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REVIEW ARTICLE

What's been Hapten-ing over the last 88 years?

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Abstract
Definition of the relationship between drug protein adduct formation (haptenation) and development of immunological adverse drug reactions (drug hypersensitivity) has been an area of active research for over 80 years. The hapten hypothesis which states that "immunogenicity of low molecular weight organic chemicals is dependent on modification of self-proteins," evolved from Landsteiner and Jacob's discovery of a correlation between the reactivity of dinitro-halogenated benzenes and their sensitization potential. The hypothesis rapidly evolved to encompass drugs that often require metabolic activation to generate electrophilic, protein-reactive intermediates. As tissue culture methods advanced, the importance of drug hapten-specific T-cells in the disease pathogenesis was defined. This led to a plethora of studies describing the uptake and processing of drug(metabolite) protein adducts by antigen presenting cells, and the subsequent surface display of hapten-modified peptides in the context of MHC molecules. Although the pathway of hapten-specific T-cell activation is now well established, several questions need to be addressed: first, what is the nature of the hapten-modified peptides displayed by MHC? Second, how many of these peptides stimulate T-cells?; third, what are the critical protein modifications involved in T-cell activation; and finally, what is the role of hapten-specific T-cells in the iatrogenic disease? These questions will become increasingly important as more and more targeted covalent binding inhibitor drugs are approved for human use. In this review, we provide a brief synopsis of hapten research and then describe the approaches used by Pharma and academia to study hapten covalent binding and the role of drug protein adducts in the activation of human T-cells.

Keywords Hapten · Drug hypersensitivity · Adverse drug reaction · Covalent binding · T-lymphocyte

Drug hypersensitivity reactions

Drug hypersensitivity reactions constitute type B reactions, meaning they are not predictable based on the primary pharmacology of the drug, but instead are dependent on specific genetic (e.g., HLA alleles, which encode antigen presenting proteins) and environmental (e.g., disease) factors, and are influenced by the status of an individual's immune system (i.e., immune regulatory thresholds). Adverse events can arise at therapeutic or subtherapeutic dosing regimens. Despite being rare, drug hypersensitivity reactions represent a major impediment in both the clinical and pharmaceutical sectors, as adverse events are not predictable during preclinical testing and predisposing factors are only discovered once the drug has been marketed. They present numerous hindrances across the clinical spectrum as they pose a serious threat to both the patients' health and compliance often resulting in hospitalization or the prolongation of the patients stay in hospital [1]. Most drug hypersensitivity reactions exhibit a delay in their initial onset, with sudden symptomatic reoccurrence upon rechallenge [2]. Mechanistic studies strongly implicate a role for the adaptive immune system, namely CD4+ and CD8 + T-cells, in the disease pathogenesis.

There are two chemical pathways (hapten or pharmacological interaction [PI] pathway) for drug-(metabolite) T-cell activation. These pathways are differentiated based on the nature of the interaction of the drug(metabolite) with their cellular target. According to the hapten concept, a drug(metabolite) binds covalently to protein, and drug(metabolite)-modified peptides derived from the conjugate associate with antigen presenting cell MHC proteins before stimulating T-cells. Generation of drug(metabolite)-

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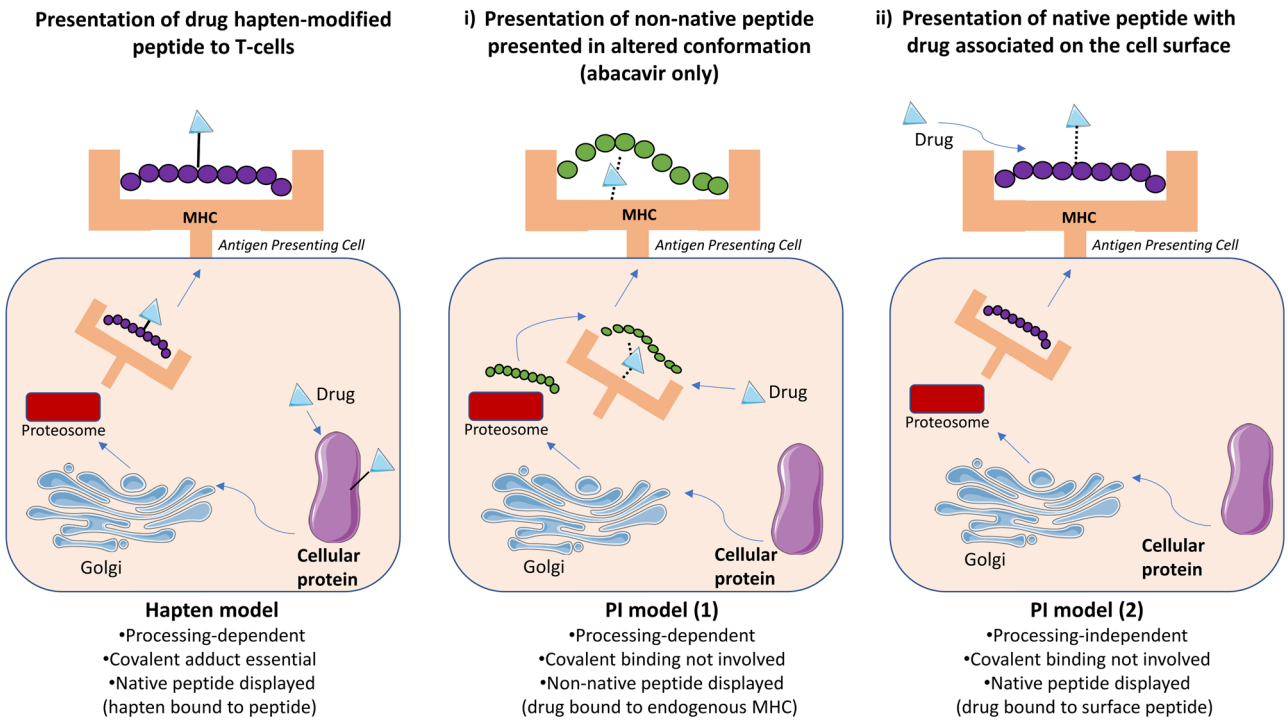

Fig. 1 Models of drug-specific T-cell activation. LHS) Hapten model: The drug or metabolite covalently binds to cellular protein, which undergoes intracellular processing. Modified peptides are loaded onto MHC for subsequent display on the cell surface (TCR exposed to drug and peptide). CENTRE) PI-model (1): Drug associates with intracellular MHC before peptide binding resulting in the loading of a non-native peptide into the binding groove (TCR exposed to peptide alone). RHS) PI-model (2): Drug associates directly with MHC bound peptide on the cell surface (TCR exposed to drug and peptide)

modified peptides is brought about through the enzymatic infrastructure within antigen presenting cells. Protein degradation results in large numbers of peptides (approx. 8–30 amino acids in length), some of which contain the adducted hapten, that interact with MHC class I or II proteins [3]. The MHC proteins then migrate to the cell surface and display peptides to the extracellular environment. If the hapten-modified peptide is distinguished by a T-cell as a "foreign entity" TCR engagement and a downstream T-cell response with the secretion of a variety of soluble mediators is instigated (Fig. 1; left hand side). In contrast, the PI concept proposes that T-cells are activated when a drug(-metabolite) binds directly (non-covalently) to MHC proteins or peptides already embedded in the MHC peptide binding cleft. In this case, antigen processing within antigen presenting cells to generate peptide sequences is not needed for T-cell activation as peptides are displayed naturally by surface MHC molecules. According to the PI concept, T-cells are triggered through their T-cell receptor after receiving signals from either the drug (e.g., carbamazepine) and MHC bound peptide [4, 5] (Fig. 1: right hand side). The drug and peptide structures available for T-cell engagement are very similar according to the hapten and PI concept (the only difference being the nature of the drug-peptide binding interaction) and this explains the cross reactivity sometimes observed between drugs and drug haptens. An alternative PI concept pathway has been described with one drug, abacavir. Abacavir binds endogenously deep within the MHC peptide binding cleft before peptide binding. This alters the peptides displayed by the MHC protein for T-cell receptor engagement (Fig. 1: center) [6–8]. A detailed discussion of the PI concept, originally described by Pichler and co-workers [9], is beyond the scope of this article; however, the implications of direct drug HLA binding is the subject of several reviews [10–13].

A brief synopsis of hapten research

The term hapten derives from the Greek word "haptein", meaning "to fasten." Below we provide a brief history of hapten research with reference to drug hypersensitivity reactions, highlighting significant advances in the field, and then discuss the tools available to detect drug protein and drug peptide adducts with increasing sensitivity. Also mentioned are key translational studies using immune cells from patients with drug hypersensitivity to describe the importance of adduct formation in clinically-relevant adverse events.

The origins of the hapten hypothesis dates back to studies conducted in the 1930s by Landsteiner and Jacobs. Using dinitrohalogenated benzenes the authors were able to

successfully sensitize guinea pigs and show that the extent of sensitization directly correlated with the electrophilicity of the halogen leaving group and protein adduction. Hence, the authors were able to hypothesize that sensitization to low molecular weight chemicals was dependent on protein conjugation [14]. The term hapten was originally defined as "a compound of less than 1000D that instigates immune reactions when conjugated to a protein via formation of a covalent bond" [15]. The concept was applied to drugs in the 1960s when researchers began to characterize the covalent interaction of β-lactam antibiotics with off-target proteins [16–18]. All β-lactam antibiotics contain a β-lactam ring joined to a 5- or 6-membered ring. Stress on the central structure of the drug results in opening of the β-lactam ring. The molecule is then susceptible to nucleophilic attack by amino acid residues in proteins [19, 20]. Unlike the β-lactam antibiotics, the majority of drugs are not directly reactive. As such metabolic activation, resulting in formation of chemically-reactive metabolites (e.g., epoxide, quinone and nitroso derivatives), is believed to be the initiating event in many forms of drug hypersensitivity reactions [21–24]. Halothane hepatitis represents the best example where metabolic activation has been shown to be directly involved in the disease pathogenesis. In the 1980's Kenna and co-workers demonstrated that drug exposure results in the production of anti-drug antibodies and presumably T-cells against the trifluoroacetyl chloride metabolite, which binds covalently to lysine residues in proteins [25–27]. Derivatives of halothane that undergo lower levels of metabolism (i.e., enflurane, isoflurane) were found to form fewer protein adducts and hepatic reactions are largely eliminated [28]. A series of 18 drug protein conjugation papers from the 1980's by Park and co-workers explored the relationship between metabolic activation and detoxification in adduct formation, and the levels of protein conjugation needed for immune activation in experimental animals [29–46]. These studies demonstrated that a threshold level of binding was required for immune recognition, but when the level of modification exceeded this threshold, the protein was directed towards degradation and immune activation was not observed. Around the same time, Ashby and Baillie introduced and discussed the concept of structural alerts with reference to carcinogenicity and immune-mediated adverse drug reactions, respectively [47, 48]. In the 1990's Baillie's team published a succession of "glutathione-trapping" papers that utilized the ability of glutathione to bind to electrophilic drug metabolites to characterize the nature of adduction reactions that occur in physiological conditions, often in the presence of drug metabolizing systems [49–54]. The term structural alert is still used widely today in medicinal chemistry to mitigate the risk of a molecule causing an adverse event [55]. The 1990's saw a plethora of functional studies with immune cells from hypersensitive human patients to demonstrate that haptens including reactive drug(metabolites) must bind covalently to proteins to stimulate specific T-cells. Friedmann explored the sensitization of human subjects against the chemical sensitizer dinitrochlorobenzene and defined the underlying mechanism of T-cell activation to the hapten [56–59]. The group of Weltzien conducted elegant studies with contact sensitizer- and penicillin-modified HLA binding peptides to show (i) the fine specificity of the hapten peptide complex MHC binding interaction and (ii) the importance of hapten positioning in the peptide sequence for T-cell activation [60–67]. Naisbitt and co-workers synthesized reactive nitroso drug metabolites for incorporation in cell culture assays to explore the relationship between drug antigenicity and immunogenicity [68–70]. These studies identified a threshold level of drug metabolite protein binding required for in vitro T-cell activation and demonstrated that drug-modified proteins released from dead/dying cells were potent immunogens. Through collaboration with Pichlers' drug allergy team in Switzerland, drug metabolite hapten-specific T-cells were detected in patients with hypersensitivity and characterized in terms of phenotype, function, and pathways of T-cell activation [71–75]. The 1990's also saw evolution of Polly Matzinger's danger theory [76] to explain the importance of innate immune signaling in the development of a pathogenic T-cell response, and around the turn of the century Park and Uetrecht applied this concept to immunological drug reactions [21, 77]. Drug-dependent danger theory states that the formation of reactive drug metabolites and protein haptenation results in cell stress and promotes the release of damage-associated molecular patterns that activate the innate immune system, disrupting the regulatory network and promoting adaptive immune responses to a drug. As the century progressed Uetrecht utilized in vitro and in vivo models to show (i) the importance of peroxidase metabolism of drugs in the generation of protein adducts and (ii) the role of the drug-primed innate immune response in adverse drug reactions [78–85]. A European ban on animal testing for new cosmetic ingredients was implemented in 2009 (Cosmetic legislation, Regulation (EC) No. 1223/2009). This prompted a research drive for in vitro alternatives that predict sensitization. In the context of this discussion, haptenation of skin proteins was defined as the key biological event in skin sensitization, and Aleksic and Gerberick developed a peptide reactivity assay to quantify the covalent binding of directly reactive and metabolite-generated haptens to lysine and cysteine amino acids [86–89]. The simple binding assay was found to have a sensitization prediction accuracy of 80–90%, when compared with rodent local lymph node assay sensitization data [90]. Martin and collaborators used an increased knowledge of drug-protein conjugation to help improve in vitro cell

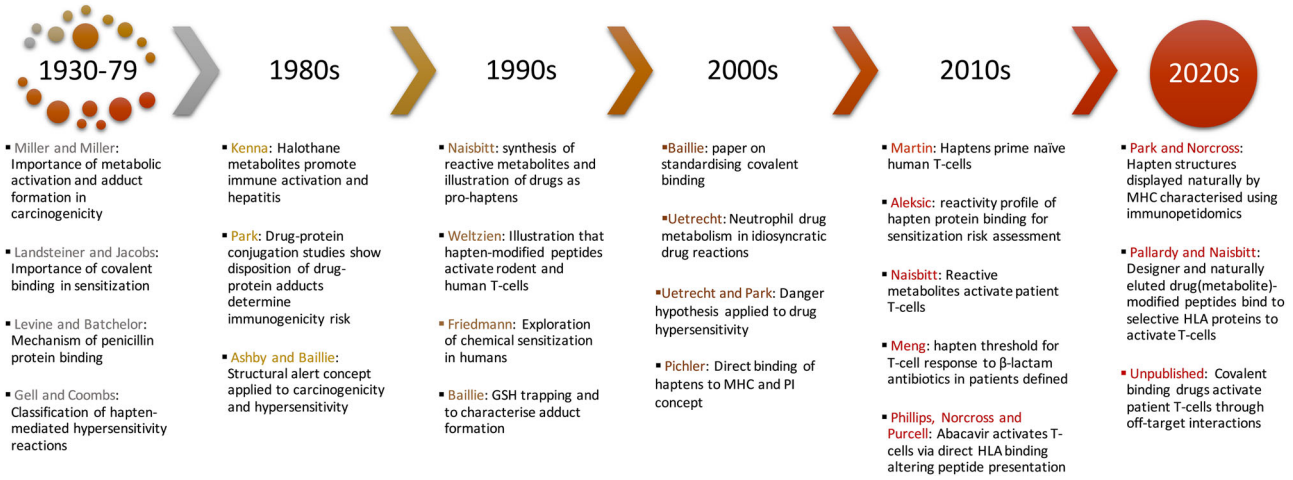

Fig. 2 Timeline of hapten research and discovery

culture assays that predict the immunogenicity of hapteneic compounds [91]. To overcome the intrinsic toxicity of hapteneic compounds, antigen presenting cells were (i) pulsed with high concentrations of hapten for short time periods, and (ii) exposed to haptens pre-conjugated to carrier proteins such as human serum albumin. Groundbreaking work by Meng in 2017 found that the level of β-lactam antibiotic albumin modification that activated T-cells was equivalent to the levels formed in hypersensitive and tolerant patients, which indicates that threshold levels of drug protein conjugate are formed in all patients [92]. Hence, the propensity to develop drug hypersensitivity is dependent on factors other than the level of conjugate formation per se. In the last 5 years, researchers have focussed on the characterization of MHC-bound hapten peptide conjugates displayed naturally by antigen presenting cells. The evolution of immunopeptidomics as a discipline led to the development of technologies and methods to elute peptides from MHC and characterization of the MHC bound repertoire. It was then down to researchers with knowledge in hapten chemistry to apply these methods to identify the "needle in the haystack", peptides with hapten modification. In this respect, Park and Norcross both characterized flucloxacillin-modified peptides eluted from HLA-B*57:01 [93, 94], the HLA protein strongly associated with flucloxacillin-induced liver injury [95], and responsible for presentation of flucloxacillin haptens to CD8 + T-cells [96]. Alongside these analytical studies, methods have been established to synthesize designer hapten-modified HLA binding peptides. Studies with penicillin-, amoxicillin- and nitroso dapsone-modified peptides [97–99] have shown (i) that the peptides bind with a degree of selectivity to HLA proteins identified in genetic association studies, (ii) the position of the hapten modification is important for stimulating T-cells, and (iii) that haptens and the hapten concept is relevant (alongside the PI concept) for adverse reactions associated with expression of a specific HLA allele. As we move towards the middle of the 2020's, the race is on to study the immunogenicity of naturally-eluted hapten-modified peptides. This will help researchers investigate whether modification of specific source proteins impacts on the development and severity of tissue injury, and tissue selectivity of an adverse event. Figure 2 provides a timeline highlighting key research showing the importance of haptenation in immune activation and the development of drug hypersensitivity. Table 1 utilizes some of the most studied drug haptens to highlight key milestones in the verification of haptenated peptides as critical antigenic determinants in drug hypersensitivity reactions.

Drug-protein adduct detection during drug development and in academic studies

There are numerous assays available throughout the drug development cascade which can be used to assess the extent and the capacity of a given asset to form adducts with biological macromolecules. It is noteworthy that none of these available assays directly correlate to the capacity of a compound to elicit toxicity, though they do permit identification of the qualitative, and to some degree quantitative, nature of the adduction chemistry pertinent to a given compound. The utility of the assays also depend on their place in drug discovery; high throughput, lower cost assays are useful early in development, where they can serve to aid in candidate optimization/selection. Other assays help inform translational aspects of risks. On the whole, (and as is often applicable in preclinical drug development), outputs of a single assay or endpoint rarely are "show stopping" by themselves. When considering the impact of results in later

Table 1 Summary of the key milestones in the verification of haptenated peptides as critical antigenic determinants in drug hypersensitivity reactions

Drug	Benzylpenicillin	Flucloxacillin	Piperacillin	Amoxicillin	Nitroso sulfamethoxazole	Nitroso dapsone
Clinical observation of hypersensitivity	(Levine and Price 1964) [236]	(Monshi et al. 2013) [96]	(El-Ghaiesh et al. 2012; Casimir-Brown et al. 2021) [237, 238]	(Kim et al. 2015) [239]	(Farrell et al. 2003; Schnyder et al. 2000; Elsheikh et al. 2011) [71, 75, 152]	(Kosseifi et al. 2006; Zhao et al. 2021; Jiang et al. 2022) [240–242]
Trapping	(Meng et al. 2011) [167]	(Jenkins et al. 2009) [166]		(Grujic et al. 2008; Pajares et al. 2020) [243, 244]	(Callan et al. 2009; Naisbitt et al. 1996) [70, 158]	(Alzahrani et al. 2017; Ali et al. 2023) [160, 245]
Model protein adducts	(De Weck and Eisen 1960; Meng et al. 2011; Yvon, Anglade, and Wal 1989) [18, 167, 246]	(Jenkins et al. 2009; Monshi et al. 2013) [96, 166]	(El-Ghaiesh et al. 2012; Whitaker et al. 2011; Jenkins et al. 2013; Amali et al. 2017; Meng et al. 2017 [92, 168, 169, 237, 247]	(Ariza et al. 2012; 2014; Garzon et al. 2014; Meng et al. 2016; Pajares et al. 2020) [170, 244, 248–250]	(Tailor et al. 2019; Callan et al. 2009; Ogese et al. 2015; Meekins, Sullivan, and Gruchalla 1994) [158, 251, 252]	(Alzahrani et al. 2017) [160]
Broader proteomic adduct characterization (western blot etc.)	(Bechara et al. 2019; Sánchez-Gómez et al. 2017) [253, 254]	(Waddington, Meng, et al. 2020; Waddington, Ali, et al. 2020; Ogese et al. 2019) [93, 255–257]	(El-Ghaiesh et al. 2012) [237]	(Ogese et al. 2019; Warbrick et al. 1995; Ariza et al. 2012; 2014) [248, 249, 256, 258]	(Ogese et al. 2019; Tailor et al. 2019; Reilly et al. 2000; Manchanda et al. 2002; Cribb et al. 1996) [251, 256, 259–261]	
Ex vivo T-cell activation by soluble drug	(Zdziarski 2002; Meng et al. 2011; Brander et al. 1995) [167, 262, 263]	(Monshi et al. 2013) [96]	(El-Ghaiesh et al. 2012; Whitaker et al. 2011; Jenkins et al. 2013; Meng et al. 2017; Sullivan et al. 2018) [92, 168, 169, 237, 264]	(Horton, Weston, and Hewitt 1998; Kim et al. 2015; Meng et al. 2016; Ariza et al. 2020) [170, 239, 265, 266]	(Farrell et al. 2003; Schnyder et al. 2000; Elsheikh et al. 2011) [71, 75, 152]	(Zhao et al. 2019; 2021) [241, 267]
De novo T-cell activation by soluble drug		(Faulkner et al. 2016; Wuillemin et al. 2014; Yaseen et al. 2015) [204, 268, 269]	(Faulkner et al. 2016) [268]	(Faulkner et al. 2016) [268]	(Faulkner et al. 2012) [200]	(Alzahrani et al. 2017) [160]
Ex vivo T-cell activation by hapten-protein conjugate	(Zdziarski 2002; Meng et al. 2011; Brander et al. 1995; Azoury et al. 2018) [98, 167, 263]		(El-Ghaiesh et al. 2012; Whitaker et al. 2011; Meng et al. 2017; Sullivan et al. 2018) [92, 168, 237, 264]	Tailor et al. unpublished		
De novo T-cell activation by hapten-protein conjugate	(Nhim et al. 2013; Azoury et al. 2018; Bechara et al. 2019 [98, 253, 270]					
Representation of haptenated peptides/ perturbed eluted immunopeptidome		(Puig et al. 2020; Waddington et al. 2020) [93, 94]				

Table 1 (continued)

Drug	Benzylpenicillin	Flucloxacillin	Piperacillin	Amoxicillin	Nitroso sulfamethoxazole	Nitroso dapsone
Ex vivo T-cell activation designer/synthetic peptides	(Azoury et al. 2018; Padovan et al. 1997) [61, 98]		(Tailor et al. 2020) [97]			Almutairi et al. 2023 [99]
De novo activation of T-cells by designer/synthetic peptides	(Azoury et al. 2018) [98]		(Ogese et al. 2019) [256]			
Identification of naturally eluted peptides which activate T-cells from drug hypersensitive individuals						

Table includes some of the most studied drug haptens

stages (go/no go decisions), integration of the various characteristics of a drug, in addition to hazard identification as defined by assays can inform decision making in a weight of evidence context. Compounding liabilities (both those related to covalent binding and other mechanisms) arising predominantly from an in vitro battery of assays to generate a drugs score in terms of safety (especially within the realm of drug-induced liver injury) has been a theme developed over the last decade or so [100–103] The study of protein adduction in hypersensitivity reactions also extends into the realm of investigative science. A key goal of academic work within the field is to determine how adduction chemistry contributes to formation of antigenic determinants which ultimately drive antibody and the pathogenic T-cell responses responsible for the clinical manifestation in a patient. Over the last decade progress has continued to have been made toward this goal, with application of sophisticated methods within this interdisciplinary space.

Structural alerts and in silico de-risking activities

Structure-activity relationships are one of the fundamentals of pharmacology and over the course of 5 decades, the work of several visionary toxicologists translated this to genotoxicity and then to applications in sensitization/toxicity. Due to space limitations only a fraction of the great contributions to this field can be overviewed.

Landmark studies of Miller and Miller identified various compounds amenable to carcinogenicity-conferring bioactivation [104, 105]. Comprehensive listing of bioactivation pathways of structures to electrophilic (toxic) reactive metabolites in drug toxicity followed in the 1980s, linking the concept to bioactivation to more direct lines of drug toxicity [106]. Work of Ashby and others codified substructures with respect to Ames mutagenicity tests [107, 108], local lymph node assays [109] and later toxicophores for idiosyncratic liver injury [110, 111]. The corroboration of structural alert presence (alongside other key factors) to clinical toxicity was evaluated in a noteworthy review of the 200 most commercially successful small molecular weight drugs circa 2009 [55]. Two themes are clear from this review. Firstly, the presence of structural alerts and the ability of compounds to form reactive metabolites does appear to be over-represented in drugs that cause clinical toxicity. Secondly, the presence of a structural alert absolutely does not preclude a drug from becoming successful (over half of compounds reviewed contained at least one) [55]. Compilation of expert knowledge on these substructural liabilities has led to the emergence and proliferation of in silico screening tools which permit presynthetic iterative design. The first of these softwares was Derek for windows, first developed in the 1980s [112], and

this field has proliferated, yielding a range of rule based predictive systems for structure-activity relationship to toxicity, including well know systems such as the OECD QSAR Toolbox [113], and Toxtree [114].

Structural alerts are useful to predict the type of reactive metabolites and thus the mode of covalent binding behavior one might expect from a compound. However, there are nuances to this; the presence of structural alerts can be trivialized by competing metabolic pathways or the molecule may simply not undergo certain expected bioactivation pathways due to enzyme substrate selectivity and the structure of the drug. Thus, where the structural alert is detected and cannot be circumvented by structural modifications due to loss of pharmacological activity or alteration to other critical parameters, emphasis is placed upon verifying that the relevant reactive metabolites are indeed formed.

The relevance of structural alerts to drug hypersensitivity is not straightforward. As illustrated well in [55], context is critical and the interrelation between chemical, pharmacokinetic and pharmacodynamic properties of a compound are all determinants of compound specific factors at play in hypersensitivity reactions. This, coupled with the incomplete resolution on antigenic determinants that drive hypersensitivity for many drugs, make it exceedingly difficult to deliver a clear composition of structural alerts that are most relevant to drug hypersensitivity.

Enzyme inactivation/mechanism-based inhibition

Where bioactivation is concerned, one might propose that proximity of formation to potential targets may be an important determinant of which proteins are adducted. The enzymes which confer bioactivity can be considered the most proximal targets in many cases, and this notion is ratified with mechanism-based inhibition assays. Aside from the potential for drug-drug interactions and altered disposition of compound itself, adduction of CYP enzymes may be immunogenic and may lead to breakdown of tolerance to the endogenous enzyme as seen with halothane [115]. For an excellent review outlining these assays and their utility in safety prediction, the authors refer to [116].

However, support for the indirect promotion of the immunogenicity of CYP proteins through their adduction has been provided through the identification of antibodies directed against the native enzyme. A classic example of which is observed with tienilic acid, with this drug formation of a thiophene reactive metabolite is linked to the high degree of adduction (and thus MBI) of CYP2C9 [117]. Anti-CYP2C9 directed autoantibodies were detected in sera of patients who were hypersensitive to tienilic acid [118, 119] (which cross reacted to rat derived CYP2C11 [120]). Other drugs for which an autoantibody response directed against the enzyme responsible for catalysis of reactive metabolite formation occurs include isoniazid (CYP2E1, CYP2C9, and CYP3A4) [121], and hydralazine (CYP1A2) [122]. The series of studies on halothane (the administration of which exhibits an association of autoantibodies to native CYP2E1 [115] by Kenna and others represented an important step in the holistic understanding of idiosyncratic liver injury. These studies linked the generation of reactive metabolites, downstream formation of covalent adducts to intracellular proteins, verification of adaptive immune response directed against respective adduct-dependent antigens, and clinical status of patients [123–128].

Though MBI is typically considered in the context of liver enzymes (particularly CYPs), this concept could theoretically be extended to other metabolizing enzymes. For example, neutrophil myeloperoxidase (MPO) has been cited as a key enzyme involved in metabolic activation of multiple drugs including sulfonamides, amodiaquine, hydralazine and clozapine [129–132]. Although the capacity for metabolism within neutrophils is dwarfed by hepatic metabolism, it is thought to be important for localized reactive metabolite generation, and provides an explanation for the formation of extra-hepatic adducts with metabolites too unstable to escape the liver [133]. Extra-hepatic metabolism and subsequent adduct formation may provide a source of antigen for the adaptive immune system. In this respect, early studies report the presence of anti-neutrophil cytoplasmic, anti-MPO and anti-nuclear (specific for the nuclei of neutrophils)-specific antibodies in drug-induced lupus, agranulocytosis and cutaneous hypersensitivity reactions [133–142]. Though not studied in great detail, antigen presenting cell peroxidase-catalysed and keratinocyte flavin mono-oxygenase/peroxidase-catalysed metabolism of sulfamethoxazole has been shown to result in the formation of a reactive nitroso metabolite that targets intracellular proteins for adduction [143–149]. The formation of reactive sulfamethoxazole metabolites locally results in dendritic cell maturation, the provision of antigens (drug-protein adducts) for T-cell activation and stress signaling when generated in excess [143, 150–152].

From the perspective of bioactivation of a parent drug to a reactive metabolite, the link between MBI and adaptive immune responses illustrates one does not always have to look far from the source of bioactivity to find potentially immunologically relevant covalent binding. A key question that remains to be addressed comprehensively from a drug hypersensitivity point of view is just how immunogenic CYP- or MPO-derived conjugates are.

Nucleophilic trapping

For several decades, nucleophilic trapping assays have featured in the drug discovery cascade, serving to identify

the reactive electrophilic species which might be responsible for covalent binding of a drug. The premise of these experiments is simple: since highly reactive electrophilic species often exist transiently (usually for brief periods of time in between bioactivation and their adduction) and are, therefore, often not stable enough to be characterized through typical analytical methods. The identity of such species can be disclosed through the provision of a surrogate target for the covalent binding chemistry. These in vitro test systems generally consist of 3 fundamental components in the incubation stage: (i) the study drug, (ii) an abundant, readily detectable nucleophile, and (iii) a metabolizing system for bioactivation of drug to potential reactive metabolites (such as cofactor fortified S9 fraction, microsomes, or hepatocytes derived from human or preclinical species). These systems are relatively simple to set up and are amenable to high throughput screening using LC-MS/MS platforms.

The classic and most common nucleophile used for these experiments is glutathione. Glutathione is a triamino acid peptide which is present endogenously at high concentrations within the liver, its physiological role has been characterized as a detoxification agent, serving to competitively "mop up" electrophilic species generated intracellularly and thus minimise the covalent binding burden on critical proteins. It behaves as a soft nucleophile and thus is best suited for detection of soft electrophiles. Typically, in MS/MS analysis, a characteristic neutral loss can be seen of 129 Daltons from conjugates, which corresponds to the γ-glutamyl moiety [153].

Trapping nucleophiles can be selected based on electrophiles they may preferentially interact with, due to their distinct chemical behaviors such as relative hardness/softness [154]. As range of other nucleophiles are utilized in trapping assays, including cysteine, N-acetylcysteine, N-acetyl lysine, and 2-mercaptoethanol in addition to glutathione for soft electrophile detection, and cyanide, semicarbazide, methoxylamine and DNA bases for hard nucleophiles. Further variations on these trapping agents which enable quantitation of adduct formation within the assay include radiolabelled nucleophiles ([35 S] cysteine and [14 C]KCN) as outlined in [155, 156], fluorescent variants such as dansylated glutathione [157] and peptides containing single nucleophilic amino acid targets [86].

Trapping assays are effective for the high throughput requirements of early drug discovery, and as such feature commonly in the small molecular weight compound development paradigm. They have been performed for a great number of agents, as exemplified well by the evaluation of glutathione trapping of using a single platform for 190 drugs [103]. Trapping assays have also been used to great effect in numerous investigative studies for resolution of the identity of electrophilic species that may form haptens. In our laboratory, glutathione trapping assays (alongside a quenching effect of addition of excess glutathione in functional T-cell assays) have featured in linking reactive metabolites to the mechanism of antigenicity for numerous drugs, with a notable series being the sulfonamide derivatives [158–161]. Overall, glutathione trapping assays serve in some way as a bridge between the theoretical chemistry of structural alerts and the practical realization that a tangible, potentially toxicologically relevant adduct may be formed, through either the intrinsic covalent binding properties or formation of a reactive metabolite by a compound.

Protein adducts

Further resolution of the adduction chemistry of a compound can be provided through the study of covalent binding to model proteins. Typical examples include glutathione S-transferase pi and human serum albumin. Both of these proteins are abundant in vivo and thus can both serve within in vitro and in vivo systems. The adduction chemistry of these proteins can be used to study the nature and density of covalent binding of a compound to a macromolecular structure, thus embodying a stepping stone between the simplistic trapping experiments outlined above and the adduction to proteins which form the critical antigenic determinants of a hypersensitivity reaction.

Two approaches are employed in the study of covalent binding of proteins. These are referred to as "top-down" and "bottom up" [162]. As applied to the study of adduction chemistry the "top down" approach consists of investigating intact proteins in their native vs adducted form as an input into a mass spectrometer. The opposite "bottom up" approach entails an initial digestion of proteins by proteolytic enzymes prior to input, thus generating peptide fingerprints of proteins which can be specifically scrutinized for adducts through various mass spectrometer modes (product ion, precursor ion, and MRM modes) [163].

Human serum albumin is one of the best studied model proteins in this regard. It is the most abundant protein in human sera, and makes up around 50–60% of all proteins within total plasma [164]. It is therefore an ideal protein to study due to its abundance, relative accessibility for in vitro and ex vivo studies in terms of necessary sampling. A dedicated review on albumin biomonitoring detailing typical assay set up, sample preparation and analysis is outlined in [165]. With regards to drug hypersensitivity reactions, the study of β-lactam antibiotic adduction a great example of the utility of human serum albumin. This class of drug typically exhibits direct covalent binding by virtue of its pharmacologically relevant β-lactam ring undergoing an atypical acylation reaction; modification at the various lysine residues of albumin is observed. The dose and time

dependence of the promiscuity of lysine modification by β-lactam antibiotics is readily apparent as seen both in vitro and in vivo with flucloxacillin [166], benzylpenicillin [167], piperacillin [92, 168], meropenem and aztreonam [169], amoxicillin and clavulanic acid [170]. With piperacillin and benzylpenicillin, synthetic albumin conjugates were shown to elicit T-cell responses with ex vivo rechallenge of drug responsive T-cell clones derived from hypersensitive patients, just as soluble drug in a lymphocyte transformation test was. This illustrated the relevance of HSA adducts as antigenic determinants of hypersensitivity [98, 168]. Further evaluation of the modified peptide sequences has also been studied in a number of cases, providing further resolution on the identity of modified sequences which serve as critical antigenic determinants [98]. In 2017, Meng et al. [92] defined the hapten threshold for piperacillin albumin conjugate T-cell activation in patients and in vitro T-cell assays. Importantly, the level of adduct formation in tolerant and hypersensitive patients was similar, and in all cases, exceeded the threshold for T-cell activation. This shows that additional factors are necessary to translate the antigen signal into an effector T-cell response.

Radiometric covalent binding studies

Covalent binding studies have long been considered the gold standard for assessment of drug discovery with regards to evaluating the capacity for, distribution of, and extent of which, covalent binding can take place. A key aspect of these assays is that they are amenable to both in vitro and in vivo studies. Through the various formats they exist in, radiometric studies can be used to study the covalent binding propensity generated via bioactivation of a compound within microsomal and hepatocyte preparations across species e.g., rat and human [171], and can also be used within in vivo studies to provide insight into covalent binding with the additional context of biodistribution. The (classic) output of such assays is a quantitative determination of total protein adduction in equivalent pmol of drug per mg of protein in the system [172]. These assays have been subject to important discussions in the field of reactive metabolites and their related toxicity for over two decades.

For several decades prototypical compounds known to cause toxicity were studied, and when considered in isolation the toxicity and bioactivation qualities were shown to be associated in a dose dependent fashion. However, inter-compound discrepancies in equivalent total covalent binding burden required to elicit toxicity made assigning a critical threshold of covalent binding required for toxicity an impossible task. In the absence of a "perfect" answer to the quantification of such a threshold, a key development for the field was the pragmatic approach of assigning a target for drug development. The first example of this was provided by Baillie et al., where the authors proposed a 50pmol per mg protein as a target for the covalent binding of development candidates across their standardized covalent binding study cascade, and identified that >200 pmol was a particularly undesirable threshold [172]. The importance of contextually qualifying any risks that covalent binding liabilities indicate was duly pointed out by the authors with factors such as anticipated daily drug dose/total exposure, detoxifying metabolism pathways and the risk/benefit across potential therapeutic indications acknowledged to play a role in impact of findings. An analysis of >200 marketed compounds focusing on drug-induced liver injury liabilities provided correlative insight that the combination of high daily dose (>100 mg/day) and high covalent binding within in vitro human liver microsomes was highly predictive of clinical drug-induced liver injury [103]. Calculation of an estimated covalent binding burden (based on in vitro covalent binding data and drug dose/pharmacokinetic parameters) was utilized alongside a panel of in vitro mechanistic toxicity assays within another group for assessment of idiosyncratic toxicity [102]. The downside of such studies is the requirement for radiolabelled material and the associated costs. This has meant that such studies are not high throughput, and are typically done at later stages of discovery such as lead optimization. Therefore, the placement of such assays does limit their potential mitigative value. In this light, it is notable that covalent binding assays were excluded from the default early hepatotoxic signal decision tree proposed by [103], indication was provided that Merck no longer routinely conduct covalent binding studies for all drug projects [173], and covalent binding was omitted as a parameter in another cumulative predictive tool for risk in early development [101]. As outlined in [155], these assays are often conducted following candidate selection at the preclinical stage of drug development, at which point the chemistry of the selected compound are often "baked in". Covalent binding studies do have their place within drug development and can serve to address key questions when the resource allocation is justified. As a conventional bioanalytical tool, in vivo radiolabelling experiments in one guise or another are a stalwart of the drug development process (autoradiography has existed within drug development for over half a century) [174]. The information gleaned from studies with radiolabelled compound is important for understanding ADME properties and therefore is routinely generated.

From adductomics to the characterization of MHC binding, hapten-modified peptides

The principle of adductomics involves the quantification of covalent adducts bound to tissues, thereby typifying the

electrophilic characteristics of a drug or its reactive metabolites [175]. Throughout everyday life humans are exposed to reactive physiochemical agents with both endogenous and exogenous origins [176]. Electrophilic haptens are commonly formed through metabolism of molecules, which in the case of drugs yields intermediates with the potential to irreversibly modify proteins. Adductomics, including the methods outlined above and below, attempts to enumerate the totality of adduct formation at nucleophilic locales characterizing human exposure to an antigen. One key example of a drug class with the facility to form drug-protein adducts is the β-lactam antibiotics. These drugs contain a cyclic amide ring making them rife for nucleophilic attack by nucleophiles present on proteins such as albumin. LC-MS analysis of β-lactam antibiotic binding characteristics expose their ability to bind favorably to lysine residues, in both a time and concentration dependent fashion. Indeed, such modifications present similar to those identified in the patient plasma i.e., piperacillin modification at Lys 190, 195, 432, and 451 and flucloxacillin modification at Lys 190 and 212 [166, 168]. Advances in mass spectrometric techniques has resulted in the characterization of a range of drug haptenated sequences on model proteins (e.g., piperacillin [169, 177], flucloxacillin [93], SMX-NO [178], and nevirapine [179]), alongside identification of favorable drug binding sites. As the sensitivity of new mass spectrometers increased there has been a drive to characterize peptides displayed naturally on the surface of antigen presenting cells by MHC molecules. A new discipline of immunopeptidomics has evolved from pioneers in the field. Major breakthroughs include the discovery of novel viral antigens, antigens displayed selectively by tumor cells and neoantigens involved in autoimmune disease [180–183].

In the context of drug hypersensitivity, immunopeptidomics was first used to define the pathway for abacavir-specific CD8 + T-cell activation. When abacavir hypersensitivity reactions were first observed in the clinic, the formation of a hapten aldehyde metabolite and subsequent protein conjugation was believed to be responsible for immune cell activation [184, 185]. However, this was difficult to reconcile with in vitro studies characterizing the selective activation of CD8 + T-cells with the parent compound [186–191]. To complicate the field further, activation of several CD8 + T-cells with abacavir was shown to be dependent on antigen processing [191, 192]; as such, the PI concept could not be used to explain the pathway of T-cell activation. In 2012, a model was put forward stating that abacavir bound with exquisite selectivity deep within the HLA-B*57:01 peptide binding cleft, effectively altering the structural space for peptide binding [6–8]. This model helped to explain why abacavir hypersensitivity is only observed in individuals expressing HLA-B*57:01 [193] and abacavir only activates CD8 + T-cells (abacavir is the only drug causing hypersensitivity reactions that doesn't activate CD4+ and CD8 + T-cells). Illing et al. utilized mass spectrometry to characterize the peptides displayed by HLA-B*57:01 in the presence and absence of abacavir. Abacavir haptenated peptides were not observed; however, when antigen presenting cells were exposed to the drug, an alternative array of peptides were presented on the surface by HLA-B*57:01 [7]. These class I binding peptides terminated in small aliphatic amino acids (I, L, V) in comparison to the canonical HLA-B*57:01 peptides which terminate in larger chain aromatic residues (W, F, P). This is because abacavir reduces the chemical space within HLA-B*57:01 that permits the binding of aromatic amino acids in the absence of the drug. With these findings, the authors proposed that the altered peptide repertoire would be misconstrued as foreign by CD8 + T-cells, initiating a T-cell response and the adverse event [7, 8, 194, 195], Recently, this same approach has been used to explore the kinetic profile of individual peptides displayed by HLA-B*57:01 and their dependence on abacavir exposure [196].

In recent years, researchers with knowledge of hapten chemistry have adopted the same mass spectrometry approach to search for and identify flucloxacillin-modified peptides displayed by HLA-B*57:01 on the surface of antigen presenting cells [93, 94]. Flucloxacillin was selected as a study paradigm as liver injury associated with flucloxacillin exposure is seen commonly in patients expressing HLA-B*57:01 [95], the same HLA associated with abacavir hypersensitivity. Multiple naturally presented flucloxacillin-haptenated peptides were identified from cell lines expressing HLA-B*57:01. These haptenated HLA-B*57:01 bound peptides were displayed (i) through antigen processing of flucloxacillin-modified intracellular proteins, and (ii) direct binding of flucloxacillin to peptides already displayed by B*57:01 on the surface of antigen presenting cells. These findings are exciting in numerous ways. First, the approach can be applied to other hapteneic drugs and metabolites to define the diversity of modified peptides displayed by HLA proteins. Second, the approach can be applied to HLA proteins with different amino acid anchor residues to determine whether the hapten is displayed preferentially at specific positions in the peptide sequence. Third, the data lay the foundations to discover critical protein targets involved in the adverse event. Finally, the identity of natural hapten peptide adducts displayed by antigen presenting cells will aid strategies to synthesize peptides for use in functional assays with T-cells from hypersensitive patients.

In vitro immunological assays using T-cells among other cellular components isolated from the venous blood of a healthy donor or, in some cases, a patient who has experienced a hypersensitivity reaction, are among the gold

standard of methods to elucidate the cellular pathophysiology of adverse reactions. These assays range from stimulating peripheral blood mononuclear cells (that contain drug-specific memory T-cells) from a hypersensitive patient with particular drug-(metabolite) and measurement of proliferation, cytokine release or cytotoxicity [197–199], to generating memory T-cells from healthy donors through the naïve T-cell priming assays [200–202]. Furthermore, drug(metabolite)responsive T-cell clones can be generated from responsive peripheral blood mononuclear cell cultures and characterized to define the nature of immune response (cytokine profile and cell surface markers), pathways of T-cell activation and cross-reactivity with related drug(-metabolite) structures [189, 203–205].

While immunological and proteomic assays together offer a greater understanding of the mechanism of the drug hypersensitivity reaction, the role of each of these aspects in tandem represents somewhat of a juxtaposition given the distinct nature of these techniques. Nonetheless, strides have been made in recent years to attempt to bridge this chasm. As discussed above, the introduction of piperacillin hapten-modified human serum albumin into T-cell in vitro assays has been pivotal in confirming T-cell antigenicity towards drug-modified proteins. Similarly, benzylpenicillin-modified albumin induced CD8 + T-cell reactivity in drug naïve donors, albeit to a lesser degree than the free drug itself [206], suggesting that a lower precursor frequency exists within a CD8 + T-cell repertoire compared to a CD4+ [207]. The design and synthesis of drug-modified peptides for incorporation into T-cell studies represents an exciting prospect with the potential to map specific antigenic peptides susceptible to modification by the drug in question. However, this process is not without its issues. Firstly, in terms of solubility it is difficult to replicate the exact peptide characterized from a drug-modified protein during M/S analysis. Often when amino acid residues are altered to enhance the solubility of the synthetic peptide this bears a knock-on effect on the peptide's affinity for the MHC in question, triggering a vicious cycle of solubility vs binding strength which is counterproductive. Next, the peptide must be reconstituted correctly to undergo HPLC analysis, which upon collection must be dried then dissolved adequately for introduction into T-cell assays. In most cases DMSO or a solvent of similar properties would be utilized, however these are difficult to incorporate into cell assays, thereby affecting the concentration ranges available for use. Naturally, one distinct drawback is the yield of peptide obtained following the modification of target amino acids with the drug-(metabolite). Such drawbacks once again affect the potential to screen designer peptides at sufficient concentrations to obtain a complete dataset, given that purchasing copious quantities is not commercially viable. Lastly, in vitro modification of synthetic peptides gives rise to the potential for side chain reactions to occur which can firstly affect the stability and quality of the modified peptide, but also may alter the reactivity of T-cells towards this antigen. These include N-terminal modifications, with beta-lactams being the main culprits, and oxidation reactions usually at cysteine and methionine, but also histidine, tyrosine, tryptophan and phenyalanine. C-terminal modification at aromatic residues has also been noted with highly reactive compounds. Finally, β-lactam antibiotics are known to undergo dimerization or in some cases trimerization which can make characterization troublesome. Despite these difficulties, in recent years several studies have managed to synthesize HLA binding drug hapten-modified designer peptides for functional studies. The group led by Pallardy used benzylpenicillin-modified albumin and synthetic HLA binding peptides containing relevant amino acid sequences from albumin to illustrate that CD4 + T-cells from healthy donors displayed a degree of specificity towards the individual peptides [207]. Furthermore, two of the penicillin haptenated peptides induced T-cell responses in hypersensitive patient PBMC, thereby confirming the role of drug-peptide conjugates in the adverse event [207]. Continuing this trend, we have developed a strategy to synthesize amoxicillin- and nitroso dapsone-modified peptides that bind to specific HLA class I and II proteins associated with adverse events. HLA class I ligands (9 mers) and class II ligands (12–15 mers) were designed by incorporating appropriate HLA binding motifs into a poly-alanine peptide. A reactive amino acid (cysteine, lysine, and arginine) was inserted within different positions to generate positional derivatives. Glutamic acid or aspartic acid was also included to improve peptide solubility. In silico prediction of the binding affinity of all designer ligands to a specific HLA was performed using the MHC binding prediction tool at www.iedb.org. Amoxicillin 12-mer peptide adducts, with the drug bound to lysine, interacted with HLA-DRB1*15:01 and DQB1*06:02 (both components of the risk haplotype) to activate CD4 + T-cell clones from patients with drug-induced liver injury. T-cells were not activated with unmodified peptides subjected to the same extraction and purification procedure or with structural variants containing the hapten at different locations in the peptide sequence [208]. The nitroso dapsone-modified peptides synthesized, with the drug bound to cysteine, were shorter and designed to interact with HLA-B*13:01. CD8 + T-cells from patients with dapsone hypersensitivity syndrome were activated with the hapten-modified, but not unmodified peptides, and the T-cell response was again restricted to the HLA risk allele [209]. These data provide a framework for synthesis and assessment of naturally HLA eluted hapten-modified peptides and for structural studies defining hapten HLA peptide interactions.

Where haptenation is intentional; targeted covalent inhibitors

Targeted covalent inhibitors that act on target proteins via a covalent mechanism have revolutionized oncology and now are also emerging for non-cancer indications, e.g., antivirals and autoimmune diseases. In contrast to reversible inhibitors that require continues systemic drug exposure to achieve sustained pharmacodynamic effect, targeted covalent inhibitors can provide exceptional potency that may translate to lower doses and reduced off-target effects, but also distinct pharmacodynamic profiles and extended efficacy resulting from the inhibition of a target under nonequilibrium kinetics [210]. For example, ibrutinib (**1**; Fig. 3), a BTK inhibitor approved for the treatment of several B-cell malignancies including chronic lymphocytic leukemia (CLL) and mantle cell lymphoma, exhibited high potency through covalent binding to a cysteine residue (Cys481) adjacent to the ATP-binding site in BTK [211]. Following the success of ibrutinib, the next generation of BTK inhibitors such as acalabrutinib (**2**) and zanubrutinib (**3**) have been designed to improve the safety and efficacy. Importantly, targeted covalent inhibitors can mitigate the development of drug resistance resulting from mutation of a binding site. Afatinib (**4**) and osimertinib (**5**) are representative targeted covalent inhibitors used for the treatment of non-small-cell lung cancer (NSCLC). These therapeutics irreversibly inhibit EGFR kinase activity by forming a covalent bond with Cys797 in the ATP-binding pocket and consequently overcoming resistance to reversible TKIs in lung cancer patients with the acquired T790M/L858R EGFR mutation [212, 213]. An additional important advantage of targeted covalent inhibitors is the inhibition of targets with shallow, undruggable binding sites. For example, KRAS, long viewed as "undruggable," has now been targeted in tumors including NSCLC, pancreatic and colorectal cancer bearing the specific mutant allele KRASG12C [214–216]. Screening of small molecules that covalently bind to KRASG12C led to the discovery of multiple highly potent and selective covalent inhibitors for the treatment of various solid tumors, for example, Sotorasib (AMG510, **6**) and Adagrasib (MRTX849, **7**) [217–221]. Through a cysteine-reactive ligand screen, Boike et al. have identified a covalent ligand, EN4 (**8**) that targets Cys171 of MYC, a major oncogenic transcription factor frequently amplified in most human cancers. MYC has often been considered "undruggable" as there are no obvious binding pockets or druggable sites for pharmacological interrogation [123]. The development of site-specific covalent inhibitors for these "undruggable" targets has opened novel ways and an exciting era of covalent drug development.

Recently, proteolysis-targeting chimeras (PROTACs) that enhance the degradation of disease-related proteins of interest have been recognized as new therapeutics in drug discovery. By forming a ternary complex between the protein of interest and E3 ligase, PROTACs facilitate ubiquitination and subsequent degradation of target proteins [222]. These bifunctional molecules offer several benefits over direct inhibition of protein of interest by small-molecule inhibitors, including reduced systemic drug exposure due to their high potencies, improved selectivity, and the complete inhibition of protein of interest and the downstream signaling cascades [223]. Covalent PROTACs that covalently bind to either the protein of interest and/or E3 ligase are expected to benefit from the advantages associated with targeted covalent inhibitors [223, 224], for example, targeting those "undruggable" proteins of interest. A PROTAC, LC-2 (**9**) based on a Adagrasib warhead and a VHL E3 ligase ligand was shown to efficiently induce KRASG12C degradation and suppresses MAPK signaling in KRASG12C cell lines [225]. To further improve the efficacy and sustainability of protein degradation, both irreversible and reversible covalent E3 ligase recruiters have been explored [226, 227]. Compared to their noncovalent analogues, these covalent PROTACs exhibit equal or greater target degradation efficacy due to more favorable PK properties [224].

Despite their potential benefits, targeted covalent inhibitors and covalent PROTACs are counter-balanced by safety concerns regarding the covalent modification of both on-target and off-target proteins. Targeted covalent inhibitors therapies are generally better tolerated than cytotoxic agents due to their high target selectivity. However, they often cause skin reactions, diarrhea, and, to a lesser extent, hepatotoxicity which impairs a patient's quality of life. For patients with an adverse event, withholding, reducing or permanently discontinuing treatment is recommended, depending on severity or persistence of the reaction; permanent discontinuation poses a dilemma if the tumour is responsive to the targeted covalent inhibitors. The adverse reactions associated with targeted covalent inhibitors appear to be dose-limiting and are likely to be caused by either inhibition of wild-type target proteins or covalent binding to off-target proteins. For example, the use of ibrutinib is associated various adverse reactions including rash, diarrhea, bleeding, and atrial fibrillation, leading to discontinuation of ibrutinib in >20% of patients administered with the drug [228]. The causative mechanisms for these adverse reactions are not fully understood but may be due to the inhibition of both on-target and alternative kinases, e.g., EGFR [229, 230]. To minimize off-target inhibition of alternative kinases, acalabrutinib (**3**) and zanubrutinib (**4**) have been designed to improve overall safety profiles. Unfortunately, the dermatological adverse reactions appeared to be similar to those observed with ibrutinib [231]. Some dose-limiting adverse reactions can be managed by closely monitoring the dose-response associated

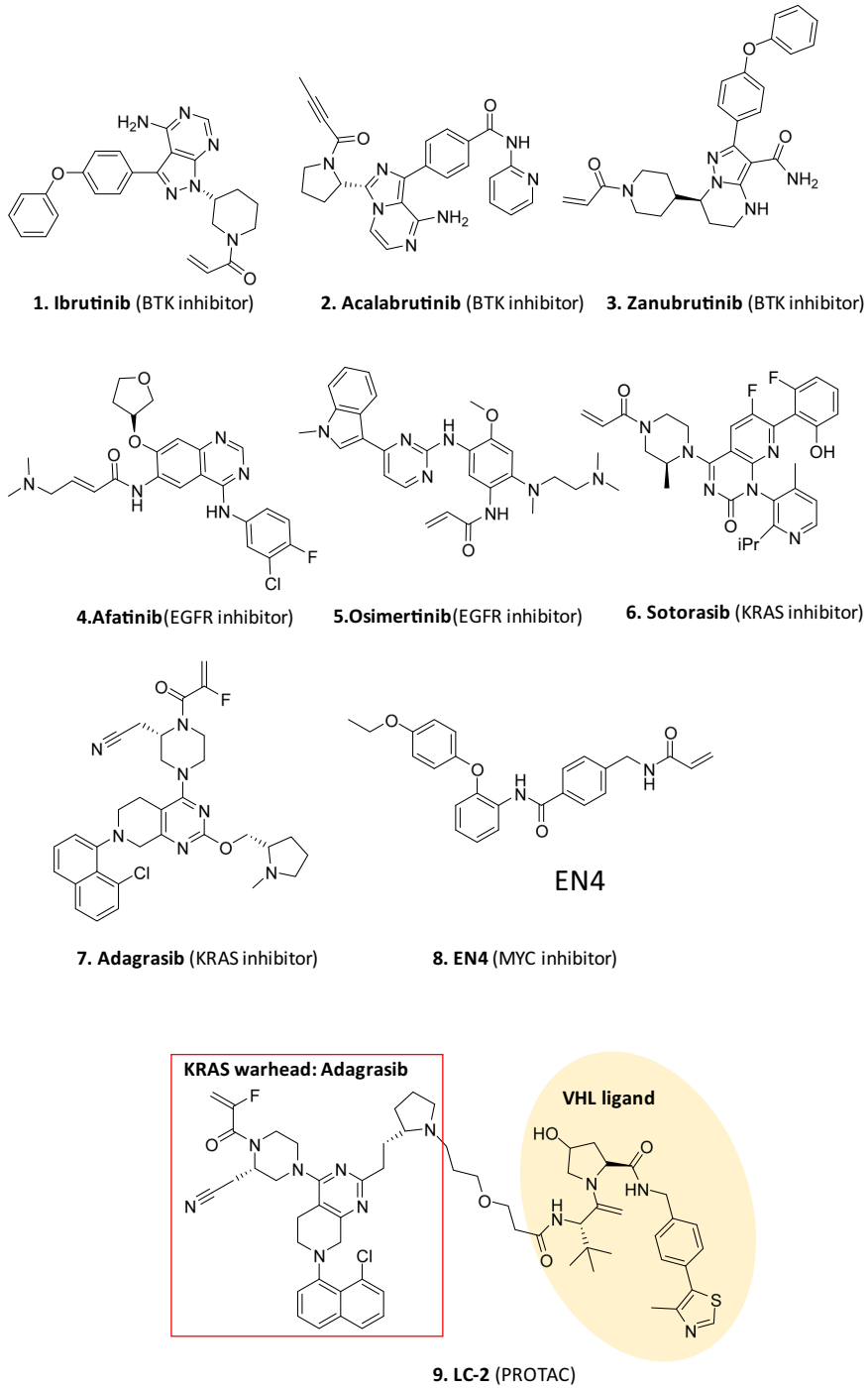

Fig. 3 Scheme showing the structure of targeted covalent inhibitor drugs

with efficacy and safety, thus creating a therapeutic window that may decouple efficacy from toxicity, leading to improved patient treatment.

However, some serious idiosyncratic adverse reactions associated with targeted covalent inhibitors are immune-mediated reactions that can lead to significant mortalities. Dermatological reactions are probably the most frequent immune-mediated reactions associated with targeted covalent inhibitors. The pathogenesis of targeted covalent inhibitor-induced immunotoxicity is yet to be determined, the excessive infiltration of lymphocytes (CD4+ and CD8+) in skin lesions detected in patients administered with either BTK of EGFR inhibitors suggested targeted covalent inhibitors are potentially involved in activating the immune system [231, 232]. Targeted covalent inhibitors can form covalent adducts with both target and off-target proteins, which can be processed and presented by antigen presenting cells as haptenated HLA ligands, leading to

activation of hapten specific T cells. It is also possible targeted covalent inhibitors can impair the proliferation and functions of different T cell population, indirectly or directly modulating immune responses. Some evidence also indicates that targeted covalent inhibitors and PROTACs can increase MHC class I presentation, leading to enhanced T cell activation [233, 234]. The observed greater and more severe toxicity associated with concurrent programmed death-ligand (PD-1/L1) plus osimertinib further confirms the important role of targeted covalent inhibitor-specific immune responses in these reactions [235]. The fear of potential immune-mediated reactions associated with this class of drugs has been an obstacle of covalent drug discovery. As no animal models are available for testing the potential immune reactions associated with targeted covalent inhibitors, developing predictive in vitro systems incorporating functional drug antigens, genetics of the susceptible patients and factors that modulate immune function is urgently needed to identify those candidates with high risk of immunogenicity at early stages of drug discovery.

Perspectives

An important question to address is how the distinct intracellular targets of adduction may contribute to the capacity of an adduct to be immunogenic. As demonstrated by the contingency of pharmaceuticals with a covalent binding mechanism of action, and their recent resurgence into the limelight, covalent binding is not always undesirable and even relatively high levels are not preclusive to the development of a successful compound without such liabilities. As indicated above, the threshold level of covalent binding necessary to elicit an adverse event may differ between prototypical compounds. This indicates that the qualitative nature of covalent binding is important in determining the quantitative threshold for toxicity. Another relevant observation in this regard is one of the recognized shortcomings of using glutathione as a trapping agent – this molecules' putative endogenous role is to serve as fodder in detoxification, and thus preserve the integrity of other proteins. Thus, one might propose that electrophilic species which target glutathione potently, are likely to be better tolerated than those which are not quenched by an equivalently large endogenous pool of protective nucleophiles.

For some time, it has been speculated that mapping the human proteome in terms of which proteins are critical to direct toxicity will be fruitful. In the context of hypersensitivity reactions there is precedence for both a role of signal 1 (antigenicity) and signal 2 (adjuvanticity) with regards to the role of covalent binding (and associated toxicity) in the governance of whether a reaction will occur in an individual [271]. How the adductome resulting from covalent binding translates to perturbation of the immunopeptidome is directly linked to antigenicity, whilst the toxicological mechanisms derived from adduction-imposed protein dysfunction/cellular stress, and any activation of pattern recognition receptors will contribute to the adjuvanticity.

Hapten theory, and in particular, the relationship between adduct formation and development of an adverse reaction, has been studied for over 80 years. Significant progress has been made throughout the decades: as we move forward, exploration of the immunogenicity of naturally MHC eluted hapten-modified binding peptides will shed light on the critical proteins involved in drug hypersensitivity. However, the hapten story does not end there. With new drugs entering the market, each with their individualized binding characteristics, there will work for researchers to perform for many decades to come.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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References

1. Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, et al. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18,820 patients. BMJ. 2004;329:15–9.
2. Schrijvers R, Gilissen L, Chiriac AM, Demoly P. Pathogenesis and diagnosis of delayed-type drug hypersensitivity reactions, from bedside to bench and back. Clin Transl Allergy. 2015;5:31.
3. Ariza A, Mayorga C, Fernandez TD, Barbero N, Martín-Serrano A, Pérez-Sala D, et al. Hypersensitivity reactions to β-lactams: relevance of hapten-protein conjugates. J Investig Allergol Clin Immunol. 2015;25:12–25.
4. Wu Y, Sanderson JP, Farrell J, Drummond NS, Hanson A, Bowkett E, et al. Activation of T cells by carbamazepine and carbamazepine metabolites. J Allergy Clin Immunol. 2006;118:233–41.
5. Ko TM, Chung WH, Wei CY, Shih HY, Chen JK, Lin CH, et al. Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome. J Allergy Clin Immunol. 2011;128:1266–76.e11.
6. Norcross MA, Luo S, Lu L, Boyne MT, Gomarteli M, Rennels AD, et al. Abacavir induces loading of novel self-peptides into HLA-B*57: 01: an autoimmune model for HLA-associated drug hypersensitivity. AIDS. 2012;26:F21–9.
7. Illing PT, Vivian JP, Dudek NL, Kostenko L, Chen Z, Bharadwaj M, et al. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. Nature. 2012;486:554–8.
8. Ostrov DA, Grant BJ, Pompeu YA, Sidney J, Harndahl M, Southwood S, et al. Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. Proc Natl Acad Sci USA. 2012;109:9959–64.
9. Pichler WJ. Pharmacological interaction of drugs with antigen-specific immune receptors: the p-i concept. Curr Opin Allergy Clin Immunol. 2002;2:301–5.
10. Yun J, Cai F, Lee FJ, Pichler WJ. T-cell-mediated drug hypersensitivity: immune mechanisms and their clinical relevance. Asia Pac Allergy. 2016;6:77–89.
11. Pichler WJ, Naisbitt DJ, Park BK. Immune pathomechanism of drug hypersensitivity reactions. J Allergy Clin Immunol. 2011;127:S74–81.
12. Pichler WJ, Daubner B, Kawabata T. Drug hypersensitivity: flare-up reactions, cross-reactivity and multiple drug hypersensitivity. J Dermatol. 2011;38:216–21.
13. Pichler WJ. The important role of non-covalent drug-protein interactions in drug hypersensitivity reactions. Allergy. 2022;77:404–15.
14. Landsteiner K, Jacobs J. Studies on the sensitization of animals with simple chemical compounds. II. J Exp Med. 1936;64:625–39.
15. Gefen T, Vaya J, Khatib S, Rapoport I, Lupo M, Barnea E, et al. The effect of haptens on protein-carrier immunogenicity. Immunology. 2015;144:116–26.
16. Levine BB, Ovary Z. Studies on the mechanism of the formation of the penicillin antigen. J Exp Med. 1961;114:875–1153.
17. Batchelor FR, Dewdney JM, Gazzard D. Penicillin allergy: the formation of the penicilloyl determinant. Nature. 1965;206:362–4.
18. de Weck AL, Eisen HN. Some immunological properties of penicillenic acid. An antigenic determinant derived from penicillin. J Exp Med. 1960;112:122–47.
19. Ariza A, Mayorga C, Fernandez TD, Barbero N, Martín-Serrano A, Pérez-Sala D, et al. Hypersensitivity reactions to beta-lactams: relevance of hapten-protein conjugates. J Investig Allergol Clin Immunol. 2015;25:12–25.
20. He Y, Lei J, Pan X, Huang X, Zhao Y. The hydrolytic water molecule of Class A β-lactamase relies on the acyl-enzyme intermediate ES* for proper coordination and catalysis. Sci Rep. 2020;10:10205.
21. Park BK, Pirmohamed M, Kitteringham NR. Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective. Chem Res Toxicol. 1998;11:969–88.
22. Park K, Williams DP, Naisbitt DJ, Kitteringham NR, Pirmohamed M. Investigation of toxic metabolites during drug development. Toxicol Appl Pharm. 2005;207:425–34.
23. Uetrecht J. Mechanisms of idiosyncratic drug-induced liver injury. Adv Pharm. 2019;85:133–63.
24. Uetrecht JP. New concepts in immunology relevant to idiosyncratic drug toxicity: the “danger hypothesis” and innate immunity. Chem Res Toxicol. 1999;12:887–95.
25. Eliasson E, Kenna JG. Cytochrome P450 2E1 is a cell surface autoantigen in halothane hepatitis. Mol Pharm. 1996;50:573–82.
26. Kitteringham NR, Kenna JG, Park BK. Detection of autoantibodies directed against human hepatic endoplasmic-reticulum in sera from patients with halothane-associated hepatitis. Br J Clin Pharm. 1995;40:379–86.
27. You Q, Cheng L, Ju C. Generation of T cell responses targeting the reactive metabolite of halothane in mice. Toxicol Lett. 2010;194:79–85.
28. Park BK, Pirmohamed M, Kitteringham NR. Role of drug disposition in drug hypersensitivity: a chemical,molecular and clinical perspective. Chem Res Toxicol. 1998;9:969–88.
29. Christie G, Breckenridge AM, Park BK. Drug-protein conjugates XVIII. Detection of antibodies towards the antimalarial amodiaquine and its quinone imine metabolite in man and the rat. Biochem Pharm. 1989;38:1451–8.
30. Christie G, Coleman J, Park BK. Drug-protein conjugates XVII. The effect of storage on the disposition and immunogenicity of benzylpenicillin in the rat. Biochem Pharm. 1988;37:4121–8.
31. Coleman JW, Foster AL, Yeung JH, Park BK. Drug-protein conjugates XV. A study of the disposition of D-penicillamine in the rat and its relationship to immunogenicity. Biochem Pharm. 1988;37:737–42.
32. Maggs JL, Park BK. Drug-protein conjugates XVI. Studies of sorbinil metabolism: formation of 2-hydroxysorbinil and unstable protein conjugates. Biochem Pharm. 1988;37:743–8.
33. Maggs JL, Tingle MD, Kitteringham NR, Park BK. Drug-protein conjugates XIV. Mechanisms of formation of protein-arylating intermediates from amodiaquine, a myelotoxin and hepatotoxin in man. Biochem Pharm. 1988;37:303–11.
34. Christie G, Coleman JW, Park BK. Drug-protein conjugates–XVII. The effect of storage on the antigenicity and immunogenicity of benzylpenicillin in the rat. Biochem Pharm. 1988;37:4121–8. p
35. Maggs JL, Park BK. Drug-protein conjugates–XVI. Studies of sorbinil metabolism: formation of 2-hydroxysorbinil and unstable protein conjugates. Biochem Pharm. 1988;37:743–8.
36. Christie G, Kitteringham NR, Park BK. Drug-protein conjugates XIII. The disposition of the benzylpenicilloyl hapten conjugated to albumin. Biochem Pharm. 1987;36:3379–85.
37. Maggs JL, Kitteringham NR, Grabowski PS, Park BK. Drug-protein conjugates–X. The role of protein conjugation in the disposition of dinitrofluorobenzene. Biochem Pharm. 1986;35:505–13.
38. Yeung JHK, Coleman JW, Park BK. Drug-protein conjugates IX. Immunogenicity of captopril-protein conjugates. Biochem Pharm. 1985;34:4005–12.
39. Kitteringham NR, Maggs JL, Newby S, Park BK. Drug-protein conjugates–VIII. The metabolic fate of the dinitrophenyl hapten conjugated to albumin. Biochem Pharm. 1985;34:1763–71.

40. Grabowski PS, Park BK. Drug-protein conjugates–VII. Disposition of [3H]-ethinylestradiol-protein conjugates in the rat. Biochem Pharm. 1984;33:3289–94.
41. Yeung JH, Breckenridge AM, Park BK. Drug protein conjugates–VI. Role of glutathione in the metabolism of captopril and captopril plasma protein conjugates. Biochem Pharm. 1983;32:3619–25.
42. Maggs JL, Grabowski PS, Park BK. Drug-protein conjugates–V. Sex-linked differences in the metabolism and irreversible binding of 17 alpha-ethinylestradiol in the rat. Biochem Pharm. 1983;32:2793–800.
43. Yeung JH, Breckenridge AM, Park BK. *Drug-protein conjugates–IV. The effect of acute renal failure on the disposition of [14C]captopril in the rat*. Biochem Pharm. 1983;32:2467–72.
44. Maggs JL, Grabowski PS, Park BK. Drug-protein conjugates–II. An investigation of the irreversible binding and metabolism of 17 alpha-ethinyl estradiol in vivo. Biochem Pharm. 1983;32:301–8.
45. Park BK, Grabowski PS, Yeung JH, Breckenridge AM. Drug protein conjugates–I. A study of the covalent binding of [14C] captopril to plasma proteins in the rat. Biochem Pharm. 1982;31:1755–60.
46. Maggs JL, Grabowski PS, Park BK. Drug protein conjugates–III. Inhibition of the irreversible binding of ethinylestradiol to rat liver microsomal protein by mixed-function oxidase inhibitors, ascorbic acid and thiols. J Steroid Biochem. 1983;19:1273–8.
47. Baillie TA, Pearson PG, Rashed MS, Howald WN. The use of mass spectrometry in the study of chemically-reactive drug metabolites. Application of MS/MS and LC/MS to the analysis of glutathione- and related S-linked conjugates of N-methylformamide. J Pharm Biomed Anal. 1989;7:1351–60.
48. Ashby J, Tennant RW, Zeiger E, Stasiewicz S. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 42 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. Mutat Res. 1989;223:73–103.
49. Tang W, Stearns RA, Bandiera SM, Zhang Y, Raab C, Braun MP, et al. Studies on cytochrome-P450-mediated bioactivation of diclofenac in rats and in human hepatotocytes: identification of glutathione conjugated metabolites. Drug Metab Dispos. 1999;27:365–72.
50. Tang W, Stearns RA, Bandiera SM, Zhang Y, Raab C, Braun MP, et al. Studies on cytochrome P-450-mediated bioactivation of diclofenac in rats and in human hepatocytes: identification of glutathione conjugated metabolites. Drug Metab Dispos. 1999;27:365–72.
51. Jin L, Baillie TA. Metabolism of the chemoprotective agent diallyl sulfide to glutathione conjugates in rats. Chem Res Toxicol. 1997;10:318–27.
52. Jin L, Davis MR, Hu P, Baillie TA. Identification of novel glutathione conjugates of disulfiram and diethyldithiocarbamate in rat bile by liquid chromatography-tandem mass spectrometry. Evidence for metabolic activation of disulfiram in vivo. Chem Res Toxicol. 1994;7:526–33.
53. Davis MR, Kassahun K, Jochheim CM, Brandt KM, Baillie TA. Glutathione and N-acetylcysteine conjugates of 2-chloroethyl isocyanate. Identification as metabolites of N,N'-bis(2-chloroethyl)-N-nitrosourea in the rat and inhibitory properties toward glutathione reductase in vitro. Chem Res Toxicol. 1993;6:376–83.
54. Pearson PG, Slatter JG, Rashed MS, Han DH, Baillie TA. Carbamoylation of peptides and proteins in vitro by S-(N-methylcarbamoyl)glutathione and S-(N-methylcarbamoyl)cysteine, two electrophilic S-linked conjugates of methyl isocyanate. Chem Res Toxicol. 1991;4:436–44.
55. Stepan AF, Walker DP, Bauman J, Price DA, Baillie TA, Kalgutkar AS, et al. Structural alert/reactive metabolite concept as applied in medicinal chemistry to mitigate the risk of idiosyncratic drug toxicity: a perspective based on the critical examination of trends in the top 200 drugs marketed in the United States. Chem Res Toxicol. 2011;24:1345–410.
56. Pickard C, et al. "Investigation of mechanisms underlying the T-cell response to the hapten 2,4-dinitrochlorobenzene." The Journal of investigative dermatology vol. 2007;127:630–7.
57. Scott AE, Kashon ML, Yucesoy B, Luster MI, Tinkle SS. Insights into the quantitative relationship between sensitization and challenge for allergic contact dermatitis reactions. Toxicol Appl Pharm. 2002;183:66–70.
58. Rees JL, Matthews JN, Friedmann PS. Quantifying anti-inflammatory agents' potency by measurement of response to dinitrochlorobenzene challenge. J Dermatol Sci. 1992;4:1–5.
59. Rees JL, Friedmann PS, Matthews JN. The influence of area of application on sensitization by dinitrochlorobenzene. Br J Dermatol. 1990;122:29–31.
60. Weltzien HU, Padovan E. Molecular features of penicillin allergy. J Invest Dermatol. 1998;110:203–6. p
61. Padovan E, Bauer T, Tongio MM, Kalbacher H, Weltzien HU. Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. Eur J Immunol. 1997;27:1303–7.
62. Preckel T, Grimm R, Martin S, Weltzien HU. Altered hapten ligands antagonize trinitrophenyl-specific cytotoxic T cells and block internalization of hapten-specific receptors. J Exp Med. 1997;185:1803–13.
63. Padovan E, Mauri-Hellweg D, Pichler WJ, Weltzien HU. T cell recognition of penicillin G: structural features determining antigenic specificity. Eur J Immunol. 1996;26:42–8.
64. Martin S, Weltzien HU. T cell recognition of haptens, a molecular view. Int Arch Allergy Immunol. 1994;104:10–6.
65. Martin S, von Bonin A, Fessler C, Pflugfelder U, Weltzien HU. Structural complexity of antigenic derminants for class I MHC-restricted, hapten-specific T cells. J Immunol. 1993;151:678–87.
66. Martin S, Ortmann B, Pflugfelder U, Birsner U, Weltzien HU. Role of hapten-anchoring peptides in defining hapten-epitopes for MHC-restricted cytotoxic T cells. Cross-reactive TNP-determinants on different peptides. J Immunol. 1992;149:2569–75.
67. Weltzien HU, Hebbelmann S, Pflugfelder U, Ruh H, Ortmann B, Martin S, et al. Antigen contact sites in class I major histocompatibility complex-restricted, trinitrophenyl-specific T cell receptors. Eur J Immunol. 1992;22:863–6.
68. Naisbitt DJ, Hough SJ, Gill HJ, Pirmohamed M, Kitteringham NR, Park BK. Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity. Br J Pharm. 1999;126:1393–407.
69. Gill HJ, Hough SJ, Naisbitt DJ, Maggs JL, Kitteringham NR, Pirmohamed M, et al. The relationship between the disposition and immunogenicity of sulfamethoxazole in the rat. J Pharmacol Exp Ther. 1997;282:795–801.
70. Naisbitt DJ, et al. Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: Implications for immune mediated toxicity. Bioorg Med Chem Lett. 1996;6:1511–6.
71. Farrell J, et al. Characterisation of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and man. Toxicology. 2003;192:83–4.
72. Naisbitt DJ, Farrell J, Gordon SF, Maggs JL, Burkhart C, Pichler WJ, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. Mol Pharmacol. 2002;62:628–37.

73. Naisbitt DJ, Gordon SF, Pirmohamed M, Burkhart C, Cribb AE, Pichler WJ, et al. Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. Br J Pharm. 2001;133:295–305.
74. Burkhart C, von Greyerz S, Depta JP, Naisbitt DJ, Britschgi M, Park KB, et al. Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. Br J Pharm. 2001;132:623–30.
75. Schnyder B, Burkhart C, Schnyder-Frutig K, von Greyerz S, Naisbitt DJ, Pirmohamed M, et al. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4(+) T cells from allergic individuals. J Immunol. 2000;164:6647–54.
76. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994;12:991–1045.
77. Uetrecht JP. New concepts in immunology relevant to idiosyncratic drug reactions: the "danger hypothesis" and innate immune system. Chem Res Toxicol. 1999;12:387–95.
78. Sernoskie SC, Jee A, Uetrecht J. The role of myeloperoxidase in clozapine-induced inflammation: a mechanistic update for idiosyncratic drug-induced agranulocytosis. Int J Mol Sci. 2023;24:1–23
79. Uetrecht J. Idiosyncratic drug reactions: a 35-year chemical research in toxicology perspective. Chem Res Toxicol. 2022;35:1649–54.
80. Kato R, Ijiri Y, Hayashi T, Uetrecht J. Reactive metabolite of gefitinib activates inflammasomes: implications for gefitinib-induced idiosyncratic reaction. J Toxicol Sci. 2020;45:673–80.
81. Metushi IG, Hayes MA, Uetrecht J. Treatment of PD-1(−/−) mice with amodiaquine and anti-CTLA4 leads to liver injury similar to idiosyncratic liver injury in patients. Hepatology. 2015;61:1332–42.
82. Uetrecht J, Kaplowitz N. Inhibition of immune tolerance unmasks drug-induced allergic hepatitis. Hepatology. 2015;62:346–8.
83. Zhu X, Li J, Liu F, Uetrecht JP. Involvement of T helper 17 cells in D-penicillamine-induced autoimmune disease in Brown Norway rats. Toxicol Sci. 2011;120:331–8.
84. Li J, Mannargudi B, Uetrecht JP. Covalent binding of penicillamine to macrophages: implications for penicillamine-induced autoimmunity. Chem Res Toxicol. 2009;22:1277–84.
85. Ju C, Uetrecht JP. Mechanism of idiosyncratic drug reactions: reactive metabolite formation, protein binding and the regulation of the immune system. Curr Drug Metab. 2002;3:367–77.
86. Aleksic M, Thain E, Roger D, Saib O, Davies M, Li J, et al. Reactivity profiling: covalent modification of single nucleophile peptides for skin sensitization risk assessment. Toxicol Sci. 2009;108:401–11.
87. Troutman JA, Foertsch LM, Kern PS, Dai HJ, Quijano M, Dobson RL, et al. The incorporation of lysine into the peroxidase peptide reactivity assay for skin sensitization assessments. Toxicol Sci. 2011;122:422–36.
88. Lalko JF, Kimber I, Dearman RJ, Gerberick GF, Sarlo K, Api AM. Chemical reactivity measurements: potential for characterization of respiratory chemical allergens. Toxicol Vitr. 2011;25:433–45.
89. Gerberick GF, Troutman JA, Foertsch LM, Vassallo JD, Quijano M, Dobson RL, et al. Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. Toxicol Sci. 2009;112:164–74.
90. Gerberick GF. The use of peptide reactivity assays for skin sensitisation hazard identification and risk assessment. Alter Lab Anim. 2016;44:437–42.
91. Martin SF, Esser PR, Schmucker S, Dietz L, Naisbitt DJ, Park BK, et al. T-cell recognition of chemicals, protein allergens and drugs: towards the development of in vitro assays. Cell Mol Life Sci. 2010;67:4171–84.
92. Meng X, Al-Attar Z, Yaseen FS, Jenkins R, Earnshaw C, Whitaker P, et al. Definition of the nature and hapten threshold of the beta-lactam antigen required for T cell activation in vitro and in patients. J Immunol. 2017;198:4217–27.
93. Waddington JC, Meng X, Illing PT, Tailor A, Adair K, Whitaker P, et al. Identification of flucloxacillin-haptenated HLA-B*57:01 ligands: evidence of antigen processing and presentation. Toxicol Sci. 2020;177:454–65.
94. Puig M, Ananthula S, Venna R, Kumar Polumuri S, Mattson E, Walker LM, et al. Alterations in the HLA-B*57:01 Immunopeptidome by Flucloxacillin and Immunogenicity of Drug-Haptenated Peptides. Frontiers in immunology, 2021;11;629399.
95. Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, et al. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nat Genet. 2009;41:816–9.
96. Monshi MM, Faulkner L, Gibson A, Jenkins RE, Farrell J, Earnshaw CJ, et al. Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. Hepatology. 2013;57:727–39.
97. Tailor A, Meng X, Adair K, Farrell J, Waddington JC, Daly A, et al. HLA DRB1*15:01-DQB1*06:02-Restricted Human CD4+ T cells are selectively activated with amoxicillin-peptide adducts. Toxicol Sci. 2020;178:115–26.
98. Azoury ME, Filì L, Bechara R, Scornet N, de Chaisemartin L, Weaver RJ, et al. Identification of T-cell epitopes from benzylpenicillin conjugated to human serum albumin and implication in penicillin allergy. Allergy. 2018;73:1662–72.
99. Almutairi M, Lister A, Zhao Q, Line J, Adair K, Tailor A, et al. Activation of Human CD8+ T Cells with Nitroso Dapsone-Modified HLA-B*13:01-Binding Peptides. J Immunol. 2023;210:1031–42. https://doi.org/10.4049/jimmunol.2200531.
100. Semenova E, Williams DP, Afzal AM, Lazic SE. A Bayesian neural network for toxicity prediction. Comput Toxicol. 2020;16:100133.
101. Williams DP, Lazic SE, Foster AJ, Semenova E, Morgan P. Predicting drug-induced liver injury with bayesian machine learning. Chem Res Toxicol. 2020;33:239–48.
102. Thompson RA, Isin EM, Li Y, Weidolf L, Page K, Wilson I, et al. In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. Chem Res Toxicol. 2012;25:1616–32.
103. Sakatis MZ, Reese MJ, Harrell AW, Taylor MA, Baines IA, Chen L, et al. Preclinical strategy to reduce clinical hepatotoxicity using in vitro bioactivation data for >200 compounds. Chem Res Toxicol. 2012;25:2067–82.
104. Miller EC, Miller JA. The presence and significance of bound aminoazo dyes in the livers of rats fed p-Dimethylaminoazobenzene*. Cancer Res. 1947;7:468–80.
105. Miller EC, Millere JA. Mechanisms of chemical carcinogenesis: nature of proximate carcinogens and interactions with macromolecules. Pharmacol Rev. 1966;18:805–38.
106. Nelson SD. Metabolic activation and drug toxicity. J Med Chem. 1982;25:753–65.
107. Ashby J, Tennant RW. Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. Mutat Res. 1988;204:17–115.
108. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res. 1975;31:347–64.
109. Ashby J, Basketter DA, Paton D, Kimber I. Structure activity relationships in skin sensitization using the murine local lymph node assay. Toxicology. 1995;103:177–94.

110. Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, Henne KR, et al. A comprehensive listing of bioactivation pathways of organic functional groups. Curr Drug Metab. 2005;6:161–225.
111. Kalgutkar AS, Soglia JR. Minimising the potential for metabolic activation in drug discovery. Expert Opin Drug Metab Toxicol. 2005;1:91–142.
112. Sanderson DM, Earnshaw CG. Computer prediction of possible toxic action from chemical structure; the DEREK system. Hum Exp Toxicol. 1991;10:261–73.
113. Schultz TW, Diderich R, Kuseva CD, Mekenyan OG. The OECD QSAR Toolbox Starts Its Second Decade. Methods in molecular biology (Clifton, N.J.), 2018;1800:55–77.
114. Patlewicz G, Jeliazkova N, Safford RJ, Worth AP, Aleksiev B. An evaluation of the implementation of the Cramer classification scheme in the Toxtree software. SAR QSAR Environ Res. 2008;19:495–524.
115. Bourdi M, Chen W, Peter RM, Martin JL, Buters JT, Nelson SD, et al. Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis. Chem Res Toxicol. 1996;9:1159–66.
116. Orr ST, Ripp SL, Ballard TE, Henderson JL, Scott DO, Obach RS, et al. Mechanism-based inactivation (MBI) of cytochrome P450 enzymes: structure–activity relationships and discovery strategies to mitigate drug–drug interaction risks. J Med Chem. 2012;55:4896–933.
117. Lecoeur S, Bonierbale E, Challine D, Gautier JC, Valadon P, Dansette PM, et al. Specificity of in vitro covalent binding of tienilic acid metabolites to human liver microsomes in relationship to the type of hepatotoxicity: comparison with two directly hepatotoxic drugs. Chem Res Toxicol. 1994;7:434–42.
118. Homberg JC, Andre C, Abuaf N. A new anti-liver-kidney microsome antibody (anti-LKM2) in tienilic acid-induced hepatitis. Clin Exp Immunol. 1984;55:561–70.
119. Beaune P, Dansette PM, Mansuy D, Kiffel L, Finck M, Amar C, et al. Human anti-endoplasmic reticulum autoantibodies appearing in a drug-induced hepatitis are directed against a human liver cytochrome P-450 that hydroxylates the drug. Proc Natl Acad Sci USA. 1987;84:551–5.
120. Robin MA, Maratrat M, Le Roy M, Le Breton FP, Bonierbale E, Dansette P, et al. Antigenic targets in tienilic acid hepatitis. Both cytochrome P450 2C11 and 2C11-tienilic acid adducts are transported to the plasma membrane of rat hepatocytes and recognized by human sera. J Clin Investig. 1996;98:1471–80.
121. Metushi IG, Sanders C, Acute Liver Study G, Lee WM, Uetrecht J. Detection of anti-isoniazid and anti–cytochrome P450 antibodies in patients with isoniazid-induced liver failure. Hepatology. 2014;59:1084–93.
122. Bourdi M, Larrey D, Nataf J, Bernuau J, Pessayre D, Iwasaki M, et al. Anti-liver endoplasmic reticulum autoantibodies are directed against human cytochrome P-450IA2. A specific marker of dihydralazine-induced hepatitis. J Clin Investig. 1990;85:1967–73.
123. Boike L, Cioffi AG, Majewski FC, Co J, Henning NJ, Jones MD, et al. Discovery of a functional covalent ligand targeting an intrinsically disordered cysteine within MYC. Cell Chem Biol. 2021;28:4–13.e17.
124. Kenna JG, Knight TL, Pelt FNAMV. Immunity to halothane metabolite-modified proteins in halothane hepatitisa. Ann N. Y Acad Sci. 1993;685:646–61.
125. Kenna JG, Neuberger J, Williams R. Specific antibodies to halothane-induced liver antigens in halothane-associated hepatitis. BJA: Br J Anaesth. 1987;59:1286–90.
126. Kenna JG, Neuberger J, Williams R. Identification by immunoblotting of three halothane-induced liver microsomal polypeptide antigens recognized by antibodies in sera from patients with halothane-associated hepatitis. J Pharmacol Exp Ther. 1987;242:733–40.
127. Kenna JG, Satoh H, Christ DD, Pohl LR. *Metabolic basis for a drug hypersensitivity: antibodies in sera from patients with halothane hepatitis recognize liver neoantigens that contain the trifluoroacetyl group derived from halothane*. J Pharmacol Exp Ther. 1988;245:1103–9.
128. Martin JL, Kenna JG, Martin BM, Thomassen D, Reed GF, Pohl LR. Halothane hepatitis patients have serum antibodies that react with protein disulfide isomerase. Hepatology. 1993;18:858–63.
129. Gardner I, Zahid N, MacCrimmon D, Uetrecht JP. A comparison of the oxidation of clozapine and olanzapine to reactive metabolites and the toxicity of these metabolites to human leukocytes. Mol Pharm. 1998;53:991–8.
130. Uetrecht JP, Shear NH, Zahid N. N-chlorination of sulfamethoxazole and dapsone by the myeloperoxidase system. Drug Metab Dispos. 1993;21:830–4.
131. Hofstra AH, Uetrecht JP. Reactive intermediates in the oxidation of hydralazine by HOCl: the major oxidant generated by neutrophils. Chem Biol Interact. 1993;89:183–96.
132. Naisbitt DJ, Ruscoe JE, Williams D, O'Neill PM, Pirmohamed M, Park BK. Disposition of amodiaquine and related antimalarial agents in human neutrophils: implications for drug design. J Pharm Exp Ther. 1997;280:884–93.
133. Hofstra AH, Uetrecht JP. Myeloperoxidase-mediated activation of xenobiotics by human leukocytes. Toxicology. 1993;82:221–42.
134. Christie G, Coleman JW, Newby S, McDiarmaid-Gordon A, Hampson JP, Breckenridge AM, et al. A study of the prevalence of penicillin-specific IgG, IgM and IgE antibodies detected by ELISA and defined by hapten inhibition, in patients with suspected penicillin allergy and in healthy volunteers. Br J Clin Pharm. 1988;25:381–6.
135. Coleman JW, Yeung JH, Roberts DH, Breckenridge AM, Park BK. Drug-specific antibodies in patients receiving captopril. Br J Clin Pharm. 1986;22:161–5.
136. Coleman JW, Yeung JH, Tingle MD, Park BK. Enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to protein-reactive drugs and metabolites. Criteria for identification of antibody activity. J Immunol Methods. 1986;88:37–44.
137. Bedard K, Smith S, Cribb A. Sequential assessment of an anti-drug antibody response in a patient with a systemic delayed-onset sulphonamide hypersensitivity syndrome reaction. Br J Dermatol. 2000;142:253–8.
138. Clarke JB, Neftel K, Kitteringham NR, Park BK. Detection of antidrug IgG antibodies in patients with adverse drug reactions to amodiaquine. Int Arch Allergy Appl Immunol. 1991;95:369–75.
139. Dansette PM, Bonierbale E, Minoletti C, Beaune PH, Pessayre D, Mansuy D. Drug-induced immunotoxicity. Eur J Drug Metab Pharmacokinet. 1998;23:443–51.
140. Nassberger L, Hultquist R, Sturfelt G. Occurrence of anti-lactoferrin antibodies in patients with systemic lupus erythematosus, hydralazine-induced lupus, and rheumatoid arthritis. Scand J Rheumatol. 1994;23:206–10.
141. Nässberger L, Johansson AC, Björck S, Sjöholm AG. Antibodies to neutrophil granulocyte myeloperoxidase and elastase: autoimmune responses in glomerulonephritis due to hydralazine treatment. J Intern Med. 1991;229:261–5.
142. Hofstra AH, Li-Muller SM, Uetrecht JP. Metabolism of isoniazid by activated leukocytes. Possible role in drug-induced lupus. Drug Metab Dispos. 1992;20:205–10.
143. Ogese MO, Jenkins RE, Maggs JL, Meng X, Whitaker P, Peckham D, et al. Characterization of peroxidases expressed in human antigen presenting cells and analysis of the covalent binding of nitroso sulfamethoxazole to myeloperoxidase. Chem Res Toxicol. 2015;28:144–54.

144. Lavergne SN, Wang H, Callan HE, Park BK, Naisbitt DJ. "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. J Pharmacol Exp Ther. 2009;331:372–81.
145. Roychowdhury S, Vyas PM, Svensson CK. Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells. Drug Metab Dispos. 2007;35:676–81.
146. Bhaiya P, Roychowdhury S, Vyas PM, Doll MA, Hein DW, Svensson CK. Bioactivation, protein haptenation, and toxicity of sulfamethoxazole and dapsone in normal human dermal fibroblasts. Toxicol Appl Pharm. 2006;215:158–67.
147. Vyas PM, Roychowdhury S, Khan FD, Prisinzano TE, Lamba J, Schuetz EG. et al. Enzyme-mediated protein haptenation of dapsone and sulfamethoxaozle in human keratinocytes—1. Expression and role of cytochromes P450. J Pharm Exp Ther. 2006;319:488–496.
148. Vyas, P. M., Roychowdhury, S., Koukouritaki, S. B., Hines, R. N., Krueger, S. K., Williams, D. E. et al . Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes: II. Expression and role of flavin-containing monooxygenases and peroxidases. The Journal of pharmacology and experimental therapeutics, 2006;319:497–505.
149. Roychowdhury S, Vyas PM, Reilly TP, Gaspari AA, Svensson CK. Characterization of the formation and localization of sulfamethoxazole and dapsone-associated drug-protein adducts in human epidermal keratinocytes. J Pharm Exp Ther. 2005;314:43–52.
150. Lavergne SN, Wang H, Callan HE, Park BK, Naisbitt DJ. "Danger" conditions increase sulfamethoxazole-protein adduct formation in human antigen-presenting cells. J Pharm Exp Ther. 2009;331:372–81.
151. Sanderson JP, Naisbitt DJ, Farrell J, Ashby CA, Tucker MJ, Rieder MJ, et al. Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling. J Immunol. 2007;178:5533–42.
152. Elsheikh A, Castrejon L, Lavergne SN, Whitaker P, Monshi M, Callan H, et al. Enhanced antigenicity leads to altered immunogenicity in sulfamethoxazole-hypersensitive patients with cystic fibrosis. J Allergy Clin Immunol. 2011;127:1543–51.e3.
153. Kumar S, Mitra K, Kassahun K, Baillie TA. Approaches for minimizing metabolic activation of new drug candidates in drug discovery. Handb Exp Pharmacol. 2010;511–44.
154. LoPachin RM, Geohagen BC, Nordstroem LU. Mechanisms of soft and hard electrophile toxicities. Toxicology. 2019;418:62–69.
155. Kakutani N, Nanayama T, Nomura Y. Novel risk assessment of reactive metabolites from discovery to clinical stage. J Toxicol Sci. 2019;44:201–11.
156. Inoue K, Shibata Y, Takahashi H, Ohe T, Chiba M, Ishii Y. A trapping method for semi-quantitative assessment of reactive metabolite formation using [35S]Cysteine and [14C]Cyanide. Drug Metab Pharmacokinet. 2009;24:245–54.
157. Gan J, Harper TW, Hsueh MM, Qu Q, Humphreys WG. Dansyl glutathione as a trapping agent for the quantitative estimation and identification of reactive metabolites. Chem Res Toxicol. 2005;18:896–903.
158. Callan HE, Jenkins RE, Maggs JL, Lavergne SN, Clarke SE, Naisbitt DJ, et al. Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation. Chem Res Toxicol. 2009;22:937–48.
159. Hammond S, Olsson-Brown A, Grice S, Gibson A, Gardner J, Castrejón-Flores JL, et al. Checkpoint inhibition reduces the threshold for drug-specific T-cell priming and increases the incidence of sulfasalazine hypersensitivity. Toxicol Sci. 2022;186:58–69.
160. Alzahrani A, Ogese M, Meng X, Waddington JC, Tailor A, Farrell J, et al. Dapsone and nitroso dapsone activation of naïve T-cells from healthy donors. Chem Res Toxicol. 2017;30:2174–86.
161. Castrejon JL, Berry N, El-Ghaiesh S, Gerber B, Pichler WJ, Park BK, et al. Stimulation of human T cells with sulfonamides and sulfonamide metabolites. J Allergy Clin Immunol. 2010;125:411–8.e4.
162. Chait BT. Mass spectrometry: bottom-up or top-down?. Science. 2006;314:65–66.
163. Tailor A, Waddington JC, Meng X, Park BK. Mass spectrometric and functional aspects of drug–protein conjugation. Chem Res Toxicol. 2016;29:1912–35.
164. Jr TP. All about albumin: biochemistry, genetics, and medical applications. Academic Press; 1995. p. 456.
165. Sabbioni G, Turesky RJ. Biomonitoring human albumin adducts: the past, the present, and the future. Chem Res Toxicol. 2017;30:332–66.
166. Jenkins RE, Meng X, Elliott VL, Kitteringham NR, Pirmohamed M, Park BK. Characterisation of flucloxacillin and 5-hydroxymethyl flucloxacillin haptenated HSA in vitro and in vivo. Proteomics Clin Appl. 2009;3:720–9.
167. Meng X, Jenkins RE, Berry NG, Maggs JL, Farrell J, Lane CS, et al. Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients. J Pharmacol Exp Ther. 2011;338:841–9.
168. Whitaker P, Meng X, Lavergne SN, El-Ghaiesh S, Monshi M, Earnshaw C, et al. Mass spectrometric characterization of circulating and functional antigens derived from piperacillin in patients with cystic fibrosis. J Immunol. 2011;187:200–11.
169. Jenkins RE, Yaseen FS, Monshi MM, Whitaker P, Meng X, Farrell J, et al. β-Lactam antibiotics form distinct haptenic structures on albumin and activate drug-specific T-Lymphocyte responses in multiallergic patients with cystic fibrosis. Chem Res Toxicol. 2013;26:963–75.
170. Meng X, Earnshaw CJ, Tailor A, Jenkins RE, Waddington JC, Whitaker P, et al. Amoxicillin and clavulanate form chemically and immunologically distinct multiple haptenic structures in patients. Chem Res Toxicol. 2016;29:1762–72.
171. Lévesque J-F, Day SH, Jones AN. Protocols of in vitro protein covalent binding studies in liver, In Drug Safety Evaluation: Methods and Protocols, J-C Gautier, editor. Totowa, NJ: Humana Press; 2011. p. 283–301.
172. Evans DC, Watt AP, Nicoll-Griffith DA, Baillie TA. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. Chem Res Toxicol. 2004;17:3–16.
173. Park BK, Boobis A, Clarke S, Goldring CE, Jones D, Kenna JG, et al. Managing the challenge of chemically reactive metabolites in drug development. Nat Rev Drug Discov. 2011;10:292–306.
174. Solon EG. Autoradiography techniques and quantification of drug distribution. Cell Tissue Res. 2015;360:87–107.
175. Preston GW, Phillips DH. Protein adductomics: analytical developments and applications in human biomonitoring. Toxics. 2019;7:29.
176. Carlsson H, Rappaport SM, Törnqvist M. Protein adductomics: methodologies for untargeted screening of adducts to serum albumin and hemoglobin in human blood samples. High Throughput. 2019;8:6.
177. Meng X, Al-Attar Z, Yaseen FS, Jenkins R, Earnshaw C, Whitaker P, et al. Definition of the nature and hapten threshold of the β-lactam antigen required for T cell activation in vitro and in patients. J Immunol. 2017;198:4217–27.
178. Naisbitt D. J, Olsson-Brown A, Gibson A, Meng X, Ogese M. O, Tailor A, Thomson P. Immune dysregulation increases the

incidence of delayed-type drug hypersensitivity reactions. Allergy. 2020;75:781–797.
179. Meng X, Howarth A, Earnshaw CJ, Jenkins RE, French NS, Back DJ, et al. Detection of drug bioactivation in vivo: mechanism of nevirapine-albumin conjugate formation in patients. Chem Res Toxicol. 2013;26:575–83.
180. Ahn R, Cui Y, White FM. Antigen discovery for the development of cancer immunotherapy. Semin Immunol. 2023;66:101733.
181. Lichti CF, Wan X. Using mass spectrometry to identify neoantigens in autoimmune diseases: the type 1 diabetes example. Semin Immunol. 2023;66:101730.
182. Shapiro IE, Bassani-Sternberg M. The impact of immunopeptidomics: from basic research to clinical implementation. Semin Immunol. 2023;66:101727.
183. Schroeder SM, Nelde A, Walz JS. Viral T-cell epitopes - identification, characterization and clinical application. Semin Immunol. 2023;66:101725.
184. Meng X, Lawrenson AS, Berry NG, Maggs JL, French NS, Back DJ, et al. Abacavir forms novel cross-linking abacavir protein adducts in patients. Chem Res Toxicol. 2014;27:524–35.
185. Walsh JS, Reese MJ, Thurmond LM. The metabolic activation of abacavir by human liver cytosol and expressed human alcohol dehydrogenase isozymes. Chem Biol Interact. 2002;142:135–54.
186. Thomson PJ, Illing PT, Farrell J, Alhaidari M, Bell CC, Berry N, et al. Modification of the cyclopropyl moiety of abacavir provides insight into the structure activity relationship between HLA-B*57:01 binding and T-cell activation. Allergy. 2020;75:636–47.
187. Lucas A, Lucas M, Strhyn A, Keane NM, McKinnon E, Pavlos R, et al. Abacavir-reactive memory T cells are present in drug naive individuals. PLoS One. 2015;10:e0117160.
188. Adam J, Wuillemin N, Watkins S, Jamin H, Eriksson KK, Villiger P, et al. Abacavir induced T cell reactivity from drug naive individuals shares features of allo-immune responses. PLoS One. 2014;9:e95339.
189. Bell CC, Faulkner L, Martinsson K, Farrell J, Alfirevic A, Tugwood J, et al. T-cells from HLA-B*57:01+ human subjects are activated with abacavir through two independent pathways and induce cell death by multiple mechanisms. Chem Res Toxicol. 2013;26:759–66.
190. Adam J, Eriksson KK, Schnyder B, Fontana S, Pichler WJ, Yerly D. Avidity determines T-cell reactivity in abacavir hypersensitivity. Eur J Immunol. 2012;42:1706–16.
191. Chessman D, Kostenko L, Lethborg T, Purcell AW, Williamson NA, Chen Z, et al. Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity. Immunity. 2008;28:822–32.
192. Bell CC, Faulkner L, Martinsson K, Farrell J, Alfirevic A, Tugwood J, et al. T-Cells from HLA-B*57:01+ human subjects are activated with abacavir through two independent pathways and induce cell death by multiple mechanisms. Chem Res Toxicol. 2013;26:759–66.
193. Mallal S, Phillips E, Carosi G, Molina JM, Workman C, Tomazic J, et al. HLA-B*5701 screening for hypersensitivity to abacavir. N Engl J Med. 2008;358:568–79.
194. Norcross MA, Luo S, Lu L, Boyne MT, Gomarteli M, Rennels AD, et al. Abacavir induces loading of novel self-peptides into HLA-B*57: 01: an autoimmune model for HLA-associated drug hypersensitivity. AIDS. 2012;26:F21–9.
195. Thomson PJ, Illing PT, Farrell J, Alhaidari M, Bell CC, Berry N. et al. Modification of the cyclopropyl moiety of abacavir provides insight into the structure activity relationship between HLA-B*57:01 binding and T-cell activation. Allergy. 2019;75:636–47.
196. Illing PT, van Hateren A, Darley R, Croft NP, Mifsud NA, King S. et al. Kinetics of abacavir-induced remodelling of the major histocompatibility complex class I peptide repertoire. Front Immunol. 2021;12:1–19.
197. Thomson PJ, Kafu L, Meng X, Snoeys J, De Bondt A, De Maeyer D. et al. Drug-specific T-cell responses in patients with liver injury following treatment with the BACE inhibitor atabecestat. Allergy. 2021;76:1825–1835.
198. Sanchez-Gonzalez MJ, Barbarroja-Escudero J, Antolin-Amerigo D, Bellon-Heredia T, Lerma-Hambleton V, Rodriguez-Rodriguez M. DRESS syndrome induced by piperacillin-tazobactam. Clin Transl Allergy. 2014;4:P96.
199. Pichler WJ, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. Allergy. 2004;59:809–20.
200. Faulkner L, Martinsson K, Santoyo-Castelazo A, Cederbrant K, Schuppe-Koistinen I, Powell H, et al. The development of in vitro culture methods to characterize primary T-cell responses to drugs. Toxicol Sci. 2012;127:150–8.
201. Ogese MO, Watkinson J, Lister A, Faulkner L, Gibson A, Hillegas A, et al. Development of an Improved T-cell assay to assess the intrinsic immunogenicity of haptenic compounds. Toxicol Sci. 2020;175:266–78.
202. Ogese MO, Lister A, Gardner J, Meng X, Alfirevic A, Pirmohamed M, et al. Deciphering adverse drug reactions: in vitro priming and characterization of vancomycin-specific T cells from healthy donors expressing HLA-A*32:01. Toxicol Sci. 2021;183:139–53.
203. Lichtenfels M, Farrell J, Ogese MO, Bell CC, Eckle S, McCluskey J, et al. HLA restriction of carbamazepine-specific T-Cell clones from an HLA-A*31:01-positive hypersensitive patient. Chem Res Toxicol. 2014;27:175–7.
204. Yaseen FS, Saide K, Kim SH, Monshi M, Tailor A, Wood S, et al. Promiscuous T-cell responses to drugs and drug-haptens. J Allergy Clin Immunol. 2015;136:474–6.e8.
205. Ye YM, Hur GY, Kim SH, Ban GY, Jee YK, Naisbitt DJ, et al. Drug-specific CD4(+) T-cell immune responses are responsible for antituberculosis drug-induced maculopapular exanthema and drug reaction with eosinophilia and systemic symptoms syndrome. Br J Dermatol. 2017;176:378–86.
206. Bechara R, Maillere B, Joseph D, Weaver RJ, Pallardy M. Identification and characterization of a naïve CD8+ T cell repertoire for benzylpenicillin. Clin Exp Allergy. 2019;49:636–43.
207. Azoury ME, Filì L, Bechara R, Scornet N, de Chaisemartin L, Weaver RJ, et al. Identification of T-cell epitopes from benzylpenicillin conjugated to human serum albumin and implication in penicillin allergy. Allergy. 2018;73:1662–72.
208. Tailor A, Meng X, Adair K, Farrell J, Waddington JC, Daly A, et al. HLA DRB1*15:01-DQB1*06:02-restricted human CD4+ T cells are selectively activated with amoxicillin-peptide adducts. Toxicol Sci. 2020;178:115–26.
209. Tangamornsuksan W, Lohitnavy M. *Association between HLA-B*1301 and dapsone-induced cutaneous adverse drug reactions: a systematic review and meta-analysis*. JAMA Dermatol. 2018;154:441–6.
210. Zhang T, Hatcher JM, Teng M, Gray NS, Kostic M. Recent advances in selective and irreversible covalent ligand development and validation. Cell Chem Biol. 2019;26:1486–1500.
211. Davids MS, Brown JR. Ibrutinib: a first in class covalent inhibitor of Bruton's tyrosine kinase. Future Oncol. 2014;10:957–67.
212. Yu HA, Pao W. Targeted therapies: Afatinib–new therapy option for EGFR-mutant lung cancer. Nat Rev Clin Oncol. 2013;10:551–2.
213. Finlay MR, Anderton M, Ashton S, Ballard P, Bethel PA, Box MR, et al. Discovery of a potent and selective EGFR inhibitor (AZD9291) of both sensitizing and T790M resistance mutations that spares the wild type form of the receptor. J Med Chem. 2014;57:8249–67.

214. Kerr KM, Dafni U, Schulze K, Thunnissen E, Bubendorf L, Hager H, et al. Prevalence and clinical association of gene mutations through multiplex mutation testing in patients with NSCLC: results from the ETOP Lungscape Project. Ann Oncol. 2018;29:200–8.
215. Luo J. KRAS mutation in pancreatic cancer. Semin Oncol. 2021;48:10–18.
216. Neumann J, Zeindl-Eberhart E, Kirchner T, Jung A. Frequency and type of KRAS mutations in routine diagnostic analysis of metastatic colorectal cancer. Pathol Res Pr. 2009;205:858–62.
217. Rathod LS, Dabhade PS, Mokale SN. Recent progress in targeting KRAS mutant cancers with covalent G12C-specific inhibitors. Drug Discov Today. 2023;28:103557.
218. Lanman BA, Allen JR, Allen JG, Amegadzie AK, Ashton KS, Booker SK, et al. Discovery of a covalent inhibitor of KRAS(G12C) (AMG 510) for the treatment of solid tumors. J Med Chem. 2020;63:52–65.
219. Strickler JH, Satake H, George TJ, Yaeger R, Hollebecque A, Garrido-Laguna I, et al. Sotorasib in KRAS p.G12C-mutated advanced pancreatic cancer. N Engl J Med. 2023;388:33–43.
220. Skoulidis F, Li BT, Dy GK, Price TJ, Falchook GS, Wolf J, et al. Sotorasib for lung cancers with KRAS p.G12C mutation. N Engl J Med. 2021;384:2371–81.
221. Hong DS, Fakih MG, Strickler JH, Desai J, Durm GA, Shapiro GI, et al. KRAS(G12C) inhibition with sotorasib in advanced solid tumors. N Engl J Med. 2020;383:1207–17.
222. Bekes M, Langley DR, Crews CM. PROTAC targeted protein degraders: the past is prologue. Nat Rev Drug Discov. 2022;21:181–200.
223. Kiely-Collins H, Winter GE, Bernardes GJL. The role of reversible and irreversible covalent chemistry in targeted protein degradation. Cell Chem Biol. 2021;28:952–68.
224. Gabizon R, London N. The rise of covalent proteolysis targeting chimeras. Curr Opin Chem Biol. 2021;62:24–33.
225. Bond MJ, Chu L, Nalawansha DA, Li K, Crews CM. Targeted degradation of oncogenic KRAS(G12C) by VHL-recruiting PROTACs. ACS Cent Sci. 2020;6:1367–75.
226. Tong B, Luo M, Xie Y, Spradlin JN, Tallarico JA, McKenna JM, et al. Bardoxolone conjugation enables targeted protein degradation of BRD4. Sci Rep. 2020;10:15543.
227. Zhang X, Crowley VM, Wucherpfennig TG, Dix MM, Cravatt BF. Electrophilic PROTACs that degrade nuclear proteins by engaging DCAF16. Nat Chem Biol. 2019;15:737–46.
228. Mato AR, Nabhan C, Thompson MC, Lamanna N, Brander DM, Hill B, et al. Toxicities and outcomes of 616 ibrutinib-treated patients in the United States: a real-world analysis. Haematologica. 2018;103:874–9.
229. Barf T, Covey T, Izumi R, van de Kar B, Gulrajani M, van Lith B, et al. Acalabrutinib (ACP-196): a covalent bruton tyrosine kinase inhibitor with a differentiated selectivity and in vivo potency profile. J Pharm Exp Ther. 2017;363:240–52.
230. Stephens DM, Byrd JC. How I manage ibrutinib intolerance and complications in patients with chronic lymphocytic leukemia. Blood. 2019;133:1298–307.
231. Sibaud V, Beylot-Barry M, Protin C, Vigarios E, Recher C, Ysebaert L. Dermatological toxicities of Bruton's tyrosine kinase inhibitors. Am J Clin Dermatol. 2020;21:799–812.
232. Holcmann M, Sibilia M. Mechanisms underlying skin disorders induced by EGFR inhibitors. Mol Cell Oncol. 2015;2:e1004969.
233. Jensen SM, Potts GK, Ready DB, Patterson MJ. Specific MHC-I peptides are induced using PROTACs. Front Immunol. 2018;9:2697.
234. Massafra V, Tundo S, Dietzig A, Ducret A, Jost C, Klein C, et al. Proteolysis-targeting chimeras enhance T cell bispecific antibody-driven T cell activation and effector function through increased MHC class I antigen presentation in cancer cells. J Immunol. 2021;207:493–504.
235. Schoenfeld AJ, Arbour KC, Rizvi H, Iqbal AN, Gadgeel SM, Girshman J, et al. Severe immune-related adverse events are common with sequential PD-(L)1 blockade and osimertinib. Ann Oncol. 2019;30:839–44.
236. Levine BB. Studies on the immunological mechanisms of penicillin allergy. I. Antigenic specificities of guinea-pig skin sensitizing rabbit anti-benzylpenicillin antibodies. Immunology. 1964;7:527–41.
237. El-Ghaiesh S, Monshi MM, Whitaker P, Jenkins R, Meng X, Farrell J, et al. Characterization of the antigen specificity of T-cell clones from piperacillin-hypersensitive patients with cystic fibrosis. J Pharmacol Exp Ther. 2012;341:597–610.
238. Casimir-Brown RS, Kennard L, Kayode OS, Siew L, Makris M, Tsilochristou O, et al. Piperacillin-Tazobactam hypersensitivity: a large, multicenter analysis. J Allergy Clin Immunol Pract. 2021;9:2001–9.
239. Kim S-H, Saide K, Farrell J, Faulkner L, Tailor A, Ogese M, et al. Characterization of amoxicillin- and clavulanic acid-specific T cells in patients with amoxicillin-clavulanate-induced liver injury. Hepatology. 2015;62:887–99.
240. Kosseifi SG, Guha B, Nassour DN, Chi DS, Krishnaswamy G. The Dapsone Hypersensitivity Syndrome revisited: a potentially fatal multisystem disorder with prominent hepatopulmonary manifestations. J Occup Med Toxicol. 2006;1:9.
241. Zhao Q, Almutairi M, Tailor A, Lister A, Harper N, Line J, et al. HLA Class-II–Restricted CD8+ T cells contribute to the promiscuous immune response in dapsone-hypersensitive patients. J Investig Dermatol. 2021;141:2412–25.e2.
242. Jiang H, Wang CW, Wang Z, Dai Y, Zhu Y, Lee YS, et al. Functional and structural characteristics of HLA-B*13:01-mediated specific T cells reaction in dapsone-induced drug hypersensitivity. J Biomed Sci. 2022;29:58.
243. Grujic S, Vasiljevic T, Lausevic M, Ast T. Study on the formation of an amoxicillin adduct with methanol using electrospray ion trap tandem mass spectrometry. Rapid Commun Mass Spectrom: RCM. 2008;22:67–74.
244. Pajares MA, Zimmerman T, Sánchez-Gómez FJ, Ariza A, Torres MJ, Blanca M, et al. Amoxicillin inactivation by Thiol-Catalyzed cyclization reduces protein haptenation and antibacterial potency. Front Pharmacol. 2020;11:189.
245. Ali S-E, Meng X, Kafu L, Hammond S, Zhao Q, Ogese M, et al. Detection of hepatic drug metabolite-specific T-cell responses using a human hepatocyte, immune cell coculture system. Chem Res Toxicol. 2023;36:390–401.
246. Yvon M, Anglade P, Wal JM. Binding of benzyl penicilloyl to human serum albumin. Evidence for a highly reactive region at the junction of domains 1 and 2 of the albumin molecule. FEBS Lett. 1989;247:273–8.
247. Amali MO, Jenkins RE, Meng X, Faulkner L, Whitaker P, Peckham D, et al. Assessment of antipiperacillin IgG binding to structurally related drug protein adducts. Chem Res Toxicol. 2017;30:2097–9.
248. Ariza A, Garzon D, Abánades DR, de los Ríos V, Vistoli G, Torres MJ, et al. Protein haptenation by amoxicillin: high resolution mass spectrometry analysis and identification of target proteins in serum. J Proteom. 2012;77:504–20. p
249. Ariza A, Collado D, Vida Y, Montañez MI, Pérez-Inestrosa E, Blanca M, et al. Study of protein haptenation by amoxicillin through the use of a biotinylated antibiotic. PloS One. 2014;9:e90891.
250. Garzon D, Ariza A, Regazzoni L, Clerici R, Altomare A, Sirtori FR, et al. Mass spectrometric strategies for the identification and characterization of human serum albumin covalently adducted by

amoxicillin: ex vivo studies. Chem Res Toxicol. 2014;27:1566–74.
251. Tailor A, Waddington JC, Hamlett J, Maggs J, Kafu L, Farrell J, et al. Definition of haptens derived from sulfamethoxazole: in vitro and in vivo. Chem Res Toxicol. 2019;32:2095–106.
252. Meekins CV, Sullivan TJ, Gruchalla RS. Immunochemical analysis of sulfonamide drug allergy: identification of sulfamethoxazole-substituted human serum proteins. J Allergy Clin Immunol. 1994;94:1017–24.
253. Bechara R, Maillere B, Joseph D, Weaver RJ, Pallardy M. Identification and characterization of a naïve CD8+ T cell repertoire for benzylpenicillin. Clin Exp Allergy. 2019;49:636–43.
254. Sánchez-Gómez FJ, González-Morena JM, Vida Y, Pérez-Inestrosa E, Blanca M, Torres MJ, et al. Amoxicillin haptenates intracellular proteins that can be transported in exosomes to target cells. Allergy. 2017;72:385–96.
255. Waddington JC, Ali SE, Penman SL, Whitaker P, Hamlett J, Chadwick A, et al. Cell membrane transporters facilitate the accumulation of hepatocellular flucloxacillin protein adducts: implication in flucloxacillin-induced liver injury. Chem Res Toxicol. 2020;33:2939–43.
256. Ogese MO, Jenkins RE, Adair K, Tailor A, Meng X, Faulkner L, et al. Exosomal transport of hepatocyte-derived drug-modified proteins to the immune system. Hepatology. 2019;70:1732–49. p
257. Ali SE, Waddington JC, Lister A, Sison-Young R, Jones RP, Rehman AH. et al. Identification of flucloxacillin-modified hepatocellular proteins: implications in flucloxacillin-induced liver injury. Toxicol Sci. 2023;192:106–116.
258. Warbrick EV, Thomas AL, Stejskal V, Coleman JW. An analysis of beta-lactam-derived antigens on spleen cell and serum proteins by ELISA and Western blotting. Allergy. 1995;50:910–7.
259. Reilly TP, Lash LH, Doll MA, Hein DW, Woster PM, Svensson CK. A role for bioactivation and covalent binding within epidermal keratinocytes in sulfonamide-induced cutaneous drug reactions. J Investig Dermatol. 2000;114:1164–73.
260. Manchanda T, Hess D, Dale L, Ferguson SG, Rieder MJ. Haptenation of sulfonamide reactive metabolites to cellular proteins. Mol Pharmacol. 2002;62:1011–26.
261. Cribb AE, Nuss CE, Alberts DW, Lamphere DB, Grant DM, Grossman SJ, et al. Covalent binding of sulfamethoxazole reactive metabolites to human and rat liver subcellular fractions assessed by immunochemical detection. Chem Res Toxicol. 1996;9:500–7.
262. Zdziarski P. Difficulties with using T lymphocyte culture as a method for diagnosing allergies to benzylpenicillin. Wiad Lek. 2002;55:81–7.
263. Brander C, Mauri-Hellweg D, Bettens F, Rolli H, Goldman M, Pichler WJ. Heterogeneous T cell responses to beta-lactam-modified self-structures are observed in penicillin-allergic individuals. J Immunol. 1995;155:2670–8.
264. Sullivan A, Wang E, Farrell J, Whitaker P, Faulkner L, Peckham D, et al. β-Lactam hypersensitivity involves expansion of circulating and skin-resident TH22 cells. J Allergy Clin Immunol. 2018;141:235–49.e8.
265. Horton H, Weston SD, Hewitt CR. Allergy to antibiotics: T-cell recognition of amoxicillin is HLA-DR restricted and does not require antigen processing. Allergy. 1998;53:83–88.
266. Ariza A, Fernández-Santamaría R, Meng X, Salas M, Ogese MO, Tailor A, et al. Characterization of amoxicillin and clavulanic acid specific T-cell clones from patients with immediate drug hypersensitivity. Allergy. 2020;75:2562–73.
267. Zhao Q, Alhilali K, Alzahrani A, Almutairi M, Amjad J, Liu H, et al. Dapsone- and nitroso dapsone-specific activation of T cells from hypersensitive patients expressing the risk allele HLA-B*13:01. Allergy. 2019;74:1533–48.
268. Faulkner L, Gibson A, Sullivan A, Tailor A, Usui T, Alfirevic A, et al. Detection of primary T cell responses to drugs and chemicals in HLA-typed volunteers: implications for the prediction of drug immunogenicity. Toxicol Sci. 2016;154:416–29.
269. Wuillemin N, Terracciano L, Beltraminelli H, Schlapbach C, Fontana S, Krähenbühl S, et al. T cells infiltrate the liver and kill hepatocytes in HLA-B∗57:01-associated floxacillin-induced liver injury. Am J Pathol. 2014;184:1677–182.
270. Nhim C, Delluc S, Halgand F, de Chaisemartin L, Weaver RJ, Claude N, et al. Identification and frequency of circulating CD4(+) T lymphocytes specific to Benzylpenicillin in healthy donors. Allergy. 2013;68:899–905.
271. In-Vitro Approaches to Predict and Study T-Cell Mediated Hypersensitivity to Drugs. Frontiers in Immunology. 2021;12. https://doi.org/10.3389/fimmu.2021.630530.