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

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Abbreviations used

ADRs: adverse drug reactions

BP: benzyl penicillin

PA: penicillenic acid

HSA: human serum albumin

LT: lymphocyte transformation test

PBMC: peripheral blood mononuclear cell

RPMI: Roswell Park Memorial Institute medium

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

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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.

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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

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(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 °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 °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

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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°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°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 μ M) was freshly dissolved in phosphate buffer and then added to a solution of HSA (40 μ M, 0.25 ml). The mixture was incubated at 37 °C for 1h, then the protein was precipitated in methanol to remove unbound drug and was then resuspended in phosphate buffer to continue the

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incubation at 37 °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°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 peroxidase 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 (0.15×10^6 cells per well in 200 μ l 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 5 μ M to 2mM. Tetanus toxoid (0.5 μ g/ml) was used as a positive control. Cell cultures were incubated in a CO₂ ventilated (5%) incubator at 37 °C for 6 days. On the fifth day 0.5 μ Ci 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 °C 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, erythematous 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.

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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 $^{198}\text{LK}^*\text{C}(\text{iodo})\text{ASLQK}^{205}$ 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

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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 -7.07 kcal/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 Lys525 observed in Figure 3A.

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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 diastereomer 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 take 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

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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 μ M. In contrast, maximal stimulation was achieved by PA at a much lower concentration (*ca* 20 μ M; 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 5.49×10^8 cps. PA-HSA conjugate generated at a molar ratio of drug to protein of 10:1 resulted in a total MRM signal of 1.35×10^9 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.

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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

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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.

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More importantly, we have demonstrated that BP can form diastereoisomeric haptens *in vitro* and in patients via its rearrangement intermediate, penicillenic 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 groove on antigen presenting cells, which may subsequently affect the binding affinity and T cell recognition, leading to differences in immunogenicity.

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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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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 °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 $^{198}\text{LK}^*\text{C(iodo)ASLQK}^{205}$ 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. Multiple 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).

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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

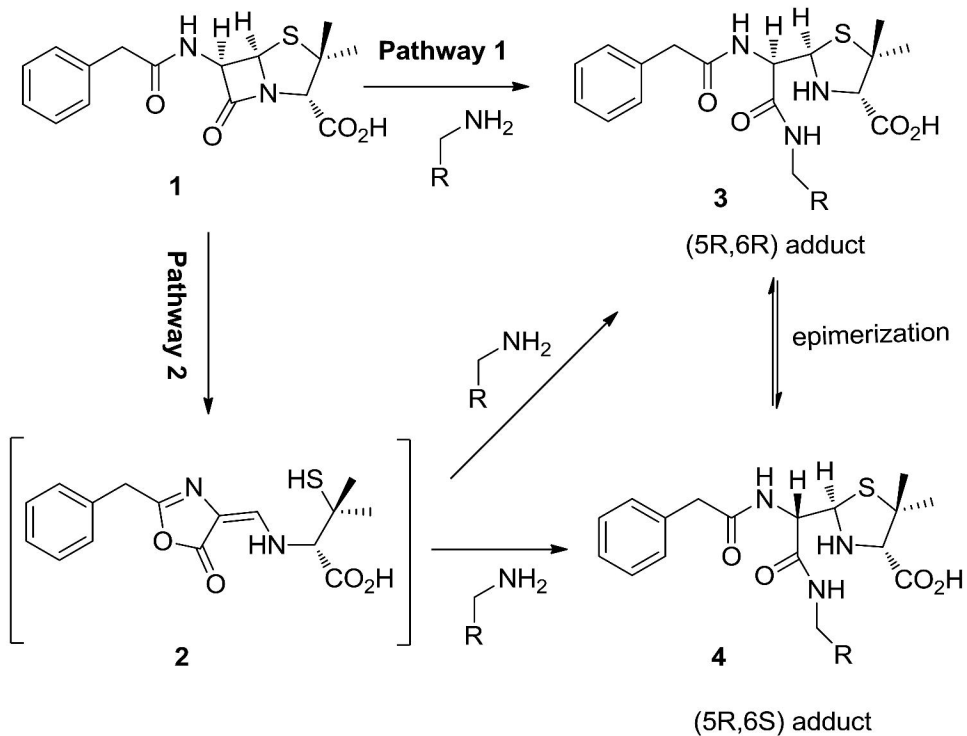


Figure 2

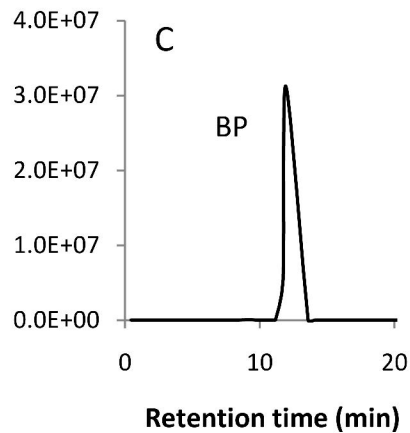
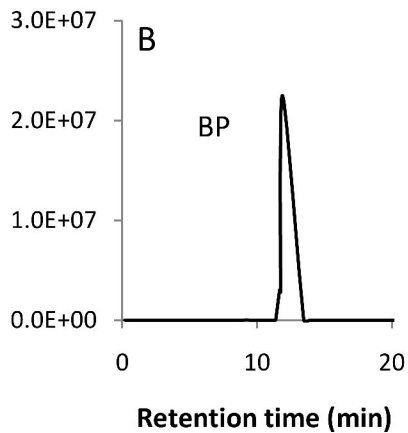
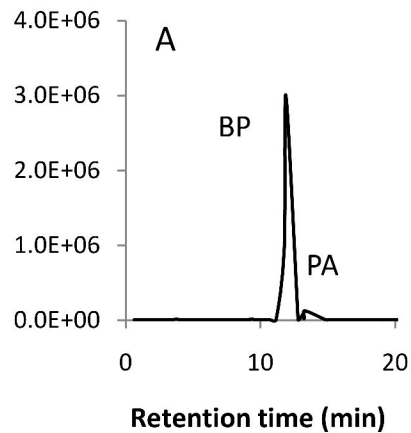
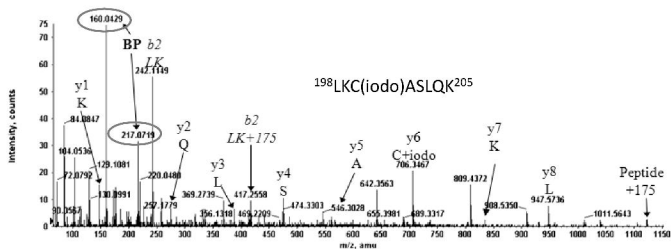
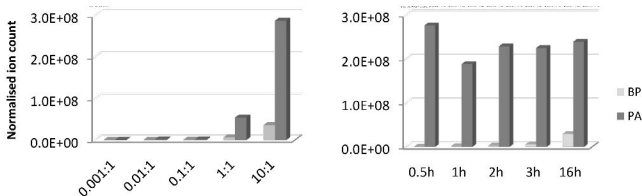


Figure 3

A



B



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

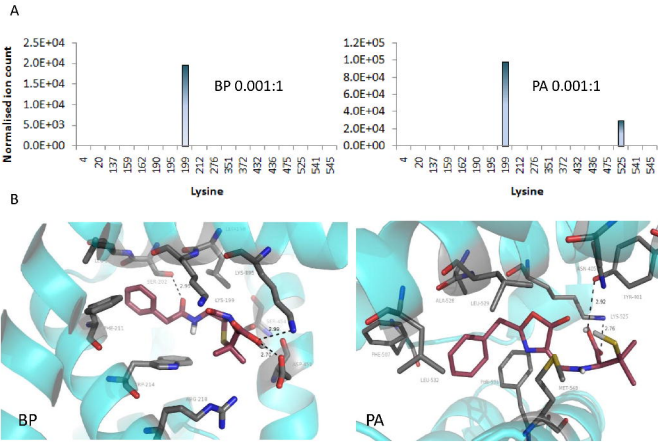


Figure 5

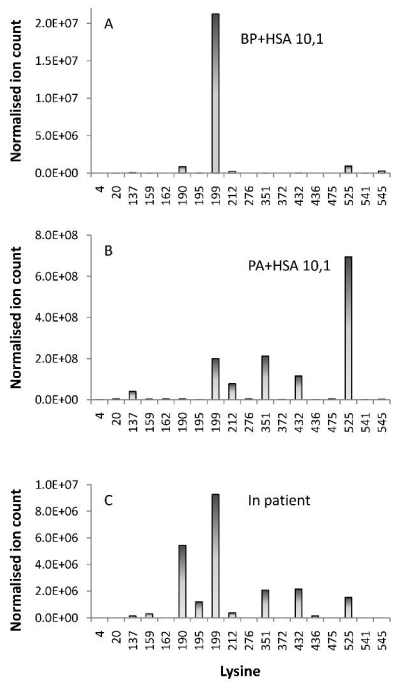
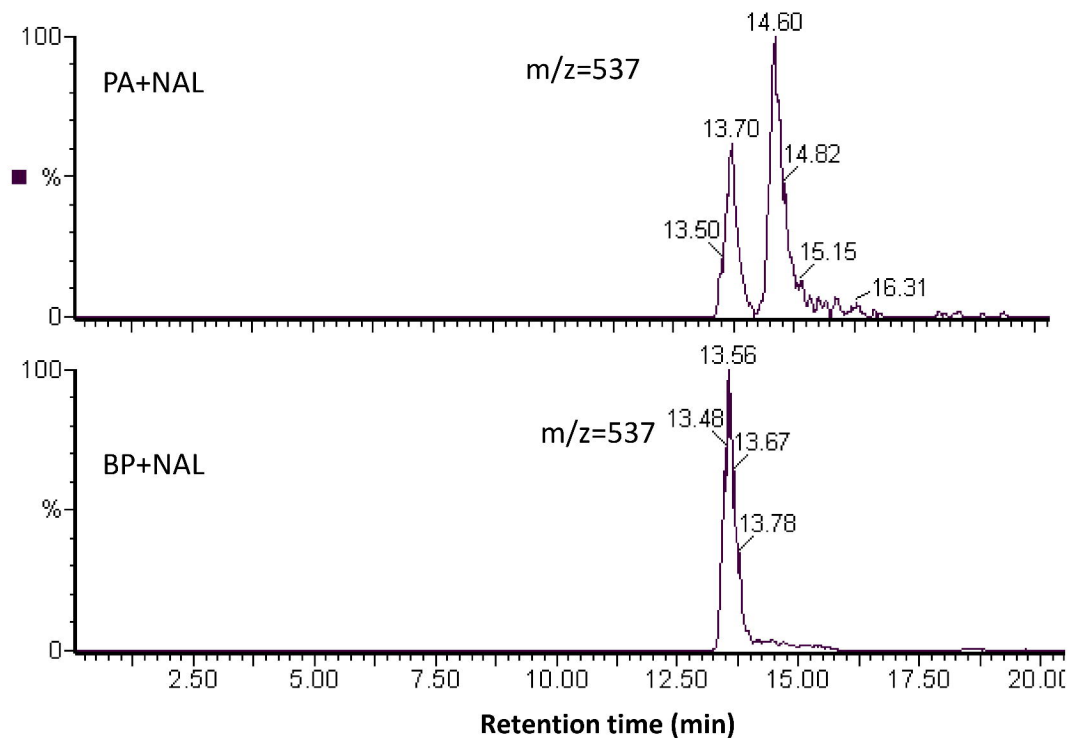


Figure 6

A



B

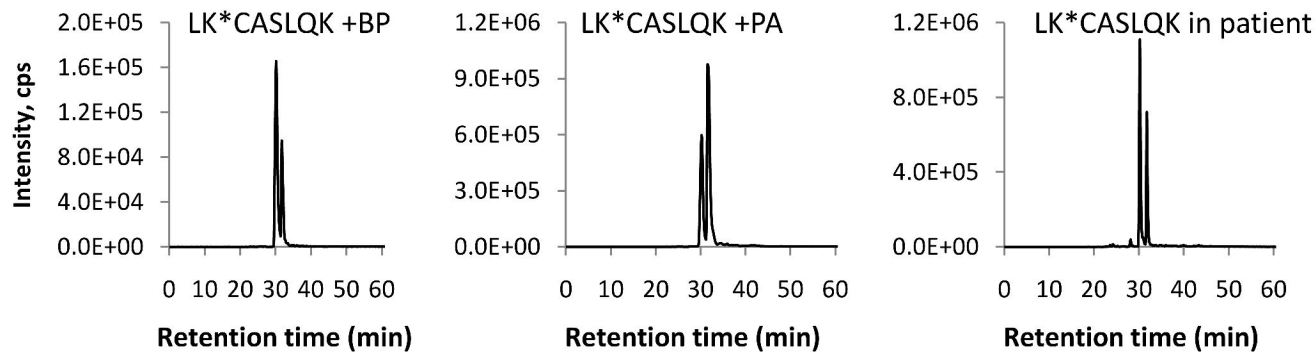


Figure 7

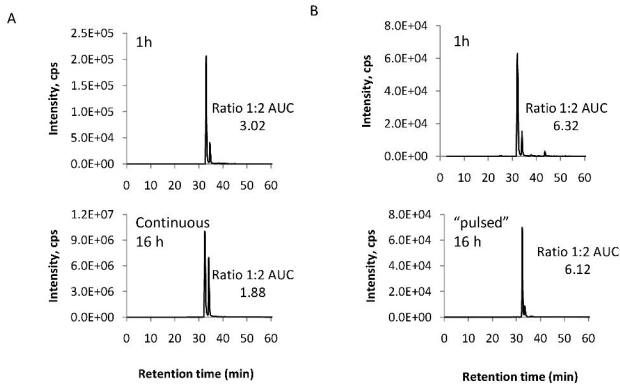


Figure 8

