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Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients

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Running Title: Diastereoisomeric benzylpenicilloyl hapten

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Abbreviations used

ADRs: adverse drug reactions

BP: benzyl penicillin

PA: penicillenic acid

HSA: human serum albumin

LTT: lymphocyte transformation test

PBMC: peripheral blood mononuclear cell

RPMI: Roswell Park Memorial Institute medium

LC-MRM-MS: liquid chromatography -multiple reaction monitoring- mass spectrometry

ABSTRACT

Covalent binding to proteins to form neoantigens is thought to be central to the pathogenesis of penicillin hypersensitivity reactions. We have undertaken detailed mass spectrometric studies to define the mechanism and protein chemistry of hapten formation from benzylpenicillin (BP) and its rearrangement product, benzyl penicillenic acid (PA). Mass spectrometric analysis of human serum albumin exposed to BP and its rearrangement product, PA *in vitro* revealed that at low concentrations (drug protein molar ratio 0.001:1) and during short time incubations, BP and PA selectively target different residues, lys199 and lys525 respectively. Molecular modelling showed that the selectivity was a function of non-covalent interaction prior to covalent modification. With increased exposure to higher concentrations of BP and PA, multiple epitopes were detected on albumin, demonstrating that the multiplicity of hapten formation is a function of time and concentration. More importantly, we have demonstrated direct evidence that PA is a hapten accounting for the diastereoisomeric BP antigen formation in albumin isolated from the blood of patients receiving penicillin. Furthermore, PA was found to be more potent than BP with respect to stimulation of T cells from patients with penicillin hypersensitivity, illustrating the functional relevance of diastereoisomeric hapten formation.

Introduction

β-lactams such as the penicillins remain a very important group of antibiotics for the treatment of a wide variety of infections. Unfortunately, penicillins can cause serious adverse drug reactions (ADRs), which vary in severity from mild skin rashes to much more severe conditions such as anaphylaxis (Gruchalla and Pirmohamed, 2006). Up to 10% of patients receiving penicillin report allergic reactions, but the incidence of genuine hypersensitivity reactions is found to be *only* 1-2% (Solensky, 2003). In the UK, 26% of fatal drug-induced anaphylaxis and 11% of all cases of fatal anaphylaxis are caused by β-lactam antibiotics (Solensky, 2003; Pumphrey, 2004; Fitzharris, 2008).It is therefore important to improve the early diagnosis of these ADRs to prevent patients from progressing to life-threatening reactions, to ensure sensitive patients are not exposed inadvertently, and to ensure that patients who are not allergic are misdiagnosed, leading to the prescribing of other more expensive antibiotics. In order to develop novel diagnostic assays with high sensitivity and specificity, we need to better understand the mechanism underlying penicillin mediated ADRs.

The mechanism of penicillin mediated ADRs has not been fully elucidated, however, it has been postulated that covalent binding to proteins to form neoantigens plays a crucial role in these ADRs (Levine and Ovary, 1961; Brander et al., 1995; Park et al., 1998). Benzyl penicillin (BP) has been shown to form protein conjugates *in vitro* and *in vivo*, and six amino acids of human serum albumin (HSA) have been claimed to be penicilloylated on the basis that these lysine residues were absent from N-terminal sequences of tryptic peptides(Yvon et al., 1989; Yvon et al., 1990). However, the precise structure of these conjugates and the chemical mechanism of this conjugation reaction were not elucidated completely. In particular, it is unclear as to whether conjugates are formed via the direct aminolysis of BP

(Pathway 1, Figure 1) (Batchelor et al., 1965; Schneider and De Weck, 1965) or via the reaction of penicillenic acid (PA), an intermediate formed from rearrangement of penicillin (Pathway 2).

PA has been postulated as a possible intermediate for the penicillin antigen formation on the basis of the observation that aqueous solutions of BP exhibited a strong and distinctive UV absorption at 322 nm, which is characteristic of PA(Neftel et al., 1982; Christie et al., 1988). PA was found to be highly immunogenic both *in vitro* and in experimental animals (Levine and Price, 1964; Christie and Park, 1989). However, substantive evidence that PA is involved in penicillin antigen formation in patients is lacking and the origins of the greater immunogenicity of PA remain to be determined. Defining the chemistry of antigen formation is a critical step in understanding of the mechanism of penicillin hypersensitivity, and full characterization of the penicillin hapten formed in patients is essential for the design and synthesis of antigens for use in a diagnostic assay. The aims of this study were therefore (1) to determine whether PA is involved in penicillin antigen formation in patients, (2) to define the precise structures of the penicillin antigenic determinants, and (3) to investigate the role of PA in penicillin hypersensitivity reactions.

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MATERIALS AND METHODS

Reagents

The following products were purchased from Sigma-Aldrich (Gillingham, UK): Hanks balanced salt solution; penicillin-streptomycin; L-glutamine; HEPES; RPMI 1640; human AB serum; HSA (97-99%); and benzyl penicillin. Invitrogen (Paisley, UK) provided fetal bovine serum. Radiolabeled thymidine was obtained from Moravek International Limited (CA, USA). Trypsin was obtained from Promega (Madison, WI, USA).

Synthesis of penicillenic acid

The synthesis of PA was achieved by coupling oxazolone with D-penicillamine as described previously (Livermore et al., 1948)with modifications (Supplemental Figure 1A). A full description of methods and characterisation of compounds (Supplemental Figure 1B) is provided in supplementary methods.

Rearrangement of benzyl penicillin to penicillenic acid

BP (4.8 mg/ml) was incubated in phosphate buffer (10 mM, pH 7.4), HSA, and denatured HSA (20 mg/ml) at 37 ^{o}C. At each time interval (0.5, 1, 3, 5, or 16 h), a 50 µL aliquot was taken out and extracted with ethyl acetate. The solution obtained was then dried in a speed vac and the products were reconstituted in absolute ethanol and analysed by LC-MS. Denatured HSA was prepared by incubating HSA with dithiothreitol at 37 ^{o}C for 15 minutes and then with iodoacetamide at room temperature for another 15 minutes. The protein was purified by methanol precipitation and was then resuspended in phosphate buffer (pH 7.4).

Preparation and isolation of modified HSA

The time and concentration dependent modification of human serum albumin was investigated *in vitro*. HSA (40 mg/ml, 1mM) in phosphate buffer (10 mM, pH 7.4) was incubated at 37^{o}C with BP or PA at molar ratios of drug to HSA of 0.01:1, 0.1:1, 1:1, 10:1 and 50:1 for 24h, and at 10:1 for 0.5h, 1h, 3h, 5h and 16h. In patients, the molar ratio of BP to HSA is about 0.1:1 following administration of a single dose of 1.2 g BP by continuous infusion over 2 hours(O'Grady et al., 1997). Thus, the conditions used in *in vitro* studies were consistent with those that would be encountered *in vivo*. The protein was precipitated by the addition of nine volumes of ice-cold methanol followed by centrifugation at 14,000g and 4^{o}C for 15min. To ensure the removal of non-covalently bound drug, the precipitation was repeated and protein pellets were washed with ice cold methanol. The efficiency of washing was confirmed as detailed in previous studies (Jenkins et al., 2009) and by using radiolabeled benzyl penicillin. The concentration of HSA was determined by Bradford assay (Bradford, 1976) and aliquots were prepared in serum-free RPMI for application in T-cell assays, in 50mM ammonium bicarbonate for mass spectrometric analysis and in Laemmli sample buffer for Western blotting. Prior to mass spectrometry, all samples were processed as described in previous studies (Jenkins et al., 2009).

A pool of the plasma samples was prepared. HSA was isolated by affinity chromatography, as described previously (Jenkins et al., 2009). In brief, a POROS anti-HSA affinity cartridge (Applied Biosystems, Foster City, CA, USA) attached to a Vision Workstation (Applied Biosystems) was used to affinity capture HSA which was then eluted with 12 mM HCl. Protein was methanol precipitated, processed as described previously, and analysed by reversed phase LC-MS.

Mass spectrometric analysis of penicillin hapten

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Analyses were performed on a 5500 QTRAP® hybrid triple-quadrupole/linear ion trap instrument with Nanospray® II source (AB SCIEX, Foster City, CA, USA). MRM transitions specific for drug modified peptides were selected as follows: the *m/z* values for all possible modified peptides with a missed cleavage at the modified lysine residue were used together with a fragment mass of 160 corresponding to the cleaved thiazolidine ring of the drug. Notwithstanding the disparity in the ionisation efficiency of the peptides, relative MRM peak heights for each of the modified peptides were determined by MultiQuant software version 2.0 (AB SCIEX) to provide an 'epitope profile' that is characteristic for each drug. The total ion count for the whole digest for each sample was normalised to that of the BP-HSA conjugate formed at a molar ratio of drug to protein of 10:1 for 16h: in this way, the MRM signals were adjusted for differences in sample loading on-column. Further details of MS method are provided in supplementary methods.

In vitro reaction of BP or PA with *N*-acetyl-lysine

BP or PA was incubated with *N*-acetyl L-lysine methyl ester in 50% ethanol in phosphate buffer (10 mM, pH7.4) at a molar ratio of 1:1 for 16h. Aliquots were extracted with ethyl acetate, and the products were analyzed by LC-MS. Further details of MS method are provided in supplementary methods.

In vitro kinetic studies of diastereomers of penicillin hapten

Drug pulsed experiments were performed as follows. BP (2 mM) or PA (400 μM) was freshly dissolved in phosphate buffer and then added to a solution of HSA (40 μM, 0.25 ml). The mixture was incubated at 37 ^{o}C for 1h, then the protein was precipitated in methanol to remove unbound drug and was then resuspended in phosphate buffer to continue the

incubation at 37 ^{o}C. At 3, 5 and 16 h time points, an aliquot (50 µl) was removed and processed for MS analysis as described above.

Detection of BP and PA antigens by Western blotting

5 µg of protein was separated by electrophoresis on a 10% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane by electroblotting. The nitrocellulose membrane was washed in deionised water and blocked in Tris/saline/Tween buffer (TST: 150 mM NaCl, 10 mM Tris-HCl, 0.05% Tween 20, pH 8.0) containing 10% non-fat dry milk for 16 h at 4^{o}C. The membrane was then washed in TST and incubated with primary anti-penicillin antibody (mouse anti-penicillin monoclonal antibody; AbD Serotec) in TST buffer and 5% non-fat dry milk for 1 h. The blot was washed repeatedly in TST buffer, and incubated with horseradish peroxidise conjugated anti-mouse IgG antibody (Abcam, Cambridge, UK) in TST buffer and 5% non-fat dry milk for a further 1 h. Following repeated washes, signal was detected by enhanced chemiluminescence (Western Lightning, PerkinElmer, Boston, USA).

Computer modelling of the non-covalent binding of BP and PA to HSA.

BP and PA were subjected to an *in silico* docking procedure using Autodock (Morris et al., 1998) and associated suite of programs. For the calculations, the structure of HSA (PDB code 2BXM with myristate and indomethacin removed) was held rigid, and BP and PA were modelled in their neutral form. The center of the volume that was searched for favourable binding poses was either Lys199 or Lys525 as appropriate. The most popular docking pose for each molecule, as clustered by their RMSD, were identified and examined. Details of the modelling method are provided in supplementary information.

Drug-specific lymphocyte transformation test

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Freshly isolated PBMCs from heparinized venous blood were dispensed into a 96-well U-bottom culture plate ($0.15x10^6$ cells per well in 200µl cell culture medium [RPMI 1640 supplemented with HEPES (25mM), L-glutamine (2mM), 10% pooled human AB serum and transferrin (12.5 mg)]). BP or PA was first tested from 5µM to 2mM. Tetanus toxoid (0.5µg/ml) was used as a positive control. Cell cultures were incubated in a CO_2 ventilated (5%) incubator at 37 ^oC for 6 days. On the fifth day 0.5 µCi of [^3H]thymidine was added to each well. Cells were finally harvested onto filter membranes, and the amount of incorporated radioactivity was measured (counts per minute, cpm) using a β-counter (MicroBeta Trilux, PerkinElmer, Cambridge, UK).

Patient details

Patients (n=8) receiving intravenous BP for cellulitis at either 1.2 g or 2.4 g four times daily were recruited. The age range of the patients was 33-87 years (median 60); there were 3 females and 5 males, and the course of treatment lasted 3-7 days. None of the patients exhibited hypersensitivity reactions to BP or any other drugs being given concurrently. Venepuncture samples were taken no more than 8 hours following a prior dose of BP and extracted into heparinised tubes. Samples were placed immediately on ice and were centrifuged at 2,000 x g and 4 ^oC within 15 min. Small aliquots were prepared and stored at -80 °C.

Patients (n=2) with clinical histories of allergic reactions to either BP or amoxicillin were recruited for the T cell stimulation studies. These patients had immediate reactions to amoxicillin, and the clinical symptoms included facial swelling, erythematory rash. The studies were approved by Liverpool Local Research Ethics Committee and informed consent was obtained from all the subjects before carrying out the studies.

Results

Rearrangement of benzyl penicillin to penicillenic acid catalyzed by HSA

The degradation of BP in different media was monitored to confirm whether PA is an intermediate involved. Our data have shown that the degradation of BP was influenced dramatically by the reaction medium employed. PA was formed in the presence of native HSA within 1 h incubation with BP, but was undetectable in phosphate buffer (pH 7.4) even after 16 h incubation (Fig 2A and B), clearly indicating the formation of PA was catalyzed by HSA. Only a small amount of PA was detected, but this may not reflect the actual amount of PA formed in the system as PA has a short half-life and could either covalently bind to HSA or hydrolyze to penicilloic acid. Further study suggested that the degree of the catalytic effect was dependent on the nature of HSA. As seen in Figure 2C, denatured HSA lost its catalytic effect on BP degradation indicating that the protein pocket of HSA is essential to facilitate this function.

Characterisation of penicillin hapten formed by BP and PA *in vitro*

Mass spectrometric analysis revealed that both BP and PA covalently bind to lysine residues in HSA through opening of the β-lactam ring, mainly forming penicilloyl adducts *in vitro*. At a molar ratio of drug to protein of 10:1, 14 penicilloylated lysine residues were detected when BP was incubated with HSA; whereas 18 penicilloylated lysine residues were detected following incubation with PA (Table 1). As an example, an MS/MS spectrum of carboxamidomethylated tryptic peptide ^{198}LK*C(iodo)ASLQK^{205} is shown with covalent modification of lys199 with a penicilloyl group (Figure 3A). The characteristic fragment ions (both circled) at *m/z* 160 (cleavage of the thiazolidine ring) and *m/z* 217 are derived from the anticipated fragmentation of a BP or PA hapten, providing firm evidence for modification. In addition, a missed cleavage at the proposed site of modification and the presence of b_2 ions

corresponding to the N-terminal dipeptide plus 175 amu (the moiety remaining after cleavage of the thiazolidine ring) provided further evidence of penicilloylation at lys199.

The penicilloylation of HSA by BP and PA was found to be time-and concentration dependent. The epitope profile measured by LC-MRM-MS revealed that, PA modification was 40-60 times greater than BP modification (Figure 3B). These findings were also mirrored by Western blot data (Figure 3C). There was an approximately linear relationship between the ratio of drug to protein and the normalised ion count for each modified peptide, which is illustrated by the data acquired for peptides containing Lys199 (Supplemental Figure 2A). A linear relationship was also observed between the level of modified peptide and incubation time (Supplemental Figure 2B).

Selective modification of HSA by BP and PA *in vitro*

BP and PA appeared to selectively target different lysine residues in HSA when incubated with HSA at low concentration (drug protein molar ratio 0.001:1) and short incubation time. BP exhibited a marked preference for Lys199 whereas PA preferentially targeted lys199 and lys525 (Figure 4A). Molecular modelling via docking of BP and PA into the structure of HSA revealed the favourable binding poses adjacent to Lys199 and Lys525, respectively (Figure 4B). Further "focused" docking experiments were performed in which the search volume was restricted around Lys199 and Lys525, respectively (see experimental for details).The most popular binding pose of BP and PA had a predicted binding energy of -7.88 kcal/mol and -7.07kcal/mol, respectively. However, at greater exposure, multiple epitopes were observed for both BP and PA (Figure 5).

Characterisation of diastereoisomeric penicillin hapten formed *in vitro* and *in vivo*

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In order to probe the mechanism of penicillin hapten formation *in vitro* and *in vivo*, the aminolysis reactions of BP or PA with N-acetyl lysine was first studied. As shown in Figure 6A, only one adduct was detected (retention time 13.6 min, *m/z* 537 $[M+H]^+$) when BP was reacted with N-acetyl lysine methyl ester at pH 7.4. We hypothesise that this adduct resulted from the direct binding of BP to N-acetyl lysine methyl ester and therefore that it retains the 5R, 6R configuration. In contrast, two adducts were formed from the reaction of PA under identical conditions (adduct 1, retention time 13.7 min, *m/z* 537 [M+H], and adduct 2, retention time 14.6 min, *m/z* 537 $[M+H]^+$).The MS fragmentation patterns observed of the two adducts are identical, indicating that they are diastereoisomers (Supplemental Figure 3). As the thiazolidine ring of PA would prefer to be 1,3-trans , which would lead to a (5R)-configuration upon ring closure of PA, we therefore postulate that adduct 1 was in 5R,6R configuration, whereas adduct 2 was in 5R,6S configuration.

Interestingly, when BP was incubated with HSA *in vitro*, two isomeric adducts at each of the modified lysine residues were observed (Figure 6B), in contrast to the single adduct that was formed with N-acetyl lysine methyl ester. The same isomeric adducts were also produced when PA was incubated with HSA. The relative amount of the two diastereomers varied: BP appeared to predominately form the diastereomer 1 at most sites, whereas PA preferentially yielded diastereomer 2. This pattern was observed for the majority of modified peptides (Supplemental Table 1 A and B). An exception was found with the modified peptide containing Lys525: diastereomer 2 was present at slightly higher abundance than diastereomer 1 irrespective of whether HSA was incubated with BP or PA (Supplemental Table 1 A and B). This may be due to competition for the site and the preferential binding of PA to ly525 observed in Figure 3A.

The diastereoisomeric penicillin hapten formation is dependent on PA

In order to determine how BP forms diastereoisomeric haptens in the presence of protein, the dynamics of diastereomer formation was monitored over the time course of incubation. During the continuous and prolonged incubation of BP with HSA, an increase in the relative abundance of diastereomer 2 was observed (Figure 7A). Diastereomer 2 could result from PA, which was formed spontaneously in solution, but could also result from epimerisation of diasteromer 1 as HSA has been shown previously to have a catalytic effect on the rearrangement of reactive metabolites(Smith et al., 1989). In order to determine which pathway has occurred in the presence of HSA, a 'pulsed' experiment was designed in which drug was removed after 1h incubation with protein, and the protein was incubated for a further 16 h in the absence of drug. Under these conditions, the ratio of diastereomer 1 to diastereomer 2 does not change over time (Figure 7B). The same profile was observed for the majority of modified peptides (Supplemental Figure 4). These data suggested that epimerisation of diastereomer 1 to diastereomer 2 does not takes place after the drug has become covalently bound to the protein. Therefore, for incubation of HSA with BP, the diastereomer 2 can only be formed via a two-step sequential reaction: rearrangement of BP to PA followed by covalent modification of lysine residues by PA (pathway 2), rather than BP modification of lysine residues followed by epimerisation of diastereomer 1 to form diastereomer 2 (pathway 1).

Penicillin hapten formed in patients

HSA was extracted by affinity chromatography from a pool of plasma samples donated by patients undergoing antibiotic therapy, and analysed on a 5500 QTRAP® instrument using the MIDAS approach (Unwin et al., 2005). Fourteen sites of penicilloylation were detected (Table 1) indicating that the qualitative profile of protein modification was similar to that

observed in the samples modified with BP and PA *in vitro*. The ion current epitope profile (Figure 5) displayed some similarity to those obtained with BP and PA but did not map exactly onto either of them. In addition, diastereoisomeric haptens were also detected on albumin isolated from plasma from patients receiving BP (Figure 6B). It must be noted that most of the patients were being treated with more than one penicillin, and indeed haptens formed by flucloxacillin and amoxicillin were detected in the pooled sample.

Drug-specific lymphocyte transformation test.

Lymphocytes from BP hypersensitive patients proliferated in the presence of BP or PA. For BP, a maximal stimulation was achieved at 1 mM, with no stimulation being observed under 100 µM. In contrast, maximal stimulation was achieved by PA at a much lower concentration (*ca* 20µM; Figure 8A), with higher concentrations of PA being toxic to the patients' T-cells. To investigate why PA appeared to be more immunogenic than BP, a normalized synthetic HSA conjugate of BP and PA were prepared. BP-HSA conjugate was generated *in vitro* at a molar ratio of drug to protein of 50:1 giving a total MRM signal for all modified peptides of 5.49 X 10^8 cps. PA-HSA conjugate generated at a molar ratio of drug to protein of 10:1 resulted in a total MRM signal of 1.35 X 10^9 cps. The PA-HSA conjugate formed at 10:1 was therefore diluted with unmodified HSA at a ratio 1:2.45 to normalise the MRM signals. In addition, the epitope profiles at these drug concentrations were similar for BP and PA (Figure 8B). Control HSA was processed in the same manner as the conjugates (overnight incubation followed by two rounds of methanol precipitation). T-cells from hypersensitive patients were then challenged with the normalised conjugates. At a concentration of 1 mg/ml and 3 mg/ml (with a background of 3.5 mg/ml HSA from the serum-supplemented medium), the stimulation of PA conjugate was still greater than BP conjugate (Figure 8C), indicating the 5R,6S diastereomer adducts formed preferentially by PA may be more immunogenic.

Discussion

Covalent binding to proteins to form neoantigens is thought to be central to the pathogenesis of penicillin hypersensitivity (Levine and Fellner, 1965; Park et al., 1998; Pichler et al., 1998). It has been postulated that penicillenic acid is a possible intermediate for the penicillin antigen formation and may contribute to the immunogenicity of benzyl penicillin in patients. However, solid evidence to support this hypothesis was lacking. In this study, we have used mass spectrometry to define the reactivity of benzyl penicillin and its rearrangement product, benzyl penicillenic acid, and have confirmed that BP and PA selectively bind to lysine residues in HSA *in vitro*. More importantly, we have proven, for the first time that penicillenic acid is a hapten accounting for the formation of diastereoisomeric penicillin antigens in patients and the diastereomer formed preferentially via PA may be the causative immunogen of BP in patients.

Studies described herein have demonstrated that BP and PA bind selectively to lysine residues on HSA through opening of the β-lactam ring, yielding penicilloyl lysine adducts. No evidence of other modified amino acid residues could be found. A possible explanation is that the adducts resulted from other nucleophiles such as serine, histidine and cysteine may be too labile to be detected under current analytical conditions, or a further transacylation may have occurred (Tsuji et al., 1975).In addition, only penicilloyl lysyl antigenic determinants were detected in this study; minor determinants, such as penicillanyl and penicillamine derivatives (Levine, 1960; Schneider et al., 1973), were not observed. It has been shown that some patients are more sensitive to the minor determinants(Weltzien and Padovan, 1998). However, the formation of minor determinants requires either further degradation of BP, for example, penicillamine derivatives, or the involvement of particular

bioactivation pathway, for example, production of the penicillanyl adducts derived from the free carboxyl group of penicillin. Thus these adducts may not be formed in plasma.

Furthermore, the penicilloylation of lysine residues by BP and PA appeared to be concentration- and time-dependent. At low concentrations, BP and PA were shown to bind selectively to lys199 and lys525, respectively. Molecular modelling via docking of BP and PA into the structure of HSA revealed favourable binding poses in the proximity of Lys199 and Lys525, respectively, closely mirroring the experimental data. It is worth noting the remarkable proximity between lys199 and the BP β-lactam carbonyl group, a pose clearly favourable to penicilloylation; while in the case of PA, the best pose reveals the key proximity between the oxazolone ring and lys525, which is conducive to covalent binding. Thus the preference of the drugs for different lysine residues in HSA is driven at least in part by the non-covalent interaction with protein, with non-covalent interaction positioning the drugs in favourable orientations to facilitate covalent binding with adjacent lysine residues (Qiu et al., 1998; Szapacs et al., 2006). The three-dimensional shape of the drug, as well as its inherent chemical reactivity, will therefore determine selectivity of covalent binding as demonstrated in this study. However, at high concentrations and with prolonged incubation, multiple epitopes were detected for both BP and PA, indicating the multiplicity of the epitope is a function of concentration and reaction time. As the half-life of human serum albumin is approximately 19 days (Muller et al., 2010), and consequently the modified protein is likely to accumulate over the course of the therapeutic intervention which is usually 7 days in duration, it is perhaps not surprising that similar multiple epitope profile was observed in patients receiving BP, establishing the physiological relevance of the *in vitro* studies.

More importantly, we have demonstrated that BP can form diastereoisomeric haptens *in vitro* and in patients via its rearrangement intermediate, penicillienic acid. Two isomeric adducts at each of the modified lysine residues were observed when BP was incubated with HSA *in vitro*, in contrast to the single adduct formed with N-acetyl lysine methyl ester in phosphate buffer (pH 7.4). As the diastereomer 2 was proven to be formed exclusively from PA, this led to the hypothesis that PA is an intermediate partially accounting for the formation of diastereoisomeric penicillin antigen. This hypothesis was further supported by the observation that BP had indeed undergone rearrangement to PA when incubated with HSA but not in phosphate buffer. Comparable diastereoisomeric penicilloyl albumin adducts were also detected in patients receiving penicillin, suggesting that the same reaction pathway could be essential for the formation of penicillin antigens in patients. It must be noted that most of the patients were on multiple medications, and so it is perhaps not surprising that the ratio of diastereomers of penicilloylated peptides observed *in vivo* is slightly different from those obtained *in vitro*.

The finding that PA is involved in penicillin antigen formation in patients is of considerable clinical significance since it has been shown that PA is highly reactive and immunogenic(Christie et al., 1988). Although only a small amount of PA may be formed in patients, the greater reactivity of PA could have a significant impact on the antigenic determinants found on protein. Furthermore, the diastereoisomeric penicilloyl haptens derived from PA could also have an impact on penicillin ADRs as the immune system can discriminate between penicillin stereoisomers (Nagata et al., 1986). These two diastereomers may bind differently to the MHC binding grove on antigen presenting cells, which may subsequently affect the binding affinity and T cell recognition, leading to differences in immunogenicity.

Our data have shown that PA is a more potent stimulator of T-cell proliferation than BP. There are several possible explanations for the observed difference: PA may form a higher level of protein adduct due to its greater reactivity; the modified protein may be processed differently because of the preference of PA for specific lysine residues; or the diastereomeric adducts formed preferentially by PA may be inherently more immunogenic than the one formed by BP (Nagata et al., 1986). To investigate the latter possibility, synthetic HSA conjugates of BP and PA were normalised to minimize the effect of lysine residue preference and the total level of antigen. Normalised PA-HSA conjugate was a more potent stimulator of T cell proliferation than BP-HSA conjugate, indicating that the diastereomeric adducts formed preferentially by PA are more antigenic.

In conclusion, we have demonstrated that BP and PA can selectively bind to lysine residues in HSA at low concentrations; whereas at higher concentrations and in patients, multiple lysine adducts have been identified. In addition, we have demonstrated direct evidence that PA is an intermediate involved in diastereoisomeric BP antigen formation in patients and that PA forms bona fide antigen(s) which could be responsible for penicillin hypersensitivity reactions. Furthermore, we have found that PA is a more potent stimulator of T cell proliferation than BP. The greater potency of PA could of course be a function of hapten density or the epitope multiplicity which has been observed *in vitro*, but could also be a function of stereochemistry.

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Authorship Contributions

Participated in research design: Meng, Jenkins, Stachulski, French, Naisbitt, Pirmohamed, and Park.

Conducted experiments: Meng, Jenkins, Berry, Maggs, and Farrell.

Contributed new reagents or analytic tools: Meng, Jenkins, and Lane.

Performed data analysis: Meng, Jenkins, Berry, Naisbitt, and Farrell.

Wrote or contributed to the writing of the manuscript: Meng, Jenkins, Berry, James, Stachulski, French, Naisbitt, Pirmohamed, and Park.

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Footnotes

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Figure Legends

Figure 1. Scheme showing the two potential pathways by which BP covalently bound to protein. Pathway 1: direct binding of BP (**1**) with epsilon amino groups of lysine residues by opening of the β-lactam ring. Pathway 2: rearrangement of BP (**1**) to PA (**2**) followed by nucleophilic attack on lysine residues.

Figure 2. Analysis of BP degradation in different aqueous media. Degradation of BP (4.8 mg/ml) at 37 ^{o}C in HSA (A), phosphate buffer (B), and denatured HSA (C) was analysed by LC-MRM-MS after 1hour incubation.

Figure 3.MS/MS and Western blotting analysis of penicilloylated HSA peptides identified *in vitro*. MS/MS spectrum of tryptic peptide ^{198}LK*C(iodo)ASLQK^{205} modified with BP at the lys199 marked with an *. Dominant fragment ions from the penicilloyl group are circled (A).Time and concentration-dependent binding of BP and PA to HSA *in vitro* analysed by mass spectrometric analysis (B) and Western blotting (C).

Figure 4. Selective binding of BP or PA to HSA identified *in vitro*. At low concentration BP preferentially bound to lys199 whereas PA bound to lys199 and lys525 (A). Molecular modelling of non-covalent interaction of drug with HSA revealed the best poses by docking BP and PA into HSA, showing the key proximity between lys199 and the β-lactam carbonyl group for BP, and lys525 and the oxazolone ring for PA (B). Protein rendered as cyan ribbons, amino acid residues close to the guest molecule rendered as sticks (carbon – grey, nitrogen – blue, oxygen – red) and guest molecule rendered as sticks (carbon – violet, nitrogen – blue, oxygen – red, polar hydrogens - white).

Figure 5. Mutiple epitope profile identified *in vitro* and *in vivo*. Notwithstanding the differences in the ionisation efficiency of the peptides, epitope profile generated by relative MRM peak heights revealed that multiple epitopes were formed at high concentration of BP (A) and PA (B) incubated with HSA *in vitro* and in patients (C).

Figure 6. MS/MS analysis of diastereoisomeric penicillin adducts. Mass chromatogram ([MH+]) of penicilloyl adducts formed by BP or PA with N-acetyl lysine methyl ester (A). Mass chromatogram of distereomers of penicilloylated HSA tryptic peptides identified in incubation of BP or PA with HSA (B). Mass chromatogram of penicilloylated peptide diastereomers identified in albumin isolated from plasma of patients receiving BP (C).

Figure 7. Kinetic profile of penicilloylated peptide diastereomers formed in the *in vitro* incubation of BP with HSA. Mass chromatogram of penicilloylated peptide diastereomers derived from continuous incubation with BP (A).In the pulsed incubation, HSA was incubated with BP for 1h , and the incubation was continued for 16h in the absence of drug (B).

Figure 8. Penicillin-specific stimulation of lymphocytes from hypersensitive patients. Positive responses were observed by stimulation of PBMCs from penicillin allergic patient 1 and patient 2 with BP and PA (A). Epitope profile of synthetic BP-HSA conjugate and normalised PA-HSA conjugate generated *in vitro*(B).Proliferation of lymphocytes with synthetic conjugate (C). RPMI supplemented with 10% HSA was used as control (R9).

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Table 1. Penicilloylated tryptic peptides of HSA identified *in vitro* and *in vivo*

Lysine	Peptide[a]	PA[b]	BP[c]	In patient
20	FK*DLGEENFK	+	+	+
137	K*YLYEIAR	+	+	+
159	HPYFYAPELLFFAK*R	+	+	+
162	YK*AAFTECCQAADK	+	-	-
190	LDELRDEGK*ASSAK	+	+	+
195	ASSAK*QR	+	+	+
199	LK*CASLQK	+	+	+
212	AFK*AWAVAR	+	+	+
351	LAK*TYETTLEK	+	+	+
372	VFDEFK*PLVEEPQNLIK	+	-	+
432	NLGK*VGSK	+	+	+
436	VGSK*CCK	+	-	+
475	VTK*CCTESLVNR	+	-	-
525	K*QTALVELVK	+	+	+
541	ATK*EQLK	+	+	+
545	EQLK*AVMDDFAAFVEK	+	+	+

[a] *indicates modification site [b] incubation at PA HSA molar ratio of 1:1, [c] incubation at BP HSA molar ratio of 10:1.

Figure 1

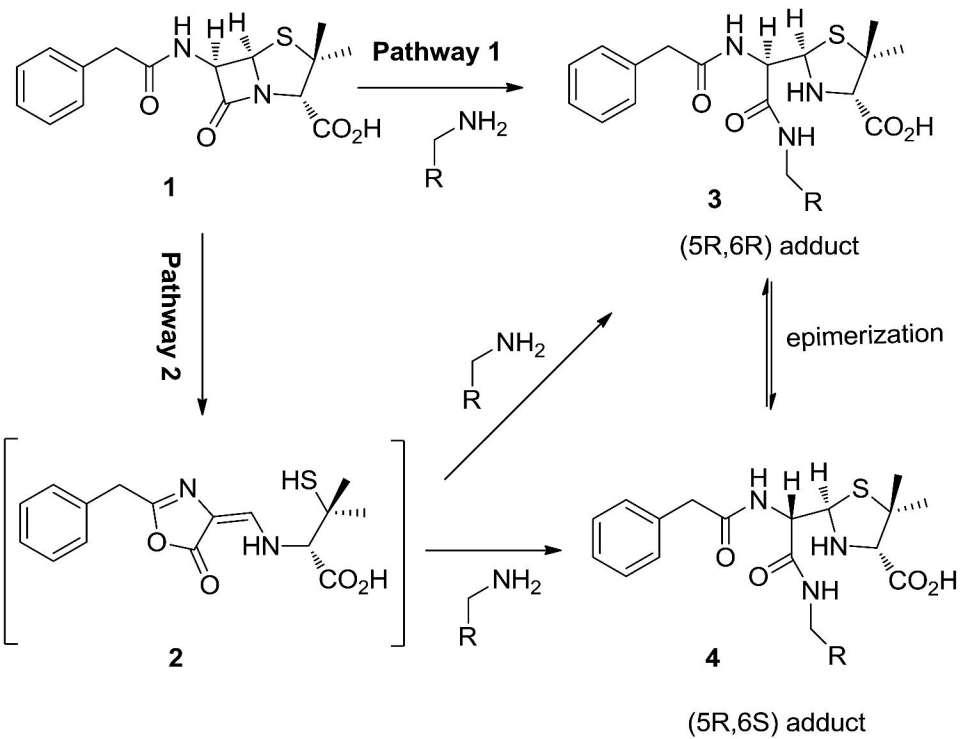

Pathway 1
Pathway 2
epimerization
1
2
3
4
(5R,6R) adduct
(5R,6S) adduct

Figure 2

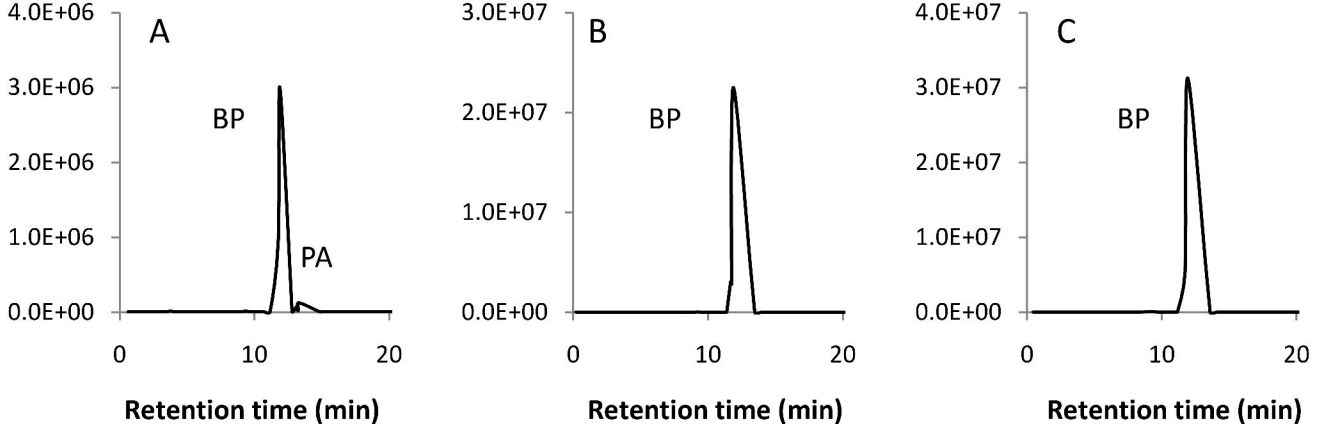

A
BP
PA
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0.0E+00
0
10
20
Retention time (min)
B
BP
3.0E+07
2.0E+07
1.0E+07
0.0E+00
0
10
20
Retention time (min)
C
BP
4.0E+07
3.0E+07
2.0E+07
1.0E+07
0.0E+00
0
10
20
Retention time (min)

Figure 3

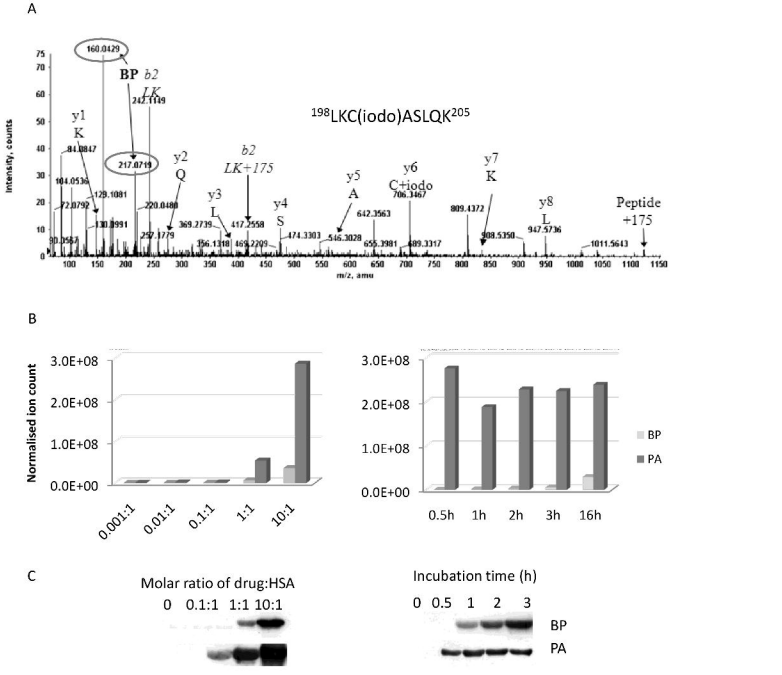

A
198LKC(iodo)ASLQK205
BP b2 LK 242.1139
160.0429
217.0719
y1 K 84.0847
y2 Q 129.1081
y3 L 369.2739
b2 LK+175 417.2558
y4 S 474.3303
y5 A 546.3028
y6 C+iodo 706.3467
y7 K 809.4372
y8 L 947.5736
Peptide +175 1011.5643
Intensity, counts
m/z, amu
B
Normalised ion count
3.0E+08
2.0E+08
1.0E+08
0.0E+00
0.001:1
0.01:1
0.1:1
1:1
10:1
0.5h
1h
2h
3h
16h
BP
PA
C
Molar ratio of drug:HSA
0 0.1:1 1:1 10:1
Incubation time (h)
0 0.5 1 2 3
BP
PA

Figure 4

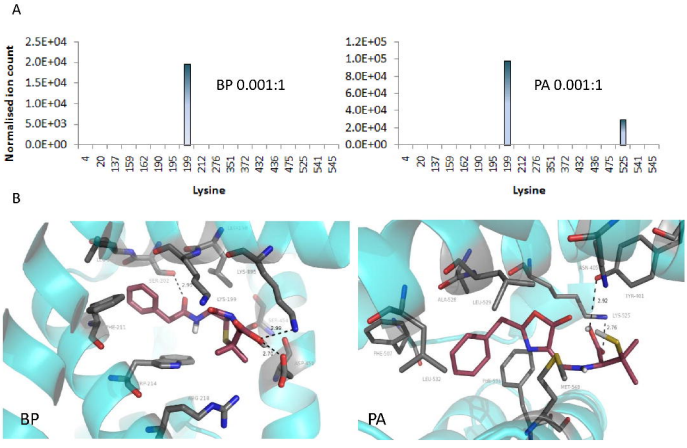

A
Normalised ion count
2.5E+04
2.0E+04
1.5E+04
1.0E+04
5.0E+03
0.0E+00
BP 0.001:1
4 20 137 159 162 190 195 199 212 276 351 372 432 436 475 525 541 545
Lysine
1.2E+05
1.0E+05
8.0E+04
6.0E+04
4.0E+04
2.0E+04
0.0E+00
PA 0.001:1
4 20 137 159 162 190 195 199 212 276 351 372 432 436 475 525 541 545
Lysine
B
BP
PA

Figure 5

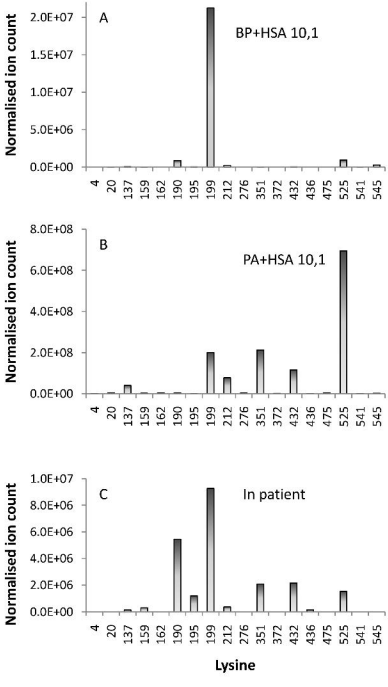
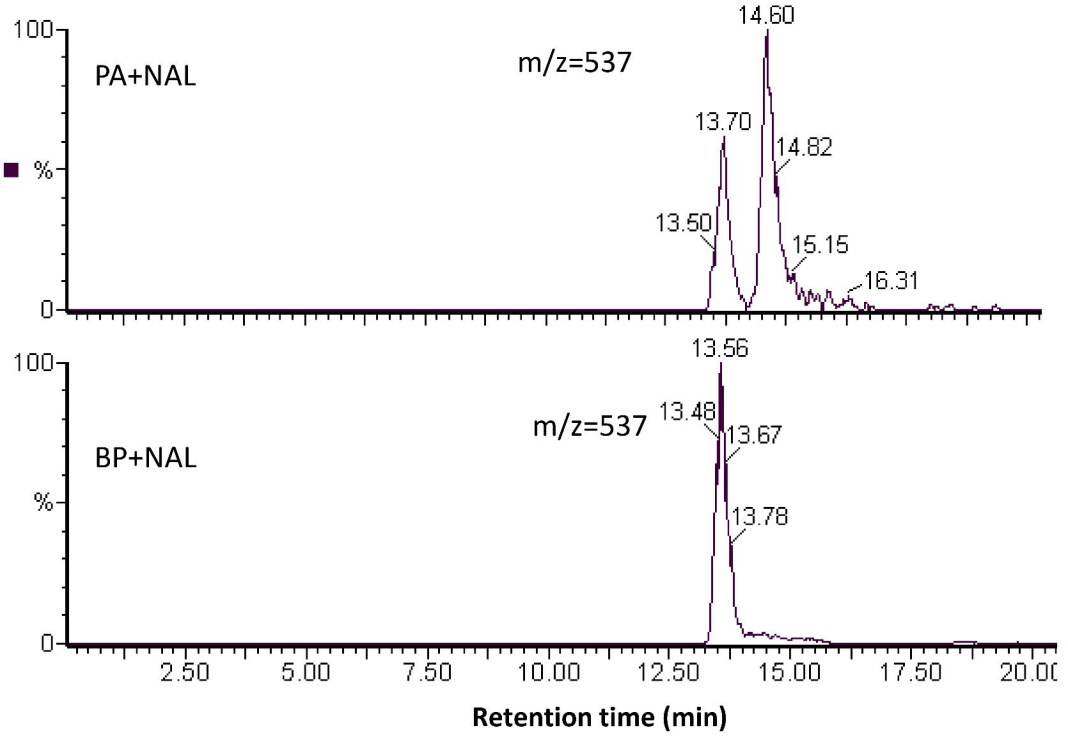

A
BP+HSA 10,1
B
PA+HSA 10,1
C
In patient
Normalised ion count
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
8.0E+08
6.0E+08
4.0E+08
2.0E+08
1.0E+07
8.0E+06
6.0E+06
4.0E+06
2.0E+06
4
20
137
159
162
190
195
199
212
276
351
372
432
436
475
525
541
545
Lysine

Figure 6

A


PA+NAL
m/z=537
14.60
13.70
14.82
13.50
15.15
16.31
BP+NAL
m/z=537
13.56
13.48
13.67
13.78
100
%
0
2.50
5.00
7.50
10.00
12.50
15.00
17.50
20.00
Retention time (min)

B

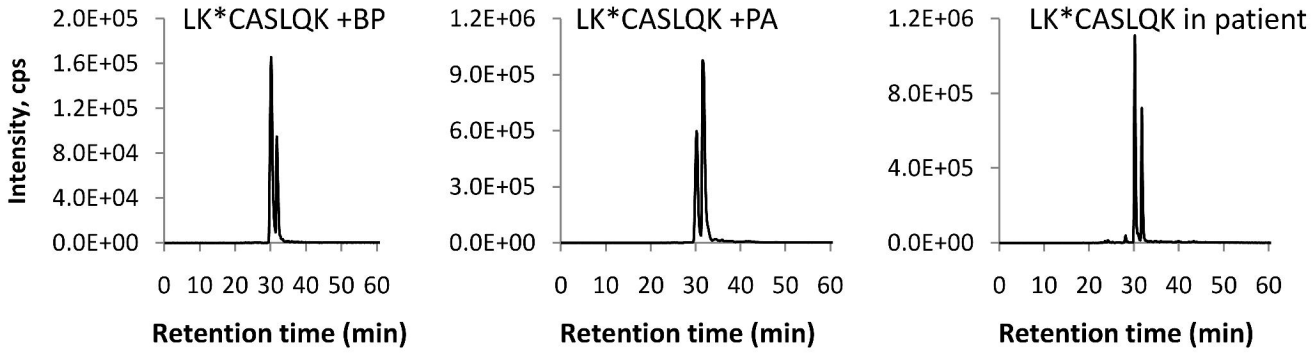

Intensity, cps
LK*CASLQK +BP
LK*CASLQK +PA
LK*CASLQK in patient
2.0E+05
1.6E+05
1.2E+05
8.0E+04
4.0E+04
0.0E+00
1.2E+06
9.0E+05
6.0E+05
3.0E+05
8.0E+05
4.0E+05
0 10 20 30 40 50 60
Retention time (min)

Figure 7

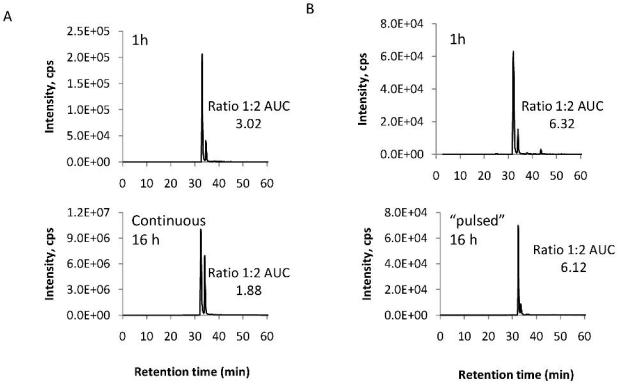
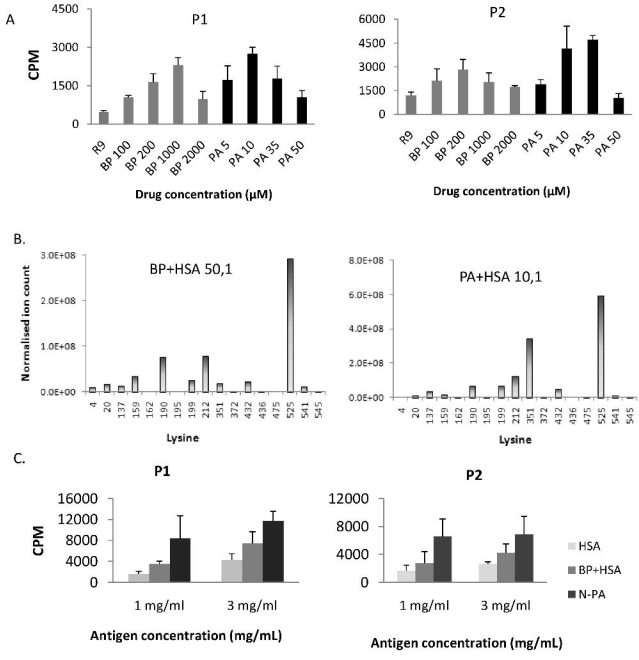

A
B
1h
Ratio 1:2 AUC
3.02
Continuous
16 h
Ratio 1:2 AUC
1.88
1h
Ratio 1:2 AUC
6.32
"pulsed"
16 h
Ratio 1:2 AUC
6.12
Intensity, cps
Retention time (min)

Figure 8


A
P1
CPM
4500
3000
1500
0
R9
BP 100
BP 200
BP 1000
BP 2000
PA 5
PA 10
PA 35
PA 50
Drug concentration (μM)
P2
6000
4500
3000
1500
0
R9
BP 100
BP 200
BP 1000
BP 2000
PA 5
PA 10
PA 35
PA 50
Drug concentration (μM)
B.
BP+HSA 50,1
Normalised ion count
3.0E+08
2.0E+08
1.0E+08
0.0E+00
4
20
137
159
162
190
195
199
212
351
372
432
436
475
525
541
545
Lysine
PA+HSA 10,1
8.0E+08
6.0E+08
4.0E+08
2.0E+08
0.0E+00
4
20
137
159
162
190
195
199
212
351
372
432
436
475
525
541
545
Lysine
C.
P1
CPM
16000
12000
8000
4000
0
1 mg/ml
3 mg/ml
Antigen concentration (mg/mL)
P2
12000
8000
4000
0
1 mg/ml
3 mg/ml
Antigen concentration (mg/mL)
HSA
BP+HSA
N-PA

Supplemental data

Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients

*Xiaoli Meng, Rosalind E Jenkins, Neil Berry, James L Maggs , John Farrell , Catherine S Lane, Andrew V Stachulski, Neil S French , Dean J Naisbitt, Munir Pirmohamed, B Kevin Park**

The Journal of Pharmacology and Experimental Therapeutics

MATERIALS AND METHODS

Reagents

The following products were purchased from Sigma-Aldrich (Gillingham, UK): Hanks balanced salt solution; penicillin-streptomycin; L-glutamine; HEPES; RPMI 1640; human AB serum; HSA (97-99%); and benzyl penicillin. Invitrogen (Paisley, UK) provided fetal bovine serum (FBS). Radiolabeled thymidine was obtained from Moravek International Limited (CA, USA). Trypsin was obtained from Promega (Madison, WI, USA).

Synthesis of penicillenic acid

The synthesis of PA was achieved by coupling oxazolone with D-penicillamine as described with modifications(Livermore et al., 1948). Briefly, the synthesis of PA **2** was achieved in four steps starting with a coupling of phenyl acetic acid **3** and glycine ethyl ester (Supplemental Figure 1A). The resulting ester **4** was then hydrolysed to acid **5** under basic conditions and then converted to the oxazolone **6**. The synthesis of oxazolone **6** was problematic due to the presence of a labile enol ether functional group. However, after much experimentation, we found that **6** can be prepared in reasonable yield and high purity by heating **5** with freshly distilled triethyl orthoformate in acetic anhydride, followed by a very rapid flash chromatographic separation. The following step, coupling of D-penicillamine with **6** in pyridine and triethylamine afforded the crude product **2**. A rapid recrystallisation with chloroform-hexane gave pure **2** as a pale yellow solid in 60% yield.

Ethyl 2-(2-phenylacetamido)acetate 6 2-phenylacetic acid (2.72g, 20mmol) was dissolved in dry DCM (30 ml) and treated with oxalyl chloride (5ml) and five drops of N,N-dimethyl formamide. The reaction mixture was stirred at room temperature for 2 hours, and then evaporated twice from dry DCM. The yellowish residue (acid chloride) was used directly in the next step.

To a solution of ethyl 2-aminoacetate (2.79g, 20mmol) in an ice cold, well stirred two phase mixture of 10% sodium bicarbonate solution (50 mL) and DCM (30 mL) was added the acid chloride solution (in 20 mL dry DCM) dropwise. Upon the completion of the addition, the reaction was allowed to stir for a further hour. Then the reaction mixture was diluted with ethyl acetate. The organic layer was washed with 1N HCl solution (20 mL), 10% sodium carbonate (20 mL) and brine, dried over anhydrous sodium sulphate and concentrated to afford **2** as a white solid (3.54 g) in 80% yield. Anal. ($C_{12}H_{15}NO_3$) Required C, 65.14; H, 6.83; N, 6.33%; Found C, 65.11; H, 6.86; N, 6.3%; ^{1}H NMR (400 MHz, $CDCl_3$) 1.26 (3 H, t, J = 7.1 Hz), 3.63 (2 H, s), 3.98 (2H, d, J = 5.2 Hz), 4.16 (2 H, q), 7.26-7.38 (5 H, m); ^{13}C NMR (100MHz, $CDCl_3$) 14.47, 41.87, 43.91, 61.86, 127.84, 129.42, 129.85, 134.89, 170.14, 171.41 *m/z* (CI, NH_3) 222 $[M+H]^+$, 100%).

2-(2-Phenylacetamido)acetic acid 7 Ethyl ester **5** was dissolved in ethanol and 1M sodium hydroxide solution (5 equiv.) and the reaction was stirred at 30ºC. When reaction was complete by TLC (3 hours), the solution was extracted with ethyl acetate (3x10 mL) to remove any organic impurities. The water phase was acidified using 1M HCl and extracted with ethyl acetate (3x20 mL). The combined organic phases were dried with anhydrous sodium sulphate and concentrated to give **6** as a white solid (g) in 80% yield. Anal. ($C_{10}H_{11}NO_3$)

Required C, 62.18; H, 5.74; N, 7.25%; Found C, 62.47; H, 5.89; N, 7.31%; ^{1}H NMR (400 MHz, MeOD) 3.59 (2 H, s), 3.92 (2H, s), 7.26-7.38 (5 H, m); ^{13}C NMR (100MHz, MeOD) 42.39, 43.96, 128.33, 129.97, 130.66, 137.05, 173.31, 174.86; *m/z* (CI, NH_3) 211 $[M+NH_4]^+$, 100%).

(E)-2-Benzyl-4-(ethoxymethylene)oxazol-5(4H)-one 6 Freshly distilled triethyl orthoformate (0.83 mL, 5mmol) was added to a mixture of acid **5** (0.486 g, 2.5 mmol) in 6 mL acetic anhydride. After stirring under N_2 at 120^{o}C for 1.5 hours, The reaction mixture was concentrated and separated by flash chromatography (10-15% ethyl acetate in hexane) to afford **6** as a pale yellow oil (0.27 g) in 47% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.6 (3H, t, J=7.2 Hz), 3.88 (2H, s), 4.32 (2H, q), 7.17 (1H, s), 7.24-7.34 (5H, m); ^{13}C NMR (100MHz, $CDCl_3$): δ_C 15.27, 35.9, 72.86, 116.77, 127.51, 128.82, 129.14, 133.38, 152.55, 162.34, 168.53; High-resolution MS (ESI) calculated for $C_{13}H_{13}NO_3$ $[M+H]^+$: 232.0974. Found: 232.0985.

(E)-2-{[2-Benzyl-5-oxooxazol-4(5H)-ylidene]methylamino}-3-mercapto-3-methylbutanoic acid 2 Triethylamine (1.6 mL) was added to a mixture of oxazolone **6** (0.226 g, 0.97 mmol) and D-penicillamine (0.145 g, 0.97 mmol) in pyridine (15 mL). The reaction was stirred under N_2 at 65^{o}C for 25 minutes and then concentrated. The residue was dissolved in chloroform (20 mL) and washed with pH 1.6 buffer (10 mL) and then pH 5.4 buffer (10 mL). The organic phase was dried over anhydrous magnesium sulphate and concentrated to afford the crude product as pale yellow oil. Recrystallization of crude product from chloroform and hexane yielded the PA **2** as a white solid (0.22 g) in 66% yield. ^{1}H NMR (400 MHz, $CDCl_3$) : δ_H 1.4 (3H, s), 1.6 (3H, s), 3.5 (2H, d, J=7Hz), 3.9 (1H,

s),7.24-7.34 (6H, m); ^{13}C NMR (100MHz, $CDCl_3$) : δ_C 28.9, 29.1, 35.1, 46.9, 73.2, 105.7, 127.6, 128.9, 129.2, 133.3, 142.6, 160.2, 167.1, 170.1;High-resolution mass (ES) calculated for $C_{16}H_{18}N_2O_4S$ $[M+H]^+$: 335.1066. Found: 335.1054.

Methods of mass spectrometric analyses

N-acetyl lysine adducts formed from BP or PA were separated on a Symmetry 5-µm C8 column (3.9 mm × 150 mm; Waters Corp, Milford, MS, USA) using a gradient of acetonitrile (10-50% over 20min) in 0.1% (v/v) formic acid with a flow rate of 0.9 mL/min. The analysis was carried out on a Quattro II instrument as described previously (Meng et al., 2007).

BP degradation products were analysed by LC-MS. Samples were separated on a prodigy 5 µm C18 column (150X4.6 mm, phenomenex) using a gradient of ACN (20%-80% over 18 min) in 0.1% formic acid with a flow rate of 1 mL/min. The samples were delivered into an API2000 triple quadrupole mass spectrometer (Applied Biosystems) and analysed as described previously (Jenkins et al., 2009). Transitions for multiple monitoring MS were selected based on experimental data. One MRM transition for BP was used, combining the m/z of BP (334 amu) with that of the dominant fragment ion (160 amu, [M+H]+ of thiazolidine ring).

Tryptic digests were reconstituted in 2% ACN/0.1% formic acid (v/v), and aliquots of 2.4-5 pmole were delivered into a QTRAP® 5500 hybrid quadrupole-

linear ion trap mass spectrometer (AB SCIEX) by automated in-line liquid chromatography (U3000 HPLC System, 5mm C18 nano-precolumn and 75 µm x 15cm C18 PepMap column [Dionex, California, USA]) via a 10µm 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 70mins was applied at a flow rate of 280 nL/min. The ionspray potential was set to 2,200-3,500V, the nebuliser gas to 18 and the interface heater to 150oC. Multiple reaction monitoring (MRM) transitions specific for drug modified peptides were selected as follows: the m/z values were calculated for all possible peptides with a missed cleavage at a lysine residue; to these were added the mass of the hapten (334 amu), the parent ion masses were then paired with a fragment mass of 160 ([M+H]+ of cleaved thiazolidine ring present in all of the haptens). MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity, they were optimised for collision energy and collision cell exit potential, and dwell time was 20ms. 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 20s. Total ion counts were determined from a second aliquot of each sample analysed by conventional LC-MS/MS and were used to normalize sample loading on column. MRM peak areas were determined by MultiQuant 1.2 software (ABSCIEX). Epitope profiles were constructed by comparing the relative intensity of MRM peaks for each of the modified lysine residues within a sample and normalization of those signals across samples.

Computer modellingof the non-covalent binding of BP and PA to albumin.

BP and PA were subjected to an *in silico* docking procedure using Autodock (Morris et al., 1998) and associated suite of programs. Autodock uses an empirical function to estimate the free energy of binding. This function contains five terms: a Lennard-Jones 12-6 dispersion/repulsion term; a directional 12-10 hydrogen bonding term; a screened Coulombic electrostatic potential; unfavourable entropy of binding due to restricted conformations and a desolvation energy term. For the calculations, the structure of HSA (PDB code 2BXM with myristate and indomethacin removed) was held rigid, and BP and PA were modelled in their neutral form. The centre of the volume that was searched for favourable binding poses was either Lys199 or Lys525 as appropriate. The grid spacing was 0.3 Å with 126 grid points in each direction. A distance dependant dielectric of -0.1465 was employed for all docking runs. A combination of a Lamarckian genetic algorithm and pseudo-Solis and Wets local search was used to generate docking poses for each molecule. The parameters used in this 'blind docking' procedure were those that have been shown to reproduce the binding mode of drugs within known structures of drug:crystal complexes with no prior knowledge of the binding site (Hetenyi and van der Spoel, 2002). The most popular docking pose for each molecule, as clustered by their RMSD, were identified and examined.

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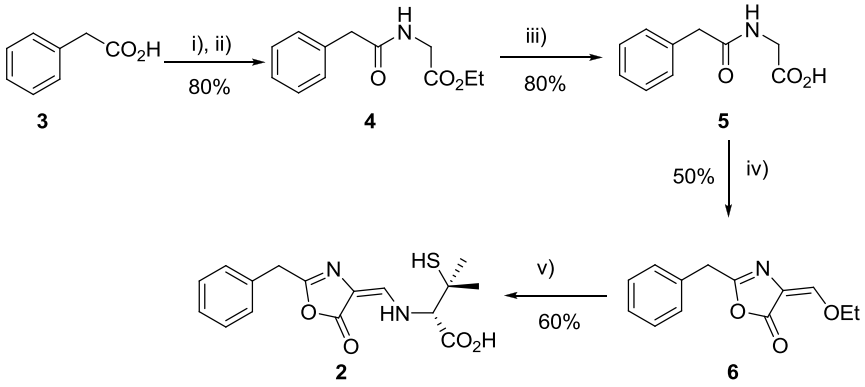
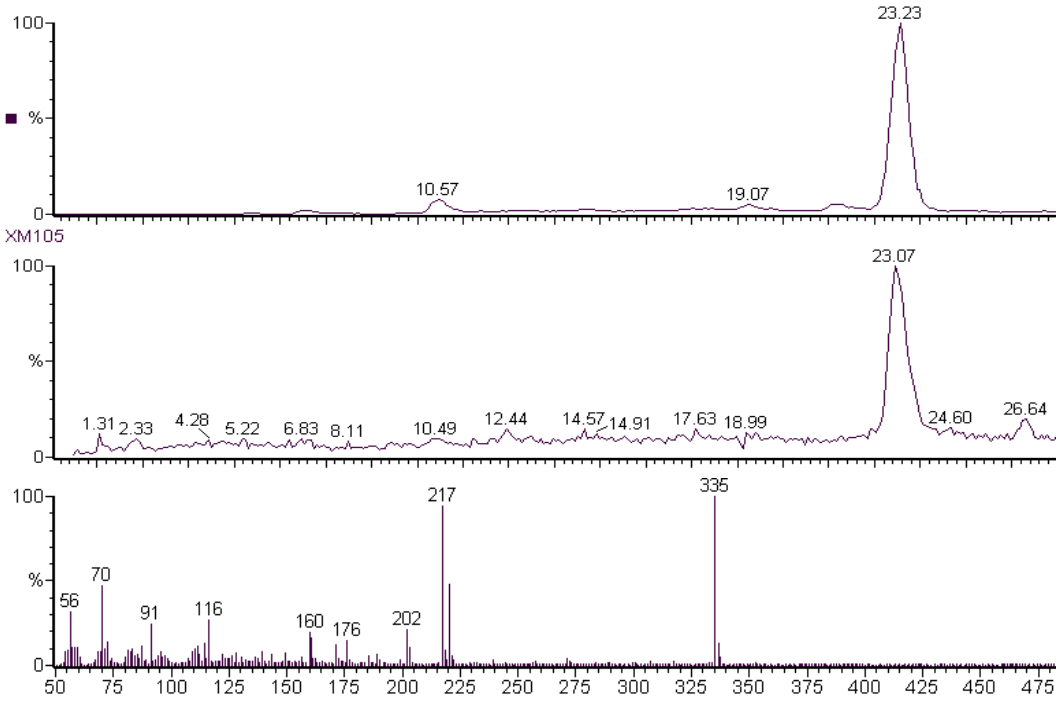


Reagents and conditions: i) $(COCl)_2$, DCM/DMF; ii) HCl $NH_2CH_2CO_2Et$; iii) 1M NaOH/ethanol, 35°C; iv) $HC(OEt)_3$, Ac2O, 120°C; v) D-penicillamine, Et_3N/pyridine

Supplemental Figure 1A. The synthesis of penicillenic acid



Supplemental Figure 1B. Characterisation of penicillenic acid

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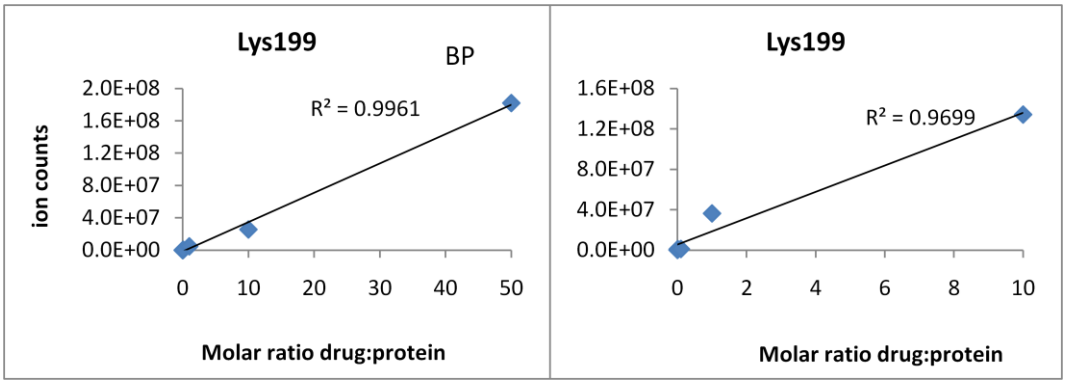
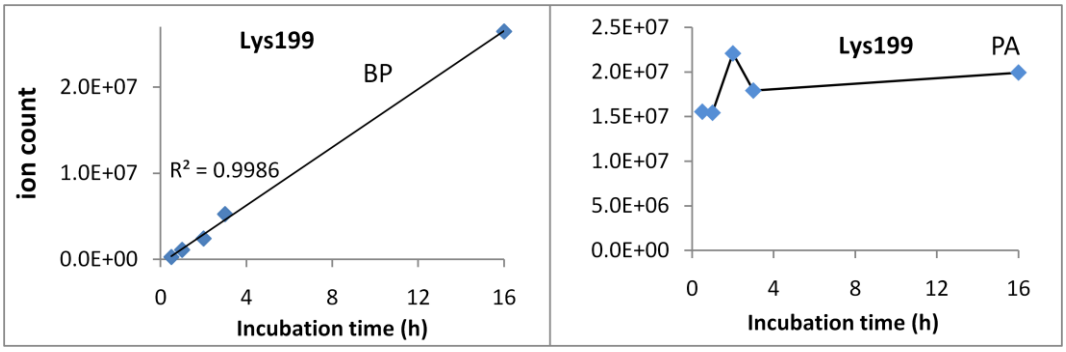
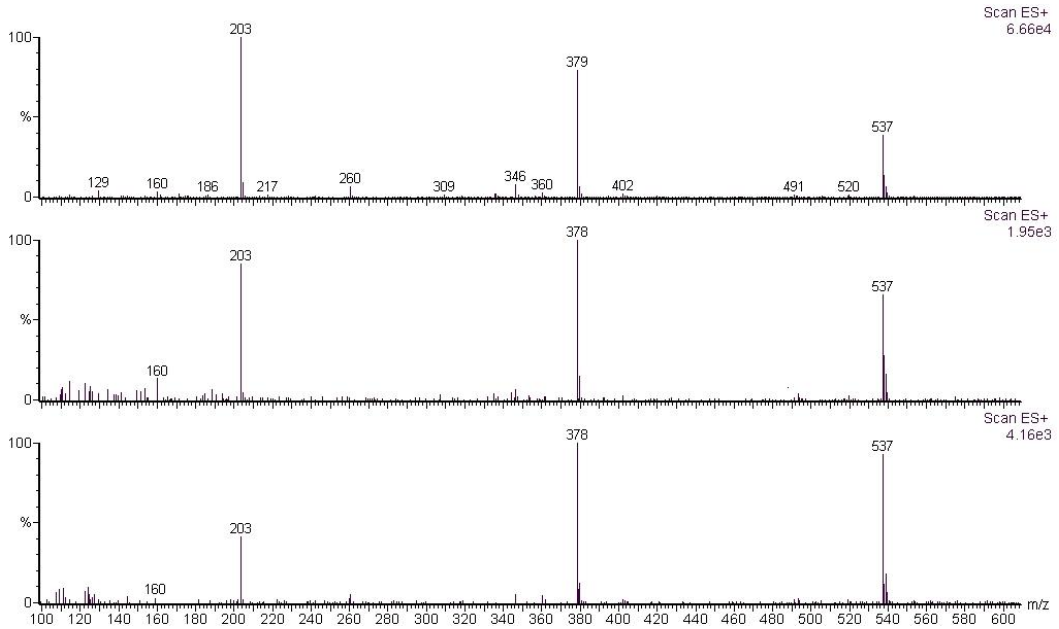
A



B



Supplemental Figure 2. Linear relationship between the level of modification and drug concentration (A) and incubation time (B).



Supplemental Figure 3. MS spectra of diastereomers of penicilloylated N-acetyl lysine methyl ester.

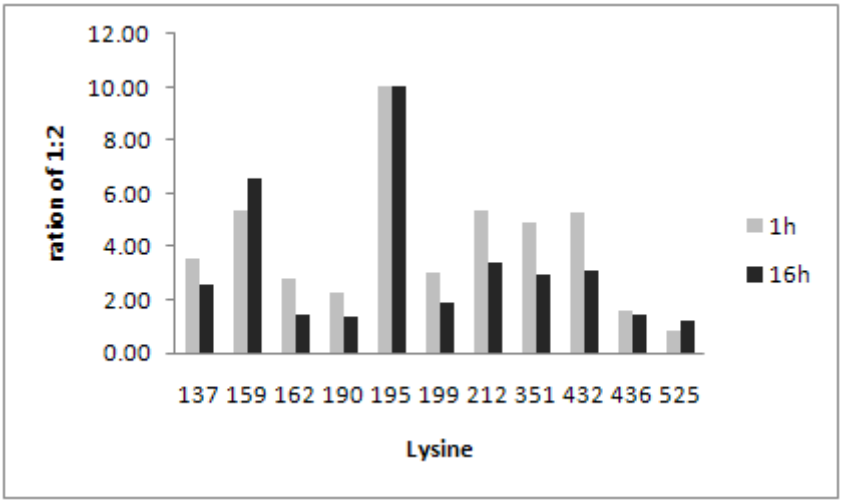
Supplemental Table 1 A. Diastereoisomeric penicillin hapten formed in the incubation of BP with HSA.

Peptide	Mass	1st peak RT	1st peak AUC	2nd peak RT	2nd peak AUC
KYLYEIAR	463.9	42.06	2.81E+05	44.09	1.14E+05
HPYFYAPELLFFAKR	745	50.6	1.90E+05	51.45	8.62E+04
YKAAFTECCQAADK	666.2	36.82	3.58E+05	38.24	1.45E+05
LDELRDEGKASSAK	618.3	31.83	1.94E+06	33.06	1.52E+06
ASSAKQR	541.2	26.7	5.58E+04	0	0
LKCASLQK	427.8	33.27	7.70E+07	34.61	3.24E+07
AFKAWAVAR	451.9	42.5	4.06E+05	44.26	1.28E+05
ATKEQLK	576.2	33.01	6.18E+05	34.5	3.65E+05
LAKTYETTLEK	544.2	37.3	3.53E+05	38.8	1.29E+05
NLGKVGSK	568.7	33.57	2.71E+05	35.53	9.85E+04
VGSKCCK	586.7	26.77	3.58E+04	28.96	1.56E+04
KQTALVELVK	488.2	43.01	7.75E+05	44.04	6.37E+05
EQLKAVMDDFAAFVEK	725.6	54.6	6.71E+05	------	0

Supplemental Table 1B. Diastereoisomeric penicillin hapten formed in the incubation of PA with HSA.

Peptide	Mass	1st peak RT	1st peak AUC	2nd peak RT	2nd peak AUC
AFKAWAVAR	451.9	42.5	4.02E+07	44.26	9.44E+07
ASSAKQR	541.2	26.7	2.93E+05	28.1	4.13E+06
ATKEQLK	576.2	32.93	1.62E+07	34.5	3.42E+07
EQLKAVMDDFAAFVEK	725.6	54.45	5.89E+05	-----	0
FKDLGEENFK	520.9	41.26	3.84E+06	42.38	1.01E+07
KQTALVELVK	488.2	43.01	7.98E+07	44.04	5.66E+08
KYLYEIAR	463.9	43.09	1.56E+07	43.96	2.46E+07
LAKTYETTLEK	544.2	37.3	7.65E+07	38.8	2.97E+08
LDELRDEGKASSAK	618.3	31.22	1.12E+07	32.73	6.44E+07
LKCASLQK	427.8	33.27	2.35E+07	34.61	5.14E+07
NLGKVGSK	568.7	33.15	9.07E+06	35.4	4.34E+07
VGSKCCK	586.7	26.25	0.00E+00	28.96	0.00E+00
VTKCCTESLVNR	600.9	35.73	5.98E+05	36.89	1.73E+06
YKAAFTECCQAADK	666.2	36.75	4.80E+05	38.1	1.32E+06
HPYFYAPELLFFAKR	586.7	50.6	5.43E+06	51.4	1.58E+07
VFDEFK*PLVEEPQNLIK	666.2	50.24	9.06E+05	51.51	1.78E+06

A



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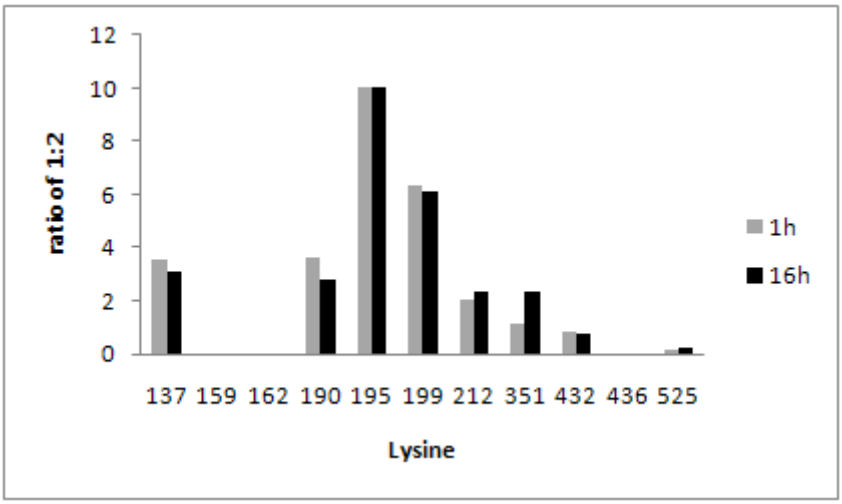

Supplemental Figure 4. The kinetic profile of distereoisomeric penicillin hapten formation in the continuous incubation of BP with HSA (A) and in the 'pulsed' incubation of BP with HSA (B).