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COMMUNICATION

Boosting the sensitivity of *in vitro* β-lactam allergy diagnostic tests

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The synthesis of structurally new haptens and development of suitable antigens is essential for boosting the sensitivity of drug allergy diagnostic testing. Unprecedented structural antigens for benzylpenicillin and amoxicillin are characterised and evaluated in a cohort of 70 subjects with a turnkey solution based on consumer electronics.

β-lactams (BLCs) are the most extensively used antibiotics for the treatment of bacterial infections[1], but also the most frequently involved in allergic reactions.[2,3] Penicillins -BLC antibiotics- represent 37% of total antibiotic consumption in the European Union. Between 2016 and 2018 the median intake was 7.1 defined daily doses per 1,000 inhabitants per day. [4]

According to the hapten hypothesis on the mechanism of antigen presentation, a drug (e.g., a hapten) covalently binds to specific lysine residues of endogenous proteins (carrier molecule) to be processed and presented by cells, eliciting an immune response[5]. The mechanism involves the production of major (–oyl) and minor (–anyl) antigens.[6,7] Approximately 95% of penicillin molecules results in the penicilloyl configuration, involved in 75 % of IgE-mediated allergic reactions. [8,9]

Quantitative inhibition methods have allowed to establish a correlation between fine structural features of haptens and allergic sensitivities in patients.[10,11] For effective development of sensitive and selective *in vitro* tests, the chemical structure of the hapten, among other critical variables, is essential to define the specific epitope that binds to the carrier molecule. Spacer arms have been widely used in the haptenization of small molecules [12–14] to increase exposure of the epitope and also to covalently link the hapten to a carrier molecule, reducing steric hindrances.[15] Experimental data on drug-derived antigens is limited. Montañez *et al*. synthetized different cephalosporin skeletons conjugated to carrier molecules to establish structure-IgE molecular recognition relationships[16], while Pastor-Navarro *et al.* synthetized various haptens for tetracyclines using spacer arms to induce heterology in their chemical structures.[17] Some results suggest that the conformational epitope should be accessible to generate antigens that are better recognized by specific IgEs.[18,19] According to certain researchers the ideal spacer arm should have[14]: (I) a proper length (at least three atoms), (II) no active centre that could lead to non-specific adsorption, and (III) bifunctional groups to react with the hapten and the carrier molecule. Another strategy to enhance drug-IgE molecular recognition is to prepare effective antigens with higher hapten:carrier ratio to increase the strength and specificity of the immune response and subsequent antibody generation.[20] This may be achieved by forming additional amines on the carrier protein by using cross-linkers to convert negatively charged carboxylates to positively charged amines.[21] Cationized proteins are known to increase the immune response compared to their native forms making them possible promising molecules to boost molecular recognition events.[22]

In this work, a collection of 13 diamine-derived haptens for benzylpenicillin (PG) and amoxicillin (AMX) was synthesized with aliphatic diamines of different alkyl chain lengths. To this end, the spacer arm was placed (1) at the carbonyl carbon after β-lactam ring opening (–oyl) and (2) at the carboxylic acid of the thiazolidine ring (–anyl), maintaining the β-lactam ring common to all BLCs, maximizing exposure to the lateral chain. Of the 13 haptens (see Supplementary nuclear magnetic resonance spectra), six were –oyl (**1-6**, Scheme S1) and seven -anyl (**7-13**) haptens, using PG (haptens **1-3** and **7-9**) and AMX (haptens **4-6** and **10-13**) as the starting material. These haptens were used to develop antigens (Scheme 1) conjugated to human serum albumin (HSA) prepared following the carbodiimide chemistry. HSA was employed due to its content of glutamic and aspartic residues, 60 and 30, respectively. Next, five antigens, one for HSA and four for histone H1, were prepared using cationized carrier proteins (Scheme 2). The effect of diamine chain length on molecular recognition of the diamine-based antigens was assessed by a multiplex microimmunoassay developed on a

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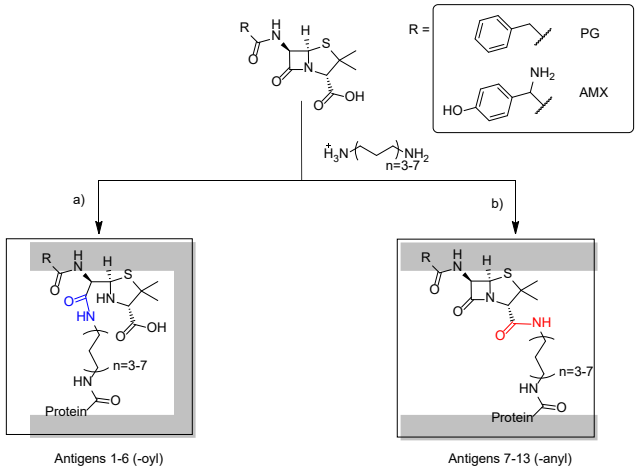
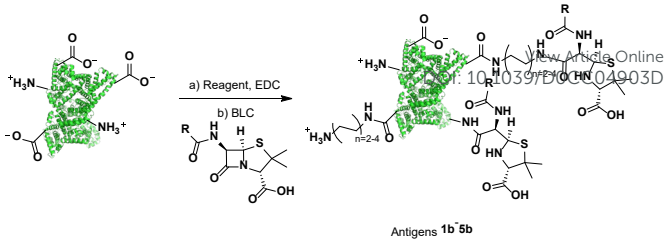
DVD with colorimetric detection (Figure S1-S2), using a hacked disc drive as detector.[23]



Scheme 1. Reaction pathway for antigen preparation
a) Protein, Na_2CO_3, pH=11, rt; b) 0.1 M DCM/DMF, then protein, 1x PBS pH=7,4, rt. PG, benzylpenicillin AMX, amoxicillin



Scheme 2. Chemical structures of the antigens using diamine cationized proteins.
[a] Reaction conditions: -oyl haptens: 0.5 M sodium carbonate, pH 11.0

Using the results of a reference antigen **14**, three comparisons were made. First, polyclonal IgGs to PG were used. These specific IgGs were obtained after rabbit immunization with the reference –lloyl antigen linked to keyhole limpet haemocyanin (Scheme S3). Sera dilutions ranging from 1/1,000 to 1/32,000 were tested and PBS-T was used as blank control. Results are plotted in Figure 1. No recognition was observed for either HSA (< 500 arbitrary units) or the antigen prepared with aztreonam (AZT) (<700 arbitrary units), used as negative controls. The term molar recognition ratio (MRR) was used to compare antigenic-specific responses, representing the ratio between the signal given by the –oyl and/or –anyl antigen(s) to the reference signal (antigen **14**) and expressed as percentages. Overall, all synthesized antigens detected polyclonal IgG to PG and the assay signals were similar to those obtained for the reference antigen. For -oyl antigens (Figure 1a), PG-based antigens **1-3** showed higher MRRs (ca. 103-123 %), while for AMX-based antigens **4-6**, MRRs ranged between 86 and 102 %.

Furthermore, the results revealed that –oyl antigens linked to 1,5-diaminopentane had the best responses, with MRR values of 123% and 102% for PG and AMX-based antigens, respectively. On the other hand, 1,7-diaminoheptane-based antigens had the lowest MRR values. For antigen **1b**, where cationized carrier molecules were used, results were similar, with a MRR of 95%. Signals of –anyl antigens (Figure 1b) were lower than for the reference. PG and AMX-based antigens **7-9** and **10-13** had MRRs between 76% and 98% and 58% and 88%, respectively. On the protective effect of the free amine on AMX, antigen **13** achieved higher MRR values (ca. 86%) than its corresponding non-protected antigen **10**, with a MRR value of 58%. Contrarily, no general agreement was reached regarding diamine alkyl chain length. Indeed, antigen **10**, linked to 1,3-diaminopropane showed the lowest serum response (ca. 58 %) for AMX, while PG antigen **9**, linked to 1,7-diaminopentane, reached higher MRR value (76%). In short, recognition of PG-modified antigens was better than for AMX.

Figure 2 shows the results of the evaluation of artificial human serum to PG. Variations were observed in PG-based antigens depending on the conjugation strategy. Antigens **1b** and **2b**, in which diamines where used to cationize the carrier, reached specific IgE (sIgE) concentrations of 23 IU/mL, ten times higher than the reference, indicating good recognition of the sera for these antigens. Regarding AMX-antigens, the best results were seen for antigens **5** and **6** with sIgE concentrations of 20 IU/mL.

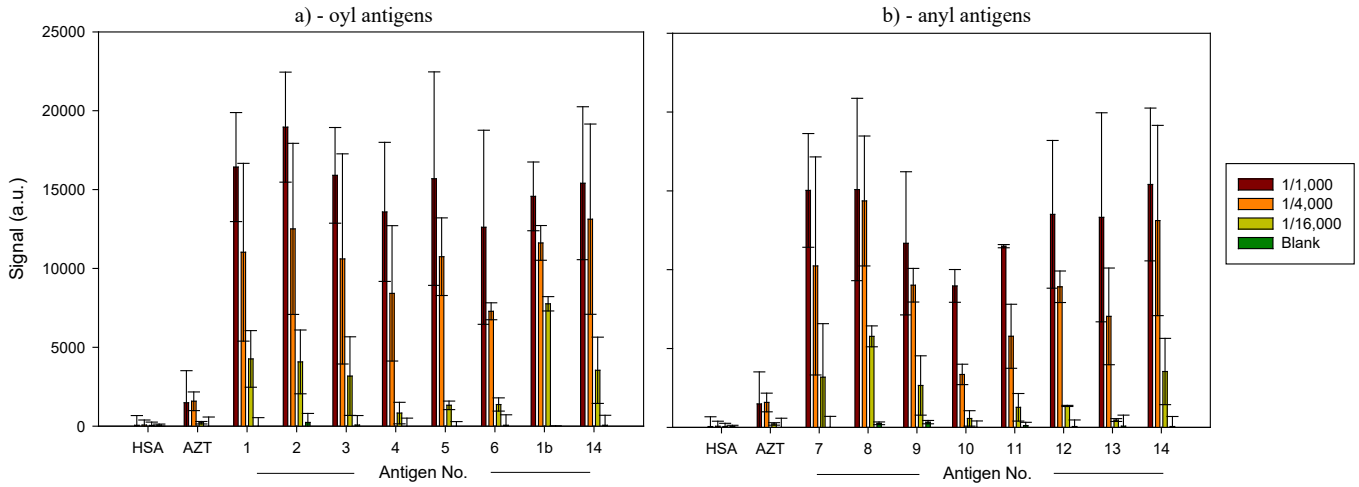


Figure 1. Signals (in arbitrary units, a.u.) for the modified β-lactams antigens with specific rabbit IgG to benzylpenicillin (n=5; dilution factors represented: 1/1,000, 1/4,000 and 1/16,000). a) –oyl antigens and b) –anyl antigens.

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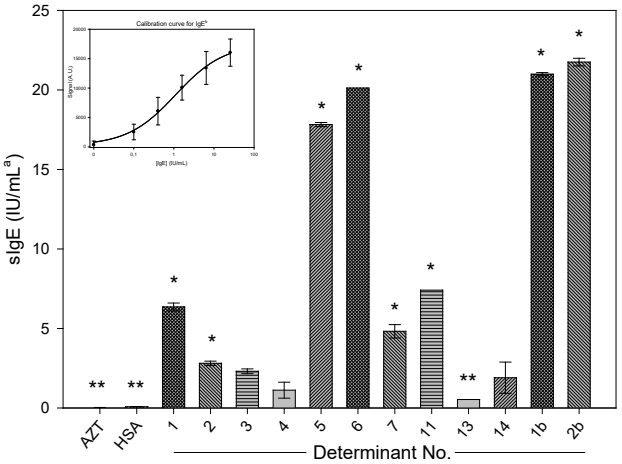

Figure 2. Evaluation of β-lactam antigens with artificial human serum specific to benzylpenicillin ([IgE] = 25 IU/mL). [a] 1 IU/mL = 2.4 ng/mL. The sensitivity measured as 50% effective concentration (EC_{50}) was 2.08 ± 0.78 IU/mL and the limit of detection, calculated as the concentration corresponding to the signal of the blank plus three times its standard deviation, was 0.07 IU/mL. The inset shows the standard curve for total IgE. The limit of quantification was 0.92 IU/mL and the relative standard deviation ranged from 3 to 15%. *indicates a statistically significant difference (p<0.001) as compared to reference antigen 14. ** indicates concentrations were below the limit of quantification.

No improvement of the results was seen when the free amine group of AMX was protected, as observed for antigens **7** and **13**, with sensitivities of 5 and 1 IU/mL, respectively. This may explain why antigens **5** and **6** were promising for *in vitro* diagnosis of BLC allergy. The conjugation of AMX could have been directed not only towards the free amine of the diamine, but also towards the free amine in its lateral chain, improving the molecular recognition yield. It may also explain the better response for AMX antigens, prepared by using diamines as spacer arms for the hapten synthesis in comparison to PG-based antigens, even in PG-specific artificial human serum. Furthermore, spacer arm length showed different patterns regarding the BLC antigen selected. This is in line with the results of previous study in which molecular recognition differed on the selected hapten.[24]

A cohort of 70 subjects, 35 of which developed an immediate reaction to β-lactams (Table S1), was tested. Prick tests (*in vivo*), ImmunoCAP, and the multiplex immunoassay (*in vitro*) results are shown in Table S2. When diamines were used as spacer arms (both –oyl and –anyl antigens) results were negative, with values below the limit of detection. Prick tests showed that four out of 31 PG patients (13 %) and 21 out of 31 AMX patients (68 %) were positive. With the *in vitro* ImmunoCAP reference test from the 35 allergic patients, seven PG (20 %) and thirteen AMX (37 %) were positive. The *in vitro* ImmunoCAP test allows sIgE to be quantified within a range of 0.01 to 100 IU/mL, with a cut-off point of 0.35 for positive results and levels above 0.10 IU/mL indicating sensitization. Furthermore, recent data indicates that the specificity of the immunoCAP system ranges from 85.7% to 100%, while sensitivity ranges from 0% to 25%, depending on initial clinical symptoms.[25] The evaluation of our antigens with the multiplex DVD-microimmunoassay in a cohort of 35 allergic patients, resulted in 21 PG (60 %) and 11 AMX (31 %) subjects positive with antigen **3b**. The 35 control patients were negative. Figure 3 shows a dot plot diagram of the clinical performances of the developed multiplex microimmunoassay.

Evaluation of the different diamines used to modify the carrier proteins with AMX (antigens **3b**, **4b** and **5b**) revealed that the shorter diamine, ethylene diamine, provided the best results. Its short chain length ensures minimal steric effects and virtually no hydrophobic interactions while the aromatic ring presented in 1,4-phenylenediamine may induce extremely steric effects and become less accessible. For instance, patient 04 had a specific IgE value to AMX of 0.44 IU/mL when ethylene diamine was used, and 0.02 IU/mL when both 1,4-diaminobutane and 1,4-phenylenediamine were used. The same was observed with patient 09 with values of 2.75, 0.04, and 0.06 IU/mL, respectively.

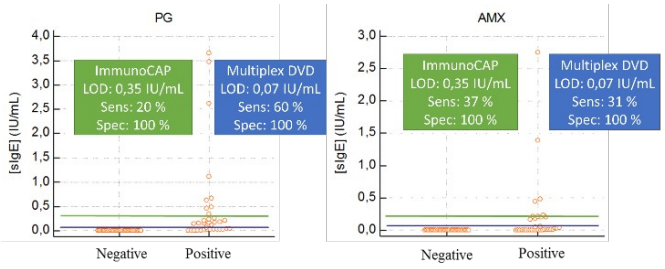

Figure 3. Dot plot of the specific IgE levels to PG and AMX determined by the multiplex DVD-assay. Positive and negative refer to samples from allergic patients and control subjects, respectively. The cut-off considered is the limit of detection of the developed multiplex DVD-assay and is represented as a blue line (>0.07). Sens and Spec stand for sensitivity and specificity using antigens **1b** and **3b** for PG and AMX, respectively.

In conclusion, the search for new antigens for allergy testing involves synthesis and screening of chemical entities to delineate the selectivity and the strength of interaction between the antigens and the IgE targets. Given the need to consider side-chain on individual β-lactam derived antigens and

the heterogeneity of the target β-lactam antibiotics, this research contributes with new approaches to synthesize structural haptens and generating specific antigens for immunoanalytical determination of IgEs, going beyond the state of the art. As we demonstrate, the synthesis of structurally new haptens to generate antigens for β-lactam allergy is key for developing highly sensitive *in vitro* tests to determine specific IgG and IgE antibodies. The purpose of our study was to synthetize a collection of haptens to produce a panel of major and minor antigens for PG and AMX to determine sIgE for these BLCs. We obtained a panel of 18 antigens, as well as a collection of different haptens (epitopes) using diamines of various length and different linkage strategies. To the best of our knowledge, this is the first report that describes the development of modified major and minor –oyl and –anyl antigens for benzylpenicillin and amoxicillin, demonstrating the potential for an improved serological diagnosis of IgE-mediated drug allergic reactions for commonly prescribed and consumed β-lactam antibiotics. We henceforth determine the presence of IgE antibodies in a defined allergic population in which Augmentin and amoxicillin were the most common inducers of immediate reactions. The results with patients showed that antigens for which diamines were used as spacer arms were not detected, although they recognized both specific IgG and IgE from immunized rabbits and artificial human sera to PG. We found a good correlation between our multiplex DVD immunoassay and ImmunoCAP for positive samples to both PG and AMX. Cationization of carrier molecules with diamines and their subsequent use to generate antigens is a suitable approach to detect specific IgE at low concentrations in allergic patients, improving the sensitivity of the existing *in vitro* tests for drug allergy. This result reveals that cationized antigens are a promising alternative to current –oyl derived antigens for boosting the sensitivity of the current *in vitro* test in antibiotic allergy. Synthesized amoxicillin –oyl haptens enhance drug-IgE molecular recognition, increasing three-fold the sensitivity of the existing *in vitro* diagnostic testing for antibiotic allergy. The current lack of clinically standardized reagents make the panel of synthesized structural haptens and the generated specific antigens a promising approach for massive detection of antibiotic allergies. In this way, better drug sensitization profiles could be defined, which will be of valuable support for allergy screening campaigns and antibiotic delabeling process.

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Conflicts of interest

The authors declare no competing financial interest.

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