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Advanced MALDI mass spectrometry imaging in pharmaceutical research and drug development

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Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) has emerged as a key technology for label-free bioanalysis of the spatial distribution of biomolecules, pharmaceuticals and other xenobiotics in tissue sections. Recent advances in instrumentation, sample preparation, multimodal workflows, quantification, analytical standardization and 'big data' processing have led to widespread utilization of MALDI MSI in pharmaceutical research. These developments have led to applications of the technology in drug discovery beyond drug disposition analysis, most notably in pharmacodynamic biomarker research and in toxicology.

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Introduction

Discovery and development of pharmaceuticals is a complex and costly process [1]. Early quantitative knowledge of drug disposition in disease-relevant tissues, of target engagement evidenced by pharmacodynamic (PD) biomarkers, of *in situ* drug metabolism, and of toxicity-related histopathology and other safety risks are fundamental for selection of safer and more efficacious drug candidates. To reduce attrition rates in pharmaceutical research and development (R&D), original and performant analytical methods are needed. Since the first analysis of an active pharmaceutical ingredient in 2003 [2], matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) has developed into the key label-free technology for quantitative spatial analysis of drugs, metabolites and formulations as well as PD biomarkers in tissues. Since then, MSI has been applied to various pharmaceuticals in different organs or whole-body animal sections (reviewed in [3••,4]). Whereas other MS imaging technologies such as desorption electrospray ionization (DESI) [5], secondary ion mass spectrometry (SIMS) imaging [6] or liquid extraction surface analysis (LESA) [7•] can be useful in spatial analysis of pharmaceuticals, we focus on recent advances in MALDI MSI in this field (Table 1). We summarize advances in instrumentation, quantification and highlight emerging MSI applications in drug metabolism and pharmacokinetics (DMPK) [8•,9,10•] as well as MSI of drug formulations or delivery systems [11••,12,13]. We also outline current trends that expand the scope of MSI into target engagement — PK–PD — as well as drug-induced toxicity studies [14,15•,16•]. As an outlook, we point to technology trends and recent MSI work on biopharmaceutical and nucleic acid analogs.

Quantitative MALDI MSI (qMSI) of drugs: calibration, internal standards and normalization

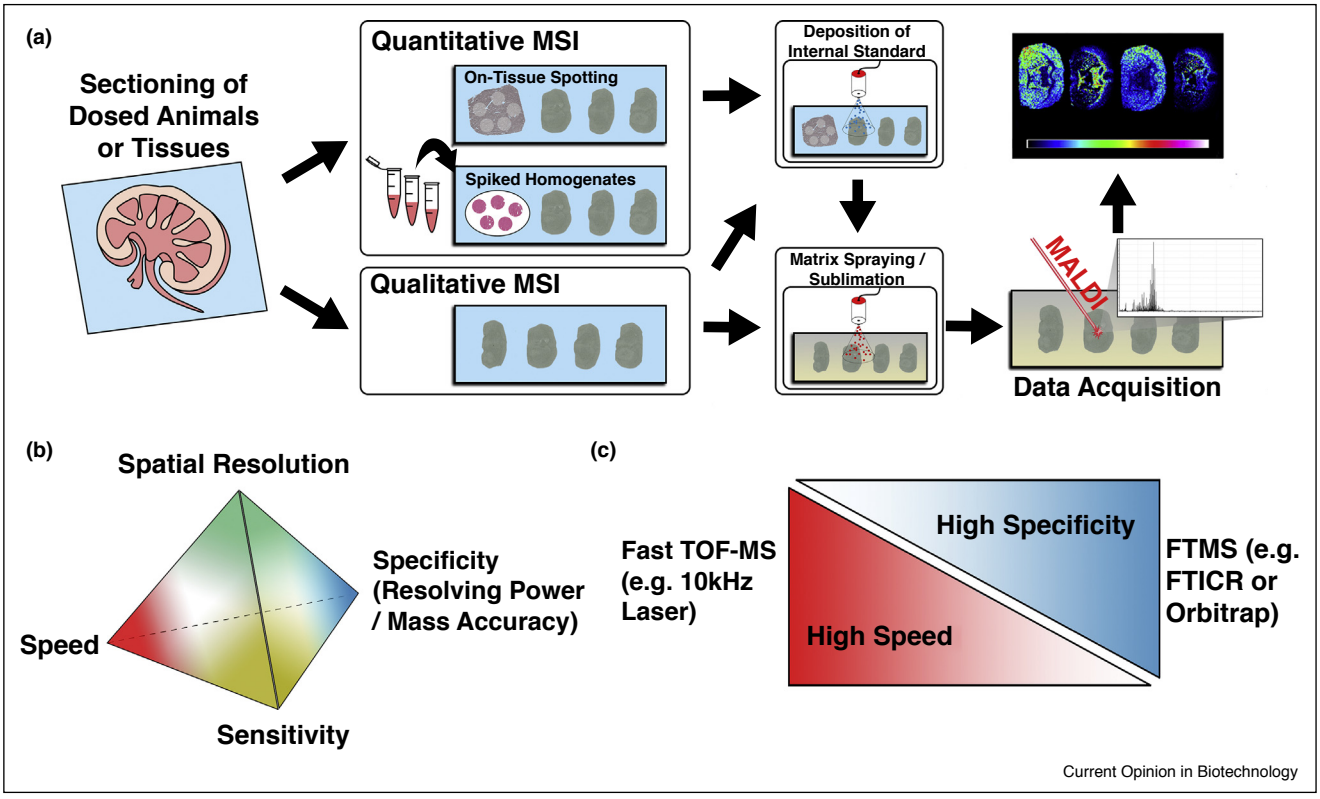
MALDI MSI has the unique ability to reveal the quantitative distribution of unlabeled drugs or biomarkers across a tissue section with high spatial resolution. Although qualitative analysis might suffice in some cases, qMSI is often required in pharmaceutical R&D. However, absolute quantification by MALDI MSI is not trivial, because, as with any MS-based analysis, the measured analyte intensity is influenced by several factors including analyte extraction efficiency, ionization efficiency and consistency of co-crystallization with matrix. Additionally, in MALDI MSI, there is no sample cleanup or chromatography step prior to ionization. Thus, ion suppression is prevalent [24•,34•], and ion intensities and absolute quantities may show limited correlation. The critical step in a qMSI experiment is to generate a relevant calibration curve of a reference standard to which the analyte's ion intensity in dosed tissue can be compared to estimate its concentration. The goal is to detect the standards under conditions that reproduce the analyte extraction and ionization effects observed from a dosed tissue [35] (Figure 1a).

Table 1

Summary of recent applications of MALDI imaging in the drug development process

	Drug	Tissue	MALDI matrix	Matrix deposition	Normalization	MSI quantification	Mass analyser	Reference
Drug distribution	Roflumilast, Tofacitinib, Ruxolitinib, LEO 29102	Skin explants (human)	DHB	Robotic sprayer	TEC approach	TEC approach; LC–MS/MS validation	FTICR	[11••]
	Tacrolimus, Tofacitinib, Ruxolitinib, LEO 29102	Skin explants (human)	DHB	In-house pneumatic sprayer	No	No	Orbitrap	[17]
	Paclitaxel (3D)	Tumour (xenografts in mouse)	TiO_2-based nanoparticle	Airbrush	Stable isotope	Spotting approach; LC validation	TOF/TOF	[18]
	Paclitaxel	Tumour (xenografts in mouse)	TiO_2-based nanoparticle	Airbrush	Stable isotope	Spotting approach; LC validation	TOF/TOF	[19]
	Epertinib, Lapatinib	Brain metastasis (mouse)	DHB	Vibrational vaporization	Stable isotope	Spotting approach; LC–MS/MS validation	LTQ ion trap	[20]
	Rifampicin, Moxifloxacin	Lung (rabbit)	THAP, DHB	Robotic sprayer	Stable isotope	No	LTQ Orbitrap	[21•]
Drug metabolism	AZD2820	Kidney (mouse)	CHCA	TLC sprayer	No	Spotting approach	TOF/TOF; FTICR	[22]
	Reserpine	Whole-body (rat)	DHB	Spray nebulizer	No	No	LTQ Orbitrap	[23]
	Erlotinib	Pancreas, tumour	CHCA	Vibrational vaporization	TIC	No	TOF/TOF; FTICR	[24•]
PK and PD	Pirfenidone	Lung, liver, kidney (mouse)	sDHB	Robotic sprayer	Stable isotope	Spotting approach	FTICR	[10•]
	Octreotide	Stomach, intestine, liver (mouse)	DHB	Robotic spotting device	Lanreotide as internal standard	Spotting approach; LC–MS/MS validation	TOF	[25]
	Alectinib	Brain (mouse)	CHCA	Sublimation + robotic sprayer (two-step)	Erlotinib-D6 as internal standard	Yes; correlation of MSI to LC–MS/MS of adjacent sections	qIT-TOF	[26]
	Panobinostat	Tumour (mouse)	sDHB	Robotic sprayer	TIC	No	TOF/TOF	[16•]
Formulation and drug delivery	Tiotropium	Lung (guinea pig)	CHCA	Vibrational vaporization	TIC	No	LTQ Orbitrap	[12]
	Theophylline/ propranolol-loaded implants	Lipid implant	DHB	Robotic sprayer	No	No	TOF	[27]
	Liposomal drug carrier with indocyanine	Liver, kidney, brain (mouse)	PhCCAA	Robotic sprayer	Median	No	TOF/TOF	[13]
	Cabotegravir	Muscle and sub-cutaneous abdominal tissue (rat)	DHB	Robotic sprayer; sublimation	No	No	FTICR	[28]
	Lipid-formulated siRNA nanoparticle	Whole-body (mouse)	CHCA	Robotic sprayer	Imatinib as internal standard	No	QqQ	[29]
Drug-induced toxicity	Polymyxin B1, colistin, polymyxin B nanopeptide	Kidney (rat)	DHB	Robotic sprayer	TIC	No	TOF/TOF	[30]
	Dabrafenib	Kidney (rat)	DHB	Robotic sprayer; Sublimation	No	Tissue mimetic model; LC–MS validation	FTICR	[31]
	Janssen R&D compound	Kidney (rabbit)	DHB	Robotic sprayer	No	No	QTOF	[14]
	RO5372709, RO4917523, RO6809959, and RO0728617	Kidney (rat, mouse, monkey)	CHCA	Robotic sprayer	RMS	No	FTICR	[15•]
Drug quantification	Rifampicin	Liver (rabbit)	THAP	TLC sprayer	Stable isotope	Spotting approach; LC–MS/MS validation	LTQ	[32]
	Rifampicin	Liver (rabbit)	THAP	Robotic sprayer	Stable isotope	Spotting approach; LC–MS/MS validation	TOF/TOF	[33]

Figure 1



Schematic MALDI MSI workflow. **(a)** In extension of simple qualitative MSI experiments, qMSI in pharmaceutical R&D requires calibration against a reference standard on the same slide and deposition of an internal (often isotope-labeled) standard on the sample tissue. **(b)** Currently, MSI methods need to compromise between the '4S-criteria for performance' (speed, specificity, spatial resolution and sensitivity). **(c)** High-specificity (mass accuracy and resolving power) FTMS-instruments, currently the best choice in pharmaceutical MSI, are complemented by much faster TOF instruments.

The most commonly used approach to generate the calibration curve is to spot a dilution series of reference standard onto the surface of a control sample [10•,22,25,32,33,36,37]. Drug concentration in dosed tissue is then estimated by comparing the ion intensity with a calibration curve generated from the standards. The approach is relatively quick and straightforward; but in practice applying standards in a uniform manner can be challenging. Mimetic tissue models (MTM) — control tissue homogenates spiked with a range of drug concentrations and frozen together — are an alternative approach [31,38•,39]. MTM cryosections are mounted next to dosed tissue sections, and a calibration curve is generated from average intensities in each region from the MTM. Although this approach mimics analyte extraction and ion suppression effects of dosed tissue more closely, generation of MTMs is laborious and requires relatively large amounts of control tissue. Each of these approaches generate reproducible drug concentrations that correlate well with LC–MS results of adjacent sections, the current gold standard for quantification. These promising results highlight the potential of MALDI MSI for spatial quantification of drugs and metabolites in tissues [10•]; however, this is still a dynamic field with a limited number of examples. Further refinement, validation and multi-site studies of the various sample preparation strategies are required and best practices, standardized approaches, and reporting guidelines are necessary.

Internal standards (IS) have been used in MALDI qMSI experiments in an effort to account for variations in ion suppression and matrix inhomogeneity in different organs [40] and pixel-to-pixel [41–46]. The most common and simple approach is to add a stable-isotope labelled (SIL) version of the analyte (or a close analog) to the matrix solution. Upon matrix application, the SIL is homogenously applied to the tissue along with the matrix. The drug's ion intensity is then normalized to the SIL intensity in each pixel/spectrum to normalize variability. Superior results were reportedly achieved by applying the IS to the tissue surface prior to matrix application [32]. As qMSI methods continue to develop, reporting of average tissue concentration may shift towards true pixel-to-pixel absolute quantification, IS will play a key

role in elucidating the advantages and limitations of different approaches.

Advances in MALDI MSI instrumentation and reagents

MALDI MSI sample preparation remains a major area of interest with the goal of improved sensitivity, specificity and experimental repeatability [47]. In recent years, novel MALDI matrices [48,49] and derivatization agents [50•,51,52] have been proposed for MSI of small molecule drugs and lipid/metabolite biomarkers with improved sensitivity and/or specificity. Most of them require more thorough evaluation to determine their widespread applicability.

In pharmaceutical R&D, reliability and ease of use are key to maximize efficiency, especially in routine studies. Therefore, commercially available solutions are preferred over custom-built systems. Recent advancements in both matrix application devices and MALDI mass spectrometers have expanded the available options for MSI practitioners. Matrix application is most critical during MSI sample preparation. The main challenge is to properly balance sensitivity and lateral resolution. Solvent composition (typically methanol or acetonitrile in water) and mode of application (incl. sprayer device, movement speed, solvent flow rate, distance between sample and target and/or nozzle temperature) influence the extraction of target molecules from tissue. Together with the matrix itself [commonly 2,5-dihydroxybenzoic acid (DHB), 9-amino acridine (9-AA) or α-cyano-4-hydroxycinnamic acid (CHCA)], these parameters govern the size of matrix crystals formed and the type of analyte compounds incorporated. Large volumes of solvent applied quickly allow for efficient extraction and favor sensitivity and improved lateral resolution, but sensitivity may be limited. By contrast, volatile solvents combined with low flow rates typically lead to small matrix crystals, limited sensitivity but good lateral resolution. The large number of key parameters offer many degrees of freedom when developing protocols, but present a challenge for standardization and definition of best practices.

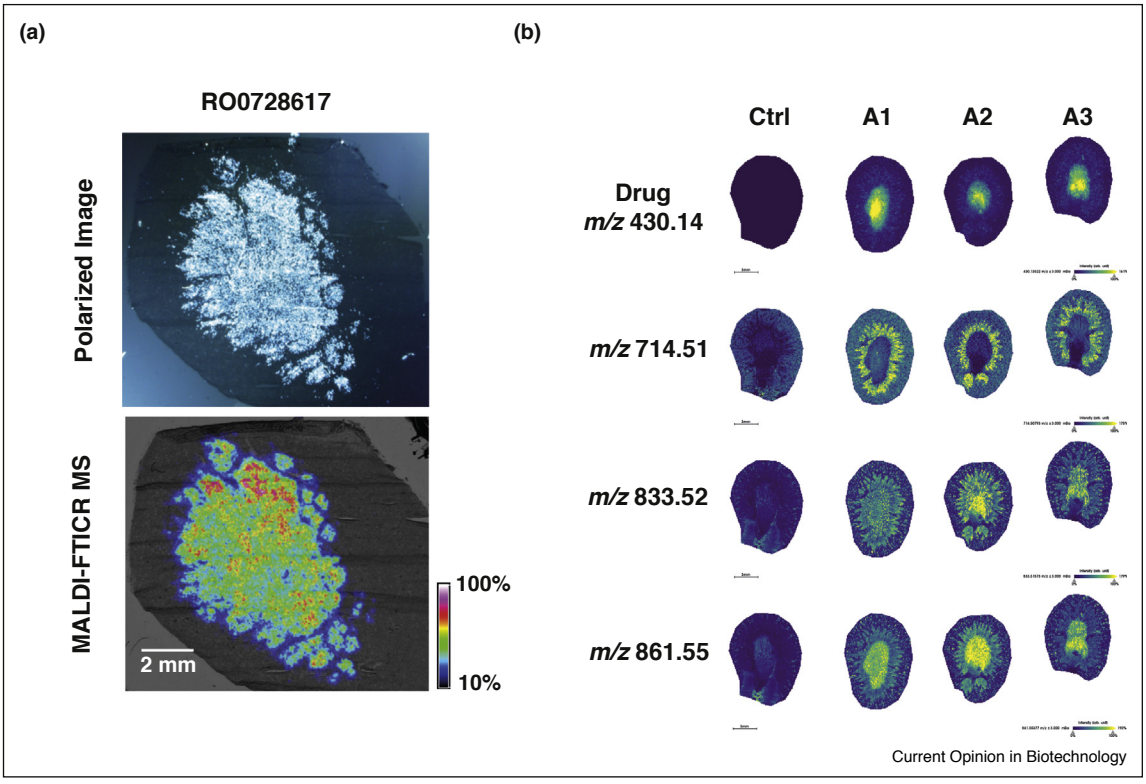
Robotic sprayers with a pneumatic spray nozzle pump-fed with matrix solution are currently the most widespread matrix application devices (Figure 1a). They also support more advanced sample preparation procedures such as on-tissue derivatization, enzymatic digests, application of matrix mixtures and IS, or simply handling of non-standard size samples (e.g. rodent whole body sections). Alternatively, solvent-free matrix deposition by sublimation using a heated vacuum device generates a homogenous layer of very small matrix crystals that enables high lateral resolution, but the absence of solvent limits sensitivity and might induce selectivity for easily diffusible analytes [53,54•]. Sublimation is therefore often followed by rehydration/recrystallization in a humidity chamber to increase sensitivity [55]. Other matrix application devices (e.g. ink jet-like matrix printing or acoustic droplet ejection) exist, but are not commonly used in pharmaceutical R&D.

The ideal mass spectrometer for MALDI MSI would satisfy the '4S-criteria for performance' (**s**peed, **s**pecificity, **s**patial resolution and **s**ensitivity). However, increases in spatial resolution will invariably lead to smaller ablation areas and thus limit sensitivity; and need for speed must be balanced against the other three criteria (Figure 1b). High resolving power and mass accuracy provided by Fourier Transform (FT) ion cyclotron resonance (ICR) or FT-orbitrap mass analyzers are key for small molecule MSI and determinants of specificity, as they enable separation of target molecules from the complex background of tissue constituents and matrix ions (Figure 1c). Currently, FT-ICR MS provides the highest resolving power (>1 000 000 at m/z 200) capable of resolving isotopic fine structure. Combined with their high mass accuracy and versatile MS^2 capabilities FT-ICR MS can provide valuable information for structure elucidation [56]. MALDI-Orbitrap FTMS also provides high resolving power (>140 000) and lateral resolution. Relatively low speed of FTMS data acquisition may be overcome in combined workflows with fast infrared microscopy as guiding modality [57•]. Some MALDI-QTOF instruments with intermediate resolving power combine MSI with ion mobility separation (IMS) as a complementary separation technique that offers the potential to separate isomeric molecules [58]. MALDI-TOF mass spectrometers are arguably the most common instruments used for MSI, but they are less suited for small molecules analysis, since they do not achieve specificity comparable to FTMS platforms. However, modern MALDI-TOF systems provide high spatial resolution (10 μm and better) and fast acquisition speed (e.g. provided by a 10 kHz laser), combined with robustness and relative ease of use (Figure 1c). For instance, an entire mouse brain can be imaged consecutively in both positive and negative ion mode with $50 \times 50\ \mu m^2$ pixels in <1 h [59].

Expanding the scope of MALDI MSI beyond drug disposition studies: target engagement, PK/PD and drug-induced toxicity

Targeted MALDI MSI for evaluation of drugs and their metabolites is a powerful tool supporting preclinical efficacy and toxicity testing. MSI is ideally suited to provide insights into the spatial distribution of a small molecule drug and its metabolites in target tissues [3••,4,35,60]. For example, the typically heterogeneous compound distribution in tumors [18,19] can be matched with tumor biomarkers in oncology drug development [8•]. Perhaps more importantly, MSI of PD biomarkers, for example, protein acetylation [16•] or changes in metabolite ratio [61], can provide spatially resolved evidence for compound action on target.

Figure 2



Drug-induced phospholipidosis and intratubular crystal deposits in kidney as examples of toxicity measured by MALDI FTICR MS. **(a)** Intratubular crystal formation in kidney after treatment of mice with RO0728617. The crystalline structures were visualized using polarized light microscopy and MALDI MSI. **(b)** Administration of 150 mg/kg of a research compound to rats (animal A1–A3) led to a significant increase of marker phospholipids in kidney. Sections were coated with 9-AA and measured in negative ion mode (100 μm raster size). Three lipids (*m/z* 714.5; 833.5; 861.6) are shown exemplarily.

MALDI MSI also provides helpful insights into unwanted drug effects such as off-target activity and toxicology [4,62–65] (Figure 2). For instance, MSI is now widely applied to investigate crystal nephropathy in preclinical studies (Figure 2a), which is caused by precipitation of compounds in kidney tubules leading to intratubular obstruction and acute kidney injury [14,15•,66•]. Importantly, whereas drugs or metabolites can be present in the regions of tubular deposits in the kidney, these deposits may be primarily composed of other molecules, for example, calcium phosphate [31]. Due to high concentrations and limited solubility, drug-related crystals in formalin-fixed paraffin-embedded kidney tissues are also uniquely suitable for MSI analysis [14,67,68], a methodology highly desired by pathologists, but in many cases not applicable. Drug-induced phospholipidosis (DIPL), a secondary lysosomal dysfunction in liver and other organs characterized by pronounced increases in phospholipids, is another example of toxicological evaluation using MSI (Figure 2b). Although no MALDI MSI work on DIPL has been published yet, amiodarone-induced DIPL has recently been studied by correlative nanoSIMS and electron microscopy [69].

Conclusion/future perspective

Advancements in instrumentation and methods have led to increasingly widespread acceptance and utilization of MALDI (q)MSI in pharmaceutical R&D. The number of reported applications where MSI technology can help drive decisions in drug candidate selection is expanding.

Industrial standardization and further improvements in instrumentation based on pure science discoveries

Further method validation and multi-site standardization of sample preparation, data acquisition and data processing strategies are needed to define (industrial) best practices and reporting guidelines. In parallel with the trend to better standardization, some exploratory innovations will likely become commercially available in the future and make their way into MALDI MSI in pharmaceutical R&D: For instance, laser-induced post-ionization may improve sensitivity [70••], combinations with other imaging modalities may eventually offer specificity of FTMS at a speed equivalent to current TOF instruments [57•], combinations of MALDI MSI with TOF-SIMS may provide complementarity for the localization and identification of lipids [71], and use of specialized

focusing objectives may increase spatial resolution close to 1 μm [72].

'Big data' processing

Serial studies featuring large numbers of samples ultimately require high performance computing capabilities. Based on the imzML data standard [73] and on statistically sound quality assurance, for example, utilizing false discovery rate calculations [74••], new machine learning platforms such as METASPACE or pyBASIS [75] will support pharmaceutical R&D and clinical biomarker discovery.

MALDI MSI of large molecule drugs: therapeutic oligonucleotides and proteins

Oligonucleotides and proteins are important classes of therapeutics that draw considerable attention in pharmaceutical research [76,77]. Compared to small molecules, their molecular mass is substantially higher, which makes MALDI MSI more challenging, since the MALDI process yields mainly singly charged ions, the mass range of current detectors is often limited to 20 kDa and the resolving power of instruments decreases with increasing m/z.

Whereas no MALDI MSI study of therapeutic oligonucleotides in tissue has been reported to date, initial studies on tissue distribution of protein drugs and antibody–drug conjugates (ADC) have recently been published [78,79]. MSI offers several advantages compared to immunohistochemistry, as there is no need for detection reagents that might not be available in early discovery. Furthermore, biotransformation products could also be identified, which would be especially relevant for fusion protein formats that are more susceptible to cleavage.

Conflict of interest statement

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