Binding of Sulfonamides to Erythrocyte Proteins and Possible Drug–Drug Interaction

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The mode of binding of sulfonamides to erythrocyte proteins and possible drug–drug interaction between those compounds in erythrocytes resulting in changes in tissue levels were studied in rats using zonisamide (a novel antiepileptic agent possessing a sulfonamide group), several other sulfonamides and some antiepileptics without a sulfonamide group.

In Michaelis–Menten plottings, the sulfonamide was found to be concentrated into erythrocytes *in vitro* and *in vivo* in a saturable high-affinity mode and in a linear low-affinity mode at ordinary therapeutic plasma levels through a simple diffusion process. Concentration in erythrocytes was affected by the presence of albumin in the extracellular medium. The cellular sulfonamide was readily replaced by extracellular sulfonamides *in vitro*. Even *in vivo*, erythrocyte levels of zonisamide were lowered by administration of other sulfonamides, although the plasma and tissue levels were not significantly changed since the plasma and tissue compartments of zonisamide were large relative to the erythrocyte compartment at ordinary therapeutic dose levels of zonisamide in animals and man. Therefore, disposition of zonisamide was not significantly influenced by other sulfonamides, but it is suggested that drug–drug interaction affecting the tissue levels may occur for a combination of sulfonamides with extremely different affinities for erythrocytes and low therapeutic plasma levels.

Keywords sulfonamide; erythrocyte; interaction; carbonic anhydrase

It is known[1] that sulfonamides with aromatic substituents have high affinity for carbonic anhydrase and thereby are significantly concentrated into erythrocytes, which contain a large amount of the enzyme. In previous studies,[2] we revealed that not only carbonic anhydrase but also other protein of erythrocytes are responsible for concentration of sulfonamides in human erythrocytes.

At present, however, the pharmacokinetic significance of the concentration of sulfonamides into erythrocytes is not fully understood, but we presume that pharmacokinetic interaction can occur between sulfonamides bound to erythrocytes. Using zonisamide, a new antiepileptic agent possessing a sulfonamide group,[3] we therefore examined the significance and possible pharmacokinetic interaction of sulfonamides bound to erythrocytes. That is, *in vitro* and *in vivo* examinations were made of the mode of concentration of the sulfonamides in erythrocytes and the replacement of intracellular zonisamide with various extracellular sulfonamides. The latter studies suggested that drug–drug interaction may be important for certain combinations of sulfonamides, although no significant change in tissue distribution of zonisamide was detected under the present conditions.

Experimental

Chemicals Zonisamide[3] and zonisamide labeled with ^{14}C at the position of the sulfamoylmethyl moiety (specific radioactivity, 24.6 or 32.4 μCi/mg; radiochemical purity, >99%[4]) were prepared in our laboratories. Sulthiame, chlorothiazide and hydrochlorothiazide were prepared from commercial tablets (Bayer A. G., Banyu Pharmaceutical Co., Ltd. and Banyu Pharmaceutical Co., Ltd., respectively). Acetazolamide, furosemide, sulfanilamide, diphenylhydantoin and phenobarbital were also commercial products (Lederle Japan, Ltd., Hoechst Japan, Ltd., Nakarai Chemicals, Ltd., Nakarai Chemicals, Ltd., and Fujinaga Pharmaceutical Co., Ltd., respectively). Human serum albumin (HSA, fr. V) was purchased commercially (Sigma Chemical Co., St. Louis, MO, U.S.A.). Other chemicals of reagent grade were purchased from commercial sources.

Animals Male Wistar rats weighing about 250 g were used without fasting.

Procedures for *in Vivo* Studies [^{14}C]Zonisamide suspended in 0.5% tragacanth solution was orally administered in rats with a gastric catheter in doses of 4.7—189 μmol/kg. Blood was sampled periodically from the tail vein and centrifuged at 3000 rpm for 15 min. After withdrawal of plasma and buffy layers, a sample of erythrocytes was taken for measurement of radioactivity. It was previously shown[4] that under these conditions, the radioactive component in plasma and erythrocytes was the unchanged zonisamide. Rats were killed by puncture of the abdominal artery under light ether anesthesia, their tissues were dissected out and tissue radioactivity was determined after solubilization in Soluene 350 (Packard Instruments Inc., Downers Grove, IL, U.S.A.) as described previously.[4]

Preparation of Erythrocytes Rat blood was taken in a heparinized syringe by puncture of the abdominal artery under light ether anesthesia and centrifuged at 3000 rpm for 15 min. Plasma and buffy layers were removed, and the erythrocytes were washed with two volumes of 0.9% sodium chloride solution.

Incubation [^{14}C]Zonisamide (0—250 nmol/ml) was dissolved in Krebs–Ringer phosphate buffer or isotonic buffers of 0.171 M potassium dihydrogen phosphate–sodium bicarbonate (pH 7 and 8)[5] or 0.123 M disodium hydrogen phosphate–0.263 M citric acid (pH 5 and 6).[5] A mixture of the drug solution and the same volume of erythrocytes washed as above was incubated at 37 °C for 15 min at the final hematocrit of 50%.

Analysis Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (model 3380, 460CD, Packard Instruments Inc., Downers Grove, IL, U.S.A.) as described previously.[4] In the experiment on drug interaction *in vivo*, zonisamide and sulthiame were estimated by high performance liquid chromatography[6] and by gas chromatography,[4] respectively.

Results

Concentration of Sulfonamides in Erythrocytes (a) Concentration *in Vivo* After oral administration of zonisamide at 4.7—189 μmol/kg (the usual pharmacological dose in animals[7] and men[8] or less) to rats, the plasma and erythrocyte levels were found to reach maxima at around 3 h with broad peak levels. When maximal levels were plotted *versus* doses (Fig. 1a), the marked concentration of zonisamide in erythrocytes was confirmed. Maximal plasma levels were linearly related to doses and maximal erythrocyte levels, curvilinearly.

When the erythrocyte levels were plotted *versus* plasma levels (Fig. 1b), the curve was found to be a composite of two components: saturable and linear ones. The saturable component reached the plateau of 76 nmol/ml of erythrocytes when the plasma level was 30 nmol/ml, at which the actual erythrocyte level was 113 nmol/ml (sum of the saturable and linear components).

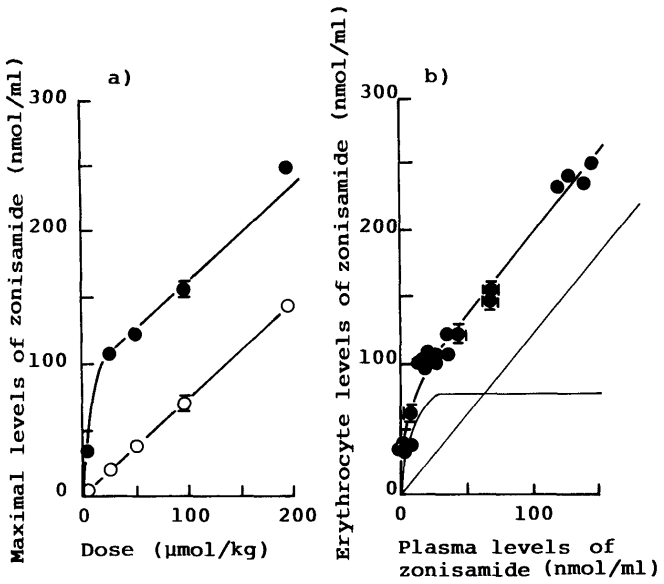

Fig. 1. Plasma and Erythrocyte Levels of [^{14}C]Zonisamide after Oral Administration in Rats

Points are means of 2—3 animals (±S.E.). a) Maximal levels *vs.* dose. ●, erythrocyte; ○, plasma. b) Erythrocyte levels *vs.* plasma levels. The bold curve represents the observed concentrations, and the thin straight line (a linear component) was drawn parallel to the linear part of the curve and passing through the origin. The thin curved line (saturable component) is the difference between the observed concentration and the linear component obtained above.

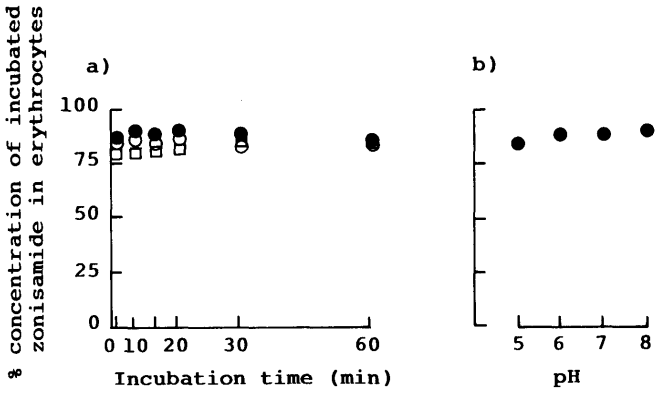

Fig. 2. Rate of Concentration of Zonisamide in Rat Erythrocytes *in Vitro*

a) Zonisamide concentration at different temperatures. b) Zonisamide concentration at different pHs. Rat erythrocytes were incubated with 66.5 nmol of zonisamide/ml of the whole incubation mixture (50% hematocrit). ●, 37 °C; ○, 20 °C; □, 0 °C.

(b) Concentration *in Vitro* Erythrocytes were incubated with zonisamide *in vitro*. Zonisamide was found to be quite rapidly concentrated into rat erythrocytes independently of temperature (Fig. 2a). The process was also found to be independent of pH (Fig. 2b).

When the levels of zonisamide in erythrocytes were plotted *versus* those in the medium, the curve was found to be a composite of two components (Fig. 3a), saturable and linear, similarly to the above *in vivo* results. A similar *in vitro* relationship was documented[9] for another sulfonamide, acetazolamide.

In Fig. 3a, the saturable component reached the plateau of 71 nmol/ml of cells when the extracellular level of zonisamide was 15 nmol/ml, at which the actual cellular level was 94 nmol/ml. It should be noted that the extracellular level of zonisamide required to attain the plateau of

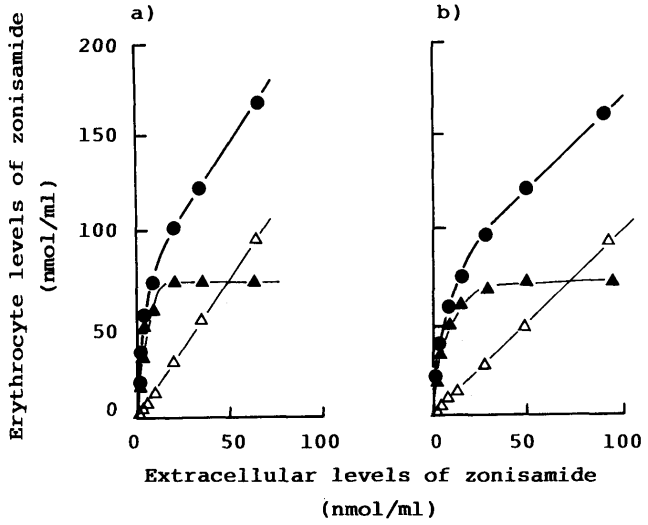

Fig. 3. Concentration of Zonisamide in Rat Erythrocytes *in Vitro* without (a) or with (b) Albumin

Rat erythrocytes were incubated with zonisamide for 15 min at 37 °C. ●, observed concentration; ▲, saturable component; △, linear component. See also the legend to Fig. 1b.

TABLE I. Release of Zonisamide from Erythrocytes by Sulfonamides and Some Antiepileptics

Agent added	Zonisamide released in medium (nmol/ml cells)
None	35.2
Acetazolamide	68.4
Sulthiame	68.0
Chlorothiazide	53.4
Hydrochlorothiazide	44.6
Furosemide	48.8
Diphenylhydantoin	35.4
Phenobarbital	33.8

Rat erythrocytes (0.5 ml) containing 200 nmol (42.4 μg)/ml of [^{14}C]zonisamide were incubated with solution (0.5 ml) of the indicated sulfonamides (0.18—0.21 mM) and antiepileptics. Radioactivity released in the medium was counted.

cellular level differs from that observed *in vivo* (see above).

When a physiological level (0.61 mM) of serum albumin was added to the incubation medium, the curve (Fig. 3b) became more similar to that observed *in vivo* (Fig. 1b). At an extracellular level of 29 nmol of zonisamide/ml, erythrocytes contained 97 nmol of zonisamide/ml. Therefore, the difference between *in vivo* and *in vitro* extracellular levels of zonisamide noted above can be ascribed to binding to protein in the extracellular fluid, reducing the amount of available zonisamide.

Possible Interaction between Sulfonamides in Erythrocytes (a) Studies *in Vitro* After one washing with saline of erythrocytes pre-labeled *in vitro* with [^{14}C]zonisamide (200 nmol/ml of cells), 35.2 nmol/ml of cellular zonisamide was found to have been released into the medium (Table I). In contrast, 44.6—68.4 nmol/ml of cellular zonisamide was released by the addition to the washing medium of acetazolamide, sulthiame, chlorothiazide, hydrochlorothiazide or furosemide. Diphenylhydantoin and phenobarbital, antiepileptics not possessing a sulfonamide group but reported[10] to bind to the erythrocyte membrane, had no effect on the release.

(b) Studies *in Vivo* Zonisamide was orally given to rats and 4 h later, sulthiame, acetazolamide, chlorothiazide or

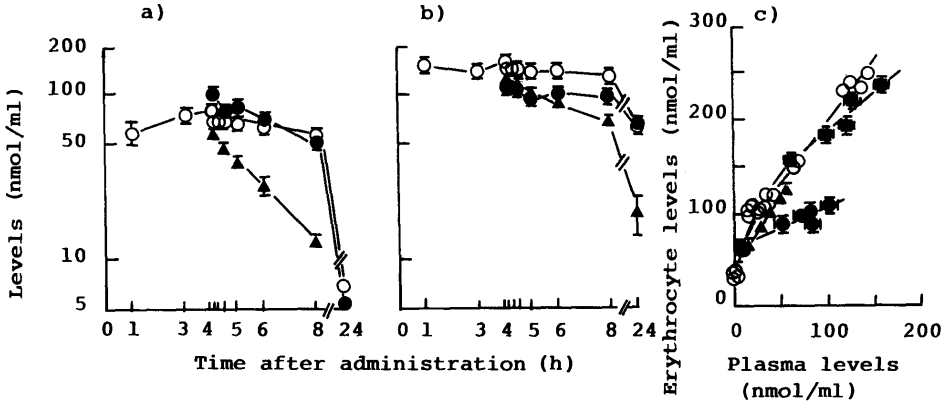

Fig. 4. Plasma and Erythrocyte Levels of Zonisamide and Sulthiame after Intravenous Administration of Sulthiame to Rats Previously Given Oral Zonisamide

a) Plasma. b) Erythrocytes. c) Erythrocyte *vs.* plasma. Points are means of 3 animals ± S.E. Sulthiame (69 μmol/kg) was administered intravenously 4 h after oral administration of zonisamide (94 μmol/kg). ○, zonisamide levels in control rats; ●, zonisamide levels in sulthiame-treated rats; ▲, sulthiame levels in treated rats; ■, levels of zonisamide plus sulthiame (c). Significantly different from erythrocytes of control rats (○) ($p<0.05$), at 4.08—8 h (●).

TABLE II. Tissue Levels of Zonisamide 0.5 h after Intravenous Administration of Sulthiame in Rats Previously Given Oral Zonisamide

Tissue	Levels of zonisamide	
	Control	Sulthiame-treated
	(μg eq/ml or g)	
Plasma	11.8 ± 0.2	11.8 ± 0.7
Erythrocytes	29.3 ± 1.1	15.3 ± 0.8[a)]
Brain	16.7 ± 0.3	15.1 ± 0.8
Liver	25.7 ± 0.6	24.0 ± 1.3
Kidney	26.5 ± 0.4	24.6 ± 1.8
Muscle	13.7 ± 0.2	13.1 ± 0.7

Values are means of 3—4 animals ± S.E. *a)* Significantly different from control ($p<0.01$). [^{14}C]Zonisamide was orally given to rats at 94 μmol/kg, and 4 h later, sulthiame was administered intravenously at 69 μmol/kg.

diphenylhydantoin was administered intravenously.

After administration of sulthiame, plasma levels (Fig. 4a) of zonisamide were not significantly affected. The half lives of zonisamide in sulthiame-treated and -untreated control rats up to 24 h were 5 and 6 h, respectively, and the disappearance of sulthiame from plasma ($t_{1/2}=1.9$ h) was more rapid than that of zonisamide.

In contrast, erythrocyte levels of zonisamide (Fig. 4b) rapidly fell to about one half after administration of sulthiame and then recovered to the control level at 24 h. Intra- and extracellular levels of zonisamide and sulthiame are shown in Fig. 4c. Levels in erythrocytes of zonisamide in rats given sulthiame were lower than those in control rats. However, when erythrocyte levels of zonisamide plus sulthiame were plotted *vs.* plasma levels, the curve became virtually the same as that of control rats.

Levels of zonisamide in the brain, liver, kidney and muscle were not significantly different from each other in the sulthiame-treated and -untreated rats (Table II).

As shown in Fig. 5, plasma levels of zonisamide were also not greatly affected by intravenous administration of acetazolamide, chlorothiazide or diphenylhydantoin in rats orally given zonisamide. However, administration of acetazolamide resulted in a significant but transient decrease of erythrocyte levels of zonisamide similarly to the case of sulthiame. The ability of chlorothiazide to release zonisamide from erythrocytes was less potent than that of sulthiame or acetazolamide. Diphenylhydantoin had no effect.

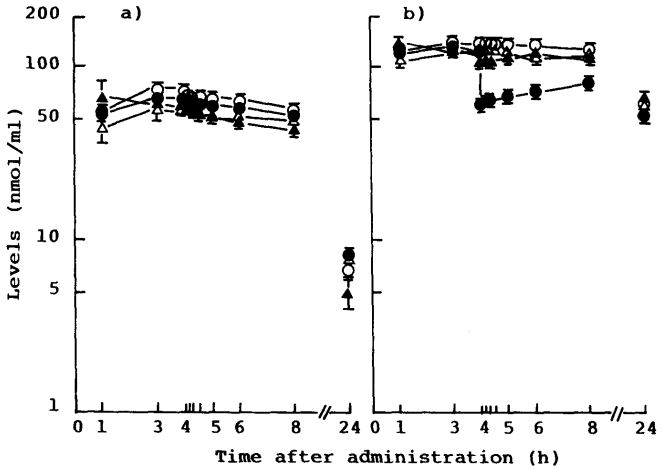

Fig. 5. Effects of Acetazolamide, Chlorothiazide and Diphenylhydantoin on the Levels of [^{14}C]Zonisamide Radioactivity in Plasma (a) and Erythrocytes (b) in Rats

Points are means of 3 animals ± S.E. ○, control; ●, acetazolamide; ▲, chlorothiazide; △, diphenylhydantoin. Acetazolamide (113 μmol/kg), chlorothiazide (68 μmol/kg) and diphenylhydantoin (99 μmol/kg) were administered intravenously 4 h after oral administration of [^{14}C]zonisamide (103 μCi/94 μmol/kg). Significantly different from erythrocytes of control rats (○) ($p<0.05$), at 4.08—8 h (●); at 4.08—4.5 h (▲).

Discussion

It has been reported[1)] that, unlike other sulfonamide antiepileptics, zonisamide does not exert anticonvulsive action through inhibition of carbonic anhydrase in the central nervous system.

In our previous studies,[2)] we found by examination of Scatchard plots that the concentration of sulfonamides into erythrocytes was due to the binding to cellular carbonic anhydrase and other proteins with higher affinities than to extracellular albumin. The dissociation constants for the binding of zonisamide to carbonic anhydrase, other erythrocyte proteins and albumin were 5.7, 77.5 and 472 μM, respectively. At ordinary pharmacological and therapeutic levels of zonisamide, the Michaelis–Menten plots presented in this paper revealed two components of binding, saturable and linear, with respect to the extracellular levels of zonisamide. The saturable, high-affinity component and the latter low-affinity linear one therefore correspond to the

binding of zonisamide to carbonic anhydrase and to other erythrocyte protein, respectively.

In the present studies it was shown that the mode of binding was similar in *in vitro* and *in vivo* systems. However, the concentration in erythrocytes was found to be affected by the presence of extracellular albumin. Under the present *in vitro* conditions, the extent of protein binding can be estimated from the difference of extracellular levels of zonisamide with and without albumin at the same erythrocyte level, *e.g.*, at the plateau level in erythrocytes, $(1-15/29)\times 100\%=48\%$, or from the difference between *in vitro* medium and *in vivo* plasma, $(1-15/30)\times 100\%=50\%$. These estimates are compatible with the extent of albumin or serum protein binding of zonisamide (49.7% (unpublished data) or 48.6%[4] on average, respectively) determined by the ultrafiltration method. Zonisamide was also found to be concentrated through a simple diffusion process.

The cellular zonisamide was shown to be readily replaced by other sulfonamides *in vitro*. Acetazolamide and sulthiame were more potent in replacing cellular zonisamide than chlorothiazide, hydrochlorothiazide and furosemide. This suggests that the potency for replacement depends on the dissociation constants of sulfonamides for carbonic anhydrase, which were previously found[2] to decrease in the above order.

Many epilepsy patients are often treated with multiple antiepileptic drugs, some of which possess a sulfonamide structure. In addition, they may be treated with other kinds of sulfonamide drug. This fact and the above findings *in vitro* raise the possibility that pharmacokinetic interaction possibly occurs between different sulfonamides in erythrocytes of the patients. In rats given zonisamide and then sulthiame, the plasma levels of zonisamide were not significantly affected. This fact together with similar elimination half lives of zonisamide in sulthiame-treated and -untreated animals, suggests that the disposition of zonisamide (including the rate) was not significantly affected by administration of sulthiame.

On the other hand, erythrocyte levels fell rapidly after administration of sulthiame, and they gradually recovered within 24 h to the control level of rats untreated with sulthiame. It was previously shown[2] that the dissociation constant of sulthiame for erythrocyte carbonic anhydrase (4.5 μM) was close to that of zonisamide, and in the present study, relationships between intra- and extracellular levels of zonisamide plus sulthiame were found to be similar to those between intra- and extracellular levels of zonisamide in the control animals. Therefore, the present study has provided evidence that cellular zonisamide is partly replaced by sulthiame even *in vivo*. Recovery of the erythrocyte level of zonisamide in sulthiame-treated rats to the control level at 24 h suggests that re-distribution of zonisamide into erythrocytes in place of sulthiame occurred again as sulthiame was eliminated faster than zonisamide. In other words, zonisamide released from erythrocytes by administration of sulthiame was taken up in tissue compartments. Thus, possible changes in tissue levels of zonisamide leading to the changes in the intensity of its pharmacological effect may occur in animals treated with both zonisamide and sulthiame. However, in the present studies in rats, interaction between zonisamide and sulthiame in erythrocytes was found not to affect the tissue levels significantly. Under the conditions of Fig. 4, erythrocytes contained 182 nmol of zonisamide/ml at the time when sulthiame was to be administered. If it is assumed that the intracellular zonisamide was completely replaced by sulthiame and the volume of erythrocytes is 3% of body weight, the replaced amount of zonisamide would be 5.5 μmol/kg. For plasma and tissue compartments other than erythrocytes, this means that the dose of zonisamide (94.3 μmol/kg) would be increased to 99.8 μmol/kg (105.8%) since excretion of zonisamide would have been negligible at 4 h because of its long half life. The increment (5.8%) is small relative to the interindividual differences of rats and experimental errors. The present findings on interaction of zonisamide and sulthiame in erythrocytes and the apparently insignificant change in tissue distribution are thus reasonable, since the actual replacement would have occurred not completely but only partially and it can be concluded that the tissue compartment of zonisamide is large relative to the erythrocyte compartment under the pressent conditions. This view is compatible with our previous finding[4] of a rather even distribution of zonisamide; levels of [^{14}C]zonisamide in most tissues were similar to the plasma level.

The findings in rats given with zonisamide and other sulfonamides were also similar. The *in vivo* findings on the potency of replacement of cellular zonisamide in Fig. 5 apparently correspond with the *in vitro* findings in Table I and therefore with the difference of dissociation constants.

Despite the findings presented in this paper, however, the above considerations strongly suggest that a sulfonamide in erythrocytes could be largely replaced by another sulfonamide with higher affinity, if their affinities for erythrocytes are extremely different from each other. The pharmacological effect of the former would be influenced if the effective level in plasma or tissue of the former is sufficiently low. Therefore, it can be concluded that interaction between some combinations of sulfonamides in erythrocytes may affect the tissue levels under certain conditions: significant drug–drug interaction may occur for such a combination of sulfonamides.

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