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REVIEW

The role of IgE recognition in allergic reactions to amoxicillin and clavulanic acid

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Summary

Betalactam (BL) antibiotics are the drugs most frequently involved in IgE-mediated reactions. The culprit BL varies according to consumption patterns, with amoxicillin (AX) more prevalent in Southern Europe and penicillin V in Scandinavian countries. Nowadays, the combination of AX and clavulanic acid (CLV) is the most highly consumed BL containing medicine worldwide. Both BLs, AX and CLV, can independently be involved in reactions, which poses a diagnostic challenge. In patients with immediate allergic reactions to AX, two patterns of responses have been described, those responding to benzylpenicillin (cross-reactors) and those selective to AX. In addition, selective reactions to CLV account for around 30% of allergic reactions to the combination AX-CLV. These patterns of IgE recognition could be related to differences in the haptenation process, in the immunological response, or in the BL involved in the first sensitization. In this regard, patients with selective responses to CLV are generally younger than those allergic to AX or benzylpenicillin. So far, no evidence of cross-reactivity between CLV and other BLs has been reported. This shows the importance of an accurate diagnosis of CLV allergy, as patients with selective reactions to CLV could take other BLs including AX. Diagnosis can be performed *in vivo* and *in vitro*, although no immunoassay currently exists. Research regarding the CLV antigenic determinants and protein conjugates is essential to improve diagnosis. BLs need to covalently bind to a carrier protein to be immunogenic. The antigenic determinant of AX is the amoxicilloyl amide, but CLV leads to unstable structures, many of which are unknown. Moreover, the nature of the BL–protein conjugates plays an important role in IgE recognition. This review aims to summarize current knowledge on the immunochemistry, diagnostic approaches as well as chemical and proteomic studies for both AX and CLV.

Introduction

Immediate allergic reactions to drugs are mediated by specific IgE immune mechanisms [1]. Beta-lactam (BL) antibiotics are the drugs most frequently involved in these reactions [2–4]. Although all BLs, including penicillins, cephalosporins, monobactams, carbapenems, clavams and oxacephems [5, 6], can induce allergic reactions, each has a different prevalence and incidence in populations [7, 8]. These estimates are partially related to historical antibiotic consumption patterns, with amoxicillin (AX) [9–11] and to a lesser extent penicillin V (PV) [12] the main culprits, followed by cephalosporins [13].

Clavulanic acid (CLV) is a BL from the clavam group that, in spite of its weak antibacterial activity, is prescribed alongside AX, due to its β-lactamase inhibitor capacity. Although it was initially shown to be non-immunogenic in experimental animal models [1], as its commercialization, IgE-mediated allergic reactions to CLV have been reported [14–17]. Thus, from the first description of two patients with immediate reactions to CLV in 1995 [14], the number of cases has progressively increased [15–18] and in fact immediate reactions to CLV are more common than to benzylpenicillin (BP) in Spain [16].

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Patients with allergic reactions after AX-CLV administration can react to either AX or CLV [16]. Although both are BLs, cross-reactivity between them has not been reported [15], probably due to differences in their chemical structures and degradation patterns [19]. This is an important issue because CLV selective patients can safely take other BLs, including AX, increasing therapeutic options and avoiding the use of inappropriate alternative treatments, which are often more expensive and have more potential adverse effects [20].

The reasons why patients develop an allergic reaction to either all penicillins (cross-reactors), to AX only or to CLV only (selective reactors) after taking AX-CLV are not fully understood. It has been hypothesized to be related to the BL involved in the primary sensitization rather than on the following contacts [15].

Understanding IgE-mediated reactions induced by AX-CLV requires an in depth analysis of recent advances in different areas. Studies in the fields of immunology, proteomics and chemistry can help to improve our knowledge about recent changes in BL allergy patterns. This can be used as a model and be applied to other BLs.

This review describes (i) changes in consumption patterns in recent years and their influence on the frequency of IgE responses to different BLs, including the appearance of CLV allergy; (ii) the well-established IgE recognition patterns of penicillins and the limited number of studies of CLV IgE recognition; (iii) the current diagnostic approaches available; (iv) how AX and CLV structures lead to different immunogenic determinants as well as the importance of the carrier protein to form conjugates, which are recognized by IgE; and (v) how all this knowledge can be used to improve the allergological work up for these patients.

Influence of BL consumption in patterns of IgE response

The true prevalence and incidence of allergic reactions to BLs in the general population is not well known. Results can be overestimated if patients are diagnosed according to clinical history alone or underestimated if patients are evaluated long after the allergic episode. The prevalence for immediate BL reactions accounts for 16.1% of all drug allergic reactions [7].

The BL consumption pattern is different among European countries and in the same country over time (Table 1). In 1997, in Belgium, the broad-spectrum penicillins (mainly AX) became the most popular penicillins and its combination with CLV represented more than 50% of penicillin used, figures extended to Austria, Belgium, Hungary, Luxemburg, Portugal and Spain in 2003 [2]. On the contrary, in 2003, narrow-spectrum penicillins (mainly PV) still represented more than 60% of total penicillin use in Norway, Sweden and Denmark, whereas in Belgium, France, Italy, Luxemburg, Portugal and Spain, they represented less than 2%. These differences in the pattern of consumption are in agreement with the specific BL involved in the allergic reactions that also vary among countries. In fact, PV is the most frequent culprit in Scandinavian countries [12], and AX in southern Europe [9–11, 15, 16, 21]. Importantly, in recent years, there has been a significant increase in the number of immediate reactions induced by AX-CLV where CLV was responsible [15, 16, 18], and considering the increasing consumption of this combination, it is expected that this pattern of selective reactions to either AX or CLV will continue [2].

Table 1. Patterns of BL consumption in European countries

Country	Year	Main BL	Consumption (%)
Belgium	1997	AX-CLV	50
Belgium Austria Hungary Luxemburg Portugal Spain	2003	AX-CLV	50
Norway Sweden Denmark	2003	PV	60
Belgium France Italy Luxemburg Portugal Spain	2003	PV	<2

There are two important findings in patients with immediate allergy to AX-CLV. The first is that those patients with selective reactions to CLV are younger on average than those with selective reactions to AX, whilst both groups of patients are younger than cross-reactors to BP [16]. This is most likely related to the dates these drugs were introduced, as older patients would not have been exposed to AX-CLV when younger, demonstrating the important role of the first sensitizer in the development of IgE-mediated responses, as has been described for other BLs [22]. The second is that those patients selective to CLV do not cross-react to other BLs. In fact, it has been recently shown that selectivity is a stable phenomenon as most subjects with immediate selective allergic responses to AX tolerate BP and PV in subsequent administrations and all subjects with selective response to CLV tolerate subsequent administrations of BP, PV and AX [15]. These observations deserve further studies.

IgE recognition studies

It is not clearly understood why a subject taking a drug becomes tolerant or allergic to it. This is challenging in the case of AX-CLV, where the same patient can develop different responses to either BL administered simultaneously, allergy to AX and tolerance to CLV or *vice versa* [16]. This could be related to differences in drug processing and recognition by the immunological system.

The antigenicity of penicillins has been evaluated *in vitro* using monoclonal antibodies identifying three different epitopes: the side chain, the thiazolidine ring and the part resulting from the conjugation of the four-member β-lactam ring to the carrier protein [23, 24]. Using the same approach with AX, it was demonstrated that the majority of the generated monoclonal antibodies recognized an epitope in which the side chain was a major constituent, although with variable contributions from other regions of the molecule [25]. The relevance of the AX side chain in IgE recognition has been recently corroborated in a study that investigated the influence of the tridimensional conformation of BP and AX on IgE recognition using synthetic nano-carriers [26]. Differences in spatial conformation were found, with the benzylpenicilloyl (BPO) group partially exposed to IgE recognition, as the benzyl side chain was oriented inside the carrier molecule, whereas the entire structure of the AXO group was outside of the carrier molecule [26].

These results may explain why patients allergic to AX who have good tolerance to BP (selective reactors) showed a specific recognition of the AX side chain structure whereas those who had clinical responses to both AX and BP (cross-reacting responders) presented IgE directed to the nuclear part of the BL [9, 22, 27–29]. Moreover, the rate of negativization of STs over time was different in both groups, being faster in those with selective reactions that in those with cross-reactions [30]. In the last ten years, the determination of sIgE to BP and AX bound to basophils using flow cytometry (basophil activation test, BAT) has provided an interesting tool for evaluating IgE immunological recognition, although initial studies indicated that this was less accurate than immunoassay for the identification of selective responders [31, 32].

Regarding CLV, the number of studies is rather limited. The first study, made in 1988, showed that although CLV was able to react with protein amino groups, it did not induce an antibody response. Moreover, it could not elicit contact sensitization in animal models or even sensitization in humans when it was prescribed in combination with AX, indicating that CLV had a very low immunogenicity [1]. In spite of this, years later, several studies showed the existence of immediate selective allergic reactions to CLV [14, 16, 18, 33, 34]. *In vitro* studies developed with BAT demonstrated evidence for the presence of specific IgE antibodies to either AX or CLV in patients with immediate reactions after AX-CLV intake [16, 18, 34]. In a more recent study performed in a series of patients with an immediate reaction after AX-CLV intake, 30% of patients showed a selective response to CLV and good tolerance to BP and AX, with BAT positivity to the combination AX-CLV and CLV, but not to AX or BP. Importantly, it was confirmed that basophil activation was mediated by specific IgE, using a specific inhibitor of the IgE cascade (the wortmannin test) [16, 35, 36], and by the decreased BAT positivity observed one year after the reaction [16]. All these studies, in contrast to previous experimental data [1], demonstrate the immunogenicity of CLV in humans and its ability to induce IgE-mediated reactions.

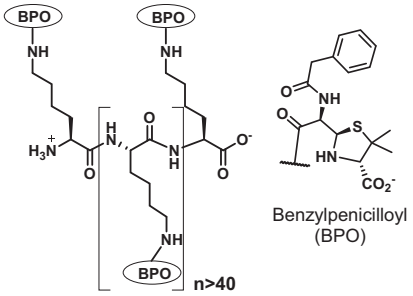
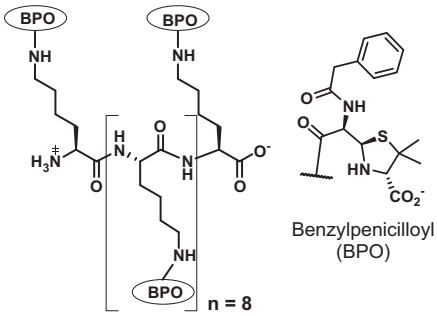
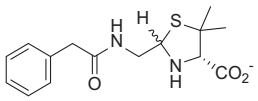
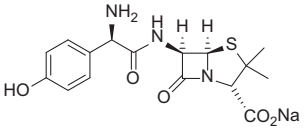
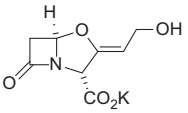
The reasons why patients have different selective responses either to AX or CLV after AX-CLV administration are unknown. Apart from interindividual differences in immunological recognition other factors can influence such as the concentration of each BL in the formula (ratio AX/CLV ranging from 4/1 to 16/1) and the first sensitizer. In this sense, as mentioned above, it has been observed that patients with selective response to CLV are younger than those allergic to AX [16] indicating the importance of establishing chronology in the initiation of consumption of each BL.

Diagnostic approaches

Skin testing is the most widely used diagnostic method to evaluate IgE-mediated allergic reactions to BLs, and commercial kits are available (Table 2). The current BP determinants consist of BPO octa-L-lysine and benzylpenilloic acid in many European countries, and of BPO poly-L-lysine in USA and Canada. However, in the case of AX, the equivalent determinant for benzylpenicilloic acid (amoxicilloic acid) and benzylpenilloic acid (amoxilloic acid) are not of value in ST, and AX itself with the intact β-lactam ring is the reagent used. According to the Landsteiner hapten-carrier hypothesis [37], after application of AX to the skin, it conjugates with a carrier protein quickly forming the AXO determinant *in situ*, leading to a positive wheal and flare ST response in just 20 min.

For the ST to work optimally, it generally requires total solubility of the drug; however, there has been some debate as to whether this is true for AX [38]. Two types of commercial AX are available, the injectable formulation (AX sodium salt) and the oral formulation (AX trihydrate); however, only the injectable sodium salt form allows the dissolution in water up to concentrations recommended for ST (20 mg/mL). As a

Table 2. Current commercial reagents for performing skin testing

Drug	Commercial Name Company Commercialization date	Reagent	Concentration mg mL^{-1}	 Molar
BP	PRE-PEN® AllerQuest LLC (Plainville, US) 2009	Benzylpenicilloyl poly-L-lysine (PPL)  		6 × 10^{-5} (of BPO)
BP	DAP® Diagnostic Allergy Penicillin Diater (Madrid, Spain) 2011	Benzylpenicilloyl octa-L-Lysine (BP-OL)  	0.04	8.6 × 10^{-5} (of BPO)
		Benzylpenilloate (PO) 	0.5	1.5 × 10^{-3}
AX	DAP® Amoxicillin Diater (Madrid, Spain) 2010	Sodium Amoxicillin 	20	5 × 10^{-2}
CLV	DAP® Clavulanic Diater (Madrid, Spain) 2010	Potassium Clavulanate 	20	8.4 × 10^{-2}

consequence, only the injectable AX sodium salt has been validated for diagnosing immediate hypersensitivity to penicillins [9, 39, 40]. Similar results have been obtained for the latest AX compound designed for ST, commercialized since 2010 [41]. In that sense, according to the EAACI guidelines, it is mandatory to use AX in ST for diagnosing immediate hypersensitivity to BL [10, 42]. However, even including AX in the ST hapten panel in patients with immediate allergic reactions to penicillins, sensitivity is still not optimal, ranging from

50% to 70% [10, 39, 43]. In an attempt to increase sensitivity, other determinants derived from AX, such as amoxicilloic acid and diketopiperazine, have been tested, but unfortunately their inclusion did not improve the diagnostic capacity of either ST or *in vitro* tests [44].

As previously mentioned, an increasing number of patients are referred to allergy unit centres with adverse reactions after intake of AX-CLV. The suspicion of CLV allergy in patients with immediate allergic reactions after AX-CLV administration has been classically based on the presence of negative response in both ST and specific IgE determination to AX and a positive response of ST to AX-CLV. However, as has been shown in CLV selective patients, ST to AX-CLV is positive in only 18% of cases, which can be attributed to the lower concentration of CLV compared to AX in the AX-CLV combination [16]. Unfortunately, when using AX-CLV, CLV is increased with a parallel increase of AX leading to concentrations that produce false-positive results [16].

The recent commercialization of CLV, in potassium salt form, for ST diagnosis has shown a sensitivity from 9 to 18.7% in skin prick test and from 63.6 to 81.2% in intradermal testing [15, 16, 18]. According to recent studies performed in Spanish populations and using ST with CLV, it has been demonstrated that patients with immediate hypersensitivity reactions after AX-CLV administration respond to BP determinants in less than 10% of cases, to AX determinants in more than 50% and to CLV in more than 30% [15, 16, 18]. All these data support the need to include not only AX but also CLV for ST in the routine diagnosis of allergic reactions to AX-CLV.

Given that ST sensitivity is not optimal, a drug provocation test (DPT) must be considered for establishing diagnosis in a non-negligible percentage of cases [11]. However, DPT requires well-trained personnel and an appropriate setting, is time-consuming and carries some risk. DPT can be used to assess AX allergy/tolerance directly [42], but its use for CLV is complicated by the fact that this BL is only available combined with AX. Therefore, assessment of CLV allergy/tolerance must be made indirectly, by assessing tolerance to AX in patients with positive DPT to the combination of AX-CLV.

Regarding *in vitro* tests, BL hypersensitivity has classically been evaluated by quantifying specific IgE in serum by immunoassay. However, commercially available tests only include BP, PV, AX, ampicillin and some cephalosporins but not CLV. Moreover, as these tests do not show an optimal sensitivity (38–66%) and specificity (52–100%) [39, 45–48], they do not help to rule out other penicillins and therefore to suggest an IgE response to CLV. On the other hand, there are no home-made immunoassays for detecting specific IgE to CLV, probably due to its chemical characteristics and the lack of knowledge of both CLV determinants and candidate carrier protein to be used in the solid phases.

Currently, only BAT has been shown to be useful for evaluating IgE-mediated allergic reactions to CLV [16, 34], as is the case for other drugs like fluoroquinolones [49] and pyrazolones [50] where immunoassay are not available. In a well-characterized group of patients with confirmed selective reactions to CLV, a good correlation with ST has been found [18], with a 50% sensitivity and 90% specificity [16]. These data demonstrate that this technique is a reliable *in vitro* method for the detection of IgE-mediated CLV allergy, especially in those patients with anaphylactic reactions and negative ST results, avoiding the need for DPT. Thus, BAT can be used to complement ST diagnosis.

Until now cross-reactivity between CLV and other BL has not been found in studies using *in vivo* or *in vitro* tests [14–16, 18, 33, 34, 51]. This suggests that allergy to CLV is selective, allowing CLV allergic patients to be treated safely with any other BL.

Formation of BL–protein conjugates

BLs are low molecular weight molecules that, according to the hapten hypothesis, do not induce an immune response unless they are covalently bound to a carrier molecule [37]. The epitope or antigenic determinant can be formed by both the BL chemical structure and a part of the carrier protein. Thus, this immune response is determined not only by the BL chemical structure but also by the nature of the carrier molecules (mostly proteins) [37], with the density and distribution of the BL on the carrier molecule having an important role [52, 53].

Chemical structure of antigenic determinants

The pattern of IgE recognition is determined by the BL chemical structure. The chemical core of both AX and CLV consists of a bicyclic structure (a four-member β-lactam ring condensed to a five-member ring) (Fig. 1). The main difference between both structures is that the five-member ring in AX contains a sulphur atom, which is substituted for an oxygen atom in CLV (Fig. 1). Other differences are the substituents at C-2, and the presence of a side chain (acylamino substituent) at C-6 in AX, whereas there is no substituent in CLV [54].

The formation of the antigenic determinants of BLs requires the nucleophilic attack at the β-lactam carbonyl by the ε-amino groups of lysine, leading to the drug–protein conjugate [55, 56]. Therefore, the immunological behaviour of BLs is determined by their intrinsic chemical reactivity. The major antigenic deter-

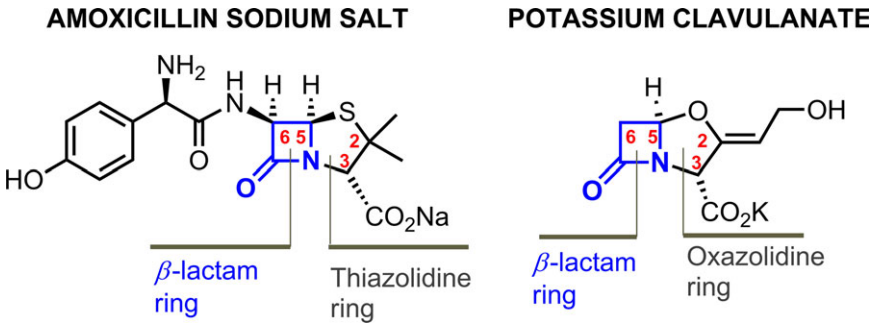


Fig. 1. Chemical structures of AX and CLV.

minant of AX is the amoxicilloyl (AXO) amide, which results from the straightforward opening of the β-lactam ring by amino groups, in the same way as other penicillins (Fig. 2a) [57, 58]. This chemical structure is stable enough to enable purification and characterization by classical spectroscopic techniques [26] and has been considered an unquestionable antigenic determinant [58–60]. Other determinants result from hydrolysis of the β-lactam ring, amoxicilloic acid or intramolecular acylation by the amino group of the AX side chain, diketopiperazine (Fig. 2b). Their stability has allowed their chemical and immunological characterization [44].

CLV has a more complex chemistry than AX [54, 61–63] in terms of its conjugation process, and relatively little is known about its immunogenicity. It is assumed that for developing an allergic response to CLV, a nucleophilic attack at the β-lactam carbonyl by a protein-associated nucleophile is required, leading to protein conjugates. However, the instability of the structure after protein conjugation may produce more complex degradation pathways, leading to multiple possible determinants, making it difficult to identify the chemical structures that make up the antigenic determinant [55]. To our knowledge, only one study, published in 1988, has described the intrinsic immunogenicity of CLV, in which the formation of small determinants is hypothesized (Fig. 3a) together with other degradation products (Fig. 3b) [1]. Protein conjugation consists of β-lactam ring opening through lysine residues leading to a CLV–protein conjugate (CLV1), a highly instable structure that undergoes degradation to form several structures (Fig. 3). The best described structure is CLV4, which consists of a very small molecule with aldehyde functionality. All described intermediates are possible antigenic determinants involved in IgE recognition.

Moreover, recent publications out of the context of allergy have described CLV binding to β-lactamases [54, 64, 65], with protein conjugation mechanisms and proposed structures in agreement with those previously described [1]. Although somewhat scarce, available data point to the formation of CLV–protein conjugates that display very small and heterogeneous epitopes with a very low density in the carrier [1]. More studies are needed to obtain insight into the true structure of the relevant antigenic determinants of CLV.

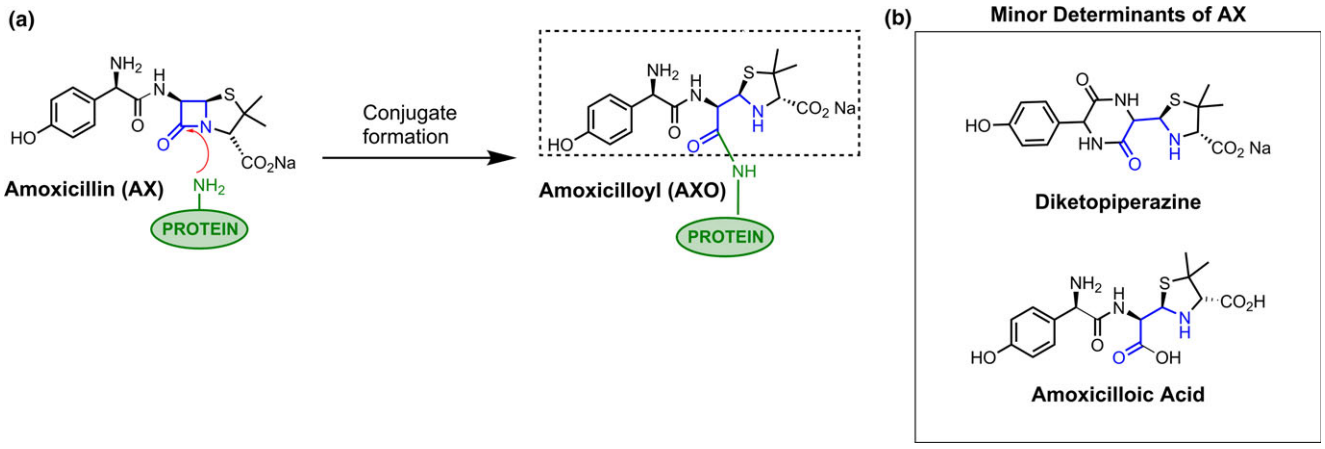


Fig. 2. Conjugation of AX to its carrier protein and the generation of its antigenic determinant: (a) the major antigenic determinant AXO formation, a stable, isolable and well-characterized structure, shown in the dotted box; (b) minor determinants of AX, which do not bind the protein.

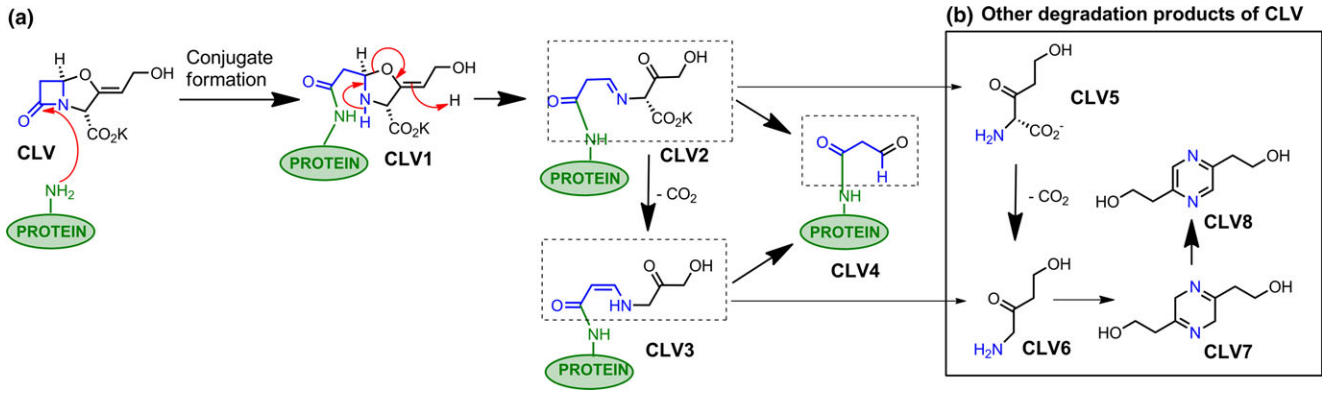


Fig. 3. Conjugation of CLV to carrier protein leads to an intermediate which is not a stable, isolable and well-characterized structure: (a) pathway to the formation of hypothesized antigenic determinant structures of CLV (shown in the dotted boxes); (b) other degradation products of CLV, which do not bind the protein.

Candidate proteins for drug conjugate formation

Knowledge of the haptenization process that occurs *in vivo* as well as the potential candidate carrier proteins for BLs is crucial for understanding the mechanisms involved and for improving the diagnostic approaches used in the evaluation of BL allergic patients. One of the main limitations of *in vivo* haptenation studies has been the difficulty to detect BL–protein conjugates generated after BL administration. In this sense, significant advances in high-resolution mass spectrometry (HRMS) have allowed the identification and characterization of proteins modified by AX and other BLs [59, 66–71]. Human serum albumin (HSA) has been considered the main target protein for BL haptenation for several reasons: it is the most abundant protein in plasma, has an extraordinary ligand-binding capacity and has a crucial role as carrier for endogenous and exogenous compounds, including several drugs [72]. Recently, the modification of HSA by AX in serum from subjects treated with AX [66] or serum proteins modified with AX *in vitro* [67] have been characterized by tandem HRMS coupled to a liquid chromatography system (HRMS-LC). AX was shown to have preference for some HSA-specific residues (Lys 190, 199, 351, 432, 541 and 545) that are common to other BL antibiotics (BP, flucloxacillin and piperacillin) [59, 66–71] (Fig. 4). Moreover, the grade of modification is dependent on the AX concentration [67], as occurs for other penicillins [68, 69, 71]. However, similar studies have not been performed for CLV and the degree of competition with AX for common haptenation targets is not known. Although the factors that determine which amino acids are modified by BLs are not well understood, the presence of a serine close to the lysine in the polypeptide chain or in the tertiary configuration of the protein could favour BL binding to lysine residues [59, 73].

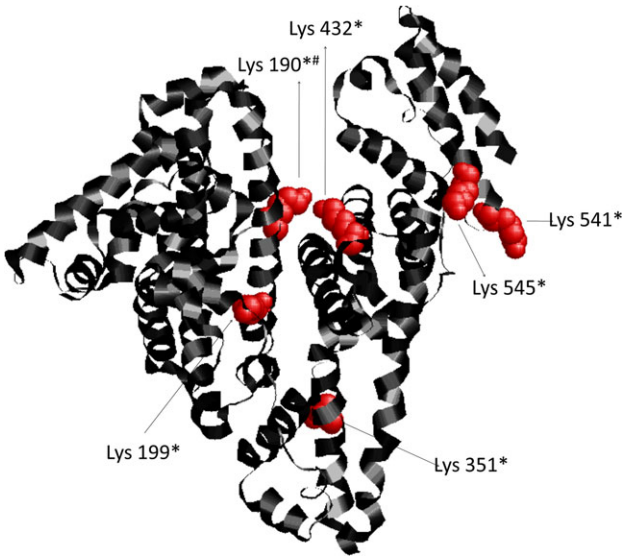


Fig. 4. Human serum albumin modified by amoxicillin model. Lysine residues modified by amoxicillin (*in vitro** [67] and *in vivo*# studies [66]) identified by mass spectrometry are remarked in red. Atomic coordinates are taken from the code 1AO6 [82] of the Protein Data Bank.

In addition to HSA, other serum proteins such as transferrin and immunoglobulins (light and heavy chains) have been identified as AX differentiated target proteins *in vitro* [67]. Furthermore, using a biotinylated AX analogue (AX-B), the presence of intracellular modified protein conjugates has been observed in cellular fractions from cell lines (monocytes, B-lymphoma cells and macrophages) with different band patterns in the Western blot, showing that the haptenation process may be cell type dependent [74]. The pharmacokinetics of AX or CLV could also influence the haptenation process and should be considered in future studies of proteins haptenation with co-administration of both AX and CLV. Both AX and CLV reach the maximum

concentration levels approximately 1 h after oral administration, with similar time–concentration profiles [75], low levels of serum protein binding with approximately 70% of the drug remaining free in serum (as described in Vademecum) and a mean bioavailability of 22.8% [76]. A great variability in the clearance of both AX and CLV has been found in critically ill patients treated with AX-CLV [77]. However, this does not occur in healthy volunteers taking AX [78–80]; unfortunately, this type of data is not available for CLV.

Conclusions

Bacterial resistance is becoming a major problem and is potentially a 'catastrophic threat' to medicine [81]. The use of β-lactamase inhibitors such as CLV can help mitigate this problem and highlights the need to be aware of CLV allergy and differentiate between selective AX and CLV reactions in patients treated with AX-CLV. However, many individuals are erroneously labelled as allergic to BL and then recommended to avoid all BLs. In countries with high consumption of AX or AX-CLV formulations, it is compulsory to find out whether patients have selective reactions and are therefore able to tolerate other BLs. In that sense, it is increasingly recommended to use AX and CLV separately for both *in vivo* and *in vitro* tests.

CLV lacks a side chain and has an oxazolidine ring bound to the β-lactam ring, differences that could contribute to the generation of antigenic determinants with little or no cross-reactivity with those generated by BP, AX or other BLs. This is important because it implies that an accurate diagnosis of a patient as selective to CLV will allow the administration of other BL derivatives including AX.

However, much work is still necessary in order to identify the chemical structures involved in the generation of the specific immunogenic epitopes, including candidate protein carriers, and to study their immunogenicity. This will permit us to improve the diagnostic methods currently available and ensure that patients are correctly diagnosed and able to receive the right medication.

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Conflict of interests

The authors declare no conflict of interest.

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