Early clinical experience with the novel proteasome inhibitor PS-519

I. M. Shah, K. R. Lees, C. P. Pien¹ & P. J. Elliott¹

University Department of Medicine & Therapeutics, Western Infirmary, Glasgow, UK., G11 6NT and ¹Millennium Pharmaceuticals Inc, Cambridge, MA, USA

Aims The main objective of this study was to investigate the safety, tolerability and pharmacodynamics of the novel proteasome inhibitor PS-519 in young male volunteers. Many pro-inflammatory mediators such as cytokines and cell adhesion molecules that are responsible for the development of the cerebral infarct are under the control of the transcription factor Nuclear Factor kappa-B (NF- κ B). The activity of NF- κ B is itself tightly regulated through the multicatalytic enzyme known as the proteasome. PS-519 is a novel and highly selective small molecule that inhibits the proteasome. An *ex vivo* assay of 20S proteasome activity allows monitoring of the drug effect in blood. PS-519 is protective in multiple animal models of cerebral ischaemia over a range of doses that achieve 20S inhibition of 40%–80%.

Methods PS-519 has been administered to healthy male volunteers as single and repeated doses up to 1.6 mg m⁻². It was given as an intravenous bolus over 20–30 s in a double blind, randomized, placebo-controlled phase I study, examining vital signs, safety, tolerability and blood 20S proteasome inhibition.

Results Thirty-nine subjects received single doses of 0.012 mg m⁻²–1.6 mg m⁻² and 28 subjects received doses of 0.5 mg m⁻²–1.6 mg m⁻² on three consecutive days. The drug was well tolerated. There was no clear treatment-emergent symptom or abnormality of laboratory tests. Proteasome inhibition in blood samples as measured by 20S assay achieved the intended maximum target level of 70–80% with 1.6 mg m⁻², and was reproducible with repeated dosing.

Conclusions This study has demonstrated that proteasome inhibition is well tolerated by healthy subjects at levels that are maximally neuroprotective in experimental conditions. Further clinical evaluation appears justified.

Keywords: neuroprotection, nuclear factor-kappa B (NF- κ B), proteasome inhibitors, safety and tolerability

Introduction

Stroke is the third commonest cause of death in the Western World and a major cause of disability. It is believed that there are three phases to the brain damage that occurs during an ischaemic stroke. The first phase involves loss of blood supply to the tissue and this causes hypoxia. There is then excessive release of glutamate from presynaptic cells which causes excitotoxicity to adjacent cells. The third phase involves an inflammatory response, characterized by the invasion of neutrophils and the activity of various cytokines. There is increasing interest in the last of these phases with respect to acute stroke

treatment, because the time window for intervention may be longer and the scope for recovery greater at this stage.

There are a number of potential ways of interfering with this inflammatory response but rather than tackle the process at a series of downstream points, there is now the prospect of controlling the common expression factors that control neutrophil invasion and cytokine activity. These factors are under the control of transcription factor, Nuclear Factor- κ B (NF- κ B), which is itself regulated via the ubiquitin–proteasome pathway [1–3]. This regulatory protein, once activated results in the transcription of pro-inflammatory gene products. In unstimulated cells, NF- κ B exists in an inactive state in the cytoplasm complexed to the inhibitory I κ B molecule. Proteasome inhibitors block the activation of NF- κ B by preventing the degradation of the inhibitory I κ B molecule, via inhibition of the 20S proteasome [4–7].

Correspondence: Dr I. M. Shah, University Department of Medicine & Therapeutics, Western Infirmary, Glasgow, G11 6NT. Tel: +44 141211 6395; Fax: +44 141211 6396; E-mail: I.M.Shah@clinmed.gla.ac.uk

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Figure 1 Structure of PS-519.

PS-519 (1R - [1S,4R,5S]) - 1 - (1 - hydroxy - 2 - methylpropyl) - 4 - propyl - 6 - oxa - 2 - azabicyclo [3.2.1.] heptane-3,7-dione) is a novel proteasome inhibitor (Figure 1) [8]. It shows neuroprotective efficacy in animal models of cerebral ischaemia [9].

Male Sprague-Dawley rats were subjected to 2 h of focal cerebral ischaemia by means of the filament method of middle cerebral artery occlusion (MCAo). PS -519 was then administered as an intravenous bolus, 2 h after MCAo, immediately after the onset of reperfusion. The doses of PS -519 ranged from 0.003 to 0.3 mg kg⁻¹. Infarct size was measured after histological staining with 2,3,5-triphenyltetrazolium chloride. Neurological function, electro-encephalographic (EEG) activity, and neutrophil infiltration were also quantified. Rats treated with 0.01–0.3 mg kg⁻¹ of PS-519 had significantly reduced volumes of infarction ranging from 183 ± 42 mm³–138 ± 30 mm³, respectively.

Dose-response analysis of infarct volume at 24 h revealed that PS -519 neuroprotection approached 50–60% with the three highest doses tested (0.1 mg kg⁻¹ to 0.3 mg kg⁻¹). In rats treated at 6 h after occlusion, only limited neuroprotection was measured with 0.10 mg kg⁻¹ of PS -519.

Evaluation of the rats showed significant improvements in neurological function and EEG activity. Neutrophil infiltration at 24 h was also significantly decreased in cortical and striatal infarcted tissue of treated rats, by 70% and 63%, respectively. This reduced neutrophil infiltration is the most likely mechanism by which this drug produces its anti-inflammatory effect [10]. Similar results were seen in ischaemic rat cardiac muscle [11]. Radio -labelled PS-519 showed no brain tissue penetration, suggesting the drug mainly acts in the vasculature around the ischaemic area.

PS-519 appears to have a favourable pharmacodynamic and toxicological profile for consideration of its use as a neuroprotective agent in acute stroke. Neuroprotection has also been shown in a model of embolic stroke, when used together with *t*-PA [12]. NF-κB concentrations have also been found to be elevated in animal

models of haemorrhagic stroke and may be responsible for peri-lesional oedema formation [13]. PS-519 has also been tested in other inflammatory conditions and found to elicit positive outcomes [14, 15].

In acute safety studies undertaken in rats, signs of toxicity were seen above 8 mg kg⁻¹. Gastrointestinal side-effects and possible lowering of blood pressure were noted. To avoid such events in other animal studies and also in man, it was decided to limit the dosing of PS-519 to a range that did not elicit 20S proteasome inhibition greater than 80%. At such levels of inhibition no evidence of toxicity is seen.

Methods

This Phase I study was designed to provide initial information on the safety, tolerability and pharmacodynamics of PS -519 in man. The study was a single centre, randomized, double-blind, placebo-controlled trial in young healthy male volunteers. Ethical approval for the study protocol was granted by the West Ethical Committee of West Glasgow Hospitals University NHS Trust and written informed consent was obtained from all volunteers. The initial phase of the study used an ascending single dose of PS-519 which was administered as an intravenous bolus. Once the therapeutic range had been established, the second part of the study examined multiple dose administration, when each subject was given a single dose on three consecutive days, with dose increments between groups up to the expected maximal therapeutic level. The primary endpoints of the study were the effects of PS-519 on blood 20S proteasome inhibition and drug toxicity.

Study population

Subjects were screened by clinical history, physical examination and laboratory tests up to 14 days prior to treatment. Male subjects in 17 groups of four were each randomly assigned to receive either a dose of PS-519 or placebo (10 groups had the single dose and 7 groups had the multiple dosing). Within each group, three subjects received the active drug and one placebo.

The dose was chosen from standard acute and chronic toxicity studies in animals. The starting dose was approximately 1% of the dose used to induce a therapeutic effect in an animal model of cerebral ischaemia (1.0 mg kg⁻¹ = 6.0 mg m⁻² in mice; starting dose = 0.012 mg m⁻² in man = 1.2% of rat dose). Subsequent doses were incremented in logarithmic steps until pharmacological effects were encountered, and then modified to achieve approximately 70–80% inhibition of blood 20S proteasome activity at 1 h after dosing (Table 1).

The inclusion criteria comprised:

Table 1 Dose escalation and groups.

Group	Number of subjects	Dose of PS -519
1	4	0.012 mg m ⁻² single dose or placebo
2	4	0.04 mg m ⁻² single dose or placebo
3	4	0.12 mg m ⁻² single dose or placebo
4	4	0.25 mg m ⁻² single dose or placebo
5	4	0.5 mg m ⁻² single dose or placebo
6	4	1 mg m ⁻² single dose or placebo
7	3	1.4 mg m ⁻² single dose or placebo
8	4	1.4 mg m ⁻² single dose or placebo
9	4	1.6 mg m ⁻² single dose or placebo
10	4	1.6 mg m ⁻² single dose or placebo
11	4	0.5 mg m ⁻² triple dose or placebo
12	4	1 mg m ⁻² triple dose or placebo
13	4	1 mg m ⁻² triple dose or placebo
14	4	1.3 mg m ⁻² triple dose or placebo
15	4	1.6 mg m ⁻² triple dose or placebo
16	4	1.6 mg m ⁻² triple dose or placebo
17	4	1.6 mg m ⁻² triple dose or placebo

Males aged 18–35 years

Body Mass Index < 30

Healthy (by medical history and physical examination)

Written informed consent

The exclusion criteria comprised:

Illicit drug use or history of alcohol abuse

Blood donation in the preceding 6 months

Smoking > 5 per day

History of any serious drug allergy

Taken any prescription medication in preceding 14 days

Clinically significant abnormality of any screening investigations

Any additional factor which, in the opinion of the investigator, would compromise the conduct, safety or validity of the study

Drugs

PS-519 or an equal volume of placebo (propylene glycol (40%), ethanol (10%) and saline (50%)) was administered to each subject by intravenous bolus injection over a period of 20–30 s, giving approximately 0.5–1 ml in volume. The pharmacist on-site, (unblinded observer) prepared the drug and each dose was labelled for the specific subject and given to the investigator for administration. Within each group, all actively treated subjects received the same dose. Escalations in dose as the study progressed were decided, in consultation between the investigator, sponsor and an independent safety advisor, following analysis of the 20S proteasome inhibition data and the tolerability data of the preceding group, with

subject identity concealed from the investigator. No concomitant therapy was permitted during the study. Subjects were fasted overnight and food was permitted 1 h post-dosing. Water was provided as required throughout the dosing day. No medications were taken by the subjects during the 7 day follow up period.

Observations

Laboratory values and haemodynamic changes were closely monitored after drug administration. General health assessment was repeated on the morning of the study and blood samples were taken pre-dose for biochemistry, haematology and 20S proteasome activity. Urinalysis was also performed. Continuous ECG monitoring was undertaken during the first hour post-dose and 12 lead ECG was taken pre- and post-dosing.

The supine BP and heart rate were recorded in triplicate at 5, 15, 30 min and 1, 2, 4, 6 and 24 h post-injection. Blood samples were taken for 20S proteasome activity at 1, 6 and 24 h post-dose. Any spontaneous complaints were recorded as adverse events and closely monitored. Subjects were detained overnight under observation, and repeat physical examination and laboratory assessments were conducted the following morning. This procedure was repeated daily for the multiple dosing groups and all subjects were seen back at the unit after seven days for repeat assessment.

Analyses

Complete blood count and full biochemical profile (urea and electrolytes, liver function, bone biochemistry and glucose) were performed using the local hospital laboratories. Dip-stick analyses of urine for blood, protein, ketones and glucose were performed in the Research Unit.

Blood 20S proteasome assay was performed at Millennium Pharmaceuticals Inc., Cambridge, MA, USA. Blood samples were frozen and stored at –80 °C, before being shipped over on dry ice. The samples (500 µl) were collected in sodium heparin containing tubes and centrifuged. The blood cells were separated and lysed with 5 mM EDTA (pH 8.0) for 1 h and then centrifuged for 10 min at 4 °C. The blood lysate samples were then used in the 20S proteasome assay. Samples (10 µl) were added to 2 ml of substrate buffer (20 mM HEPES, 0.5 mM EDTA, 0.05% SDS and 60 mM Ys substrate-Suc-Leu-Leu-Val-Tyr-AMC, Bachem, King of Prussia PA) and the rate of substrate cleavage per 20S proteasome activity was determined using a Coomassie protein assay.

Fluorogenic kinetic assays for both the chymotryptic and tryptic activities of the proteasome have been opti-

mized for both whole blood and blood cells. Using the ratio of these activities, the percentage proteasome inhibition was calculated. This ratio method was demonstrated to be sensitive (detection limit of 13% inhibition with 10 µg of cell lysate), specific to the proteasome (proteasome inhibitor PS-341 provides > 98% inhibition), accurate (112% analyte recovery), and precise (0% ± 5% inhibition at 0 nmol l⁻¹ PS-341 and 74.5% ± 1.7% inhibition at 200 nmol l⁻¹ PS-341) [16].

The units for blood 20S proteasome activity are nmol of AMC (the fluorescent tag attached to the substrate that is broken down by active proteasome) per second (rate of activity of proteasome) per mg of protein (in the assay – from the blood sample taken) i.e. nmol AMC s⁻¹ mg⁻¹.

Results

Sixty-seven subjects completed the study in accordance with the protocol. There was no withdrawal. The mean age of the volunteers was 22.7 years (s.d. 3 years), mean weight was 76.1 kg (s.d. 5.8 kg) and the mean surface area was 1.93 m² (s.d. 0.084 m²).

Blood pressure and heart rate

There was no significant effect of PS-519 on blood pressure or heart rate during the trial.

ECG analysis

There were no significant differences between pre-dose and post-dose ECG parameters (heart rate, PR and QT intervals). No significant changes in ECG morphology or rhythm were observed during the ECG monitoring period or on any subsequent 12 lead ECGs.

Laboratory determinations

All subjects completed assessment at 7 days following the study. No significant abnormalities in biochemistry, haematology or urinalysis were identified.

Biochemistry

Two subjects had persistent mild elevation of bilirubin attributed to Gilbert's Syndrome and this was unchanged by the study drug.

Haematology

One subject showed a fall in his neutrophil count from $4.3 \times 10^9/l$ to $1.98 \times 10^9/l$ during the study period. He had been complaining of a head cold a few days before the start of the study, resulting in a relatively elevated white cell count at baseline, and this had returned back to the screening level at the end of the study. Follow-up haematology was stable.

Pharmacodynamics

Table 2 summarizes the blood 20S proteasome activity achieved at each dose level. The maximum observed effect was at 1 h post-dosing and activity generally returned to normal within 24 h (Figure 2).

Due to the rapid vascular clearance of PS-519 it has not been possible to provide pharmacokinetic data. In animal studies, using radio-labelled material, a greater than 85% of the label was removed from the vasculature within the first 10 min. The disappearance of the drug was likely to be into the blood cells and endothelium. The pharmacodynamic data is therefore, of more value

Table 2 Blood 20S proteasome activity.

Dose of PS-519	Baseline (Mean)	1 h (Mean)	6 h (Mean)	24 h (Mean)	48 h (Mean)
0.012 mg m ⁻²	1.39	1.43	1.49	1.56	1.46
n	3	3	2	3	3
s.d.	0.16	0.22	0.23	0.24	0.06
0.04 mg m ⁻²	1.82	1.27	1.19	1.30	1.11
n	3	3	3	3	3
s.d.	0.07	0.03	0.05	0.07	0.37
0.12 mg m ⁻²	0.84	0.78	0.74	0.9	0.94
n	3	3	3	3	3
s.d.	0.09	0.19	0.08	0.14	0.2
0.25 mg m ⁻²	1.3	1.06	1.17	1.27	1.13
n	3	3	3	3	3
s.d.	0.12	0.07	0.11	0.14	0.03
0.5 mg m ⁻²	1.37	0.83	1.03	1.32	1.32
n	3	3	3	3	3
s.d.	0.18	0.14	0.04	0.04	0.21

Table 2 continued

Dose of PS-519	Baseline (Mean)	1 h (Mean)	6 h (Mean)	24 h (Mean)	48 h (Mean)
1 mg m ⁻²	1.32	0.43	0.75	1.16	1.50
<i>n</i>	3	3	3	3	3
s.d.	0.03	0.21	0.01	0.18	0.42
1.4 mg m ⁻²	1.44	0.42	0.61	1.02	1.14
<i>n</i>	5	5	5	5	5
s.d.	0.15	0.05	0.12	0.28	0.22
1.6 mg m ⁻²	0.31	0.60	1.15	1.29	
<i>n</i>	6	6	6	6	6
s.d.	0.06	0.13	0.08	0.12	0.15
0.5 mg m ⁻² ×3	1.6	1.06	1.14	1.46	
<i>n</i>	3	3	3	3	
s.d.	0.07	0.11	0.14	0.22	
Day 2	1.46	1.03	1.26	1.31	
	(0.22)	(0.08)	(0.23)	(0.18)	
Day 3	1.31	1.01	1.34	1.45	
	(0.18)	(0.07)	(0.27)	(0.13)	
Day 4	1.54	–	–	–	
	(0.09)				
1 mg m ⁻² ×3	1.54	0.73	0.89	1.28	
<i>n</i>	6	6	6	6	
s.d.	0.14	0.14	0.13	0.12	
Day 2	1.28	0.63	0.84	1.06	
	(0.12)	(0.3)	(0.19)	(0.17)	
Day 3	1.06	0.46	0.77	1.01	
	(0.17)	(0.11)	(0.08)	(0.20)	
Day 4	1.06	–	–	–	
	(0.33)				
1.3 mg m ⁻² ×3	1.77	0.52	0.86	1.4	
<i>n</i>	3	3	3	3	
s.d.	0.25	0.25	0.27	0.2	
Day 2	1.4	0.56	0.86	1.31	
	(0.2)	(0.22)	(0.24)	(0.08)	
Day 3	1.31	0.47	0.65	1.18	
	(0.08)	(0.11)	(0.19)	(0.18)	
Day 4	1.39	–	–	–	
	(0.22)				
1.6 mg m ⁻² ×3	1.46	0.45	0.76	1.22	
<i>n</i>	9	9	9	9	
s.d.	0.66	0.19	0.27	0.56	
Day 2	1.22	0.39	0.74	1.09	
	(0.56)	(0.23)	(0.51)	(0.58)	
Day 3	1.09	0.29	0.59	1.04	
	(0.58)	(0.15)	(0.32)	(0.48)	
Day 4	1.44	–	–		
	(0.57)				

Units of activity are nmol AMC s⁻¹ mg⁻¹.

since this provides a measure of biological activity of the drug.

Adverse events

28% of the active group reported minor adverse events compared with 41% of the placebo group. These were not drug dose related. The main adverse events reported

were transient altered taste sensation and discomfort in the injection arm post-dosing (Table 3).

Discussion

This was a Phase I trial of PS-519, the first administration to human subjects. The study was commenced at a dose which was predicted to have no detectable pharma-

Figure 2 Single dose PS-519:20S proteasome activity. ◆ baseline; ■ 1 h post-dose; ▲ 6 h post-dose; ○ 24 h post-dose.

Table 3 Adverse events.

Adverse effect	Number of subjects affected	Dose group (Placebo or drug)	Onset time	Duration	Severity
Altered taste sensation	11	0.5 mg m ⁻² (2 drug) 1 mg m ⁻² (1 placebo, 4 drug) 1.4 mg m ⁻² (1 placebo, 1 drug) 1.6 mg m ⁻² (1 placebo, 1 drug)	Immediately after giving bolus	2–3 min	Mild
Discomfort in injection arm	13	0.5 mg m ⁻² (4 drug) 1.3 mg m ⁻² (3 drug) 1.6 mg m ⁻² (5 placebo, 1 drug)	During drug administration	10–20 s	Mild
Headache	5	0.5 mg m ⁻² (1 placebo, 1 drug) 1.3 mg m ⁻² (1 drug) 1.4 mg m ⁻² (1 drug) 1.6 mg m ⁻² (1 drug)	> 8 h after giving drug	1–2 h	Mild
Flu-like symptoms	3	1 mg m ⁻² (1 placebo, 1 drug) 1.6 mg m ⁻² (1 drug)	One before study Two: 2 days after study.	1–3 days	Mild

colological effect, and in the expectation that the blood 20S proteasome inhibition would be minimal. Subsequent doses were intended to reach potentially therapeutic plasma concentrations measured by blood 20S proteasome assay. Intensive monitoring was maintained for purposes of safety and the number of subjects treated was deliberately small. A placebo group was included to look for any laboratory or symptomatic abnormalities.

Thirty-nine subjects received single doses of 0.012–1.6 mg m⁻² (*n* = 29 active, 10 placebo) and 28 subjects received doses of 0.5–1.6 mg m⁻² on 3 consecutive days (21 active, 7 placebo), with 9 subjects receiving PS-519

at the top dose. Proteasome inhibition as measured by blood 20S assay and achieved the maximum target level of 70–80% with 1.6 mg m⁻². This was reproducible with repeated dosing (Figure 3).

There was a potential that excessive proteasome inhibition could lead to a compromised cellular function, and that is the reason why we chose to limit the degree of inhibition (maximum 80%) and the length of this effect (maximum 3 days).

The drug was administered as a bolus, as both the efficacy and toxicity effects of PS-519 observed in animals were not due to a *C*_{max} effect but resulted from

Figure 3 Triple dose PS-519 (1.6 mg m^{-2}): 20S proteasome activity.

excessive ($> 95\%$) proteasome inhibition for extended ($> 24 \text{ h}$) periods of time. Infusions of the compound would have been unlikely to limit the toxicity or improve the efficacy.

No drug related abnormality was identified on laboratory safety tests. One volunteer had a decrease in his neutrophil count but this was unlikely to be due to a drug effect, as this was a sudden drop over 24 h. No ECG or haemodynamic abnormalities were detected after administration of the drug bolus. No neurological or liver abnormalities were detected.

In terms of these safety tests, there was no evidence of acute or delayed toxicity. The main side-effects encountered were altered taste sensation and arm discomfort after administration of the bolus. These symptoms developed immediately after the bolus was given and resolved within a few minutes. As the placebo subjects also reported these symptoms, they are unlikely to be due to a drug effect and probably caused by the diluent.

Pharmacokinetic data normally reflects the presence of the drug in the blood rather than the duration of its biological activity. This would be misleading for this study since it would imply that the drug is cleared within 30 min and hence should be given more frequently. Under this condition PS-519 would induce severe toxicity due to excessive proteasome inhibition. Since the effect of PS-519 is most likely to be derived from its action on blood cells and the endothelium, then the pharmacodynamic activity measured is most relevant. Here we have attempted to monitor the effects of the drug in its biologically relevant target tissue to help provide data to support dosing regimens that are likely to be effective. The rapid removal of the drug and its wide distribution were also critical factors in deciding to dose in mg m^{-2} rather than mg kg^{-1} .

Therefore the data derived from the pharmacodynamic assay is more reflective of the biological activity of the drug and demonstrates that recovery to basal activity of

the proteasome is seen within 24 h. While inhibition of the proteasome may last up to 24 h, the effects of proteasome inhibition (i.e. on cytokine expression or cell adhesion molecule expression) may last for different periods of time.

The degree of inhibition at 1 h is likely to be maximum. Since PS-519 enters cells very rapidly and due to the time taken to sample cells, lyse them and analyse enzyme activity it is difficult to examine time points earlier than 1 h with levels of certainty. The 1 and 6 h time points were easy times to sample in the study and provide a consistent data set which can be compared with others generated in separate trials.

Further, the rate at which inhibition returns to baseline is very gradual over the first 24 h and sampling at other time points would not provide us with any other additional information. Inflammation of the cerebral tissue tends to continue up to 72 h after the initial ischaemic event. As proteasome activity returned to baseline after 24 h, then repeated triple dosing was performed, to try and mimic the potential clinical treatment regime. There was no evidence of accumulation of the pharmacodynamic effect with repeated dosing.

NF- κ B translocation is thought to play a significant role in both the early and late phases of inflammation, after an ischaemic stroke. This stimulates the expression of cellular adhesion molecules on endothelial cells, which promote leucocyte adherence and their subsequent migration into the brain parenchyma. Increased levels of cytokines and TNF- α , further exacerbate this response. The main affect of PS-519 seems to be via the reduction in the expression of cell adhesion molecules and cytokines on the ischaemic endothelium. This prevents the diapedesis of the leucocytes and modifies the inflammatory response.

Conclusions

In animal models, PS-519 has been shown to have a potent neuroprotective effect against acute focal brain ischaemia. On the basis of this effect, it could potentially be used as an agent for the treatment of acute cerebral ischaemia in humans. This Phase I study of the proteasome inhibitor PS-519 showed that proteasome inhibition is well tolerated by healthy subjects at levels that are maximally neuroprotective in experimental conditions, and also on repeat dosing. Clinical experience with this drug still remains limited and we need to expand both the exposure and extend testing to elderly subjects, and those with cerebrovascular disease and comorbidity.

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