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Cite this: *Med. Chem. Commun.*, 2012, **3**, 938

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CONCISE ARTICLE

Sangamides, a new class of cyclophilin-inhibiting host-targeted antivirals for treatment of HCV infection†

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Received 6th September 2011, Accepted 2nd October 2011
DOI: 10.1039/c1md00227a

Sangamides are amide derivatives of sanglifehrin A, a cyclophilin-binding polyketide natural product which is structurally distinct from cyclosporine A. Cyclosporine A is the starting point for the synthesis of cyclophilin inhibitors such as alisporivir, currently in development for the treatment of HCV infection. We report here initial results of the optimisation program which led to identification of the sangamides, compounds that exhibit significantly improved potential for the treatment of chronic HCV infection.

Introduction

As the name would imply, host-targeted antivirals (HTAs) interact with cellular factors essential for viral replication, and as such, might be anticipated to give a higher barrier to the selection of resistance than agents that specifically target viral proteins. The key to successful host target identification is the discovery of cellular factors that are essential to the virus, but can be targeted without disabling important cellular processes. In HIV treatment, this has been successfully exploited with CCR5 receptor antagonists such as maraviroc.[1] In HCV therapy the cyclophilins fit these criteria, and initial clinical validation has been seen with the non-immunosuppressive cyclosporine A (CsA) analogues alisporivir (DEBIO-025) and SCY-635 (Scheme 1). Cyclophilins act as peptidyl-prolyl isomerases, catalysing the *cis-trans* isomerisation of the peptide bond preceding prolyl residues, an activity which is critical for HCV replication.[2] A number of knockout studies in various species, including mice and human cells, have shown that cyclophilins are non-essential for cellular growth and survival,[3] validating their potential as therapeutic targets. There are seven major cyclophilins in humans,[4] and whilst the question as to which is most important for the HCV life-cycle has been the subject of some debate, recent evidence would suggest that it is cyclophilin A,[2,5–8] with other cyclophilins such as cyclophilin B and cyclophilin 40 also relevant under certain conditions.[9,10]

The non-immunosuppressive CsA analogues alisporivir[11–13] and SCY-635[14] have shown promise in clinical trials. These compounds are active against multiple HCV genotypes and have been reported to exhibit the highest hurdle for the selection of resistance of HCV therapies tested.[13,15,16] However, the published synthetic route is a complex 13-step (longest linear route 9 steps) synthetic route from CsA.[17] Adverse events such as reversible hyperbilirubinemia have been seen in a number of patients with the most advanced candidate alisporivir.[17,18] CsA class-related drug-drug interactions (DDIs) *via* inhibition of drug transporters such as MDR1, MRP2, OAT1B1 and OAT1B3[19–21] may also be a concern, potentially limiting certain combinations and use in some patients undergoing treatment for co-infections such as HIV.[22]

Sanglifehrin A (**1**) and its natural analogues are mixed non-ribosomal peptide/polyketides produced by the soil bacterium *Streptomyces* sp. A92-308110. They were originally discovered after screening for compounds with high affinity to cyclophilin A (CypA). **1** is the most abundant component in fermentation broths and exhibits significantly higher affinity for CypA compared to CsA.[23] This led to the suggestion that **1** could be useful for the treatment of HCV.[24] However, recent studies have shown that although the natural sanglifehrins have potent antiviral activity, they have additional properties unsuited for HCV drug candidates including immunosuppressive activity and poor physicochemical properties for oral dosing.[25]

With the aim of shifting the focus away from the current development of cyclophilin inhibitors based on CsA, **1** was used

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† Electronic supplementary information (ESI) available: Experimental data for compounds **1** and **3a–3p**, materials and methods for *in vitro* and *in vivo* assays, data analysing *in vivo* liver exposure after acute dosing and data showing combination with DAAs in HCV replicon assays. See DOI: 10.1039/c1md00227a

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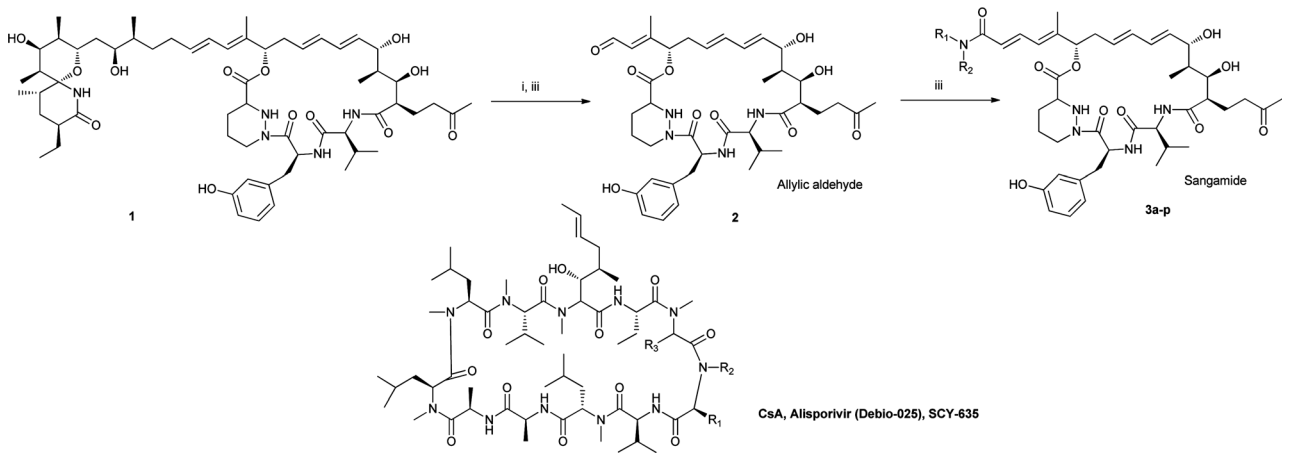

Scheme 1 Synthetic route to generate sangamides and structure of cyclosporine A and derivatives. i) OsO_4 in *tert*-BuOH, $(DHQ)_2PHAL$, $MeSO_2NH_2$, $K_3[Fe(CN)_6]$, K_2CO_3, H_2O, 1 h, room temperature (rt); ii) $NaIO_4$, 2 : 1 THF/H_2O, 2 h, rt; iii) $R_1R_2NCOCH_2P(O)(OEt)_2$, Cs_2CO_3, CH_3CN, 0 °C to rt, 30 min or $R_1R_2NCOCH_2P(O)(OEt)_2$, NaH, THF, 0 °C to rt, 30 min. CsA: $R_1 = CH_2CH(CH_3)_2$, $R_2 = CH_3$, $R_3 = H$; Alisporivir: $R_1 = CH(CH_3)_2$, $R_2 = CH_2CH_3$, $R_3 = CH_3$; SCY-635: $R_1 = CH_2C(CH_3)_2OH$, $R_2 = CH_3$, $R_3 = SCH_2N(CH_3)_2$; NIM-811: $R_1 = CH(CH_3)CH_2CH_3$, $R_2 = CH_3$, $R_3 = H$).

as a starting point for a medicinal chemistry program aimed at improving physicochemical properties to make molecules more suitable for oral administration, and removing or reducing the immunosuppressive activity, whilst retaining or improving potency against HCV replicons.

Results and discussion

Generation of macrocylic analogues of sanglifehrin A and B

Previous observations showed that piperazic acid-containing macrocycles derived from **1** retain cyclophilin binding,[26] leading us to generate several new chemical series lacking the western spiro-ketal fragment. Following an established route we generated the allylic aldehyde (**2**) by modified Sharpless asymmetric dihydroxylation and oxidative cleavage to provide a reactive handle for selective modification and parallel synthesis. This allowed identification of several amide derivatives (termed sangamides) as potential advanced leads worthy of further study. The sangamides (**3a-p**) were generated by Horner–Wadsworth–Emmons coupling of **2** and a suitable α-amidophosphonate in moderate to good yield (Scheme 1). The sangamides tested include primary and secondary amides, with alkyl and aryl substituents in a set of compounds designed to probe the steric and lipophilic tolerance of substitutions at this position. We also profiled a potential intermediate to a further series, the N,O-dimethylhydroxyamide (Weinreb amide, **3n**). Based on the profile of **3n** (vide infra) this series of compounds was expanded by tethering the methyl groups together in a 1,2-oxizinane to give **3o**.

Sangamides selectively inhibit genotype 1b replicons

Initial screening of sangamides was carried out by analysing inhibition of the luciferase activity of HCV genotype 1b I389luc-ubi-neo/NS3-3′/5.1 subgenomic replicons in Huh5.2 cells *via* measurement of relative luciferase luminescence, along with concurrent measurement of cytostatic/cytotoxicity/anti-metabolic effects using MTS followed by measurement of absorbance at 498 nm. Table 1 shows data for the sangamides tested, and reveals that several are more potent inhibitors of HCV replication than **1** and CsA, and in most cases were seen to be less cytotoxic.

Sangamides potently block the interaction between CypA and HCV NS5A

Inhibition of the interaction between CypA and the HCV NS5A protein has been proposed as an important antiviral target for cyclophilin inhibitors.[7] Consistent with this we confirmed that sangamides inhibit CypA-NS5A complex formation. This was achieved by coating Nunc Maxisorb 8-well strip plates with GST or GST-CypA, then titrating increasing concentrations of compound along with recombinant NS5A-His. Levels of captured NS5A-His were subsequently measured by use of mouse anti-His antibodies and rabbit anti-mouse-horseradish peroxidase phosphatase (HRP) antibodies.[27] All sangamides inhibited complex formation more potently than CsA with **3n** being the most potent inhibitor (Table 1). Differences in rank order between this data and replicon inhibition probably largely being due to variation in cell permeability. Inhibition of CypA PPIase activity was then confirmed for a selection of compounds, and gave a similar rank order to that for inhibition of complex formation, as follows: **3n** (1.1 nM), **1** (2.4 nM), **3o** (2.7 nM), **3j** (4.1 nM) and CsA (9.6 nM).

Sangamides display varying metabolic stability and solubility

Natural sanglifehrins have poor drug-like properties, which is a concern for future development as an oral therapy. To aid selection of candidate molecules to take forward, solubility in PBS, stability in human liver microsomes (HLM), mouse liver microsomes (MLM), and human hepatocytes was measured (Table 1). Many of the sangamides tested were more soluble than both **1** and CsA. Microsome stability was variable, with $t_{1/2}$ values between 1–37 min. However, stability in hepatocytes was better - this is unlikely to be due to poor cellular

Table 1 Data from initial biological evaluation of sangamides. Replicon data is an average of at least 6 replicates.

Compound	R_1	R_2	Huh5.2 **1b** Replicon EC_{50} (nM)	Huh5.2 **1b** Replicon EC_{90} (nM)	Huh5.2 **1b** Replicon CC_{50} (μM)	Huh5.2 **1b** Replicon SI_{50}	NS5A-CypA Disruption/ ELISA IC_{50} (μM)	Solubility, PBS, pH7.4 (μM)	Liver microsome stability $t_{1/2}$ (min) Mouse	Liver microsome stability $t_{1/2}$ (min) Human	Human hepatocyte stability $t_{1/2}$ (min)
1	n/a	n/a	318 ± 56	5500[a]	9.1	29	0.35 ± 0.11	45	4.0	7.9	145
CsA	n/a	n/a	306 ± 141	1000 ± 500	4.4	14	0.95 ± 0.05	51	33.7	25.6	>240
3a	Me	H	8000 ± 1700	60200 ± 2400	>100	>13	0.5 ± 0.04	>100	22.5	37.2	192
3b	Me	Me	349 ± 328	62000 ± 2050	>100	>287	0.63 ± 0.03	>100	24.7	48.0	128
3c	iPr	H	560 ± 442	4600 ± 1200	>100	>179	0.38 ± 0.04	12	6.4	16.9	59
3d	allyl	H	1600 ± 400	8500 ± 2000	48	30	0.36 ± 0.05	96	10.6	23.9	139
3e	Et	Et	204 ± 90	1200 ± 400	>100	>490	0.35 ± 0.06	>100	8.4	16.8	94
3f	iBu	iBu	309 ± 52	1100 ± 200	2.1	6.8	0.39 ± 0.02	33	2.5	21.6	98
3g	C_6H_{11}	H	336 ± 166	4230 ± 1200	>100	>298	0.45 ± 0.06	29	2.7	6.5	>240
3h	C_6H_{11}	Me	148 ± 49	1900 ± 500	66	446	0.42 ± 0.03	48	1.9	3.3	>240
3i	4-biphenyl	H	208 ± 78	1150 ± 500	>100	481	0.52 ± 0.03	2	1.3	2.3	>240
3j	2-pyridyl	H	167 ± 32	740 ± 119	40	240	0.39 ± 0.03	61	8.8	4.4	95
3k	2-pyridyl	Me	2013 ± 435	11500 ± 3630	>100	>50	0.33 ± 0.02	17	9.8	19.1	150
3l	3-pyridyl	H	2483 ± 516	5410 ± 850	>100	>40	0.46 ± 0.03	18	3.3	7.5	116
3m	4-pyridyl	H	8065 ± 904	31900 ± 900	>100	>12.4	0.55 ± 0.03	15	15.2	32.2	>240
3n	OMe	Me	162 ± 85	920 ± 190	>100	>617	0.2 ± 0.02	>100	17.5	26.5	>240
3o	$-O(CH_2)_4-$		125 ± 22	690 ± 174	>100	>800	0.36 ± 0.03	>100	5.8	10.6	220
3p	$-(CH_2)_2O(CH_2)_2-$		1400 ± 400	7400 ± 2100	7400	>72	0.33 ± 0.02	>100	18.0	50.3	>240
Telaprevir	—		235 ± 191	838 ± 294	>74	>315	—	—	—	—	—
DMSO	—		20500 ± 2560	N/A	30.7	1.5	—	—	—	—	—
BSA	—		—	—	—	—	—	>500	—	—	—

[a] 90% viral inhibition only reached at <10% metabolic inhibition in one replicate.

permeability, as the replicon assays were also carried out in hepatocytes, and may well be due to cellular localisation of the molecules (possibly due to their high affinity for cellular cyclophilins). Notably, analogues with bulky substituents were most stable.

Based on the combination of potent inhibition of NS5A-CypA complexes, selectivity in genotype 1b replicons, good solubility and hepatocyte stability, **3n** and **3o** were chosen for further profiling.

Sangamides show potent cross-genotype replicon activity

One of the more compelling arguments for development of cyclophilin inhibitors is their activity across HCV genotypes.[17] We therefore determined whether the sangamides **3n** and **3o** exhibit this property, and confirmed the potent replicon inhibition observed for the Huh5.2 system, using Huh7 cells stably expressing luciferase subgenomic genotype 1a (H77), genotype 1b (Con1) and genotype 2a (JFH-1) replicons (Table 2). Both compounds were more potent than **1** and CsA, and showed similar high potency across different genotypes.

Sangamides show reduced immunosuppression compared to 1 and CsA

One of the aims in developing sanglifehrin analogues for HCV was to reduce or remove the immunosuppressant activity. To examine this, the inhibition of human mixed lymphocyte reactions (MLR) was analysed (Table 2). **3n** and **3o** were both seen to have lower potency in these assays when compared to CsA and **1**. The observed IC_{50} values are notably higher than those reported for alisporivir (840 nM) in a similar MLR assay, a value which is classed as non-immunosuppressive.[28]

Table 2 Profiling data for sangamides in comparison to **1**, CsA, telaprevir (positive controls) and DMSO (negative control)

Compound	**1a** replicon H77 EC_{50} (nM)[a]	**1b** replicon Huh7 EC_{50} (nM)[a]	**2a** replicon JFH-1 EC_{50} (nM)[a]	logD, pH7.4[b]	Human PPB, fraction bound (%)	Inhibition of human MLR IC_{50} (nM)
1	301 ± 13	222 ± 10	247 ± 6	>3.7	>99	215
CsA	198 ± 12	137 ± 4	455 ± 6	4.4	>99	3
3n	156 ± 5	128 ± 8	247 ± 8	2.3	27	1060
3o	63 ± 8	58 ± 6	89 ± 6	2.6	72	1450
Telaprevir	—	720 ± 14	—	—	—	—
DMSO	>100000	>100000	>100000	—	—	—

[a] Replicon data generated are representative of 3 independent experiments and are the median values of triplicates. [b] logD 10mM potassium phosphate buffer, pH7.4/Octanol.

Sangamides display *in vivo* pharmacokinetics potentially suitable for once a day therapy

To further analyse their potential for use as oral therapies, **3n** and **3o** were tested for plasma protein binding (PPB) and mouse pharmacokinetics (10 mg kg^{-1} p.o. and 1 mg kg^{-1} i.v. dosing) (Table 2 and Fig. 1). Both compounds have low volume of distribution and long half-lives similar to **1**[25] although **3o** was more orally bioavailable (see the ESI for PK parameters†). **3o** and **3n** also exhibit lower plasma protein binding as compared to CsA and **1** (Table 2).

Based on the combination of potency and selectivity in cross-genotype replicons, reduction in the immunosuppressive potency, and mouse oral pharmacokinetics, **3o** was chosen for further detailed profiling. Confirmation of liver exposure was carried out by administration of a single oral (5 mg kg^{-1}) dose to CD-1 mice, followed by measurement of blood and liver concentrations for 24 h post dose. Liver : blood ratios of around 3 : 1 were present after oral dosing, and analyte was present in both matrices above 20 ng ml^{-1} or 20 ng g^{-1} for at least 24 h post dose (see the ESI†).

3o is able to clear HCV replicons from hepatoma cells

Huh7-Con1 cells were passaged seven consecutive times in the presence of **3o** or CsA, then drugs were removed and the cells passaged for two further rounds. Analysis of viral RNA at each stage revealed that CsA (at 0.5 and 1 μM) was unable to clear HCV from hepatoma cells, whilst **3o** at similar concentrations led to no viral rebound (see Fig. 2). This is consistent with data published previously for alisporivir.[11]

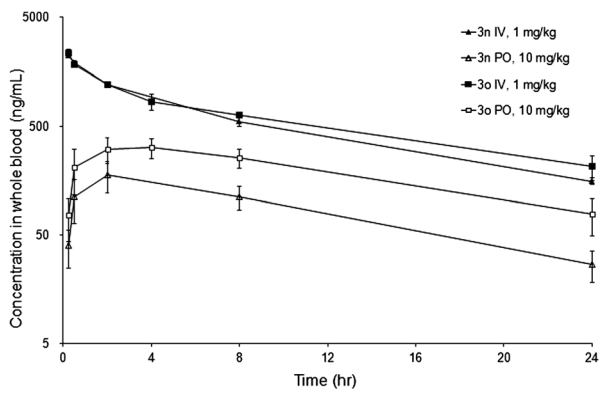

Fig. 1 Mean blood concentration-time profiles of sangamides after IV dose of 1 mg kg^{-1} or PO dose of 10 mg kg^{-1} in CD1 mice (n = 3).

3o is (at least) additive when used in combination with DAAs

Initial combination studies were carried in Huh7 cells carrying the genotype 1b subgenomic replicon using a single IC_{50} concentration of a protease inhibitor (telaprevir), a polymerase inhibitor (R-1479), and an NS5A inhibitor (BMS-790052) to assess the potential of a future combined treatment regime. **3o** was seen to decrease replicon IC_{50} values with at least an additive effect (see the ESI†).

3o shows no significant inhibition of MRP2

Clinically observed CsA and alisporivir treatment-associated elevation in bilirubin levels may be caused (at least in part) by drug-mediated inhibition of Multidrug Resistance Protein 2 (MRP2), the primary transporter for excretion of organic anions and their conjugates. We therefore assessed MRP2 inhibition

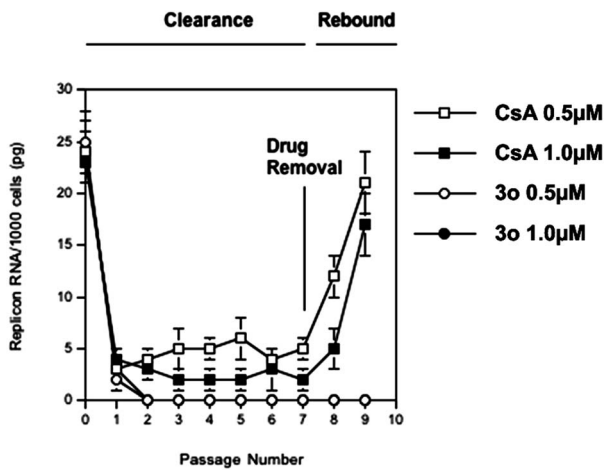

Fig. 2 Huh7-Con1 cells were passaged seven consecutive times without G418 in the presence or absence of two concentrations of CsA or **3o** (0.5 and 1 μM). At each passage, ten clones were collected, RNA extracted and analyzed by RT-qPCR for replicon content. After seven passages, drugs were removed and cells were analyzed for viral RNA content by qPCR for two additional passages. Data are representative of two independent experiments.

using an *in vitro* ATPase inhibition system. Previous data have shown that CsA and alisporivir inhibit MRP2 at similar levels.[20] Benzbromarone was included as a positive control for MRP2 inhibition. Whilst benzbromarone and CsA inhibited MRP2 at low μM concentrations (14 and 9 μM), no significant inhibition was seen with **3o** at concentrations up to 50 μM.

3o shows no significant inhibition of MDR1

Cyclosporine A is known to inhibit MDR1/P-glycoprotein,[19] a major xenobiotic transporter, inhibition of which can lead to DDIs with drugs which are MDR1 substrates. We therefore assessed MDR1 inhibition using an *in vitro* MDCK MDR1-expressing cell line. As expected, CsA showed potent inhibition of MDR1 (IC_{50} 0.729 μM), whilst **3o** showed no significant inhibition at concentrations tested up to 50 μM.

Conclusions

Treatment of chronic hepatitis C virus (HCV) infection is currently undergoing a revolution. Until 2011, standard of care (SOC) treatment for all genotypes was a combination of subcutaneous pegylated interferon alpha and oral ribavirin, administered for a period of 24–48 weeks. In May 2011, the first of the new Direct Acting Antivirals (DAAs) to complete clinical development, the NS3-4A protease inhibitors telaprevir (Incivek™) and boceprevir (Victrelis™) were approved for treatment of patients infected with genotype 1, when used in combination with SOC. However, 10–50% of patients still do not achieve a sustained virological response (SVR) and interferon is contraindicated in many patient populations. All of these therapies are associated with serious side effects, leading to discontinuation in up to 20% of patients.[29] Further classes of DAAs are in clinical development, including NS5B polymerase inhibitors and NS5A inhibitors. It is clear that a future therapy will probably consist of a combination of compounds with different mechanisms of action, in particular those showing high barriers to generation of resistance with a lack of cross-resistance.[15,30]

HTAs such as the cyclophilin inhibitors currently in clinical trials for treatment of chronic HCV infection (alisporivir and SCY-635), offer a compelling alternative to the numerous DAAs in development. Host cyclophilin is required for the HCV lifecycle and is thus a suitable target for HTA therapy. Removal of the spiroketal moiety from the **1** structure leads to compounds of significantly reduced molecular weights with a concomitant improvement in drug like properties and similar or improved inhibitory activity against CypA. The sangamides, α,β,γ,δ-unsaturated amides connected to the piperazic acid containing macrocycle of sanglifehrin, were explored as a novel class of compounds that selectively inhibit the replication of HCV. Surprisingly, the *N*,*O*-dimethylhydroxylamide (**3n**) displayed good potency and selectivity that warranted further study. The synthesis of the 1,2-oxezinane amide (**3o**) resulted in an improved potency as compared to **3n** and generated a compound displaying pharmacokinetics potentially suitable for once-a-day oral treatment for chronic HCV infection. These properties, in combination with a lack of immunosuppressive activity, led to **3o** being chosen as an advanced lead for further optimization towards a candidate drug for the treatment of HCV infection.

Acknowledgements

We would like to thank Stephen Wrigley at Hypha Discovery and Gerhard Schlingmann for technical support, Chen Weiqing, David Xie, Yan Liping, Qian Mingxin, Chen Ping and Huang Jianfeng for *in vitro*, ADME, physicochemical, and mouse PK analysis, Will Parish and Neil Williams at KWS Biotest for MLR studies, Sally Lee at Cyprotex for MDR1 inhibition studies and Dawn Evans and Steve Clifton at Biofocus DPI for MRP2 inhibition studies.

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