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ORIGINAL ARTICLE

Usefulness of competitive inhibitors of protein binding for improving the pharmacokinetics of ^{186}Re-MAG3-conjugated bisphosphonate (^{186}Re-MAG3-HBP), an agent for treatment of painful bone metastases

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Abstract
Purpose We have developed a ^{186}Re-mercaptoacetylglycylglycylglycine complex-conjugated bisphosphonate (^{186}Re-MAG3-HBP) for the treatment of painful bone metastases. We assumed competitive inhibitors of protein binding to be useful for procuring a favorable biodistribution of ^{186}Re-MAG3-HBP for the palliation of bone pain because it has been reported that the concurrent administration of ^{99m}Tc-MAG3 and drugs with high affinity for serum protein produced competitive displacement at specific binding sites and enhanced total clearance and tissue distribution.
Methods The displacement effects of several protein-binding inhibitors on the protein binding of ^{186}Re-MAG3-HBP were investigated. Biodistribution experiments were performed by intravenously administering ^{186}Re-MAG3-HBP into rats with ceftriaxone as a competitive protein-binding inhibitor or saline.
Results The protein binding of ^{186}Re-MAG3-HBP in rat serum, human serum, and a human serum albumin solution was significantly decreased by the addition of ceftriaxone, which has high affinity for binding site I on serum albumin. In the biodistribution experiments, pretreatment with ceftriaxone enhanced the clearance of the radioactivity of ^{186}Re-MAG3-HBP in blood and nontarget tissues but had no effect on accumulation in bone.
Conclusions The findings suggested that the use of protein-binding competitive inhibitors would be effective in improving the pharmacokinetics of radiopharmaceuticals with high affinity for serum protein.

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Keywords Protein binding · Absorbed radiation dose · Palliation · Bisphosphonate · ^{186}Re

Introduction

Bone metastases often present with severe bone pain, which has a significant impact on patients' quality of life [1]. Systemic radionuclide therapy using specifically localized bone-seeking radiopharmaceuticals is preferable because of the few side effects and long-lasting therapeutic effects brought about by a single injection [2]. Previously, based on the concept of bifunctional radiopharmaceuticals, we have developed ^{186}Re complex-conjugated bisphosphonate analogs for the palliation of painful bone metastases and have demonstrated the utility of these compounds [3–7]. Among these compounds, ^{186}Re-mercaptoacetylglycylglycylglycine (MAG3) complex-conjugated bisphosphonate, [[[[(4-hydroxy-4,4-diphosphonobutyl)carbamoylmethyl] carbamoylmethyl]carbamoylmethyl]carbamoylmethanethiolate] oxorhenium(V) (^{186}Re-MAG3-HBP) showed superior biodistribution characteristics as a therapeutic agent for the palliation of painful bone metastases [3]. However, ^{186}Re-MAG3-HBP may have high affinity for serum protein because ^{99m}Tc-MAG3-HBP had a high rate of binding to serum protein, which might be attributed to the high protein-binding ratio of the ^{99m}Tc-MAG3 complex [8–10]. We synthesized a ^{99m}Tc-6-hydrazinopyridine-3-carboxylic acid (HYNIC)-conjugated bisphosphonate, which had a lower protein-binding ratio and improved biodistribution compared with that of ^{99m}Tc-MAG3-HBP [11]. However, since HYNIC cannot form a stable complex with ^{186}Re, in this study, we tried a different approach to reducing the protein binding.

The binding of drugs to serum proteins has important pharmacokinetic consequences because it influences their distribution, metabolism, and excretion. Many of the interactions occur in relatively well-defined regions on human serum albumin (HSA) and on α1-acid glycoprotein (AGP). Sudlow et al. reported the presence of two primary drug-binding sites on HSA namely, site I (also referred to as the warfarin-binding site) and site II (the indole-benzodiazepine-binding site) [12, 13]. As a result, coadministration of drugs with high protein-binding affinity might produce competitive displacement at the binding site or might influence the simultaneous binding of another drug by electrostatic effects and/or binding-induced conformational changes in the protein, causing higher free concentrations of drugs in plasma than with the administration of the drugs alone [14–17]. Kawai et al. applied a strategy for the competitive displacement of protein bindings to radiopharmaceuticals for the first time and reported that treatment of a drug with high protein binding affinity increased the free fraction of ^{99m}Tc-MAG3, achieving rapid accumulation in the kidney and fast clearance from the blood [18].

In this study, the displacement effects of several drugs with high affinity for HSA-binding site I and site II on ^{186}Re-MAG3-HBP were evaluated in vitro. Biodistribution experiments of ^{186}Re-MAG3-HBP with or without pretreatment with an inhibitor were performed to investigate the validity of employing a competitive inhibitor of protein binding.

Materials and methods

Materials

^{186}Re was supplied by the Japan Atomic Energy Agency (Tokai-mura, Japan) as $^{186}ReO_4^-$ [19]. ^{186}Re-MAG3-HBP and nonradioactive Re-MAG3 were synthesized as described previously [3]. HSA (essentially fatty acid free albumin), AGP, ceftriaxone, and cefazolin were purchased from Sigma Chemical (St. Louis, MO, USA). Bucolome (5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine, Grelan Pharmaceutical, Tokyo, Japan) was obtained as a pure substance from the manufacturer. Ibuprofen was purchased from Nakalai Tesque (Kyoto, Japan). Other reagents were of reagent grade and used as received.

In vitro protein-binding studies

The protein-binding ratios of ^{186}Re-MAG3-HBP were evaluated by ultrafiltration (Centrifree; Millipore, Billerica, MA, USA). ^{186}Re-MAG3-HBP (74 kBq in 33.3 μL saline) was added to 300 μL of rat or human serum (or HSA, AGP solution). Mixtures (280 μL) of ^{186}Re-MAG3-HBP and serum were centrifuged at 1,000×*g* for 20 min at room temperature. The radioactivity of the part of the initial solutions (30 μL) and the filtrates (30 μL) was measured with an auto-well gamma counter (ARC-2000; Aloka, Tokyo, Japan). The free fraction and protein-binding ratio were determined as follows:

$$\text{Free fraction}(\%) = (\text{radioactivity of filtrate})/(\text{radioactivity of initial solution}) \times 100.$$

$$\text{Protein-binding ratio}(\%) = 100 - (\text{free fraction}).$$

The HSA (740 μM) and AGP (17 μM) solutions were prepared at normal human serum concentrations.

In order to investigate the moiety with high affinity for HSA in ^{186}Re-MAG3-HBP, nonradioactive Re-MAG3 and bisphosphonate (1-hydroxyethylidene-1,1-diphosphonate; HEDP) at concentrations of 0.4, 1, or 2.5 mM were added to mixtures of ^{186}Re-MAG3-HBP and HSA. Then, the

protein-binding ratios of ^{186}Re-MAG3-HBP were evaluated as described above.

The effects of each competitive protein-binding inhibitor were examined in rat serum, human serum, and HSA solution. Bucolome [20, 21], ceftriaxone [22], and cefazolin [23] as displacers of HSA binding site I and ibuprofen [13] as a displacer of HSA binding site II were added to each serum at 400 μM before the addition of ^{186}Re-MAG3-HBP. Then, the protein binding of ^{186}Re-MAG3-HBP was evaluated as described above.

In vivo studies

Animal experiments were conducted in accordance with our institutional guidelines; the experimental procedures were approved by the Kyoto University Animal Care Committee. Biodistribution experiments were performed by intravenous injection of 250 μL of the diluted tracer solution into male Wistar rats (190–230 g, Japan SLC, Hamamatsu, Japan). Ceftriaxone (50 mg/kg), as a competitive protein-binding inhibitor, was administered intravenously just before the tracer was injected. Saline was administered to the rats in a control group instead of ceftriaxone. Groups of four or five rats each were killed by decapitation at 5, 10, 30, 60, and 180 min postinjection. Tissues of interest were removed and weighed. The entire left femur was isolated as a representative bone sample. The levels of radioactivity in the tissues were determined with an auto-well gamma counter and corrected for background radiation and physical decay during counting.

To determine detailed blood time–activity curves, blood samples of the male Wistar rats (190–230 g) were taken. Specifically, a polyethylene cannula (inner diameter 0.5 mm, outer diameter 0.8 mm; Dural Plastics, Dural, Australia) filled with heparin in saline was inserted into the femoral artery of each rat anesthetized with sodium pentobarbital. After intravenous injections via the tail of the tracer and ceftriaxone (50 mg/kg) or saline, blood samples were taken at 2, 5, 7, 10, 15, 20, 30, 40, 50, and 60 min postinjection. Blood samples were weighed, and the radioactivity was determined with an auto-well gamma counter. The area under the blood time–activity curve was calculated by numeral integration using a linear trapezoidal formula from the time of tracer injection to the time of last blood sampling and extrapolation to infinity based on a monoexponential equation [24].

Radiation dose estimates

For estimation of the radiation dose absorbed by the bone and bone marrow, the level of activity in the bone marrow was assumed to be 30% of that in the blood [25]. Red marrow mass was assumed to be 25% of blood volume [25]. Blood volume was calculated using the following formula [26].

$$\text{Blood volume(mL)} = 0.06 \times \text{body weight (g)} + 0.77$$

The bone mass of the rats was assumed to be 10% of body weight [27]. According to the International Commission on Radiological Protection, an equal distribution of the radionuclide to trabecular and cortical bones was assumed [28]. The no-decay-corrected activity from each source organ was converted to a percentage of the injected dose (ID). A time–activity curve was created for each organ by plotting the percentage of ID versus time. The area under an organ's activity curve from time zero to infinity was calculated by the abovementioned method. According to the values, absorbed radiation doses were calculated for an adult patient using OLINDA 1.0 software (Vanderbilt University) [29].

Statistical analysis

An unpaired Student's *t* test was used for the biodistribution experiments. A one-way analysis of variance followed by Dunnett's post-hoc test compared to the control group was used for in vitro protein-binding experiments. Results were considered statistically significant at $p<0.05$.

Results

Binding of ^{186}Re-MAG3-HBP to serum protein

The percentages of ^{186}Re-MAG3-HBP bound to rat serum and human serum were 97.4±1.1% and 97.0±0.3%, respectively. In the case of the purified human serum proteins, ^{186}Re-MAG3-HBP bound to HSA strongly (98.2±0.1%) and bound to AGP weakly (11.0±0.5%).

In the experiments in which nonradioactive Re-MAG3 or HEDP was added to a mixture of ^{186}Re-MAG3-HBP with HSA, Re-MAG3 appreciably inhibited the binding of ^{186}Re-MAG3-HBP to HSA on a dose-dependent basis (Fig. 1).

Effects of several drugs on the binding of ^{186}Re-MAG3-HBP to serum protein

The effects of several drugs as competitive inhibitors on serum protein binding of ^{186}Re-MAG3-HBP were evaluated. Data were expressed relative to the free fraction value for the control group (without inhibitor). On the addition of bucolome or ceftriaxone, the free fraction of ^{186}Re-MAG3-HBP in rat serum increased significantly compared with that of the control (Fig. 2a). In human serum, the addition of ceftriaxone significantly increased the free fraction of

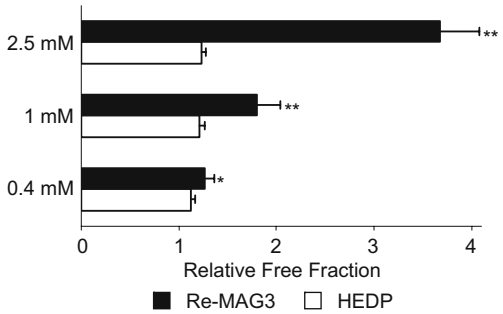

Fig. 1 Displacement of ^{186}Re-MAG3-HBP from protein in HSA. Re-MAG3 and HEDP were loaded at 0.4, 1, and 2.5 mM (final concentration). Data are expressed relative to the free fraction for the control group (mean±SD for three experiments). Significant differences from control (without inhibitor) were identified with Dunnett's test (*asterisk*, $p<0.05$; *double asterisk*, $p<0.01$)

^{186}Re-MAG3-HBP (Fig. 2b). In HSA solution, the addition of bucolome, ceftriaxone, cefazolin, or ibuprofen significantly increased the free fraction of ^{186}Re-MAG3-HBP (Fig. 2c). The increase on the addition of bucolome, ceftriaxone, or cefazolin was more remarkable than that on the addition of ibuprofen (significance difference determined with the Tukey–Kramer test).

In vivo studies

Table 1 lists the biodistribution of ^{186}Re-MAG3-HBP with or without pretreatment with ceftriaxone in normal rats, expressed as a percentage of the ID per gram tissue (%ID/g). As to the accumulation of radioactivity in the target tissue, bone, there was no difference between the ceftriaxone-pretreated group and the control group. At the same time, the pretreated group had lower levels of radioactivity than did the control group in nontarget tissues such as the kidney.

The blood time–activity curves of ^{186}Re-MAG3-HBP with or without pretreatment with ceftriaxone in rats, expressed as %ID/g, are presented in Fig. 3. The clearance value in the ceftriaxone-pretreated group and the control group calculated from the area under the curve was 172.3±11.8 and 115.8±14.4 mL/h, respectively.

Dosimetry

Table 2 lists the estimated absorbed radiation doses for ^{186}Re-MAG3-HBP with and without pretreatment with ceftriaxone. We assumed that the radiation dose absorbed by the osteogenic cells is an index of the therapeutic effects. There was no difference in the absorbed radiation dose versus that without the ceftriaxone. Although the dose absorbed by red marrow, a dose-limiting factor of bone-seeking radiopharmaceuticals, did not differ between the two groups, that absorbed by other nontarget tissues such as the kidney was markedly decreased by pretreatment with ceftriaxone.

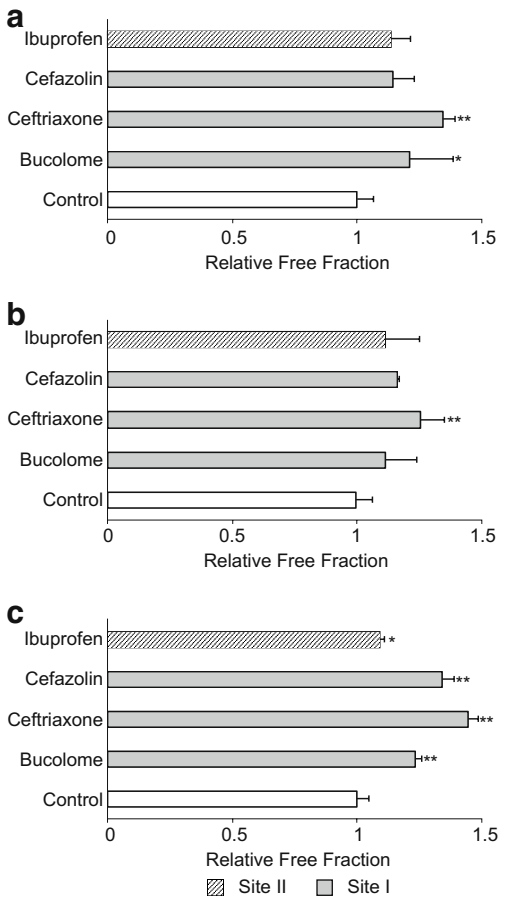

Fig. 2 Displacement of ^{186}Re-MAG3-HBP from protein in **a** rat serum, **b** human serum, and **c** HSA. Each competitive protein-binding inhibitor was loaded at a final concentration of 400 μM. Data are expressed relative to the free fraction for the control group (mean±SD for three experiments). Significant differences from control (without inhibitor) were identified with Dunnett's test (*asterisk*, $p<0.05$; *double asterisk*, $p<0.01$)

Discussion

In the protein-binding experiments in vitro, ^{186}Re-MAG3-HBP showed very high ratios of protein binding in human and rat serum. These high ratios of protein binding are probably due to the strong binding of ^{186}Re-MAG3-HBP to serum albumin because ^{186}Re-MAG3-HBP exhibited extensive binding to HSA and little binding to AGP. In experiments using several inhibitors of protein binding, the HSA site I inhibitors were more effective than the HSA site II inhibitor. A previous study showed that ^{99m}Tc-MAG3 binds specifically with binding site I on HSA [18]. If the ^{186}Re-MAG3 structure contributes to the binding of ^{186}Re-MAG3-HBP, the result of the experiments using an inhibitor could be reasonable. Then, to investigate the moiety with high affinity for HSA in ^{186}Re-MAG3-HBP, the binding to HSA was examined when a large excess of nonradioactive Re-MAG3 or bisphosphonate (HEDP) was

Table 1 Biodistribution of radioactivity after intravenous administration of ^{186}Re-MAG3-HBP with or without pretreatment with ceftriaxone in rats

Tissue	Time after administration				
	5 min	10 min	30 min	60 min	180 min
Pretreatment of ceftriaxone					
Blood	0.98** (0.19)	0.57 (0.06)	0.19* (0.02)	0.05** (0.00)	0.02** (0.00)
Liver	0.32** (0.03)	0.13 (0.01)	0.08** (0.00)	0.07** (0.01)	0.06* (0.01)
Kidney	2.76 (1.15)	1.22 (0.13)	0.85** (0.02)	0.62** (0.06)	0.58 (0.10)
Intestine	0.17* (0.03)	0.09 (0.01)	0.08 (0.02)	0.14 (0.04)	0.26 (0.10)
Spleen	0.27 (0.03)	0.12 (0.05)	0.06* (0.01)	0.05 (0.01)	0.05 (0.01)
Femur	1.73 (0.20)	1.88 (0.08)	3.47 (0.14)	3.89 (0.24)	4.01 (0.28)
Muscle	0.15 (0.02)	0.12 (0.06)	0.06 (0.01)	0.01 (0.00)	0.01 (0.01)
Control					
Blood	1.32 (0.10)	0.77 (0.22)	0.27 (0.06)	0.07 (0.01)	0.03 (0.00)
Liver	0.44 (0.06)	0.16 (0.04)	0.09 (0.00)	0.07 (0.00)	0.08 (0.01)
Kidney	4.35 (2.40)	1.56 (0.38)	1.40 (0.08)	0.85 (0.11)	0.73 (0.14)
Intestine	0.25 (0.07)	0.10 (0.03)	0.10 (0.02)	0.11 (0.02)	0.30 (0.16)
Spleen	0.28 (0.02)	0.15 (0.03)	0.08 (0.01)	0.05 (0.00)	0.04 (0.01)
Femur	1.67 (0.22)	1.91 (0.10)	3.43 (0.33)	4.15 (0.14)	4.14 (0.65)
Muscle	0.17 (0.02)	0.11 (0.01)	0.06 (0.02)	0.01 (0.01)	0.02 (0.02)

Each value represents the mean (SD) for four to five rats. Data are expressed as percent of injected dose per gram. Significance was determined by Student's *t* test.
*$p<0.05$, **$p<0.01$

added to a mixture of ^{186}Re-MAG3-HBP and HSA. As a result, the addition of Re-MAG3 strongly inhibited the binding of ^{186}Re-MAG3-HBP to HSA, indicating that the binding of ^{186}Re-MAG3-HBP was attributable to the ^{186}Re-MAG3 moiety.

Ceftriaxone was the most effective of the HSA site I inhibitors used in this study. At the same time, we consider ceftriaxone, one of the antibiotics, to be effective for clinical applications because it can be administered clinically at a comparable dose from the point of view of safety. Previously, Haradahira et al. reported that the uptake in the brain of a radioligand for the glycine-binding site of the *N*-methyl-D-aspartic acid receptor, [^{11}C]L-703,717, was increased by the use of warfarin, which inhibits plasma protein binding in mice [30]. The strategy is similar to that of our study, but the ID of warfarin in that study was quite different from a clinical dose. In this study, in vitro experiments were performed under conditions where the final concentration of inhibitors was 360 μM. It was reported that the early plasma concentration of ceftriaxone was over 400 μM after a single intravenous administration of 2 g of ceftriaxone to an adult [31]. The administration of 2 g of ceftriaxone as a clinically available dose should be useful for the competitive inhibition of protein binding because ceftriaxone significantly elevated the free fraction of ^{186}Re-MAG3-HBP at the lower concentration in vitro. Another advantage of ceftriaxone is its long elimination half-life (6 to 9 h) [31, 32]. Maintaining a high concentration in the blood seems to be favorable for competitive inhibition at serum-binding sites. Thus, in vivo experiments were performed with pretreatment of ceftriaxone as an inhibitor of protein binding. As expected, the clearance from blood of ^{186}Re-MAG3-HBP was greater with the pretreatment than without. These results suggest that ceftriaxone could be also useful in vivo as a competitive protein-binding inhibitor. Furthermore, the ceftriaxone-pretreated group also showed lower radioactiv-

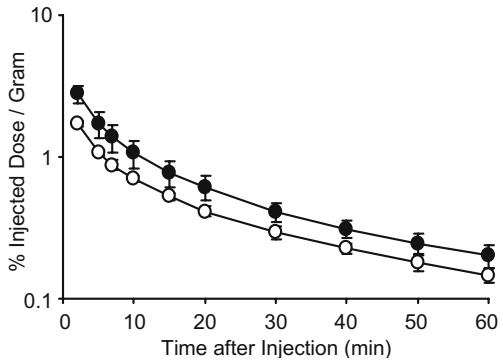

Fig. 3 Radioactivity in blood after injection of ^{186}Re-MAG3-HBP in rats. Ceftriaxone (*open circles*) (50 mg/kg) or saline (*closed circles*) as a control was loaded just before ^{186}Re-MAG3-HBP was injected. Data are expressed as a percentage of the injected dose per gram (mean±SD for seven rats) on a log scale

Table 2 Absorbed dose estimates of ^{186}Re-MAG3-HBP with or without pretreatment with ceftriaxone

Organ	Pretreatment with ceftriaxone	Control
Osteogenic cells[a]	8.75	8.87
Red marrow[a]	3.01	3.05
Liver[a]	0.03	0.04
Kidneys[a]	0.42	0.77
Small intestine[a]	0.01	0.01
Spleen[a]	0.02	0.03
Effective dose equivalent[b]	0.69	0.72
Effective dose[b]	0.50	0.51

[a] Expressed as mGy/MBq
[b] Expressed as mSv/MBq

ity levels than did the control group in nontarget tissues, such as the kidney. These decreases in radioactivity might be attributed to an enhanced distribution to the tissues with the decrease in protein binding. However, there was no significant change between the groups in the accumulation in bone. We suppose that it is the reason that ^{186}Re-MAG3-HBP binds to the hydroxyapatite in the bone and is retained for a long term without excretion. Accordingly, pretreatment with ceftriaxone could be useful for improving the biodistribution of ^{186}Re-MAG3-HBP from the point of view of decreasing the radiation dose to nontarget tissues. The results from the estimates of absorbed doses indicate that the dose absorbed by the kidney was certainly decreased and that absorbed by osteogenic cells was almost the same. We assumed that the dose of radiation absorbed by the osteogenic cells is an index of the therapeutic effects. Although osteogenic cells should not be the exact target of this type of internal radionuclide therapy, we cannot describe a direct index of the therapeutic effect because we used normal rats and calculated the radiation dose to normal humans by extrapolation. The therapeutic (palliation) effects must occur by binding radiopharmaceuticals with hydroxyapatite in or near the metastatic bone. Because the accumulations of radiopharmaceuticals in the metastatic bone are not specific, it is predicted that the radiation dose of osteogenic cells would correlate with the therapeutic effect. The results of the radiation dose indicate that it could decrease toxicity to kidney tissue while obtaining the same therapeutic effect. However, when the usual therapeutic dose of ^{186}Re-MAG3-HBP is injected, the strategy might have little clinical merit because the initial radiation dose to the kidneys is not very high. For example, when a much higher dose was injected with an autologous peripheral blood stem cell transplant [33, 34], the strategy should be more effective. At the same time, the dose absorbed by red marrow, which is known as a dose-limiting factor of bone-seeking radiopharmaceuticals, was not decreased. Since almost the entire dose absorbed by red marrow is from the radioactivity accumulated in bone, accelerated blood clearance would not affect the absorbed dose in red marrow.

In conclusion, pretreatment with ceftriaxone enhanced the clearance of the radioactivity of ^{186}Re-MAG3-HBP in blood and nontarget tissues, although it had no effect on accumulations in the bone. These findings suggested that treatment with a protein-binding competitive inhibitor would be effective for radiopharmaceuticals with high affinity for serum protein to improve their pharmacokinetics, and this strategy might have even greater effects for other radiopharmaceuticals.

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