

# Ranibizumab and Aflibercept: Intraocular Pharmacokinetics and Their Effects on Aqueous VEGF Level in Vitrectomized and Nonvitrectomized Macaque Eyes

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Submitted: May 16, 2015  
Accepted: August 28, 2015

Citation: Niwa Y, Kakinoki M, Sawada T, Wang X, Ohji M. Ranibizumab and aflibercept: intraocular pharmacokinetics and their effects on aqueous VEGF level in vitrectomized and nonvitrectomized macaque eyes. *Invest Ophthalmol Vis Sci.* 2015;56:6501-6505. DOI:10.1167/iovs.15-17279

**PURPOSE.** We evaluated the pharmacokinetics of intravitreally injected ranibizumab and aflibercept, and their effects on VEGF in the aqueous humor of vitrectomized and nonvitrectomized macaque eyes.

**METHODS.** Intravitreal ranibizumab (IVR; 0.5 mg/50  $\mu$ L) or intravitreal aflibercept (IVA; 2 mg/50  $\mu$ L) was injected into the previously vitrectomized right eyes of three macaques and nonvitrectomized right eyes of three macaques. The left eyes served as controls (nonvitrectomized, noninjected). Aqueous humor was obtained from both eyes just before injection and on days 1 and 3, and weeks 1 to 8 after IVR and IVA. The ranibizumab, aflibercept, and VEGF concentrations were measured using enzyme-linked immunosorbent assays.

**RESULTS.** The half-lives in aqueous humor of nonvitrectomized and vitrectomized eyes were, respectively, 2.3 and 1.4 days for ranibizumab, and 2.2 and 1.5 days for aflibercept. Concentration of VEGF was decreased below the limit of detection (LOD) by IVR for 3 weeks in nonvitrectomized eyes and 1 week in vitrectomized eyes, respectively, and by IVA for 6 weeks in nonvitrectomized eyes and 4 weeks in vitrectomized eyes, respectively. In the untreated control eyes, the ranibizumab and aflibercept concentrations were below the LOD, and the VEGF aqueous concentrations remained unchanged after IVR and decreased for 3 days after IVA.

**CONCLUSIONS.** Intravitreally injected ranibizumab and aflibercept have similar half-lives in aqueous humor and shorter half-lives in vitrectomized eyes. Compared to IVR, IVA suppresses VEGF level for a longer time period.

**Keywords:** VEGF, ranibizumab, aflibercept

Ranibizumab (Lucentis; Genentech, Inc., South San Francisco, CA, USA) and aflibercept (Eylea; Regeneron Pharmaceuticals, Tarrytown, NY, USA) are VEGF inhibitors used for the treatment of age-related macular degeneration (AMD), myopic choroidal neovascularization, macular edema following retinal vein occlusion, and diabetic macular edema. Ranibizumab is a recombinant, humanized monoclonal antibody fragment that neutralizes all biologically active forms of VEGF-A.<sup>1</sup> Aflibercept is a recombinant fusion protein comprised of portions of human VEGFR1 and VEGFR2 extracellular domains fused to the Fc portion of human immunoglobulin G1.<sup>2</sup> Ranibizumab and aflibercept are used widely. There are many reports about their efficiency,<sup>3-11</sup> but only a few about their pharmacokinetics. We previously reported the intraocular pharmacokinetics of bevacizumab and its effect on aqueous VEGF concentration in vitrectomized and nonvitrectomized macaque eyes.<sup>12,13</sup> Christoforidis et al.<sup>14</sup> and Ahn et al.<sup>15</sup> reported the intraocular pharmacokinetics of ranibizumab in vitrectomized and nonvitrectomized rabbit eyes. Gaudreault et al.<sup>16</sup> reported the pharmacokinetics of ranibizumab in monkey eyes. To our best

knowledge, no one has reported the pharmacokinetics of aflibercept in monkey eyes. Therefore, we measured concentrations of ranibizumab, aflibercept, and VEGF over time in the aqueous humor of vitrectomized and nonvitrectomized eyes after the injection of intravitreal ranibizumab (IVR) and intravitreal aflibercept (IVA) in macaques.

## METHODS

All treatments were in accord with principles of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the animal research protocol was approved by the Animal Experimentation Committee at Shiga University of Medical Science. Six male cynomolgus macaques were used in this study. A procedure consisting of pars plana vitrectomy with lensectomy was performed in the right eyes of three macaques, at least 6 months before the experiments. Each macaque was anesthetized with 5 mg/kg intramuscular ketamine hydrochloride and 1 mg/kg intramuscular xylazine hydrochloride.

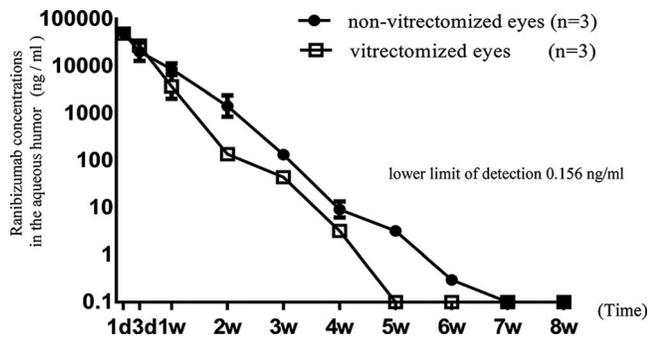


FIGURE 1. Ranibizumab concentrations in the aqueous humor of nonvitreotomized eyes and vitreotomized eyes after intravitreal injection.

Povidone iodine was placed on the conjunctiva of each eye. Using a 29-gauge needle, ranibizumab (0.5 mg/50  $\mu$ L) was injected into the vitreous cavity of the right eye of each macaque. The left eyes received no intravitreal injections and served as controls. Using a 29-gauge needle, aqueous humor samples (150  $\mu$ L) from both eyes were obtained just before injection and on days 1 and 3, and weeks 1 to 8 after IVR. At least 6 months after IVR, aflibercept (2 mg/50  $\mu$ L) was injected into the vitreous cavity of the right eye, and aqueous humor samples (150  $\mu$ L) from both eyes were obtained just before injection and on days 1 and 3, and weeks 1 to 8 after IVA. The depth of the anterior chamber remained normal at all times after the samples were obtained. The samples were stored in a freezer at  $-80^{\circ}\text{C}$  until analysis.

### Measurement of VEGF

Aqueous VEGF concentrations were measured using an ELISA (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, MN, USA). According to the manufacturer's instructions, the detection limit of VEGF was 9.0 pg/mL. A VEGF concentration less than the limit of detection (LOD) was considered as 0 in the calculation of the mean.

### Measurement of Ranibizumab and Aflibercept Concentrations

Aqueous ranibizumab and aflibercept concentrations were measured by ELISA. A total of 96 well plates were coated with recombinant human VEGF165 (R&D Systems) at a concentration of 1.0  $\mu$ g/mL overnight at  $4^{\circ}\text{C}$  (100  $\mu$ L/well). After washing three times with PBS containing 0.05% Tween-20, the wells were blocked with 3% BSA/PBS overnight at  $4^{\circ}\text{C}$  (200  $\mu$ L/well), washed five times with PBS containing 0.05% Tween-20, and stored dry at  $4^{\circ}\text{C}$  for later use. Aqueous humor diluted in 0.1% BSA/PBS (50  $\mu$ L/well) was added to the plates, which were incubated overnight at  $4^{\circ}\text{C}$ . Ranibizumab and aflibercept were detected by incubation with horseradish peroxidase-goat anti-human IgG (H+L) conjugate (1  $\mu$ g/mL; Invitrogen Corporation, Carlsbad, CA, USA) for 2 hours at room temperature, five washes, color development with a 100  $\mu$ L 3,3',5,5'-tetramethyl benzidine substrate, and addition of 1 M HCl (100  $\mu$ L) to stop the reaction. The optical density was measured at 450 nm with correction at 570 nm. A standard curve was prepared with ranibizumab and aflibercept concentrations ranging from 7.8 to 4000 pg/mL. The minimal quantifiable concentration of ranibizumab and aflibercept was 15.6 pg/mL. Because the sample volumes were small, they were diluted 10 times before measurement. Therefore, the LODs of ranibizumab and aflibercept in aqueous humor were 156 pg/mL (0.156 ng/mL). This sandwich ELISA assay only measured free ranibizu-

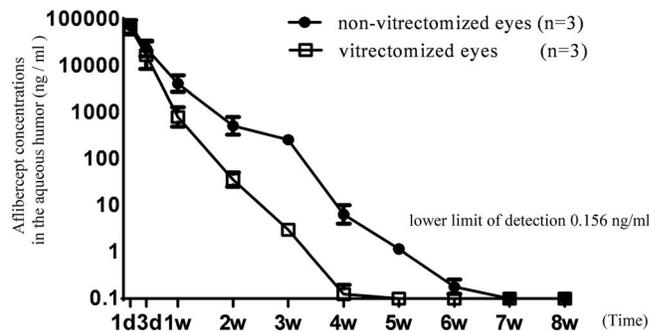


FIGURE 2. Aflibercept concentrations in the aqueous humor of nonvitreotomized eyes and vitreotomized eyes after intravitreal injection.

mab/aflibercept rather than total concentration of free and VEGF-bound ranibizumab/aflibercept.

### Pharmacokinetic Analysis

The pharmacokinetic properties of ranibizumab and aflibercept were determined using a one-compartment model as described in our previous report on bevacizumab.<sup>12,13</sup> This method assumes that drug clears from the eye monoexponentially. The experimental concentration data of ranibizumab and aflibercept were fit to the following equation with Standard Microsoft Excel 2010 software (Microsoft, Redmond, WA, USA):

$$C(t) = C_0 \exp(-kt),$$

where  $C$  ( $\mu$ g/mL) and  $C_0$  ( $\mu$ g/mL) denote concentration at any time  $t$  (in days), and at  $t = 0$ , respectively.  $C_0$  is the baseline concentration, and  $k$  indicates the elimination rate constant. The half-life ( $t_{1/2}$ ) was calculated as  $0.693/k$ .

The areas under the curve (AUC) were estimated by a linear-trapezoidal method.

### Statistical Analysis

All statistical analyses were done using PASW Statistics 18 (SPSS Japan, Tokyo, Japan).

### RESULTS

In the injected nonvitreotomized eyes, mean ranibizumab concentrations in the aqueous humor peaked at 51,300 ng/mL ( $n = 3$ ; range, 45,700–61,500) the day after IVR injection and fell below the LOD 6 weeks later. The half-life of 0.5 mg IVR in the aqueous humor was 2.3 days ( $n = 3$ ; range, 2.0–2.4). In the injected vitreotomized eyes, mean ranibizumab concentrations in the aqueous humor peaked at 41,800 ng/mL ( $n = 3$ ; range, 34,200–58,200) the day after IVR and fell below the LOD 4 weeks after IVR. The half-life was 1.4 days ( $n = 3$ ; range, 1.0–2.0; Fig. 1). Ranibizumab was not detected in the untreated fellow eyes.

In the injected nonvitreotomized eyes, the mean aflibercept concentration in the aqueous humor peaked at 74,000 ng/mL ( $n = 3$ ; range, 65,300–88,800) the day after IVA injection and fell below the LOD 6 weeks after IVA injection. The half-life of 2 mg intravitreally injected aflibercept in the aqueous humor was 2.2 days ( $n = 3$ ; range, 2.2–2.3). In the injected vitreotomized eyes, aflibercept concentrations in the aqueous humor peaked at 68,000 ng/mL ( $n = 3$ ; range, 40,100–97,900) the day after IVA and fell below the LOD 4 weeks after IVA. The half-life was 1.5 days ( $n = 3$ ; range, 1.3–1.8; Fig. 2). Aflibercept was not detected in the untreated fellow eyes.

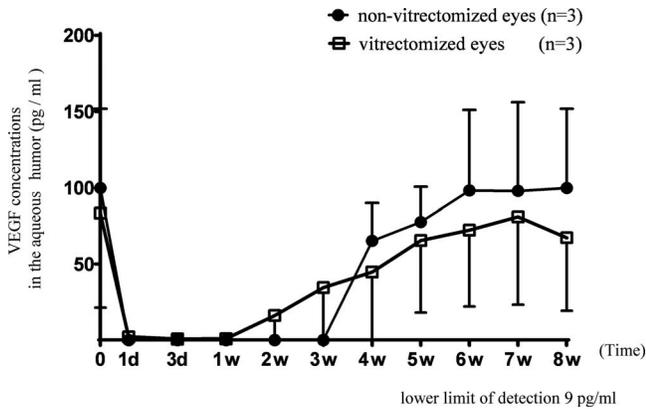


FIGURE 3. Vascular endothelial growth factor concentrations in the aqueous humor of nonvitrectomized eyes and vitrectomized eyes after intravitreal ranibizumab injection.

The mean aqueous VEGF concentration of the injected nonvitrectomized eyes was 99.7 pg/mL ( $n = 3$ ; range, 63.1–159.4) before IVR injection. One day after the IVR, the aqueous VEGF concentrations decreased to less than 9.0 pg/mL (the LOD) in all injected eyes. The concentration decreased below the LOD for 3 weeks in nonvitrectomized eyes. The mean aqueous VEGF concentration of the injected vitrectomized eyes was 78.1 pg/mL ( $n = 3$ ; range, 16.5–139.2) before IVR. One day after IVR, the aqueous VEGF concentrations decreased to the LOD in all injected eyes and concentration below the LOD was maintained for 1 week in vitrectomized eyes (Fig. 3). The mean aqueous VEGF concentration of noninjected fellow eyes was 93.1 pg/mL ( $n = 3$ ; range, 14.8–169.0) before IVR and remained unchanged throughout the experiment (Fig. 4).

The mean aqueous VEGF concentration of the injected nonvitrectomized eyes was 82.0 pg/mL ( $n = 3$ ; range, 53.2–132.6) before IVA injection, decreased to less than the LOD in all injected eyes 1 day after IVA, and remained below the LOD for 6 weeks. The mean aqueous VEGF concentration in injected vitrectomized eyes was 74.7 pg/mL ( $n = 3$ ; range, 28.7–110.7) before IVA injection, decreased to less than the LOD in all injected eyes, and remained below the LOD for 4 weeks (Fig. 5). The mean aqueous VEGF concentration of the noninjected fellow eyes was 59.5 pg/mL ( $n = 6$ ; range, 24.5–110.2) before IVA, decreased significantly to 15.0 pg/mL 1 day after IVA, and decreased to 22.6 pg/mL 3 days after IVA (Fig. 6).

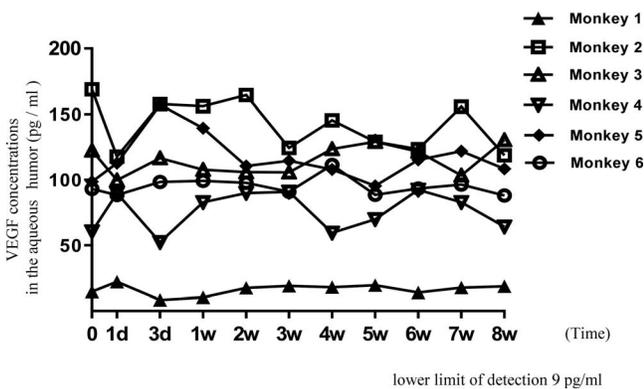


FIGURE 4. Vascular endothelial growth factor concentrations in the aqueous humor of noninjected fellow eyes after ranibizumab intravitreal injection.

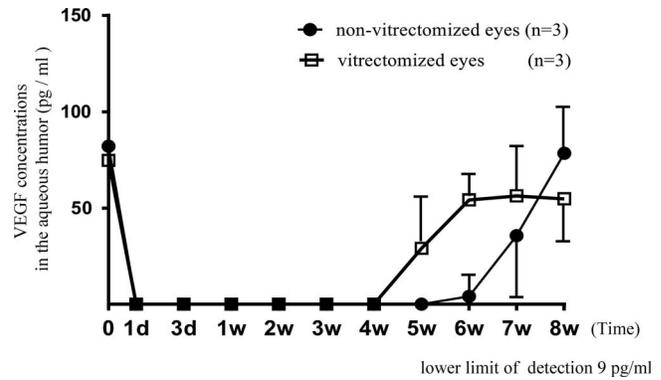


FIGURE 5. Vascular endothelial growth factor concentrations in the aqueous humor of nonvitrectomized eyes and vitrectomized eyes after intravitreal aflibercept injection.

The Table shows the maximum drug concentration, AUC, and half-life of ranibizumab and aflibercept in the aqueous humor of the injected eyes.

No complications, such as uveitis or endophthalmitis, developed after ranibizumab and aflibercept were injected.

DISCUSSION

In the current study, the half-life of 0.5 mg intravitreally injected ranibizumab in the aqueous humor was 2.3 days ( $n = 3$ ) in nonvitrectomized eyes and 1.4 days ( $n = 3$ ) in vitrectomized eyes, and the half-life of 2 mg intravitreally injected aflibercept in the aqueous humor was 2.2 days ( $n = 3$ ) in nonvitrectomized eyes and 1.5 days ( $n = 3$ ) in vitrectomized eyes. Previously, we showed a similar half-life for 1.25 mg intravitreally injected bevacizumab in the aqueous humor (i.e., 2.8 days in nonvitrectomized eyes and 1.5 days in vitrectomized eyes).<sup>12,13</sup> In previous studies, the aqueous humor half-life of ranibizumab was 2.54 days in monkey eyes<sup>16</sup> and 7.15 days in human eyes.<sup>17</sup> Although to our knowledge no reported studies have determined the half-life of intravitreal aflibercept injection in monkey and human eyes, Stewart<sup>18</sup> estimated a half-life for aflibercept of 7.13 days in human eyes. Together with our current results, the data seem to indicate a similarity between ranibizumab and aflibercept in intraocular pharmacokinetics. However, it is expected that ranibizumab and aflibercept would be effective for different periods because their affinities differ.

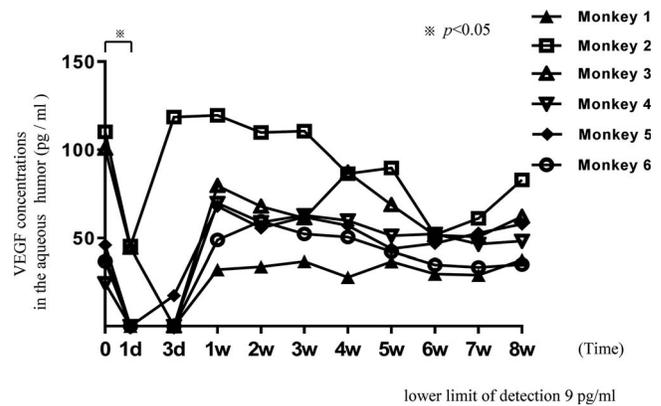


FIGURE 6. Vascular endothelial growth factor concentrations in the aqueous humor of noninjected fellow eyes after intravitreal aflibercept injection.

TABLE. Comparison of the Pharmacokinetics Parameters in Nonvitrectomized and Vitrectomized Macaque Eyes

|                                    | Ranibizumab          |                      | Aflibercept          |                      |
|------------------------------------|----------------------|----------------------|----------------------|----------------------|
|                                    | No PPV               | PPV                  | No PPV               | PPV                  |
| $C_{\max} \pm$ SD, ng/mL           | 51,300 $\pm$ 8800    | 41,800 $\pm$ 13,200  | 74,000 $\pm$ 13,000  | 68,000 $\pm$ 29,000  |
| AUC $\pm$ SD, d <sup>n</sup> ng/mL | 171,000 $\pm$ 65,500 | 154,000 $\pm$ 42,400 | 174,000 $\pm$ 35,200 | 124,000 $\pm$ 64,300 |
| $t_{1/2} \pm$ SD, d                | 2.3 $\pm$ 0.2        | 1.4 $\pm$ 0.6        | 2.2 $\pm$ 0.1        | 1.5 $\pm$ 0.3        |

PPV, pars plana vitrectomy;  $C_{\max}$ , maximum drug concentration;  $t_{1/2}$ , half-life.

In the current study, the half-lives of ranibizumab and aflibercept in the aqueous humor were shorter in vitrectomized than nonvitrectomized eyes. These results are consistent with our previous bevacizumab results.<sup>12,13</sup> Some studies have suggested that vitrectomy with lensectomy decreases the half-life of intravitreal molecules. The vitreous half-life of amphotericin-B was reduced from 9.1 to 1.4 days after vitrectomy and lensectomy in rabbit eyes,<sup>19</sup> 5-fluorouracil clearance was increased two times in aphakic vitrectomized rabbit eyes compared to phakic vitrectomized eyes,<sup>20</sup> and the intravitreal half-life of bevacizumab in rabbits was decreased from 4.22 to 2.08 days after vitrectomy and lensectomy.<sup>14</sup> Thus, vitrectomy with lensectomy may increase intravitreal drug clearance. On the other hand, Ahn et al.<sup>21</sup> compared the pharmacokinetics of intravitreal bevacizumab (IVB) in lens-sparing vitrectomized versus nonvitrectomized control rabbit eyes and reported no significant differences in IVB pharmacokinetics between them. They recently reported similar findings in eyes injected with ranibizumab.<sup>15</sup> The significant differences between vitrectomized and nonvitrectomized eyes in the current study may be due not only to vitrectomy, but also lensectomy.

In the current study, IVR decreased the VEGF concentration for 1 week in vitrectomized eyes ( $n = 3$ ) and 3 weeks in nonvitrectomized eyes ( $n = 3$ ). Our previously study of bevacizumab showed IVB decreased VEGF level below the LOD for 1 week in vitrectomized eyes and 4 weeks in nonvitrectomized eyes.<sup>12,13</sup> Both IVR and IVB maintained VEGF levels below the LOD for comparable periods. The CATT Research Group showed that IVB and IVR had equivalent effects on visual acuity when administered on the same schedule.<sup>22</sup> Our results supported this conclusion.

In the current study, IVA decreased the VEGF concentration for 4 weeks in the vitrectomized ( $n = 3$ ) and 6 weeks in the nonvitrectomized ( $n = 3$ ) eyes. Also, IVA decreased VEGF level below the LOD for a longer period compared to IVR. The VEGF concentration fell below the LOD, while the aflibercept remained detectable. On the other hand, the VEGF concentration recovered even though a small amount of ranibizumab still was detectable. Binding of VEGF to aflibercept exceeds that to bevacizumab and ranibizumab. According to a previous report, the equilibrium dissociation constant ( $K_D$ ) of the aflibercept-VEGF- $A_{165}$  complex is 0.490 pM, while those for ranibizumab and bevacizumab are 46 and 58 pM, respectively.<sup>23</sup> The higher binding affinity of aflibercept may account for its more persistent blockade of VEGF.<sup>24</sup> Investigation of Efficacy and Safety in Wet AMD (VIEW) 1 and VIEW 2 studies showed that bimonthly intravitreal injection of aflibercept after 3 loading doses was noninferior to monthly injection of ranibizumab.<sup>6</sup> Our results supported the need for less frequent dosing with aflibercept than ranibizumab.

Although the baseline VEGF level seems to be lower in the IVA study than in the IVR study, the difference was not statistically significant. We believe that it was simply due to experimental variability. In the current study, the aqueous VEGF concentrations in noninjected fellow eyes ( $n = 6$ ) were decreased significantly for 3 days after IVA even though no aflibercept was detectable. In our previous study of bevacizu-

mab, the VEGF concentrations in the aqueous humor of fellow eyes remained unchanged throughout the experiment, although small amounts of bevacizumab were detectable. Wang et al.<sup>25</sup> reported that serum and plasma VEGF concentrations are significantly decreased by IVA, but not affected by IVR. Very small amounts of aflibercept may escape into the systemic circulation, and because of its strong VEGF binding activity, suppress VEGF level in fellow eye.

In conclusion, the half-lives of intravitreally injected ranibizumab and aflibercept in the aqueous humor are similar. Their aqueous humor half-lives are shorter in vitrectomized eyes with lensectomy than in eyes without surgery. Compared to IVR, IVA suppressed VEGF level in injected eyes for a longer period, and in noninjected fellow eyes for a short time period.

### Acknowledgments

Supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#24592668 and 15K10862), and a grant from the Ministry of Health, Labour and Welfare. The authors alone are responsible for the content and writing of the paper.

Disclosure: **Y. Niwa**, None; **M. Kakinoki**, None; **T. Sawada**, None; **X. Wang**, None; **M. Ohji**, Novartis (F, R, C), Santen (F, R, C), Bayer (R, C)

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