

β -Lactam Antibiotics Form Distinct Haptenic Structures on Albumin and Activate Drug-Specific T-Lymphocyte Responses in Multiallergic Patients with Cystic Fibrosis

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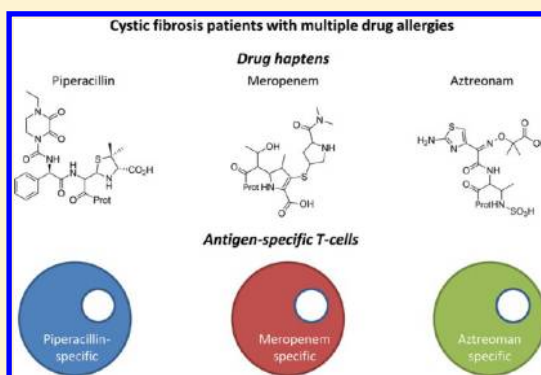
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S Supporting Information

ABSTRACT: β -Lactam antibiotics provide the cornerstone of treatment for respiratory exacerbations in patients with cystic fibrosis. Unfortunately, approximately 20% of patients develop multiple nonimmediate allergic reactions that restrict therapeutic options. The purpose of this study was to explore the chemical and immunological basis of multiple β -lactam allergy through the analysis of human serum albumin (HSA) covalent binding profiles and T-cell responses against 3 commonly prescribed drugs; piperacillin, meropenem, and aztreonam. The chemical structures of the drug haptens were defined by mass spectrometry. Peripheral blood mononuclear cells (PBMC) were isolated from 4 patients with multiple allergic reactions and cultured with piperacillin, meropenem, and aztreonam. PBMC responses were characterized using the lymphocyte transformation test and IFN- γ /IL-13 ELISpot. T-cell clones were generated from drug-stimulated T-cell lines and characterized in terms of phenotype, function, and cross-reactivity. Piperacillin, meropenem, and aztreonam formed complex and structurally distinct haptenic structures with lysine residues on HSA. Each drug modified Lys190 and at least 6 additional lysine residues in a time- and concentration-dependent manner. PBMC proliferative responses and cytokine release were detected with cells from the allergic patients, but not tolerant controls, following exposure to the drugs. 122 CD4⁺, CD8⁺, or CD4⁺CD8⁺ T-cell clones isolated from the allergic patients were found to proliferate and release cytokines following stimulation with piperacillin, meropenem, or aztreonam. Cross-reactivity with the different drugs was not observed. In conclusion, our data show that piperacillin-, meropenem-, and aztreonam-specific T-cell responses are readily detectable in allergic patients with cystic fibrosis, which indicates that multiple β -lactam allergies are instigated through priming of naïve T-cells against the different drug antigens. Characterization of complex haptenic structures on distinct HSA lysine residues provides a chemical basis for the drug-specific T-cell response.



INTRODUCTION

Cystic fibrosis is one of the commonest lethal inherited disorders of the Caucasian population. In the UK, over 9000 people have the condition, and about 1 in 25 people are carriers.¹ In 1985, the disease was linked to the long arm of chromosome 7;^{2,3} subsequently, the gene sequence which encoded a 1480 amino acid protein called the cystic fibrosis transmembrane conductor regulator (CFTR) was discovered.⁴ To date, over 1,900 mutations have been reported, and the number continues to increase. CFTR functions as a cAMP-

activated ATP-gated anion channel that transports chloride across epithelial cell membranes. Defective CFTR results in reduced chloride secretion across the apical airway epithelial membrane and increased sodium absorption basolaterally. This results in reduced airway surface liquid and defective ciliary function.⁵ These thick viscid secretions provide a favorable environment for bacterial infection or colonization. Ultimately,

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this leads to frequent pulmonary exacerbations, airway destruction, and bronchiectasis.⁶

Intravenous drug therapy, usually an aminoglycoside combined with a β -lactam antibiotic (aztreonam, ceftazidime, meropenem, or piperacillin-tazobactam), provides the foundation for treatment of respiratory exacerbations in patients with cystic fibrosis. Unfortunately, the choice of therapy is often limited by a high incidence of allergic reactions. Several studies report a reaction prevalence of 26–50%, compared with 1–10% in the general population.^{7–10} β -Lactam allergy in patients with cystic fibrosis is usually nonimmediate, with a mean time of onset of 9.1 days.¹¹ Reactions consist of rashes, fever, and/or flu-like symptoms. In the Leeds Adult Cystic Fibrosis Unit, only 6% of 302 β -lactam reactions were identified as being immediate. Severe skin reactions and systemic involvement were not encountered.¹² If an allergic reaction to one β -lactam antibiotic is observed, it is replaced by another drug often from a different class. However, approximately 20% of patients develop allergic reactions to multiple antibiotics.¹⁰ Patients who have exhausted all conventional therapeutic options are usually older and sicker and require more frequent treatment. This results in prolonged hospital admissions for observation of treatment.

Clinical data suggest that the extent of cross-reactivity between different classes of β -lactam antibiotics is low. Moss et al.¹³ reported that 19 out of 20 patients with previous penicillin or cephalosporin reactions safely tolerated aztreonam. Cross-reactivity rates between penicillin and meropenem in patients with immediate reactions are estimated at less than 1%. However, several patients became sensitized against the alternative drug following repeated exposure.^{13–16} The factors that drive multiple delayed reactions in β -lactam allergic patients are not known. Thus, there is an urgent need for a clearer understanding of the drug-specific immune response to inform drug selection and drug use.

In this study, we looked to (1) characterize the haptenic structures piperacillin, meropenem, and aztreonam generate on human serum albumin (HSA) lysine residues and (2) isolate antigen-specific T-cell clones from patients with multiple β -lactam allergies to provide a biological read-out to analyze drug cross-reactivity.

■ EXPERIMENTAL PROCEDURES

Chemicals. Sterile intravenous preparations of lyophilized piperacillin, meropenem, and aztreonam were purchased from Wyeth Pharmaceuticals (Maidenhead, UK), AstraZeneca (Luton, UK), and Bristol-Myers Squibb Pharmaceuticals (Dublin, Ireland), respectively. HSA (approx 97% pure, lyophilized), DTT, and other standard chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK). Modified trypsin was purchased from Promega (Southampton, Hampshire, UK). LC/MS grade solvents were purchased from Fisher (Loughborough, Leicestershire, UK).

Time- and Concentration-Dependent Modification of Human Serum Albumin by Meropenem, Aztreonam, and Piperacillin. HSA (66 mg/mL, 1 mM) was incubated with meropenem, aztreonam, or piperacillin (0.1 mM, 1 mM, 10 mM, 20 mM, or 50 mM) in 200 μ L of phosphate buffer, at pH 7.4 and 37 °C for 24 h. In separate experiments, the drugs were incubated at a molar ratio of drug to protein of 20:1 for 24, 48, 72, and 96 h under the same experimental conditions. The drug was removed by precipitation of the protein with 9 volumes of ice-cold methanol followed by centrifugation at 14000g and 4 °C for 10 min. This was repeated once more, and the protein pellet was reconstituted in 50 μ L of phosphate buffer. The protein was reduced by incubation with 10 mM DTT (w/v) at room temperature for 15 min and alkylated by

incubation with 55 mM iodoacetamide (w/v) for a further 15 min at room temperature. The samples were again subjected to methanol precipitation and were reconstituted in 50 mM ammonium bicarbonate buffer, and the concentration was determined by the Bradford assay. The modified HSA was diluted to 1.6 mg/mL in 50 μ L of ammonium bicarbonate buffer. Trypsin (2 μ g) was added, and the samples were incubated overnight at 37 °C. The digestions were desalted using C₁₈ Zip-Tips (Millipore) and dried prior to LC/MS/MS analysis.

LC/MS Analysis of Drug-Modified Peptides. Analysis of the free drugs by LC/MS is reported in Supporting Information. For multiple reaction monitoring (MRM) detection of modified peptides, samples were reconstituted in 2% ACN/0.1% formic acid (v/v), and aliquots of 2.4–5 pmol were delivered into a QTRAP 5500 (AB Sciex, Framingham, MA, USA) fitted with a NanoSpray II source by automated in-line liquid chromatography (U3000 HPLC System, 5 mm C₁₈ nanoprecolumn and 75 μ m $\times$ 15 cm C₁₈ PepMap column [Dionex, California, USA]) via a 10 μ m inner diameter PicoTip (New Objective, Massachusetts, USA). A gradient from 2% ACN/0.1% FA (v/v) to 50% ACN/0.1% FA (v/v) in 60 min was applied at a flow rate of 300 nL/min. The ion spray potential was set to 2,200–3,500 V, the nebulizer gas to 19, and the interface heater to 150 °C.

In order to acquire unequivocal MS/MS characterization of modified peptides, samples were also analyzed using a Q-TOF instrument. Samples were delivered into a Triple TOF 5600 mass spectrometer (AB Sciex) by automated in-line reversed phase liquid chromatography, using an Eksigent NanoUltra cHiPLC System (AB Sciex) mounted with a microfluidic trap and analytical column (15 cm $\times$ 75 μ m) packed with ChromXP C₁₈–CL 3 μ m. A NanoSpray III source was fitted with a 10 μ m inner diameter PicoTip emitter (New Objective). Samples were loaded in 0.1% formic acid onto the trap, which was then washed with 2% ACN/0.1% FA for 10 min at 2 μ L/min before switching in-line with the analytical column. A gradient of 2–50% (v/v) ACN/0.1% (v/v) FA over 90 min was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive ion mode using information-dependent acquisition powered by Analyst TF 1.5.1. software, using mass ranges of 400–1600 amu in MS and 100–1400 amu in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approximately 10 Hz) using a threshold of 100 counts per s, with dynamic exclusion for 12 s and rolling collision energy. Sequence coverage was determined using ProteinPilot software, v4.0, with the Paragon algorithm¹⁷ and the most recent version of the SwissProt database. Modified peptides were identified by filtering for specific fragment ions in PeakView 1.2.0.3 (AB Sciex) and manual inspection of the spectra.

To detect drug haptens, raw LC/MS/MS data were perused in order to identify peptides known to be modified with other β -lactam antibiotics,^{11,18,19} which were present with unexplained mass additions in samples exposed to meropenem or aztreonam. These data were used to design transitions specific for drug modified peptides. For meropenem, the *m/z* values were calculated for all possible peptides with a missed cleavage at a Lys residue. To these were added the masses of the proposed haptens (339, 384, 397, and 440 amu), and the parent ion masses were then paired with the proposed fragment masses of 175, 205, and/or 232 amu. For aztreonam, the mass of the proposed hapten (435 amu) was added to the peptide masses and paired with the proposed fragment ion mass of 237 amu.

MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity; they were optimized for collision energy and collision cell exit potential, and dwell time was 20 ms. MRM survey scans were used to trigger enhanced product ion MS/MS scans of drug-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20 s. Total ion counts were determined from a second aliquot of each sample analyzed on the same instrument by conventional LC/MS/MS. Peak areas for drug-modified peptides were determined by MultiQuant 1.2 software (AB Sciex). Epitope profiles were constructed by comparing the relative intensity of peaks for each of the modified peptides within a sample and normalization of those signals across samples using the total ion counts. In order to confirm the identity of the fragment ions

Table 1. Clinical Details of the Allergic and Tolerant Patients

Allergic Patients							
id	age/sex	drug reaction	details of the reaction	time to reaction (days)	time since reaction (years)	courses prior to reaction	LTT ^a (maximum SI)
1	21/M	piperacillin	MPE, ^b fever	9	7	3	13.3
		aztreonam	MPE	4	5	2	<2
		meropenem	MPE	5	5	3	8
2	26/M	piperacillin	MPE	5	2	3	31.1
		aztreonam	urticarial rash	2	1	4	2.4
		meropenem	MPE	3	3	5	4.4
3	28/F	piperacillin	fever, arthralgia	9	6	7	6.8
		aztreonam	fever, arthralgia	6	5	4	4.1
		meropenem	flu-like symptoms, vomiting	6	4	5	5.8
4	22/M	piperacillin	MPE/fever	2	1	4	62.5
		aztreonam	urticarial rash	1	1	2	<2
		meropenem	lip swelling	1	2	3	4
Tolerant Patients							
id	age/sex	drug reaction	drug exposure				LTT (maximum SI)
1	28/M	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years				<2 ^c
2	24/F	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years				<2
			In 1 year prior to his death, he required 73 days of antibiotic treatment.				
3	21/M	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years				<2
			averaged 81 days of antibiotic treatment per year				
4	29/M	none	exposed to piperacillin, aztreonam and meropenem in the last 3 years				<2
			averaged 14 days of antibiotic treatment per year				

^aLTT, lymphocyte transformation test. ^bMPE, maculopapular drug eruption. ^cLTT SI < 2 with each drug (0.1–2 mM).

used in the MRM transitions, MS³ experiments were performed on selected peptides modified with each of the proposed haptens: MRM-triggered enhanced product ion MS/MS scans of drug-modified peptide were in turn used to trigger information-dependent MS³ scans, with two MS/MS scans and four MS³ scans per cycle. Piperacillin modified peptides were analyzed as described previously.¹¹

Human Blood Donors. Four patients with cystic fibrosis and allergies to piperacillin, meropenem, and aztreonam, and four drug-exposed tolerant patients with cystic fibrosis were recruited. Table 1 summarizes the patients' demographics and the clinical features of the reactions. Approval for the study was acquired from the local research ethics committee, and informed written consent was obtained from each donor.

Medium for PBMC Culture and T-Cell Cloning. Culture medium consisted of RPMI-1640 supplemented with pooled heat-inactivated human AB serum (10%, v/v), HEPES (25 mM), L-glutamine (2 mM), transferrin (25 µg/mL), streptomycin (100 µg/mL), and penicillin (100 U/mL). Penicillin was removed from the culture medium prior to testing.

Detection of Drug-Specific PBMC Responses. Proliferation of patient PBMCs (0.15×10^6 cells/well) induced by piperacillin, meropenem, and aztreonam (0.1–2 mM) and tetanus toxoid (5 µg/mL) was measured using the lymphocyte transformation test (LTT).²⁰ IFN-γ secreting PBMC were visualized using ELISpot (MabTech AB, Nacka Strand, Sweden) by culturing PBMC (0.5×10^6 cells/well; 200 µL) with the drugs (0.5–2 mM) or PHA as a positive control (5 µg/mL) for 48 h. Patients with cystic fibrosis are exposed to high doses of the drugs, and maximum plasma concentrations reach 0.5 mM.

Generation of T-Cell Clones from Drug Allergic Patients. PBMC (1×10^6 cells/well; 0.5 mL) from the allergic patients were cultured with piperacillin, meropenem, and aztreonam (1 mM) in culture medium that was supplemented with 200 IU/mL rhIL-2 (PeproTech EC Ltd., London, UK) on days 6 and 9. On day 14, the expanded T-cell cultures were cloned by serial dilution using previously described methods.²⁰

Epstein–Barr virus (EBV)-transformed B-cell lines were created from PBMC by transformation with supernatant from the virus-producing cell line B9.58. Lines were maintained in RPMI 1640

supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 100 mM L-glutamine, 100 µg/mL penicillin, and 100 U/mL streptomycin and used as a source of autologous antigen presenting cell.

Phenotype and Specificity of T-Cell Clones. Drug specificity was assessed by culturing autologous irradiated EBV-transformed B-cells (1×10^4 cells/well) and piperacillin, meropenem, or aztreonam (1–2 mM) with T-cell clones (5×10^4 cells/well; 200 µL) for 48 h. Proliferation was measured by the addition of [³H]thymidine (0.5 µCi/well, 5 Ci/mmol, Morovek Biochemicals Ltd., Brea, CA, USA) for the last 16 h of culture followed by scintillation counting. Clones with a stimulation index of greater than 2 were expanded by repetitive stimulation with irradiated allogeneic PBMC (5×10^4 cells/well; 200 µL) and 5 µg/mL PHA in IL-2 containing medium (250 IU/mL). Drug specificity (piperacillin, meropenem, and aztreonam [0.1–2 mM]) of the T-cell clones was assessed by proliferation and ELISpot for IFN-γ (Mabtech AB). Our previous studies with piperacillin-responsive clones from monoallergic patients revealed that the vast majority secreted Th1 (IFN-γ and TNF-α) and Th2 (IL-4, IL-5, and IL13) cytokines following drug stimulation;^{11,26} thus, IFN-γ secretion was deemed a suitable second readout to study cross-reactivity against the different drug antigens. Cell phenotyping was performed by flow cytometry on a BD FACSCanto II using CD4-FITC and CD8-PE antibodies (BD Biosciences).

Statistical Analysis. Student's *t*-test was used to analyze the data.

RESULTS

Elucidation of Haptenic Structures by Mass Spectrometric Analysis of Modified Peptides. The fragmentation properties of the free drugs were determined, but they did not coincide with the fragmentation characteristics of the covalently bound moieties (see Supporting Information, Figures 1 and 2).

The haptens formed by piperacillin binding to HSA have been described previously,¹¹ but the MS/MS spectrum of peptide ¹⁸²LDELRLDEGKASSAK¹⁹⁵ modified at Lys190 with the cyclized form of the drug has been included here for

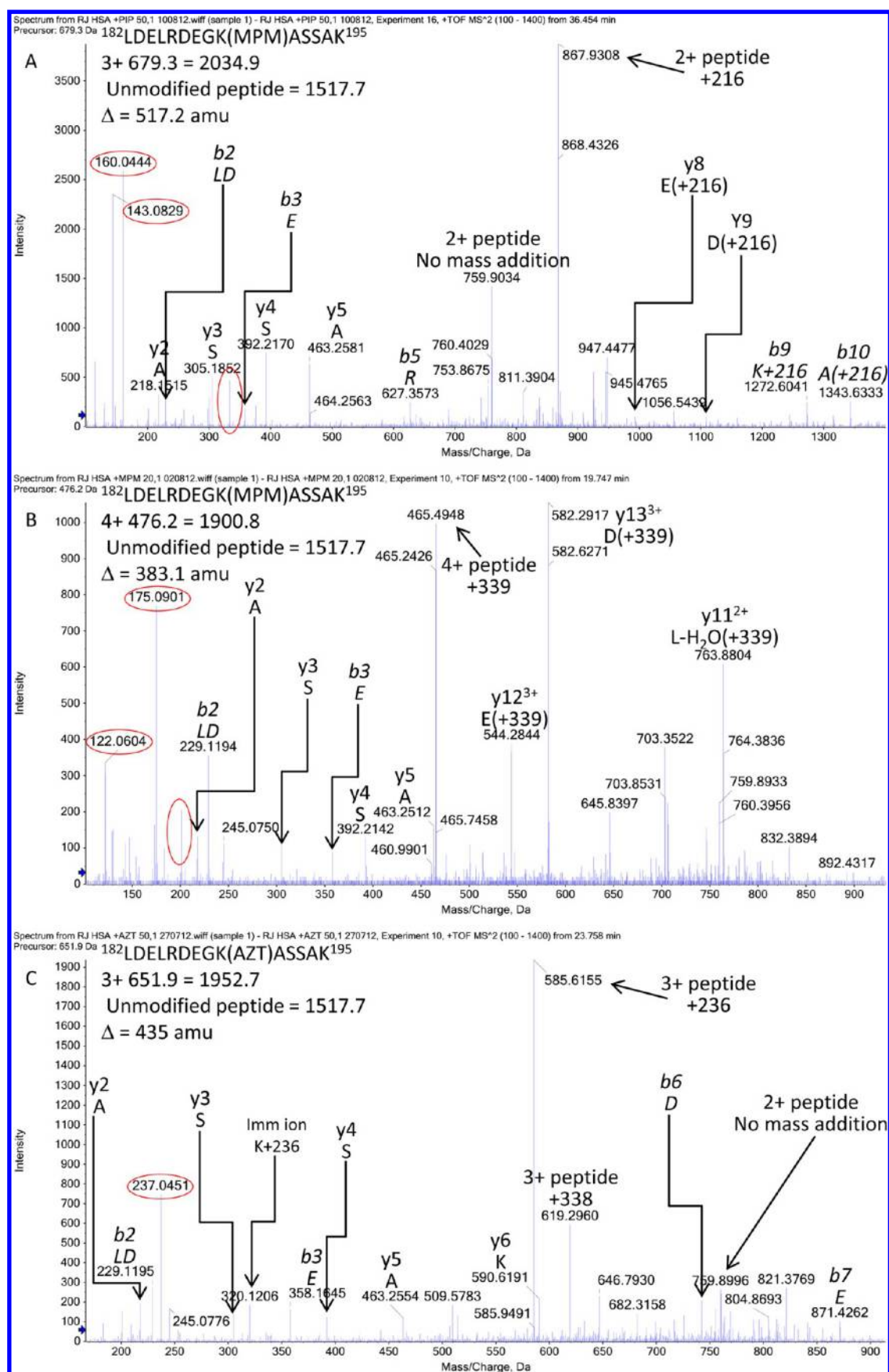


Figure 1. continued

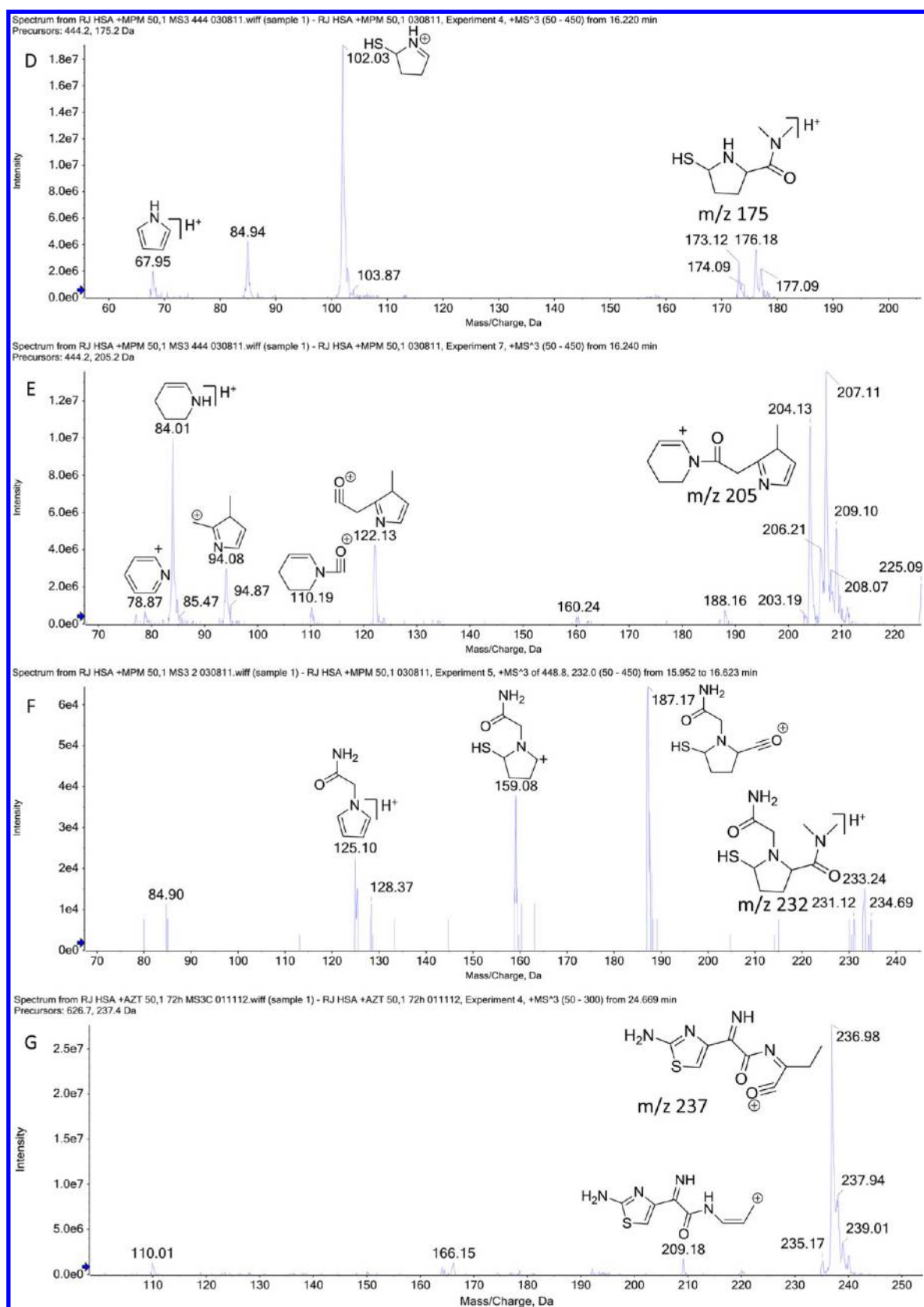
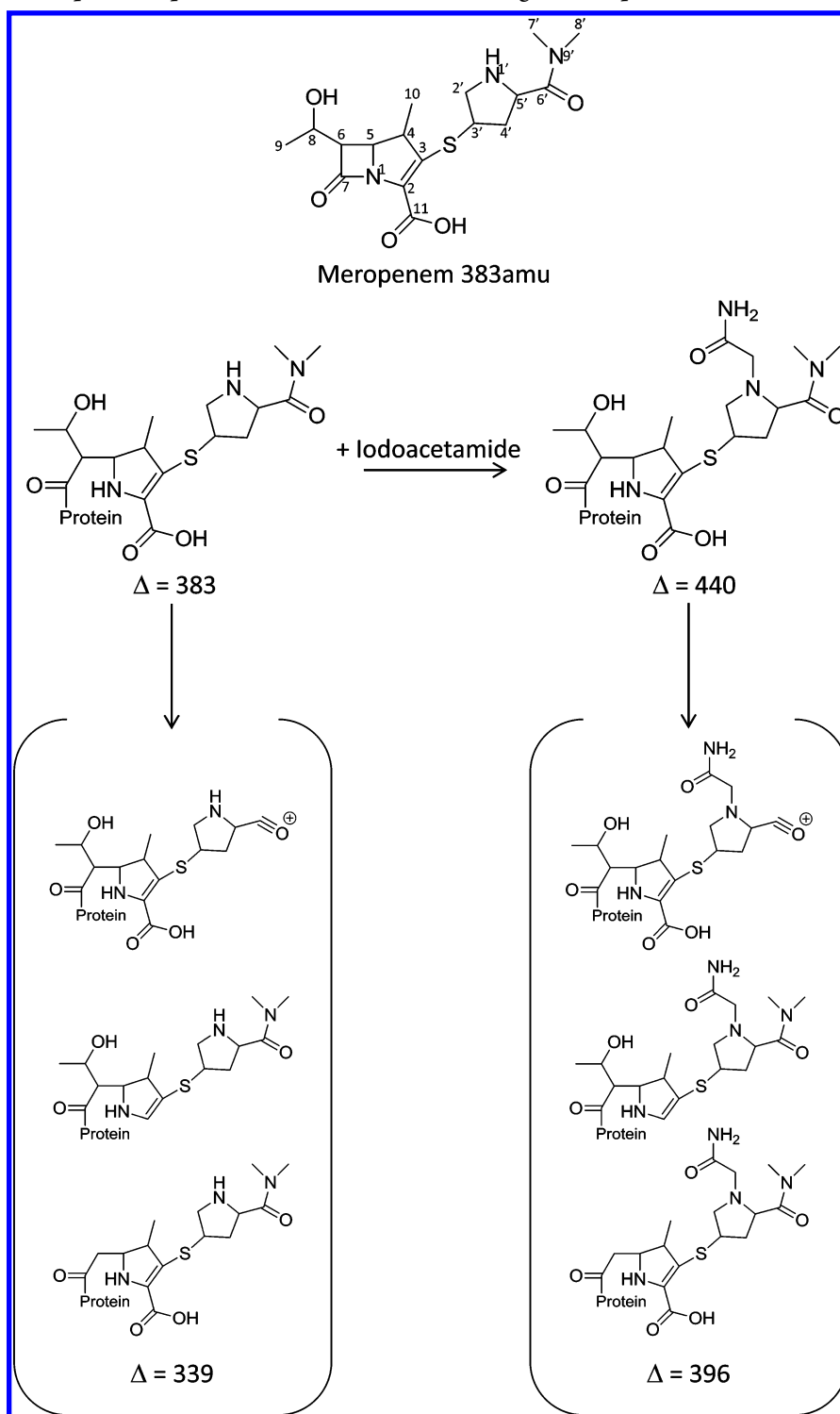


Figure 1. Mass spectrometric characterization of piperacillin, meropenem, and aztreonam haptens formed on HSA. Representative MS/MS spectra of peptide ¹⁸²LDELRLDEGKASSAK¹⁹⁵ modified on Lys190 with (A) piperacillin, (B) meropenem, and (C) aztreonam. (D, E, and F) Representative MS³ spectra of characteristic fragment ions from meropenem-modified peptides. (G) Representative MS³ spectrum of characteristic fragment ions from aztreonam-modified peptides.

Scheme 1. Structure of Proposed Haptens Formed on Covalent Binding of Meropenem to HSA^a

^aMeropenem forms a hapten by opening of the β -lactam ring and nucleophilic attack by protein lysine residues, resulting in a mass increase of 383 amu for each modified lysine residue. In-source fragmentation of this hapten results in the loss of the propanol at position 6, the carboxylic acid at position 2, or the trimethylamine group at position 9' (loss of 44 amu; mass increase of 339 amu). The chemistry is complicated by the methods conventionally used to process proteins for mass spectrometry analysis, namely, iodoacetylation of cysteine residues. The drug itself was shown to be iodoacetylated, resulting in two further haptens of 440 and 396 amu.

comparison (Figure 1A). HSA exposed to meropenem *in vitro* at a molar ratio of drug to protein of 20:1 was used to characterize drug haptens. Careful examination of raw MS/MS data revealed the addition of four different masses (339, 383,

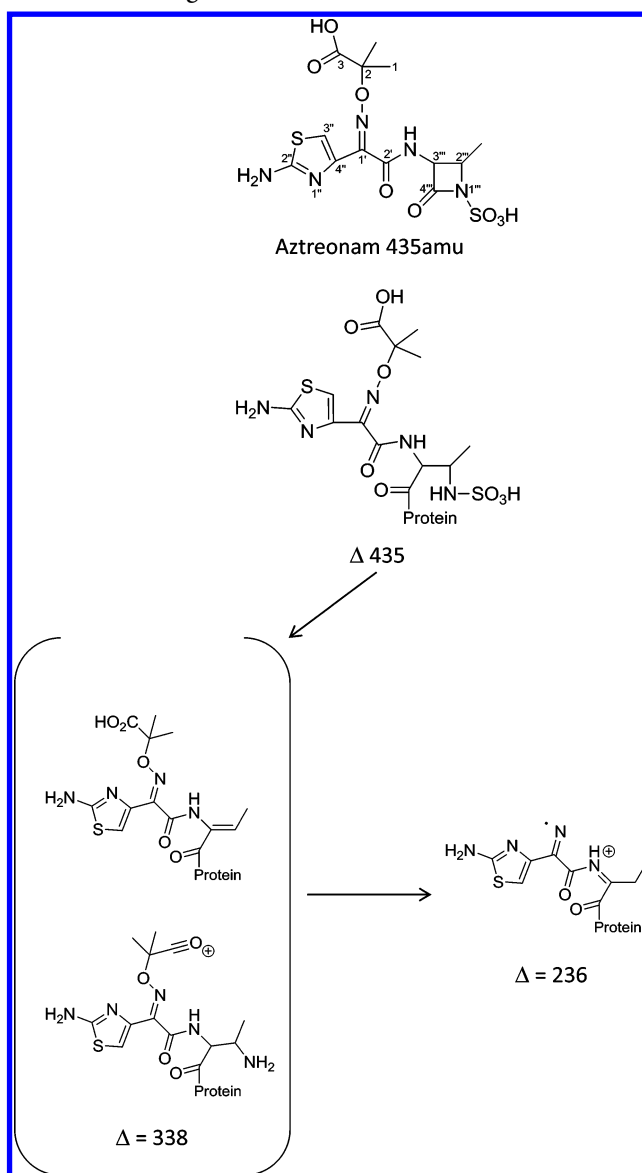
396, and 440 amu) to tryptic peptides exhibiting a missed cleavage at lysine residues: peptide ¹⁸²LDELRDEGKASSAK¹⁹⁵ exhibiting a mass increase of 383 amu is shown as an example (Figure 1B) (spectra for ¹⁹⁸LKASLQK²⁰⁵ modified with each

of the four haptens are shown in Supporting Information, Figure 3A–D). The retention times of the four different peptide species were, however, identical suggesting that they had all been derived from in-source fragmentation of the peptide plus 440 amu (Supporting Information, Figure 3E). Mass additions of 339 and 383 amu were associated with drug-specific fragment ions of m/z 175 and 205, whereas mass additions of 396 and 440 were associated with fragment ions of m/z 205 and 232. These data together with MS³ spectra for the three fragment ions (Figure 1D, E, and F) were interpreted as follows. The drug hapten of 440 amu corresponded to the covalent binding of the parent drug iodoacetylated on the nitrogen at the 1' position (Scheme 1). This gave rise to the immonium ion of Lys modified with the 3-methyl-3H-pyrrole ring of meropenem (m/z 205) and to the fragment ion of m/z 175 plus acetamide (232 amu; S-(5-(dimethylcarbamoyl)-pyrrolidin-2-yl) carbamothioate). In-source cleavage of the acetamide group from the drug resulted in the drug hapten of 383 amu (440 minus 57 amu). In-source cleavage of the propanol at position 6, the carboxylic acid at position 2, or the trimethylamine group at position 9' from the iodoacetylated drug hapten gave rise to the observed mass addition of 396 amu (440 minus 44) and the same fragment ions as observed for the hapten of 440 amu. Finally, the mass addition of 339 amu (Scheme 1, and equating to structures labeled m/z 340^a, 340^b, and 340[#] in Supporting Information, Figure 1A) was derived from the cleavage of both the acetamide group and the propanol, carboxylic acid, or trimethylamine group from the hapten of 440 amu (440 minus 57 minus 44), and the same fragment ions as observed for the hapten of 383 amu.

HSA was modified with aztreonam at a molar ratio of drug to protein ratio of 50:1 *in vitro* in order to characterize potential hapten structures. Nucleophilic attack by protein lysine residues and opening of the β -lactam ring was the predicted pathway for covalent binding of aztreonam, which would lead to a mass increase on the peptides of 435 amu (Scheme 2). The MS/MS spectrum of ¹⁸²LDELRLDEGKASSAK¹⁹⁵ modified with aztreonam shows this to be the case (Figure 1C), with a mass addition to the peptide of 435 amu plus the presence of partial drug haptens of 338 and 236 amu (Scheme 2). We propose that the former is formed following the loss of the sulfonic acid and amino groups from position 1''' and that the latter is formed following the further loss of 2-hydroxy-2-methylpropanoic acid (Scheme 2). Analysis of the cleaved 236 amu partial hapten by MS³, despite the ion being recalcitrant to fragmentation, supports this interpretation of the hapten chemistry (Figure 1G). As further illustration of aztreonam hapten formation and ionization characteristics, the MS/MS spectrum of peptide ⁵³⁹ATKEQLK⁵⁴⁵ modified with the drug is also shown (Supporting Information, Figure 4).

Time and Concentration Dependency of Human Serum Albumin Modification with Piperacillin, Meropenem, and Aztreonam. We have previously characterized the time and concentration dependency of the modification of HSA with piperacillin using LC-MRM-MS.¹¹ After normalization for the total ion count, the MRM peak areas for the modified peptides were determined in order to assess changes in the relative abundance of those peptides: the epitope profile. This revealed that the levels of covalent binding increased with increased molar ratio of drug to protein and with incubation time up to 72 h (Figure 2, panels C and D). Piperacillin bound consistently to Lys190, Lys432, and Lys541 *in vitro* and *in*

Scheme 2. Structure of Proposed Haptens Formed on Covalent Binding of Aztreonam to HSA^a



^aAztreonam forms a hapten by β -lactam ring-opening, resulting in a mass increase of 435 amu for each modified lysine residue. The hapten is labile in the mass spectrometer leading to the detection of partial haptens in the MS/MS spectra of modified peptides of 338 and 236 amu.

vivo,¹¹ and drug modifications were detected at an additional 10 of the 59 Lys residues in HSA *in vitro* (Figure 2, panel B).

A similar approach was adopted here to characterize meropenem and aztreonam HSA binding profiles. For meropenem, the MRM peak areas for all four hapten signals were summed for each of the modified peptides. Up to 7 of the 59 Lys residues of HSA were modified with the drug after 24 h (Figure 2, panel B), but no further modification occurred with increasing incubation times (Figure 2, panel D). The stability of meropenem in aqueous solution at 37 °C is limited^{21,22} so that after 8 h, little active drug will be present in the incubations. Similarly, increasing the molar ratio of drug to protein up to 20:1 resulted in an increase in signal for each modified peptide, but no further increase in covalent binding could be detected at

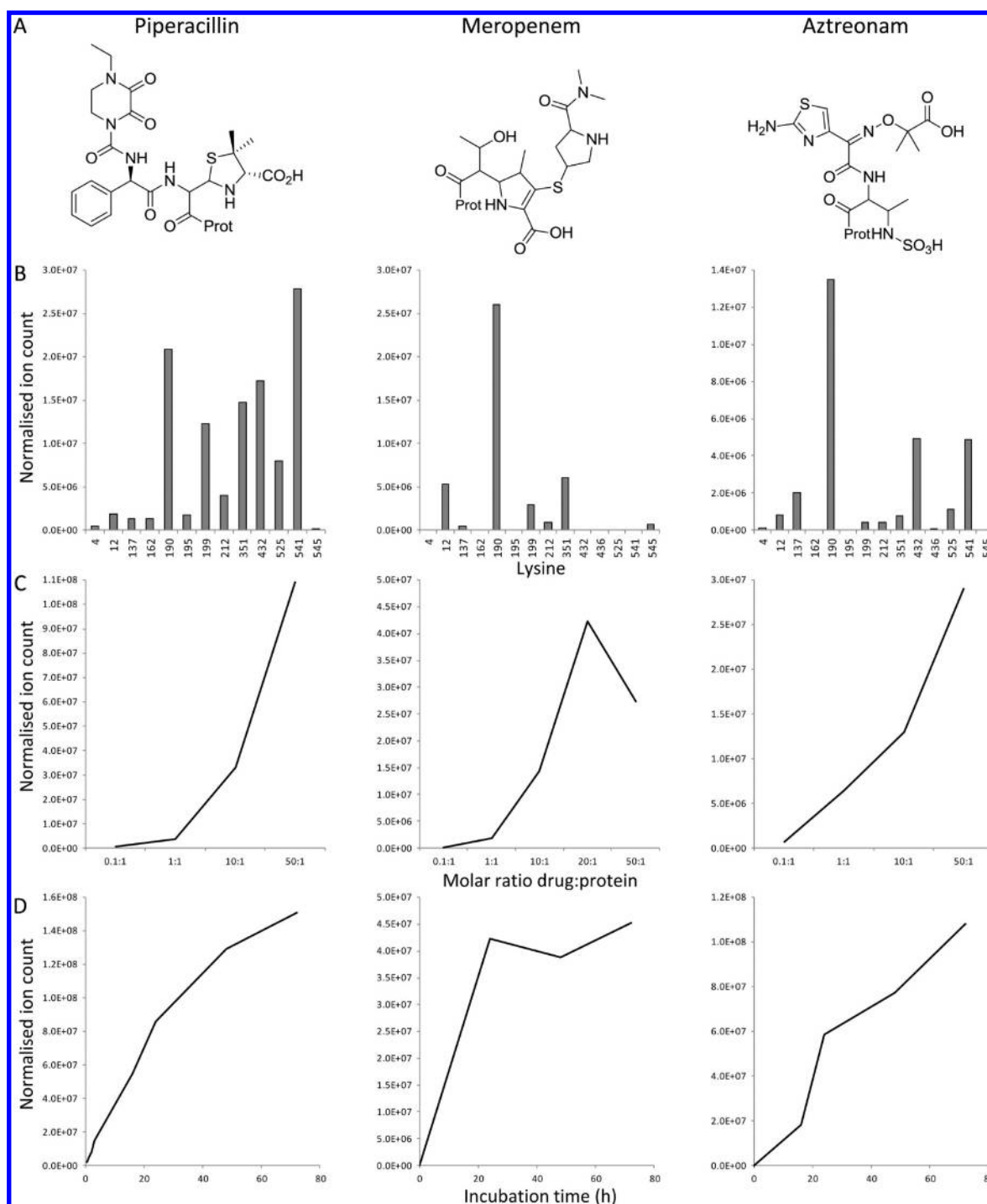


Figure 2. Chemical characterization of piperacillin, meropenem, and aztreonam HSA covalent binding profiles. (A) Hapten structures of the three drugs. (B) Epitope profiles showing the lysine residues in HSA covalently modified by the three drugs *in vitro*, as determined by LC-MRM-MS/MS. (C) Concentration dependency of total drug modification of HSA, as determined by LC-MRM-MS/MS. (D) Time dependency of total drug modification of HSA, as determined by LC-MRM-MS/MS.

molar ratios above 20:1 (Figure 2, panel C). Meropenem is sparingly soluble in water, limiting the maximal drug concentration available for reaction with the protein in aqueous solution. Aztreonam modification of HSA was detected at 11 Lys residues after 24 h (at a drug/protein ratio of 20:1), and the levels of modification increased progressively with time (Figure 2, panel D) and drug concentration (Figure 2, panel C). While the same subset of Lys residues are targeted by all the β -lactam drugs we have investigated to date,^{11,18,19} the relative

abundance of modification at each lysine differs between the drugs. Here, Lys190 was a primary target for modification with piperacillin, meropenem, and aztreonam: the high signal observed was probably not due to an innate greater ionization efficiency of the peptide since the same peptide modified with benzylpenicillin¹⁹ or ampicillin (unpublished observation) was detected at low relative signal.

PBMC from Allergic Patients Are Stimulated with Piperacillin, Meropenem, and Aztreonam. PBMC from all

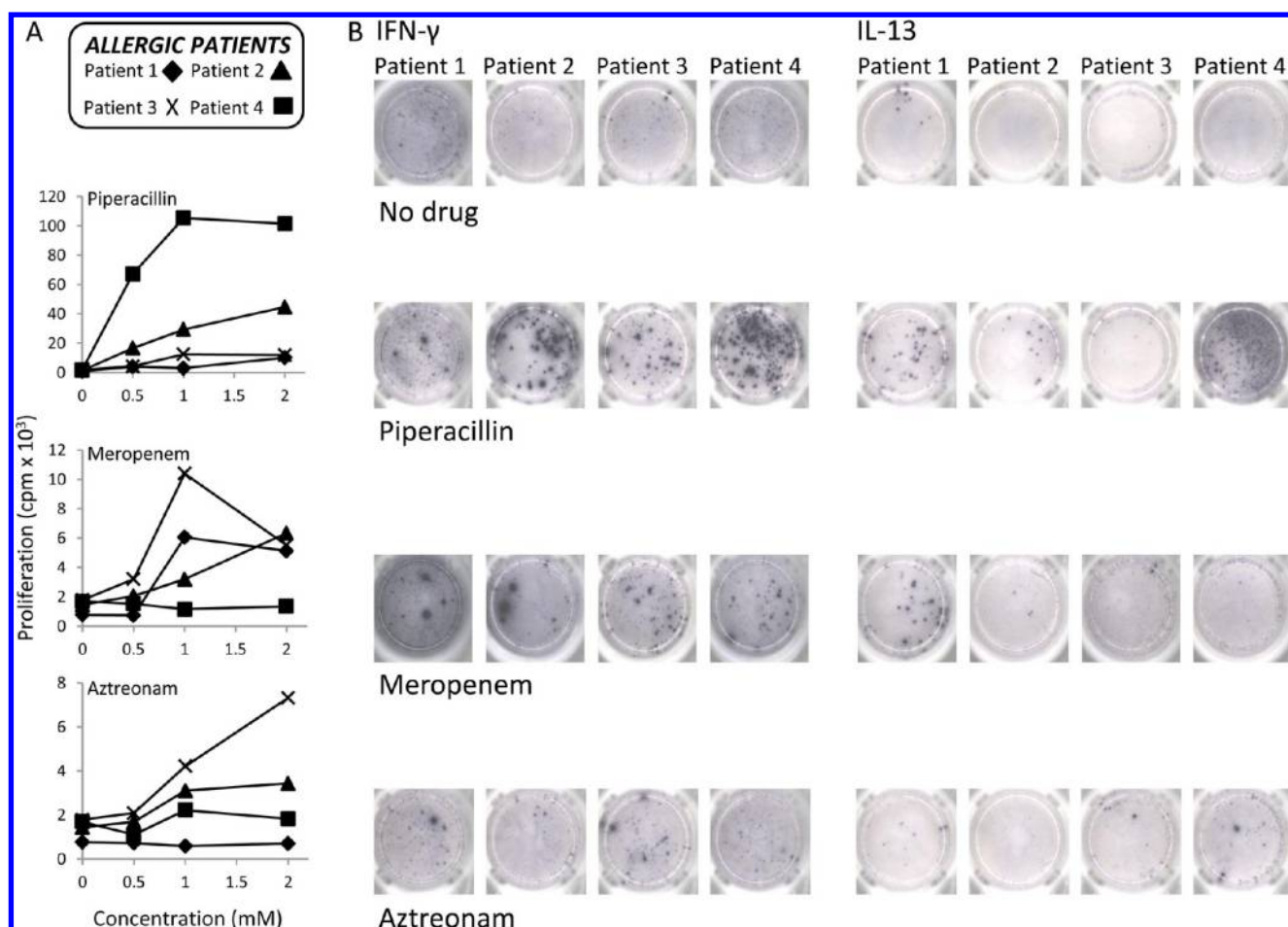


Figure 3. Drug-specific stimulation of PBMC from allergic patients. (A) PBMC were cultured in the presence of piperacillin, meropenem, or aztreonam. Proliferation was measured by the addition of [^{3} H]thymidine for the final 16 h of the experiment. Results are shown as the mean cpm of triplicate cultures. Coefficient of variation consistently was less than 20%. (B) Drug-specific IFN- γ and IL-13 ELISPOT. The figure shows results from patient PBMC stimulated with the drugs for 48 h.

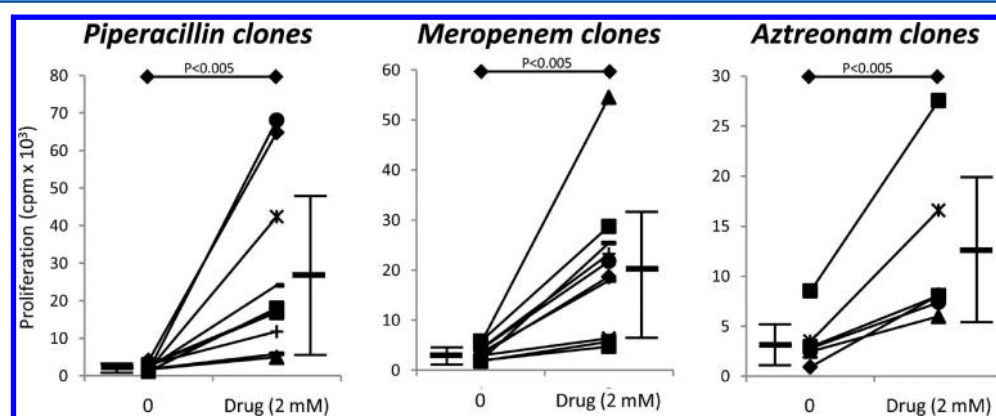


Figure 4. Activation of T-cell clones with piperacillin, meropenem, and aztreonam. Clones were cultured with antigen presenting cells and the drugs (2 mM) for 72 h. Proliferation was measured by the addition of [^{3} H]thymidine for the final 16 h of the experiment. The figure shows the proliferative response of representative piperacillin ($n = 10$), meropenem ($n = 6$), and aztreonam ($n = 10$) clones.

four multiallergic patients were stimulated to proliferate in the presence of piperacillin, meropenem, and aztreonam (Table 1). The proliferative response was drug concentration-dependent and associated with the release of IFN- γ and/or IL-13 (Figure 3). In contrast, PBMC from patients exposed to several courses of the three drugs with no apparent allergies were not stimulated to proliferate or release cytokines (Table 1).

Generation of Piperacillin, Meropenem, and Aztreonam-Responsive T-Cell Clones. One thousand six hundred and sixty-three T-cell clones were generated from the 4 allergic patients following PBMC stimulation with piperacillin, meropenem, or aztreonam and tested for drug specificity. A total of 122 were found to display reactivity against piperacillin ($n = 89$), meropenem ($n = 14$), or aztreonam ($n = 22$) (Figure

Table 2. Origin, Phenotype, and Specificity of T-Cell Clones from Allergic Patients

id	drug	clones tested (<i>n</i>)	specific clones (<i>n</i>)	proliferation (cpm)		phenotype (%)		
				control	drug (2 mM)	CD4+	CD8+	CD4+CD8+
1	piperacillin	58	2	1422 ± 365	10512 ± 7907	100		
	aztreonam	111	2	2043 ± 788	6683 ± 2042	100		
	meropenem	111	5	4340 ± 3988	11744 ± 11173	80	20	
2	piperacillin	67	23	5477 ± 4137	36004 ± 23732	78	9	13
	aztreonam	160	6	3529 ± 1761	15711 ± 19623	65	17	17
	meropenem	100	4	1046 ± 441	2399 ± 938	100		
3	piperacillin	144	16	8133 ± 4353	53954 ± 18612	50	50	
	aztreonam	216	13	8984 ± 5963	23323 ± 12356	84	8	8
	meropenem	72	1	3504	9550	100		
4	piperacillin	216	48	2134 ± 2412	24461 ± 18542	88	6	6
	aztreonam	208	1	6867	33942	100		
	meropenem	200	5	3795 ± 2827	11493 ± 6948	100		

4 shows the proliferative response of representative clones). Although the majority of drug-responsive clones were CD4+, CD8+ and CD4+CD8+ (dual+) clones were also generated from the allergic patients. The CD4+CD8+ clones expressed high levels of both proteins. The number of piperacillin, meropenem, and aztreonam-responsive clones generated from each patient and the phenotype is summarized in Table 2. Fifty-five well-growing CD4+ and CD8+ clones selected from all 4 allergic patients were used to characterize concentration-dependent proliferative responses, cytokine release, and cross-reactivity.

Drug-Antigen Specificity of T-Cell Clones. Proliferation of piperacillin, meropenem, and aztreonam-responsive CD4+ and CD8+ clones was drug concentration-dependent; maximal responses were detected between 0.5 and 2 mM (Figure 5A). Fifty-five clones were tested for reactivity against piperacillin, meropenem, and aztreonam, and the response to each drug was found to be highly specific. Clones were stimulated to proliferate (Figure 5A) and release IFN- γ (Figure 5B) in the presence of the drug PBMC were cultured with to generate the clones, but not the other β -lactam antibiotics.

DISCUSSION

β -Lactam antibiotics form covalent bonds with lysine residues on protein in drug-exposed patients to generate antigenic determinants.^{11,18,23,24} Several studies have shown that processing of β -lactam HSA conjugates liberates peptide sequences that stimulate T-cells,^{11,25–27} although it must be noted that naturally occurring drug-modified peptides eluted from MHC have yet to be characterized. Under *in vitro* conditions, β -lactams bypass this requirement for protein processing by binding directly to MHC-associated peptides.^{25,28} Cross-reactivity studies using PBMC and T-cell clones from penicillin allergic patients reveal a plethora of drug response profiles.^{29–31} In most allergic patients, the peptide-bound penicilloyl ring structure is thought to represent the main structural determinant for T-cells, as β -lactams that contain a different core structure do not stimulate a response. However, individual T-cells display a range of side-chain reactivity. Cross-reactive clones can be stimulated with several penicillins, some of which the allergic patient will have not been exposed to. In contrast, other clones are incredibly drug antigen-specific. Our recent studies with monoallergic patients revealed that piperacillin-responsive PBMC and clones proliferated and secreted a mixed panel of cytokines including IFN- γ and IL-13 following drug stimulation. Interestingly, piperacillin-

responsive clones were not stimulated with other penicillins, whereas several penicillins stimulated flucloxacillin-responsive T-cells.^{11,26,32} Collectively, these data suggest that different antigenic determinants derived from penicillins can be accommodated within the MHC binding cleft without interfering with the T-cell receptor binding interaction. However, the degree of cross-reactivity observed is drug- and patient-specific and might be influenced by previous drug exposure. Thus, there is a need to further delineate the structural features that determine T-cell receptor cross-reactivity.

Our investigation focused on patients with cystic fibrosis and a history of allergic reactions to piperacillin, meropenem, and aztreonam. Piperacillin is a member of the penicillin class of antibiotics, which contains β -lactam and thiazolidine ring structures. Meropenem, a carbapenem licensed by the FDA in 1996, contains similar ring structures, but the sulfur in the thiazolidine ring has been replaced with a carbon atom, and unsaturation has been introduced. Finally, monobactam aztreonam is unusual in that the β -lactam ring is not fused to another ring structure. Superficially, the free drugs would appear to show some structural similarities, most notably the presence of the β -lactam ring, and these have been used previously to explain the observed immunological cross-reactivity within this class of antibiotic.^{28,33–35} However, characterization of the piperacillin, meropenem, and aztreonam haptens formed following ring-opening and binding to protein lysine residues reveals markedly different structures (Figure 2, panel A), which would be difficult to argue could drive a cross-reactive response. The chemical differences are further highlighted by the fact that the drugs favor different covalent binding sites in a model protein (Figure 2, panel B).

T-cells from multiallergic patients were used as a biological read-out of chemical cross-reactivity. Our objectives were to determine whether it was possible to detect T-lymphocyte responses to each drug in the allergic patients and to analyze T-cell receptor cross-reactivity through the generation of T-cell clones. Symptoms of the drug-induced immune reactions were classified according to patients' medical records. Patients 1, 2, and 4 developed maculopapular exanthema and/or non-immediate urticarial eruptions against the 3 drugs (Table 1). Patient 3 developed less-classical symptoms of drug allergy (arthralgia, nausea, and vomiting). Reactions were relatively mild, but in every case, treatment had to be discontinued. Each patient received and tolerated several courses of the individual drugs prior to the allergic reaction.

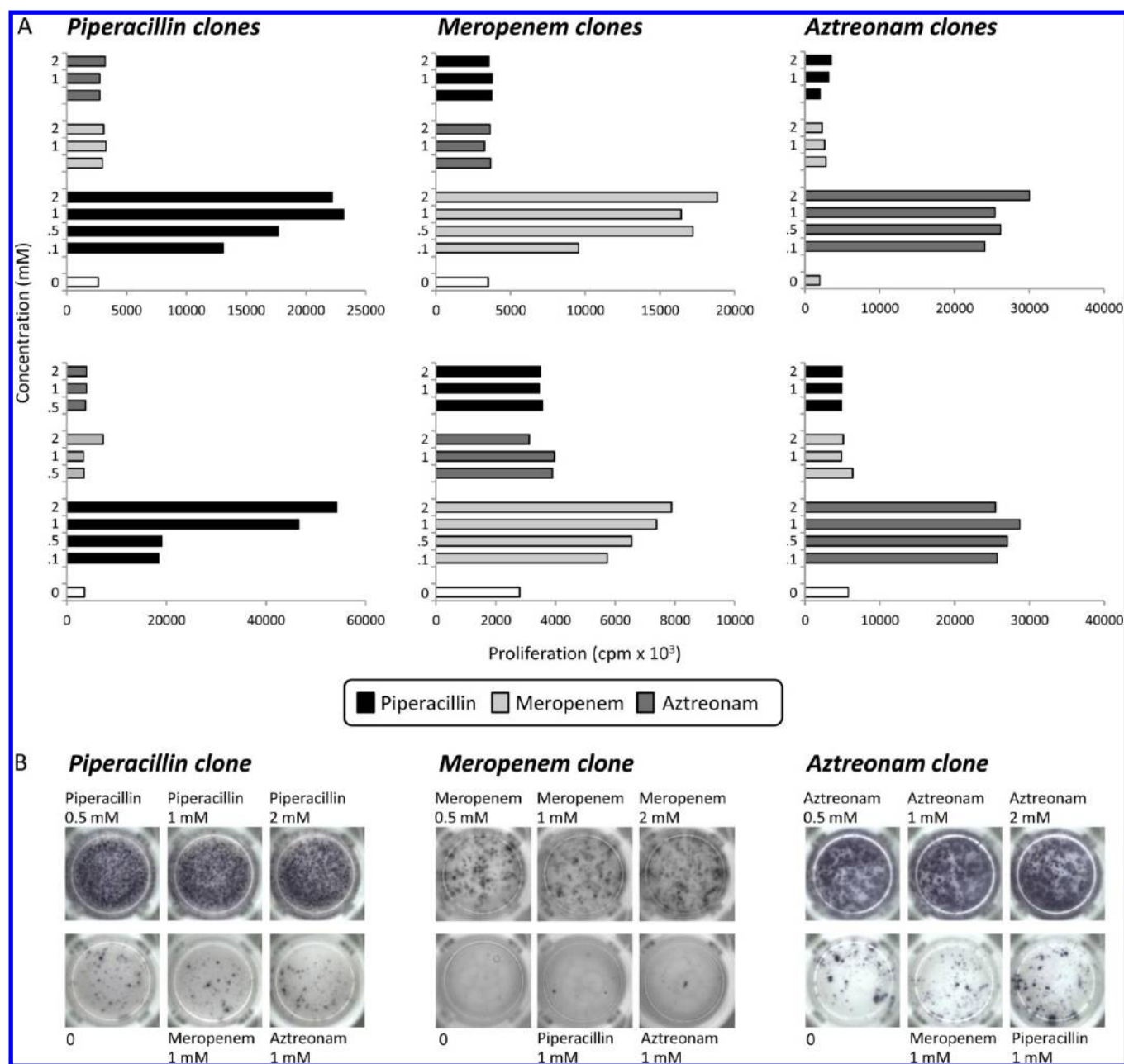


Figure 5. Cross-reactivity of piperacillin, meropenem, and aztreonam-responsive T-cell clones. Clones were cultured with antigen presenting cells and the drugs (0.1–2 mM). (A) Proliferative responses were analyzed by incorporation of [³H]thymidine of the final 16 h of the experiment. Results are shown as the mean cpm of triplicate cultures. Coefficient of variation consistently was less than 20%. (B) Drug-specific IFN- γ ELISpot. The figure shows results from patient clones stimulated with the drugs for 48 h.

The LTT and IFN- γ ELISpot are the biological assays most commonly applied to diagnose drug allergy *in vitro*.^{31,36} As our previous study with piperacillin (mono) allergic patients revealed that almost all drug-responsive T-cells proliferate and secrete IFN- γ following stimulation,²⁶ we utilized both approaches to show that piperacillin, meropenem, and aztreonam stimulate PBMC from allergic patients but not drug-exposed tolerant controls. These data confirm our previous findings with piperacillin allergic patients and demonstrate that T-cell responses are also readily detectable with meropenem and aztreonam. The drug-specific response was dose-dependent and reproducible on repeated testing. PBMC were stimulated with similar concentrations of the drugs. For three of the patients, piperacillin stimulated the

strongest proliferative response and the highest levels of IFN- γ secretion, when the three drugs were compared (Table 1 and Figure 3). Although the number of patients examined is limited, it is interesting to speculate that this may relate to intrinsic chemical reactivity and differences in the epitope profile of drug protein conjugates formed *in vitro* (Figure 2, panel B). The absolute level of binding of each of the drugs to protein cannot be defined from the experiments presented here; however, our data show that piperacillin is more ubiquitous in its binding profile suggesting that a higher epitope density may be achieved (Figure 2, panel B).

As described in other forms of drug allergy,²⁰ CD4+, CD8+, and CD4+CD8+ T-cell clones were isolated from piperacillin-, meropenem-, and aztreonam-stimulated PBMC. The

CD4+CD8+ clones expressed high levels of both proteins indicating that they somehow evaded thymic deletion. Interestingly, the number of CD4+CD8+ T-cells is known to increase in certain pathogenic conditions including autoimmune disease and chronic inflammatory disorders.³⁷ CD4+, CD8+, and CD4+CD8+ clones were used to explore T-cell receptor cross-reactivity. Antigen-specificity was measured using readouts for proliferation and cytokine release, and no cross-reactivity was observed (Figure 5). Over 50 clones were analyzed. It is theoretically possible that the clones were driven to become more specific to one drug during the cloning procedure, but this phenomenon has not been noted previously. If anything, T-cells are more likely to cross-react with other related drug structures through epitope spreading. Thus, our data indicate that nonimmediate reactions to different classes of β -lactam antibiotic in patients with cystic fibrosis are instigated not by cross-reactive T-cells but through priming T-cells against the different drug antigens. Daubner et al.³⁸ have recently demonstrated that drug-responsive T-cells in certain patients with a history of multiple drug allergies reside in an *in vivo*-activated (CD4+CD25dim, with elevated CD38 and PD-1) T-cell fraction. Thus, we are currently exploring whether a similar phenotype of drug-responsive T-cells exists in patients with cystic fibrosis and multiple drug allergies, and indeed whether increased numbers of such T-cells might explain why multiple drug allergy is such a common occurrence in this patient group.

■ ASSOCIATED CONTENT

■ Supporting Information

Additional analysis of the free drugs and protein adducts by mass spectrometry. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PBMC, peripheral blood mononuclear cells; SI, stimulation index; HSA, human serum albumin; LTT, lymphocyte transformation test; CFTR, cystic fibrosis transmembrane conductor regulator

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Determination of fragmentation properties of free drugs

It was believed that gaining an understanding of how the free drug ionised and fragmented in the mass spectrometer would guide identification of peptides modified with drug *in vitro*. To this end, meropenem and aztreonam were dissolved in 50% methanol/0.1% formic acid to concentrations of 0.25 μ M and 2.5 μ M, respectively, and infused at 5 μ l/min directly into a QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer (AB Sciex, Framingham, MA, USA) fitted with a turboion source. The ionspray potential was set to 5500 V and the interface heater was turned off. For meropenem, an ion of m/z 384 was selected manually for fragmentation to generate a full product ion scan, plus the resulting fragment ions of m/z 254 and 340 were further fragmented (Supplementary Figure 2A-C). For aztreonam, an ion of m/z 436 was fragmented to generate a full product ion scan, and the fragment ion of m/z 356 was further fragmented (Supplementary Figure 2D and E). The structures of the fragments were deduced (Supplementary Figure 1A and B).²¹⁻²³ The MS³ scans were acquired to aid identification and reveal potential diagnostic fragment ions to facilitate the detection of covalently bound drug in studies of modified protein. The observed fragment ions were used to set up precursor ion scanning methods for screening for modified peptides, but failed to detect any targets: it is clear that the fragmentation properties of the free drugs cannot be extrapolated to the covalently bound moieties.

Supplementary Figure Legends.

Supplementary Figure 1. Proposed degradation/fragmentation products of free drugs.

A. Meropenem. ^a Mouton *et al.* ^b Sun *et al.* [1-2]. B. Aztreonam. ^cCohen *et al.* [3].

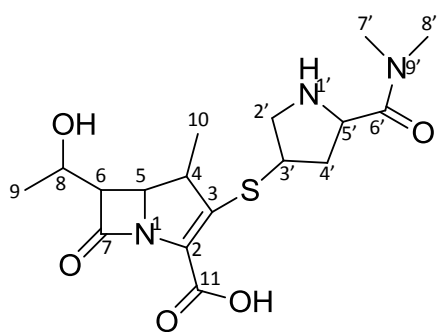
[#]Alternative structures for fragment ions proposed here.

Supplementary Figure 2. Mass spectrometric analysis of free drugs. A. The MS/MS spectrum of meropenem revealed fragment ions of m/z 141, 175, 267 and 340, previously shown to be degradation products of the drug (Supplementary Figure 1A). In addition, dominant fragment ions of m/z 114, 254 and 298 were observed, the proposed structures of which are shown in Supplementary Figure 1A and/or 2A. B. MS3 of fragment ion with m/z 340. The ions of m/z 267 and 295 could be derived from all proposed forms of m/z 340, whereas ions of m/z 141 and 175 could only be derived from 340^a or 340^b , and the ion of m/z 312 could only be derived from $340^\#$ (Supplementary Figures 1A). C. MS3 of fragment ion with m/z 254. Fragment ions of m/z 143 and 173 could only have originated from m/z 340^a or 340^b , whereas others of m/z 124 and 156 could have been the result of fragmentation of m/z $340^\#$. These data suggest that the m/z 340 fragment ion from meropenem exists in all 3 forms proposed in Supplementary Figure 1A. D. The MS/MS spectrum of aztreonam revealed fragment ions of m/z 356 and 313 previously shown to be fragment ions of aztreonam (Supplementary Figure 1B). In addition, dominant fragment ions of m/z 210, 182 and 126 were observed, the proposed structures of which are shown in Supplementary Figure 1B and 2D. E. MS3 of fragment ion with m/z 356.

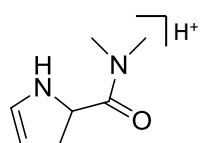
Supplementary Figure 3. Mass spectral analysis of peptide $^{198}\text{LKASLQK}^{205}$ modified with meropenem. MS/MS spectra of peptide modified with hapten of A. 339 amu. B. 383 amu, C. 396 amu and D. 440 amu. The parent ion masses and drug-specific fragment ions are circled. E. Extracted ion counts of peptide $^{198}\text{LKASLQK}^{205}$ modified with each of the meropenem haptens revealing identical retention times.

Supplementary Figure 4. MS/MS spectrum of peptide $^{539}\text{ATKEQLK}^{545}$ modified with aztreonam. The parent ion masses and drug-specific fragment ions are circled.

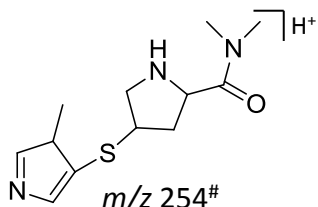
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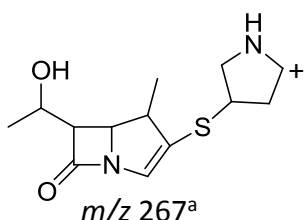
Meropenem 383amu



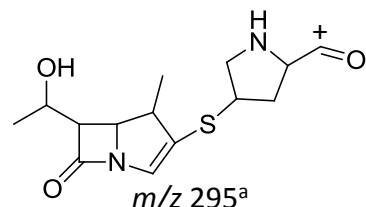
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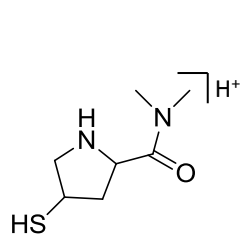
m/z 254[#]



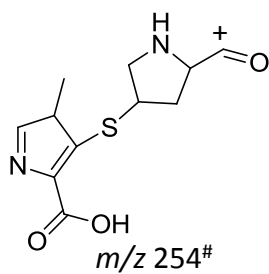
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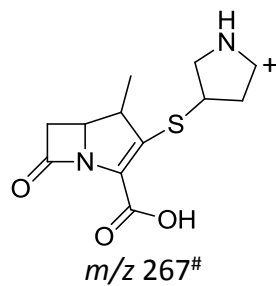
m/z 295^a



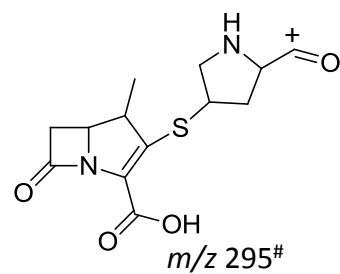
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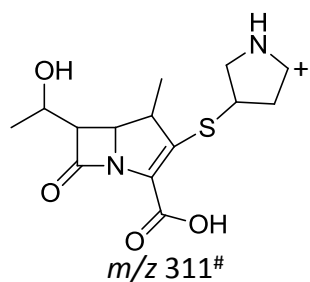
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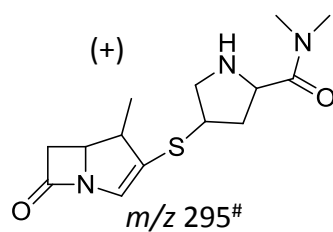
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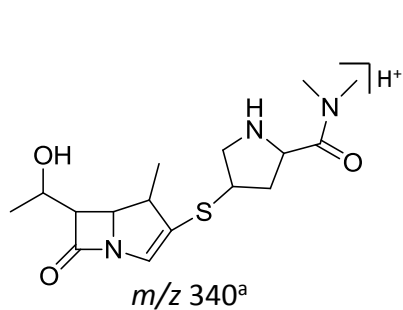
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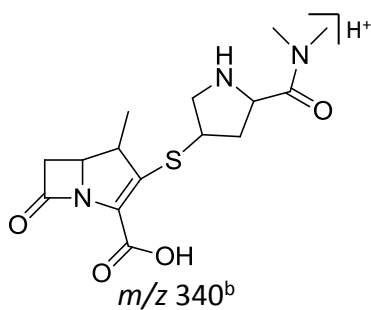
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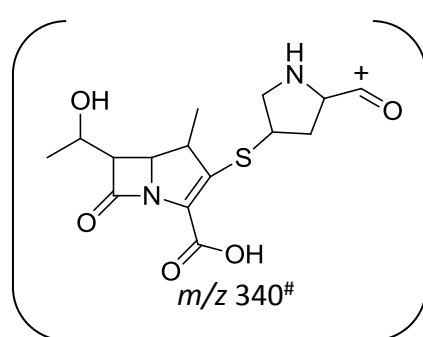
m/z 295[#]



m/z 340^a

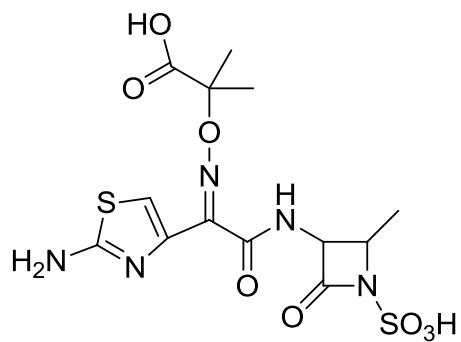


m/z 340^b

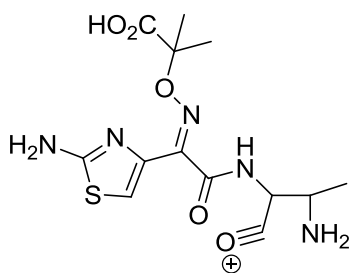


m/z 340[#]

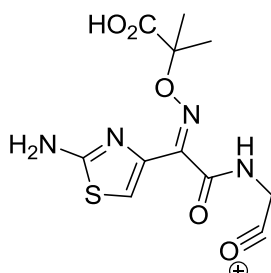
Supplementary Figure 1A



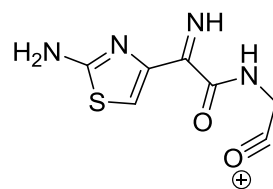
Aztreonam 435amu



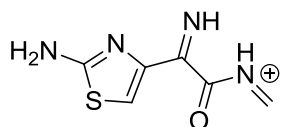
m/z 356^c



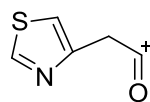
m/z 313^c



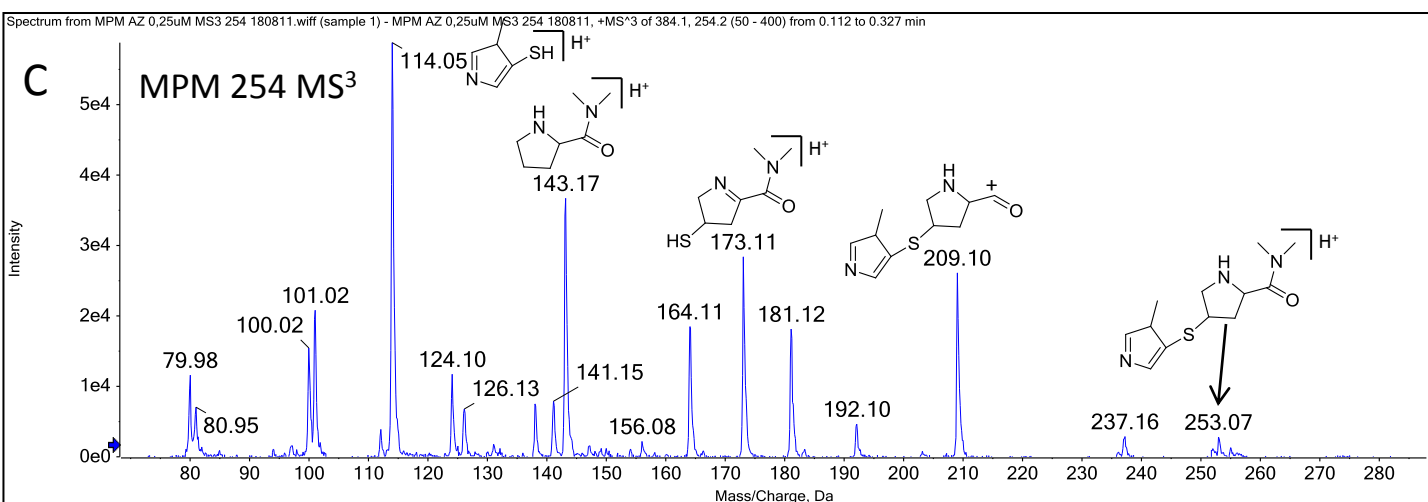
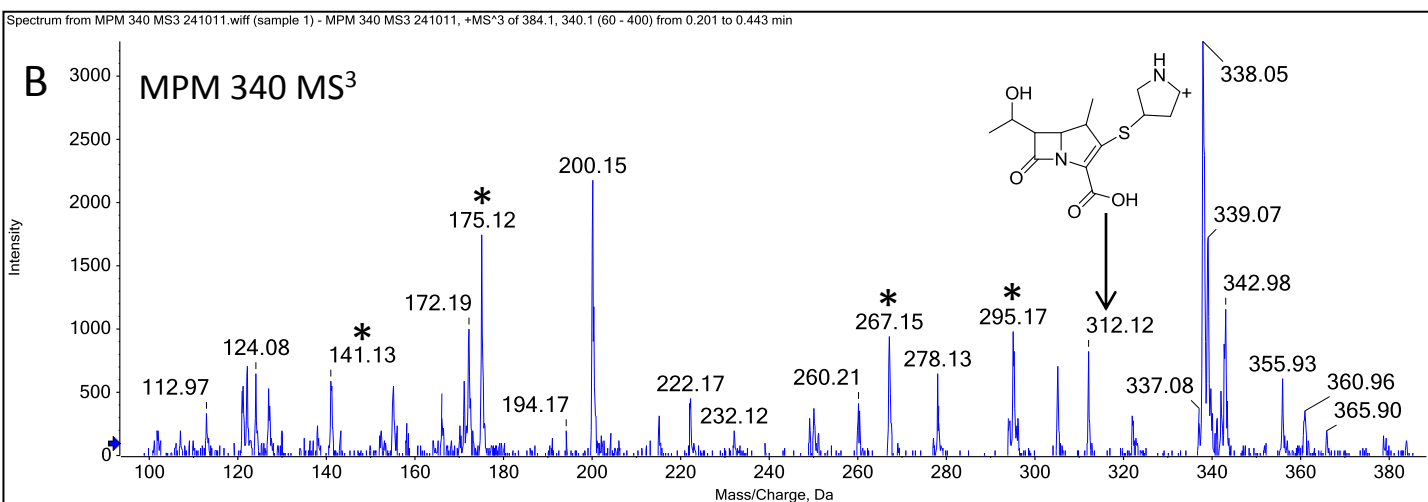
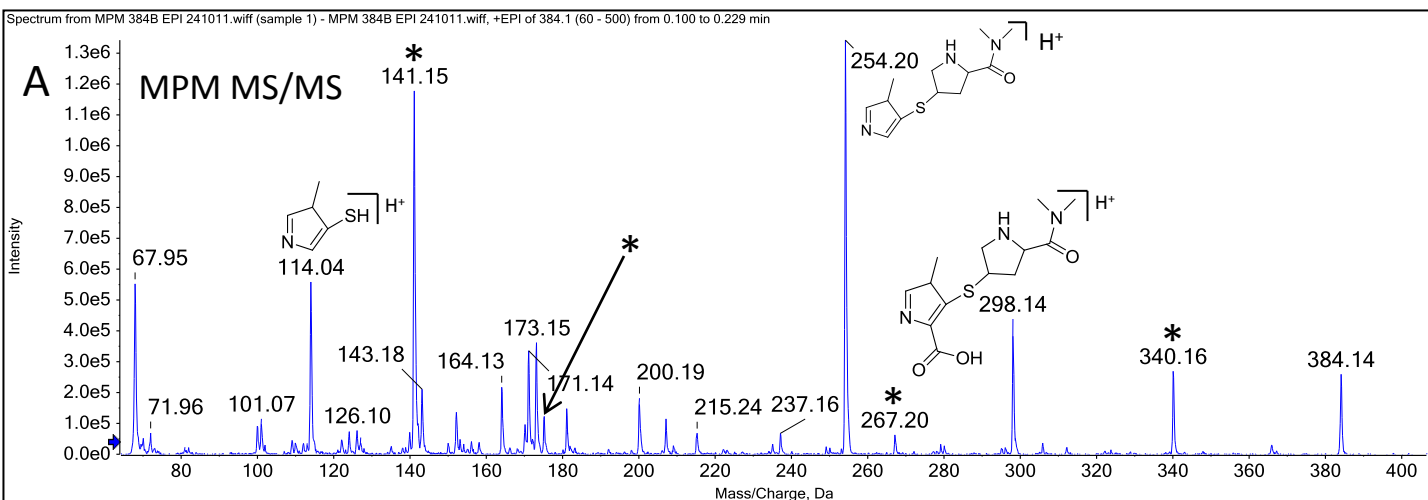
m/z 210[#]



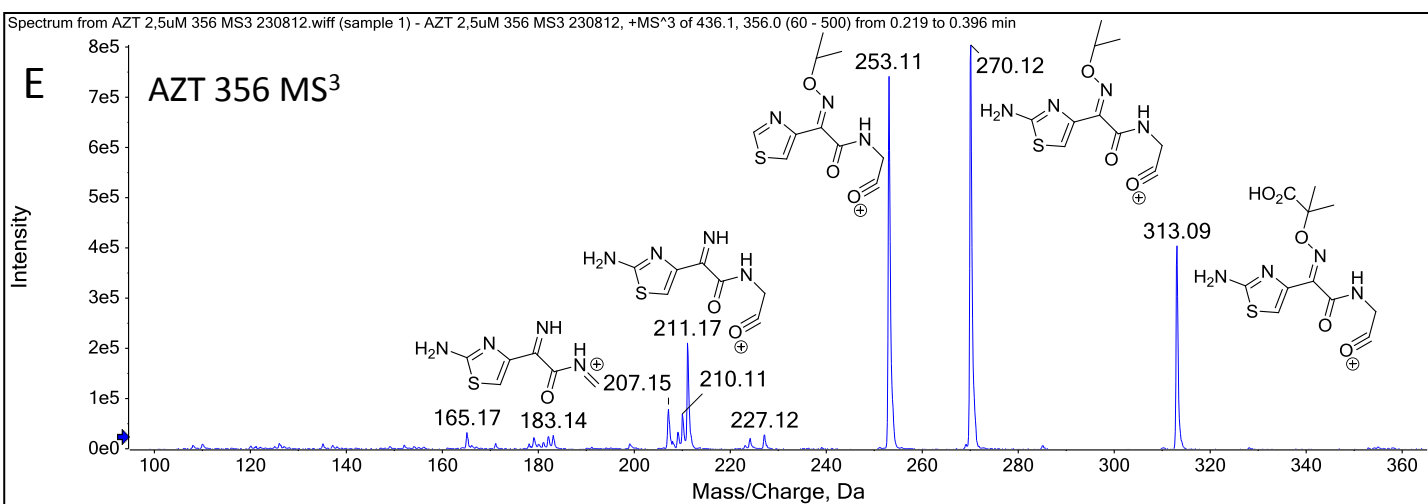
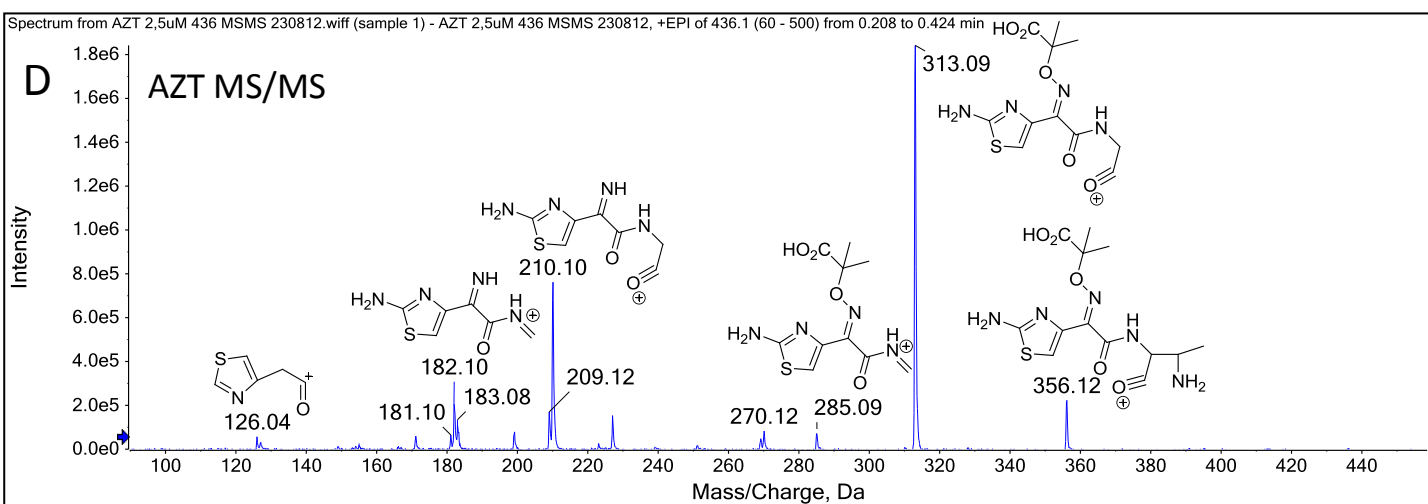
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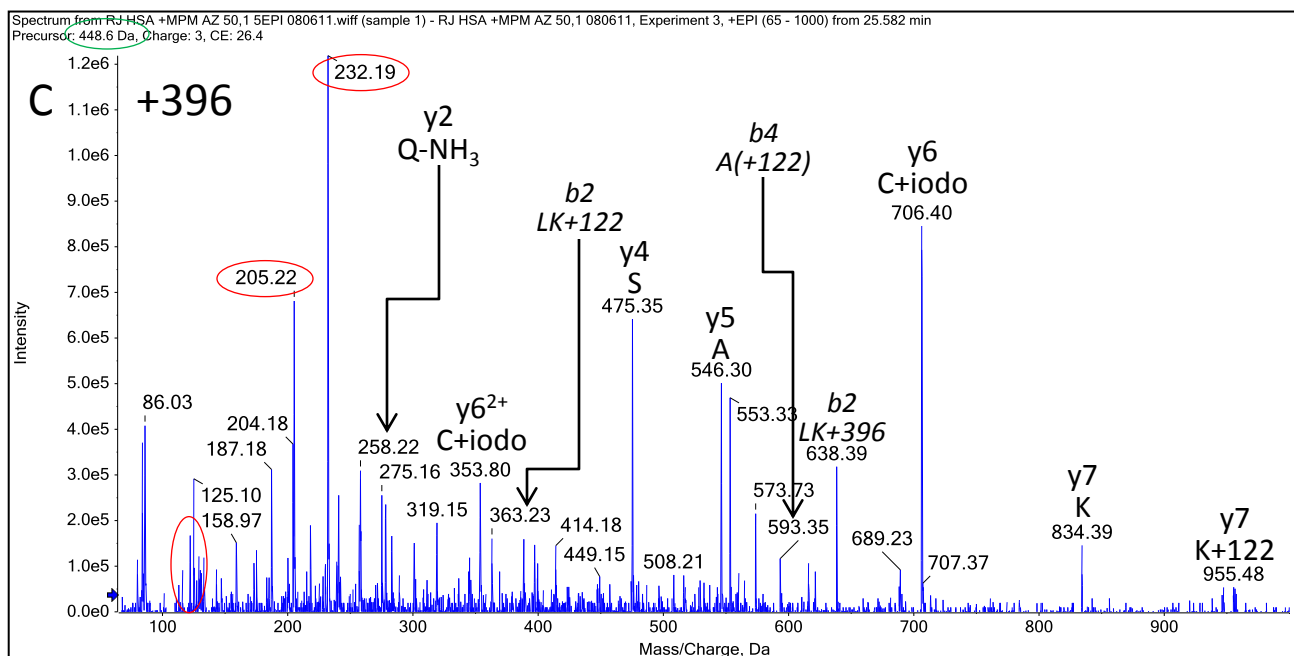
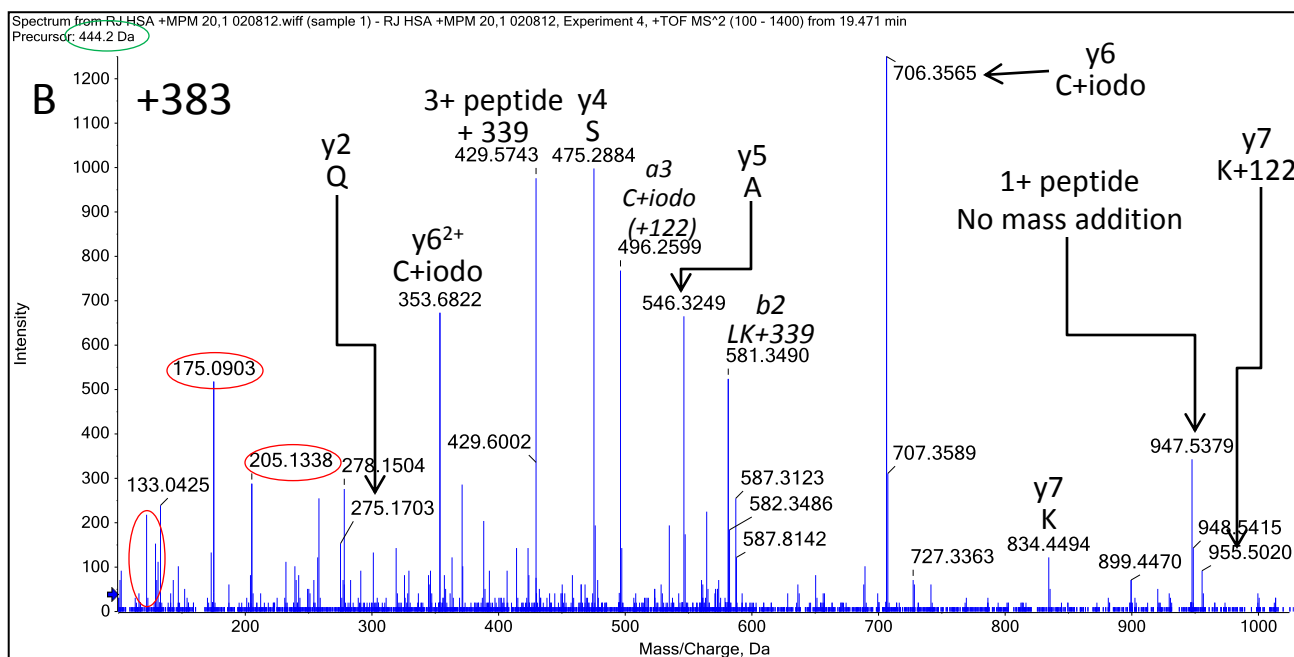
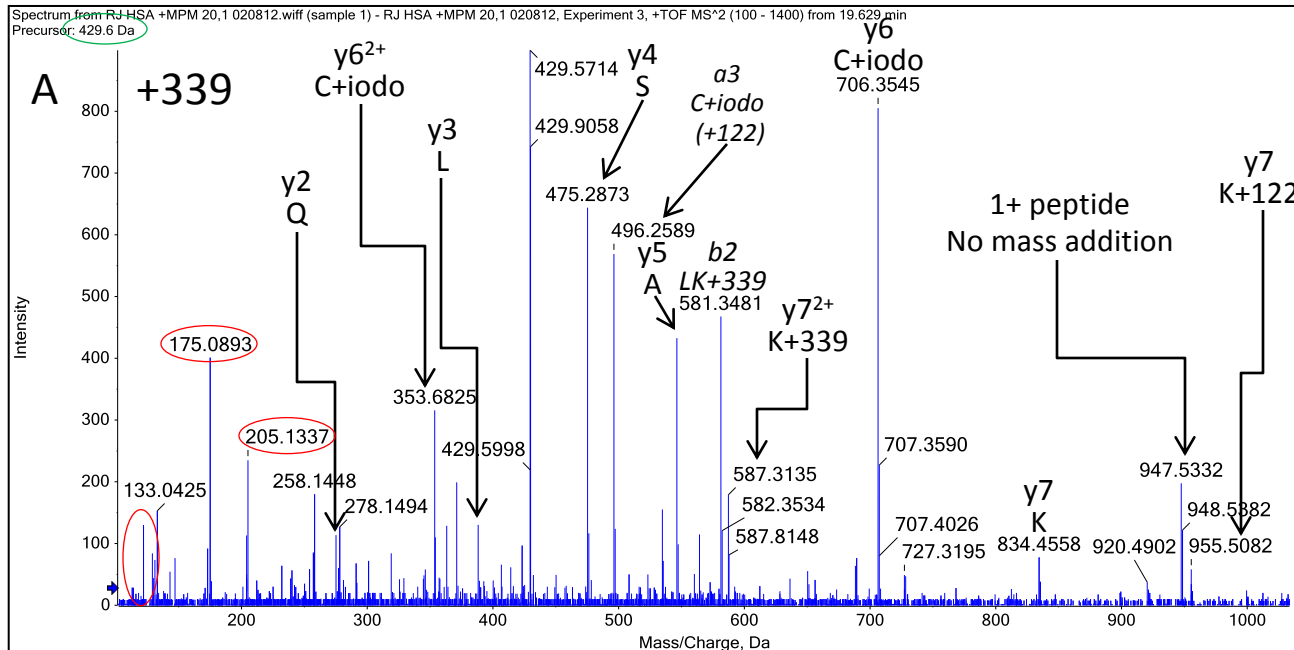


m/z 126[#]

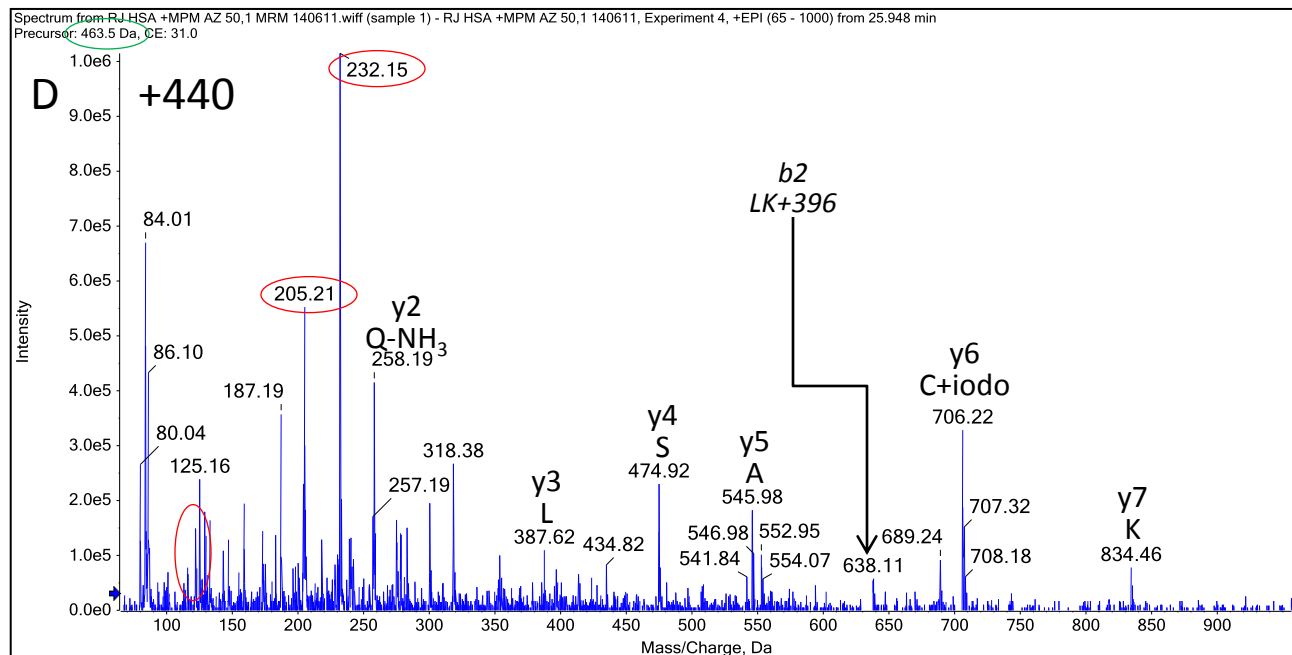


Supplementary Figure 2

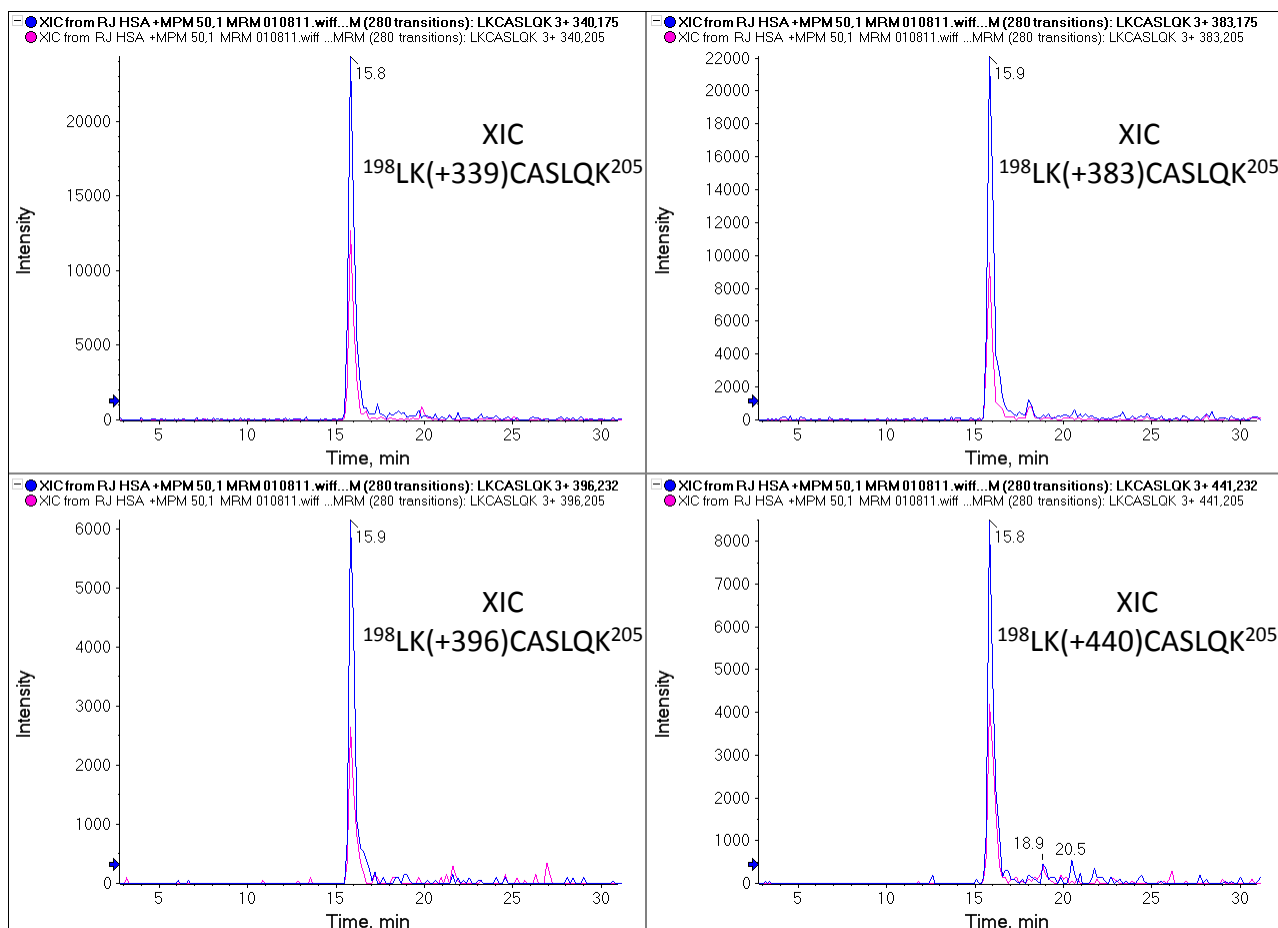


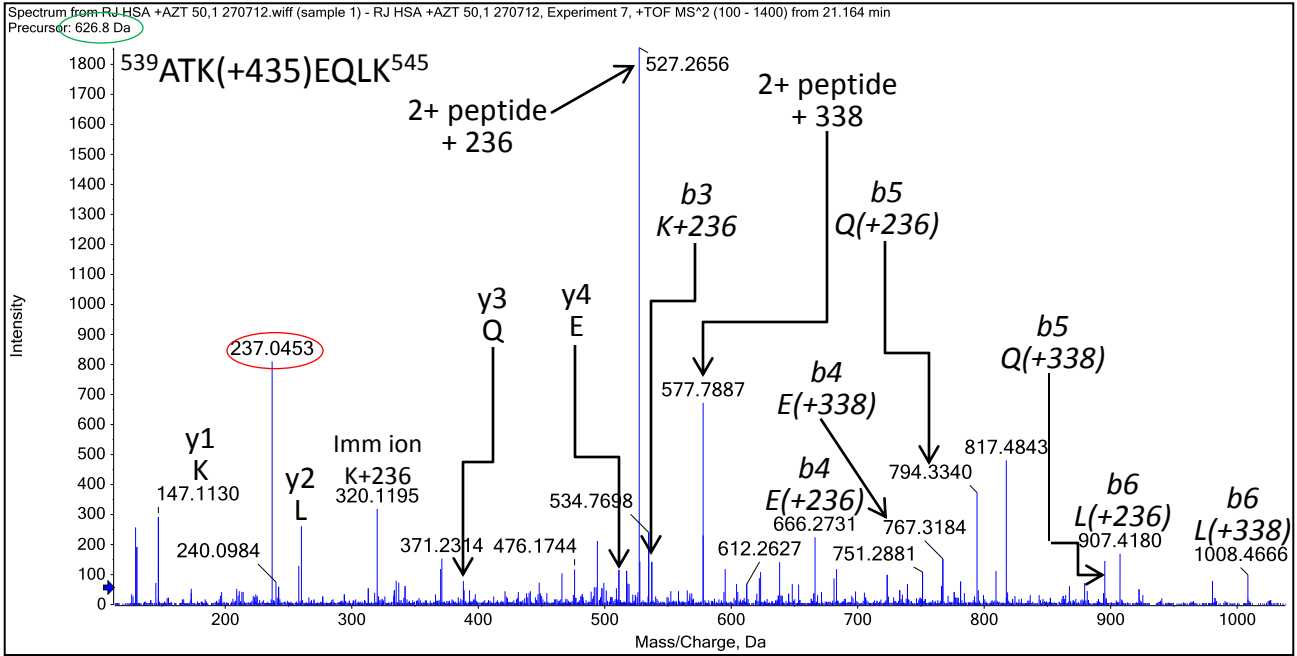


Supplementary Figure 3



E





Supplementary Figure 4