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Crystal structures of factor Xa specific inhibitors in complex with trypsin: structural grounds for inhibition of factor Xa and selectivity against thrombin

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Abstract Crystal structures of DX9065a and a related bis-amidino-aryl inhibitor specific for the blood-clotting factor Xa have been solved in complex with bovine β-trypsin to a resolution of 1.9 Å. Each inhibitor exhibits an extended conformation along the active site, in contrast to the compact folded structures observed for thrombin specific inhibitors. Few direct contacts (predominantly in the S1 pocket) are made between trypsin and the inhibitors. Transfer of the inhibitors to the active site of factor Xa suggests a three-site interaction: salt bridge formation at the base of the primary specificity pocket, extensive hydrophobic surface burial and a weak electrostatic interaction between the distal basic component of the inhibitor and an electronegative cavity of factor Xa formed by three backbone carbonyl oxygens. Additivity of these three interactions is the basis for the observed strong inhibition of factor Xa and provides a framework for the design of novel factor Xa inhibitors. A propionic acid group of the inhibitor would clash with the thrombin specific '60-insertion loop', thus conferring selectivity against thrombin.

Key words: Factor Xa inhibitor; Anticoagulant; Coagulation; X-ray crystal structure; Binding mode

1. Introduction

The prevention of blood coagulation is of primary importance in a variety of pathological situations. Conventional anticoagulant treatment includes the administration of heparin and coumarins (warfarin). A number of limitations are associated with these, however [1]. Heparin acts indirectly as an anticoagulant by promoting the inhibition of α-thrombin by antithrombin and is, therefore, unable to inhibit clot bound thrombin; warfarin disrupts the vitamin K-dependent synthesis of many of the coagulation enzymes and must, therefore, be monitored very carefully. The development of directly acting low molecular weight inhibitors of the coagulation factors has, therefore, become attractive.

Much emphasis has been put upon inhibitors of thrombin, the ultimate protease of the coagulation cascade. The X-ray crystal structure of human α-thrombin [2,3] has been of value in identifying structural requirements for its inhibition [4]. The central role played by thrombin in haemostasis in both coagulatory and anticoagulatory processes (see [5]) suggests that its inhibition may not be desirable in all clinical settings, however.

One attractive line of attack is to disrupt the thrombin catalyzed amplification reactions. This could be achieved through inhibition of factor Xa, the enzyme directly responsible for thrombin activation. Inhibition of factor Xa interrupts both the extrinsic and intrinsic pathways of thrombin production, whilst allowing the thrombin catalyzed activation of protein C. The efficacy of factor Xa inhibition is demonstrated by the anticoagulant properties of the factor Xa specific inhibitors antistasin [6] and tick anticoagulant peptide [7] derived from haematophagous parasites.

Synthetic factor Xa specific inhibitors have been described in the literature [8–10]. The most potent inhibitors consist of two basic moieties separated by a spacer of appropriate length (Fig. 1). Daiichi compound DX9065a ((+)-2-[4-[((3*S*)-1-acetimidoyl-3-pyrrodinyl)oxy]phenyl]-3-(7-amidino-2-napthyl)propionic acid) exhibits a K_i of 41 nM for factor Xa, no activity against thrombin and displays some oral activity [11]. The inhibitor has been modelled to the active sites of factor Xa, thrombin and human trypsin [10,12–13]. In each case, the models suggest an extended conformation of the inhibitor. The inherent flexibility of the molecule precludes an exhaustive search of possible conformational space, however, and, therefore, the interaction mechanism is still a matter of debate. Experimental crystallographic data are necessary to establish the binding mode unequivocally.

The recent crystallographic structure determination of factor Xa [14] provides an opportunity to apply a structure-based approach to the search for specific inhibitors. However, the active site of factor Xa is blocked in the crystal form examined and no crystals of inhibited factor Xa have been reported as yet. The success in determining the mode of action of thrombin specific inhibitors by introducing them into crystals of trypsin [15–17], subsequently verified by their crystal structures in complex with thrombin [18,19] prompted us to investigate the binding of factor Xa inhibitors to the active site of trypsin. In this communication, we present three structures of compounds of the Daiichi class in complex with bovine β-trypsin, indicating structural requirements for the inhibition of factor Xa for the first time.

2. Experimental

Bovine β-trypsin was separated from commercial trypsin (Merck,

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Abbreviations: DX9065a, (+)-2-[4-[((3*S*)-1-acetimidoyl-3-pyrrodinyl)oxy]phenyl]-3-(7-amidino-2-napthyl) propionic acid; BX5633, (+)-2-[4-[(-1-acetimidoyl-4-piperidinyl)oxy]phenyl]-3-(7-amidino-2-napthyl) propionic acid; DABE, 1,2-di-(5-amidino-2-benzofuranyl) ethane; BABCH, 2,7-bis-(4-amidinobenzilidene)-cycloheptan-1-one; NAPAP, N^{α}-(2-napthyl-sulphonyl-glycyl)-DL-*p*-amidinophenylalanyl-piperidine; PDB, Protein Data Bank.

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Darmstadt, Germany) by ion-exchange chromatography. Crystals for soaking experiments were grown from small seeds of the 'open' form in 1.7–1.8 M ammonium sulphate, pH 6.0, as described [16]. Crystals were soaked for 2 days in approximately 2 mM inhibitor solution, 2.5 M ammonium sulphate, pH 8.0. Due to problems of insolubility of some of the inhibitors in this high salt buffer, cocrystallization experiments were also carried out. Trypsin with approximately 1 mM inhibitor was crystallized by vapour diffusion against 20–30% polyethylene glycol 8000, 0.1–0.3 M ammonium sulphate, pH 7–8.

Data were collected on a Centronics area detector (Siemens) and processed using XDS [20]. Starting coordinates [16,21,22] were taken from the Protein Data Bank [23] according to the space group (Table 1). Conventional crystallographic refinement (rigid body, positional and temperature factor) was carried out using XPLOR [24,25]. Molecular models of the inhibitors were constructed using SYBYL (Tripos Associate) and model building performed using O [26]. Coordinates of the inhibitors were transferred to those of factor Xa [14] (PDB code 1hcg.pdb) and human α-thrombin [27] (PDB code 1fph.pdb) through superposition of conserved residues around the active sites of trypsin, factor Xa and thrombin using the programme O [26]. Data collection and refinement statistics are given in Table 1.

3. Results

BX5633 was successfully soaked into the 'open' trypsin crystal form. As attempts to soak DX9065a were unsuccessful, cocrystallization experiments were also performed. Two space groups were obtained: one orthorhombic (DX9065a), related to the crystal form observed for *p*-amidinophenylpyruvate-trypsin [21] and one trigonal (BX5633), isomorphous to free bovine trypsin [22] (Table 1). The conformation of trypsin

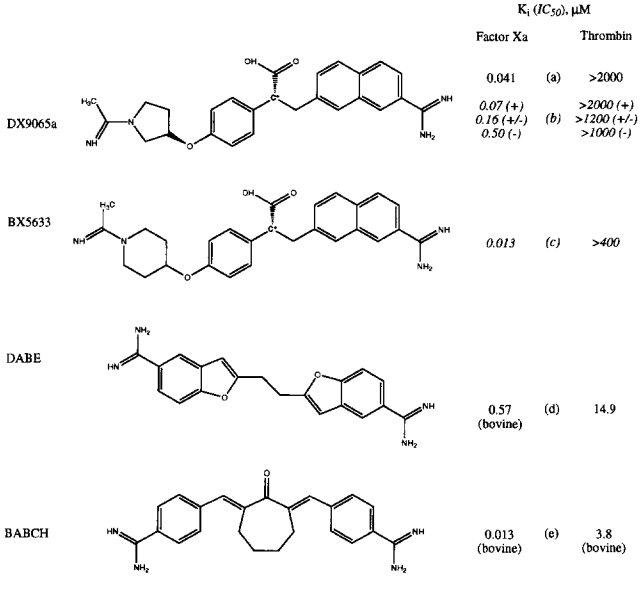

Fig. 1. Chemical formulae and inhibition constants for potent factor Xa specific inhibitors. DX9065a: (+)-2-[4-[((3*S*)-1-acetimidoyl-3-pyrrodinyl)oxy]phenyl]-3-(7-amidino-2-napthyl) propionic acid; BX5633: (+)-2-[4-[(-1-acetimidoyl-4-piperidinyl)oxy]phenyl]-3-(7-amidino-2-napthyl) propionic acid; DABE: 1,2-di-(5-amidino-2-benzofuranyl) ethane; BABCH: 2,7-bis-(4-amidinobenzilidene)-cycloheptan-1-one. DX9065a and BX5633 were supplied as racemic mixtures at the chiral carbon atom marked with an asterisk. K_i values (for human enzyme unless otherwise stated) are taken from (a) Hara et al. (1994) [11], (d) Tidwell et al. (1980) [8] and (e) Stürzebecher et al. (1989) [9]; IC_{50} values (italic) are given for (b) the + and − epimers and the racemic mixture of DX9065a, Nagahara et al. (1994) [28] and (c) for BX5633, Nagahara, T., Kanaya, N., Inamura, K. and Yokoyama, Y. (1992), European Patent Application No. 0 540 051 A1.

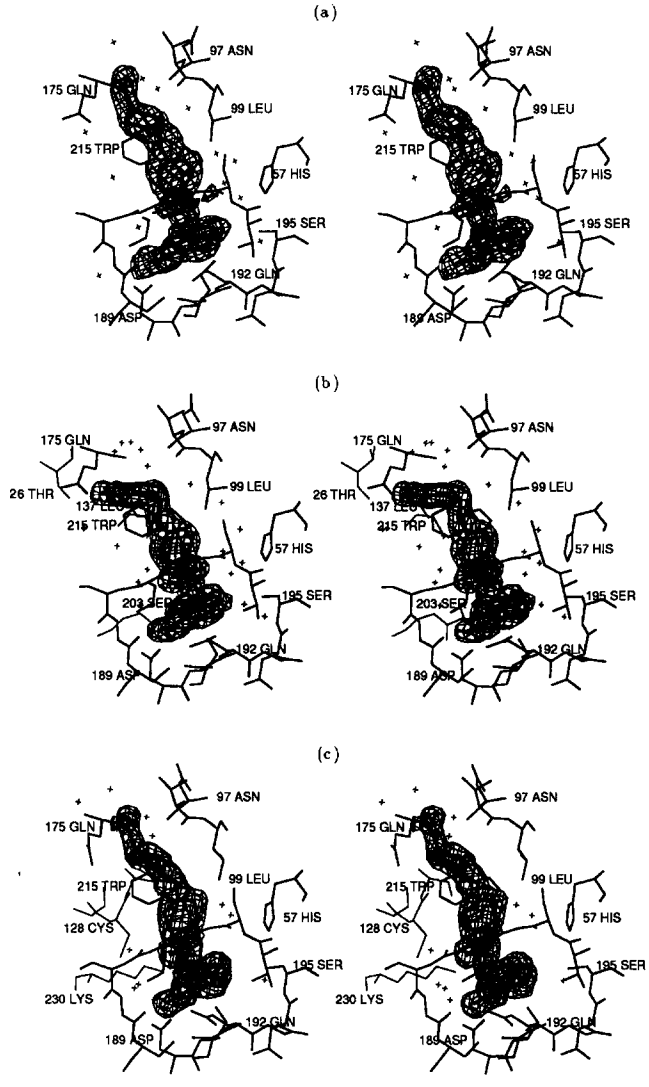

Fig. 2. 'Omit' electron densities for factor Xa specific inhibitors in complex with bovine β-trypsin. (a) Electron density for BX5633 soaked into the 'open' trypsin form. Broken density at the attachment for the propionic acid reveals that both enantiomers are bound in the crystal, both of which can be modelled into the density. Thick connections denote the (+) epimer, medium lines the (−) epimer. (b) Cocrystal of DX9065a; note that the pyrrolidinyl ring makes a contact to a symmetry related molecule (thin lines). (c) Cocrystal of BX5633. The propionic acid moiety makes a salt bridge to a symmetry related lysyl side chain, presumably giving rise to preference for this stereoisomer.

showed minor perturbations, largely associated with crystal packing. The only significant differences observed were in the side chain conformations of Gln-192 (which was partially disordered) and Gln-175.

The inhibitors are in each case well-defined by electron density (Fig. 2). Both compounds were supplied initially as racemic mixtures at the C15 atom (Fig. 1) and it is clear from the density for the soaked crystal that both enantiomers are present in the crystal (Fig. 2a). In the two cocrystal forms, only one stereoisomer is observed bound to the enzyme (Fig. 2b,c). In both cocrystals, the inhibitor is stabilised by crystal contacts to the propionic acid group: via an ionic contact to a symmetry related lysine side chain in the trigonal form (Fig. 2c) or via

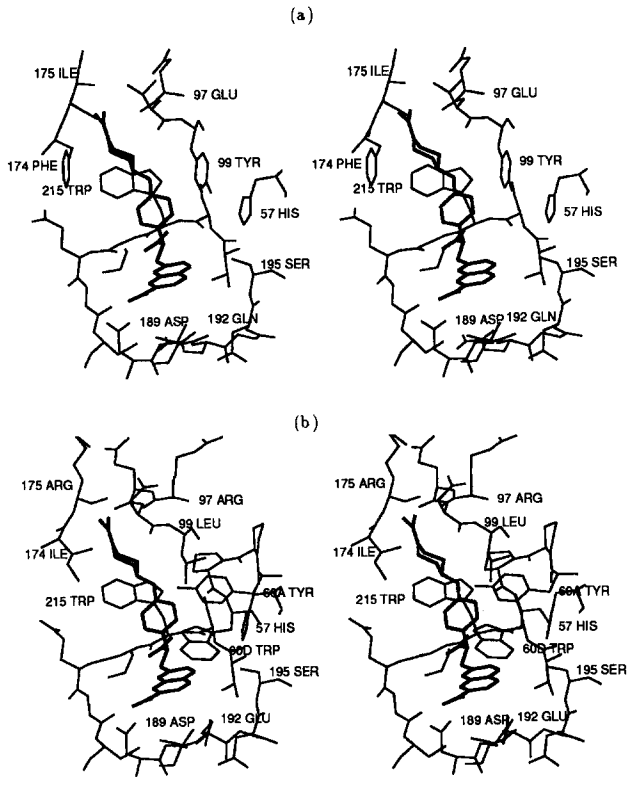

Fig. 3. (a) Transfer of BX5633 (cocrystal) to the active site of factor Xa. The interaction is divided over three sites. The napthalene amidino group makes a salt bridge to Asp-189 of factor Xa, while the propionic acid moiety makes no contacts to the enzyme. The hydrophobic box formed by the side chains of Trp-215, Tyr-99 and Phe-174 is occupied by the benzyl spacer and the hydrophobic side of the piperidinyl ring. The distal basic group points towards an electronegative cavity on the surface of factor Xa, at the conjunction of carbonyl groups of Glu-97, Thr-98 and Ile-175. (b) Transfer to the active site of human α-thrombin. The propionic acid 'selectivity filter' would clash with thrombin's rather rigid 60 loop; in addition, the 'electronegative cavity' is less open due to the insertion in thrombin of Glu-97A.

hydrogen bonds made with backbone amide groups in the orthorhombic form (Fig. 2b). The free acetimidoyl group of DX9065a is further stabilised through contacts with a symmetry related carbonyl group (Fig. 2b). Comparison of the conformation of BX5633 in both the open and trigonal forms reveal minimal influence of crystal packing forces on the overall interaction; on the other hand, the conformation of the pyrrolidine group of DX9065a is clearly crystal related.

The inhibitors bind in extended conformation, filling the 'unprimed' sites of trypsin. The bulky napthamidine moiety occupies the S1 site, filling it almost completely, with its amidino function forming a salt bridge with Asp-189. The rigid nature of the napthalene group results in a considerable distance (>3.5 Å) between the enzyme surface and the aromatic benzyl 'spacer', which is oriented perpendicular to the napthalene group and parallel to the indole moiety of Trp-215. This conformation results in the propionic acid moiety pointing into solution away from the enzyme (Fig. 2).

Outside the S1 pocket, the majority of contacts between the enzyme and inhibitors are made between the indole moiety of Trp-215 and the hydrophobic edge of the pyrrolidine (piperidine) ring, the plane of which is more or less perpendicular to that of the benzyl spacer. For DX9065a, the pyrrolidine ring exhibits an envelope conformation, whilst the piperidine ring of BX5633 exhibits a chair conformation (Fig. 2). The ring nitrogens and acetimidoyl groups (Fig. 1) are all coplanar (Fig. 2), suggesting electron delocalization within this group, and it is most probably protonated. The nitrogen atoms of the pyrrolidine (piperidine) ring and the acetimidoyl group approach Gln-175 Oε1, and are in the vicinity of the carbonyl oxygen of Asn-97. As mentioned above, the pyrrolidine group of DX9065a is involved in additional contacts to a symmetry related molecule.

The inhibitors were transferred to the active site of factor Xa after superposition of its active site residues with those of trypsin (Fig. 3a). BX5633 fitted well to the surface of factor Xa without rearrangement. The pyrrolidine ring of DX9065a, on the other hand, would clash with the benzyl ring of factor Xa residue Phe-174. As discussed above, this conformation is influenced by crystal packing; DX9065a could follow a similar course to BX5633 through flipping of the envelope conformation (cf. the endo-/exo-transition of furanose). In the following, the interaction with factor Xa and thrombin will be based on the cocrystal structure of trypsin with BX5633.

The interaction appears dominated by the burial of the napthamidine group in the S1 pocket, which is slightly more hydrophobic due to the substitution of trypsin Ser-190 for Ala-190 in factor Xa. The benzyl 'spacer' of the inhibitor makes weak van der Waal's contacts to the longer phenolic side chain of Tyr-99, whose perpendicular arrangement could marginally enhance binding through edge-on stacking (Fig. 3a).

The environment of the acetimidoyl-piperidinyl moiety is

Table 1
Data collection and refinement statistics

Compound	Crystallization conditions	Space-group	Cell constants	Observed/ unique reflections	R_{sym}	Reflections $>3\sigma$ (completeness)	Number of non-hydrogen atoms			r_{ms}		R_{fac}
							Protein	Solvent	Inhibitor	Bonds	Angles	
BX5633[1]	1.8 M AS, pH 6.0	$P2_12_12_1$	63.3 69.3 63.8 90. 90. 90.	49006/22069	2.9%	21173 (94.2%)	1628	163	34	0.008	1.75	17.9
DX9065a[2]	0.3 M AS, pH 8.0 30% PEG 8000	$P2_12_12_1$	54.9 61.0 64.3 90. 90. 90.	25539/16195	3.2%	14950 (86.3%)	1628	290	33	0.009	1.99	16.9
BX5633[3]	0.1 M AS, pH 7.0 20% PEG 8000	$P3_12_1$	54.8 54.8 109.7 90. 90. 120.	26673/14594	3.7%	13599 (88.4%)	1628	245	34	0.008	1.78	16.9

[1] Soak crystal; isomorphous to NAPAP-trypsin [16] (PDB code 1ppc.pdb).
[2] Cocrystal; similar to *p*-amidinophenylpyruvate-trypsin (54.9 58.5 67.8 90. 90. 90.) [21] (PDB code 1tpp.pdb).
[3] Cocrystal; isomorphous to free trypsin [22] (PDB code 3ptn.pdb).

largely hydrophobic. The interaction between the edge of the piperidine ring and the side chain of Trp-215 is further enhanced by the presence of the hydrophobic side chain of Phe-174. It is striking, however, that this basic group maps to an electronegative cavity on the surface of factor Xa formed by the juxtaposition of the carbonyl groups of Glu-97, Thr-98 and Ile-175.

Transfer of BX5633 to the active site of thrombin reveals that the propionic acid group would clash with the relatively rigid '60 insertion loop' of thrombin (Fig. 3b). Furthermore, the electronegative cavity identified for factor Xa is less accessible due to the sequence insertion of residue Glu-97A in thrombin. Selectivity of these inhibitors against thrombin is, therefore, effected via steric hindrance.

4. Discussion

The compounds investigated here are amongst the most potent synthetic inhibitors of factor Xa known (Fig. 1). In common with the simple symmetric molecules DABE and BABCH, these compounds exhibit a dibasic character. Structure–activity relationships for DX9065a have shown that the distal basic moiety plays a major role in the inhibition of factor Xa, whilst the propionic acid group is responsible for its lack of activity against thrombin [10]. Molecular modelling has attributed the basicity requirement variously to interaction with factor Xa Glu-97 [10], the carbonyl group of Glu-97 [12] or to a novel cation-π interaction with the aromatic rings of Trp-215, Tyr-99 and Phe-174 [13]. Modelling studies have further proposed that selectivity against thrombin is due to electrostatic repulsion between the side chain of thrombin Glu-192 (Gln-192 in factor Xa) and the propionic moiety [10]. The distal basic moiety also has a (slight) detrimental effect on the inhibition of human trypsin [12], which possesses lysyl residues at both residues 97 and 175.

Comparing our structures with the published models, we note the following:

(i) the propionic acid moiety faces towards Gly-219 in the models of Katakura et al. [10,12], instead of away from the enzyme. This results in an alternative approach of the distal basic group to the surface of factor Xa. In their most recent model [12], the dominant interaction of this group is to the carbonyl group of Glu-97, with fewer hydrophobic contacts;

(ii) the overall inhibitor conformation proposed by Lin and Johnson [13] is much closer to that which we see. Due to a slight inclination of the napthalene group, however, the pyrrolidinyl group does not extend as far along the enzyme surface. In their model, therefore, the distal basic group does not reach the 'electronegative cavity', but resides in the hydrophobic box formed by Tyr-99, Phe-174 and Trp-215, leading them to propose an interaction between this group and the π electrons of these aromatic groups.

Although these modelling studies give an idea about the general nature of the interaction, they possess an inherent uncertainty in the conformation of the inhibitor and cannot be totally objective. Despite the obvious differences between trypsin and factor Xa, if it is possible to transfer the coordinates from one active site to the other without major rearrangements, one can expect that the positioning of the inhibitor will be similar in each. A model derived in this fashion gains stature if improved interactions are made in factor Xa than in trypsin. This has been shown to be the case for thrombin specific inhibitors [16–19], even though thrombin and trypsin exhibit even weaker similarity.

Our results show that, in contrast to the compact folded conformations observed for thrombin specific inhibitors [4,15–19], DX9065a stretches along the active site cleft in an extended manner. This conformation is stabilised by the expected salt bridge formation of the napthamidine group to Asp-189, by hydrophobic interactions between the benzyl spacer and the side of the pyrrolidinyl group with the enzyme surface and by a weak electrostatic interaction of the basic pyrrolidinyl/acetimidoyl group with a previously unidentified electronegative cavity of factor Xa. Indirectly, the structural studies in trypsin presented here show that this distal basic group searches for and is able to recruit hydrogen bonding partners.

We propose that the additivity of these three interactions form the basis for the observed tight binding of DX9065a to factor Xa. Anchoring the pyrrolidinyl/acetimidoyl group as described could have the added benefit of directing and thereby strengthening the hydrophobic interaction. This binding mode could also be adopted by the other potent factor Xa inhibitors shown in Fig. 1, DABE [8] and BABCH [9].

A striking feature of DX9065a is its selectivity for factor Xa over thrombin. Katakura et al. [10] have shown that this is predominantly a function of the propionic group, leading them to propose that this would cause electrostatic repulsion with the acidic side chain of thrombin Glu-192. In our model, these groups are sufficiently separated that this is not a major effect; in any case, thrombin Glu-192 exhibits enhanced flexibility [27]. We suggest that the propionic group would clash with the thrombin specific '60-insertion loop', whilst the distal basic group cannot be comfortably accommodated due to the insertion of thrombin residue Glu-97A. An ethyl ester derivative of DX9065a is able to inhibit thrombin [10]. We propose that this derivative adopts an alternative binding mode, inserting the ethyl side chain into the hydrophobic aryl-binding site of thrombin, with a concommitant reorientation of the benzyl spacer and distal basic group. Thrombin is then inhibited by a combination of strong interactions in the S1 and aryl-binding sites with weaker (undetermined) interactions elsewhere.

Notwithstanding the inadequacies of examining factor Xa inhibitors in the context of trypsin, these structural studies provide an expedient and plausible model for understanding the specificity determinants of factor Xa. It should be noted, however, that crystal structures present a static, enthalpic picture: other factors such as changes in entropy and / or solvation might play an important role in achieving a low K_i.

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