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Running title: Unique Amoxi-Clav Antigen Determinants for T cells

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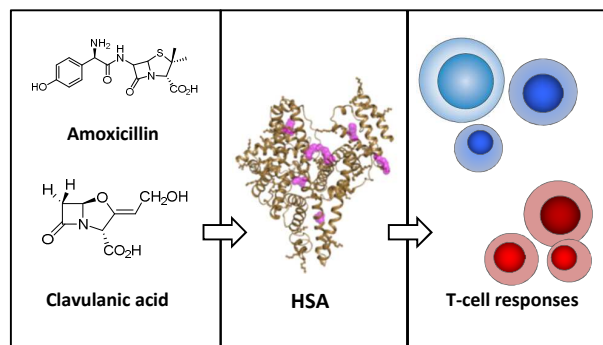Telephone: (+) 44 151 7945559

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TOC

Amoxicillin		
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ABSTRACT

Amoxicillin-clavulanate (AC) is one of the most common causes of drug induced liver injury

(DILI). The association between AC-DILI and HLA alleles and the detection of drug-specific

T cells in patients with AC-DILI indicate that the adaptive immune system is involved in the

disease pathogenesis. In this study, mass spectrometric methods were employed to

characterize the antigen formed by AC in exposed patients and the antigenic determinants

that stimulate T cells. Amoxicillin formed penicilloyl adducts with lysine residues on human

serum albumin (HSA) *in vitro*, with K190 and K199 being the most reactive sites.

Amoxicillin-modified K190 and K199 have also been detected in all patients, and more

extensive modification was observed in patients exposed to higher doses of amoxicillin. In

contrast, the binding of clavulanic acid to HSA was more complicated. Multiple adducts were

identified at high concentrations *in vitro*, including those formed by direct binding of

clavulanic acid to lysine residues, novel pyrazine adducts derived from binding to the

degradation products of clavulanic acid, and a cross-linking adduct. Stable adducts derived

from formylacetic acid were detected in all patients exposed to the drug. Importantly,

analysis of hapten-protein adducts formed in cell culture medium revealed that the highly

drug-specific T-cell responses were likely driven by the markedly different haptenic

structures formed by these two drugs. In this study the unique haptenic structures on albumin

in patients formed by amoxicillin and clavulanic acid have been characterized and shown to

function as chemically distinct antigens which can stimulate separate, specific T-cell clones.

INTRODUCTION

Amoxicillin-clavulanate (AC) is a broad-spectrum antibiotic composed of amoxicillin and the β-lactamase inhibitor, clavulanic acid. AC is one of the most common associations with drug induced liver injury (DILI) in Europe and the US, [1, 2] and the incidence is considerably higher for AC combination prescriptions than for amoxicillin alone, [3, 4] indicating liver injury is directed by the clavulanic acid component. AC is typically associated with cholestatic injury, although mixed and hepatocellular patterns can occur, particularly in younger patients. Risk factors for hepatotoxicity include age (greater than 65 years), gender (female) and repeated course of the antibiotic. [4, 5]

The mechanism of amoxicillin-clavulanate–induced liver injury (AC-DILI) has not been fully defined. However, liver histology has shown infiltrates of lymphocytes, neutrophils and eosinophils in portal tracts in patients with cholestatic hepatitis following AC treatment. [6] Several groups have also shown that AC-DILI was associated with expression of multiple HLA class I and II alleles [7-9] and the phenotypic expression of DILI can be influenced by a specific HLA genotype. For example, HLA class I alleles A*3002 and B*1801 were associated with hepatocellular injury, while the class II allele combination DRB1*1501-DQB1*0602 was associated with cholestatic/mixed type of liver damage. [9, 10] In addition, recent studies by Kim *et. al* [11] have demonstrated that both amoxicillin- and clavulanic acid-specific T lymphocytes were detectable in patients who developed DILI. Collectively, these findings suggested that immune-mediated mechanisms might be involved in the disease. Importantly, amoxicillin and clavulanic acid responsive CD4(+) and CD8(+) T-cell clones were generated from DILI patients with and without HLA risk alleles, and mechanistic analysis suggested that all clones were activated with drug-protein adducts via a pathway dependent on the antigen presenting cells processing machinery. [11]

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Studies by Ariza *et. al* have demonstrated that amoxicillin can form covalent adducts with serum proteins *in vitro*, such as human serum albumin (HSA), transferrin and immunoglobulin heavy and light chains, [12, 13]. Lys190, Ly199 and Lys541 appeared to be the most reactive sites on HSA. However, amoxicillin-modified proteins have not been detected in patients exposed to the drug. Clavulanic acid has been shown to form covalent adducts with *Mycobacterium tuberculosis* β-lactamase, leading to unique and irreversible inhibition. A stable adduct formed by clavulanic acid with a serine residue on the enzyme has been detected, along with several further degradation adducts. [14] However, formation of antigenic adducts that can drive drug specific immune responses and adducts that are actually formed in exposed patients have not been investigated.

Thus, the antigenic determinants of AC formed in patients that could trigger immune responses and subsequently lead to liver injury remain to be determined. The purpose of this study was to (a) explore the molecular basis of amoxicillin and clavulanic acid binding to HSA *in vitro*; (b) define the precise haptenic structures formed by amoxicillin and clavulanic acid in cell culture; (c) determine whether similar adducts could be detected in patients; and (d) provide an immunochemical rationale for independent drug-specific T-cell responses to the two drugs in patients.

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EXPERIMENTAL PROCEDUREs

Chemicals. HSA (97-99% pure) and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO), trypsin from Promega (Madison, WI), liquid chromatography-mass spectrometry (LC-MS) grade solvents from Fisher Scientific UK Ltd (Loughborough, Leicestershire), and all other standard reagents from Sigma-Aldrich.

Patients. Plasma samples were isolated from four patients without adverse drug reactions (ADRs) to AC, and stored immediately at -80℃. Table S1 summarizes the patients' demographics and the clinical features of the ADRs. Approval for the study was acquired from the Liverpool and Leeds local research ethics committees; informed written consent was obtained from each patient.

Concentration-Dependent Modification of HSA by AC *In Vitro*. Amoxicillin or clavulanic acid potassium salt freshly dissolved in potassium phosphate buffer (0.06-12 mM, pH 7.4) was incubated with HSA (0.6 mM, 50 µL) in sealed Eppendorf tubes at 37℃ for 16 h. The molar ratios of drug to protein were 0.1:1, 1:1, 10:1, and 20:1. Protein was precipitated twice with 9 volumes of ice-cold methanol to remove free drug, resuspended in 50 µL phosphate buffer and then reduced with 10 mM dithiothreitol (15 min) and alkylated with 55 mM iodoacetamide (15 min) at room temperature. The protein was precipitated with methanol once more and finally dissolved in 100 µL 50 mM ammonium hydrogencarbonate, and 165 µg (1.25 nmol) of protein was digested with 1.6 µg trypsin overnight at 37℃. The samples were processed for LC-MS/MS analysis as described previously.[15]

Time-Dependent Modification of HSA by AC *In Vitro*. Amoxicillin or clavulanic acid potassium salt freshly dissolved in potassium phosphate buffer (10 mM, pH 7.4) was incubated with HSA (0.6 mM, 300 µL) at 37℃ (final drug concentration, 6 mM). Aliquots of

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50 µL were removed after 0.5, 1, 2, 3, 16 and 24 h and processed for LC-MS/MS analysis as described above.

Mass Spectrometric Characterization of Clavulanic Acid NAL Conjugates. Freshly prepared clavulanic acid solution (10 mM in phosphate buffer, pH 7.4) was incubated with N-acetyl lysine (10 mM) in the presence or absence of NaCNBH$_3$ (1 mM) at 37°C for 16 h. The resulting products were analyzed by LC-MS. Samples were delivered into a 4000 QTRAP (AB Sciex, Framingham, MA,) coupled with a 1260 Infinity Quaternary Pump HPLC system (Agilent Technologies, Santa Clara, CA) and Kinetex C18 column (2.6 µm C18, 100 mm × 2.1 mm, Phenomenex, Macclesfield, Cheshire, U.K.). A gradient from 1% acetonitrile /0.1% formic acid (v/v) to 50% acetonitrile/0.1% formic acid (v/v) in 12 min was applied at a flow rate of 150 µL/min. Data were analyzed using Analyst software, version 1.5.1 (AB Sciex).

Isolation of HSA from Plasma by Affinity Chromatography. HSA was isolated from patients' plasma by affinity chromatography as described previously [16] with slight modifications. An Affinity Removal Column HSA Only (Agilent Technologies) attached to a Vision Workstation (Applied Biosystems) was used to affinity-capture HSA from 50 µL of freshly thawed plasma. The HSA was eluted with 12 mM HCl and precipitated with 9 volumes of ice-cold methanol. The eluted protein was processed for LC-MS/MS analysis as described above.

Mass Spectrometric Characterization of AC-Modified HSA. In order to characterize all potential amoxicillin or clavulanic acid-modified peptides, samples were analyzed using a Q-TOF (AB Sciex) instrument by previous established methods. [17] Modified peptides were identified by filtering for specific fragment ions in PeakView 1.2.0.3 (AB Sciex) and manual inspection of the spectra.

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For the semi-quantitative analysis of amoxicillin or clavulanic acid-modified peptides,

samples were delivered into a QTRAP 5500 (AB Sciex) fitted with a NanoSpray II source by

automated in-line liquid chromatography (U3000 HPLC System, 5 mm C18 nano-precolumn

and 75 μm × 15 cm C18 PepMap column; Dionex, Sunnyvale, CA) via a 10 μm inner

diameter PicoTip (New Objective, Woburn, MA). Multiple reaction monitoring (MRM)

transitions specific for amoxicillin-modified peptides were selected as follows: the *m/z* values

of precursor ions were calculated for all possible peptides containing a lysine residue with a

mass addition of 365 amu together with the characteristic fragment ion of *m/z* 160 derived

from amoxicillin as the product ion. MRM transitions for clavulanic acid-modified peptides

were selected as follows: the *m/z* values of precursor ions were calculated for all possible

peptides containing a lysine residue or histidine with a mass addition of 199, 155, 137, 70,

and 222 amu together with appropriate "*b*" or "*y*" sequence ions as the product ions. 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 the

dwell time was 20 ms. Total ion counts were determined from a second aliquot of each

sample analyzed by conventional LC-MS and were used to normalize sample loading on the

column. Relative quantification of modified peptides was performed by comparing the

relative normalized intensity of MRM peaks for each of the modified residues against total

ion counts across samples. Data were analyzed using Analyst software (AB Sciex).

Western blotting. Aliquots of 5 μg proteins were separated by electrophoresis on a 10%

SDS-polyacrylamide gel, and electroblotted onto nitrocellulose membrane. Non-specific

binding was blocked using Tris/saline/Tween buffer (TST: NaCl, 150 mM; Tris-HCl, 10 mM;

Tween 20, 0.05 %; pH 8.0) containing 10% non-fat dry milk for 16 h at 4°C. The blot was

incubated with primary anti-penicillin antibody (mouse anti-penicillin monoclonal antibody;

AbD Serotec) diluted 1:20,000 in 5% milk/TST for 1 h, followed by incubation with horse

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radish peroxidise conjugated anti-mouse IgG antibody (Abcam) diluted 1:10,000 in 5 %

milk/TST for a further 1 h. Signal was detected by enhanced chemiluminescence (Western

Lightning, PerkinElmer, Boston, USA) using autoradiography film and a GS800 calibrated

scanning densitometer (BioRad, Hemel Hempstead, UK).

Generation of T-cell Clones from Patients with AC-induced Liver Injury. PBMCs were

isolated from patients with AC-DILI. Approval for the study was acquired from the Liverpool

local research ethics committee. PBMCs were cultured at $1x10^6$ cells/well with amoxicillin,

clavulanic acid and amoxicillin-clavulanate (0.1-1.0 mM) in RPMI 1640 supplemented with

10% AB serum (Class A: Innovative Research Inc., Novi, MI), 25 mM HEPES, 10 mM of L-

glutamine and 20 µg/ml of transferrin (Sigma-Aldrich, Gliingham, UK). On days 6 & 9, 200

IU/ml of recombinant interleukin (IL)-2 was added (PeproTech, London, UK). On day 13, T

cells were cloned by serial dilution, as described previously. [18] Epstein-Barr virus (EBV)-

transformed B-cells were used as antigen presenting cells (APCs). APCs were transformed by

adding supernatant from the EBV-producing cell line, B95.8. Clones were cultured for 48 h

and cell proliferation was measured by the addition of [^{3}H]thymidine for the final 16 hours of

the assay (0.5 uCi/well, 5 Ci/mmol; Morovek Biochemicals, Brea, CA). Clones with a

stimulation index of 2 or above were determined to be drug specific.

Concentration-dependent Response of AC-specific T-cell Clones. T-cell clones were

tested for IFN-γ secretion after drug stimulation using ELIspot. T cells ($5x10^4$ cells/well)

were cultured with autologous irradiated APC ($1x10^4$ cells/well) and amoxicillin or

clavulanic acid for 48 h. Prior to developing the ELIspot, cell supernatants were removed for

LC/MS/MS analysis.

RESULTS

Characterization of Amoxicillin-HSA Adducts *In Vitro*. Amoxicillin, like other penicillins, was found to form penicilloyl adducts with lysine residues on proteins (Figure 1A). The MS/MS spectrum of peptide [182]LDELRDEGKASSAK[195] (Figure 1B) confirmed the formation of amoxicillin-HSA adducts *in vitro*. The presence of characteristic fragment ions of *m/z* 160 and *m/z* 349, corresponding to the thiazolidine ring and the loss of amino group of amoxicillin, respectively, and a series of amoxicillin-adducted *b* and *y* ions provided substantial evidence of modification. This is consistent with previous findings. [12] Up to 12 amoxicillin-modified Lys residues on HSA were detected after 24 h (at a drug/protein molar ratio of 20:1), with Lys190 and Lys 199 being the most reactive sites (Figure 1C). The level of modification increased progressively with drug concentration and incubation time (drug/protein molar ratio of 1:1) (Figure 1D).

Characterization of Clavulanic Acid-N-Acetyl Lysine Adducts *In Vitro*. To probe the chemical basis of clavulanic acid's covalent binding to nucleophiles, clavulanic acid was incubated with NAL with or without $NaCNBH_3$ in phosphate buffer (10 mM, pH 7.4). The resulting mixture was analyzed by LC-MS/MS. A total of ten types of adduct were detected when clavulanic acid was incubated with NAL at high concentration (Scheme 1 and 2), including adducts formed by direct opening of beta-lactam ring, novel pyrazine adducts derived from binding to the degradation products of clavulanic acid, and a cross-linking adduct.

The initial attack of the beta-lactam ring of clavulanic acid by NAL would generate an adduct with a mass addition of 199 amu (adduct **A**), however it is quickly decarboxylated to generate the +155 adduct (adduct **B**: *m/z* 344, Figure S1a, Supporting Information). Further reduction of the +155 adduct by $NaCNBH_3$ confirmed the presence of imine functional group (*m/z* 346, Figure S1b, Supporting Information). Adduct **B** can be subsequently hydrolyzed or

dehydrated to generate adduct **C** and **D** respectively (*m/z* 362 and *m/z* 326, Figure S1c and d,

Supporting Information). Further degradation of adducts **A-D** provided either adduct **E**

(Δm=70 amu, *m/z* 259, Figure S1e) or cross-linking adduct **H** that can be stabilized by

NaCNBH$_3$ (Δm=54 amu, *m/z* 431, Figure S1f). Another two adducts with mass addition of 70

amu were also detected (adduct **G/F**, *m/z* 259, Figure S1g), which can be reduced by

NaCNBH$_3$ (*m/z* 261, Figure S1h), indicating the presence of an imine functional group.

Further mass spectrometric fragmentation suggested that these adducts were formed by the

reaction of NAL with formylacetic acid, a degradation product derived from clavulanic acid.

Clavulanic acid is known to undergo spontaneous hydrolysis under neutral or basic

conditions, and pyrazine end-products can be formed via the reactive amino ketone (Scheme

2). A pyrazine product (*m/z* 223, Figure 2A) was detected when calvulanic acid was

incubated with NAL under neutral conditions (10 mM phosphate buffer, pH 7.4). In addition,

two types of NAL-pyrazine adducts were also detected: adduct **I** with mass addition of 222

amu and adduct **J** with mass addition of 310 amu (*m/z* 411 and *m/z* 499, Figure 2B and C).

Characterization of Clavulanic Acid-HSA Adducts *In Vitro*. To characterize the putative

protein adducts formed by clavulanic acid, HSA was incubated with a range of molar ratios

of clavulanic acid (1:1 to 50:1). LC-MS/MS analysis of the tryptic digests revealed seven

types of adducts, including direct binding of clavulanic acid to lysine residues, subsequent

degradation products, and pyrazine adducts on lysine and histidine residues (Table I).

The initial addition of clavulanic acid to lysine in HSA resulted in an adduct with a mass

addition of 199 amu. Figure 3A shows a representative MS/MS spectrum for a triply charged

ion at *m/z* 573.28, corresponding to the tryptic peptide [182]LDELRDEGKASSAK[195] with an

additional mass of 199 amu. The peptide sequence was confirmed by fragmentations that

generated partial singly charged *y* and *b* series ions. The modification site was confirmed by

*b*12* (*m/z* 750.89) and *y*13*(*m/z* 794.39) with adduction of 199 amu. The presence of a

fragment ion of *m/z* 182 that derived from dehydrated clavulanic acid provided further

evidence of the modification. In addition to modification on Lys190, two more sites (Lys414

and 525) were also modified with full mass addition (Table I). The +199 adducts had

undergone further degradation to form the +154 and +137 adducts, which were detected at

multiple sites on HSA (Figure S2a and b, Table I). The end adducts appeared to be quite

stable and have a mass addition of 70 amu. A typical MS/MS spectrum representing the

tryptic peptide [525]KQTALVELVK[534] with a mass addition of 70 amu is shown in Figure 3B.

The mass increment of 70 amu could be attributed to adduct **E** or **F/G**, however,

simultaneous observation of the reduced +72 adducts indicates that the +70 adducts are most

likely derived from formylacetic acid (Figure S2c). A total of eleven lysine residues and one

histidine residue were found to be adducted with 70 amu when clavulanic acid was incubated

with HSA at a molar ratio of 20:1 (Table I).

Similar to the modifications observed with NAL, stable adducts derived from pyrazine were

also detected on multiple sites including eleven lysine residues and two histidine residues

(Table I). Figure 3C shows a representative MS/MS spectrum for a triply charged ion at *m/z*

707.72, corresponding to the tryptic peptide [145]RHPYFYAPELLFFAK[160] with an additional

mass of 222 amu. The observation of a fragment ion of *m/z* 223 and the subsequent fragment

ions (*m/z* 205 and 193) indicates the presence of pyrazine modification. In addition, the

modification site was confirmed by *b*4* (2+, *m/z* 388.7), *b*5* (2+, *m/z* 462.3), and *b*5* (*m/z*

1086.6), all with a mass addition of 222 amu, giving evidence of modification at His146

(Figure 3C).

Concentration Dependence of HSA Modification with Clavulanic Acid. The modification

of HSA by clavulanic acid was found to be concentration-dependent. A semi-quantitative

analysis of modification at each site was performed by determining the area under the curve

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for the extracted masses of the modified peptides, followed by normalization of the ion

intensity against the total ion count for the sample. Notwithstanding the disparity in the

ionization efficiency of the peptides, the epitope profile (the relative abundance of clavulanic

acid-modified peptides) revealed that the pyrazine adducts were preferentially formed on

Lys159, 212 and 525; whereas Lys190 was more likely targeted by formylacetic acid (Figure

4A and B). The total level of the pyrazine adducts increased with increased molar ratio of

drug to protein, whereas the total level of the +70 adducts declined when the concentrations

of clavulanic acid increased, indicating pyrazine adducts were formed predominately with

high concentration of clavulanic acid (Figure 4C and D). A concentration-dependent increase

in normalized ion counts was also observed for each modified peptide (Figure 4E and F),

except for the +70 Lys190 adduct.

Effects of Clavulanic Acid on Covalent Binding of Amoxicillin to HSA. In order to

determine the effect of clavulanic acid on amoxicillin covalent binding, clavulanic acid was

co-incubated with amoxicillin and HSA at different concentrations (0.625-2.5 mM). There

was only a slight difference with respect to the epitope profiles of amoxicillin binding to

HSA in the presence of clavulanic acid (Figure S3A). The optimal binding of amoxicillin to

HSA was found when incubated with amoxicillin and clavulanic acid at the molar ratio of

2:1, indicating there was a balance in the role of clavulanic acid between preventing

amoxicillin hydrolysis and competitive binding to proteins. These findings were also

confirmed by Western blot data (Figure S3B).

Modification of HSA by Amoxicillin-Clavulanic Acid in Patients. We next investigated

whether the same adducts could be formed in patients receiving AC therapy. HSA was

isolated from four patients receiving AC either orally or intravenously, and AC-modified

albumin was detected in all patients (Table II). Amoxicillin modification of HSA was

detected at up to 8 lysine residues in patients receiving higher doses of AC, and amoxicillin-

modified K190 was detected in all patients. Interestingly, MS/MS analysis also revealed a

novel adduct that was formed by amoxicillin dimer (Figure 5A). The observed triply charged

ion of *m/z* 750.34 corresponds to peptide [182]LDELRDEGKASSAK[195] with a mass increment

of 730 amu, which could be assigned as two molecules of amoxicillin. The presence of

characteristic fragment ions derived from amoxicillin (*m/z* 160, *m/z* 217, and *m/z* 349, Figure

1A) confirmed the modification. The modification was further substantiated by the observed

triply charged ion of *m/z* 744.68, corresponding to 2 amoxicillin adducted peptide with

elimination of amino group, and doubly charged ion of *m/z* 1045.49 that could be assigned as

2 amoxicillin adducted peptide after losing the thiazolidine group.

The profile of clavulanic acid-modified peptides in patients is less complicated than *in vitro*.

Only the +70 adducts were detected, with Lys190 adducts being detected in all patients

(Table II). Figure 5B shows a typical MS/MS spectrum for a triply charged ion of *m/z*

530.27, corresponding to peptide [182]LDELRDEGKASSAK[195] with modification at Lys 190.

Interestingly, several piperacillin-modified peptides have also been detected in these patients

who had stopped the medication long before the bloods were taken (Table II and Figure S4),

indicating drug protein adducts can remain in the body for a long time period.

Drug-Antigen Specificity of T-Cell Clones. Proliferation of amoxicillin and clavulanic acid-

responsive CD4+ and CD8+ clones and the release of IFN-γ was drug concentration-

dependent. Responses were detected between 0.5 and 2 mM for amoxicillin, while the level

of clavulanic acid required to trigger responses was much lower than that of amoxicillin,

ranging from 50-200 μM (Figure 6A and B). Clones were tested for reactivity against

amoxicillin and clavulanic acid, and the response to each drug was found to be highly

specific (results not shown). Mass spectrometric analysis of covalent binding of drugs to

albumin in cell culture medium demonstrated that amoxicillin bound the same group of lysine

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residues that were identified *in vitro* and in patients (Figure 6C). In addition, the +70 adduct

and pyrazine adducts derived from clavulanic acid were also detected when cells were

exposed to clavulanic acid (Figure 6D).

DISCUSSION

Accumulative data suggests that immune-mediated mechanisms are involved in AC-DILI. In particular, recent findings indicated that AC responsive T-cell clones are activated by a hapten mechanism involving the formation of hapten protein adducts followed by antigen processing.[11] To probe the molecular and immunochemical mechanisms involved in AC-DILI we have investigated the haptenation of HSA by amoxicillin and clavulanic acid *in vitro* and in patients. Using advanced mass spectrometric techniques, we have demonstrated that amoxicillin and clavulanic acid form distinct multiple haptenic structures with HSA *in vitro*. More importantly, similar adducts were detected in patients receiving AC therapy, and AC responsive T-cell clones were activated by the same adducts formed in cell culture.

The chemistry of amoxicillin binding to proteins is relatively straightforward and similar to other penicillins that have been investigated by mass spectrometry both *in vitro* and *in vivo*. [17, 19-21] Amoxicillin is prone to nucleophilic attack at the β-lactam ring to form covalent adducts with several, but not all, of the lysine residues in albumin. Amoxicillin lysine adducts were detected in various serum proteins *in vitro,* [12] however, neither the amoxicillin adducts formed in exposed patients nor the functions of these protein adducts has been investigated. In this study, up to eleven amoxicillin-modified lysine residues in albumin were detected in exposed patients. Furthermore, adducts formed by an amoxicillin-dimer were seen in the same patients. This observation is important since amoxicillin polymers could possess strong antigenic properties. [22, 23] Following antigen processing, a range of amoxicillin-modified HLA class I (8-12mers) and class II binding peptides (9-25mers) will be generated that may interact preferentially, but not exclusively, with proteins encoded by the HLA risk alleles identified in genetic studies (e.g., HLA-A*02:01, DRB1*15:01 and DQB1*06:02). [9]

In contrast, the formation of potential antigenic adducts with clavulanic acid is much more complex. Clavulanic acid also contains a beta-lactam ring; however, it has important chemical differences to amoxicillin: first, it lacks a side chain; and second, the thiazolidine ring has been substituted by an oxazolidine. The structural differences may result in the formation of antigenic determinants, which are, in an immunochemical sense, quite distinct from those formed from β-lactam antibiotics. Clavulanic acid undergoes hydrolysis under neutral or basic condition, at a rate that is approximately 10 times faster than the hydrolysis of penicillin G. [24] The initial hydrolysis product is unstable: further decomposition products might be formed by the fission of the C-O bond on the oxapenam ring, decarboxylation, and/or hydrolytic elimination of formylacetic acid. [25] The degradation of clavulanic acid is self-accelerated at higher concentration because clavulanic acid and its decomposition products act as similarly strong general-base catalysts. [26] This is consistent with our observation that the pyrazine-type products were preferentially formed at high concentrations of clavulanate. Similar to the hydrolysis, the initial attack of a lysine residue on the beta-lactam ring of clavulanate results in an unstable adduct, which would generate multiple haptenic adducts following further decomposition. These adducts are unique and have markedly different structures to amoxicillin haptenic adducts. Analysis of the proliferative response and cytokine release from T-cell clones revealed that there was no cross-reactivity between amoxicillin- and clavulanic acid. Our mass spectrometric data showing divergent haptenic structures on protein provides a chemical explanation for the drug-specific T-cell responses.

Notably, a total of seven types of clavulanate-HSA adducts were identified in *in vitro* incubations. Interestingly, both the +70 adducts and pyrazine adducts were also identified in cell culture medium. Furthermore, the +70 adducts were also detected in exposed patients, reaffirming the relevance of *in vitro* studies. However, it is important to bear in mind that

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although *in vitro* incubation is necessary for identification of all possible adducts, the *in vitro*

data do not always mirror what occurs in patients. Considering the relatively long half-life of

HSA *in vivo* (21 days), [27] the multiple doses of AC taken by patients, and the stability of the

adducts, it is plausible that only adducts with great plasma stability would have accumulated

in the plasma, leading to different binding profiles than that occurring *in vitro*. Likewise, the

clavulanate-HSA adducts may be different from the acute binding of clavulanate to hepatic

proteins, which may be responsible for AC-DILI. It is therefore important to develop

sensitive methods for characterization of clavulanate adducts formed in liver and investigate

the role of these adducts in AC-DILI.

The hydrolysis of beta-lactam antibiotics by bacterial beta-lactamase is the major cause of

bacterial resistance to this group of compounds, and the combination of amoxicillin and

clavulanic acid has an increased spectrum of action and restored efficacy against amoxicillin-

resistant bacteria that produce β-lactamase. However, the addition of clavulanic acid to

amoxicillin increases the risk of DILI. [28] The most probable explanation for the increased

DILI risk is that the addition of clavulanic acid generates novel antigenic determinants that

are distinct from those generated by amoxicillin alone, thus increasing the chemical repertoire

of antigenic structures in patients which in turn may increase the possibility of immune

recognition in an HLA restricted manner. Additional mechanisms suggested by the present

study include selective targeting of the liver by clavulanic acid (or amoxicillin), and increased

hepatic stress signaling by reactive degradation products of clavulanic acid, such as

formylacetic acid, which could promote co-stimulatory signaling and the recruitment of

inflammatory cells. Future studies will require novel model systems to investigate the

relationship between amoxicillin and/or clavulanic acid antigen formation in the liver and the

activation of cytolytic T cells that target the liver. However, it is important to note that the

greater the varieties of antigens that are generated by a drug (combination) there will be a

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paradoxical decrease in the potential for highly selective HLA restriction and thus the

possibility of genotyping patients to predict those at enhanced risk of an adverse drug

reaction.

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Notes

The authors declare no competing financial interest.

Supporting Information

Additional figures (Figure S1-S3) show the formation of clavulanic acid N-acetyl lysine

adducts and amoxicillin and protein adducts. This material is available free of charge via the

Internet at http://pubs.acs.org.

ABBREVIATIONS

AC, Amoxicillin-clavulanate; DILI, drug-induced liver injury; NAL, N-α-acetyl-lysine; LC-

MS/MS, liquid chromatography-tandem mass spectrometry; HSA, human serum albumin;

ADR, adverse drug reactions; MRM, multiple reaction monitoring.

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Table 1. Amoxicillin and Clavulanic acid-modified albumin peptides detected *in vitro*[a]

Lysine	Peptides	Amox adducts	Clavulanic acid adducts				
			A Δm=199/181	B Δm=155	D Δm=137	E/F Δm=70	I Δm=222
K12	FK*DLGEENFK	√			√	√	√
K137	K*YLYEIAR	√		√	√	√	√
K159	RHPYFYAPELLFFAK*R					√	√
K162	YK*AAFTECCQAADK	√					
K190	LDELRDEGK*ASSAK	√	√	√	√	√	√
K195	ASSAK*QR						√
K199	LK*CASLQK	√			√	√	√
K212	AFK*AWAVAR	√			√	√	√
K351	LAK*TYETTLEK	√			√	√	√
K414	K*VPQVSTPTLVEVSR		√		√	√	√
K432	NLGK*VGSK	√				√	√
K436	VGSK*CCK	√					
K475	VTK*CCTESLVNR	√					
K525	K*QTALVELVK	√	√		√	√	√
K541	ATK*EQLK	√				√	√
H146	RH*PYFYAPELLFFAK					√	√
H338	RH*PDYSVVLLLR						√

[a]HSA was incubated with amoxicillin or clavulanic acid at drug protein molar ratio of 20:1 for 24 h, tryptic peptides of HSA were characterized by LC-MS/MS

Table 2. Amoxicillin and Clavulanic acid-modified albumin peptides detected in patients[a]

Patient	Modification detected		
	Amoxicillin	piperacillin	Clavulanic acid
P3 (ML)	K190	K190, K541	K190
P1 (ST)	K190	K190	K190
P2 (TS)	K541, K432, K190, K351, K525, K199	K541, K190	K190, K199, K525
P4 (79/F)	K541, K432, K190, K351, K525, K199, K137	K541, K190, K195, K432	K190, K199, K525

[a]The drug-modified tryptic peptides of HSA isolated from the patients' plasma were characterized by LC-MS/MS

FIGURE LEGENDS

Figure 1. Characterization of amoxicillin-HSA adducts formed *in vitro*. Chemical structure of penicilloyl adduct formed by amoxicillin and HSA (A); representative MS/MS spectrum of the albumin peptide [182]LDELRDEGKASSAK[195] modified at Lys190 with amoxicillin, characteristic fragment ions derived from partial cleavage of the hapten are circled (B); epitope profile showing the lysine residues of albumin modified after 24 h incubation at drug/protein molar ratio of 10:1 (C); the level of modification is concentration- and time-dependent (drug/protein molar ratio 1:1) (D).

Figure 2. LC-MS/MS analysis of clavulanic acid degradation products and N-acetyl lysine adducts identified *in vitro* incubation in phosphate buffer (pH 7.4). MS/MS spectra showing the formation of pyrazine degradation product (A); a N-acetyl lysine adduct with mass addition of 222 amu (B); a N-acetyl lysine adduct with a mass addition of 310 amu (C).

Figure 3. LC-MS/MS analysis of clavulanic acid modified HSA peptides identified *in vitro*. MS/MS spectra showing peptide [182]LDELRDEGKASSAK[195] was modified by clavulanic acid at Lys190 with a mass addition of 199 amu, indicating a full molecule was attached to Lys190 (A); peptide [525]KQTALVELVK[534] was modified at Lys525 with a mass addition of 70 amu (B); peptide [145]RHPYFYAPELLFFAK[159] was modified at His146 with a mass addition of 222 amu, indicating the formation of a pyrazine adduct (C).

Figure 4. LC-MS/MS analysis of clavulanic acid-modified albumin peptides identified *in vitro* incubation with HSA. Clavulanic acid was incubated with HSA in phosphate buffer (10 mM, pH7,4) at molar ratio of 10:1. Epitope profile showing the amino acid residues of albumin modified in vitro with a mass addition of 222 amu (A) and 70 amu (B); the total level of modification is concentration dependent (C and D for the +222 and the +70 adducts,

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respectively); concentration-dependent modification of individual amino acid residue was

also observed (E and F)

Figure 5. LC-MS/MS analysis of modified HSA peptides identified in patients receiving

amoxicillin-clavulanic acid therapy. MS/MS spectra showing peptide

[182]LDELRDEGKASSAK[195] was modified at Lys190 with a mass addition of 730 amu,

indicating an amoxicillin dimer was attached to Lys190 (A); clavulanic acid-modified peptide

[182]LDELRDEGKASSAK[195] with a mass addition of 70 amu (B).

Figure 6. **Activation of T-cell clones with amoxicillin and clavulanic acid and analysis of**

drug-protein binding in culture medium. IFN-γ ELIspot of amoxicillin and clavulanic acid

specific clones were generated from patient PBMCs. Drug specific clones were selected

based upon a stimulation index of 2-fold or higher. Clones A6, A18, C23 and C30

demonstrated IFN-γ cytokine release and proliferation in a dose dependent manner (A,B); the

epitope profile shows the lysine residues of albumin modified by amoxicillin (C) and

clavulanic acid (D) under cell culture conditions

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SCHEME LEGENDS

Scheme 1. Proposed pathways for the formation of clavulanic acid-protein adducts

Scheme 2. Pathways for the degradation of clavulanic acid (A)[24, 25] and the formation of

pyrazine adducts in phosphate buffer (B).

Scheme 1. Proposed pathways for the formation of clavulanic acid-protein adducts
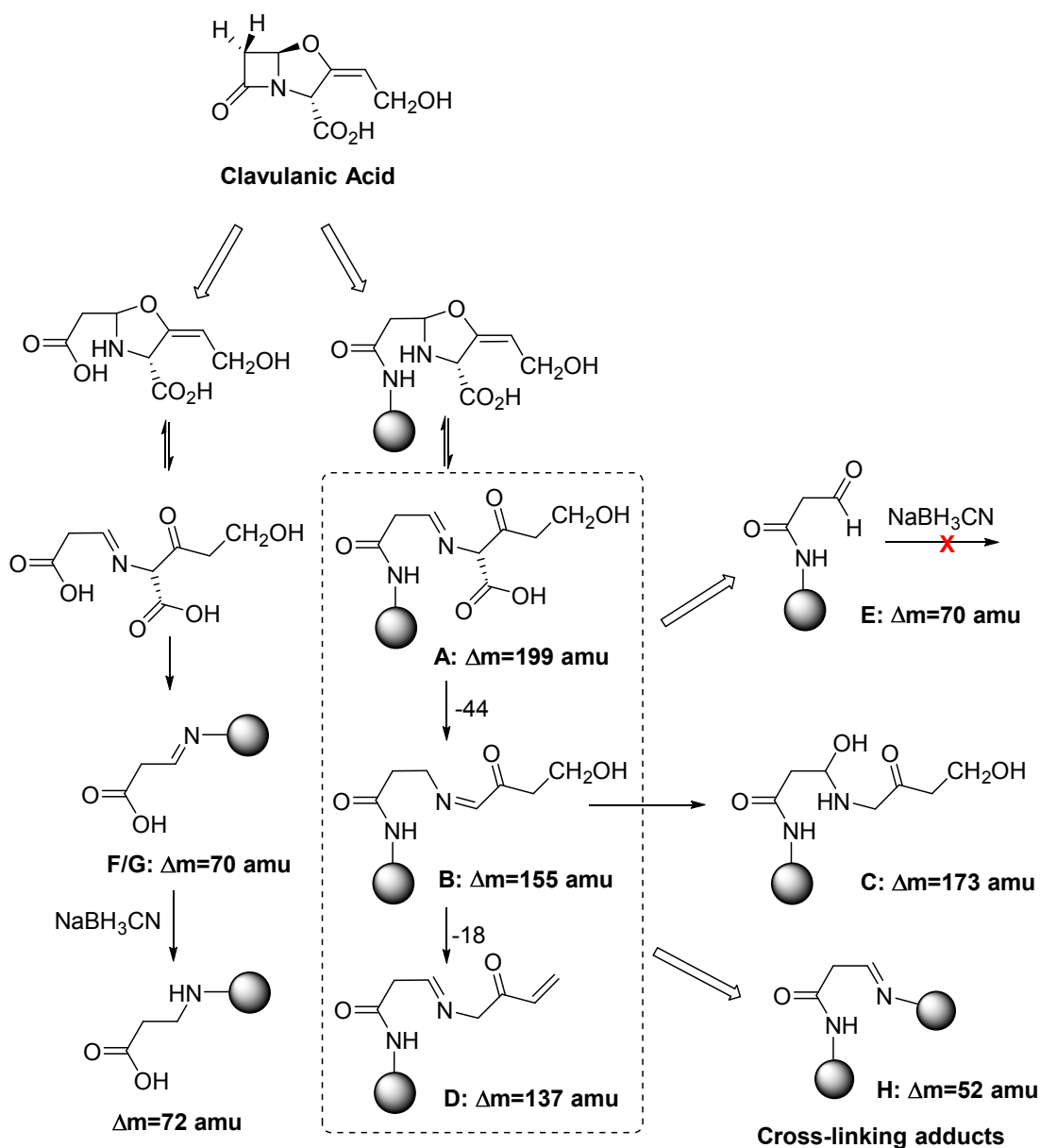
Scheme 2. Pathways for the degradation of clavulanic acid (A)[24, 25] and proposed pathways

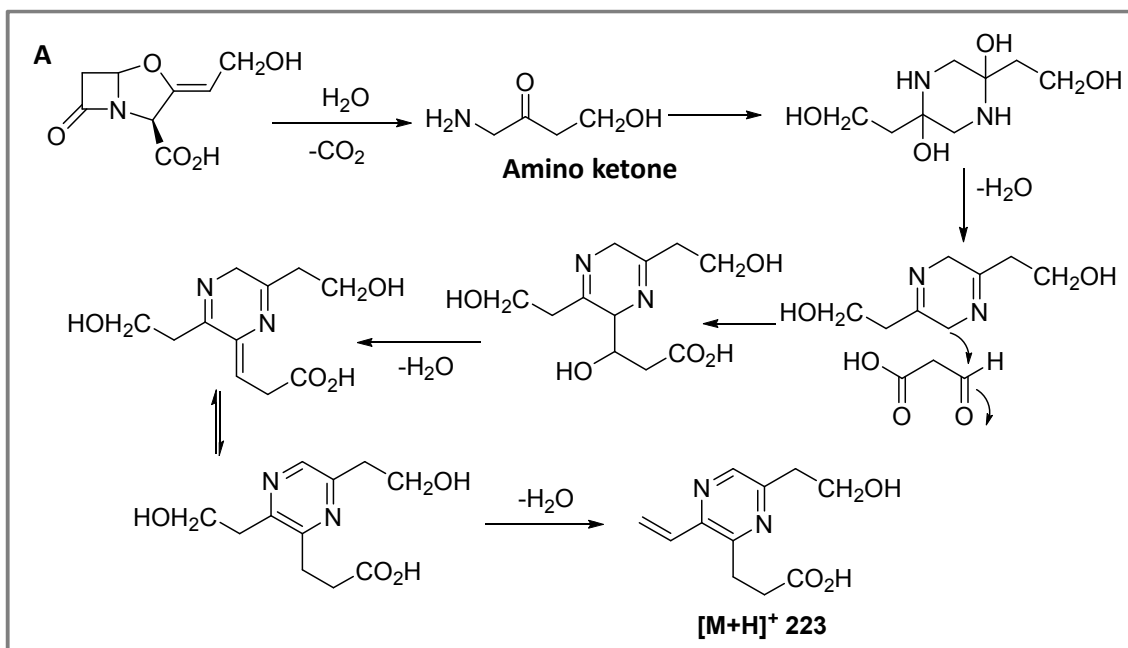for the formation of pyrazine N-acetyl lysine adducts in phosphate buffer (B).
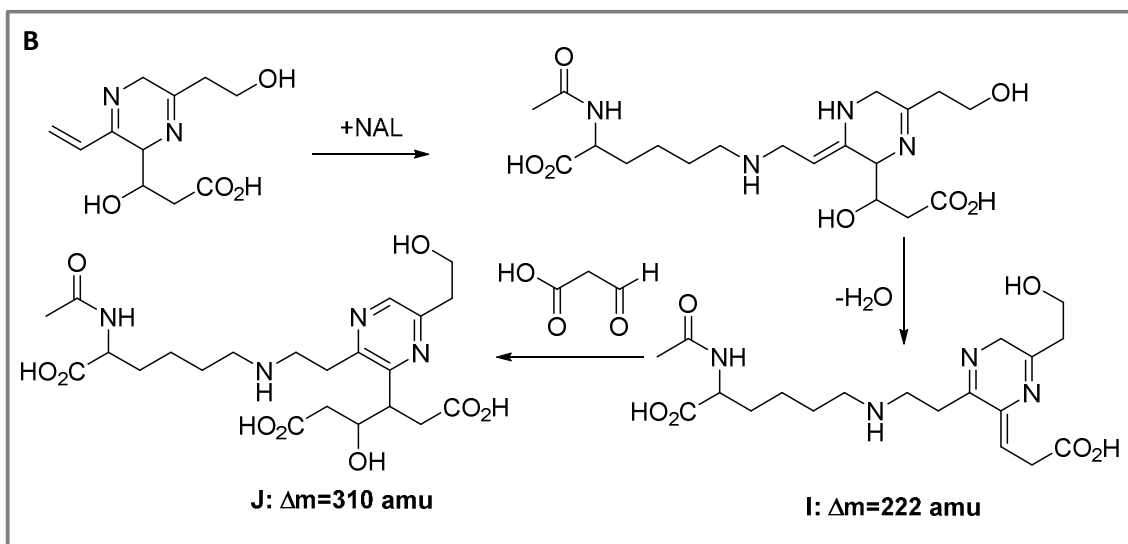
Figure 1

A

Amoxicillin
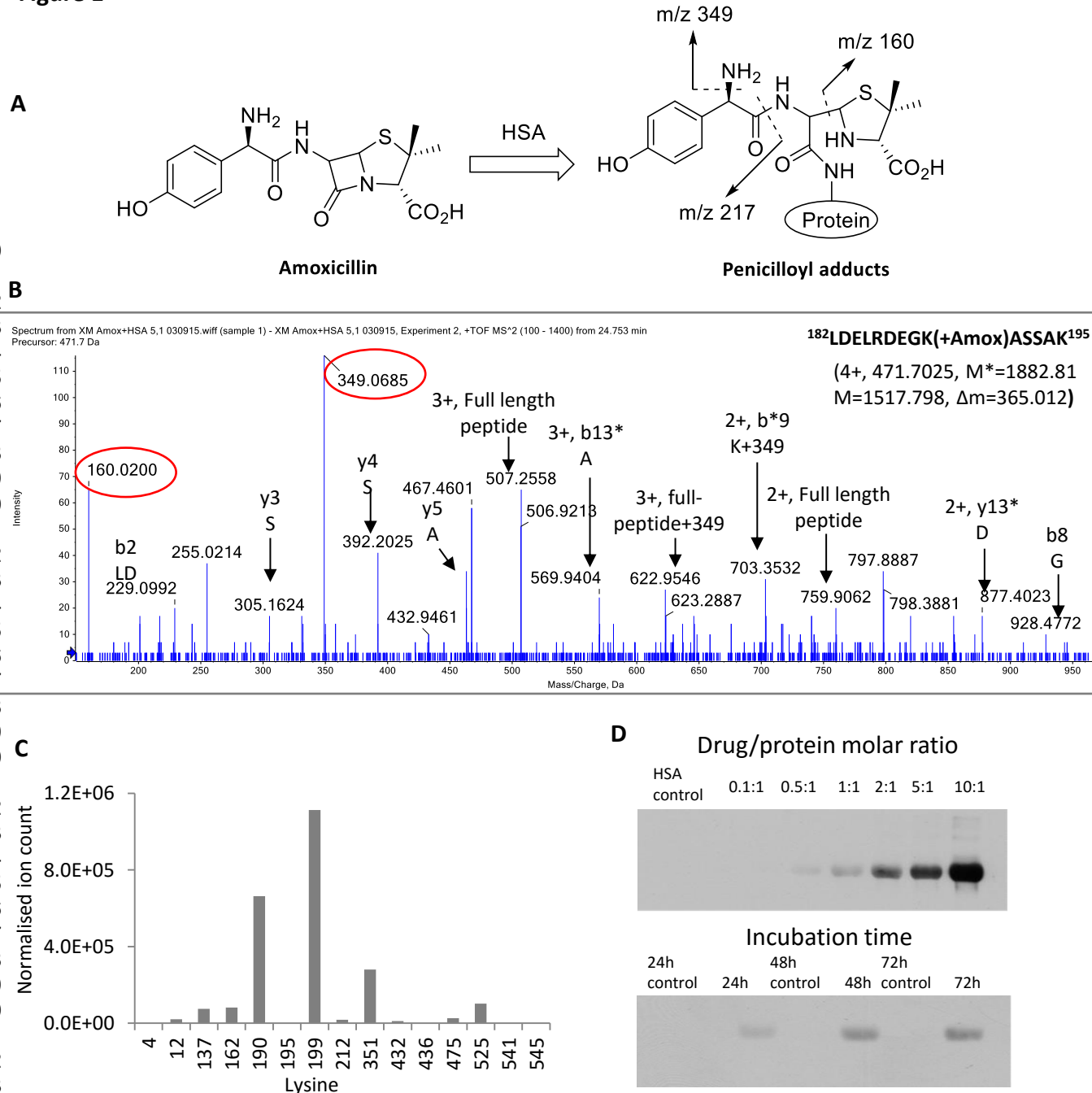
Penicilloyl adducts

B

C

D

Figure 1. Characterization of amoxicillin-HSA adducts formed *in vitro*. Chemical structure of penicilloyl adducts formed by amoxicillin and HSA (A); representative MS/MS spectrum of the albumin peptide [182]LDELRDEGKASSAK[195] modified at Lys190 with amoxicillin, characteristic fragment ions derived from partial cleavage of the hapten are circled (*indicates drug modification) (B); epitope profile showing the lysine residues of albumin modified after 24 h incubation at drug/protein molar ratio of 10:1 (C); the level of modification is concentration-and time-dependent (drug/protein ratio of 1:1) (D).
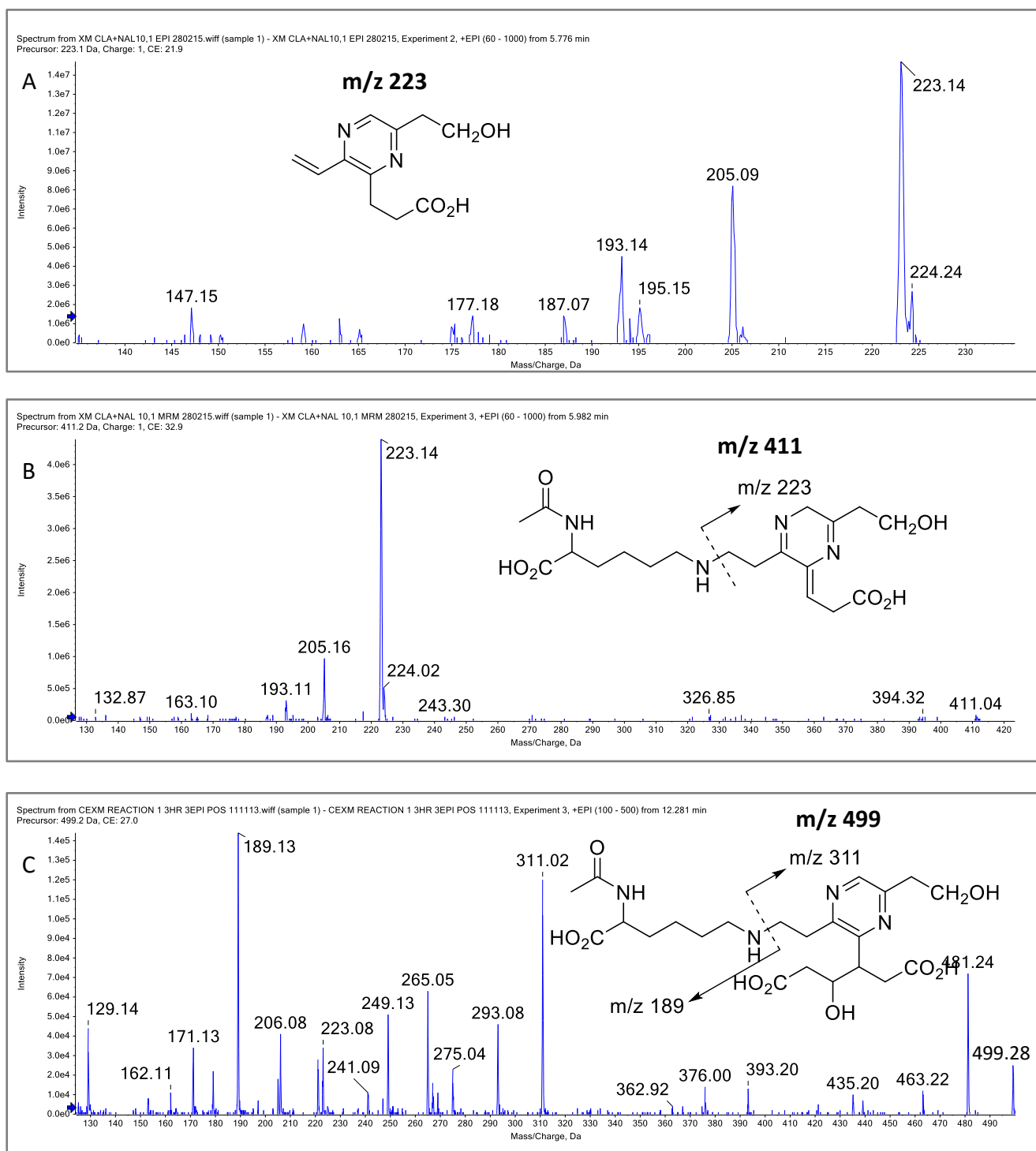
Figure 2

Figure 2. **LC-MS/MS analysis of clavulanic acid degradation products and N-acetyl lysine adducts identified *in vitro* incubation in phosphate buffer (pH 7.4).** MS/MS spectra showing the formation of pyrazine degradation product (A); a N-acetyl lysine adduct with mass addition of 222 amu (B); a N-acetyl lysine adduct with a mass addition of 310 amu (C).
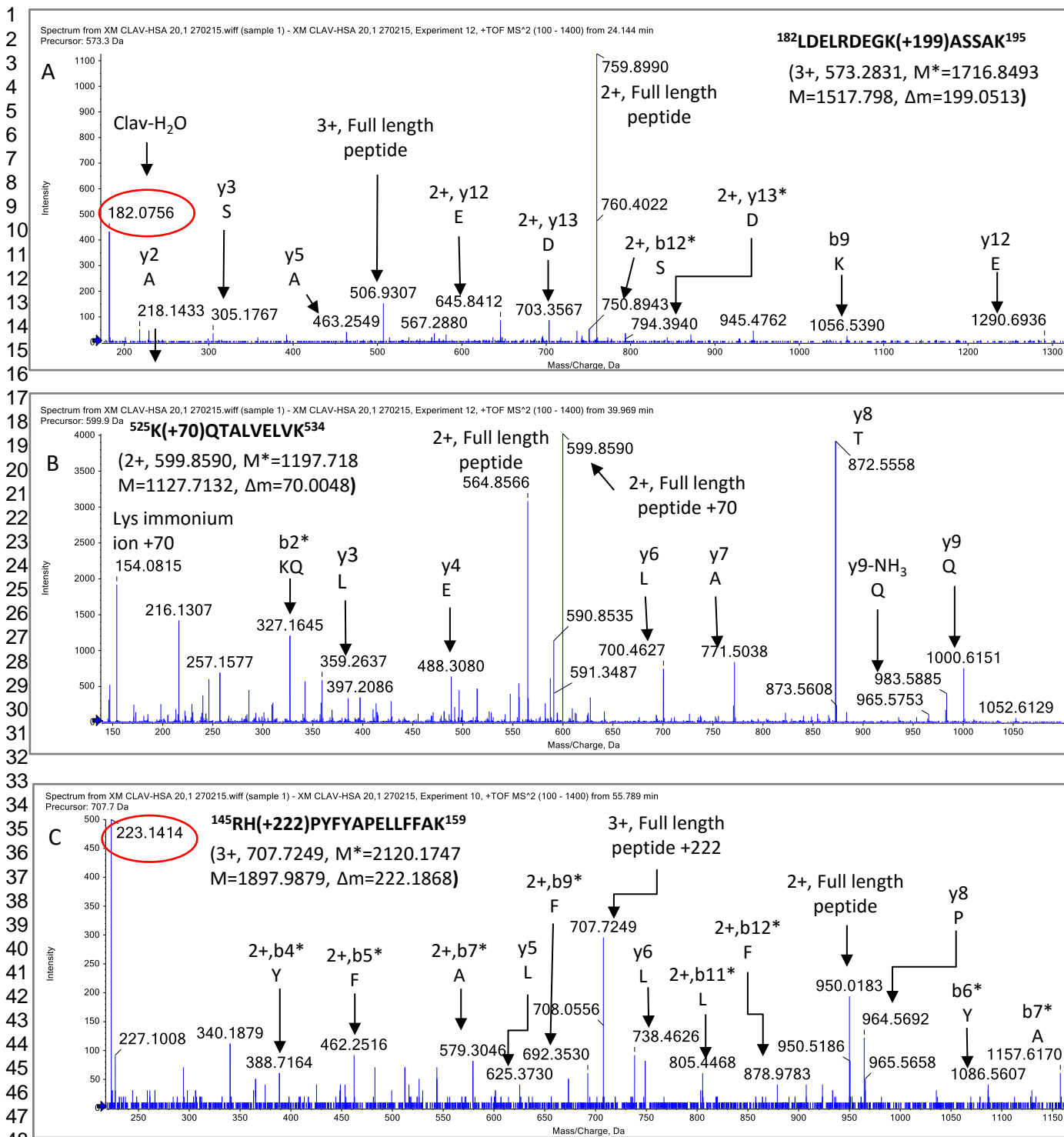
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[145]RHPYFYAPELLFFAK[159] was modified at His146 with a mass addition of 222 amu, indicating the
formation of a pyrazine adduct (C). (* indicates drug modification).

Figure 4

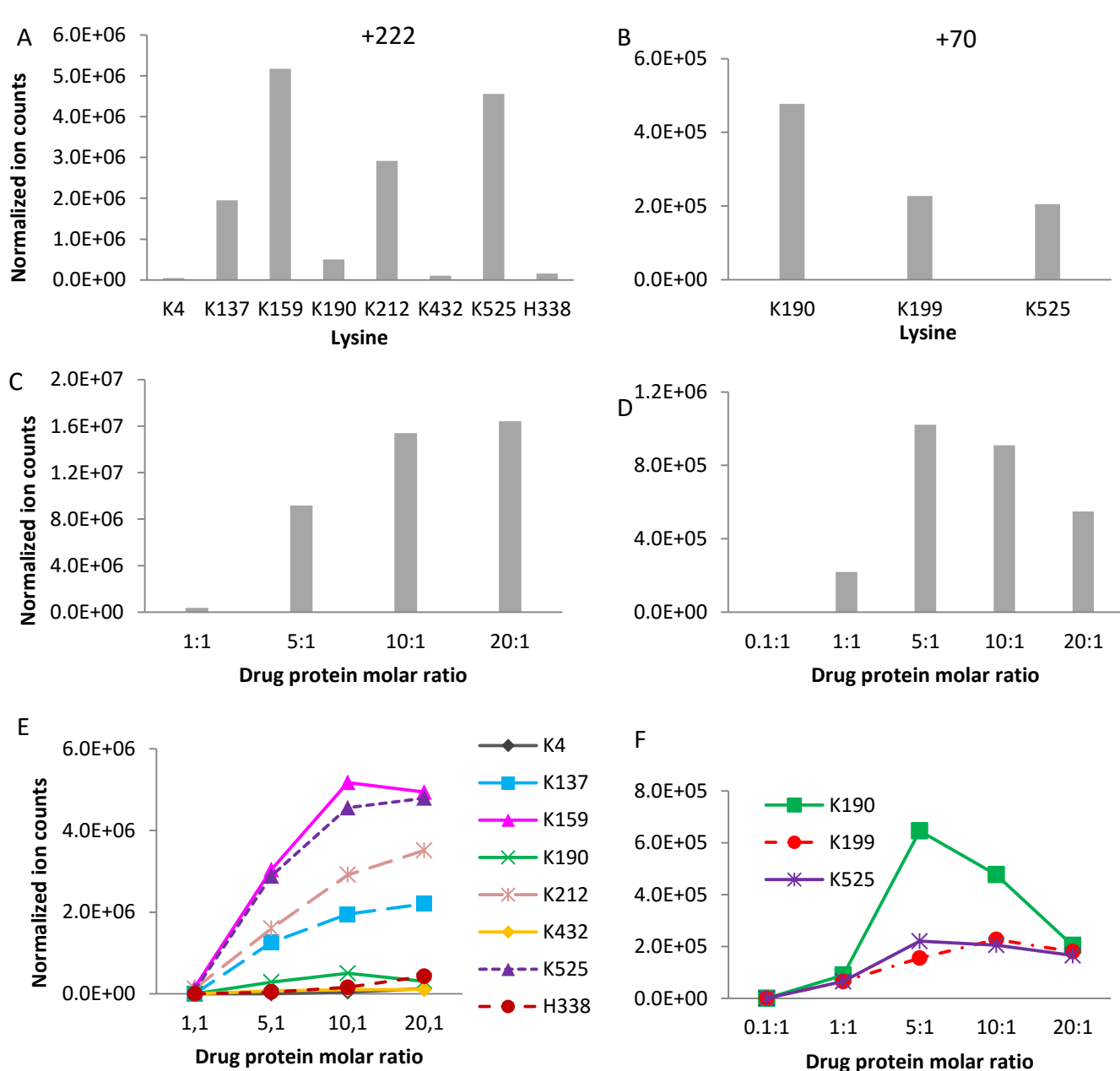Chemical Research in Toxicology

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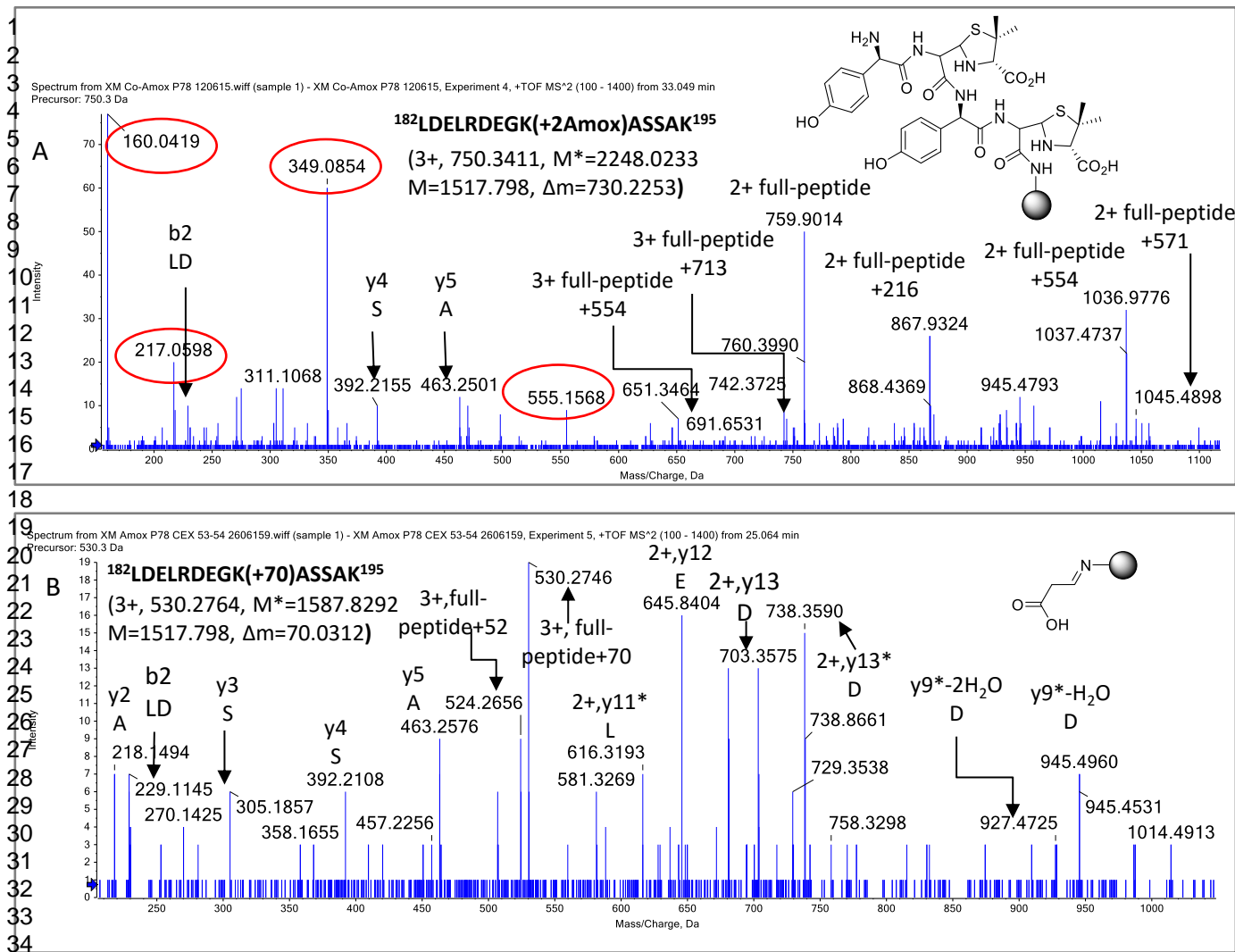
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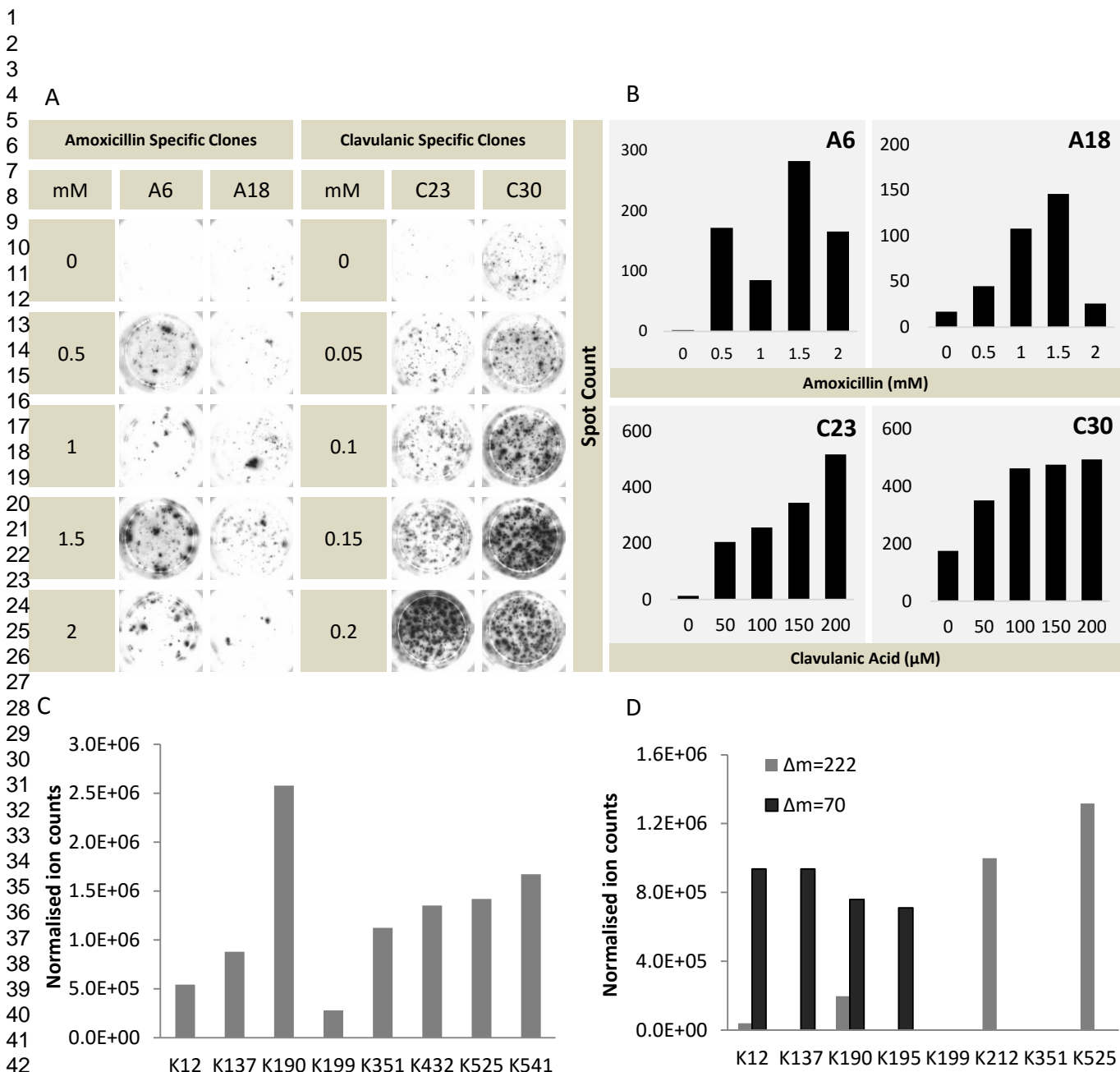
Figure 6

Figure 6. **Activation of T cell clones with amoxicillin and clavulanic acid and analysis of drug-protein binding in culture medium.** IFN-γ ELIspot of amoxicillin and clavulanic acid specific clones were generated from patient PBMCs. Drug specific clones were selected based upon a stimulation index of 2-fold or higher. Clones A6, A18, C23 and C30 demonstrated IFN-γ cytokine release and proliferation in a dose dependent manner (A, B); the epitope profile shows the lysine residues of albumin modified by amoxicillin (C) and clavulanic acid (D) under cell culture conditions.