

Proteomics in immunological reactions to drugs

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Purpose of review

To discuss the avenues that proteomic techniques are opening for the study of the chemical basis and cellular mechanisms of immunological reactions to drugs.

Recent findings

Technical developments in recent years are allowing a detailed characterization of drug–protein interactions. In addition, novel metabolic pathways for drug biotransformation are being uncovered and potential targets for protein haptentation are being proposed that may help in the understanding of these complex processes.

Summary

Immunological reactions to drugs pose important clinical problems. Since early works exploring drug–protein interactions, there has been steady progress in this field. However, the mechanisms involved remain incompletely understood. The availability of proteomic techniques with high resolution and sensitivity presents a unique opportunity to tackle this subject from a broad perspective, integrating work in model systems and in patients. Chemical and metabolic characterization of immunological reactions to drugs may also help in the prevention, diagnosis and/or treatment of these processes.

Keywords

drug allergy, protein haptentation, proteomics

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Introduction

Immunological reactions to drugs pose an important problem in clinical practice. Clinically, these entities may range from a mild cutaneous rash to severe anaphylactic shock and death. Importantly, these reactions reduce the therapeutic options for many patients. Particularly, patients in need of chronic treatment with anti-inflammatory drugs face problems finding well tolerated drugs [1,2]. In addition, rising incidence of immunological reactions to antibiotics becomes an important issue in the context of increasing development of antimicrobial resistance [3,4]. Although less frequent, allergic reactions to radiocontrast agents and anesthetic agents constitute important risks for diagnostic and surgical procedures [5].

Although the immunological processes underlying allergic reactions have been exhaustively studied, we are still far from understanding the molecular and chemical processes involved in allergic reactions to drugs. Particularly, it is especially intriguing how and what genetic and environmental factors trigger an allergic reaction to develop at a precise moment in life. Epidemiological and genetic studies are yielding important clues into the mechanisms and factors involved in drug sensitization [6]. The development of systems biology strategies, the massive sequencing capabilities, multiplexing technologies and the development in recent years of

high-sensitivity and high-throughput proteomic tools are expected to promote significant advances in our understanding of these processes. In this review, we attempt to present the early work and the recent advances in proteomic approaches exploring the mechanisms of immunological reactions to drugs.

General description of immunological reactions and mechanisms of immunological reactions to drugs

Adverse drug reactions (ADRs) are defined by the World Health Organization as any noxious, unintended and undesired effect of a drug that occurs at doses used for prevention, diagnosis or treatment. ADRs constitute an important public health issue, causing 5% of hospital admissions [7]. ADRs with an immunological basis or allergic drug reactions account for 6–10% of all ADRs. These reactions require a sensitization period, during which the patient is exposed to the drug that stimulates the immune system by producing antibodies, sensitized T cells or both. Allergic drug reactions are reproduced with a new drug exposure and are elicited at drug doses far below the therapeutic range, usually subsiding after drug discontinuation [8].

As with hypersensitivity reactions in general, drug allergic reactions can be classified according to the categories

defined by Gell and Coombs, which have been updated [9] and are as follows: type I or immediate hypersensitivity reactions [mediated by drug-specific immunoglobulin (Ig) E antibodies]; type II or cytolytic or cytotoxic reactions; type III or immune complex-mediated reactions; and type IV or delayed hypersensitivity reactions (mediated by drug-specific T lymphocytes). Some allergic reactions to drugs cannot be easily classified due to lack of mechanistic information, but most of them are IgE or T-cell mediated.

Although any drug can induce an immune response, the IgE-mediated immediate hypersensitivity reactions to β -lactams constitute the most frequent allergic reactions. In addition, NSAIDs such as pirazolones, arylpropionics and arylacetics are widely prescribed, and the incidence of hypersensitivity reactions toward these drugs is increasing. The drug causing more cases of liver failure is paracetamol (acetaminophen). Although this is not a classical 'allergic' reaction, the immune system may play a key role in acetaminophen-induced liver toxicity [10].

Mechanistically, two types of hypothesis attempt to explain immunological reactions to drugs (Fig. 1). According to the 'hapten hypothesis' [11], small chemical agents, such as drugs, would be too small to elicit allergic reactions by themselves, but would do so when

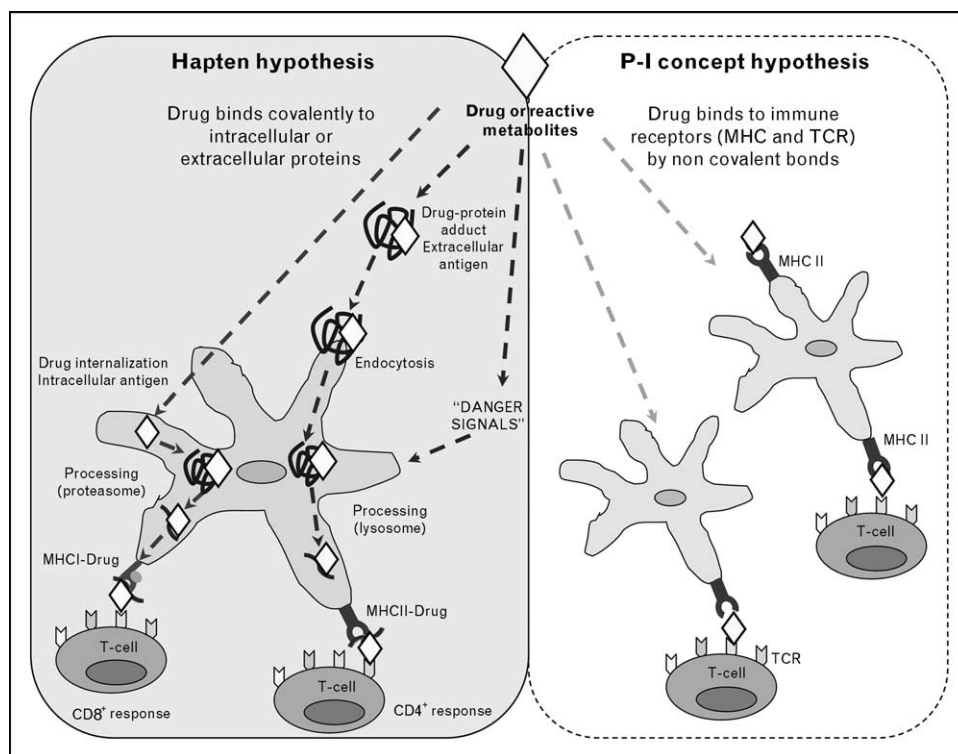
Key points

- Usefulness of proteomic approaches in the characterization of drug–protein complexes.
- Importance of biotransformation in drug reactivity.
- Proteomic identification of novel intracellular and extracellular drug targets.

conjugated to proteins. Therefore, chemically reactive drugs or their metabolites would act as haptens and form covalent adducts with extracellular or intracellular proteins. This process is known as haptenation. Drugs or metabolites bound to intracellular proteins are processed via proteasomal degradation, presented by major histocompatibility complex I (MHC I) and elicit a $CD8^+$ cell response. However, drugs or metabolites bound to extracellular proteins are processed through lysosomal proteolysis, presented by MHC II and elicit a $CD4^+$ cell response [12]. Some drugs can directly modify MHC molecules and stimulate a T-cell response [13].

High-affinity noncovalent interactions could also be involved in the pathophysiology of immunological reactions. According to the p-i concept [14,15], activation of the immune system would be mediated by binding of drugs to immune receptors, such as T-cell receptors or MHC, by van der Waals forces, electrostatic and

Figure 1 Proposed mechanisms of adverse reactions to drugs



MHC, major histocompatibility complex; TCR, T-cell receptor.

hydrogen bonds (Fig. 1). A more detailed discussion on drug-induced hypersensitivity can be found in the excellent review by Park *et al.* [16].

Chemical aspects of protein haptentation by drugs

Formation of drug–protein adducts plays an important role in drug toxicity in general and in immunological reactions to drugs in particular (Fig. 2). Drugs may react via acylation, alkylation or Michael addition, among other reaction types [17]. Benzylpenicillin was the first hapten studied in detail and has traditionally been considered as the model hapten for extending comprehension of allergic reactions. Benzylpenicilloyl (BPO) is the major antigenic determinant for penicillins, formed by the nucleophilic opening of the β -lactam ring by free amino groups on proteins. In some patients, this conjugate leads to an immune response against the penicillin–protein adduct [10]. Early works described the attachment of BPO groups to six of 59 lysine residues of human serum albumin (HSA) [18]. In the context of hepatotoxicity, adducts of flucloxacillin have been detected by western blot in the liver of rats treated with this β -lactam antibiotic [19].

Unlike the well established structures of penicillin conjugates, the chemical structures derived from cephalosporins that interact with specific antibodies are still unknown [20]. By analogy with penicillins, it is accepted that the conjugation process of cephalosporins follows the scheme depicted in Fig. 2 [21]. However, the resulting conjugate is unstable, hampering the identification of the structures responsible for immediate allergic reactions [20].

Importance of drug metabolism or biotransformation in protein haptentation

Many drugs are not chemically reactive *per se*, but their metabolism may lead to reactive compounds [10,22]. For instance, sulfonamides and sulfones can be bioactivated to metabolites capable of forming drug–protein conjugates (Fig. 2), which mediate hypersensitivity reactions to these drugs [23] and have been employed to diagnose sulfamethoxazole allergy [24]. In the case of the antiepileptic drug felbamate, the reactive metabolite 2-phenylpropenal forms covalent adducts with HSA potentially involved in antigen formation [25]. The reactive metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine, forms adducts with cysteine residues [26] and is hepatotoxic. The correlation between acetaminophen–protein adducts and toxicity supports their use as specific biomarkers of drug toxicity in patients with acute liver injury [27].

Reactive metabolites may also arise by mechanisms involving oxidation or photoactivation. Procainamide

was shown to be *N*-oxidized in macrophages, leading to the formation of neometabolites that may sensitize T-cells [28]. In other cases, light-activated drugs or drug metabolites may react with proteins forming covalent photoadducts capable of interacting with the immunological system and induce photoallergic dermatitis. Photoactivatable drugs include NSAID such as ketoprofen, antibiotics such as fluoroquinolones and platelet aggregation inhibitors, such as triflusal, in which the active metabolite 2-hydroxy-4-trifluoromethylbenzoic acid suffers a nucleophilic attack by protein amino groups (Fig. 2) [29*].

Regarding NSAIDs, propionic acid-derived NSAIDs, such as ibuprofen, and arylacetic acid-derived NSAIDs, such as diclofenac, form acyl glucuronides, and other xenobiotics are metabolized to carboxylic acids (phase I metabolites), which subsequently undergo phase II conjugation to form acyl glucuronides. The electrophilic properties of acyl glucuronides enable them to couple to nucleophilic sites on proteins giving rise to immunogenic adducts [30,31]. Additionally, quinoneimine derivatives produced through autoxidation of the 5-hydroxy diclofenac metabolite provide an antigenic determinant for immune cell activation in the mouse [32].

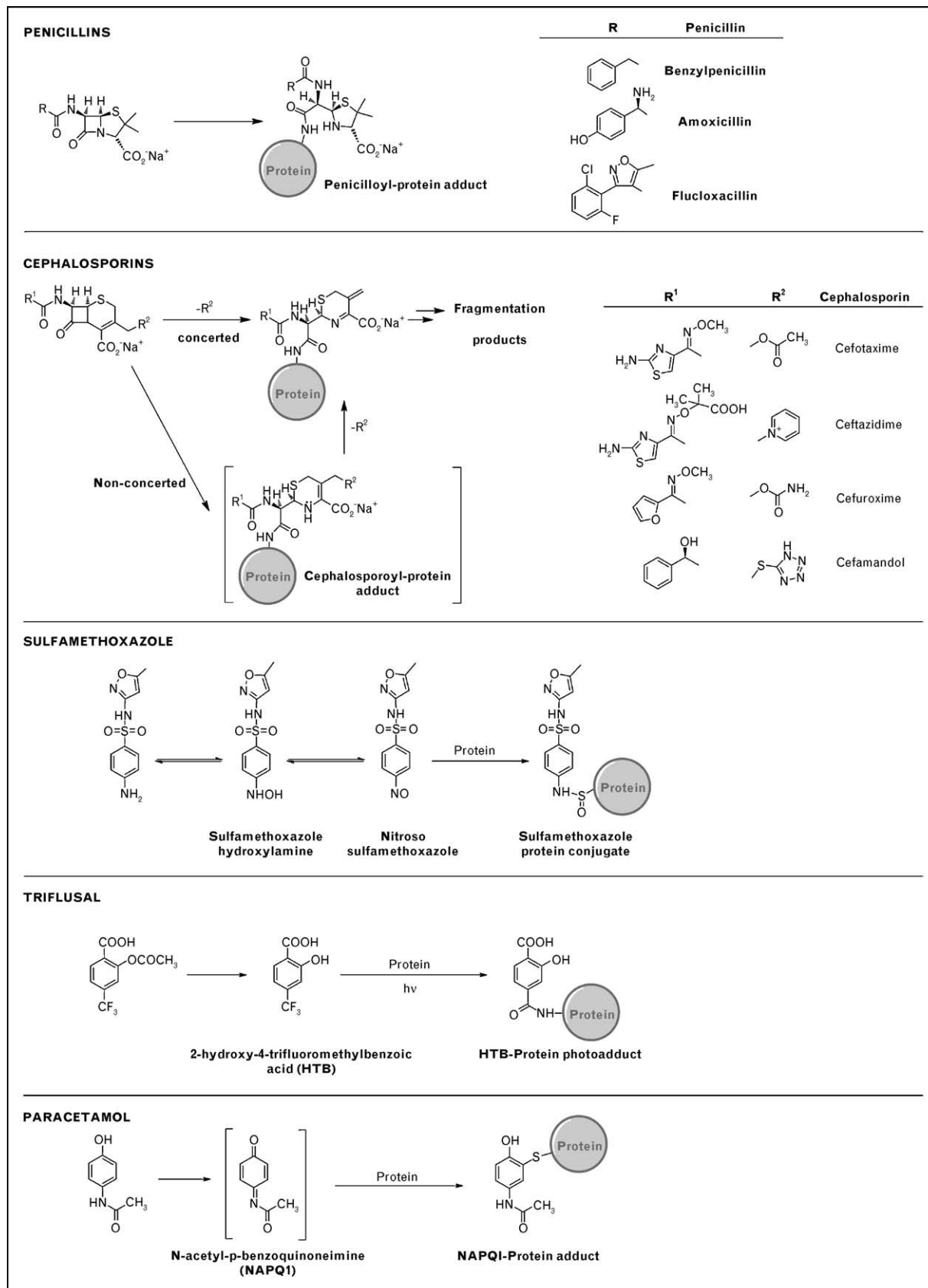
Importance of ‘danger signals’

It should be noted that drug–protein adducts are usually detected in tolerant patients. Therefore, an open question remains as to why only some patients develop drug allergy. In the current view, costimulatory signals must contribute to the activation of the immune system. These signals could consist of concurrent infections, endotoxin exposure, cytokine stimulation, metabolic alterations or drug toxicity, among others, and have been referred to as ‘danger signals’ [33]. In this scenario, drug–protein adducts could play both a direct and an indirect role in the immune response by providing antigens for recognition by immune cells and through cytotoxic effects, which could contribute to the generation of costimulatory signals for dendritic cell activation. Moreover, danger signals increase the formation of drug–protein adducts in antigen-presenting cells [33]. In the case of drugs reacting with thiol residues, factors affecting the intracellular redox status may also be important determinants in the levels of adduct formed [34*] and, therefore, in the final antigenicity.

Basic aspects of proteomic techniques

Proteomics can be defined as the array of techniques that attempt to obtain a general view of the protein composition of a given biological system or ‘proteome’, the analysis of its variations in response to diverse agents and its chemical characteristics in terms of

Figure 2 Some of the mechanisms involved in the formation of adducts of common drugs with proteins



posttranslational modifications (PTMs). In general, separation techniques are employed to resolve the complex mixture of proteins into its components. These techniques include chromatographic procedures, gel electrophoresis-based methods and/or immunoaffinity procedures, among others. Protein identification and characterization relies on immunological, and mainly, mass spectrometry (MS) approaches. In MS analysis, the ratio of mass:charge of a molecule is measured after its ionization and transfer into gas phase by various methods [35^{••}]. Full-length proteins, or more frequently, peptides arising from their enzymatic digestion can be analyzed by MS. Protein identification from the peptide fingerprint obtained is achieved by database searching. Digestion of all proteins present in a mixture and analysis of the resulting peptides is known as shotgun proteomics [36]. Peptide sequence information can be obtained by fragmentation, which is usually carried out by collision with an inert gas, and subsequent analysis of the fragments obtained by MS, in what is called MS/MS or tandem MS.

Quantitative proteomic approaches attempt to accurately determine differences in protein or peptide patterns in different experimental conditions. In DIGE (differential in gel electrophoresis), labeling of the proteins present in each sample with different fluorescent dyes, resolution of pooled samples by two-dimensional electrophoresis and image analysis of the differences in the intensities of the two fluorescent signals provide relative quantitation. Isotopic labeling of protein samples provides quantitative information through liquid chromatography (LC)-MS approaches. In SILAC (stable isotope labeling amino acids in cell culture), the metabolic incorporation into proteins of amino acids bearing heavy atoms induces a mass shift in the corresponding peptides. In iTRAQ (isobaric tags for relative and absolute quantitation), chemical labeling of peptides with isobaric tags gives rise to 'reporter ions'. Precise identification and quantitation of specific peptides in proteins or in mixtures can also be achieved by multiple reaction monitoring (MRM), taking advantage of the features of the triple quadrupole [36].

MALDI (matrix-assisted laser desorption ionization) is widely used for protein and peptide analysis, but also for analysis of complex specimens. MALDI imaging technologies allow the direct analysis of tissue sections, which after processing provides the distribution of molecules in the sample. This technology has been used to assess the localization of toxic metabolites in tissue samples [37] or the distribution of specific proteins as toxicity biomarkers, thus providing pathogenic information [38].

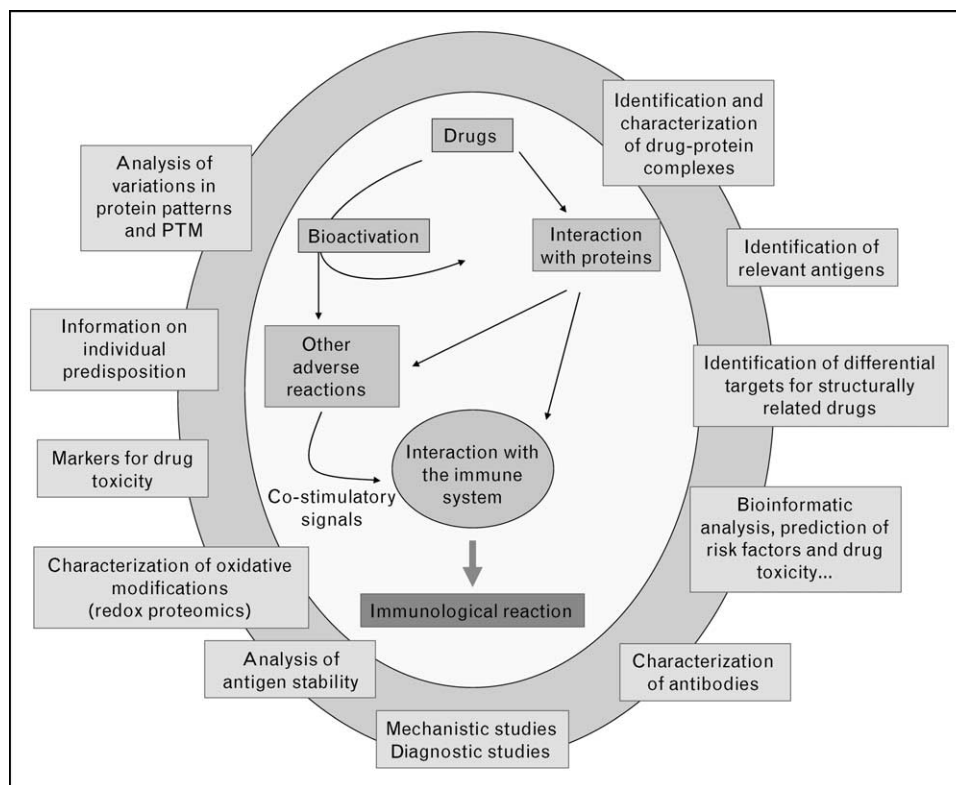
The techniques based on 'arrays' provide a different approach for protein analysis. Arrays consist of panels

of immobilized proteins or antibodies and allow assessment of abundance, reactivity or functional parameters.

Applications of proteomics to the study of immunological reactions to drugs

Proteomic techniques provide powerful tools for the study of allergic reactions (Fig. 3). In combination with immunological detection, proteomic approaches allow the identification of novel allergens and the characterization of antigenic determinants. Moreover, arrays of allergens are becoming widely used in diagnosis and profiling of allergic patients [39,40]. With respect to drug allergy, proteomic studies based on HPLC-MS or MRM analysis of serum or tissue proteins may provide markers for drug toxicity [41]. MS is a sensitive and accurate method for the determination of PTMs and, thus, for the study of protein haptentation. Using two-dimensional electrophoresis and immunological detection, HSA and transferrin were identified as penicilloyl-modified proteins in plasma from ampicillin-treated patients [42]. More recently, the modification of HSA by flucloxacillin has been studied *in vitro* and in tolerant patients by LC-MS/MS, and Lys 190 and 212 of HSA have been identified as the main sites for addition of this antibiotic [43]. Identifying functionally relevant antigens is essential for the accuracy of diagnostic tests [24]. Various studies have shown that not only HSA, long considered the main target for haptentation, but also other plasma or cellular proteins participate in the allergic reactions. Recently, it has been proposed that functional antigens may arise by metabolism of sulfamethoxazole in immune cells and binding of the reactive metabolites to intracellular proteins [44^{••}]. This raises the importance of extrahepatic metabolites in allergic reactions. In cells of the immune system, oxidative stress can influence adduct formation. Therefore, advances in 'redox proteomics' may be critical in achieving a full understanding of the immunological responses to drugs. In certain organs, such as skin, metabolites produced by air oxidation or photoactivation are especially relevant [29[•],45]. The platelet anti-aggregant triflusal produces photoallergy. The triflusal metabolite 2-hydroxy-4-trifluoromethylbenzoic acid binds covalently to HSA upon photoactivation, thus providing a mechanism for protein haptentation and likely photoallergy [46]. The phenothiazine fluphenazine may also be photoactivated and form adducts potentially involved in photosensitivity and in agranulocytosis [47].

Drug-protein binding may be influenced by competitive and synergistic mechanisms with other protein-binding agents [48] with consequences on protein antigenicity. Drug binding is structure dependent. Model electrophiles with related structures may modify both common and selective subsets of protein targets [34[•],49]. Paracetamol and its regioisomer *N*-acetyl-*m*-aminophenol,

Figure 3 Potential applications of proteomic approaches in the study of immunological reactions to drugs

PTM, posttranslational modification.

which is less hepatotoxic, bind to different targets in the liver (reviewed in [13[•]]). Identification of the selective protein targets for drugs with similar structure but differential toxicity could provide important clues for the mechanism of immunological reactions to drugs. Moreover, adducts formed by drugs with different structures may display different stability in biological systems [50]. Interestingly, by proteomic analysis of the endolysosomal fractions of dendritic cells, it has been proposed that allergens resistant to proteolysis persistently provide peptides for MHC binding, thus favoring presentation to T cells and, thus, high immunogenicity [51[•]].

Proteomic studies can also provide information on individual predisposition or protective factors, as it has been shown in animal models of drug-induced liver disease [52]. Proteomics studies in patients with allergic dermatitis have shown alterations in lipid metabolism proteins and in PTMs [53]. Potentially, proteomic studies in different phases of the immune response could aid in the identification of pathogenic factors.

The power of proteomic approaches may lead to the generation of massive amounts of data that need computational resources for analysis, including database searching for protein identification, protein modeling

or docking studies. In addition, databases can be searched to find information on drug targets or biomarkers of different processes [54], to predict proteins with affinity for various drugs or to predict drug toxicity and sensitization risks [55,56]. In the broader context of immunological reactions, the emerging area of immunoinformatics may provide valuable tools for understanding drug interactions, potentially leading to a more personalized drug design [57].

Conclusion

Proteomics is gaining increasing importance in the study of drug allergy, providing insight into the precise mechanism of protein conjugation and its subsequent metabolism, aiding in the identification of pathogenic and risk factors and providing valuable tools for the clinical diagnosis of allergy. Multidisciplinary approaches involving clinicians, chemists, geneticists and proteomics experts will be required to scrutinize the correlates between complex clinical entities and the underlying biochemical processes.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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