# Internalization of $sst_2$, $sst_3$, and $sst_5$ Receptors: Effects of Somatostatin Agonists and Antagonists

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The uptake of radiolabeled somatostatin analogs by tumor cells through receptor-mediated internalization is a critical process for the in vivo targeting of tumoral somatostatin receptors. In the present study, the somatostatin receptor internalization induced by a variety of somatostatin analogs was measured with new immunocytochemical methods that allow characterization of trafficking of the somatostatin receptor subtype 2 ($sst_2$), somatostatin receptor subtype 3 ($sst_3$), and somatostatin receptor subtype 5 ($sst_5$) in vitro at the protein level. **Methods:** Human embryonic kidney 293 (HEK293) cells expressing the $sst_2$, $sst_3$, or the $sst_5$ were used in a morphologic immunocytochemical internalization assay using specific $sst_2$, $sst_3$ and $sst_5$ antibodies to qualitatively and quantitatively determine the capability of somatostatin agonists or antagonists to induce somatostatin receptor internalization. In addition, the internalization properties of a selection of these agonists have been compared and quantified in $sst_2$-expressing CHO-K1 cells using an ELISA. **Results:** Agonists with a high $sst_2$-binding affinity were able to induce $sst_2$ internalization in the HEK293 and CHO-K1 cell lines. New $sst_2$ agonists, such as Y-DOTA-TATE, Y-DOTA-NOC, Lu-DOTA-BOC-ATE (where DOTA is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; TATE is [$Tyr^3$, $Thr^8$]-octreotide; NOC is [1-$Nal^3$]-octreotide; and BOC-ATE is [$BzThi^3$, $Thr^8$]-octreotide), iodinated sugar-containing octreotide analogs, or BIM-23244 were considerably more potent in internalizing $sst_2$ than was DTPA-octreotide (where DTPA is diethylenetriaminepentaacetic acid). Similarly, compounds with high $sst_3$ affinity such as KE108 were able to induce $sst_3$ internalization. In $sst_2$- or $sst_3$-expressing cell lines, agonist-induced receptor internalization was efficiently abolished by $sst_2$- or $sst_3$-selective antagonists, respectively. Antagonists alone had no effect on $sst_2$ or $sst_3$ internalization. We also showed that somatostatin-28 and somatostatin-14 can induce $sst_5$ internalization. Unexpectedly, however, potent $sst_5$ agonists such as KE108, BIM-23244, and L-817,818 were not able to induce $sst_5$ internalization under the same conditions. **Conclusion:** Using sensitive and reproducible immunocytochemical methods, the ability of various somatostatin analogs to induce $sst_2$, $sst_3$, and $sst_5$ internalization has been qualitatively and quantitatively determined. Whereas all agonists triggered $sst_2$ and $sst_3$ internalization, $sst_5$ internalization was induced by natural somatostatin peptides but not by synthetic high-affinity $sst_5$ agonists. Such assays will be of considerable help for the future characterization of ligands foreseen for nuclear medicine applications.

**Key Words:** somatostatin receptors; receptor internalization; antagonist; tumor targeting; receptor immunocytochemistry

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Interest in somatostatin and somatostatin analogs is increasing, largely because of the success of in vivo targeting of somatostatin receptors in tumors (*1*). In this clinical application, not only is binding of the radiolabeled somatostatin analogs to the receptor important but also internalization of the receptor–ligand complex for successful in vivo targeting of tumoral peptide receptors using radiopeptides (*1,2*). Therefore, during the course of optimal development of new radiopeptide analogs for in vivo receptor targeting, peptides need to be tested not only for receptor binding and biodistribution but also for their receptor internalization properties.

Most of the internalization studies performed with radiopeptides, including radiolabeled somatostatin analogs, have been done with methods that measure internalization of the radioligand but not of the receptor itself (*3–8*). Although such methods give a good indication of the internalization capability of a given radioligand, they are not always easy to interpret because of the extremely complex mechanisms of intracellular receptor trafficking and intracellular processing of the internalized radioligand (*9*); furthermore, in these radioligand internalization studies, receptor internalization can be quantitated only at subsaturating ligand concentrations, rather than at a large range of agonist

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concentrations and receptor occupancies. Finally, the role of agonists versus antagonists in the internalization process could not be thoroughly investigated in the previous studies: Although there is a consensus that antagonists generally do not trigger the internalization of G-protein–coupled receptors (*10*), examples exist of peptide receptor antagonists that do stimulate internalization, such as cholecystokinin-, 5-$HT_{2A}$-, endothelin-, and neuropeptide Y–analogs (*10–13*). In the somatostatin receptor field, a recent report indicated that somatostatin receptor agonists, but not somatostatin receptor antagonists, are able to internalize the somatostatin receptor subtype 2 ($sst_2$) (*14*). Many studies describing new radiopeptides for in vivo targeting do not give experimental evidence of whether these radioligands are agonists or antagonists.

Somatostatin action is mediated by 5 somatostatin receptors (*15*). However, not all will equally internalize on agonist binding (*15,16*). $sst_2$, somatostatin receptor subtype 3 ($sst_3$), and somatostatin receptor subtype 5 ($sst_5$) are internalized to a much higher extent than is somatostatin receptor subtype 1 ($sst_1$) or somatostatin receptor subtype 4 ($sst_4$) (*15,16*). Up to now, clinically relevant radioligands were predominantly tested for internalization on $sst_2$ model systems (*3–7,14*) because of their predominant $sst_2$-binding affinity. Recently, however, increasing numbers of reports have been published on the development of somatostatin analogs with distinct affinity profiles for $sst_2$, $sst_3$, and $sst_5$ such as DOTA-NOC (where DOTA is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid and NOC is [1-$NaI^3$]-octreotide) (*4*), BIM-23244 (*17*), or even analogs with a pansomatostatin profile such as KE108 (*18*). Thus, a thorough investigation of the internalization properties of these analogs is required at each of the somatostatin receptor subtypes.

One aim of the present study was to evaluate a variety of somatostatin analogs, either in clinical use or in development, for their capability to induce somatostatin receptor internalization, using morphologic or nonmorphologic immunocytochemical assays that are able to identify the receptor protein of the 3 somatostatin receptor subtypes: $sst_2$, $sst_3$, and $sst_5$. The principle of the morphologic immunocytochemical assay is, first, to allow the living cells to interact for a given time with the nonradioactive agonists or antagonists to be tested. The cells are then fixed and made permeable for immunostaining of the somatostatin receptor of interest using specific somatostatin receptor antibodies. Localization of the receptor (cell surface, intracellular) can then easily be detected morphologically using an immunofluorescence microscope. In addition, a nonmorphologic ELISA has been used allowing the precise quantitation of the agonist-induced internalization process. These 2 assays therefore directly measure the internalization of the receptor itself rather than the bound ligand detected in radioligand internalization studies. Tested compounds included representatives of clinically used drugs, such as [$Tyr^3$]-octreotide (TOC), lanreotide, and vapreotide (*19–23*); of pansomatostatins such as KE108 (*18*); or of analogs selective for $sst_2$ (L-779,976) (*24*), for $sst_5$ (L-817,818) (*24*), or for both NOC-ATE ([1-$NaI^3$, $Thr^8$]-octreotide) (*25*) and BIM-23244 (*17*). A number of chelated analogs of the first generation (DTPA-octreotide [where DTPA is diethylenetriaminepentaacetic acid] or DOTA-lanreotide) (*26*) or second generation (Y-DOTA-NOC, Y-DOTA-TATE, Lu-DOTA-BOC-ATE, or Lu-DOTA-NOC-ATE) (where TATE is [$Tyr^3$, $Thr^8$]-octreotide and BOC-ATE is [$BzThi^3$, $Thr^8$]-octreotide) (*4,26*) have also been tested, as well as several iodinated, sugar-containing octreotide analogs (*27*). For comparison, established $sst_2$ or $sst_3$ antagonists have been used (*28,29*). This study focused on $sst_2$ internalization, because $sst_2$ is the most important somatostatin receptor from a clinical point of view (*1*) and because most of the clinically available somatostatin analogs have a strong $sst_2$ affinity (*15,26*). However, $sst_3$ and $sst_5$ internalization has also been investigated because many of the newly developed compounds have affinities for somatostatin receptors other than the $sst_2$ subtype.

## MATERIALS AND METHODS

### Reagents

All reagents were of the best grade available and were purchased from common suppliers. The R2-88 antibody to the $sst_{2A}$ was generated as previously described and has been extensively characterized (*30,31*). The $sst_3$-specific antibody (SS-850) and the corresponding C-terminal antigen peptide (S-851) were purchased from Gramsch Laboratories. The $sst_5$-specific antibody (6005) and the corresponding antigen peptide (amino acids 12−20 of the human $sst_5$) were provided by Dr. Stefan Schulz. The secondary antibody Alexa Fluor 488 goat antirabbit IgG (H+L) was from Molecular Probes, Inc. The rabbit polyclonal hemagglutinin epitope antibodies were purchased from Covance or from Sigma-Aldrich. The horseradish peroxidase substrate kit and the goat antirabbit IgG (H+L)-horseradish peroxidase conjugate were purchased from Bio-Rad Laboratories, Inc.

### Peptides

Peptides were obtained as follows: somatostatin-14, somatostatin-28, KE108 (*18*), Coy-14 (BIM-23A760) (*28*), and $sst_3$-ODN-8 (*29*) were synthesized at the Salk Institute and were provided by Dr. Jean Rivier; [$Tyr_3$]-octreotide (TOC) (*19*) was from Novartis Inc.; vapreotide (RC160) (*20*) was from Calbiochem, somatostatin-28 was from Bachem; lanreotide (BIM-23014) (*21–23*) and BIM-23244 (*17*) were provided by Biomeasure Inc.; NOC-ATE (*25*), Y-DOTA-lanreotide (*26*), Y-DOTA-NOC (*4*), Lu-DOTA-BOC-ATE, and Lu-DOTA-NOC-ATE were provided by Dr. Helmut R. Mäcke; L-779,976 and L-817,818 (*24*) were from Merck Pharmaceuticals; DTPA-octreotide (MP2321) (*26*) and DOTA-lanreotide (MP2353) (*26*) were from Mallinckrodt; Y-DOTA-TOC (*26*), Y-DOTA-TATE (*26*), I-Gluc-TOC (*27*), I-Gluc-TATE (*27*), I-Gluc-S-TATE (*27*), and I-Gal-S-TATE (*27*) (where Gluc is glucose, S is mercaptopropionyl spacer, and Gal is galactose) were provided by Dr. Hans-Jürgen Wester. All peptides were dissolved in 10 mmol/L acetic acid except L-817,818, which was dissolved in 1:3 DMSO:$H_2O$.

### Cell Lines

The HEK293 cell lines expressing either the T7-epitope–tagged human $sst_{2A}$ (HEK-$sst_2$), the human $sst_3$ (HEK-$sst_3$), or the human $sst_5$ (HEK-$sst_5$) were provided by Dr. Stefan Schulz and were cultured at 37°C and 5% $CO_2$ in Dulbecco's modified eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 500 μg/mL G418. The clonal CHO-K1 cell line expressing the hemagglutinin-epitope–tagged rat $sst_{2A}$ receptor (CHO-$sst_2$) was generated by transfection of CHO-K1 cells as previously described (*14*) and grown at 37°C and 5% $CO_2$ in Ham's F12 medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 μg/mL G418. All culture reagents were from Gibco BRL.

### Binding-Affinity Measurements

The $sst_2$, $sst_3$, and $sst_5$ binding affinity of the various compounds was measured as described previously using in vitro receptor autoradiography with 20-μm-thick sections from membrane pellets of the respective transfected cells (*26*).

### Immunofluorescence Microscopy

Immunofluorescence microscopy–based internalization assay for $sst_2$, $sst_3$, and $sst_5$ was performed as previously described by Liu et al. (*14*) for $sst_2$. The internalization assay was extensively validated through a whole series of experiments in HEK-$sst_2$ cells showing that $sst_2$ is membrane bound in the absence of agonist; that there is a time, temperature, and agonist concentration dependency for $sst_2$ internalization; and that $sst_2$ is internalized via clathrin-coated vesicles and localizes to mannose 6-phosphate receptor–positive intracellular compartments, most likely the trans-Golgi network (TGN)/late endosome. Moreover, the internalization property of the HEK-$sst_2$ (HEK293 cells expressing the T7-epitope tagged human $sst_{2A}$) was found to be comparable to that of HEK293 cells expressing the wild-type $sst_{2A}$ in the internalization assay.

HEK-$sst_2$, HEK-$sst_3$, and HEK-$sst_5$ cells were grown on poly-D-lysine (10 μg/mL) (Sigma-Aldrich)–coated 35-mm 4-well plates (Cellstar; Greiner Bio-One GmbH). Cells were treated either with the various agonists listed in Table 1 at concentrations ranging from 1 to 10,000 nmol/L, or with the agonists in the presence of an excess of antagonist (Coy-14, $sst_3$-ODN-8; 50–100 times the concentration of the agonist), or with antagonist alone for 30 min at 37°C in growth medium and then rinsed twice with 100 mmol/L phosphate buffer containing 0.15 mol/L sucrose (PS). After the cells were fixed and made permeable for 7 min with cold methanol (−20°C), they were rinsed twice with PS, and nonspecific binding sites were blocked by incubating the cells in PS containing 0.1% bovine serum albumin for 60 min at room temperature. The cells were subsequently incubated for 60 min at room temperature with either the $sst_{2A}$-specific primary antibody (R2-88) diluted 1:1,000 in PS, the $sst_3$-specific primary antibody (SS-850) diluted 1:1,000 in PS, or the $sst_5$-specific primary antibody (6005) diluted 1:2,000 in PS. After the antibody incubation, the cells were washed 3 times for 5 min with PS containing 0.1% bovine serum albumin and then incubated for 60 min at room temperature in the dark with the secondary antibody, Alexa Fluor 488 goat antirabbit IgG (H+L), diluted 1:600 in PS. Subsequently, the cells were washed 3 times for 5 min each with PS containing 0.1% bovine serum albumin, embedded with 1:1 PS:glycerol, and covered with a glass slip. No immunostaining was observed in HEK-$sst_2$ cells with R2-88 (1:1,000) preabsorbed with antigen peptide, consistent with the known specificity of this receptor antibody (*30,31*). Similarly, no immunostaining was observed in HEK-$sst_3$ cells with the $sst_3$-specific antibody SS-850 preabsorbed with the corresponding antigen peptide S-851 or in HEK-$sst_5$ cells with the $sst_5$-specific antibody 6005 preabsorbed with the corresponding antigen peptide. The cells were imaged using a Leica DM RB immunofluorescence microscope and an Olympus DP10 camera.

### Quantitation of Internalized Somatostatin Receptors by Immunofluorescence Microscopy

The internalization assay for $sst_2$ and $sst_3$ was performed as described previously by Liu et al. (*14*). Single cells were then analyzed for the amount of internalized somatostatin receptors after agonist stimulation. The immunofluorescence intensity of the labeled cells was densitometrically determined using a Zeiss Axioskop microscope equipped with a Roper CoolSNAP cf monochrome camera. The relative optical density (ROD) of the total area of the cell and the ROD of the area of the internalized somatostatin receptors were determined, and the percentage of internalized somatostatin receptors after agonist stimulation was then calculated according to the following equation:

$$\%\,\text{internalized receptor} = \frac{\{(\text{ROD (internalized receptor)} - \text{ROD (background)}) \times \text{area (internalized receptor)}\} \times 100\%}{(\text{ROD (total cell)} - \text{ROD (background)}) \times \text{area (total cell)}}.$$

The data were analyzed using the MCID Basic 7.0 program (Imaging Research Inc.). For each tested agonist concentration, 10–12 cells were analyzed and the mean value was used for the graph. The GraphPad Prism program, version 3.0, was used to create the graphs.

### Quantitation of Internalized Somatostatin Receptors by ELISA

Receptor internalization was also quantitatively assessed in another cell line, CHO-K1 cells expressing the hemagglutinin-epitope tagged rat $sst_{2A}$ receptor (CHO-$sst_2$), using an ELISA as described in detail previously (*14*).

## RESULTS

Table 1 lists the somatostatin analogs tested for their ability to induce somatostatin receptor internalization using immunocytochemical detection methods. Apart from the 2 natural peptides, somatostatin-14 and somatostatin-28, they can be divided into 4 groups: a series of well-established peptide and nonpeptide analogs of current preclinical or clinical interest, which are nonchelated and, when tested previously, reported to be agonists; various peptide analogs linked to a chelator, for use in nuclear medicine, expected to be agonists but not systematically tested for agonism;

**TABLE 1**

$sst_2$, $sst_3$, and $sst_5$ Binding Affinities and Internalization of Somatostatin and Somatostatin Analogs

| Compound | Binding affinity | | | Receptor internalization | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | $sst_2$ | | | $sst_3$ | | $sst_5$ |
| | $sst_2$ | $sst_3$ | $sst_5$ | HEK* | CHO† | Antagonized | HEK* | Antagonized | HEK* |
| Natural somatostatins | | | | | | | | | |
| Somatostatin-28 | 2.3 ± 0.1 (39) | 3.7 ± 0.3 (38) | 2.4 ± 0.2 (36) | + | + (0.71 ± 0.05) | Yes | + | Yes | + |
| Somatostatin-14 | 0.7 ± 0.2 (5) | 3.3 ± 1.7 (4) | 10 ± 4 (4) | + | ND | Yes | + | ND | + |
| Various synthetic analogs | | | | | | | | | |
| [Tyr$^3$]-octreotide (TOC) | 2.8 ± 0.6 (4) | 225 ± 82 (4) | 9.9 ± 1.8 (4) | + | + (0.51 ± 0.07) | Yes | − | ND | − |
| Vapreotide acetate (RC160) | 1.8 ± 0.3 (5) | 233 ± 34 (4) | 4.8 ± 0.9 (5) | + | + (4.3 ± 1.4) | ND | − | ND | − |
| Lanreotide (BIM-23014) | 1.6 ± 0.4 (2) | 438 ± 39 (2) | 7.4 ± 1.2 (2) | + | + (1.6 ± 0.1) | ND | − | ND | − |
| KE108 | 0.9 ± 0.1 (7) | 1.5 ± 0.2 (7) | 0.7 ± 0.1 (7) | + | ND | Yes | + | Yes | − |
| NOC-ATE | 3.6 ± 1.6 (3) | 302 ± 137 (3) | 17 ± 10 (3) | + | ND | ND | − | ND | − |
| BIM-23244 | 0.18 ± 0.02 (3) | 53 ± 7 (3) | 0.5 ± 0.2 (3) | + | + (0.08 ± 0.01) | ND | − | ND | − |
| L-779,976 | 0.6 ± 0.1 (4) | >1,000 (4) | >1,000 (4) | + | + (0.73 ± 0.18) | ND | − | ND | − |
| L-817,818 | >1,000 (3) | 164 ± 17 (2) | 1.6 ± 0.4 (4) | − | — | ND | − | ND | − |
| Chelated analogs | | | | | | | | | |
| DTPA-octreotide (MP2321) | 13 ± 2 (5) | 376 ± 84 (5) | 299 ± 51 (6) | + | + (23 ± 1) | Yes | − | ND | − |
| DOTA-lanreotide (MP2353) | 26 ± 3 (6) | 771 ± 229 (6) | 73 ± 12 (6) | + | + (4.5 ± 0.4) | Yes | − | ND | − |
| Y-DOTA-TOC | 11 ± 2 (6) | 389 ± 135 (5) | 114 ± 29 (5) | + | + (0.72 ± 0.14) | Yes | − | ND | − |
| Y-DOTA-lanreotide | 23 ± 5 (4) | 290 ± 105 (4) | 16 ± 3 (4) | + | ND | Yes | − | ND | − |
| Y-DOTA-NOC | 3.3 ± 0.2 (3) | 26 ± 2 (3) | 10 ± 2 (3) | + | + (0.41 ± 0.01) | Yes | + | Yes | − |
| Y-DOTA-TATE | 1.6 ± 0.4 (3) | >1,000 (3) | 187 ± 50 (3) | + | + (0.61 ± 0.08) | Yes | − | ND | − |
| Lu-DOTA-BOC-ATE | 2.4 ± 0.3 (2) | 11 ± 1 (2) | 8.3 ± 0.4 (2) | + | + (0.46 ± 0.11) | Yes | + | ND | − |
| Lu-DOTA-NOC-ATE | 3.6 ± 0.3 (2) | 31 ± 2 (2) | 15 ± 1 (2) | + | + (1.0 ± 0.3) | Yes | + | ND | − |
| I-Gluc/Gal-compounds | | | | | | | | | |
| I-Gluc-TOC | 2.2 ± 0.7 (3) | 357 ± 22 (3) | 64 ± 24 (3) | + | + (0.78 ± 0.12) | Yes | − | ND | − |
| I-Gluc-TATE | 2.0 ± 0.5 (3) | >1,000 (3) | 521 ± 269 (3) | + | ND | Yes | − | ND | − |
| I-Gluc-*S*-TATE | 2.0 ± 0.7 (3) | 398 ± 19 (3) | 310 ± 156 (3) | + | + (0.73 ± 0.21) | Yes | − | ND | − |
| I-Gal-*S*-TATE | 2.0 ± 0.8 (3) | 491 ± 63 (3) | 413 ± 167 (3) | + | + (0.89 ± 0.31) | Yes | − | ND | − |
| Antagonists | | | | | | | | | |
| Coy-14 (BIM-23A760) | 10 ± 4 (4) | 61 ± 14 (3) | 53 ± 19 (2) | — | — | ND | ND | ND | ND |
| $sst_3$-ODN-8 | >1,000 (3) | 6.7 ± 2.6 (3) | >1,000 (3) | ND | ND | ND | − | ND | ND |

*For immunofluorescent internalization assay in HEK cells, + = internalized at agonist dose of at least 100 nmol/L, − = not internalized at agonist dose of greater than 100 nmol/L, and ND = not determined.

†For internalization measured with ELISA in CHO-$sst_2$ cells, + = internalized, − = not internalized, and ND = not determined. Values in parentheses are median effective concentration, $EC_{50}$ (±SEM, $n \geq 2$).

Binding affinities were measured using in vitro receptor autoradiography, as reported previously. Values were taken from previous reports (*4,18,25–27,38,39*) or represent our unpublished data. Binding affinity values are inhibitory concentration of 50% ($IC_{50}$), in nmol/L (mean ± SEM); number of independent studies is given in parentheses. *Antagonized* means abolition of receptor internalization in presence of excess concentrations of receptor-specific antagonist, either Coy-14 for $sst_2$ or $sst_3$-ODN-8 for $sst_3$, performed with immunofluorescent internalization assay in HEK-$sst_2$ or HEK-$sst_3$ cells.

several iodinated, sugar-containing analogs; and established somatostatin receptor antagonists. All compounds in Table 1 have a high affinity for one or more somatostatin receptors. The binding affinity data presented in Table 1 were determined using receptor autoradiography with membrane pellets (*26,29*); the results generally agree with previously published data generated with different methods (*17,24,28*).

All compounds in Table 1 were analyzed by immunofluorescence microscopy for internalization of $sst_2$, $sst_3$, and $sst_5$ using a concentration ranging from 1 to 1,000 nmol/L, or even up to 10,000 nmol/L when the analogs were not of the highest affinity (e.g., chelated analogs at $sst_5$). A compound was considered active when it induced somatostatin receptor internalization at an agonist concentration of at least 100 nmol/L. The results in Table 1 show that all agonists with a high affinity for $sst_2$ induce internalization of $sst_2$. The compounds that do not induce internalization of $sst_2$ are the $sst_5$-selective L-817,818 and the $sst_2$ antagonist Coy-14. Figure 1 illustrates the $sst_2$ internalization triggered by various analogs. Compared with the control (no peptide added), for which the $sst_2$ is localized exclusively to the cell surface, each of the tested compounds can efficiently induce $sst_2$ internalization, detectable as prominent

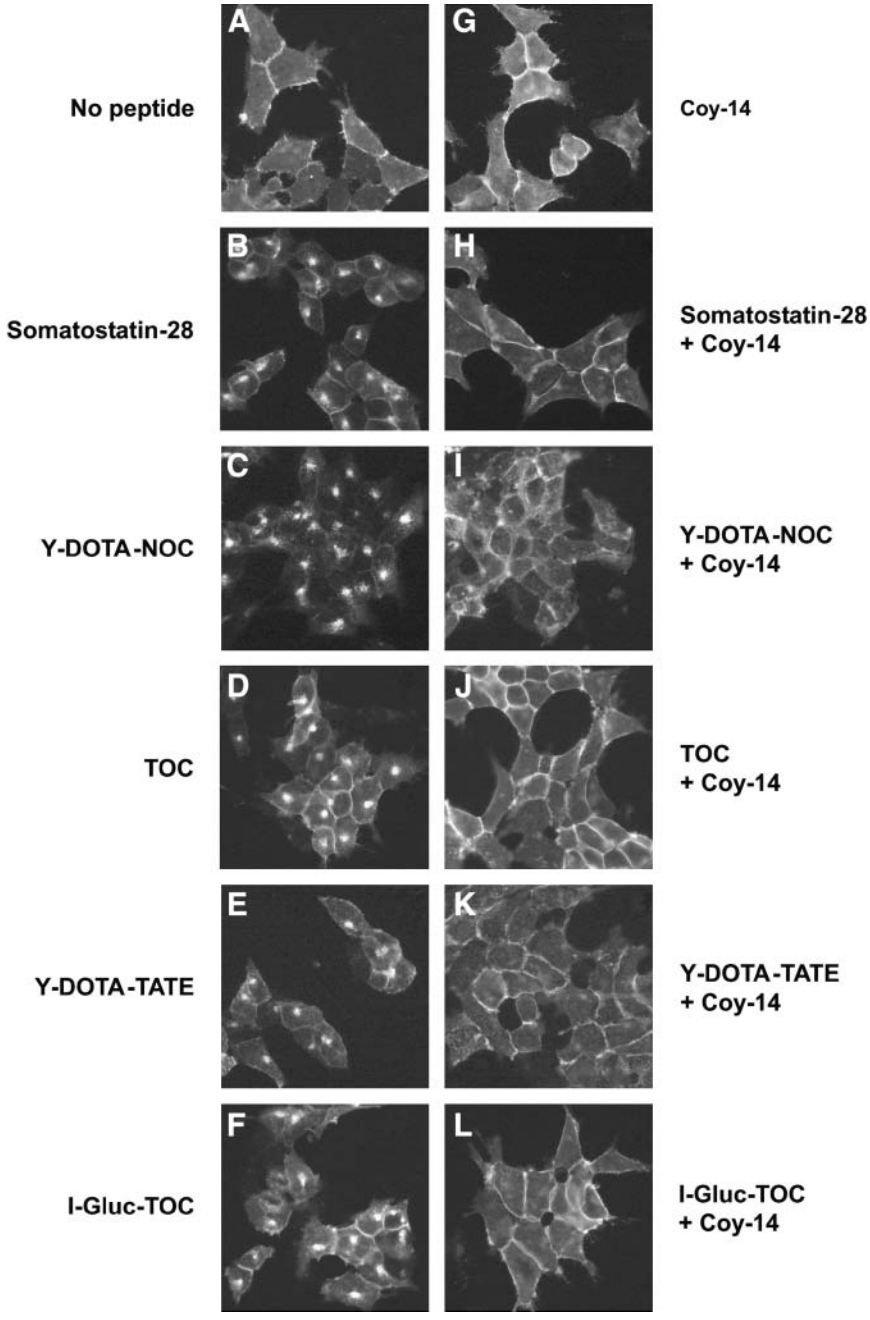


**FIGURE 1.** $sst_2$ internalization is induced by various agonists and is abolished by antagonist Coy-14. HEK-$sst_2$ cells were treated for 30 min either with vehicle (no peptide [A]) or with various agonists at concentrations inducing submaximal internalization effect (100 nmol/L [B and C] and 10 nmol/L [D–F]). (H–L) Cells treated with same agonists at same concentrations as in B–F but in presence of excess of specific $sst_2$-antagonist Coy-14 (5 [H], 10 [I], and 1 [J–L] μmol/L). Effect of antagonist alone (10 μmol/L [G]) is also shown. After incubation with peptides, cells were processed for immunocytochemistry. Clear punctate perinuclear staining is detectable for all tested agonists. This punctate staining is efficiently abolished by excess of antagonist Coy-14. Antagonist alone has no effect on internalization.

punctate perinuclear staining as shown in Figure 1. This intracellular, perinuclear $sst_2$ staining was shown previously (*14*) to colocalize with the TGN/late endosome marker protein mannose 6–phosphate receptor. Figure 1 also shows that agonist-induced $sst_2$ internalization can be abolished by the $sst_2$-specific antagonist Coy-14. Table 1 summarizes all the cases in which Coy-14 was used to antagonize $sst_2$ internalization after agonist stimulation. These results indicate that $sst_2$ internalization can be triggered only by somatostatin agonists, but not by the antagonist Coy-14. Figure 2A shows the potency of second-generation somatostatin analogs such as TOC, Y-DOTA-TATE, or I-Gal-S-TATE to elicit $sst_2$ internalization, as compared with DTPA-octreotide. Abolition of TOC-induced $sst_2$ internalization by Coy-14 is illustrated as well. Figure 2B illustrates the dose-response experiment and clearly shows that TOC is almost 2 orders of magnitude more potent in stimulating $sst_2$ internalization than is DTPA-octreotide. In addition and for comparison, Table 1 also shows the internalization properties of the clinically relevant somatostatin analogs in another cell line, CHO-$sst_2$, as measured by ELISA. Potencies (median effective concentration, $EC_{50}$) for stimulation of endocytosis varied by more than 280 times between DTPA-octreotide and the best of the tested compounds, the $sst_2$/$sst_5$-selective BIM-23244. Among the chelated analogs, the most efficient internalization was found for Y-DOTA-TOC, Y-DOTA-TATE, Y-DOTA-NOC, and Lu-DOTA-BOC-ATE. Moreover, all tested iodinated sugar-containing octreotide analogs showed a highly efficient $sst_2$ internalization as well.

Table 1 further shows that analogs with high $sst_3$ affinity are able to trigger $sst_3$ internalization. In addition to somatostatin-14 and somatostatin-28, this effect is observed for KE108, Y-DOTA-NOC, Lu-DOTA-NOC-ATE, and Lu-DOTA-BOC-ATE. Other compounds with comparatively low $sst_3$ affinity were unable to induce $sst_3$ internalization at a dose of 100 nmol/L. Figure 3 illustrates the effects of somatostatin-28, Y-DOTA-NOC, and KE108 that can be antagonized by the $sst_3$-selective antagonist $sst_3$-ODN-8. Figure 4 shows a dose-response curve with somatostatin-28

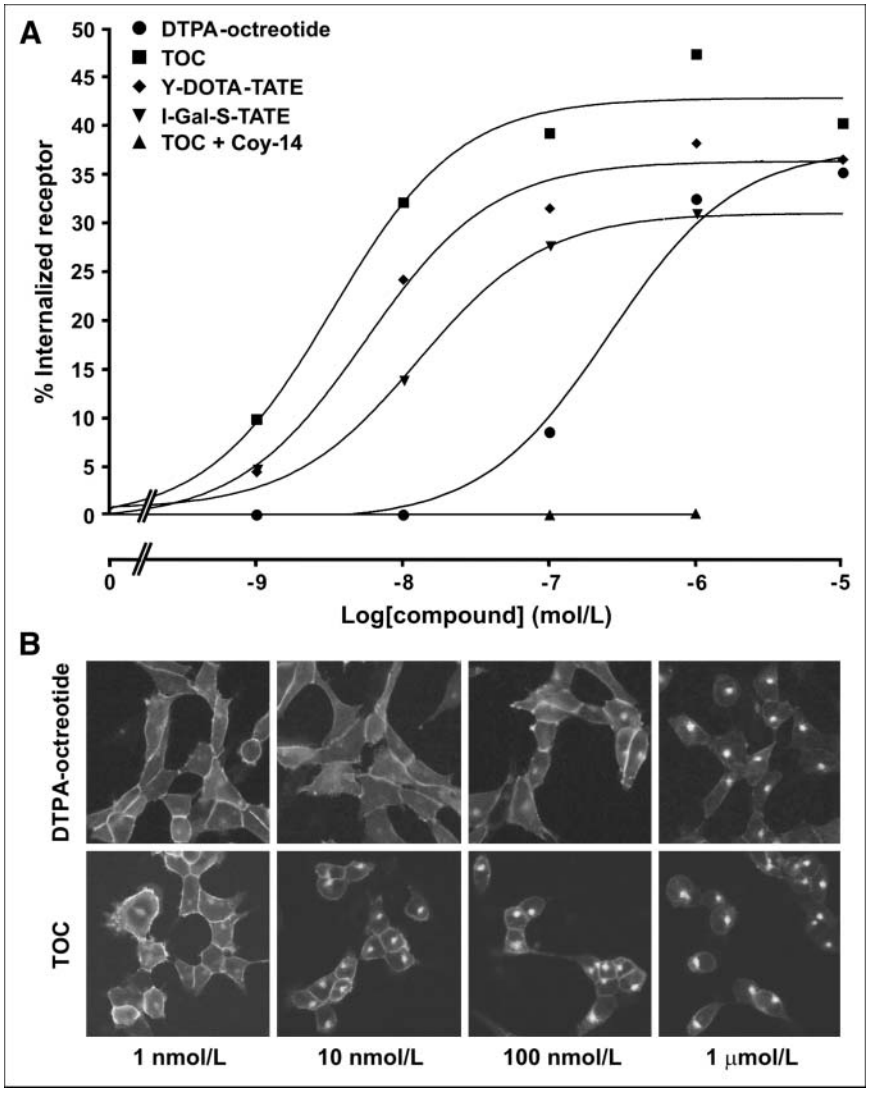


**FIGURE 2.** Dose response of agonist-induced $sst_2$ internalization. HEK-$sst_2$ cells were treated with 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, or 10 μmol/L of various agonists. Also included are cells treated with 100 nmol/L TOC plus 5 μmol/L Coy-14 or with 1 μmol/L TOC plus 50 μmol/L Coy-14. After 30 min of incubation with peptides, cells were processed for immunocytochemistry, and internalized $sst_2$ was quantified. (A) Representative experiment with dose-response curves for various agonists inducing $sst_2$ internalization. TOC, Y-DOTA-TATE, and I-Gal-S-TATE are considerably more potent in inducing $sst_2$ internalization than is DTPA-octreotide. TOC-induced internalization is efficiently abolished by excess of Coy-14. (B) Representative immunofluorescence images of agonist-induced internalized $sst_2$ using DTPA-octreotide and TOC. TOC is almost 2 orders of magnitude more potent in inducing $sst_2$ internalization than is DTPA-octreotide.

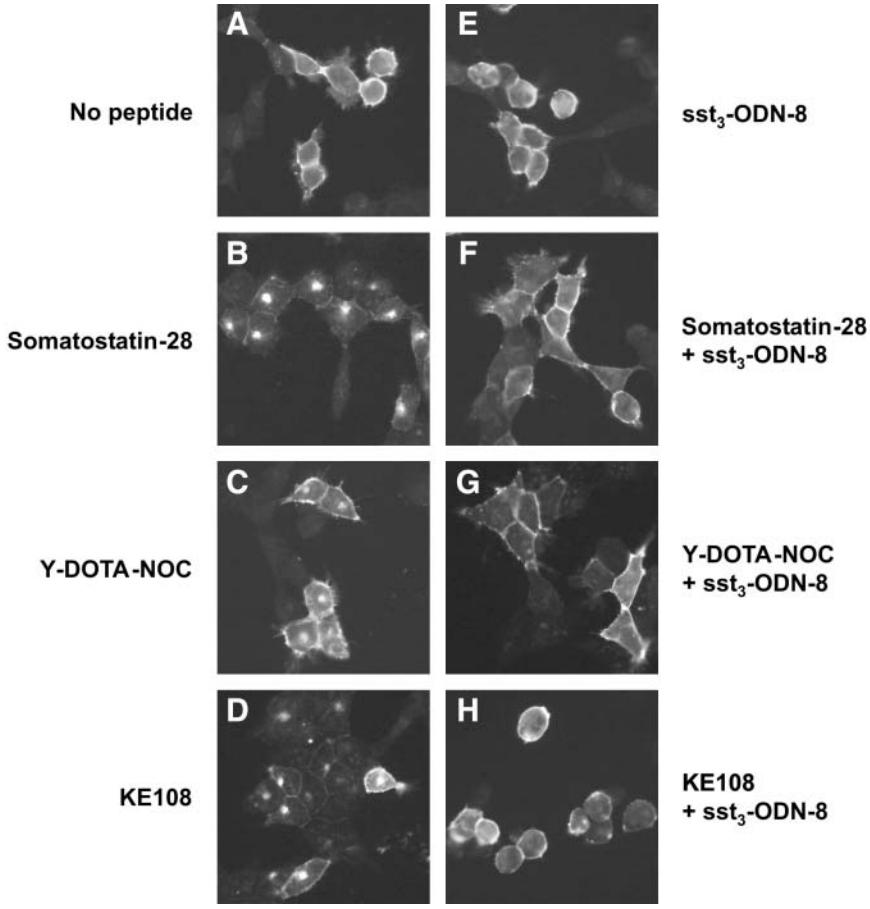


**FIGURE 3.** $sst_3$ internalization is induced by various agonists and is efficiently abolished by $sst_3$-specific antagonist $sst_3$-ODN-8. HEK-$sst_3$ cells were treated either with vehicle (no peptide [A]) or with various agonists at concentrations inducing submaximal internalization effect (100 nmol/L [B–D]). Cells treated with same agonists at same concentrations as in B–D but in presence of excess of specific $sst_3$-antagonist $sst_3$-ODN-8 (5 μmol/L [F–H]). Effect of antagonist alone (50 μmol/L [E]) is also illustrated. After incubation with peptides, cells were processed for immunocytochemistry. Clear perinuclear staining is detectable for all agonists tested. This staining is efficiently abolished by 50-fold excess of antagonist $sst_3$-ODN-8. Antagonist alone has no effect on internalization.

and KE108 and also shows that the somatostatin-28–induced internalization of $sst_3$ is abolished by the $sst_3$-specific antagonist $sst_3$-ODN-8.

Finally, Table 1 and Figure 5 show that both somatostatin-14 and somatostatin-28 trigger $sst_5$ internalization. The $sst_5$ internalization is less pronounced than is observed with $sst_2$ or $sst_3$, partly because, in contrast to $sst_2$- or $sst_3$-expressing cells, even untreated $sst_5$ cells have an intracellular pool of $sst_5$, as shown in Figure 5. Interestingly, somatostatin-28 induces greater $sst_5$ internalization at 100 nmol/L than does somatostatin-14, perhaps as a result of the higher binding affinity of somatostatin-28 than of somatostatin-14 to $sst_5$. This finding is also summarized in Table 1. Because well-characterized $sst_5$ antagonists are not available, blocking studies with an $sst_5$ antagonist could not be performed. However, we showed that the internalization process was abolished in the presence of 0.45 mol/L sucrose, a result that strongly supports the specificity of the observation. Unexpectedly, several high-affinity synthetic $sst_5$ agonists such as L-817,818, BIM-23244, or KE108 are unable to elicit an $sst_5$ internalization response, even at doses of up to 1,000 nmol/L, as shown in Figure 5. Therefore, in contrast to $sst_2$ and $sst_3$, not all agonists are able to stimulate $sst_5$ receptor endocytosis.

## DISCUSSION

In this study, sensitive immunocytochemical methods were applied to examine $sst_2$, $sst_3$, and $sst_5$ receptor internalization after agonist or antagonist treatment, using a variety of somatostatin analogs with established or potential interest for nuclear medicine. To our knowledge, this was the first time that a method monitoring receptor trafficking rather than radioligand trafficking was used to preclinically evaluate new G-protein–coupled receptor ligands for potential use in nuclear medicine. The immunocytochemistry-based internalization assay has several important advantages over assays using radiolabeled ligands. Because we are monitoring receptor trafficking, we are not restricted to the use of radiolabeled ligands but can test any nonlabeled compound. Further, using this method, receptor internalization can be monitored at a broad range of agonist and antagonist concentrations rather than at subsaturating concentrations, as usually occur for radioligands. Moreover, unlike radioactive isotopes, ligands to be tested for internalization will not experience alteration, which might affect the structure of the ligand and thus its biologic activity.

The study also showed that $sst_2$ agonists, but not $sst_2$ antagonists, can trigger $sst_2$ internalization, in agreement

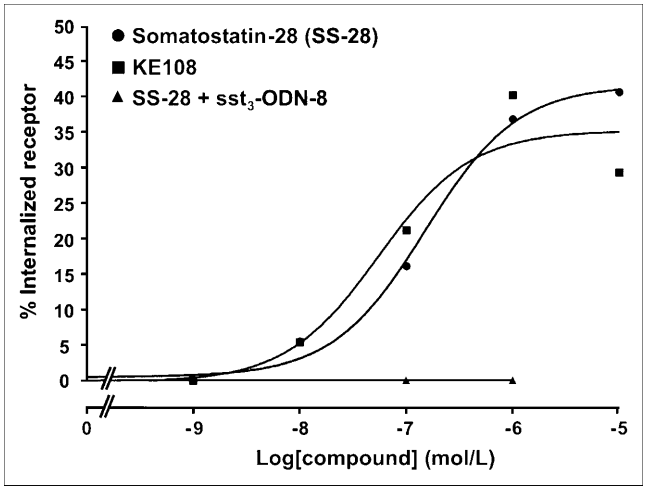


**FIGURE 4.** Dose response of agonist-induced $sst_3$ internalization. HEK-$sst_3$ cells were treated with 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, or 10 μmol/L of somatostatin-28 or KE108. Also included are cells treated with 100 nmol/L somatostatin-28 plus 5 μmol/L $sst_3$-ODN-8 or with 1 μmol/L somatostatin-28 plus 50 μmol/L $sst_3$-ODN-8. After incubation with peptides, cells were processed for immunocytochemistry, and internalized $sst_3$ was quantified. This experiment shows that somatostatin-28 and KE108 are of similar potency in inducing $sst_3$ internalization. Somatostatin-28–induced internalization effect is efficiently abolished by excess of $sst_3$-ODN-8

with our earlier study (*14*). Moreover, the study demonstrated that $sst_2$ antagonists can selectively antagonize the $sst_2$ agonistic effect on internalization. It thus seems likely that compounds such as Y-DOTA-NOC, Y-DOTA-TATE, Lu-DOTA-BOC-ATE, Lu-DOTA-NOC-ATE, or the sugar-containing octreotide analogs, each of which induces internalization, are agonists at the $sst_2$.

The study further showed that high-affinity $sst_2$ binding is a prerequisite for an agonist to trigger $sst_2$ internalization. Thus, the $sst_5$-selective L-817,818, with a low affinity for $sst_2$, is unable to trigger $sst_2$ internalization. Conversely, all agonists with high-affinity $sst_2$-binding properties were able to internalize $sst_2$. The agonist with the highest $sst_2$-binding affinity, BIM-23244, had the highest $sst_2$ internalization potency. We should emphasize that the second-generation compounds foreseen for in vivo $sst_2$ targeting, such as octreotides or octreotates modified in position 3 and linked to DOTA or sugars, often have considerably better internalization capabilities than do the first-generation compound DTPA-octreotide.

Factors other than ligand binding also play an important role in receptor internalization, and these can have cell-specific effects. Previous studies have demonstrated that $sst_2$ and $sst_3$ are rapidly phosphorylated on agonist binding, most probably by G-protein–coupled receptor kinases (*32–34*). Receptor phosphorylation is followed by the recruitment of β-arrestins to the receptor (*14,35*), and the bound arrestins then link the receptors to the endocytosis machinery. The nature and concentration of G-protein–coupled receptor kinase subtypes, as well as the relative concentrations of the 2 arrestins, β-arrestin-1 and β-arrestin-2, are known to vary among cell types and are thought to produce differences in the efficiency and extent of receptor internalization (*36*). Therefore, in an attempt to generalize the

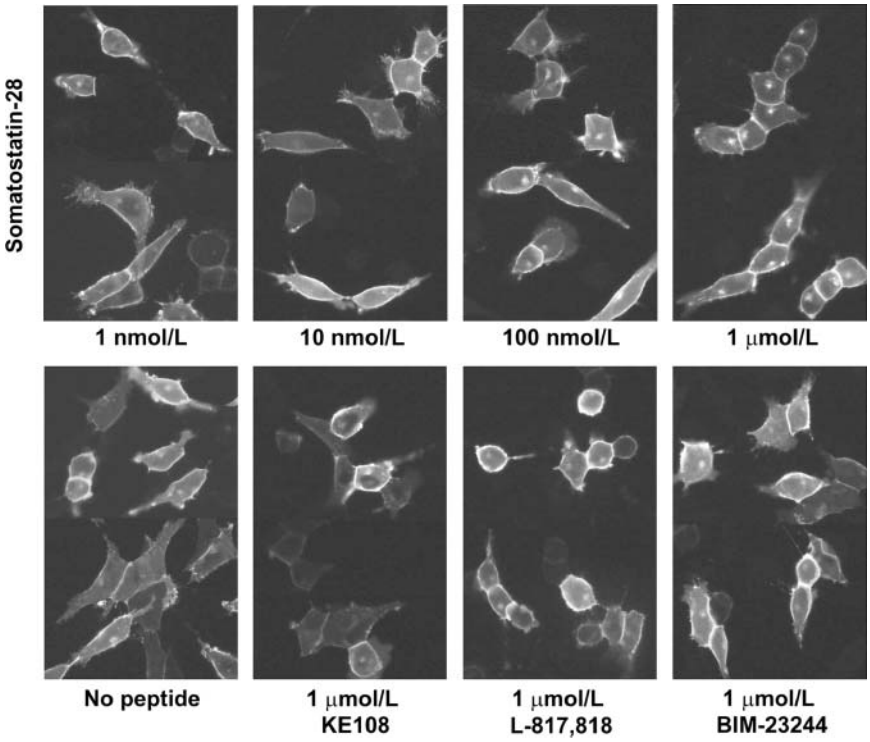


**FIGURE 5.** $sst_5$ internalization is induced by native somatostatin-28 but not by high-affinity synthetic $sst_5$ agonists. HEK-$sst_5$ cells were treated either with vehicle (no peptide); with 1 nmol/L, 10 nmol/L, 100 nmol/L, or 1 μmol/L somatostatin-28; or with 1 μmol/L KE108, 1 μmol/L BIM-23244, or 1 μmol/L L-817,818. After incubation with peptides, cells were processed for immunocytochemistry. Dose-response experiment with somatostatin-28 shows that it can elicit $sst_5$ internalization. This is, however, not true for $sst_5$ agonists KE108, BIM-23244, and L-817,818. Note also presence of intracellular $sst_5$ even when cells were treated with vehicle alone (no peptide). All images are composed of 2 single pictures.

data obtained in HEK-$sst_2$ cells, we have tested several clinically relevant compounds for their ability to stimulate receptor internalization in a different cell line, namely in CHO-$sst_2$ cells, using our previously described quantitative ELISA method (*14*). Our results demonstrated that all tested compounds produced internalization in both cell types. However, the interesting and clinically important possibility exists that receptor internalization will differ in certain tumor types.

To our knowledge, this was the first time that the internalization capability of novel somatostatin receptor ligands potentially useful in nuclear medicine has been examined for $sst_3$ and $sst_5$. Highly promising drugs with high binding affinity to $sst_3$, such as KE108, Y-DOTA-NOC, Lu-DOTA-BOC-ATE, and Lu-DOTA-NOC-ATE, show excellent $sst_3$ internalization properties. Moreover, these compounds can be antagonized by the selective $sst_3$ antagonist $sst_3$-ODN-8, strongly indicating that Y-DOTA-NOC, Lu-DOTA-BOC-ATE, and Lu-DOTA-NOC-ATE are agonists at $sst_3$—information that was not previously available.

In contrast to $sst_2$ and $sst_3$, the cellular distribution characteristics of $sst_5$ are unusual. Even in untreated cells, a distinct intracellular perinuclear staining of $sst_5$ is observed in addition to cell-surface $sst_5$ staining. One possible reason for this combined cell surface and intracellular staining may be the particular cellular distribution and trafficking of $sst_5$, which was first reported by Stroh et al. (*37*). They elegantly showed, by biochemical, confocal, and electron microscopy methods, that in COS-7 cells exogenously expressing the rat $sst_5$, functional $sst_5$ is maintained at the cell surface even in the presence of somatostatin, both because of the rapid recycling of the internalized receptor to the cell surface and because of a massive recruitment of $sst_5$ to the cell surface from an intracellular $sst_5$ reserve pool. In our HEK-$sst_5$ cells, the balance between depletion of the intracellular $sst_5$ pool and $sst_5$ internalization in the presence of somatostatin resulted in a net internalization, because the intracellular staining became more prominent after somatostatin treatment. Whether the internalized receptor colocalizes with the intracellular receptor pool is unknown. Although the presence of an intracellular $sst_5$ pool makes the evaluation of an agonist-triggered internalization more difficult for $sst_5$ than for $sst_2$ or $sst_3$, somatostatin-28 and somatostatin-14 are, without doubt, able to induce $sst_5$ internalization. The better $sst_5$ internalization by 100 and 1,000 nmol/L of somatostatin-28 than by somatostatin-14 may be explained by the significantly higher binding affinity of somatostatin-28. However, the observation that some of the most potent $sst_5$ agonists, such as BIM-23244, KE108, or L-817,818, with binding affinities below 1 nmol/L were unable to trigger any net $sst_5$ internalization was not expected. We previously showed a similar dissociation between the binding of certain somatostatin analogs and their efficacy at promoting $sst_2$ internalization (*14*). Further, we observed that the $sst_2$–arrestin complex was less stable in the presence of an analog that was a poor inducer of internalization than in the presence of somatostatin-14, which promoted internalization effectively (*14*). These results support the conclusion that the ability of analogs to facilitate the formation of a stable receptor–arrestin complex determines their effectiveness for stimulating endocytosis. Although arrestins are not recruited as strongly to $sst_5$ as to $sst_2$ (*34*), it is possible that receptor–arrestin interactions are also important for determining the ability of $sst_5$ analogs to stimulate receptor internalization. The differences among analogs for producing $sst_5$ endocytosis, described here for what is to our knowledge the first time, may not only help explain the biologic actions of these analogs but also demonstrate the importance of individual assessment of agonists for their effect on somatostatin receptor internalization.

## CONCLUSION

These present data are likely to be important for the preclinical evaluation of the internalization properties of new ligands aimed at tumor targeting and for the interpretation of future imaging data using labeled somatostatin analogs. The described methodology may be used in future to screen such novel peptide ligands for their agonistic and antagonistic properties on internalization. One of the most novel and unexpected results from these studies is that compounds with high affinity at both $sst_2$ and $sst_5$, such as BIM-23244 or KE108, or the chelated analogs Y-DOTA-NOC or Lu-DOTA-BOC-ATE, show distinct internalization properties at these 2 receptors, namely a strong $sst_2$ but no $sst_5$ internalization. Such data will have to be considered when one is interpreting clinical studies using these compounds for tumor targeting.

## ACKNOWLEDGMENTS

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