Monitoring therapeutic monoclonal antibodies in brain tumor

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Abbreviations: BBB, blood-brain barrier; CRC, metastatic colorectal cancer; CSF, cerebrospinal fluid; ¹; ⁵DAN, ¹; ⁵-diaminonaphtalene; EMA, European Medicines Agency; FDA, Food and Drug Administration; GBM, glioblastoma multiforme; IMS, imaging mass spectrometry; ISD, in-source decay; ITO, indium tin oxide; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; mAbs, monoclonal antibodies; MALDI, matrix-assisted laser desorption/ionization; NSCLC, non-small cell lung cancer; pE, pyroglutamate; RMS, root mean square; RP-HPLC, reversed phase high-performance liquid chromatography; TOF, time of flight; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VH, variable domain of the heavy chain; VL, variable domain of the light chain; WHO, world health organization

Bevacizumab induces normalization of abnormal blood vessels, making them less leaky. By binding to vascular endothelial growth factor, it indirectly attacks the vascular tumor mass. The optimal delivery of targeted therapies including monoclonal antibodies or anti-angiogenesis drugs to the target tissue highly depends on the blood-brain barrier permeability. It is therefore critical to investigate how drugs effectively reach the tumor. In situ investigation of drug distribution could provide a better understanding of pharmacological agent action and optimize chemotherapies for solid tumors. We developed an imaging method coupled to protein identification using matrix-assisted laser desorption/ionization mass spectrometry. This approach monitored bevacizumab distribution within the brain structures, and especially within the tumor, without any labeling.

Targeted therapies rely on the use of monoclonal antibodies (mAbs) or anti-angiogenesis drugs. These therapies have been designed to overcome some conventional chemotherapy side effects or pitfalls, such as toxicity, resistance, local variations in blood flow or non-effective delivery to the target.¹ Hybridomas were first described in 1975, and more than 15 y later mAbs obtained clinical validation in the mid-1990s.² There are currently more than 50 mAbs and related products approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for therapeutic use.³ In addition, over 30 more are being investigated in late stage clinical trials.⁴ Two main categories of therapeutic antibodies are in development: antibodies targeting cell markers and antibodies targeting cytokines. One of the most targeted cytokine is the vascular endothelial growth factor (VEGF), a critical mediator of angiogenesis in normal and disease states. It can be directly secreted by cancer, endothelial, stromal, or blood cells and can be a component of the extracellular matrix surrounding these cellular components.⁵ VEGF and its receptors, VEGFRs, are the target of many anti-angiogenic therapies that try to prevent angiogenesis and vascular leakage, cell proliferation, survival and migration.⁶

Bevacizumab (Avastin⁷) was the first anti-VEGF therapy approved, in combination with cytotoxic chemotherapy, for the first line treatment of metastatic colorectal cancer (CRC),⁷,⁸ recurrent or metastatic non-small cell lung cancer (NSCLC),⁹ metastatic breast cancer,¹⁰ and renal cancer.¹¹ According to the World Health Organization (WHO) classification 2007, glioblastoma multiforme (GBM) or grade IV astrocytoma is the most invasive type of gliomas associated with high mortality and morbidity. GBM is characterized by pronounced hypercellularity, pleomorphism, numerous mitoses, foci of central necrosis, and excessive vascularization.¹²

It is widely known that tumor blood vessels are leaky, tortuous and dilated, especially in malignancies such as GBM. Assuming that the anti-angiogenic therapy induces normalization of these abnormal blood vessels, making them less leaky,¹³ bevacizumab is used as a single agent for the treatment of recurrent GBM.¹⁴-¹⁷

An increasing knowledge of the pharmacokinetics properties (absorption, distribution, metabolism and elimination parameters) is necessary to estimate how effectively the therapeutic agents reach their targets, and to tailored the next generation of optimized antibodies (OptimAbs).¹⁸ Up to now, in situ concentration measurements have been almost impossible; plasma levels are easier to obtain, offering a proportional relationship with the unbound drug concentration in tissues.¹⁹ In the brain, concentrations in micro dialysates and abscesses are not frequently available for humans,²⁰ so the concentration of therapeutic agents is often

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followed by measuring levels in cerebrospinal fluid (CSF). This approach, however, does not take into account tumor anatomic and metabolic heterogeneity, or other impediments to drugs access.

Principally in GBM, the interpretation of antibody levels is hampered by the difficulty of taking local measurements, which depend on awareness of the blood-brain barrier (BBB). The molecular distribution of large molecules (antibodies) has primarily been investigated using widespread approaches of radio- or fluorescence-labeled probes like electron microscopy, intravital fluorescence videomicroscopy, autoradiography, micellarelectrokinetic capillary and positron emission tomography. Imaging mass spectrometry (IMS) provides complementary features that overcome many labeling imaging pitfalls, such as the unspecific reactions, the change of affinity due to labeled probes and the inability to distinguish between the parent compound and metabolites. IMS detects label-free analytes, measuring the distribution of administered compounds and their metabolites in the same experiment. However, a mass range limitation prevents the detection of large biomolecules, and, therefore, of therapeutic antibodies. To overcome this problem, two solutions can be envisioned. The first one is the usual bottom-up approach, where the proteins are extracted and digested by proteases into peptides and then analyzed by LC-MS/MS. This procedure can, however, induce protein modifications and delocalization. Second one is the top down approach that consists of a direct in situ fragmentation of the protein without digestion. This “top-down” approach has been previously used for extensive characterization of post-translational modifications. In-source decay (ISD) is the top-down approach occurring during the matrix-assisted laser desorption/ionization (MALDI) steps. This pseudo MS/MS technique, MALDI ISD, uses hydrogen radical transfer from the matrix to analyte molecules to provide fragmentation and sequencing of protein N and C termini. Using the appropriate matrix, MALDI ISD can be associated with MALDI IMS, and therefore provides identification and location of exogenous or endogenous molecules at the same time. In this work, we developed a novel method coupling top-down in source decay fragmentation with matrix assisted laser desorption/ionization imaging mass spectrometry (MALDI ISD IMS). This new strategy was performed to obtain in situ distribution of bevacizumab on treated glioblastoma-bearing mouse brain tissue sections.

The main result expected of bevacizumab treatment is to block the angiogenesis. As noted below, angiogenesis and its major regulator, VEGF, represent one of the most important therapeutic targets in GBM treatment. VEGF receptors (VEGFR1/2) are expressed on endothelial, but few other, cells wherein it stimulates several cell responses, including proliferation, migration, survival, and secretion of matrix-degrading enzymes. Through the VEGFR2 and having the stronger kinase activity, it represents the predominant mediator of VEGF-induced angiogenic signaling. This signaling pathway can be inhibited by bevacizumab, which binds to and neutralizes all human VEGF-A isoforms (Fig. 1A). Top-down in source decay, using 1, 5-diaminonaphtalene (DAN) as a matrix, is a radical pathway allowing the identification of the immunoglobulin amine and carboxyl terminal parts. ISD generally leads to generation of a long series of c- and z- ions after cleavage of N-Cα bonds on the peptide backbone (Fig. 1B).

In order to verify the correct NH2- and COOH terminal portions and to identify the set of virtual precursors that are specific to each mAb, we assessed the fragmentation of bevacizumab and palivizumab, two humanized IgG1 therapeutic mAbs. Palivizumab has a totally different epitope and mechanism of action than bevacizumab, and it was therefore chosen as a negative control. It blocks both cell to cell and virus to cell fusion, and targets viral proteins, not angiogenesis cytokines.

ISD mass spectra were obtained from a mixture of 1, 5 DAN matrix with one of the two purified antibodies (bevacizumab or palivizumab) directly spotted on MALDI target. The best fragmentation range was obtained between m/z 1000–8000 for palivizumab and m/z 2000–8000 for bevacizumab. A sequence of 30 residues corresponding to the variable domain of the heavy (VH) and light (VL) chains of bevacizumab (Fig. 2A) and palivizumab (Fig. 2B) was obtained. Noteworthy, the VH domain of palivizumab carried a pyroglutamate (pE) modification occurring through the rearrangement of the originally synthesized glutamine residue. It is known that both glutamine and glutamate at the N termini of recombinant mAbs can cyclize spontaneously to pyroglutamate (pE) in vitro, making the antibodies more acidic. Also, in vivo cyclization can occur as a stabilization mechanism for proteins not impacting their turnover. For therapeutic mAbs, like palivizumab, pE can be one of many post-translational modifications observed during production and storage. Apparently, this cyclized residue is resistant to amino peptidases. RP-HPLC is a chromatographic method that was able to show and to quantify post-translational modifications which were also identified by peptide mapping, mass spectrometry and microsequencing. The same in source decay fragmentation studies were performed on healthy brain tissue section to obtain the most representative fragments of the studied mAbs in these particular conditions. Wherever the antibody was spotted on the tissue slice, fragmentation occurred and fragment ions were detected showing that ion suppression effect has no impact on our study. Ion suppression usually occurs when an ion suppresses the signal of another species in the sample.

Antibody fragmentation was measured on tissue using a lowest quantity of 41.75 pmol. In this case, three ions (c20, c21 and c22) corresponding to N termini fragments of bevacizumab were measured (Fig. 2C). To confirm the identity of these ions, a second sequencing step called T3-sequencing was performed directly on tissue sections (Fig. 2D). The generated precursor ions, including the N- or C-terminal sequence generated by ISD fragmentation, were selected in the timed ion gate of a MALDI TOF/TOF mass spectrometer for MS/MS analysis. This new fragmentation (T3-sequencing) generates principally b- and y-ions, allowing the proper sequencing of both N- and -C termini, respectively, and confirmation of suspected terminal modifications.

We then achieved analysis on mouse xenografts brains after stereotactic cortical tumor U87 cells injection, which was
performed as previously described.\textsuperscript{41} To provide protein identification in addition to their localization directly on brain tissue sections, we performed ISD of the entire tissue slice at 80 μm spatial resolution. Most of the obtained signals were protein fragments. Some of them bear the exact mass as bevacizumab c-ions previously measured using the purified antibody (Fig. 3). T3-sequencing confirmed that they were (c\textsubscript{20}, c\textsubscript{21} and c\textsubscript{22}) ions (results not shown).

The intensities of these three ions were summed to provide the image of antibodies distribution within the brain slices. Bevacizumab is mainly detected on the tumor area, whereas palivizumab is distributed in all the tissue (Fig. 4). It is very difficult to compare palivizumab and bevacizumab quantities as we are not sure that they fragment exactly with the same efficiency. We will have to develop absolute quantitation methods using in source decay.

Notably, the image obtained with three ion is quite similar to the one gathered with the most abundant fragment only (c\textsubscript{21}bevacizumab = m/z 2081), but it is more relevant on a statistical basis. It could be interesting to use both c- and z- ions from protein termini for the identification and imaging, however DAN fragmentation, via the radical pathway, rather produce c- fragments from the N- terminus of big proteins than z- fragments from the C- terminus.\textsuperscript{50}

The pharmacokinetic properties of bevacizumab are currently measured in plasma. This drug is associated with a low clearance (CL), a limited volume of distribution (Vc), and a long elimination half-life (21 d) that maintains target therapeutic bevacizumab plasma levels with a range of administration schedules (such as 5–10 mg/kg once every 2 or 3 wk). It is clearly important to obtain direct in situ measurement for a direct pharmacokinetic view closer to the site of action. Such a measurement within the tumor will account for blood flow through the tumor, extravasation, convection and diffusion, binding, and internalization. Qualitative and quantitative mathematical models were constructed previously to predict the antibody diffusion through tumors taking into consideration some of these parameters, such as the effect of antibody internalization.\textsuperscript{51-54} Macro distribution models predict the role of elevated interstitial fluid pressure, tumor convection, spatial variation in extravasation, antibody binding and demonstrated a detrimental effect of antibody internalization.\textsuperscript{51} Micro distribution studies described the phenomena where antibodies are immobilized close to their site of entry and the factors that influence the location of the moving front, such as diffusivity, antigen density, permeability, and dose.\textsuperscript{52} Micro distribution also analyzed the influence of cellular pharmacology, like binding, internalization, and recycling on the distribution around blood vessels.\textsuperscript{54}

Glioblastoma is one of the most vascularized human tumors.\textsuperscript{5} This abnormal tumor vasculature is upregulated by VEGF, which serves as a major angiogenic factor in normal vascular development.\textsuperscript{5,55} Angiogenesis is a critical step during glioblastoma development and growth in which tumor requires more oxygen and nutrients levels for its survival and proliferation. Anti-VEGF treatment can reduce vascular permeability, but this may not necessarily reflect tumor cell death. Thus, the macro distribution and penetration of the bevacizumab into the tumor mass is in agreement with the tumor characteristics and treatment knowledge.

The purpose of coupling MALDI IMS and top-down in source decay analysis is to get localization in addition to the protein identification, at the same time and directly on the same tissue section. In situ molecular identification was performed by MALDI ISD, a top-down approach that allows identification of the proteins by their N- and C-termini\textsuperscript{39,41} fragmentation. Although limited to the most abundant species, MALDI ISD IMS was previously shown to be useful for glioma marker characterization\textsuperscript{51} and post-translational modifications identification.\textsuperscript{56} We now strengthen its impact with the close investigation of therapies in situ.
Further improvement of antibody quantitation directly on tissue is a possibility under development, in combination with quantitation with triple quadrupole mass spectrometry.

New coming sprayer systems will improve spatial resolution of the MALDI imaging combined with in source decay analysis. Going down to 10 μm spatial resolution or less could be important to distinguish antibody location within the tumor cells and their microenvironment.

MALDI imaging mass spectrometry could therefore provide both the macro and the micro distribution view described by the mathematical models, as described above.

MALDI imaging mass spectrometry may in this way compensate for many conventional imaging modalities limitations by combining a number of real advantages: sensitivity, label free, speed, and reproducibility. Thus, it could play an important role for cancer imaging combined with other modalities in preclinical and clinical practice.

Materials and Methods

Reagents

1. 5-diaminonaphthalene (DAN) was purchased from Sigma-Aldrich, Saint-Quentin Fallavier, France. Bevacizumab (Avastin®, Roche, Grenzach-Wyhlen, Germany) and palivizumab (Synagis®, Abbott, Berkshire, UK) were provided by the Centre d’Immunologie Pierre Fabre.

Tissues

Harvesting of tissues

The U87 cells were obtained from a malignant glioma of a female patient by explant technique. Five-weeks-old nude FOXN1 male mice (25–30 g athymic mice) were purchased from Harlan Laboratories, Gannat, France. Glioblastoma cells were injected stereotaxically using: Leica MS5-MZ6 stereomicroscopic device (Leica Microsystems SAS, France), WPI Stereotaxic...
Frame 18 deg with Ear Bars (World Precision Instruments, Germany) and Hamilton Syringe 10 μl, Model 701 SN On-Column Injection SYR, Cemented NDL, 32 ga, 3.35 in, point style 45° mounted on microinjection unit (Hamilton Bonaduz AG, GR, Switzerland). This system enabled inoculation of 10^5 glioblastoma cells suspended in 2 μl solution using the manual control of the injection system. The striatum on the right frontal lobe at interaural, lateral and depth coordinates of 0.0, 3.0, and 3.0 mm to the bregma was targeted. Eight days after cells injection, the mice were separated to 4 groups: control mice, bevacizumab treated mice, palivizumab treated mice and bevacizumab added to palivizumab treated mice. The intraperitoneal drug injection was done at 200 mg/mouse three times per week during 1 (for bevacizumab and palivizumab) or 3 wk (only for bevacizumab). Mice were anesthetized by the intraperitoneal injection of 6.5 μl/g of anesthetic solution, composed of 50 μl of 2% Rompun with 200 μl of Imalgene 1000 in a final volume of 1000 μl of 0.9% NaCl. Mice were then dissected and the total brain blocks extracted and snap-frozen in liquid nitrogen for 15 s. The brains were kept at −80°C until use. The drop weight of mice was monitored and the sacrifice was done one week after the latest treatment. Three mice per group were used for MALDI imaging experiment. The animal manipulations have been performed in accordance with international guidelines. Experimental protocols were reviewed and approved by the Institutional Animal Care Committee of the School of Medicine and performed in accordance with INSERM and Aix Marseille Université School of Medicine polices regarding the use of laboratory animals.

**Tissues preparation and staining**

Mouse brain was placed at −18°C for 15 min before use. Tissue sections were cut using a Leica CM 1900 UV Microsystems cryostat (Leica Microsystems SAS, France) with a microtome chamber and a specimen holder chilled at −18°C. The 12 μm
thick coronal and horizontal sections were thaw mounted onto Indium Tin Oxide ITO-coated microscopic slides (Bruker Daltonics, Wissembourg, France) adapted for MALDI imaging mass spectrometry. These MALDI target slides were placed in a desiccator for 1 h. ITO slides were washed with isopropanol (twice at 70% for 2 min and once at 96% for 2 min) for MALDI in source decay imaging MS analyses. Following washing, ITO slides were dried in a desiccator for 30 min before matrix deposition.

After MALDI imaging analysis, the used slides were washed with pure ethanol and acetone to remove the matrix before histological staining. For Hematoxylin and Eosin staining, the tissue sections were immersed in a filtered Harris Hematoxylin solution for 1 min and rinsed with tap water. Then, the sections were immersed in Eosin solution for 30 s and rinsed with tap water. The slices were dehydrated in various alcohol solutions (50%, 70%, 80%, 95%, 100%) and cleared with xylene (2x). They were mounted onto a labeled glass slide with Permount. The pictures of H&E stained histological sections were obtained at specific regions with Olympus BH-2 microscope at magnification, ×40 objective. The nuclei and other basophilic structures were colored in blue while cytoplasm and acidophilic structures were colored light to dark red.

Matrix deposition for imaging mass spectrometry analyses

Brain tissue sections were always scanned before matrix deposition with a histology slide scanner (Opticlab H850 scanner, Plustek). The scanned histological image was used for the teaching in order to obtain a perfect superposition between the histological and the ion images. For MALDI in source decay imaging
1. 5-diaminonaphthalene (DAN), 10 mg/ml in 50% Acetonitrile 0.2% TFA, matrix deposition was performed with the automatic matrix sprayer device (ImagePrep, BrukerDaltonics) equipped with the new spray head. The spray method was optimized adding more nebulization and drying cycles to obtain a thick matrix deposit compatible with in source decay analysis.

Data Acquisition

**MALDI in source decay MS**

MALDI mass spectra were obtained on an Ultraflextreme TOF/TOF mass spectrometer controlled by the FlexControl 3.3 software (BrukerDaltonics). ISD analyses were acquired in a reflectron positive mode with an accelerating potential of 20 kV; laser power was increased up to 20% above the ionization threshold to increase fragmentation without losing window (laser power at 50%). The spectra were internally calibrated by using parent and c10-serie ion fragment of bovine serum albumin (BSA) masses.

**MALDI in source decay imaging MS**

MALDI in source decay imaging was performed using an Ultraflextreme TOF/TOF mass spectrometer controlled by the FlexControl 3.3 software (BrukerDaltonics). The MALDI ISD IMS experiments were performed at a spatial resolution of 80 μm. Spectra were acquired in positive reflectron ion mode with 1500 laser shots accumulated at each spot and the laser power was optimized at the start of each run and then fixed for the overall MALDI ISD IMS experiment with a frequency of 500 Hz. The mass spectrometer parameters were set according manufacturer’s settings for optimal acquisition performance. Laser spot size was medium (corresponding approximately to 30–50 μm laser spot diameters). Sequences were created on FlexImaging software, version 3.0 (BrukerDaltonics) after the downloading of scanned images. Polygon measurement regions were manually defined using these histological images. A minimum of two images was acquired to ensure reproducibility.

**Data Analysis**

**MALDI in source decay imaging for monoclonal antibodies distribution**

IMS data were acquired and normalized with root mean square (RMS) (vector norm) algorithm that provides a very uniform distribution of intense signals. Raw spectra were analyzed with FlexImaging software version 3.0 after baseline subtraction. Masses were selected with a mass precision of ± 0.1 Da on the overall average spectrum. Ion density map was created for each signal present on the whole sample average mass spectrum. For signals corresponding to c-ions fragments of mAbs, the spectra lists of interest were exported in .xml format and then, the intensities of all the fragment ions were summed. Finally, we reimported the new spectra list data in FlexImaging software to obtain the image corresponding to the ion sum.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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