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Platelet Binding and Biodistribution of ^{99m}Tc -rBitistatin in Animal Species and Humans

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Abstract

INTRODUCTION— ^{99m}Tc -rBitistatin is a radioligand for the $\alpha\text{IIb}\beta 3$ (GPIIb/IIIa) receptor on platelets and is being developed as a diagnostic radiopharmaceutical for in vivo imaging of acute thrombi and emboli. Prior to the first administration of ^{99m}Tc -rBitistatin to human subjects, its biodistribution and effects on platelets were evaluated in animals. This paper reports the findings of the animal studies in comparison with the initial findings in normal human subjects.

METHODS— ^{99m}Tc -rBitistatin was administered to mice, guinea pigs and dogs to assess time-dependent organ distribution, urinary excretion and blood disappearance rates. Blood samples were analyzed to determine radioligand binding to circulating platelets and the extent of plasma protein binding. The effect of ^{99m}Tc -rBitistatin on circulating platelet count was determined. These factors were also determined in normal human subjects who received ^{99m}Tc -rBitistatin as part of a Phase I clinical trial.

RESULTS—The main organs which accumulated ^{99m}Tc -rBitistatin were kidneys, liver and spleen in all animal species and humans. The main organs seen on human images were kidneys and spleen. Liver uptake was fainter, and soft tissue background was low. ^{99m}Tc -rBitistatin bound to circulating platelets in blood, with a higher percentage of binding to platelets in guinea pigs and dogs compared with humans. Plasma protein binding was low and of little consequence in view of the platelet binding. The main route of excretion was through the urine. ^{99m}Tc -rBitistatin did not affect platelet counts in humans or dogs.

CONCLUSIONS— ^{99m}Tc -rBitistatin when administered at low doses for imaging has no adverse effects on platelets and has the qualitative biodistribution predicted by animal studies. ^{99m}Tc -rBitistatin was found to bind to circulating platelets in humans, suggesting that it will be able to bind to activated platelets in vivo in patients with acute thrombi.

Keywords

^{99m}Tc -rBitistatin; thrombus imaging; platelets; $\alpha\text{IIb}\beta 3$ integrin; GP IIb/IIIa receptor

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INTRODUCTION

Venous thrombosis is a common, potentially life-threatening clinical problem which cannot be reliably diagnosed based on clinical signs (1). If DVT is left untreated, there is a significant risk of pulmonary embolism. Although therapies for treating vascular thrombi are available, they are also associated with significant risks. Therefore, an objective diagnosis of thromboembolic disease is desirable before therapy is started. In patients with suspected pulmonary embolism, computed tomographic angiography (CTA) has become a new imaging standard for making this diagnosis (2). Many patients however are not clinical candidates for CTA because of contrast allergy, renal impairment, or a desire to limit the radiation burden to the patient. Pulmonary embolism (PE) and deep venous thrombosis (DVT) are two clinical manifestations of the same pathology as most patients with PE also have DVT. There is still therefore a role for an alternative, noninvasive imaging study which can detect both PE and DVT. Although other imaging modalities are available for detecting DVT, they have limitations. Contrast venography is highly accurate for locating thrombi, but is painful and considered invasive since there may be a reaction to the contrast medium (3). Compression ultrasound has proved to be highly sensitive and specific for detecting proximal DVT in outpatients (4). However, it is insensitive to calf thrombi and may not be reliable in patients with recurrent DVT owing to intimal thickening and scarring which may give a false positive result. Color duplex flow imaging exams of the calf veins have been shown to be technically inadequate in 40% of patients because of profound calf swelling, multiple collateral vessels or obesity (5). Moreover, as with contrast venography, accuracy can be achieved only if the test is performed by a skilled, experienced operator.

CTA and ultrasound techniques are used for imaging vascular morphology, but cannot reliably distinguish between a thrombus that is acute and hematologically active (i.e., with exposed fibrin or platelets on its surface) and therefore likely to propagate and/or embolize, versus an older resolving thrombus, which has become covered with endothelial cells and is no longer thrombogenic (6). A radiopharmaceutical which binds to exposed platelets on the surface of an acute thrombus could selectively locate hematologically active thrombi. This could be of value in distinguishing between acute thrombi and chronic thrombi or other types of blockage which are not treatable with anticoagulants.

Recently, a family of GP IIb/IIIa ligands called disintegrins has been found in viper venoms (7). Disintegrins are single-chain peptides ranging in length from 47 to 84 amino acids. Many contain an Arg-Gly-Asp (RGD) tripeptide sequence, and they contain multiple disulfide linkages in highly conserved positions. In all of the disintegrins for which the 3-dimensional structures have been determined, the RGD sequence is presented to the receptor at the end of a loop structure. In vivo studies with disintegrins have shown that they have potential utility for prevention of platelet aggregation following angioplasty or thrombolytic therapy (8). We have investigated radiolabeled disintegrins as potential thrombus imaging radiopharmaceuticals, and found that one disintegrin, bitistatin, offered advantages over previous tracers for imaging both peripheral thrombi and pulmonary emboli in a standardized canine model (9). Bitistatin is now produced by recombinant expression in bacterial culture (10). ^{99m}Tc -labeled Bitistatin was found to concentrate in DVT and PE in a canine model to provide focally positive gamma camera images of the thrombi/emboli (11). Compared to other GPIIb/IIIa targeted radiotracers, labeled autologous platelets or fibrin-targeted radiopharmaceuticals which were tested in the same animal model, ^{99m}Tc -Bitistatin had a higher percentage of the injected dose bound per gram of thrombus/embolus, and a higher target-to-background ratio with surrounding tissues.

^{99m}Tc -rBitistatin is being developed as a diagnostic radiopharmaceutical for in vivo imaging of acute thrombi and emboli. No snake venom disintegrin has previously been administered to

humans. Because rBitistatin binds to the GPIIb/IIIa receptor on platelets, there was concern that effects on platelet number or platelet function might occur at sufficiently high doses. Studies in three animal species were done to assess the biodistribution of ^{99m}Tc -rBitistatin, its binding to platelets and plasma proteins in vivo, and its effect on platelet counts. The findings are compared here with initial results from a Phase I clinical trial of ^{99m}Tc -rBitistatin in normal human subjects.

METHODS

Preparation of ^{99m}Tc -HN-rBitistatin

Recombinant bitistatin was produced in bacterial culture as a reduced polypeptide, purified by reversed-phase HPLC, and oxidatively refolded as previously published (10). The final form had the same disulfide crosslinking pattern as native bitistatin and comparable biologic activity. Recombinant bitistatin (rBitistatin) was modified with a 20:1 molar ratio of hydrazino nicotinamide (HYNIC) groups (12). The product, which contained approximately two HYNIC groups per molecule, was purified by reversed-phase HPLC and was freeze-dried in 50 μg aliquots in amber vials and stored at -70°C . Labeling with ^{99m}Tc was accomplished using a tricine intermediate (13) by adding up to 1 mL [^{99m}Tc] pertechnetate (up to 3700 MBq/mL) to a freeze-dried vial containing 54 mg tricine and 75 μg stannous chloride dihydrate, pH adjusted to 7.3. After 2 minutes at room temperature, a portion (up to 1850 MBq) of the ^{99m}Tc -tricine was drawn up and mixed with 50 μg HYNIC-rBitistatin. Radiochemical purity was assessed after 20 minutes using instant thin layer chromatography (ITLC-SG developed in saline).

Ethics in Animal Studies

The experiments reported in this paper were carried out in compliance with the relevant national laws relating to the conduct of animal experimentation. All studies were reviewed and approved by the Institutional Animal Care and Use Committee.

In vitro binding to platelets

Binding of radiolabeled bitistatin to platelets in vitro was tested with both human and canine platelets. Labeled Bitistatin was added to anticoagulated fresh canine or human blood at a final concentration comparable to what would be achieved in blood immediately after injection into a 70 kg human (1 nM). The blood was incubated at 37°C for up to 1 hour. Samples of blood were fractionated by layering over 30% sucrose and centrifuging at 13,000xg to separate plasma from cells. Equilibrium binding to platelets was typically achieved within 20–30 minutes after mixing.

Biodistribution in mice and dogs

Studies in mice and dogs were performed to determine the biodistribution and kinetics of ^{99m}Tc -rBitistatin.

Groups of five mice each were administered a single bolus IV injection (tail vein) of ^{99m}Tc -rBitistatin (0.71 $\mu\text{g}/\text{kg}$). Each mouse received 7 μCi bound to 0.02 μg (0.09 nmol/kg). Mice were euthanized at various time points (15 and 30 minutes, 1, 2, 4 and 24 hours post injection). Tissues and fluids were collected including: blood, bone, brain, heart, intestines, kidneys, liver, lungs, muscle, pancreas, spleen, stomach, testes, urine and feces. Samples were counted in a well counter along with a diluted standard of the injected dose.

Male and female mongrel dogs were administered single bolus IV doses (foreleg vein) of ^{99m}Tc -rBitistatin. Each dog received 10 mCi bound to 17 μg rBitistatin plus 3 μg ^{125}I -rBitistatin (total of 2.2 nmol per animal, approximately 0.14 nmol/kg). During injection and

intermittently for four hours post injection, planar gamma camera images of the dogs were acquired. Blood samples were drawn from the foreleg contralateral to the injection site. Urine was collected through a catheter at hourly intervals. At four hours post injection, the animals were euthanized. Tissues and fluids were collected including: blood, bone, bile, heart, intestines, kidneys, liver, lungs, muscle, ovaries, spleen, stomach, testes, uterus, urine and feces.

Effect of ^{99m}Tc -rBitistatin on circulating platelet counts in guinea pigs and dogs

We investigated the effect of a bolus dose of rBitistatin on platelet counts in vivo. Three male Hartley guinea pigs were anesthetized with 2% isoflurane in O_2 . A bolus dose of ^{99m}Tc -rBitistatin ($0.74 \mu\text{g/kg}$) was administered via the penile vein. Serial blood samples were drawn by cardiac puncture and were anticoagulated with heparin. Three dogs, anesthetized with 2% isoflurane in O_2 received ^{99m}Tc -rBitistatin ($1.2 - 2.7 \mu\text{g/kg}$) as a bolus IV dose. Serial blood samples were drawn from the contralateral foreleg vein and anticoagulated with heparin (50 units/mL blood). Platelet counts were performed by diluting aliquots of blood in an ammonium oxalate dilution vial (Unopette, Becton Dickinson, Franklin Lakes, NJ) and counting the diluted platelets in a hemacytometer.

Blood kinetics in vivo and binding to cells in blood

After administration of ^{99m}Tc -rBitistatin as an IV bolus in dogs and guinea pigs, serial blood samples were drawn and anticoagulated with heparin (50 units/mL blood). Triplicate 100 μL aliquots were layered over 30 % sucrose in a microfuge tube and centrifuged at 13,000 $\times g$ for 5 minutes to separate plasma from cells. The plasma from each tube was separated from the cell pellet and both were counted for radioactive content. The percent of the total counts found in the cell fraction was calculated as the % platelet-bound peptide. In previous studies, the cells were sub-fractionated into different types and it was determined that ^{99m}Tc -rBitistatin binds to platelets but not to white blood cells or erythrocytes.

The remaining blood was centrifuged at 13,000 $\times g$ for 5 minutes to obtain plasma. Aliquots of 50 μL each were applied to MicroSpin S-400 centrifugal microcolumns (Amersham Biosciences) designed to separate by molecular size to determine plasma protein binding by separating plasma protein from free bitistatin.

Human studies

Under a physician-sponsored IND with local IRB approval, normal human subjects were recruited for a Phase I trial of safety and biodistribution of ^{99m}Tc -rBitistatin. Each subject underwent a screening history and physical to exclude major illnesses, drug abuse, a history of bleeding, stroke, prior use of a GPIIb/IIIa antagonist, or aspirin use within 2 weeks. A negative pregnancy test was required for female subjects. In addition, laboratory tests were required to be normal: complete blood count with differential and platelet count, standard blood chemistry profile, platelet aggregometry in response to 10 μM ADP, urinalysis, bleeding time, PT/PTT and EKG.

Biodistribution in humans

Biodistribution studies were performed on four normal human subjects who each received a single intravenous injection of $0.2 \mu\text{g/kg}$ of ^{99m}Tc -rBitistatin in a peripheral vein (labeled with 7.6 – 14 mCi ^{99m}Tc). Excreted urine was collected in separate bottles with the time of collection noted. All urine was collected for the first 24 hr after dose administration. Whole body anterior and posterior planar images were acquired at 15 and 30 min, 1, 2, 4 and 6 hr after injection using a whole body dual-headed camera (Maxxus, General Electric Medical Systems, Milwaukee). The first two subjects were also imaged at 24 hours. A lateral image was acquired

at 1 hr for determination of body thickness and organ thickness. The camera was calibrated for counts/min/microcurie using a calibrated source of ^{99m}Tc in a Petri dish as recommended by Siegel et al (14). The radioactive content in each visible organ (liver, spleen, kidneys, thyroid, heart) was determined by drawing regions of interest on anterior and posterior views, correcting for local background, and correcting for attenuation based on thickness (14). These data were used to calculate radiation dosimetry estimates using the MIRD schema.

Blood distribution in Humans

Venous blood samples were drawn at 15 and 30 min, 2, 6 and 24 hr after injection, into heparinized evacuated tubes. Triplicate aliquots of 100 μL were layered over 100 μL of 30% sucrose in microfuge tubes, then centrifuged at 13,000 $\times g$ for 5 minutes to separate cells from plasma. Plasma and cells were counted separately in a well-type scintillation counter (Wallac, Perkin-Elmer). The percent of total counts found with the cell fraction was taken to be the percent of circulating radiotracer bound to platelets at the time the sample was drawn. The total counts per 100 μL of blood was used with the efficiency of the counting instrument for ^{99m}Tc to estimate the remaining radioactivity (% of injected dose) per milliliter of blood in the subject. The subject's total blood volume was estimated to be 70 mL per kg of body mass.

The remaining blood in each sample was centrifuged at 13,000 $\times g$ to generate plasma, which was used to assess plasma protein binding. Fifty μL of plasma was applied to MicroSpin 400S to separate plasma-protein bound from free ^{99m}Tc -rBitistatin in plasma.

Platelet Counts in Humans

Blood samples were drawn from human volunteers at screening (within 10 days before radiopharmaceutical injection), at 30 min post injection, and also at approximately 2, 6 and 24 hours post dosing into lavender top (EDTA) vacutainers. Platelet counts were determined by the hospital clinical laboratory.

Platelet Aggregometry in Humans

A separate blood sample was drawn from human volunteers at screening, immediately pre-injection, and at 2–3 hours post dosing. This sample was not drawn with an evacuated tube but was allowed to flow freely through an 18-ga infusion set into a polystyrene tube containing 1/10 volume 3.5% sodium citrate. Each sample was centrifuged at 180 $\times g$ for 10 min and the top two-thirds of the platelet-rich plasma was transferred with a plastic pipet to a clean plastic tube. The platelet suspension was diluted with the patient's own cell-free plasma to a concentration of 300,000 per μL (counts determined with hemacytometer and microscope). The study was performed by stirring 400 μL of adjusted platelet-rich plasma at 37°C in an aggregometer (Payton Scientific, Buffalo, NY) and recording light transmission during and for 5 min after addition of 1.5 μL of 3 mM ADP. The maximal change in light transmission was determined from each sample. The measurements were performed in triplicate. The change in light transmission for blood samples obtained after ^{99m}Tc -rBitistatin administration were compared with the baseline (pre-injection) samples.

RESULTS

Radiolabeling

Up to 1850 MBq (50 mCi) ^{99m}Tc was bound to 50 μg of bitistatin with radiochemical purity of >90%. This corresponds to a specific activity of up to 9 Ci/ μmol for the 9.08 kDa peptide.

In vitro binding to platelets

Approximately 33% of added ^{99m}Tc -rBitistatin bound to canine platelets in citrated whole blood, compared with approximately 36% in human citrated whole blood.

Blood kinetics in vivo and binding to blood components

In dogs, blood sampling studies indicated that ^{99m}Tc -rBitistatin leaves the blood pool in a biexponential fashion: approximately 57% leaves the blood with a half-life of 20 minutes and 43% leaves the blood with a half-life of 5.1 hr. An example of one dog's blood curves are shown in Figure 1A. By 20 minutes after injection, most of the blood activity was bound to platelets. Plasma radioactivity levels declined with half-lives of 3.8 minutes (18.5% ID) and 144 minutes (1.5% ID). Fractionation of blood samples from the dogs indicated that 81% to 87% of the blood activity is bound to platelets at 1 hour and 4 hours, respectively (Figure 1C).

An example of blood kinetics in one human subject is shown in Figure 1B. Blood disappearance of ^{99m}Tc -rBitistatin followed 2 phases: approximately 83% left the blood with a half-life of 6.6 minutes and 17% left the blood with a half-life of 3.8 hr. In humans, less of the injected dose was bound to platelets in comparison with dogs. At 1 hr post injection, $44.3 \pm 0.4\%$ of blood activity was platelet-bound, increasing to $75 \pm 5\%$ at 6 hr and $85 \pm 4\%$ at 24 hr as plasma activity was cleared (Figure 1C).

In guinea pigs, a higher extent of binding to platelets was observed, with 88, 92 and 95% of total blood activity bound to platelets at 1, 2 and 4 hours (data not shown).

Plasma protein binding—Although some plasma protein binding occurred after incubation of ^{99m}Tc -rBitistatin with plasma in vitro, this appeared to be of little consequence in vivo as most of the circulating radioactivity is platelet-bound. Attempts to perform HPLC analysis of plasma activity from blood samples drawn from experimental animals were hampered by inadequate counts in the plasma to permit detection by HPLC. Centrifuged microcolumns designed to separate by molecular size indicated that an average of 12, 18 and 19% of dog plasma radioactivity was bound to plasma proteins at 10 minutes, 1 and 4 hours after injection, respectively. Plasma protein binding in guinea pigs was similar to that in dogs (12–15%). In humans, plasma protein binding averaged 5, 6, and 6% at 15 min, 1 and 6 hr after injection.

Effect of ^{99m}Tc -rBitistatin on circulating platelet counts

The effect of the injection on platelet counts varied among the animals (Figure 2). In dogs which received up to $2.7 \mu\text{g/kg}$, platelet counts rose slightly above the pre-injection value but did not decline. In guinea pigs, platelet counts declined to 65% of pre-injection values, and remained at 70 – 80% of pre-injection values for the remainder of the test period. In humans, platelet counts remained in the normal range ($140,000 - 400,000/\mu\text{L}$) over the entire 24 hour test period. It was noted that the dogs have a much higher normal platelet count than guinea pigs or humans.

Platelet Aggregometry

In blood samples obtained from human subjects, there was no change in platelet aggregability in response to $10 \mu\text{M}$ ADP compared with pre-dosing samples.

Biodistribution

Mouse and dog organ distribution data are shown in Table 1 and Table 2, respectively. In the two species, the same major organs accumulated most of the radiotracer: blood, liver, spleen, kidneys and lung. The same major organs of uptake were observed in guinea pigs by gamma camera imaging (data not shown).

In human subjects kidneys, liver and spleen, which contained an average of 5.3%, 4.1% and 2.7% ID at 1h, were the main organs of uptake aside from urinary bladder. The time-activity curves for these organs are shown in Figure 3. An example anterior/posterior image of a typical subject is shown in Figure 4. Background in lung and other soft tissue was low. A small amount of thyroid activity was observed, presumably from breakdown of the radiopharmaceutical in plasma.

The estimated Effective Dose Equivalent based on human data was lower than had been predicted based on animal data. The estimated EDE for 10 mCi ^{99m}Tc -rBitistatin is 199 mrem for adult males and 262 mrem for adult females.

Urine excretion

Cumulative urine excretion in dogs averaged 23 % of the administered dose by 4 hr. In humans, an average of 30% and 42 % of the administered dose had been excreted in the urine by 4 hr and 24 hr, respectively. The chemical form of ^{99m}Tc excreted in the urine was not determined.

DISCUSSION

Preclinical studies in animal species were necessary to obtain predictions of radioligand distribution and safety prior to administering the first dose of ^{99m}Tc -rBitistatin to a human subject. These studies demonstrated that ^{99m}Tc -rBitistatin, when administered to humans, binds to platelets in the blood. While this was qualitatively similar to the behavior of the radiotracer in dogs and guinea pigs, the percentage of the administered dose that bound to platelets was lower in humans than in dogs and guinea pigs.

Radioligand which was in the plasma (not bound to cells) exhibited a small amount of plasma protein binding, but in general non-cell-bound radioligand was excreted rapidly in the urine. There was concern originally about the potential for plasma protein binding because of the use of a HYNIC linker for ^{99m}Tc labeling with tricine intermediate and no coligand. Studies in our laboratory and others (15) have shown that significant plasma protein binding can occur under these conditions, possibly because coordination sites not filled with coligand can be filled with groups on plasma proteins. We were concerned that unwanted plasma protein binding could result in excessive uptake by liver and possibly hepatobiliary excretion. Attempts at introducing a coligand were not successful in part because bitistatin could not tolerate the elevated temperatures required to achieve high labeling efficiencies in the presence of coligand (data not shown). It appears that the level of plasma protein binding in humans is small and probably not of concern.

The major organs which accumulated radiotracer (in addition to urinary bladder) were kidneys, spleen and liver. This was true for mice, guinea pigs, dogs and humans. However, the percentage of the administered dose which accumulated in these organs was different among species. The differences in spleen uptake may be related to differences in platelet binding in blood. The lung uptake observed in some of the animals may be related to anesthesia. For further studies in which ^{99m}Tc -rBitistatin will be tested for efficacy in providing a hot spot imaging agent for acute platelet deposits, the low background in lung and other soft tissue, and moderately low liver uptake, is a desirable attribute.

^{99m}Tc -rBitistatin, when administered to humans in low doses (0.2 $\mu\text{g/kg}$) did not affect the ability of platelets to aggregate and did not cause a drop in circulating platelet count. Higher doses (1.2 – 2.7 $\mu\text{g/kg}$) in dog also had no effect. In guinea pigs, however, doses of 0.74 $\mu\text{g/kg}$ did cause an apparent drop in platelet count. Platelet counts can be affected by other factors, however, and the guinea pig data were variable among the three animals.

For preclinical studies, it is important to select animal species that provide relevant information to predict human physiology and biochemistry. There are documented differences between animal species with respect to platelet response to GPIIb/IIIa antagonists. For example, rhesus macaques and guinea pigs have been reported to have responsiveness similar to that of human platelets, whereas platelets from rat and hamster are less responsive by a factor of about 100 (16). Rabbit platelets are also less responsive to the peptide RGDS (17). The appropriateness of canine platelets may depend on the breed, and some highly inbred lines have platelet abnormalities (18). In all animal studies, the environment of blood collection can have a profound effect on platelet function studies, as any stress or anxiety in the animal can alter platelet counts as well as responsiveness (18). Similarly, many drugs used as anesthetics can affect platelet function (19). In these studies, isoflurane was used because we found that it did not affect platelet function, whereas other drugs such as ketamine adversely affected the study measurements.

The concentration of platelets in the blood varies across species also. We found approximately 700,000 platelets / μ L in canine blood, in agreement with published data (18). Mice also have high platelet counts of up to 4 times the normal human platelet count (19). This may have the effect of simulating a lower dose of peptide, since so many more receptors are available for binding. It is unlikely that any of these studies approached the level of receptor occupancy needed for measurable inhibition of platelet aggregation.

Bitistatin appears to be able to differentiate somewhat between stimulated and resting platelets, with an approximate 2x higher affinity for stimulated platelets (20). This differentiation was not observed with canine platelets (11). ^{99m}Tc -rBitistatin bound approximately equally to human and canine resting platelets in a sample of citrated blood ex vivo, however in vivo there was an observed difference in the percent of dose that bound to circulating platelets. It remains to be demonstrated whether ^{99m}Tc -rBitistatin will have high binding to stimulated platelets in vivo in patients with acute thrombi.

Radiopharmaceuticals which have previously been tested for imaging of deep vein thrombosis (DVT) have suffered from various limitations (21). For example, radiolabeled platelets and fibrinogen exhibited very slow blood clearance and poor binding to mature thrombi, which precluded their ability to image pre-existing thrombi in a rapid manner. Murine antifibrin antibodies may bind to mature thrombi, but even Fab' fragments have blood clearance which is too slow for reliable thrombus imaging within 6 hours after injection. Most of the proteins and cells studied to date exhibit significant uptake by the liver, making visualization of PE difficult at the lung base adjacent to the liver. A promising approach for targeting platelet deposits has taken advantage of a receptor found on the surface of activated platelets, comprised of a complex between glycoproteins IIb and IIIa (GP IIb/IIIa). This receptor is known to undergo a conformational change upon platelet stimulation (e.g., with ADP, collagen, or thrombin) so that the altered form is able to bind fibrinogen to facilitate platelet aggregation. Studies have shown that there are approximately 100,000 copies of the GP IIb/IIIa receptor on the surface of each platelet, of which at least 80% are exposed on resting platelets (22). Macromolecular ligands for the receptor contain a common tripeptide motif, Arg-Gly-Asp (RGD), in their binding domains.

Short peptides containing the RGD tripeptide sequence or structural analogues thereof have been investigated as therapeutic drugs for inhibiting platelet aggregation and deposition in vivo. These peptides typically have reversible binding to platelets and a short duration of binding to platelets: aggregability returns to normal shortly after cessation of IV infusion. These peptides were not designed with thrombus imaging in mind. For successful imaging, a radioligand must bind in high concentration to its intended target while binding minimally to

other tissues, and it should remain bound to the intended target long enough to permit imaging while clearing from the background.

Radiolabeled versions of short synthetic peptides containing analogues of RGD have been radiolabeled and tested for their ability to image DVT. These peptides did not possess adequate targeting or lesion-to-background contrast in a standard animal model (23,24) and clinical images were of similar appearance. Although they exhibit rapid blood disappearance, radiolabeled small peptides appear to have pronounced soft tissue background (25) which may necessitate computer contrast enhancement to detect the relatively weak thrombus uptake. The rapid blood disappearance suggests that the small peptides did not bind substantially to resting platelets in the circulation, although the receptor on resting platelets is competent to bind peptides such as these (26). The reported IC_{50} for inhibition of human platelet aggregation was 79 nM for apcitide (24) compared with 130 nM for rBitistatin, but these numbers obtained in vitro do not predict the tissue distribution or bioavailability. Although rBitistatin has twofold higher affinity for activated platelets compared with resting platelets, and apcitide was reported to have a threefold difference (27), the differences in blood kinetics are greater than this might predict. A major difference between bitistatin and small cyclic peptides is not just molecular weight, but perhaps the more complex interaction of bitistatin with the platelet surface. Bitistatin has, in addition to its RGD-containing binding loop, another loop structure at the N-terminus of the molecule (11), which likely interacts with the platelet surface and thereby stabilizes binding (28). Labeled peptides which are not bound to platelets in blood are apparently susceptible to rapid clearance by excretory organs or to diffusion into extravascular fluid spaces. The initial human studies with ^{99m}Tc -rBitistatin, despite the limitation that they were done in normal humans without thrombi, showed low background in soft tissues and liver. It is anticipated that any binding of ^{99m}Tc -rBitistatin to platelet deposits would be easily detectable over this low background.

In conclusion, ^{99m}Tc -rBitistatin when administered at low doses for imaging has no adverse effects on platelets and has the qualitative biodistribution predicted by animal studies. ^{99m}Tc -rBitistatin was found to bind to circulating platelets in humans, suggesting that it will be able to bind to activated platelets in vivo in patients with acute thrombi.

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APPENDIX

Estimation of percentage of platelets labeled and amount of radiotracer which might accumulate in a thrombus:

Amount of bitistatin injected = $0.2 \mu\text{g}/\text{kg} \times 70 \text{ kg} = 14 \mu\text{g}$ bitistatin

Molecules bitistatin injected

$= 14 \mu\text{g} \times (1000 \text{ nmol} / 9080 \text{ ug}) \times 6.02 \times 10^{14} \text{ molec}/\text{nmol} = 9.28 \times 10^{14}$ molecules bitistatin

Platelets in the blood of a 70 kg human (4.9L blood)

$= 250,000 \text{ plts}/\mu\text{L} \times 4.9 \times 10^6 \mu\text{L}$

$= 1.23 \times 10^{12}$ platelets

If injected bitistatin binds uniformly to circulating platelets, the number of molecules bitistatin per platelet = $9.28 \times 10^{14} \text{ molecules bitistatin} / 1.23 \times 10^{12} \text{ platelets}$

= 754 molecules bitistatin per platelet. (Platelets have 80,000 to 100,000 copies of the receptor per cell so there is plenty of room).

Calculate atoms ^{99m}Tc per molecule of bitistatin = $50\text{mCi} / 50\text{ }\mu\text{g} = 14\text{mCi per } 14\mu\text{g dose}$

$$A = \lambda N = 0.693 \times N / T_{1/2}$$

$$N = A \times T_{1/2} / 0.693$$

Atoms ^{99m}Tc in 14 mCi

$$= 14\text{ mCi} \times 6.03\text{h} \times 3600\text{s/h} \times (3.7 \times 10^7\text{ atoms/s}) / 0.693 = 1.62 \times 10^{13}\text{ atoms } ^{99m}\text{Tc}$$

Molecules bitistatin injected = 9.28×10^{14} molecules (calculated above)

$$\text{Ratio} = 1.62 \times 10^{13}\text{ atoms } ^{99m}\text{Tc} / 9.28 \times 10^{14}\text{ molecs bitistatin}$$

$$= 1\text{ } ^{99m}\text{Tc} / 57\text{ molecs bitistatin}$$

If platelets from 1 cc blood deposit in a thrombus (not unreasonable out of 4900 mL in the body), then the amount of deposited ^{99m}Tc would be:

$$(250,000,000\text{ platelets/mL}) \times (754\text{ molecs bitistatin/platelet}) \times (1\text{ } ^{99m}\text{Tc}/57\text{ molecs bitistatin})$$

$$= 3.3 \times 10^9\text{ atoms } ^{99m}\text{Tc in thrombus}$$

$$A = \lambda N = 0.693 N / T_{1/2}$$

$$A = 0.693 \times 3.3 \times 10^9\text{ atoms } ^{99m}\text{Tc} / 6.02\text{h} / 60\text{m/h} = 6.3 \times 10^6\text{ dpm in thrombus}$$

After injection of ^{99m}Tc -bitistatin, the background clears out more rapidly than with labeled autologous platelets, so this level of accumulation in thrombus can be imaged over background.

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Figure 1.

Blood disappearance curves obtained from counting fractionated blood samples from A) dog and B) human subject. Data shown are representative of several experiments and are corrected for ^{99m}Tc decay. Solid squares = whole blood. Dashed curve with diamonds = Cell fraction. Dashed curve with open triangles = Plasma fraction. C. Cell-bound radioactivity in blood as a function of time after injection. The percentage of blood radioactivity which is bound to cells is reported at each sampling time. Open diamonds = dogs (n=2). Solid triangles = humans (n=4).

Figure 2. Effect of ^{99m}Tc -rBitistatin on circulating platelet counts in three species. A single dose of radioligand was administered at time = 0. Open diamonds = dogs (n=2). Solid triangles = humans (n=4). Open squares = guinea pigs (n=3).

Figure 3.

Time-activity curves of major organs after administration of ^{99m}Tc -rBitistatin to four human subjects. A. Liver. B. Spleen. C. Kidneys. Data are corrected for ^{99m}Tc decay.

Figure 4. Anterior (left) and posterior (right) whole body images of a female human subject 2 hr after administration of ^{99m}Tc -rBitistatin.

Table 1

Biodistribution of ^{99m}Tc-rBitistatin in mice

organ	0.25 hr	0.5 hr	1 hr	2 hr	4 hr	24 hr
%ID/g						
blood	7.152	2.748	2.256	1.862	1.15	0.835
brain	0.238	0.134	0.104	0.102	0.072	0.054
heart	2.486	3.306	1.556	1.193	1.292	0.636
lung	8.021	8.91	6.697	5.244	4.809	1.814
liver	4.834	4.175	3.769	3.911	3.875	2.345
spleen	10.604	8.601	8.782	6.169	5.35	3.054
kidneys	90.916	95.376	104.27	90.826	86.49	39.712
stom	1.742	2.541	2.837	4.489	4.732	0.835
pancreas	1.904	1.569	1.183	1.236	0.926	0.532
SI	2.988	2.781	2.571	2.757	4.028	0.757
LI	1.779	1.531	1.569	2.449	5.711	0.899
muscle	1.599	1.17	0.683	0.644	0.503	0.226
bone	3.354	2.859	2.166	1.873	1.329	0.876
testes	0.992	0.942	0.712	0.736	0.581	0.409
%ID/organ						
organ	0.25 hr	0.5 hr	1 hr	2 hr	4 hr	24 hr
blood	9.568	3.617	3.005	2.467	1.567	1.162
brain	0.09	0.057	0.044	0.042	0.029	0.022
heart	0.23	0.216	0.144	0.111	0.101	0.064
lung	1.38	1.136	0.963	0.867	0.724	0.352
liver	6.952	4.54	4.28	4.601	3.736	2.874
spleen	0.978	0.814	0.772	0.538	0.535	0.375
kidneys	24.125	27.122	29.332	26.287	23.964	11.819
stom	1.016	1.375	1.598	2.116	2.126	0.48
pancreas	0.194	0.173	0.137	0.137	0.11	0.068
SI	4.716	4.078	3.47	4.027	2.539	1.311
LI	1.114	1.034	1.025	1.482	3.615	0.73
muscle	0.422	0.346	0.216	0.186	0.127	0.052
bone	0.426	0.384	0.299	0.296	0.222	0.13
testes	0.104	0.106	0.084	0.093	0.067	0.043

Table 2Biodistribution of ^{99m}Tc -rBitistatin in dogs

<u>organ</u>	4 hr male %ID/org	4 hr female %ID/org
Gallbladder contents		0.04
LLI	0.01	0.13
Sml Int	1.30	2.32
Stomach	0.00	0.00
ULI	0.01	0.13
Heart Wall	0.33	0.30
Kidneys	40.30	34.60
Liver	12.50	14.90
Lungs	6.49	3.80
Muscle	4.08	2.91
Ovaries		0.02
Bone	7.33	9.89
Spleen	3.75	18.07
Testes	0.05	
Urine (excreted)	31.10	16.70
Uterus		0.02
blood	23.42	28.96