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Cationic drug analysis using matrix-assisted laser desorption/ionization mass spectrometry: Application to influx kinetics, multidrug resistance, and intracellular chemical change

DARRYL RIDEOUT†, ANDRÉS BUSTAMANTE†, AND GARY SIUZDAK*‡

Departments of *Chemistry and †Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

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ABSTRACT **Highly sensitive and convenient analysis of intracellular cationic drugs has been achieved by applying matrix-assisted laser desorption/ionization mass spectrometry (MALD-MS). Tetraphenylphosphonium cation was readily identified and quantified (using methyltriphenylphosphonium cation as an internal standard) at subpicomole levels in crude lysate from $< 4 \times 10^3$ FaDu human hypopharyngeal carcinoma cells. A quantitative MALD-MS time course for tetraphenylphosphonium cation accumulation into FaDu cells was comparable to a time course using scintillation counting with tritiated tetraphenylphosphonium. MALD-MS was also capable of demonstrating the reduced accumulation of the cationic drug rhodamine-123 by Dox^R MCF7, a multiply drug-resistant human breast adenocarcinoma cell line, relative to the nonresistant parent line MCF7. In addition, MALD-MS was used to follow a chemical reaction inside intact FaDu cells: the formation of a hydrazone (II-51) from benzaldehyde and an acylhydrazide, 5-[tris(4-dimethylaminophenyl)phosphonio]pentanoyl hydrazide (II-25). These results suggest that MALD-MS may provide a rapid and practical alternative to existing methods for the analysis of cationic drugs, toxins, and their metabolites in cells and tissues.**

The ability to detect and quantify exogenous drug and toxin molecules introduced into cells and tissues is crucial for drug design, preclinical and clinical pharmacology, and toxicology. Detection and quantification are inherently difficult because the wide variety of naturally occurring molecules in cells and tissues can confuse the analysis of exogenous molecules (1). Current methods for identification and quantitative analysis of exogenous molecules in cells and tissues include scintillation counting of radiolabeled molecules, fluorimetry, HPLC, and secondary-ion mass spectrometry (1, 2). However, these methods have considerable limitations, including extensive preparation or optimization time (radiolabeling, HPLC), high material cost (radiolabeling), and limited applicability (fluorimetry). The hazards and waste-disposal problems associated with radiolabeling and the requirement for secondary ion mass spectrometry that a drug contain an element not present at competing concentrations in the biological matrix further limit the utility of these methods. Consequently, the application of matrix-assisted laser desorption/ionization mass spectrometry (MALD-MS) to the analysis of intracellular drugs and other exogenous molecules may be a viable alternative to existing analytical techniques.

MALD-MS analysis is carried out by suspending or dissolving a sample in a solid or liquid matrix that allows for efficient and directed energy transfer during a laser-induced desorption process (3, 4). Once the molecules in the sample are vaporized and (for neutral molecules) ionized, time-of-flight mass analysis is typically used to separate the ions according to their mass-to-charge ratio (m/z). MALD-MS has proven useful for both qualitative (5–10) and quantitative (5, 21) analysis of biomolecules and for the analysis of heterogeneous biological samples (3). This method has proven to be a versatile analytical tool, given its exceptional range and sensitivity; however, only limited work has been done to take advantage of its capacity to screen complex biological mixtures, and MALD-MS has not been previously used to directly analyze and quantify drugs or toxins in such samples. We now report results demonstrating that MALD-MS is superior to existing techniques in terms of reproducibility and ease of application for the analysis of intracellular cationic drugs.

MATERIALS AND METHODS

Compounds. Tetraphenylphosphonium (TPP) chloride, methyltriphenylphosphonium (MTPP) chloride, rhodamine-123 (Rh123) chloride, and benzaldehyde were purchased from Aldrich. Compounds II-25 and II-51 were synthesized as described in ref. 11. Structures are depicted in Fig. 1.

Cell Preparation. The wild-type cell line MCF7 (ATCC HTB22), derived from a human breast adenocarcinoma, and FaDu human hypopharyngeal squamous carcinoma cells (ATCC HTB43) were purchased from the American Type Culture Collection. Doxorubicin-resistant Dox^R MCF7 multiply drug-resistant human breast adenocarcinoma cells were purchased from the Division of Cancer Treatment Tumor Repository (National Cancer Institute, Frederick, MD). All three lines were cultured in RPMI 1640 medium (BioWhittaker)/10% (vol/vol) fetal calf serum (BioWhittaker)/2 mM L-glutamine without antibiotics at 37°C in 7% CO_2/93% air. After trypsinization and before each experiment, cells were washed and suspended for 1 hr at 37°C in Hepes buffer {135 mM NaCl/5 mM KCl/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/50 mM Hepes [4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid]/5.5 mM dextrose, pH 7.4}. Cell lines were tested every 2 months and shown to be free of mycoplasmas by using a service of the American Type Culture Collection.

MALD-MS. For the experiments described here, we used 2,5-dihydroxybenzoic acid as the solid matrix and a model VT2000 laser desorption mass spectrometer (Vestec, Houston) with the third harmonic of a Nd:yttrium/aluminum-garnet laser, $\lambda = 355$ nm. Once the ions were generated, they were accelerated with a potential of 30 kV in the ion source. A model 9400 transient recorder (LeCroy, Chestnut Ridge, NY) was used to process the analog signals. The resolution (full-width, half-maximum) was typically 150. Spectra shown in this report are the average of 50 acquisitions, whereas each

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Abbreviations: MALD-MS, matrix-assisted laser desorption/ionization mass spectrometry; TPP, tetraphenylphosphonium; MTPP, methyltriphenylphosphonium; Rh123, rhodamine-123; Dox^R, doxorubicin resistant.

‡To whom reprint requests should be addressed.

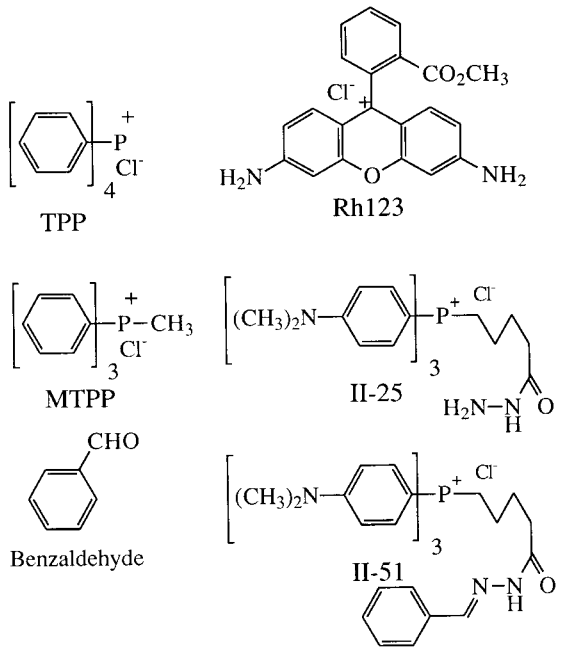

FIG. 1. Structural formulae of cations and benzaldehyde.

data point for quantitative analysis was based on three independent sets of 50 acquisitions taken with different samples. Background ions from the matrix (below $m/z = 200$) and cell lysate (m/z = 650–900) posed no problems in this study: lysate background from a variety of cell lines has been reported in this mass range (6). Sample preparation, data acquisition, and data analysis typically took 30–40 min. Neither smoothing nor background subtraction has been used for this MALD-MS data.

Cell lysate was prepared from FaDu cells (5×10^6 cells per ml) to determine its effects on the quantitation of TPP (Fig. 2). Samples of cell lysate were compared to solutions without cell lysate. MTPP was used as an internal standard at 10 μM,

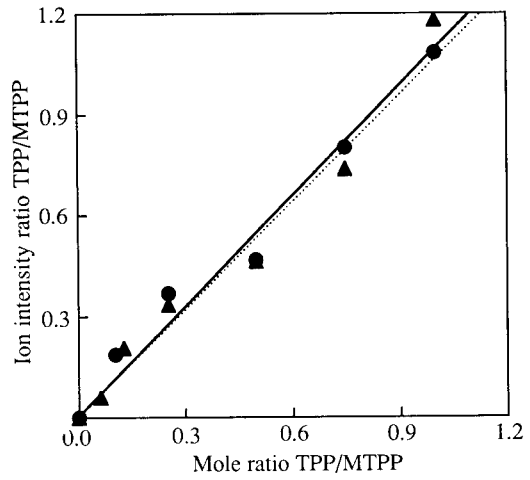

FIG. 2. Effect of material from hypotonic lysis of FaDu human hypopharyngeal carcinoma cells on linear correlation of the MALD-MS ion-intensity ratios (TPP/MTPP) versus the mole ratios (TPP/MTPP) with 10.0 μM MTPP used as internal standard. Approximately 1 μl of solution or suspension containing 0.5–10.0 pmol of TPP was used for each analysis. Averages of results from triplicate analyses with (●) and without (▲) cell lysate are shown (5×10^6 cells per ml). The lines are based on linear least-squares fitting of each of these sets of data points: ···, with lysate ($r^2 = 0.99$); ——, without lysate ($r^2 = 0.98$).

and TPP concentrations ranged from 0.5 to 10.0 μM (≈0.5–10 pmol per sample tested by MALD-MS).

When quantities >10.0 pmol of TPP were tested by MALD-MS against the internal standard, 10.0 pmol of MTPP, a saturation effect occurred, requiring that the samples be diluted in spectral-grade water to obtain a concentration <10.0 pmol/μl of TPP and reach the linear range of quantification. Thus, an intensity ratio (peak-height measurement) of <1.0 ensured that the concentration range was within the linear response; an intensity ratio >1.0 required that the sample be diluted.

A micropipette was used to remove excess buffer coating the cell pellet after each wash step for both the MALD and scintillation counting studies. This technique helped to increase MALD-MS sensitivity by minimizing background levels of sodium and potassium ions. It also ensured elimination of extracellular tritiated TPP from samples subject to scintillation counting, thus minimizing background β count levels.

For the TPP-influx kinetic studies (Fig. 3), 5×10^5 cells were exposed to 5.0 μM TPP in 10 ml of Hepes buffer, washed twice with phosphate-buffered saline (pH 7.4, 4°C), and centrifuged ($250 \times g$, 3 min). The pellet was then suspended in 150 μl of spectral-grade water and frozen to lyse the cells. After the pellet was thawed, MTPP was added to the cell lysate to yield 10.0 μM MTPP solution. The resultant ion-intensity ratio (TPP/MTPP, measured from the peak height) allowed for quantitation. Each MALD-MS analysis was done with 1 μl of the final cell lysate—i.e., $\approx 3.3 \times 10^3$ cells.

MALD-MS was also used to study the effect of multidrug resistance on Rh123 uptake (Fig. 4). Semi-confluent MCF7 and Dox^R MCF7 cells were harvested (trypsin/EDTA), centrifuged ($250 \times g$, 3 min), and suspended in 10 ml of Hepes buffer (10^5 cells per ml). Rh123 (1 mM in spectral-grade water) was then added to obtain a concentration of 10 μM Rh123 in the cell stocks. After a 1-hr incubation, cells were washed twice with cold phosphate-buffered saline, centrifuged, and subjected to hypotonic lysis by suspending them in 200 μl of spectral-grade water. The lysate was kept frozen and in the dark until analyzed by MALD-MS. TPP (500 fmol) was added before analysis as an internal standard.

To study the intracellular reaction between the acylhydrazide II-25 [tris(4-dimethylaminophenyl)phosphonio]pen-

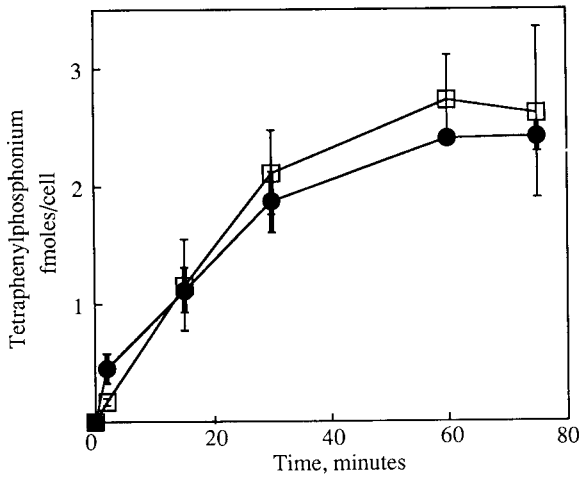

FIG. 3. Uptake of TPP by FaDu cells as a function of exposure time, as observed for unlabeled TPP with MALD-MS (●) and for tritiated TPP with scintillation counting (□). Each MALD-MS data point is an average of three independent determinations, and each scintillation data point is an average of four or five independent determinations. Error bars (thick lines, MALD-MS data; thin lines, scintillation data) represent 95% confidence limits.

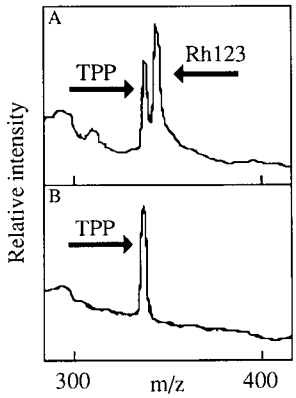

FIG. 4. MALD-MS spectra of lysates from MCF7 cells (*A*) and multiply drug-resistant Dox^R MCF7 cells (*B*) (12) after exposure to 10.0 μM Rh123 for 1 hr. The Rh123 cation peak is at m/z = 345; TPP (m/z = 339, 500 fmol) was added as an internal standard just before analysis.

tanoyl hydrazide and benzaldehyde (Fig. 5), FaDu cells were harvested and incubated in Hepes buffer for 1 hr. After incubation, the cells were suspended at 5×10^4 cells per ml and divided into four equal samples of 10 ml. To each sample was added a 10× aqueous drug stock to achieve the following final concentrations: 10 μM II-25, 100 μM benzaldehyde, 10 μM II-51, and 10 μM II-25 followed by 100 μM benzaldehyde. Samples exposed to (10 μM) II-25 or II-51 alone were incubated for 1 hr with the drugs (37°C, 7% CO_2), washed three times with 5 ml of cold (4°C) phosphate-buffered saline, and lysed with cold spectral-grade water. The samples exposed to 100 μM benzaldehyde alone were initially incubated for 1 hr free of benzaldehyde, washed three times, incubated with benzaldehyde for 1 hr, washed, and lysed. Samples exposed to a combination of II-25 and benzaldehyde were incubated for 1 hr with 10 μM II-25, washed three times with phosphate-buffered saline, incubated in 100 μM benzaldehyde for 1 hr, washed, and lysed. Lysates were frozen until analyzed by MALD-MS. No internal standard was used for these experiments.

Scintillation Counting. [^{3}H]TPP (≈30 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham and diluted with unlabeled TPP solution to a specific activity of ≈0.4 Ci/mmol and a total TPP concentration of 5.0 μM.

For the [^{3}H]TPP-uptake studies (Fig. 3), 1×10^6 cells in 5 ml of Hepes buffer were exposed to 5.0 μM [^{3}H]TPP (0.4 Ci/mmol) and placed in a 37°C water bath. At specified times, 1-ml aliquots (2×10^5 cells) were transferred into Eppendorf tubes (2-ml capacity) and centrifuged ($600 \times g$). Cells were

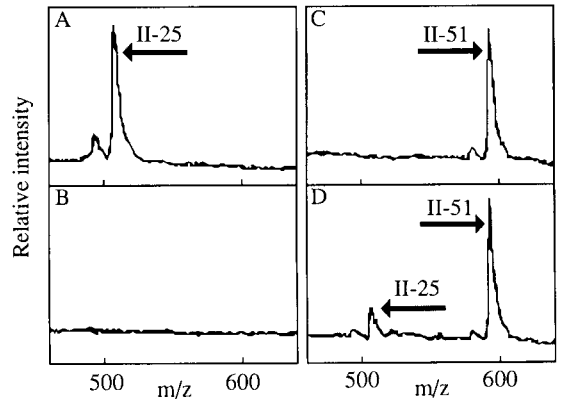

FIG. 5. FaDu cells exposed to 10 μM II-25 for 1 hr (*A*), 100 μM benzaldehyde for 1 hr (*B*), 10 μM II-51 for 1 hr (*C*), and 10 μM II-25 for 1 hr followed by 100 μM benzaldehyde for 1 hr (*D*).

washed twice with cold (4°C) Hepes buffer. Cell pellets were lysed with a 1-ml vol of cold deionized water and combined with high-flash scintillation mixture (Fisher Scintiverse BD, 15 ml) in borosilicate scintillation vials; radioactivity was then counted by using a Beckman model LS3801 instrument.

Fluorimetry. For fluorimetric quantification of Rh123, treated (10.0 μM Rh123, 1 hr, two washes) samples containing ≈ 3×10^6 cells were lysed in 1.5 ml of H_2O containing Triton X-100 at 0.5 mg per ml. Rh123 fluorescence was measured with a Shimadzu model RF5000U spectrofluorimeter (excitation 510 nm, emission 528 nm) and compared with a standard curve generated in aqueous Triton X-100.

RESULTS AND DISCUSSION

We initially tested the effect of crude mammalian cell lysate on the ability of MALD-MS to determine the aqueous concentration of TPP, a molecule that preferentially inhibits the proliferation of carcinoma cells relative to certain untransformed epithelial cells (11, 13). These analyses measured levels of TPP, with and without lysate from FaDu human hypopharyngeal carcinoma cells, using MTPP as an internal standard (chemical structures are depicted in Fig. 1). The results clearly illustrate the linear correlation (from 0.5 to 10.0 pmol of TPP) between the relative ion intensity and the mole ratio of TPP to MTPP and further demonstrate that this relationship is not affected by cell lysate (Fig. 2).

In a second experiment, live, intact FaDu carcinoma cells were exposed to TPP to follow drug-influx kinetics with MALD-MS. The influx kinetics followed by MALD-MS were compared with influx kinetics followed by scintillation counting of tritium-labeled TPP. In the MALD-MS experiments, MTPP (10.0 μM) was introduced to the lysate as an internal standard after TPP exposure, washing, and hypotonic cell lysis. MALD-MS analysis of untreated and drug-treated cell lysates indicated that TPP could be readily identified in the drug-treated cells to the subpicomole level. The time course of TPP influx, as determined with MALD-MS, compares well with the time course determined with scintillation counting and [^{3}H]TPP: the maximum discrepancy between the two methods is 0.33 fmol per cell (Fig. 3). In addition, the MALD-MS data (95% confidence limits ±0.0 to ±29% of the mean) were more reproducible than the scintillation count data (±17 to ±34% of the mean).

Further applicability of MALD-MS to intracellular drug analysis was demonstrated by examining the effect of the multiple-drug-resistance phenotype on intracellular drug accumulation. The degree of uptake of the experimental antineoplastic agent Rh123 (12, 14) was determined by MALD-MS for MCF7, a cell line derived from a human breast adenocarcinoma, and for Dox^R MCF7, a multidrug-resistant variant of MCF7 that exhibits diminished intracellular accumulation of doxorubicin and other lipophilic drugs (15). Uptake of Rh123, which is known to be excluded by multiply drug-resistant cells (16), was observed readily using MALD-MS for MCF7 but was not observed for Dox^R MCF7 cells (Fig. 4). To ensure that Rh123 is excluded by Dox^R MCF7, we also carried out independent experiments using fluorescence spectroscopy which verified that the intracellular concentration of Rh123 was dramatically lower in Dox^R MCF7 drug-resistant cells (4.5 ± 2 amol per cell; SD, n = 3) than in MCF7 wild-type cells (270 ± 60 amol per cell) after a 1-hr exposure to 10.0 μM Rh123. These results indicate that MALD-MS can be used to distinguish drug-sensitive and drug-resistant cells when the mechanism of resistance leads to diminished intracellular drug concentrations.

We explored the feasibility of using MALD-MS to monitor intracellular chemical changes in drug molecules without the need for fluorescence, HPLC, or radiolabeling. Previous studies have described the chemical formation of hydrazones

occurring from aldehydes and hydrazine derivatives inside cells, with indications that this assembly could result in the synergistic inhibition of cell growth (17, 18). However, direct evidence of those reactions required that the hydrazone be fluorescent (19). We carried out MALD-MS studies to determine whether the antineoplastic acylhydrazide II-25 (20) and benzaldehyde could combine inside intact FaDu cells to form the hydrazone II-51, as they do in aqueous solution (11). Cells were exposed to II-25 at 10.0 μM for 1 hr followed by three washings with cold phosphate-buffered saline at pH 7.4 to remove extracellular II-25. The intact cells were then exposed to 10.0 μM of benzaldehyde for 1 hr and washed three times with cold phosphate-buffered saline. The peak corresponding to II-51 (m/z = 594) was readily observed upon mass analysis of the cell lysate from cells treated with II-25 followed by benzaldehyde (Fig. 5*D*). In control experiments, cells treated with II-25 alone or benzaldehyde alone produced no evidence of the II-51 ion (Fig. 5 *A* and *B*). These results suggest that MALD-MS will be useful for the study of other chemical reactions, including the metabolism of cationic drugs and toxins in cells and tissues.

Although many cells (typically 5×10^5) were used in these MALD-MS experiments, only a small fraction ($\approx$1%) of the cell lysate was used for each spectrum. It is likely that drugs could be quantified from $<10^4$ cells when a smaller volume of water is used to make the lysates. This technique may be useful for determining drug concentrations in very small pieces of tissues or in cells difficult to culture in large numbers.

In conclusion, our results demonstrate that MALD-MS is a practical approach for quantifying cationic drug uptake by intact cells, characterizing biodistribution-based drug resistance, and following intracellular chemical reactions of cationic drugs. These studies show the potential applications of MALD-MS for the study of cationic drugs in cultured cells and suggest its prospect for measurement of drugs, toxins, and their metabolites in plasma, tissue biopsies, and other biological matter. MALD-MS may be superior to other methods in terms of analytical specificity and feasibility because the sample preparation is simple and the technique provides information on the molecular weight of the molecules analyzed. Recent quantitative results with heterogeneous mixtures of carbohydrates (21) and the ability to observe picomole quantities of anionic molecules using negative-ion MALD-MS (8, 9) suggest that detection of uncharged and anionic drugs and toxins in cells may also be possible with MALD/MS. Because sensitivity at the single-femtomole level has been seen (4) and the relevant conditions used for our studies have yet to be fully optimized, it is likely that considerable improvements can be made in the sensitivity of MALD-MS for quantification of intracellular cationic drugs.

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