Free Somatostatin Receptor Fraction Predicts the Antiproliferative Effect of Octreotide in a Neuroendocrine Tumor Model: Implications for Dose Optimization

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Abstract

Somatostatin receptors (SSTR) are highly expressed in well-differentiated neuroendocrine tumors (NET). Octreotide, an SSTR agonist, has been used to suppress the production of vasoactive hormones and relieve symptoms of hormone hypersecretion with functional NETs. In a clinical trial, an empiric dose of octreotide treatment prolonged time to tumor progression in patients with small bowel neuroendocrine (carcinoid) tumors, irrespective of symptom status. However, there has yet to be a dose optimization study across the patient population, and methods are currently lacking to optimize dosing of octreotide therapy on an individual basis. Multiple factors such as total tumor burden, receptor expression levels, and nontarget organ metabolism/excretion may contribute to a variation in SSTR octreotide occupancy with a given dose among different patients. In this study, we report the development of an imaging method to measure surface SSTR expression and occupancy level using the PET radiotracer 68Ga-DOTATOC. In an animal model, SSTR occupancy by octreotide was assessed quantitatively with 68Ga-DOTATOC PET, with the finding that increased occupancy resulted in decreased tumor proliferation rate. The results suggested that quantitative SSTR imaging during octreotide therapy has the potential to determine the fractional receptor occupancy in NETs, thereby allowing octreotide dosing to be optimized readily in individual patients. Clinical trials validating this approach are warranted. Cancer Res; 73(23); 6865–73. ©2013 AACR.

Introduction

Neuroendocrine tumors (NET) are a heterogeneous group of malignancies that are thought to originate from endocrine progenitor cells located in various organ systems including the lung, pancreas, and gastrointestinal tract. Very commonly, these NETs secrete a variety of biologically active peptides and amines that can lead to symptoms of wheezing, nausea, abdominal pain, flushing, and diarrhea, among others (1). Somatostatin receptor (SSTR) agonists have been employed with great success for controlling these symptoms (1, 2). With 80% to 100% of well-differentiated NETs expressing high levels of SSTR (3), somatostatin analogs, such as octreotide, have become the treatment of choice for symptomatic relief through the reduction of NET hormone production.

Over the past several years, it has further been demonstrated that SSTR agonists may also have an antiproliferative effect on NETs (4, 5) and may have a role as antineoplastic therapy for NETs. It was shown in a double-blind randomized controlled trial that patients with metastatic well-differentiated mid-gut NETs who received monthly intramuscular injections of a standard dose (30 mg) of long-acting octreotide (octreotide LAR) had a significantly increased progression-free survival (PFS) compared to those who received placebo (15.6 vs. 5.9 months, respectively; ref. 6). The patients in this study benefited from octreotide LAR therapy regardless of tumor functional status. The results of this trial suggest that octreotide may possess antiproliferative properties on NETs that are enacted through SSTR-mediated signaling, irrespective of activation of pathways involved in bioactive peptide and amine production. This paradigm shift in the use of SSTR agonists for their antitumor effects has been empiric in nature, without an understanding of what fraction of the somatostatin receptors were bound during agonist therapy, how this changed over the course of a monthly treatment cycle, and the association with proliferation. The development of a noninvasive approach to quantify the concentration of SSTR in tumors, the change in free receptor fraction with therapy, and the resultant downstream effects on proliferation would provide a method to optimize this treatment, both at the individual level and across treatment populations.
Radionuclide imaging of SSTR through 111In-octreotide or 68Ga-DOTATOC has largely been qualitative in nature, using the high contrast gained from the images to locate foci of disease not apparent on other imaging modalities or to monitor disease progression by the assessment of tumor volume changes over time (7–9). In fact, tumor imaging with these agents is typically performed either for primary staging before initiation of octreotide LAR therapy or monitoring of disease progression during the expected nadir blood levels of octreotide LAR to aid in tumor visualization (10, 11). The objective in this study was to use the quantitative nature of 68Ga–DOTATOC PET imaging to develop a technique that allows one to compute the receptor density in a tumor volume and monitor the fraction that is occupied with agonist treatment at a given time, based on changes in the available (unbound) receptor density. In combination with another positron-emitting radiotracer, 18F-fluoro-3-deoxy-3-fluorothymidine (18F-FLT), a proliferation marker (12), we were able to correlate the changes in unoccupied SSTR with proliferation status in an animal model. The imaging techniques and quantitation methods thus developed have the potential to be readily translated to patients with NETs to more effectively monitor treatment and improve dosing regimens.

Materials and Methods

Cell culture

AR42J [American Type Culture Collection (ATCC)], an SSTR expressing rat pancreatic carcinoma, was cultured in F-12K medium (ATCC), supplemented by 20% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. A549 (ATCC), an SSTR-negative human alveolar basal epithelial carcinoma cell line, was cultured in F-12K medium (ATCC), supplemented by 10% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in a humidified incubator at 37°C, 5% CO2. Subculturing was performed employing a 0.25% trypsin/0.1% EDTA solution. The cell lines were obtained from ATCC and were used in this study for less than 6 months after resuscitation. Cell lines undergo comprehensive quality control and authentication procedures by ATCC before shipment. These include testing for mycoplasma by culture isolation, Hoechst DNA staining, and PCR, together with culture testing for contaminant bacteria, yeast, and fungi. Authentication procedures used include species verification by DNA barcoding and identity verification by DNA profiling.

68Ga labeling of DOTATOC

A 68Ge/68Ga generator (iThemba Labs) was eluted with 6 mL of 0.6 N HCl. The eluant was added to a buffer system of 2 mol/L HEPES at pH 3.5 to 4.0 with 5 μg of DOTATOC. The reaction solution was heated at 100°C for 20 minutes. The reaction product was loaded on a reverse-phase C18 Sep-Pak mini cartridge and eluted with 200 μL of 200-proof ethanol. The final formulation was adjusted to 10% ethanol in saline. The chemical and radiochemical purity of 68Ga-DOTATOC was measured through radio thin-layer chromatography (TLC; refs. 13, 14).

Competitive binding study

To evaluate the specific binding of 68Ga-DOTATOC, a competitive binding assay using a fixed concentration of radiotracer and increasing concentration of octreotide acetate was performed. AR42J (SSTR2-expressing) and A549 (SSTR-negative) cells were seeded in 24-well plates (2.5 × 104/well) and allowed to grow to 80% confluence. Wells were incubated for 1 hour with 0.01 to 1,000 μmol/L concentration of octreotide acetate (Abbiotec). Then, 25 μCi 68Ga-DOTATOC (~9.5 nmol/L DOTATOC peptide) was added to each well and plates were incubated at 37°C for 1 hour. The medium was removed and wells were washed 3 times using 4°C PBS. Cells were collected after trypsin treatment, and the number of cells in each well was counted using an automated cell counter (Countess, Invitrogen). 68Ga activity in the cells in each well was assayed using an automated gamma counter (Wizard 2480, Perkin Elmer) and decay corrected for the beginning of incubation with 68Ga-DOTATOC.

In vitro cell-cycle assay

To assess the effect of octreotide on cell-cycle progression, AR42J and A549 were seeded in 6-well plates and incubated at 37°C for 24 hours (A549) or 2 days (AR42J) in cell culture medium. The medium was then removed and fresh medium was added to each well. Wells were randomized to receive octreotide acetate at a concentration of 1 μmol/L or no octreotide acetate and all wells were incubated at 37°C for 24 hours before the addition of Edu (5-ethyl-2'-deoxyuridine; Click-it EDU kit, Invitrogen), a fluorescent DNA analog that is incorporated during DNA synthesis. Treated cells were then sorted for cell-cycle phase using fluorescence-activated cell sorting (FACS) and the percentage of the cells in S-phase determined in octreotide treated versus nontreated groups for each cell line. Studies were performed in triplicate.

Western blotting for SSTR2 expression

Nude (nu/nu) mice were injected subcutaneously with 106 AR42J cells suspended in Matrigel (BD Biosciences) in the left upper flank. After PET imaging, AR42J tumors were removed and extracted and whole protein extract purification performed. Protein samples (30 μg) were loaded onto SDS-PAGE and run at 120 V and 14 mA for 1.5 hours. Gels were blotted on polyvinylidene difluoride (PVDF) membrane and the blots incubated overnight at 4°C with SSTR2 monoclonal antibody (Abcam) at 1:500 dilution. β-Actin monoclonal antibody (Santa Cruz) at 1:1,000 dilution was used as an internal control. Detection was performed using the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit; Roche) and imaged on the Carestream In-Vivo Multispectral FX Imaging System. Quantitation of SSTR2 and β-actin expression was performed by drawing a region of interest around the protein bands on chemiluminescence images acquired with the Carestream Molecular Imaging Software. The acquired data were normalized using β-actin expression and corrected for tumor weight.

In vivo imaging studies

AR42J-bearing mice were divided randomly in 4 groups (n = 3 in each group) that received vehicle, 1.25, 2.5, or 10 mg/kg octreotide acetate, delivered via intraperitoneal injection every 6 hours for a total of 5 injections to reach steady-state blood levels. Five hours following injection of the fifth
dose (trough blood level of octreotide acetate), the mice underwent dynamic PET imaging with 68Ga-DOTATOC. Immediately following, the mice were imaged in static mode and then a sixth dose of octreotide was administered. Five hours after receiving the last dose of treatment solution the mice were imaged using 18F-FLT PET in static mode.

Static PET imaging protocol
Approximately 400 μCi of 68Ga-DOTATOC prepared as described above and diluted to a final volume of 150 to 200 μL that was injected intravenously via tail vein, and 1 hour later, static PET images were acquired for 15 minutes in 2 bed positions using the Sedecl Argus microPET. Static PET imaging with 18F-FLT was performed 2 hours after intravenous injection of 400 μCi 18F-FLT. Images were reconstructed using 2D-OSEM (4 iterations, 16 subsets) and were corrected for scatter and randoms. The mean standard uptake value (SUV-mean) for each tumor was calculated in a 3-dimensional (3D) region of interest autodrawn around the tumor using a 30% isocontour threshold.

Dynamic PET imaging protocol and compartmental modeling
Mice were placed under anesthesia with 2% isoflurane in O2 and positioned on the scanner such that heart and tumor were both in the field of view. Dynamic PET data were acquired in list mode for 60 minutes beginning immediately before injection of 400 μCi 68Ga-DOTATOC in 150 to 200 μL of volume via tail vein. The list mode data were then reframed in 40 fifteen-second, 20 thirty-second, 16 sixty-second, and 16 ninety-second frames. Scans were reconstructed and a 3D region of interest was set around the tumor, as described above. The input function was measured from a spherical region of interest with a 3-mm diameter over the center of the mouse heart. Time activity curves were plotted for tumor and blood pool. To determine the best compartmental model fit for 68Ga-DOTATOC binding, an octreotide challenge study was performed during a dynamic 68Ga-DOTATOC PET study in AR42J-bearing mice. The octreotide dose (150 μg) was injected via tail vein 10 minutes after the scan start and the administration of 68Ga-DOTATOC.

Figure 1. In vitro studies. A, 68Ga-DOTATOC demonstrated significantly higher binding to AR42J compared to A549 cells (P < 0.0001). B, in vitro competitive binding studies demonstrate binding affinity of 68Ga-DOTATOC to SSTR. Octreotide concentration ≥ 10 μmol/L completely binds all sites, whereas doses of <1 μmol/L allow for 68Ga-DOTATOC accumulation in graded manner in AR42J cells. A549 serve as a negative control with minimal expression of SSTR. C, gated FACS EDU binding data demonstrates that treatment with octreotide significantly decreased (P < 0.001) the number of AR42J cells in S-phase, whereas it had no effect on the number of A549 cells in S-phase. D, gated FACS EDU graph shows decreased proliferation in octreotide-treated versus control cultures for AR42J (top row), compared to A549 cells (second row), which showed no difference. Arrows, the cells in the S-phase.
Ki-67 staining

To determine tumor cell proliferation changes in response to octreotide therapy in vivo, Ki-67 staining was performed. Tumor samples from AR42J tumor–bearing mice from the different treatment groups described above were excised and kept in frozen tissue-embedding fixative (Fisher Scientific) at −80°C for further immunofluorescent staining. Briefly, slide-mounted 5-μm-thick sections were prepared and fixed using ice-cold acetone for 10 minutes followed by 3× wash with PBS (5 minutes each). Tissue sections were blocked for 30 minutes with 1% bovine serum albumin in PBS with Tween, washed with PBS, and incubated overnight at +4°C with Ki-67 antibody (Abcam). The slides were washed again with PBS, mounted with mounting medium for fluorescence-containing DAPI (Vector Laboratories), and visualized by confocal fluorescent microscopy (Nikon). Ki-67–stained and -unstained cells in the resulting images were segmented using ImageJ software and the percentage of cells stained for Ki-67 was determined.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5. Unpaired t test was used to compare the number of cells in S-phase in control and treatment groups. One-way ANOVA was employed to discern the differences in SUVmean cells in S-phase in control and treatment groups. One-way Prism 5. Unpaired Statistical analysis determined.

Correlation of imaging findings with SSTR2 protein levels

There was a consistent ratio between SSTR2 expression level and 68Ga-DOTATOC quantitative imaging measures irrespective of tumor size (tumors with diameter 3.9–8.2 mm) in AR42J tumors. The total 68Ga-DOTATOC uptake of the tumors as measured by the molecular tumor burden (MTB; ref. 15) on PET studies, which were acquired in the static mode, strongly correlates with the total SSTR2 content in tumors (R² = 0.99, P < 0.0001), as seen in Fig. 2A. MTB is the product of SUVmean and molecular tumor volume (total volume of the voxels in the region of interest with 68Ga-DOTATOC uptake above the defined threshold; ref. 16). Moreover, the mean 68Ga-DOTATOC uptake in tumors (SUVmean) also strongly correlates (Fig. 2B) with the expression of SSTR2 normalized for β-actin expression (R² = 0.85, P < 0.0004). These findings demonstrate that the noninvasively measured 68Ga-DOTATOC uptake is a true reflection of the SSTR2 levels in the tumors.

Dynamic 68Ga-DOTATOC PET imaging of AR42J tumors

Octreotide challenge studies showed that 68Ga-DOTATOC is partially displaced by octreotide but a large fraction was not.
displaceable following competitive challenge. The displaceable fraction was the $^{68}$Ga-DOTATOC bound to SSTR but not yet internalized, whereas the remaining component was already internalized and non-displaceable (Fig. 3A and C). These findings were compatible with an irreversible 2-compartment tissue model (Fig. 3E; ref. 17). Thus, on the basis of the equations in Fig. 3F, we employed a Patlak graphical plot and calculated the tumor influx constant ($K_i$; refs. 18, 19). We noted that for all studies, steady state was achieved in less than 25 minutes, and thus a 25-minute cutoff was used for fitting the $K_i$. The net $^{68}$Ga-DOTATOC influx rate, measured using a Patlak plot, following IV challenge of octreotide decreased to 0.0 (mL plasma)/(mL tissue) $^{-1}$/min $^{-1}$ (Fig. 3B), whereas the influx rate was approximately 0.7 (mL plasma)/(mL tissue) $^{-1}$/min $^{-1}$ without an octreotide challenge (Fig. 3D).

The parameter $K_i$ which is the net rate of $^{68}$Ga-DOTATOC influx, independent of tumor perfusion and reflects the number of available receptors as well as the rate of receptor trafficking (17–19). Measurement of the $K_i$ in AR42J tumors demonstrated that octreotide treatment significantly decreased the rate of tracer influx in all treatment groups compared to control and that the mean $K_i$ monotonically decreased with higher doses. The mean $K_i$ was $0.67 \pm 0.02$, $0.61 \pm 0.03$, $0.23 \pm 0.02$, and $0.17 \pm 0.03$ (mL plasma)/(mL tissue) $^{-1}$/min $^{-1}$ in vehicle, 1.25, 2.5, and 10 mg/kg treatment.
groups, respectively, as shown in Fig. 4. There was a significant decrease in the $K_i$ of all treatment groups compared to the vehicle group. The decreasing $K_i$ reflects the number of receptors occupied by octreotide and that are unavailable to bind $^{68}$Ga-DOTATOC.

**Static $^{68}$Ga-DOTATOC PET imaging of AR42J tumors**

Static scan results confirmed and paralleled the results of dynamic $^{68}$Ga-DOTATOC PET imaging. Treatment with increasing doses of octreotide acetate led to progressively significant decreases in tumor SUVmean compared to controls. Representative images are shown in Fig. 5. The mean SUVmean was 0.96 ± 0.05, 0.88 ± 0.08, 0.42 ± 0.03, and 0.21 ± 0.04 in vehicle, 1.25, 2.5, and 10 mg/kg treatment groups, respectively (Fig. 6B). As shown in the Fig. 6D, there was a very strong correlation between tumor SUVmean and $K_i$ measured by static and dynamic $^{68}$Ga-DOTATOC PET imaging ($R^2 = 0.95$, $P < 0.0001$). The high agreement of quantitative parameters between static and dynamic $^{68}$Ga-DOTATOC PET in our study suggests that SUVmean of the tumors in static PET, although not as accurate as $K_i$ measured by dynamic PET for free receptor density, can be effectively employed to monitor SSTR occupancy with octreotide treatment.

**Static $^{18}$F-FLT PET imaging and its correlation with $^{68}$Ga-DOTATOC PET imaging**

Treatment with octreotide acetate decreased the SUVmean of $^{18}$F-FLT tumor uptake in all treatment groups compared to the control group. As with dynamic and static $^{68}$Ga-DOTATOC PET imaging, higher doses of octreotide led to monotonically decreasing $^{18}$F-FLT SUVmean. The mean SUVmean was 1.40 ± 0.10, 1.30 ± 0.08, 0.34 ± 0.06, and 0.18 ± 0.03 in vehicle, 1.25, 2.5, and 10 mg/kg treatment groups, respectively, as seen in Fig. 6. There was a strong correlation between the SUVmean of the tumors in $^{18}$F-FLT PET scans and both SUVmean and $K_i$ of tumors in $^{68}$Ga-DOTATOC PET scans ($R^2 = 0.95$, $P < 0.0001$ and $R^2 = 0.97$, $P < 0.0001$, respectively; Fig. 6E and F). This demonstrates that increased occupancy of SSTR with octreotide results in a reduced rate of tumor proliferation in vivo, assessed by static $^{18}$F-FLT PET; the magnitude of reduction in tumor proliferation rate is directly correlated with and dependent on the level of SSTR occupancy and thus can be potentially monitored using $^{68}$Ga-DOTATOC PET imaging.

**Ki-67 staining results**

Treatment of AR42J tumors with increasing doses of octreotide led to a reduction in the percentage of cells staining for Ki-67. The mean percentage of cells stained for Ki-67 was 25 ± 1.2, 23 ± 0.8, 11 ± 0.9, and 5 ± 1.1 in the vehicle, 1.25, 2.5, and 10 mg/kg treatment groups, respectively. Increase in the SSTR occupancy results in a corresponding decrease in the rate of tumor proliferation shown by the decrease in relative number of cells stained for Ki-67 (Fig. 5). These results are consistent with the results obtained by noninvasive $^{18}$F-FLT PET imaging and further confirm the enhanced antiproliferative effects of octreotide on tumor cells with increasing level of SSTR occupancy.
Discussion

Octreotide continues to play a key role in the treatment of patients with metastatic NET, both for the control of symptoms of hypersecretion and, more recently, for control of tumor growth (1, 20–22). In an efficacy clinical trial called the PROMID trial (6), it was shown that a fixed monthly injection of octreotide LAR significantly increased the time to tumor progression in patients with metastatic mid-gut carcinoids by 8.3 months compared to placebo, demonstrating the antiproliferative effect of octreotide LAR on well-differentiated mid-gut NETs. However, the potential for optimized individual dosing of octreotide to lead to further significant improvements in PFS has never been explored. Quantitative noninvasive assessment of somatostatin receptor occupancy and downstream pharmacodynamic assessment of proliferation, as demonstrated in this study, could directly guide such personalized dosing optimization.

This paradigm shift from the use of imaging for disease detection to disease characterization, including the direct and downstream molecular effects of therapy on specific tumors, provides an opportunity to use such assessment to prospectively guide tailored therapy rather than retrospective reporting on treatment effectiveness measured by tumor volume changes. Such paired imaging of upstream intracellular signaling from cell surface receptors and downstream effects such as proliferation changes or alterations in apoptosis rates could be applied to a broad range of targeted therapies for individual patient drug dosing optimization. This includes therapies targeted at receptor tyrosine kinases, estrogen receptors, and androgen receptors, among other targets.

The quantitative nature of the $^{68}$Ga-DOTATOC PET measurements allows an indirect assessment of fractional SSTR occupancy. Our kinetic model is similar to that developed by Henze and colleagues (17) for characterization of the kinetics of $^{68}$Ga-DOTATOC uptake in brain meningiomas. Using dynamic PET imaging, we established that $^{68}$Ga-DOTATOC and octreotide directly compete for binding to SSTR and that binding of somatostatin analogs results in irreversible internalization (23) of the ligand receptor complex. Using this tracer kinetic model, we could then reliably calculate SSTR free fraction with increasing doses of octreotide. We demonstrated a highly significant correlation between the net tracer influx rate ($K_i$) and the SUV measurement. The net tracer influx rate ($K_i$) is measured by Patlak graphical analysis of dynamic PET data based on an irreversible 2-compartment kinetic model, which removes the effects of perfusion from the calculated receptor-mediated uptake values, whereas SUV, which is calculated from static PET data, is more routinely employed in the clinic and does not separate perfusion effects from the receptor mediated uptake. For clinical translation, both the net tracer influx rate ($K_i$) and SUV measurements could be determined in patients. Given the typical enhancement pattern seen on CT scanning of carcinoid tumors (24, 25) suggestive of high tumoral perfusion, these may correlate clinically as well as in preclinical assessment performed in this study, providing a means to more simply translate this approach for patient assessment.

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Figure 5. Imaging of AR42J tumors using static $^{68}$Ga-DOTATOC (A-D), $^{18}$F-FLT (E-H), and staining for Ki-67 in tumors samples (I-L) in 4 groups of mice treated with vehicle, 1.25, 2.5, or 10 mg/kg groups. There is high $^{68}$Ga-DOTATOC and $^{18}$F-FLT uptake as well as 25% cells stained for Ki-67 in tumors in the control group; there is a graded decrease across all the biomarkers with increased dose of octreotide treatment. The 10 mg/kg octreotide dose shows near background levels of $^{68}$Ga-DOTATOC and $^{18}$F-FLT uptake and 5% staining for Ki-67.
One possible limitation to measuring receptor occupancy level using PET imaging is that it is most useful when the receptor-targeted therapy such as octreotide is in the subsaturating range. If the administered dose of octreotide is high enough to completely saturate receptors, then there will be no tracer uptake in the intracellular compartment; in these circumstances, receptor quantitation using PET imaging shows complete occupancy and the degree of excess octreotide dose cannot be assessed. In practice, the majority of carcinoid patients is given subsaturating doses of octreotide (26) and may benefit from dose adjustment using the proposed approach.

The association between SSTR occupancy and tumor proliferation observed in the animal models used in our study suggests that dose optimization of octreotide based on receptor occupancy measurements in individual patients may be clinically beneficial. Specifically, quantitative PET imaging of the free somatostatin receptor fraction measured with $^{68}$Ga-DOTATOC, coupled with PET imaging of proliferation measured with $^{18}$F-FLT, provides a multiparametric means for potentially optimizing octreotide LAR dosing in patients with carcinoid tumors on an individual basis. Such personalized treatment may provide additional benefit over standard uniform dosing approaches currently employed. This image-guided approach to individualized drug dosing may also be employed for other receptor-targeted therapies in cancer treatment in the future. Validation of this approach in clinical trials is warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Heidari
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Heidari, U. Mahmood
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