Proteomics in Medicinal Chemistry

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Abstract: Proteomics is becoming an important research area for studying protein expression patterns induced by different external stimuli. An important aspect of proteomics is to identify and quantify proteins. Many new technologies and techniques have been developed in this field and have been applied to various aspects of drug discovery.

Keywords: Proteomics, 2-D electrophoresis, high performance liquid chromatography (HPLC), isotope-coded affinity tag (ICAT), stable isotope labeling by amino acid in cell culture (SILAC), mass spectrometry (MS), drug discovery.

INTRODUCTION

Proteomics is the analysis of proteomes, and has undergone a tremendous period of growth in the past few years. A proteome is the set of proteins expressed by a cell, tissue, or organism under a specific conditions. Proteomic research is typically designed to analyze many proteins in a single analysis and provides a global view of changes in protein expression that occur in different cellular growth states or when the cell is treated with a given agent or regimen. Because proteomics is rapidly becoming an important research area, this mini-review focuses on how proteomics can play a role in medicinal chemistry.

One objective of proteomics is to examine the quantity or identity of proteins of interest within cells to elucidate the distinctions between two different states, for example, healthy and diseased tissue, or tissue treated and untreated with a drug, or treated with different drugs (derivatives). Such research is very helpful for medicinal chemistry. Although these studies can be carried out from genomics information to some extent, as exemplified in application of mRNA expression profiles, several studies indicate significant discrepancies between the results of mRNA expression profiles and the actual protein expression [1-3]. These discrepancies illustrate the importance of quantifying protein expression at the proteome level. Posttranslational modifications, the presence of isoforms, direct determination of protein expression levels, and investigation of protein-protein interactions can be determined at the protein level. Proteomics provides the opportunity to observe the proteins actually expressed and post-translationally modified, and the amount of proteins directly relates to the real expression. Proteomics provides information that is, therefore, closer to the real nature of the disease and/or potential drug action than genomics does.

In the area of drug discovery, for example, by comparing proteins expressed following treatment with a given drug with those present under untreated conditions, it is possible to identify changes in biochemical pathways via observed alterations in sets of proteins that may be related to the drug's efficacy or toxicity. These proteins may be used as efficacy or toxicity markers in high throughput screening assays to test large sets of lead compounds. Such a task is often referred to as "finding a needle in a haystack." At the heart of this field are many new technologies and techniques that are being developed reaching such daunting goals.

DEVELOPMENT OF METHODOLOGY FOR QUANTITATIVE ANALYSES OF PROTEOMES

Because of the nature of proteomics, it is essential to develop efficient methodologies for quantitative analyses of proteomes expressed under different physiological conditions. Classical methods for quantification of proteins can be exemplified as two-dimensional (2-D) densitometry of the gel [4] by extracting the Coomassie blue-stained gel spots followed by spectrophotometry [5], or by metabolic radioisotope labeling of the total proteins followed by counting the incorporated radioactivity [6]. These methods are laborious and apt to induce experimental errors. Recently, more sophisticated methods using stable-isotope labeling followed by mass spectrometry (MS) analysis have been emerging as a powerful technology for more accurate quantification of proteins [7-14].

Some of the earliest methods used metabolic labeling, where cells are cultured in isotope-enriched or in normal media and the relative abundance of specific proteins is quantitatively analyzed from peak intensities of each species in the mass spectra [15-20]. Although these metabolic labeling methods have generally been limited to relatively simple microorganisms that can be grown in these media, recently, Mann *et al*. have reported a method in which isotope-labeled essential amino acids were quantitatively incorporated into cell lines [21]. This method is referred to as SILAC (stable isotope labeling by amino acid in cell culture) and is applicable to even mammalian systems (Scheme **1**).

While this SILAC is becoming a popular method, there are several limitations in metabolic labeling in general, such as inability to use the techniques on human subjects and a relatively long time required for cell culturing. A more efficient approach, which can expand the applicability of stable-isotope labeling to a considerable extent, involves chemical modifications by covalent labeling on specific amino acid residues using isotope-labeled reagents followed by mass spectrometry analysis.

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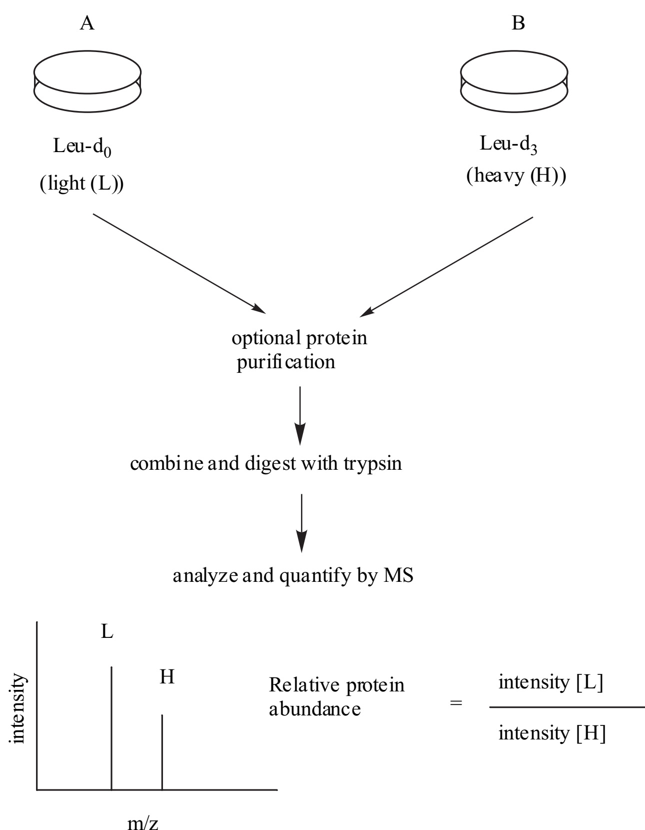


Scheme 1.

There are numerous proteomic technologies. However, there is no single method that is universally applicable to all types of projects, and the choice of technologies is dependent on the purpose of the projects. There are two major technologies for separating protein mixtures and obtaining protein expression profiles for development of such proteomic methodologies of quantification of proteins: (1) 2-D electrophoresis combined with MS and (2) multiplexed high performance liquid chromatography coupled with MS (HPLC/MS).

Mass spectrometry (MS) has also been a driving technology supporting proteomic investigations for analyses and characterization of expressed proteins due to its high sensitivity and its ability to rapidly identify proteins through peptide mass fingerprinting. Recent inventions of new soft ionization methods and mass analyzers have greatly extended overall sensitivity and the applicability to analysis of proteins and other macromolecular targets. In general, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are two major ionization techniques for protein or peptide samples.

(1) 2-D electrophoresis-based method

Separation of proteins by 2-D electrophoresis is among the most well-developed biochemical methods, and it is still the cornerstone for obtaining protein expression patterns in cells. In this technique, proteins are separated first via isoelectric focusing, followed by standard polyacrylamide sodium dodecyl sulfate gel electrophoresis. The 2-D polyacrylamide gel electrophoresis (2-D PAGE) has traditionally been the standard discovery-based tool for proteomics. A quantitative examination of global changes in protein expression in tissues, cells, or body fluids can be conducted using 2-D gels and image analysis. This method has the advantages of direct determination of protein abundance and detection of post-translational modifications such as glycosylation or phosphorylation, which result in a shift in mobility. Because thousands of proteins are imaged in one experiment, a picture of the protein profile of the sample at a given point in time is obtained, thus enabling comparative proteome analysis. Protein expression changes may give clues to the role of certain proteins in disease. Recently 2-D differential in-gel electrophoresis (2D-DIGE) is becoming a useful tool for proteomics [22]. This electrophoresis is accomplished by running two fluorescently-tagged samples with two different dyes on the same 2-D gel.

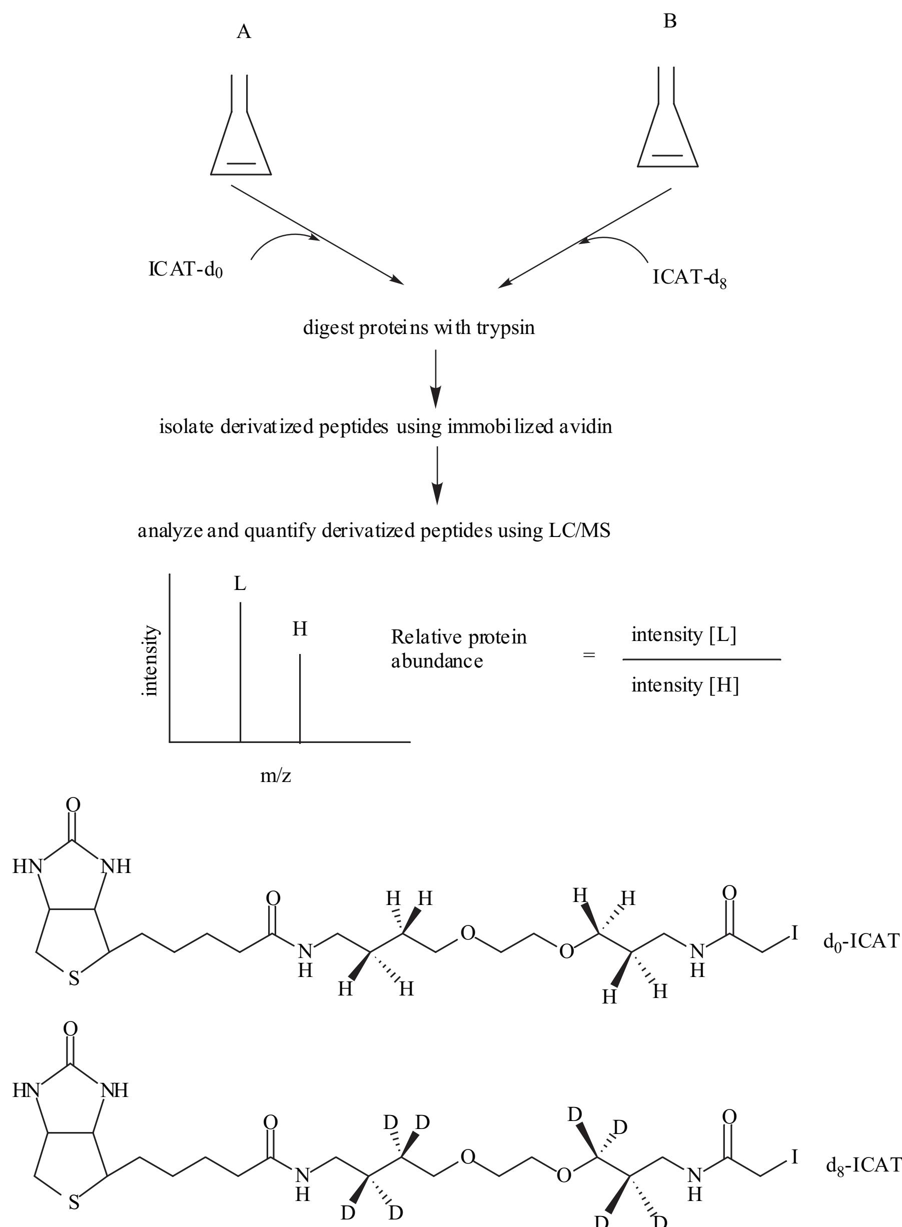
Our group has been developing methodologies for quantitative analysis of proteins by a combination of isotope-labeled and unlabeled chemical modification of specific amino acid residues followed by 2D-electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [23-26]. Our method applies small organic molecule-tagging reagents on cysteine residues, such as *N*-ethylmaleimide and iodoacetanilide, which are less hydrophobic than the isotope-coded affinity tag (ICAT) reagents described below. Some other researchers report a combination of 2-D electrophoresis and other kinds of small organic molecule-tagging reagents such as acrylamide [27-31].

The major problems with this 2-D electrophoresis are that it does not display all the proteins. In particular, the technology is not suitable for membrane proteins, very high or low molecular weight proteins, extremely acidic or basic proteins. In addition, low abundance proteins are difficult to detect, as the detection limit by silver staining is approximately 100 fmol.

(2) HPLC/MS-based method

In order to overcome some drawbacks in 2-D electrophoresis, several alternative methods using high performance liquid chromatography (HPLC) for separation of proteins have been developed.

Some of the most pioneering work, reported by Aebersold *et al*. applies deuterium-labeled and unlabeled isotope-coded affinity tags (ICATs) (Scheme **2**) [32]. The ICAT reagent is a set of reagents that consists of a biotin conjugate of a well-known cysteine-modifying reagent, iodoacetamide, and its deuterated version. The molecular weight of the deuterated version is 8 Da heavier and its chemical behavior is identical to that of the non-deuterated version. In this method, proteins are modified with the cysteine-specific reactive group (derived from iodoacetamide), and the biotin tag in the ICAT reagent allows the specific isolation of the modified Cys-containing peptides by immobilized avidin. Changes in the relative abundance of peptides from distinct proteome samples are accomplished by the use of isotopically labeled ICAT reagents. The derivatized proteomes are pooled and digested with trypsin, and the labeled Cys-polypeptides are isolated by avidin affinity chromatography. The peptide mixture can



Scheme 2.

be analyzed by LC/MS and tandem mass spectrometry (MS/MS). This method significantly reduces the complexity of the polypeptide mixture to be analyzed.

Some major problems have arisen from this ICAT method, largely due to the use of large hydrophobic organic molecules. These problems include primary isotope effects, decreased solubility, and fragmentation of the labels during collision-induced dissociation (CID) conditions, complicating the interpretation of tandem mass spectra. Several improvements have been made to the later versions of ICAT, such as acid- or photo-cleavable versions [33-35], and other different types of labeling reagents [36-41] have also been reported, but a large amount of aqueous media for purification by LC still must suffer from these problems.

Despite these problems, this ICAT method and other LC-based methods are better suited for low-molecular weight and membrane proteins as well as low-abundance proteins and have been complementary to 2-D electrophoresis. Recent advancement in multidimensional liquid chromatography has also been an effective technology for invention of LC-based methods.

(3) Mass Spectrometry

Mass spectrometry (MS) has played an increasingly important role in proteomics. A mass spectrometer consists of (1) an ionization source (2) a mass analyzer and (3) a detector. In particular, ionization is the heart of mass spectrometry, and matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are two major ionization techniques for analyses of macromolecules including proteins and peptides.

In ESI, a sample solution is sprayed from the tip of a metal nozzle at atmospheric pressure through a 3-5kV

potential, and the resulting droplets are electronically charged and ionized for analyses. ESI-MS is useful for a wide range of biological applications as a detector for HPLC and capillary zone electrophoresis. The limitations of ESI are that it is not very tolerant to the presence of salts, and it is not practical for analyses of multicomponent samples. Recently-invented nanoelectrospray ionization method achieves a two-order higher sensitivity in the low femtomole to sub-femtomole range due to the low flow rate of samples [42]. As this method is also more tolerant of salts, it has been playing an important role in proteomics.

The principle of MALDI includes bombardment of a mixed matrix of solutes by ultraviolet laser pulse in an electric field to desorb and ionize a cocrystalized sample/matrix from a metal surface. Once ions are formed in the gas phase, they can be electrostatically directed to a mass analyzer. Resolution of the technique has improved since its initial introduction and MALDI-MS has emerged as an effective bioanalytical tool having unique capabilities in handling complex mixtures such as proteolytic digests. It is also highly sensitive, allowing femtomole or even sub-femtomole measurements.

Examples of common mass analyzers include quadrupole, time-of-flight (TOF), ion trap (IT), and magnetic-sector. Tandem mass spectrometry (MS/MS) is a mass spectrometer that has more than one analyzer, and common combinations include quadrupole-quadrupole, quadrupole-time-of-flight (Q-TOF), and time-of-flight-time-of-flight (TOF-TOF). Tandem mass spectrometry has facilitated analyses of complex mixtures and elucidation of structures of complex big molecules such as peptides.

APPLICATION OF QUANTITATIVE PROTEOMICS TO MEDICINAL CHEMISTRY

Mass spectrometry-based proteomics provides general or global screens to identify proteins and to measure their relative abundances as well as specific protein subsets and their interactions in cells and tissues. Such technologies have begun to be applied to various aspects of drug research, although examples are still relatively limited.

Lin *et al*. elucidated the cellular effects of p38 mitogen-activated protein kinase (MAPK) inhibitor, SB203580 (**1**) by the ICAT method and cDNA microarray analysis using SB203580-treated transformed follicular lymphoma cells. This study revealed a number of up-regulated or down-regulated proteins involved in p38 MAPK-dependent or independent signaling pathways [43]. This approach is anticipated to be useful in the comprehensive identification of genes/proteins involved in disease pathogenesis signaling pathways.

Acetaminophen (**2**), a widely-used analgesic and antipyretic, is a common substitute for aspirin because of its lower incidence of side effects. However, its overdose can cause acute, fatal liver necrosis in humans and animals, although its mechanism still remains uncertain. Using 2-D electrophoresis, Fountoulakis *et al*. identified and quantified 35 proteins from acetaminophen-treated mice, which are believed to drive acetaminophen-induced hepatotoxicity [44]. Some of them are known targets of covalent modification by the main active metabolite *N*-acetyl-*p*-benzoquinoneimine. Such proteomic studies, which allow analyses of proteins of treated animals in comparison to the control map to reveal the differences induced by the treatment, are therefore expected to be valuable tools.

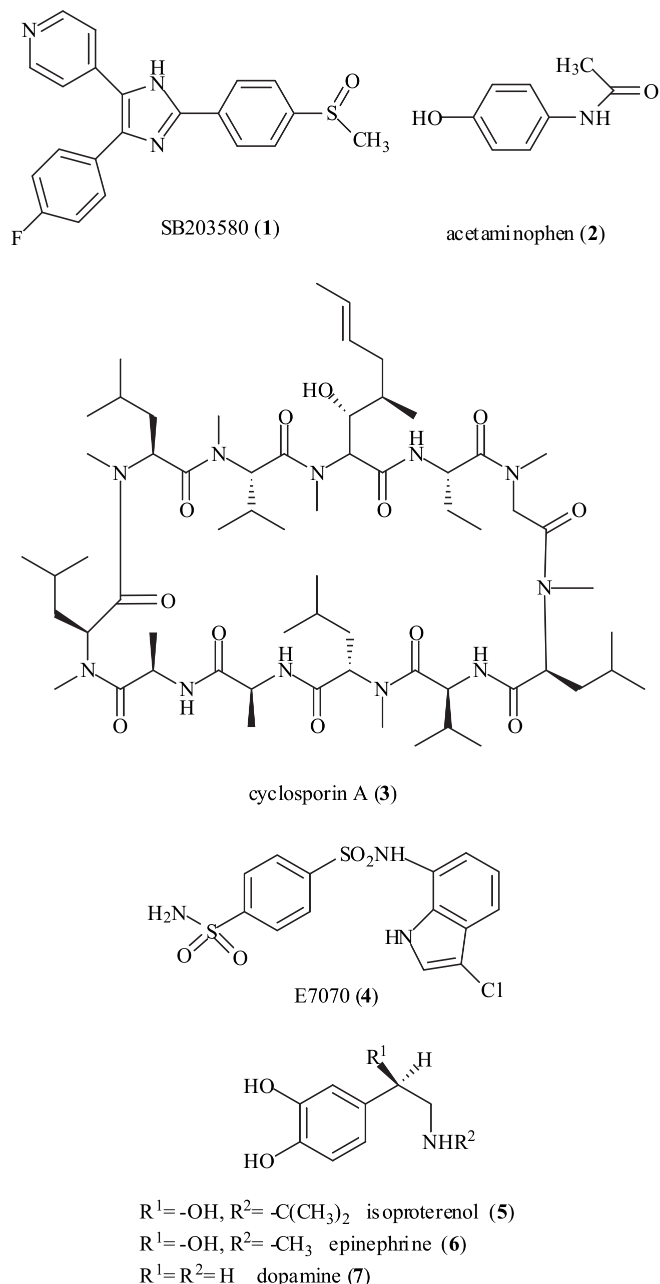
Proteomics also played a key role in finding novel molecular mechanisms involved in the nephrotoxicity of the widely-used, potent immunosuppressant, cyclosprorine A (**3**). Cyclosporine A has been shown to exert its activity by preventing T-cell proliferation via the inhibition of a Ca^{2+}-dependent event required for induction of transcription of the IL-2 gene. However, its nephrotoxicity limits its application. Based on comparative protein expression profiling on 2D gel, Steiner *et al*. found that a protein involved in calcium transport, calbindin D, decreases in a cyclosporine-A dependent manner [45-47]. They demonstrated that calbindin D is a marker for cyclosporine A-nephrotoxicity in animals and humans.

While many proteomics analyses for drug discovery have focused on comparing cells or tissues in two different states, for example, normal versus disease, some studies report application of the quantitative proteomics to identification of drug targets. Oda *et al*. identified a primary binding protein of a novel class of anticancer agents, E7070 (**4**), using the ICAT approach in combination with 2-D differential in-gel electrophoresis (2D-DIGE) [48]. A commonly-applied method of affinity chromatography using compound-conjugated affinity matrices revealed more than two hundred binding proteins. A quantitative proteomic approach that applied cleavable ICAT reagents for these proteins yielded binding ratios between the E7070 and a derivative of a negative control, and the best primary candidate was identified based on the ratios. Such approaches are expected to be of wide utility for the identification of the target proteins of pharmaceuticals in particular with relatively low affinity and specificity.

Meneses-Lorente *et al*. applied proteomics to the detection of potential toxicities of a compound in its preclinical development by combining histological and clinical chemistry studies [49]. They monitored rat protein expression patterns in liver tissue after administration of the compound by 2-D differential in-gel electrophoresis (2D-DIGE) in order to identify up-regulated and down-regulated proteins as early markers of toxicity. These proteomics data were found to correlate with the clinical and histological data. While toxicities of drugs often do not become apparent until the later stages of development, such studies are expected to help identify dose-related markers that can be correlated with the initiation of efficacy or the severity of toxicity, and therefore ultimately achieve optimal efficacy without side effects.

G-protein coupled receptors (GPCRs) are an important class of drug targets that exist as proteins on the surface membranes of all cells. The GPCRs, a superfamily of proteins accounting for approximately 1% of the human genome, are associated with a variety of therapeutic categories, including asthma, inflammation, obesity, cancer, cardiovascular, metabolic, gastrointestinal and central nervous system diseases. Ligand-induced posttranslational modifications of GPCRs such as the β-2-adrenoceptor play a critical role in controlling the functional activity of receptors. By the SILAC approach, von Zastrow *et al*.

examined effects of small molecule agonists, isoproterenol (**5**), epinephrine (**6**), and dopamine (**7**), on posttranslational modifications, such as phosphorylation and palmitoylation, of the β-2-adrenoceptor [50]. Their results indicate differences among the agonists' ability to promote multiple phosphorylation of a proximal portion of the carboxyl-terminal cystoplasmic domain. The results also suggest that their methods can be a general approach to studying regulated posttranslational modifications of GPCRs in intact cells.



Scheme 3.

CONCLUSION

Proteomics is rapidly becoming an important research area for studying the global events occurring within a cell or tissue under a specific set of conditions, although it has only started appearing in the public literature of medicinal chemistry. Since proteomics provides a global view of changes in protein expression and enables comprehensive understanding of the differences in biological effects induced by pharmaceuticals, it is anticipated to be a valuable tool for drug discovery.

ACKNOWLEDGEMENTS

The author thanks Texas Tech University for financial support (start-up funds).

ABBREVIATIONS

MS	=	Mass spectrometry
SILAC	=	Stable isotope labeling by amino acid in cell culture
HPLC	=	High performance liquid chromatography
2-D PAGE	=	2-Dimensional polyacrylamide gel electrophoresis
2-D DIGE	=	2-Dimensional differential in-gel electrophoresis
ICAT	=	Isotope-coded affinity tag
MS/MS	=	Tandem mass spectrometry
CID	=	Collision-induced dissociation
MALDI	=	Matrix-assisted laser desorption/ionization
ESI	=	Electrospray ionization
TOF	=	Time-of-flight
IT	=	Ion trap
q-TOF	=	Quadrupole-time-of-flight
MAPK	=	Mitogen-activated protein kinase
GPCR	=	G-protein coupled receptor

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Received: June 30, 2005 Revised: August 23, 2005 Accepted: August 24, 2005

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