SUPPLEMENTAL MATERIALS AND METHODS

Chemicals and Reagents

Primary fresh human hepatocytes, which had been isolated from surgical waste tissues obtained from patients undergoing partial liver resections, were obtained from Hepacult. Acetonitrile (Chromasolv LC-MS, Fluka), formic acid (98–100%, Merck), water (Millipore) and Williams Medium E (Sigma-Aldrich) were used for incubation and liquid chromatography–mass spectrometry (LC-MS) analyses. The reference compound, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), was purchased from Aldrich. The precursor, DOTA-4-amino-1-carboxymethylpiperidine-*D*-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH_2 (BAY86-7547), was obtained from Bayer HealthCare Pharmaceuticals. All the other reagents were purchased from commercial suppliers and were either synthesis or analytical grade.

Radiochemistry

The synthesis was performed with a fully automated synthesis device (Modular Lab, Eckert & Ziegler). ^{68}Ga was obtained from a ^{68}Ge/^{68}Ga generator (IGG-100, 1850 MBq (50 mCi), Eckert & Ziegler) by eluting the generator with 7 mL of 0.1 M HCl. The most radioactive fraction of the ^{68}Ga eluate (1.6 mL) was collected into the reaction vial. The BAY86-7547 precursor (17 nmol) was added to the reaction vial in a mixture containing 160 μL of 2.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 400 μL water (trace select, Fluka). The reaction mixture was first heated at 120°C for 6 minutes and then at 100°C for 6 minutes. Between the two heating periods, the reaction mixture was mixed with nitrogen gas for 30 seconds. In addition, the reaction mixture was mixed with a magnetic stirrer throughout the whole incubation period. After heating, the reaction mixture was diluted with sterile water (6 mL). The crude product was purified by loading it onto a C-18 cartridge (SepPak Light tC18, Waters) and washing the cartridge with sterile water (10 mL). BAY86-7548 was eluted with ethanol (1 mL, 50% (*v/v*)) through a non-

pyrogenic 0.22 μm sterile filter into the sterile final product vial. In the last step, the product was formulated with a sterile phosphate buffered saline (PBS, 10 mL). The overall reaction time was approximately 25 minutes.

The radiochemical purity of the product was evaluated by high-performance liquid chromatography (radio-HPLC; LC-20A Prominence HPLC System, Shimadzu; on-line radioactivity detector Flow-Count, Bioscan) using an analytical Jupiter C-18 column (5 μm, 300 Å, 150 × 4.6 mm, Phenomenex) at a flow rate of 1.0 mL/minutes and a gradient of 0.1% trifluoroacetic acid (TFA)/water (A) and 0.1% TFA/acetonitrile (B) as follows: 82% A and 18% B for 0−2 min, 40% A and 60% B from 11−14 min, and 82% A and 18% B from 15−20 min.

Blood Analyses

Venous blood samples were collected into heparinized tubes. The blood samples were drawn at 1, 10, 20, 40, 65, 100, 150, 200 and 250 min after the injection of BAY86-7548. The radioactivity of whole blood was measured with an automatic gamma counter (1480 Wizard 3", PerkinElmer). Plasma was separated by centrifugation (2,100×*g* for 5 min at 4°C) and the plasma radioactivity was measured. The blood-plasma ratio of ^{68}Ga radioactivity was calculated.

In order to examine the stability of BAY86-7548 *in vivo*, the plasma samples were analyzed by radio-HPLC. The radio-HPLC was performed using semi-preparative Luna C-18 column (5 μm, 250 × 10 mm, Phenomenex) at a flow rate of 5.0 mL/minutes and a gradient of 0.1% TFA/water (A) and 0.1% TFA/acetonitrile (B) as follows: 0−9.5 minutes with 100% A and 0% B, 9.5−10 minutes with 20% A and 80% B, 11−15 minutes with 100% A and 0% B. The fraction of ^{68}Ga metabolites in plasma was subtracted from the total radioactivity in order to estimate the plasma concentrations of unchanged tracer.

The metabolite-corrected plasma concentration was used for the calculation of pharmacokinetic parameters. Pharmacokinetic parameters were obtained using non-compartmental

analysis and adjusted for body weight, when applicable. Following the intravenous administration of BAY86-7548 of 140 MBq, which corresponds to a mass dose of ≤ 28 μg/volunteer, the pharmacokinetic parameters were determined for total radioactivity (%ID/mL) and the intact tracer (pmoL/L). Prior to any pharmacokinetic evaluation, all radioactivity values in blood and plasma were decay corrected using a ^{68}Ga physical half-life of 68 min.

Identification of Metabolites of BAY86-7549 in Human Hepatocytes and Plasma

Human hepatocytes were cultured in suspension and incubated with 10 μM BAY86-7549 (containing stable $^{69/71}$Ga isotopes instead of ^{68}Ga). The incubation reaction was initiated by the addition of 236 μL hepatocyte suspension to 3,764 μL of medium and 16 μL of 5 mM stock solution of test compound in acetonitrile. Samples were incubated in a continuously gently shaking water bath at 37°C. The reactions were stopped by addition of 200 μL of acetonitrile to 500 μL of suspension after 0, 1, 2 and 4 hours of incubation.

The incubation and control samples were centrifuged for 10 minutes at 16,000×g (4°C) to pelletize the protein. The supernatant was analyzed by LC-MS/MS for metabolite identification.

Structural characterization was carried out by accurate mass and tandem mass spectrometry using a LTQ-FT Ultra mass spectrometer connected to an Accela UPLC system (Thermo Fisher Scientific). Separations were performed on a Luna C-18 column (Phenomenex) with various dimensions: 100 × 2.1 mm, 3 μm for enhanced LC-MS analyses (flow rate 0.3 mL/minutes) and 250 × 4.6 mm, 5 μm (flow rate 1 mL/minutes) for upscaling and better correlation to radioanalyses of human plasma. The mobile phase consisted of 2.5% formic acid (A) and 2.5% formic acid/acetonitrile (B) as a linear gradient from 100% A to 100% B during 10–12 minutes.

The LTQ-FT mass spectrometer was equipped with an electrospray ionization source operating in negative ion mode. The source settings were as follows: capillary temperature 320°C, sheath gas flow 90, auxiliary gas and sweep flow 10, source voltage 3.5 kV, capillary voltage -31 V,

tube lens -70 V. Full scan MS data were obtained over the mass range of 100-1,900 Da at a peak resolution of 70,000. Targeted MS/MS experiments were acquired using fragmentation in the LTQ (linear ion trap), isolation width 2 Da, and normalized collision energy 25–30.

The relative retention times of metabolite peaks obtained from the LC-MS chromatograms of human hepatocyte incubations were compared with those of the radiochromatograms in human plasma.

SUPPLEMENTAL RESULTS

Blood Analyses

The whole blood/plasma ratio of radioactivity increased rapidly from zero to approximately 0.6 and then remained relatively constant over the 250-min duration of the PET imaging (Supplemental Fig. 1). The radio-HPLC method employed in the analysis succeeded in separating BAY86-7548 and its radioactive metabolites. The retention time of intact tracer was 7.3 minutes (range 7.2–7.4 minutes) and those of its radioactive metabolites were 3.7 minutes (range 3.6–3.8 minutes), 6.2 minutes (range 6.0–6.3 minutes) and 6.7 minutes (range 6.4–6.9 minutes).

Identification of Metabolites

Metabolite M-1 ([^{68}Ga]DOTA). The reference compound DOTA was analyzed using the LC-MS method applied for human hepatocyte incubation of BAY86-7549. The compound peak with [M-H] = 403 eluted with a relative retention time (RRT, based on the retention time of parent compound) of 0.42. The metabolite peak in the human plasma radiochromatograms eluted with RRT of 0.48 (mean value) indicating that the structure of M-1 corresponds to [^{68}Ga]DOTA and M-1 is formed by cleavage of the amide bond between DOTA and the 4-amino-piperidinyl moiety.

Metabolite M-2. After incubation of BAY86-7549 in human hepatocytes, a metabolite peak with [M-H] = 1141 at a RRT of 0.89, was detected. On the basis of accurate mass measurement and

isotope pattern, this apparent metabolite was determined to have a monoisotopic mass of 1141.4234 (M-H), which corresponds to the molecular formula of $C_{51}H_{68}N_{12}O_{14}Ga$. The molecular formula, as well as the MS/MS fragmentation pattern, indicates the cleavage of the amide bond at valine. The RRT = 0.87 (mean value) of the metabolite peak M-2 in human plasma corresponds to the RRT = 0.89 found in human hepatocytes, indicating that M-2 is formed by the cleavage of the amide bond between alanine and valine (Fig. 3).

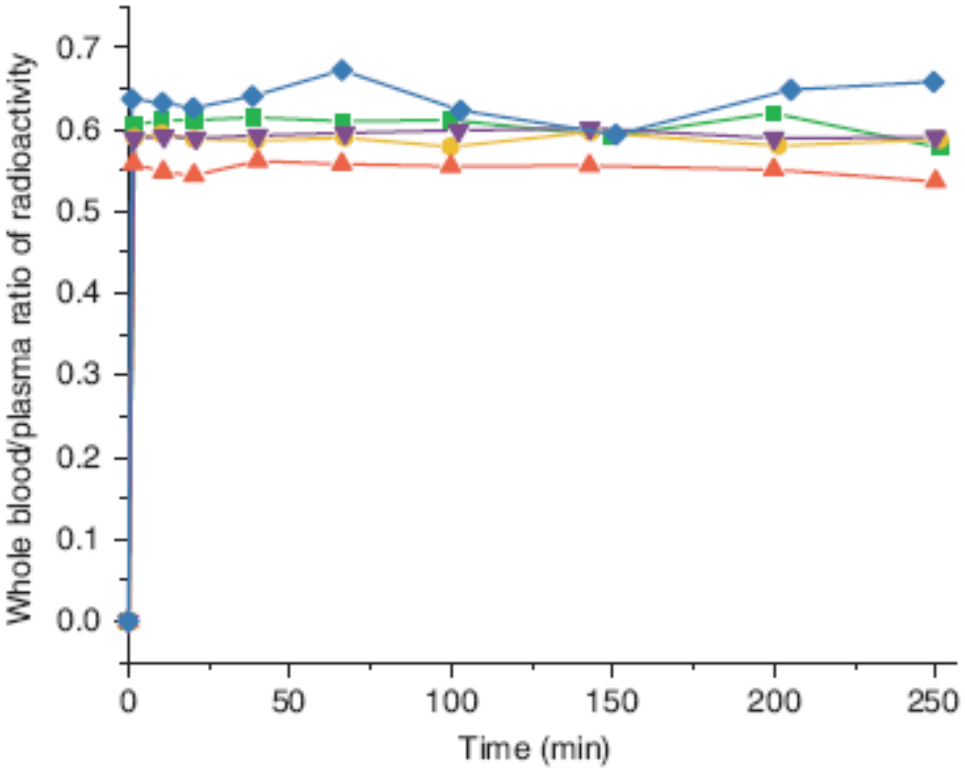
Metabolite M-3. After incubation of BAY86-7549 in human hepatocytes, a metabolite peak, with [M-H] = 1704 at a RRT of 0.95, was detected. On the basis of accurate mass measurement and isotope pattern, this apparent metabolite was determined to have a monoisotopic mass of 1704.7665 (M-H), which corresponds to the molecular formula of $C_{78}H_{113}N_{19}O_{20}Ga$. The molecular formula, as well as the MS/MS fragmentation pattern, indicates the cleavage of glutamine residue forming the glutamic acid derivative. The RRT = 0.9 (mean value) of the metabolite peak M-2 in human plasma corresponds to the RRT = 0.95 found in human hepatocytes, indicating that M-3 is formed by hydrolysis of the glutamine residue (Fig. 3).

SUPPLEMENTAL TABLE 1. Exposure of Metabolites in Human Plasma After Intravenous Administration of BAY86-7548

	Total*	M-1	M-2	M-3
n	5	5	5	5
AUC(0-t_{last}) (Bq*min/mL) †	970000	333000	37100	47300
% of Total †	100	34.3	3.80	4.80
Min (% of Total)		28.2	3.10	4.40
Max (% of Total)		44.1	5.00	6.50

* Sum of AUC(0-t_{last}) of unchanged BAY86-7548 and metabolites M-1, M-2 and M-3

† Arithmetic mean of five subjects



SUPPLEMENTAL FIGURE 1. The ratio of radioactivity in whole blood versus plasma as a function of time in five healthy males.