptor (GPCR) Agonist and Antagonist by Two Photon Fluorescence Laser Microscopy

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Membrane proteins are highly interesting and important drug targets since many of them serve as receptors and are involved in intercellular communication and in control of intracellular functions critical for all aspects of health and disease. The melanocortin hormone system consists of melanotropin peptides derived from the proopiomelanocortin gene, five melanocortin receptors, two endogenous antagonists, and two ancillary proteins.[1] Recent pharmacological and genetic studies have affirmed the role of melanocortins in pigmentation,[1] inflammation,[2] energy homeostasis,[3−5] sexual function,[6,7] and other fundamental biofunctions. Development of selective agonists and antagonists will further facilitate the investigation of these complex physiological functions and provide an experimental basis for new pharmacotherapies.

Recent studies have demonstrated that the hMC4R (human melanocortin 4 receptor) is associated with obesity and sexual dysfunction,[8] whereas the hMC3R plays a pivotal role in the energy homeostasis.[9,10] Selective peptide or non-peptide agonists of the hMC4 and hMC3 receptors can be excellent drug candidates, and many research groups in both industry and academia are making enormous progress toward this goal. Currently, screening of melanocortin-based drugs use radiolabeled ligands or indirect methods based on reporter genes. To overcome the limitations of these methods, we have developed a rapid, sensitive, and direct method using two-photon fluorescence laser microscopy (TPFLM) and confocal microscopy to differentiate agonists and antagonists of melanocortin receptors in real time.

In the past decade, TPFLM has been developed[11] and recently has been used in studies of cellular signaling. Since the rate of a molecule absorbing two photons simultaneously is proportional to the square of the incident intensity, the probability of two-photon excitation falls off as the fourth power of distance from the focal plane. Thus, it is possible to independently image planes at different depths and produce three-dimensional images of cells. TPFLM has several advantages over a confocal scanning laser or conventional epifluorescence microscopy because it reduces out-of-focal-plane photobleaching of the fluorophore, obviates the need for a confocal aperture to enhance the signal-to-noise ratio, and uses near-infrared light to excite a chromophore, which can penetrate deeper into specimens than visible or UV light.[11]

For our studies, a functionalized rhodamine dye (tetramethylrhodamine-5-(and-6)-isothiocyanate, (5(6)-TRITC) mixed isomers) was introduced at the N-terminal of the universal melanotropin agonist for melanocortin receptors MTII (Ac-Nle-Asp-c[His-D-Phe-Arg-Trp]-NH_2) and at the N-terminal of SHU-9119[12−15] (Ac-Nle-Asp-c[His-D-Nal (2′)-Arg-Trp]-NH_2) an antagonist for the hMC3R and hMC4R, but an agonist for the hMC1R and hMC5R. To reduce potential interactions between the dye and the pharmacophore of melanocortin receptors, a 6-aminohexanoic acid linker was used (Figure 1). Labeled drugs were synthesized using standard SPPS (solid-phase peptide synthesis) with an N^{α}-Fmoc strategy.[16,17] Conformational profiles of the peptides with the dye were investigated by Macromodel's (MacroModel 8.1) large-scale low mode (LLMOD) procedure.[18] It was evident that the rhodamine dye was quite separated from the core pharmacophore sequence (His-D-Phe/D-Nal(2′)-Arg-Trp) of Rho-MTII/Rho-SHU9119 (Figure 2). Furthermore, pharmacological binding and functional assays were performed on transfected HEK293 cells which stably express the melanocortin receptors.[19,20] For the assay, a dose−response curve was obtained using unlabeled MTII and SHU-9119, and rhodamine-labeled MTII (Rho-MTII) and SHU9119 (Rho-SHU-9119). The dose response curves for the unlabeled and labeled drugs gave binding constants as follows (MTII/Rho-MTII for hMC1R IC_{50}: 0.8 nM/1 nM; hMC3R IC_{50}: 1.3nM/1.4nM; hMC4R IC_{50}: 2.8nM/

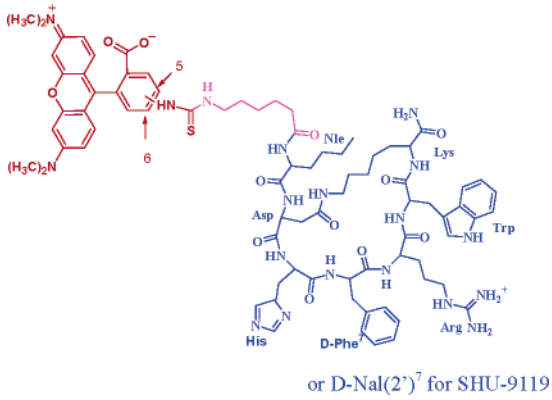

Figure 1. Structure of super-potent agonist Rho-MTII. Blue is MTII, purple is linker, and red is rhodamine. In case of the antagonist (SHU-9119) D-Nal $(2')^7$ is substituted for D-Phe^7 of MTII.

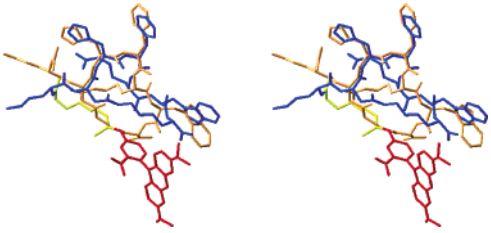

Figure 2. Large-scale low mode (LLMOD) conformations of superimposed MTII (orange) and Rho-MTII (blue) show that the dye stays on the other side of the core sequence (His-D-Phe-Arg-Trp) of MTII. Similar results were seen in the superimposed conformations of SHU-9119 and Rho-SHU-9119.

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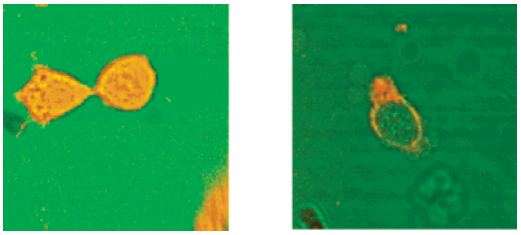

Figure 3. Real-time TPFLM images of HEK-293 cells which stably expressed human melanocortin receptor 4 (hMC4R) with its agonist (Rho-MTII) within 10-min treatment with the drug (2 nM) (left) and antagonist (Rho-SHU9119) 30 min after treatment with the drug (2 nM) (right). Laser excitation was at 830 nm.

3nM; hMC5R IC_{50}: 7.4 nM/7.5 nM) all of which are within experimental error, which demonstrates that the rhodamine dye does not significantly modify the biological activities of the ligands at the human melanocortin receptors. Comparable results were obtained for SHU9119/Rho-SHU9119 (not shown).

Two-photon fluorescence laser scanning microscopy imaging was performed on various transfected HEK293 cells, which have stably expressed melanocortin receptors, by using the labeled drugs Rho-MTII or Rho-SHU9119. Control experiments performed with HEK293 cells that were not transfected with hMCRs showed no labeling of cells. In addition, free rhodamine dye also was tested with the cloned melanocortin receptor cell lines as a further control, and these experiments also showed no significant labeling. Images were obtained at 1-min intervals and after 30 min, *Z*-scan images were taken. It was found that the fluorescence labeled agonist Rho-MTII was distributed throughout the cells expressing hMC4R, within 10 min, whereas the antagonist Rho-SHU9119 remained completely on the cell surface even after 30 min (Figure 3). Similar results were observed using other subtypes of melanocortin receptors (hMC1R, hMC3R, and hMC5R) (data not shown). Interestingly, Rho-SHU-9119, which is an agonist for both the hMC1R and hMC5R, internalized in these subtypes of MC receptors (not shown) as was observed for the universal agonist MTII. This demonstrates that indeed SHU9119 is an agonist at the hMC5R, which was the reverse at hMC3R and hMC4R. Experiments also have been done with other GPCRs (e.g., human opioid delta receptor), and similar observations were made. The results of these studies suggest that it is possible to differentiate agonists and antagonist of the melanocortin system or other GPCRs in real time by the versatile technique of TPFLM. It is important to note that the fluorescent intensity and the concentration of the labeled peptide followed in a manner analogous to a dose−response.

Experiments were also performed on HEK293 cells transfected with β-arrestin 1 fused with green fluorescent protein (β-Arr-1−GFP), and β-arrestin 2 fused with green fluorescent protein (β-Arr-2−GFP) along with the hMC4R. Confocal laser fluorescence imaging of the excited GFP emission in cells transfected with hMC4R and β-Arr-2−GFP, with and without MTII, is shown in Figure 4. Prior to the addition of MTII, β-Arr-2−GFP was observed to be distributed throughout the cell; however, 10 min after addition of MTII the β-Arr-2−GFP was highly localized on the cell surface. Similar results were obtained with β-Arr-1−GFP. Imaging of transfected cells after treatment with SHU9119 showed no change in the spatial distribution of the β-Arr-1−GFP and β-Arr-2−GFP.

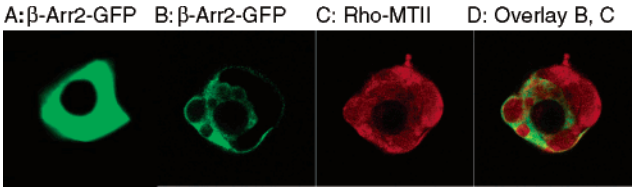

Figure 4. Agonist-mediated relocalization of β-Arr2−GFP at the cell surface in HEK293 transfected with hMC4R and β-Arr2−GFP. (A) Transient transfected β-Arr2−GFP along with hMC4R in HEK293 cells. (B−D) Split view of transient transfected β-Arr2−GFP along with hMC4R in HEK293 cells induced by treatment with agonist Rho-MTII (2 nM) after 10 min.

These results suggest that imaging of β-Arr−GFP expressed by transfected cells also can be used to differentiate agonists from antagonists of GPCRs. The imaging results indicate that β-arrestin mediates internalization of the hMC4 receptor, similarly to what has been observed for β-arrestin in other 7-trans membrane protein receptors.[21] Additional studies including internalized (acid-resistant) binding assays[22] as well as the imaging of the cytosolic protein β-arrestin and G-protein receptor kinase provide information on the mechanism for the agonist-mediated internalization, the details of which will be reported separately.

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