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Short communication

Metabolic oligosaccharide engineering of *Plasmodium falciparum* intraerythrocytic stages allows direct glycolipid analysis by mass spectrometry

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ABSTRACT

A recent addition to the arsenal of tools for glycome analysis is the use of metabolic labels that allow covalent tagging of glycans with imaging probes. In this work we show that *N*-azidoglucosamine was successfully incorporated into glycolipidic structures of *Plasmodium falciparum* intraerythrocytic stages. The ability to tag glycoconjugates selectively with a fluorescent reporter group permits TLC detection of the glycolipids providing a new method to quantify dynamic changes in the glycosylation pattern and facilitating direct mass spectrometry analyses. Presence of glycosylphosphatidylinositol and glycosphingolipid structures was determined in the different extracts. Furthermore, the fluorescent tag was used as internal matrix for the MALDI experiment making even easier the analysis.

No new chemical class of antimalarials has been introduced into clinical practice since 1996. This fact joined to the widespread resistance to most antimalarial drugs and the unavailability of an effective vaccine has fuelled the emergence of malaria in recent years as a major global health and economic burden [1]. The *Plasmodium falciparum* glycome has not been completely deciphered yet. However, there are evidences suggesting that carbohydrates are involved in key recognition events that may be blocked leading to useful clinical therapies. Moreover, interference of some of the key steps of *P. falciparum* glycobiology may provide new targets to control infection. It is still not clear whether these targets are the enzymes or the receptors or if the blocking agents will be carbohydrates or analogs, even monoclonal antibodies or small molecules acting as mimotopes may constitute a profitable alternative. Therefore all this development requires a first step of structural determination.

Lipid metabolism has been attracting a lot of attention with respect to basic biology and applications for malaria chemotherapeutic purposes [2]. Two candidates for putative GPI-anchor precursors to malarial GPI-membrane proteins with the structures ethanolamine phosphate-6(Manαl-2)Manαl-2Man-αl-6Manα1-4GlcN-PI (Pfα) and ethanolamine phosphate-6Manαl-2Manαl-6Man-αl-4-GlcN-PI (Pfβ) were identified [3,4]. Only Pfα has been found as anchor for MSP-1 and MSP-2 [5]. The GPI membrane anchor precursor Pfβ is synthesized and transferred to other, until now uncharacterized, proteins essentially in trophozoite stages [3,6]. Interestingly, the core glycans of nine *P. falciparum* isolates from different geographic regions show a universal core glycan structure [7]. An analysis of the acyl substituent on C-2 of the inositol of the non-labelled GPIs of *P. falciparum* showed palmitic acid (<90%) and myristic acid (<10%). The diacylglycerol moiety contains predominantly C18:0 and C18:1, minor proportions of C14:0, C16:0, C18:2, C20:0, C22:0 and unidentified acids, with C18:1 and C18:2 at the sn-2 position [8].

On the other hand, glycosphingolipids seem to be a general feature of eukaryotic cells. Synthesis of sphingomyelin by the parasite has been demonstrated [9,10], however its metabolic pathway in parasite cells, is poorly understood. Although there was data suggesting that *Plasmodium* has limited ability to synthesize lipids *de novo*, incorporation of [^{3}H]serine and [^{3}H]glucosamine in sphingolipids could be shown [11], the synthesis of chloroplast galactolipids in apicomplexan parasites was reported [12], *de novo* biosynthesis of glycosphingolipids [13] was proven, and the biosynthesis of sulfoglycosphingolipids was also determined [14].

A recent addition to the arsenal of tools for glycome analysis is the use of metabolic labels that allow covalent tagging of glycans with imaging probes [15]. In an attempt of getting deeper into plasmodial glycoconjugate structures, we show herein that the approach of parasite metabolic labelling using azido sugars enables the study of glycolipids that were previously hardly accessible. The ability to tag glycoconjugates selectively with a fluorescent reporter group permits TLC detection of glycolipids, providing a new method to quantify dynamic changes in the glycosylation

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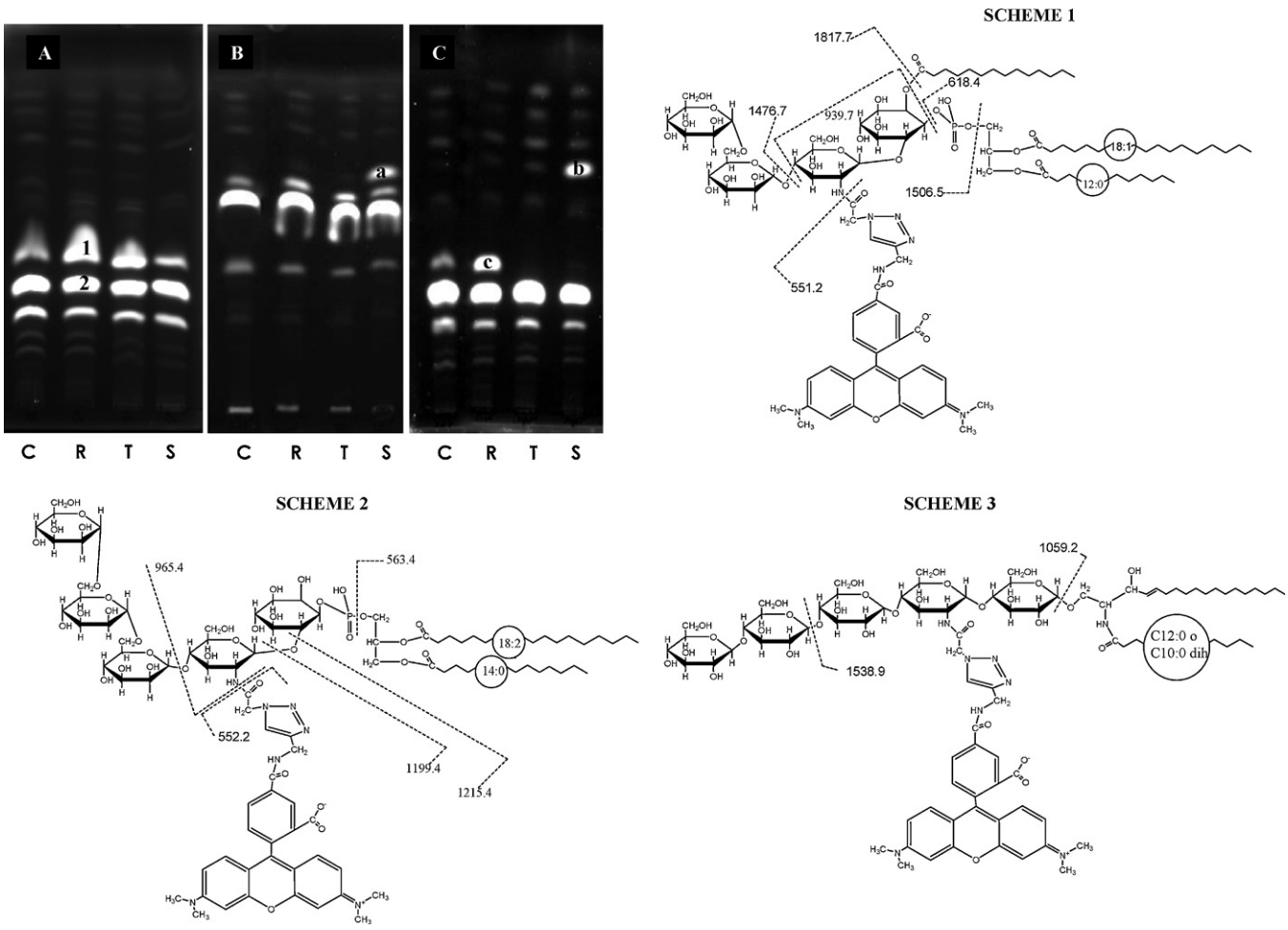

Fig. 1. *P. falciparum* clone 3D7 was cultured according to the protocol described by Trager and Jensen with modifications [20]. Parasites were grown in a 40 ml volume under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The cultures were initially synchronized in ring stage (1–20 h after reinvasion) by two treatments with 5% (w/v) D-sorbitol solution in water, for subsequent maintenance in culture until the differentiation to trophozoite (20–30 h after reinvasion) or schizont (30–35 h after reinvasion) stages. Parasite development and multiplication were monitored by microscopic evaluation of Giemsa-stained thin smears. After purification by a Percoll gradient, each stage was lysed, centrifuged and extracted with chloroform: methanol: water (3× 0.5 ml). TLC analysis of OP I (A) and OP II using chloroform:methanol:water (40:10:1) for A and B and OP II in chloroform:methanol:water (40:10:0.3) for C. Numbers and small letters indicate spots that were further analyzed by mass spectrometry. R, ring; T, trophozoite; S, schizont; C, uninfected erythrocyte. Scheme 1: One of the GPI structures determined in Fig. 2A. Scheme 2: One of the GPI structures determined in Fig. 2B and C. Sugars and linkages were adapted from the known structures of GPIs present in *P. falciparum*. Scheme 3: Glycosphingolipid structure determined in Fig. 3. Sugars and type of linkages have not been determined.

pattern and facilitating direct mass spectrometry analyses. Furthermore, the fluorescent tag is used as matrix for the MALDI experiment without the use of an external matrix making the analysis even easier. This application is particularly appealing because it decreases the analysis time and increases sensitivity with minimal sample preparation.

P. falciparum intraerythrocytic stages were metabolically incorporated with tetra-acetylated β-*N*-azidoacetylglucosamine (Invitrogen). Different conditions were tried but best incorporation was obtained using 0.26 mg/ml of culture during 24 h. After purification by a Percoll gradient, each stage was lysed, centrifuged and extracted with chloroform: methanol: water (3× 0.5 ml). The organic phases (OP I) were separated by centrifugation from precipitated proteins and each fraction was labelled with TAMRA (Click-iT^{TM} Tetramethylrhodamine, Invitrogen) under conditions suggested by the manufacturer. The labelled precipitate coming from each stage was further extracted with chloroform: methanol: water (3× 0.5 ml) and the organic phases separated and dried (OP II). In all cases non-infected red blood cells were treated in parallel and used as control.

Organic phases I were analyzed by TLC using chloroform: methanol:water (40:10:1) Interestingly, although non-infected red blood cells presented labelled lipids, quantitative differences in the fast migrating component of ring stage and trophozoites were shown. The spots corresponding to the ring stage (1 and 2) components were separately extracted from the plate with chloroform:methanol:water (10:10:1) dried and subjected to UV-MALDI-TOF mass spectrometry analysis without external matrix (Fig. 2).

The spectrum of the fast migrating spot 1 performed in the positive ion mode without addition of an external matrix (Fig. 2A) showed a signal at *m*/*z* 1817.7 (calc. *m*/*z* 1816.8) corresponding to the protonated ion of a glycosylphosphatidylinositol structure containing a diacylglycerol formed by C18:1 and C12:0 and two hexose units linked to the TAMRA-labelled HexN-Inositol moiety (see Fig. 1, Scheme 1). Further addition of a C14:0 gives rise to signal at *m*/*z* 2026.3 (calc. *m*/*z* 2027.0), in accordance with the fact that myristic acid constitutes the preferred fatty acid on the inositol ring of plasmodial GPIs [16]. Signals at *m*/*z* 1200.5 and 1216.3 (calc. 1199.4 and 1215.4; Δ O) correspond to the release of the phosphatidyl moiety from *m*/*z* 1817.7. Moreover, while peak at *m*/*z* 1506.5 (calc. 1505.6) corresponds to the loss of diacylglycerol moiety from *m*/*z* 2026.3, peak at *m*/*z* 1476.7 (calc. 1475.9) may be ascribed to the loss of two hexose units from *m*/*z* 1817.9.

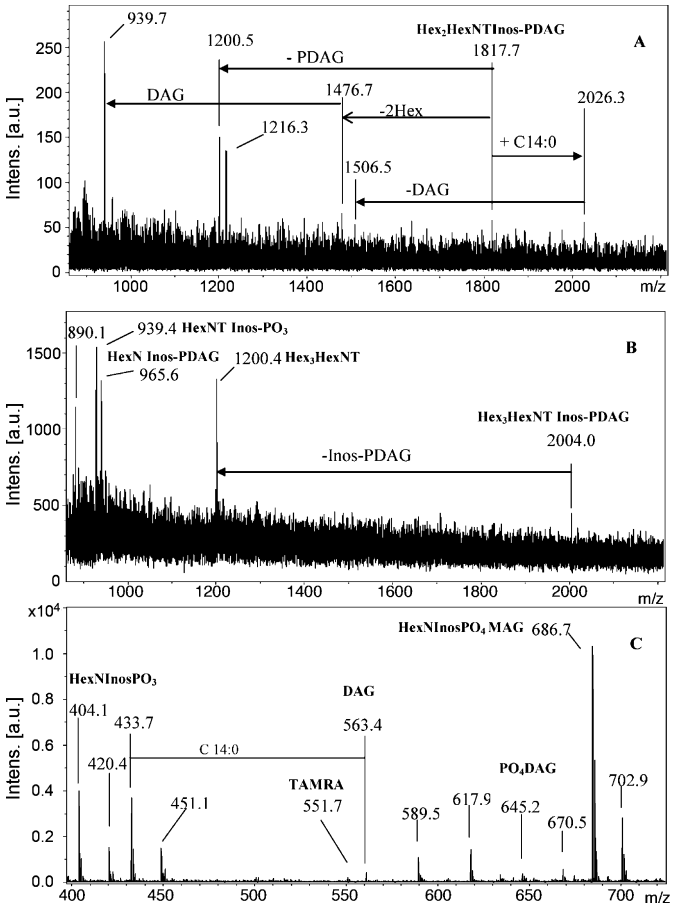

Fig. 2. Mass spectrometry analysis performed in a Ultraflex II TOF/TOF mass spectrometer equipped with a high-performance solid-state laser (λ = 355 nm) and a reflector, operated by the Flexcontrol 2.4 software package (Bruker Daltonics GmbsH, Bremen, Germany) without using external matrix of: (A) Spot **1**; (B) and (C), Spot **2** from Fig. 1A; B corresponds to expanded range *m*/*z* 850–2200. HexNT: TAMRA labelled hexosamine; DAG: phosphatidyldiacylglycerol, MAG: monoacylglycerol.

Additionally, peak at *m*/*z* 939.7 (calc. 939.3) corresponds to the loss of the diacylglycerol group from *m*/*z* 1476.5.

Spectrum of spot 2 is shown in Fig. 2B and C. In this case peak ion at *m*/*z* 2004.0 (calc. *m*/*z* 2004.9) may be ascribed to a GPI structure containing a diacylglycerol moiety formed by C 14:0 and C18:2 and a glycosyl structure containing three hexose units linked to the TAMRA labelled HexN-Inositol unit (Fig. 2B). Main cleavage between the glucosamine and the inositol units gives rise to *m*/*z* 1200.4 (calc. *m*/*z* 1199.5).The loss of two hexose units from the latter accounts for *m*/*z* 890.1 (calc. *m*/*z* 890.3). Additionally, as in the previous spectra, peak at *m*/*z* 939.7 (calc. *m*/*z* 939.4) corresponds to the HexN-Inositol moiety bearing the phosphate group. On the other hand, loss of the three hexosyl units and the phosphatidyl diacylglycerol moiety gives rise to signal *m*/*z* 890.1 (calc. *m*/*z* 889.3) while the loss of the three hexosyl units and the TAMRA group accounts for signal at *m*/*z* 965.6 (calc. *m*/*z* 966.5). In the lower mass region (Fig. 2C), loss of the TAMRA group and C18:2 fatty acid would give rise to signal at *m*/*z* 686.7, *m*/*z* 702.9 and *m*/*z* 670.5 (calc. *m*/*z* 686.3, 702.3 and 670.3 respectively, Δ O). Furthermore, the phosphatidyldiacylglycerol unit gives rise to *m*/*z* 645.2 (calc. *m*/*z* 644.4), the phosphatidyl monoacylglycerol bearing C18:2 fatty acid accounts for *m*/*z* 433.7 (calc. *m*/*z* 433.2) while the diacylglycerol moiety accounts for peak *m*/*z* 563.4 (calc. *m*/*z* 564.4). Minor peaks at *m*/*z* 617.9, 589.5 (Δ 28 mu from *m*/*z* 646.4) would correspond to micro heterogeneities in the fatty acid composition (see Fig. 1, Scheme 2).

When OPII was analyzed by TLC in chloroform:methanol:water (40:10:1) (Fig. 1 B), schizont stage presented a fast migrating spot that is present neither in the other stages nor in the control sample. The same differences but with a better resolution were detected when the samples were analyzed in chloroform:methanol:water (10:10:0.3) (Fig. 1C). Spots designed as a, from Fig. 1B, b and c from Fig. 1C were extracted from the plate using chloroform:methanol:water (10:10:1) and analyzed by mass spectrometry without external matrix. As expected spectra performed in the positive ion mode corresponding to spot a and b were undistinguishable and were ascribed to a glycosphingolipid structure (Fig. 3). Peak at *m*/*z* 1706.1 (calc *m*/*z* 1705.8) may be attributed to the $[M+Na]^+$ ion belonging to a ceramide composed by a d18:1 acylated with C 10:0 dih and glycosylated with three hexose units and one TAMRA-labelled hexosamine (Scheme 3). Moreover peak at *m*/*z* 1364.0 (calc. *m*/*z* 1364.7) would correspond to the loss of two hexose units from the latter. Similarly, *m*/*z* 1862.9 (calc *m*/*z* 1863.9) corresponds to a d18:1 acylated with a C12:0 and glycosylated with four hexose units and one TAMRA-labelled hexosamine. In the same way, peak at *m*/*z* 1538.9 (calc *m*/*z* 1538.8) would be ascribed to the loss of two hexose units from the latter. Further loss of the ceramide moiety from *m*/*z* 1538.9 would give rise to *m*/*z* 1059.4 and 1075.5 (calc *m*/*z* 1058.3 and 1074.4, Δ O). In the lower mass region, major peak at *m*/*z* 764.3 (calc. *m*/*z* 765.4) corresponds to the $[M+H]^+$ ion ascribed to the loss of the TAMRA group concomitant with the loss of the fatty acid from 1364.0 as well as to the loss of the TAMRA group, the fatty acid and one hexose unit from *m*/*z* 1538.9. Further loss of a hexose unit would give rise to *m*/*z* 603.9 (calc. *m*/*z* 604.4). Additionally, signal at *m*/*z* 684.1 (calc. *m*/*z* 685.2) corresponds to the three hexoses and the hexosamine unit. These structures are in accordance with glycosphingolipid structures previously described in *P. falciparum* [13].

On the other hand, when spots c and d were analyzed by mass spectrometry the same pattern of structures described for spots 1 and 2 from Fig. 1A was obtained (not shown) indicating that GPI structures were not completely isolated in the first organic phase.

The metabolic oligosaccharide engineering technique involves an unnatural monosaccharide with a subtle structural modification that is processed by the cells' biosynthetic pathways and incorporated into glycoconjugates analogously to the natural sugar. After its metabolic installation, the azido sugar used in this case can be chemically modified by exploiting the unique reactivity of the azide *via* the Staudinger ligation allowing the attachment of a chemical probe to the azide containing glycans [15]. In the present work we have shown that *N*-azidoglucosamine was successfully incorporated into glycan structures of *P. falciparum* intraerythrocytic stages. TLC analysis of the lipid extracts indicated qualitative and quantitative differences among the extracted lipids present in the different stages of the parasite and with uninfected red blood cells, at least one component of the organic phase I was mainly present in ring and trophozoite stages and one component of the organic phase II was only present in the schizont stage of the parasite Mass spectrometry analysis indicated that these two lipids labelled with the fluorescent tag correspond to GPI and glycosphingolipidic structures respectively. Accordingly, both types of lipids have been described as parasitic components.

GPIs are the major carbohydrate modification in plasmodial proteins and either free or linked to surface antigens have been shown to be involved in the pathogenicity of the parasite [17]. GPIs are produced through the coordinated action of a multi-component biosynthetic pathway. Based on the known structures of *P. falciparum* GPI, Delorenzi et al. proposed nine steps, initiated by the transfer of GlcNAc to phosphatidylinositol. The fact that labelled GPI structures could be determined after *N*-azidoacetylglucosamine incorporation shows that the parasite tolerates analogues to the

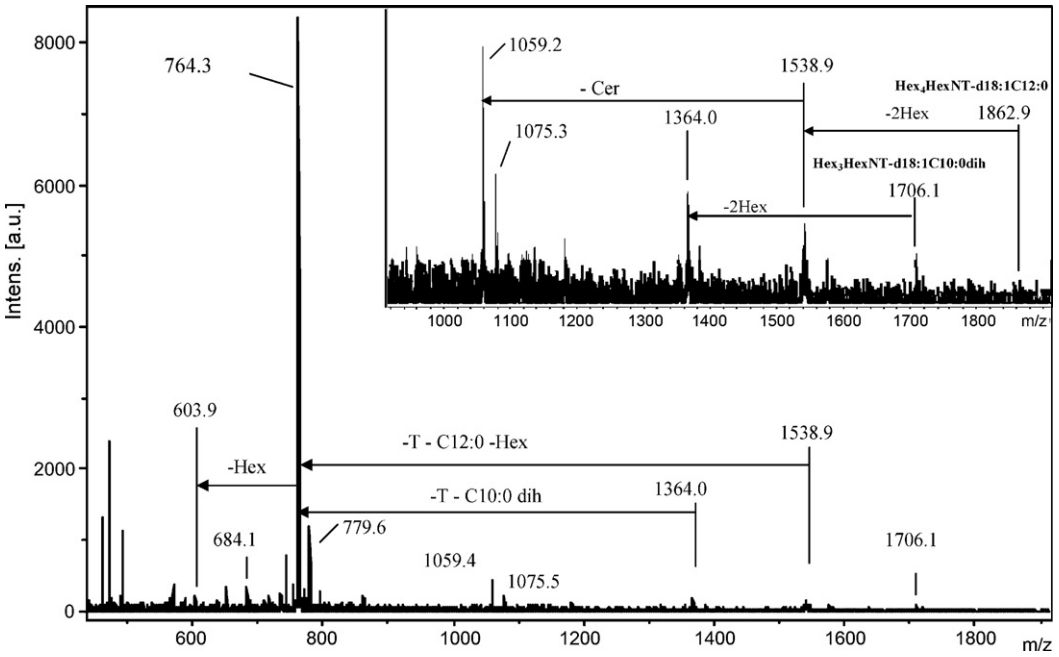

Fig. 3. UV-MALDI-TOF mass spectra of spot a from Fig. 1C. Inset corresponds to expanded range *m*/*z*: 950–1900 Da.

natural substrate. The second step would correspond to the de-*N*-acetylation of the GlcNAc unit, however our data show that the *N*-azidoacetylglucosamine stayed unmodified and was further mannosylated. This result would indicate two possibilities: either this step may be dispensable at least to a very low extent or the azido group mimics well enough the natural sugar so that the mannosylation step may take place anyway. In this sense, although eight genes for GPI synthesis by database mining and mRNA sequence and expression in *P. falciparum* were described (PIG-A, PIG-B, PIG-M, PIG-O, GPI1, GPI8, GAA-1, and DPM1), only preliminary evidence for the PIG-L gene encoding the *N*-acetylglucosaminylphosphatidylinositol de-*N*-acetylase responsible for the de-*N*-acetylation step, has been shown [18]. Further work is needed to unravel this point.

In summary, we have investigated for the first time, the incorporation of *N*-AzGlcNH$_2$ in glycolipids of the intraerythrocytic stages of *P. falciparum*. Labelling, isolation and detection was easily completed, the TAMRA fluorescent tag provided a very sensitive detection and furthermore the same tag was used as internal matrix for the mass spectrometry process. Actually, the fact that no external matrix was added indicates that a laser desorption ionization (UV-LDI) process is taking place [19]. However, although LDI is a strong ionization method, clean spectra were obtained. As far as we know this is the first report on the analysis of glycolipids by metabolic labelling with azido sugars. Finally, our results show that this strategy may become a useful tool for both biosynthetic studies and elucidation of specific *P. falciparum* glycoconjugates.

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