**Drug hapten-specific T-cell activation: Current status and unanswered questions**

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**Abstract**

Drug haptens are formed from the irreversible, covalent binding of drugs to nucleophilic moieties on proteins, which can warrant adverse reactions in the body including severe delayed-type, T-cell mediated, drug hypersensitivity reactions (DHRs). While three main pathways exist for the activation of T-cells in DHRs, namely the hapten model, the pharmacological interaction model and the altered peptide repertoire model, the exact antigenic determinants responsible have not yet been defined. In recent years, progress has been made using advanced mass spectrometry-based proteomic methods to identify protein carriers and characterise the structure of drug-haptenated proteins. Since genome-wide association studies discovered a link between human leukocyte antigens (HLA) and an individual’s susceptibility to DHRs, much effort has been made to define the drug-associated HLA ligands driving T-cell activation, including the elution of natural HLA peptides from HLA molecules and the generation of HLA-binding peptides. In this review, we discuss our current methodology used to design and synthesise drug-modified HLA ligands to investigate their immunogenicity using T-cell models, and thus their implication in drug hypersensitivity.

**KEYWORDS**

drug hypersensitivity, haptenated HLA ligands, LC-MS/MS, peptide synthesis, T-cell activation

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**1 | DRUG HYPERSENSITIVITY**

Drug hypersensitivity reactions (DHRs) account for approximately 10–15% of adverse drug reactions (ADRs) and are a major healthcare concern as they are unpredictable and life-threatening [1]. The classification of hypersensitivity reactions is divided into two categories: immune mediated (allergy) and non-immune mediated (pseudoallergy). Allergy is defined as an ‘inappropriate’ or exaggerated immune response to the exposure of low-weight chemicals resulting in diverse clinical manifestations, such as anaphylactic shock or injury to targeted organs [2]. The original Gell and Coombs classification subdivides allergic hypersensitivity reactions into four categories Type I–IV, according to their immune pathophysiology (Figure 1). Type I–III are immediate or rapid reactions occurring within 24 h, which are mediated by antibodies and can manifest clinically as urticaria or anaphylaxis. Type IV is a non-immmediate reaction; however, antibodies are not the main effectors in the disease pathology, rather this reaction is a T-cell mediated response.
1.1 Delayed-type hypersensitivity reactions

This review focuses on the mechanisms underlying delayed-type, T-cell mediated DHRs. The T-cell subset and effector mechanism involved can differ in these reactions, allowing further subdivision into four categories as shown in Table 1. Type IVa is a Th1 response with monocytic inflammation whereas Type IVb is a Th2 response with eosinophilic inflammation. Cytotoxic CD8+ T-cells and helper CD4+ T-cells mediate the cell death in Type IVc and Lastly, a T-cell response causing neutrophilic inflammation is evident in Type IVd [3]. Although different types of DHRs have been classified, it is important to note that there is likely an overlap between each type meaning many patients will show symptoms from each. Moreover, drug responses will exhibit interindividual variability [4].

DHRs have multiple clinical manifestations and can involve different organ systems such as the skin, liver and kidneys. With that said, the skin is the organ that is mostly commonly affected in DHRs [5]. It remains unknown why certain organs are targeted over others but it is possible the skin is more susceptible to drug reactions due to the large vascular network and abundance of dendritic cells and macrophages that survey the local environment for signs of stress and antigenic material [6].

1.1.1 Cutaneous reactions

Cutaneous reactions vary in appearance and severity. While more than half of cutaneous ADRs may present as a mild cutaneous reaction known as maculopapular rash [7], a proportion of cutaneous reactions are deemed more serious and are potentially fatal. Severe cutaneous adverse reactions include Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and drug reaction with eosinophilia and systemic symptoms (DRESS) [8].

Maculopapular exanthema

Maculopapular exanthema (MPE) is typically skin eruptions of both flat and raised lesions with a diameter of approximately 1–5 mm known as macules and papules, respectively, which often merge forming a patch or plaque. This condition distributes symmetrically and bilaterally affecting the face, neck, upper trunk and limbs, and may occur alongside a fever [9]. MPE studies have demonstrated the presence of CD4+ and CD8+ T-cells in drug-induced cutaneous eruptions, particularly in the dermo-epidermal junction and epidermis of the skin [10, 11]. Several drugs were involved in the development of these eruptions including amoxicillin, carbamazepine (CBZ) and cefazoline. Moreover, sulphanmethoxazole (SMX)-specific T-cells generated from a patient who developed a skin eruption following SMX therapy were capable of killing keratinocytes [12]. This combined evidence suggests immune involvement for the basal cell vacuolisation and keratinocyte death seen in drug-induced MPE [13]. Consistent throughout literature, CD4+ T-cells are the dominant effector cells in MPE [14–16], as they are present in higher numbers compared to CD8+ T-cells and clinical MPE symptoms can occur in the almost absence of CD8+ T-cells showing that CD4+ cells are adequate to trigger this reaction. Activation of CD8+ T-cells predominantly appears to be typical with more severe reactions such as bullous drug exanthems or those involving the liver [15].

DRESS

DRESS is a serious, multi-organ condition characterised by a high fever, skin eruption, haematological abnormalities and mucosal/organ involvement. The majority of patients will experience a skin eruption covering over 50% of the body surface area. Eosinophilia is the most common haematological abnormality in patients as indicated by the name, followed by atypical lymphocytes, which confirms immune involvement. Two or more internal organs are affected in over half of cases. Overall, the liver appears to be the main internal target in DRESS followed by the kidneys and lungs [17]. Approximately 50% of patients have been shown to cause DRESS. CBZ appears to be the most frequently involved followed by allopurinol (ALP), an anti-gout drug [18]. ALP is known to cause DRESS notably with greater kidney involvement in comparison to CBZ [17], therefore those with underlying kidney problems may be at greater risk. Other risk drugs include dapsone (DDS) and sulphonamides [19]. Distinctly, DRESS has an extreme delay in onset often 2–8 weeks after the initial exposure to the causative drug. Although the immunopathogenesis is not entirely clear, there is an association between DRESS and viral reactivation. Enhanced replication of herpes viruses including human herpesvirus (HHV)-6, HHV-7 and Epstein–Barr virus by culprit drugs have been reported, as well as the expansion of activated CD8+ T-cells directed against the virus [20, 21].

SJS/TEN

SJS/TEN are life-threatening skin conditions characterised by epidermal necrosis resulting in the epidermis-dermis separation, mucosal erosions and lesions, and are often accompanied with a fever. SJS and TEN are classed according to a single disease severity spectrum. SJS patients have 10% or less total skin detachment, whereas more severe detachment of 30% or more is considered as TEN [22]. Similar to DRESS, internal organs may be involved including the liver or, more commonly in SJS, oral cavity and eye involvement [21]. SJS/TEN is predominantly drug induced; common culprits include antibiotics, non-steroidal anti-inflammatory drugs, ALP and antiepileptic drugs [23].

Statement of significance

Understanding of the mechanisms underlying hapten-specific T-cell activation remains elusive, especially with respect to the antigenic determinant responsible for the reaction. Our article reviews the current status of drug-haptenated protein characterisation and their implication in drug hypersensitivity. Current methodology and challenges in depicting the antigenic epitope have been addressed.
Hypersensitivity reactions (Type B) are further classified into four categories (I–IV). Type I reactions involve IgE activation of mast cells and histamine release causing inflammation of tissues. Type II is a cytotoxic response initiated by IgG/IgM complement activation. Type III is mediated by immune complex formation between antibodies and soluble antigens that activate neutrophils and complement system. Type IV, delayed, hypersensitivity is mediated by T-lymphocytes. Sensitised T-cells are re-stimulated by an antigen initiating the activation of effector T-cells and macrophages.
TABLE 1  Classification of Type IV hypersensitivity reactions—further categorisation of Types IVa–d based on T-cell subset and effector molecules involved and their clinical manifestations

| Classification | T-cell type | T-cell effector molecule | Pathomechanism | Clinical manifestation |
|----------------|-------------|--------------------------|----------------|------------------------|
| IVa            | Th1         | Interferon gamma (IFN-γ) | Inflammation of monocytes | Eczema               |
| IVb            | Th2         | Interleukin-4 (IL-4) and interleukin-5 (IL-5) | Inflammation of eosinophils | Maculopapular exanthema, DRESS |
| IVc            | Cytotoxic T-cells | Granzyme B, perforin and FasL | Cell death mediated by CD8+/CD4+ T-cells | Maculopapular exanthema, SJS/TEN |
| IVd            | T-cells     | Interleukin-8 (IL-8) and CXCL8 | Inflammation of neutrophils | Acute generalised exanthematous pustulosis |

Literature suggests cytotoxic immune involvement, whereby keratinocyte death is mediated by the secretion of granulysin and Fas-ligand from cytotoxic T lymphocytes and natural killer cells [24].

1.1.2  | Idiosyncratic drug-induced liver injury

Drug-induced liver injury (DILI), also referred to as hepatotoxicity, is an ADR causing liver dysfunction or abnormal liver serology [25]. The major metabolic function of the liver makes it highly susceptible to direct drug-induced damage. In fact, adverse hepatic events are more likely to occur with drugs that are majorly metabolised in the liver [26]. DILI is generally classified as either dose dependent and predictable or idiosyncratic (IDILI) with a complex dose relationship [27]. The former refers to reactions such as acetaminophen (APAP) toxicity that has been studied extensively [28–30]. It is widely understood that APAP is oxidised to a reactive intermediate N-acetyl-p-benzoquinoneimine (NAPQI) by the microsomal P-450 system, which under normal doses is detoxified and eliminated by glutathione (GSH) conjugation. In APAP overdose cases, GSH is depleted, therefore the excess NAPQI reacts with cysteine residues in cellular proteins, leading to APAP-protein adducts. The binding of APAP to proteins in the mitochondria induces oxidative stress and subsequent cell necrosis [31]. On the other hand, IDILI accounts for approximately 10–15% cases of acute liver injury [32]. The pathogenesis of IDILI is a lot more obscure and due to the lack of preclinical in vivo evidence of hepatotoxicity in IDILI drugs, animal models have not been readily obtainable. It is possible that co-stimulatory innate signalling is augmented by mitochondrial injury causing oxidative stress and/or drug-induced inhibition of pumps that are responsible of exporting bile, resulting in the build-up of antigenic determinants at the site of tissue injury [33]. Moreover, drug-specific T-cells are observed in patients with IDILI caused by flucloxacinilin and co-amoxiclav [34, 35], providing evidence of immune involvement.

2  | HOW DRUGS ACTIVATE T-CELLS

The activation of T-cells is predominately determined by two signals. Firstly, the interaction between the T-cell receptor (TCR), major histocompatibility complex (MHC) and an antigen, which triggers the activation of antigen-specific CD4+ helper T-cells and/or CD8+ cytotoxic T-cells. Of particular interest to our discussion is the role of the MHC-associated peptide in T-cell activation and indeed the nature of the interaction between the peptide and the drug or metabolite [36]. MHC molecules exist on the surface of antigen presenting cells (APCs) and are responsible for the processing and presentation of antigens to the appropriate T-cells. While natural peptides are presented on the MHC, these are recognised as self and therefore do not provoke an immune response in usual circumstances. However, the invasion of a pathogen will result in the presentation of non-self-peptides and thus stimulate T-cell clonal expansion [37]. The same concept applies to T-cell activation by drugs in delayed-type DHRs. The second signals are co-stimulatory, which contribute to the activation of T-cells, for example the CD28/B7 pathway involving T-cells and APCs [38]. Although these secondary signals are not antigen specific, they augment the proliferation of T-cells that detect the antigen and contribute to the overall immune response. With that being said, secondary signals can also negatively control T-cell responses through inhibitory pathways. This is evident by the upregulation of CTLA4 following the initial T-cell activation that downregulates the proliferation of T-cells, suppressing the immune response [39].

2.1  | Pathways of T-cell activation

The pathway by which T-cells are activated by drugs is not fully defined and complicated by the different binding interactions of drugs. In addition to the presence of T-cells specific to drugs, models have been developed to define the pathway of T-cell activation by compounds involved in drug hypersensitivity, which revolve around 1) antigen processing and 2) antigen presentation. The kinetics of TCR engagement can be measured by TCR internalisation and Ca2+ mobilisation, while the requirement of antigen processing and presentation can be explored using glutaraldehyde fixation of APCs to block intracellular antigen processing mechanisms, and APC drug-pulsing to investigate whether a compound is covalently (or tightly) bound to the MHC for antigen presentation. T-cell responses in the presence of a compound are measured by proliferation or cytokine secretion to postulate which pathway is involved.

Currently, three main pathways have been proposed for the activation of T-cells: the hapten model, the pharmacological interaction (p-i) model and the altered peptide model (Figure 2). However, which takes
FIGURE 2  Models of drug hypersensitivity. Three pathways of T-cell activation in drug hypersensitivity exist. The hapten model explains the covalent binding of drugs like the penicillins to proteins that are intracellularly processed to drug-haptenated peptides and subsequently presented on the MHC as antigens for TCR interaction. In the pharmacological interaction (p-i) model, drugs including sulphamethoxazole (SMX) can interact directly with TCR via non-covalent binding and without the requirement of antigen processing. In the altered peptide repertoire model, abacavir sits in the F pocket of the MHC binding groove resulting in the binding of unconventional peptides. Abacavir can bind HLA ligands 1) intracellularly or 2) on the cell surface, which are presented to the TCR as neoantigens.

precedence is still viewed differently among drug hypersensitivity experts. In fact, it is possible for a drug to activate T-cells via the three different pathways in the same individual.

2.1.1  Hapten

The earliest proposed pathway is the hapten hypothesis discovered in 1930s [40], using chemical sensitisers and since described as the classical explanation for DHRs. The term hapten describes low molecular weight compounds that are incapable of initiating an immune response alone. However, when covalently bound to circulating proteins, these low molecular weight compounds are able to form an antigenic determinant that can activate T-cells. A drug is considered a prohapten if its derived metabolite covalently binds to proteins and becomes antigenic, as seen with SMX and its nitroso metabolite (SMX-NO). It is assumed that these protein adducts are endogenously processed into drug-modified peptides and presented by MHC molecules on the
The p-i concept

Discovery of drug-associated antigens that altered peptide the previously obscure mechanisms of T-cell activation by parent drugs usually take place over several days. Moreover, this concept explains bypassing the T-cell kinetics of a classic primary response that would upon the first exposure and within a few hours of administration [51], such as iodinated contrast media that can cause positive skin reactions [52]. The concept justifies hypersensitivity reactions to chemically inert drugs directly through the detection of patient T-cells that are responsive drug hapten protein binding in T-cell activation has been demonstrated directly through the detection of patient T-cells that are responsive towards drug-HSA adducts [42, 43] and synthetic drug-haptenated peptides [44–46]. Haptenic metabolites such as SMX-NO can activate T-cells in a processing-independent manner [47, 48]; however, SMX-NO also forms covalent bonds with nucleophilic amino acid residues in peptides associated with MHC molecules expressed on the surface of APC, which complicates delineation of pathways of drug-specific T-cell activation. This concept also applies to the penicillins since BP-T-cell clones have been shown to be activated in a processing-independent manner [49]. Therefore, there are limitations to using antigen-processing-blocking assays to distinguish between the hapten and p-i pathways.

2.1.2 | The p-i concept

Challenging the requirement for antigen processing and covalent binding of drugs to proteins for the development of T-cell response, Pichler et al. proposed the p-i concept. This novel concept suggests drugs can activate immune cells through a direct and reversible interaction with immune receptors, TCR or MHC, thus mimicking the classical p-i between a drug and a non-immunological receptor [50]. The p-i concept justifies hypersensitivity reactions to chemically inert drugs such as iodinated contrast media that can cause positive skin reactions upon the first exposure and within a few hours of administration [51], bypassing the T-cell kinetics of a classic primary response that would usually take place over several days. Moreover, this concept explains the previously obscure mechanisms of T-cell activation by parent drugs including SMX, CBZ, ALP, lidocaine and lamotrigine, which do not conform to the hapten concept.

Evidence

SMX-specific T-cell responses are observed in the presence of SMX with fixed APCs but not with drug-pulsed APCs, collectively indicating that SMX activates T-cells in a processing-independent manner, without binding covalently to a peptide or an immune receptor [52, 53]. SMX responses are abrogated in the absence of APCs and exhibit MHC restriction, suggesting that SMX interacts with the MHC rather than directly activating the TCR. Similar observations of processing-independent activation of specific T-cells and labile binding have been observed with CBZ, ALP and its metabolite oxypurinol (OXY) [54, 55]. Interestingly, unlike SMX, CBZ can activate T-cells in the absence of APCs suggesting a direct TCR interaction. Furthermore, the down-regulation of the TCR and the influx of Ca^{2+} which is indicative of TCR engagement have been observed rapidly in SMX-, lidocaine- and ALP/OXY-specific T-cells clones following drug exposure. This is inconsistent with the kinetics of antigen processing, which usually requires 4–6 h [55, 56].

It is important to note that T-cell activation by a drug may not be restricted to a sole mechanism. This phenomenon is best described with SMX hypersensitivity since T-cells are activated by both p-i and hapten pathways as mentioned previously [57].

2.1.3 | Altered peptide

The altered peptide hypothesis arose following the report of human leukocyte antigen (HLA)-B*57:01 restricted T-cells in abacavir-induced hypersensitivity [58], and is based on the structural elucidation that a drug can bind directly to the pockets of the MHC binding groove. This changes the conformation and chemistry of the binding groove, and subsequently, the repertoire of peptides that are presented. It is worth emphasising that the peptides presented are not drug-modified peptides as seen in the hapten pathway. Alternatively, they are self-peptides that would not usually be presented by that particular HLA allele, as a shift in amino acid residues occurs to accommodate the bound drug. Drug-HLA binding may occur in the endoplasmic reticulum prior to intracellular loading or on the cell surface after a peptide-HLA complex is formed [59]. The altered peptide repertoire is considered a third independent model of drug hypersensitivity by many [60, 61]; however, this has been challenged in literature and rather viewed as a branch of the p-i model [62].

Evidence

To date, abacavir is the only drug reported to activate T-cells via this pathway. Novel HLA-B*57:01 peptides have been identified in the presence of abacavir, with no change in the nature of peptides presented by closely related haplotypes, HLA-B*57:03 or HLA-B*58:01, which are not associated with abacavir hypersensitivity. The presence of abacavir results in a shift in P2 residues of HLA-B*57:01 presented peptides from the usual tryptophan and phenylalanine to smaller residues including leucine, isoleucine and valine, which accommodate the confined space in the F-pocket caused by the bound abacavir [59, 61].

2.2 | Discovery of drug-associated antigens that activate T-cells

Many classes of drugs have been studied and used to describe the role of protein haptenation in drug hypersensitivity. Haptenation is dependent upon chemical reactivity whereby electrophilic chemicals interact with nucleophilic moieties on proteins. The nature of protein carriers is dependent upon the route of administration and tissue targets therefore varying between circulating, intracellular and
tissue-specific proteins. Advanced mass spectrometry (MS) methods have facilitated the identification of the protein carriers responsible for drug adduct formation and the haptenic structures that attach to the proteins have been characterised. HSA is deemed the preferential carrier protein for many drugs, although due to the high abundance of albumin in patient samples it is possible that other proteins are not detected by MS because of the relatively low signal produced. The identification of protein adducts relies heavily on the sensitivity and reliability of the instrument and detection method. While matrix-assisted laser desorption/ionisation-MS is advantageous for quick data acquisition and easier manual operation, the detection of drug-modification sites may be reduced in comparison to using nano-LC-MS/MS in high collision dissociation mode, as observed with BP-HSA analysis [63].

2.2.1  β-Lactams

β-Lactam antibiotics are considered the most frequent cause of DHRs. This is somewhat debated in literature due to the high prevalence of allergy mislabelling [64]. Nonetheless research to improve the understanding of β-lactam hypersensitivity is imperative due to the detrimental effects for susceptible patients, along with the burden on healthcare and the challenges faced by pharmaceutical industries. β-Lactams have been shown to target the skin, liver and kidney in the event of an adverse reaction [65–67]. Indeed, 30% of patients prescribed multiple courses of piperacillin have been reported to develop reactions including maculopapular rashes [68], while co-amoxiclav and flucloxacillin are deemed two of the most common causes of IDILI [69]. Immediate reactions to β-lactams have been reported, evident by the presence of circulating drug-specific IgE and IgG in patient plasma and peripheral blood mononuclear cell (PBMC) cultures [70–72], which is suggestive of a type I, II or III DHR. Furthermore, circulating and tissue-resident β-lactam-specific T-cells have been discovered in patients and healthy donors [70–72] indicating a delayed T-cell mediated response that has led to the development of T-cell models in drug allergy.

β-Lactams possess a cyclic amide that is susceptible to nucleophilic attack by nucleophiles on proteins, thus are capable of forming drug-protein adducts. It is proposed that β-lactams preferentially bind to extracellular proteins as opposed to cellular proteins, particularly HSA [42]. Indeed, HSA adducts with flucloxacillin, piperacillin and amoxicillin have been discovered in patients’ plasma and synthetically generated for in vitro T-cell assays and MS characterisation [42, 73–75]. It has been reported that penicillin-specific patient T-cells are activated by penicilloylated HSA [45] and more recently piperacillin-HSA adducts have been shown to activate patient T-cells [42]. LC-MS/MS studies have revealed that β-lactams preferentially bind to lysine residues in a concentration and time-dependent manner. Selective modification of lysine residues in vitro is consistent with those detected in patient plasma, including piperacillin modifications on Lys190, Lys195, Lys432 and Lys541 and flucloxacillin modifications on Lys190 and Lys212 [73, 76] which has led to the generation of T-cell stimulatory peptides [44]. The analysis of BP-treated patient plasma detected the binding of BP at fourteen different sites and similar findings are observed with synthetic BP-HSA bioconjugates [63, 77], confirming the reproducibility of drug-protein conjugates in vitro T-cell assays. Although β-lactam-specific T-cells are activated by penicilloylated HSA [45] and more recently β-lactam-specific T-cells as they bind preferentially to different sites on proteins due to the distinct side chains, generating distinct antigenic determinants.

Delving further into this characterisation, drugs can form more than one hapten. In recent years, Meng et al. [42] discovered two distinguishable piperacillin-HSA adducts with the drug moiety present in cyclised and hydrolysed forms. One adduct possessed an intact dioxopiperazine ring of the piperacillin structure (cyclised) whereas on the other this ring had been hydrolysed [42], providing evidence that the environment in which adduction occurs has an effect on the hapten-protein complex. The sole detection of the hydrolysed piperacillin hapten in hypersensitive patient plasma suggests that this hapten may be responsible for interacting with TCRs on drug-specific T-cells. However, this is difficult to confirm since hydrolysis is a reversible reaction therefore the hapten is interchangeable and may take on different forms when samples are processed and prepared. Due to the identification of drug-protein adducts, the synthetic HSA-drug T-cell model is extensively used to delineate the hapten activation of T-cells in DHRs.

2.2.2  SMX-NO

SMX hypersensitivity can affect multiple organs including injury to the skin, liver or kidney. Reactive metabolites of SMX bind to protein thiols [47] therefore they are capable of forming the antigenic determinants that are likely implicated in SMX hypersensitivity. Defining the mechanism is more difficult for a prohapten since the metabolite is not readily available and an in vitro metabolising system does not exist. However, in some cases, the reactive metabolites have been successfully synthesised. SMX oxidation generates two oxidative metabolites, a hydroxylamine intermediate (SMX-NHOH) and the previously mentioned SMX-NO [78]. Early studies confirm SMX-NO protein adducts in human plasma [79] and cellular models [80–82]. While the presence of the drug modification was confirmed, the exact haptenic structure was not characterised. SMX-NO adducts have been detected with Ig and albumin in mouse serum models and cysteine capping revealed that SMX-NO was interacting with cysteine residues in mouse serum albumin (MSA) [83]. Later LC-MS/MS analysis has revealed that SMX-NO forms multiple adducts with proteins. An N-hydroxysulphinamidin-HSA adduct has been detected with the modification on Cys34. SMX-NO also forms N-hydroxysulphinamidin adducts with human GSH S-transferase pi (GSTP), as well as sulphynilamide and N-hydroxysulphinamidin adducts. Interestingly, GSTP was modified on Cys47 residue exclusively. Further protein adducts have been detected with GSH and synthetic peptides containing cysteine residues [84]. Recently, novel HSA adducts were discovered on lysine

and tyrosine residues including a Schiff base adduct and arylazoalkane adduct, and unlike any previous studies, a SMX-lysine adduct was detected in patient sera [85].

2.2.3 DDS-NO

DDS is a sulphone antibiotic used for the treatment of infectious diseases including leprosy. While the majority of patients are tolerant, a small proportion develops severe reactions known as DDS-hypersensitivity syndrome that has similar clinical manifestations to DRESS [86]. Like SMX, DDS undergoes bioactivation to form a DDS-hydroxylamine metabolite that can be further oxidised to a protein-reactive nitroso metabolite (DDS-NO). Skin cells are capable of metabolising DDS and subsequent protein haptenation has been detected in normal human epidermal keratinocytes (NHEKs), normal human dermal fibroblasts and PBMC [80, 87, 88], indicating the role of protein adducts in cutaneous reactions. Immunocytochemical analysis revealed adduct formation is exclusive to intracellular proteins of NHEKs, as no protein adducts are detected on the cell surface [89]. In recent years, DDS-NO protein adducts have been characterised using a synthetic metabolite. DDS-NO reacts with GSH forming two detectable adducts, a sulphonamide and an N-hydroxysulphonamide adduct. Furthermore, DDS-NO reacts with cysteine residues on MSA, particularly on Cys34, forming the sulphonamide adduct [90]. T-cell clones have also been generated to DDS and DDS-NO in mouse models in this study, which has revealed that DDS-NO T-cells are activated via the hapten pathway, whereas DDS-specific T-cell clone activation is dependent upon the p-i mechanism.

While the importance of drug-protein haptenation in DHRs is not to be dismissed considering the detection and generation of hapten-specific T-cell clones in patients, the question remains why not all individuals who are administered the culprit drugs are susceptible to a reaction. The presence of drug-protein adducts is not enough to elicit an immune response in some individuals evident by the detection of HSA adducts in tolerant patients [42, 73]. Other factors are currently being explored including immunoregulatory pathways and HLA associations.

2.3 Drug-HLA associations

Genome-wide association studies have depicted that certain HLA alleles increase an individual’s susceptibility to DHRs. In 2002, the relationship between carrying the HLA-B*57:01 allele and the diagnosis of abacavir hypersensitivity emerged [91, 92], whereby the abacavir-specific CD8+ T-cell activation is restricted to HLA-B*57:01 presentation. Since this report, HLA-B*57:01 screening has been used to identify patients who are susceptible to a reaction prior to abacavir treatment. While screening has a 100% negative predictive value, the positive-predictive value is less (49.7%), meaning not all patients carrying the HLA-B*57:01 allele will have a DHR [93]. In 2004, an odds ratio (OR) of 960 was reported for this association, which increases when both the HLA-B*57:01 and Hsp70-Hom M493T allele are present (OR, 3893) [94], indicating one of the strongest drug-HLA associations. Despite not having a similar structure to abacavir, flucloxacinil-induced liver injury is also associated with HLA-B*57:01 with an OR of 80. Since it is estimated that only one in 500–1000 carriers will develop DILI with flucloxacinil treatment, using genetic screening to predict a DHR in this case is not as effective for patient treatment or costs due to the high false-positive rates [95]. Another significant association is between HLA-B*15:02-CBZ-induced SJS with high prevalence in Asian populations. This association was identified in 2004 with a reported OR of 2504 in the Han Chinese population [96, 97]. Genetic testing for this association holds a 100% negative-predictive value and a positive-predictive value ranging from 0.73% to 100%. Interestingly, a later study concluded that this allele had no relation with other CBZ-induced hypersensitivity reactions including MPE and hypersensitivity syndrome [98]. The discovery of these associations has led to the development of in vitro assays to define the mechanisms underlying drug-HLA associations.

3 IDENTIFICATION OF DRUG-ASSOCIATED HLA LIGANDS

The identification of peptide epitopes is crucial to the understanding of a cellular immune response and is significant in the development of peptide-based vaccines and cancer immunotherapy [99, 100]. The critical epitope in DHRs has not yet been defined since structural studies investigating peptide-MHC and TCR interactions are limited. Challenges are faced due to the highly polymorphic nature of HLA coding genes particularly at TCR contact sites, reducing peptide-binding specificity. This allows for the binding of a multitude of peptides, as well as the diverse TCR repertoire of drug-specific T-cells, facilitating polyclonal T-cell activation in response to the culprit drug. Nevertheless, immunopeptidomics has rapidly integrated into the drug hypersensitivity research field and advanced our understanding of mechanisms underlying HLA-drug associations. To date, no natural drug-modified HLA ligands have been reported to activate drug-specific T-cells in DHR patients, although progress has been made.

Two approaches including naturally presented drug-HLA ligands and the ‘designer’ HLA ligands have been used to define drug-specific T-cell responses.

3.1 Naturally processed HLA ligands

The identification of natural HLA-peptides involves the immunoaffinity capture of naturally processed MHC-peptide complexes (pMHC) from cell lines expressing the HLA allele of interest, and the subsequent dissociation of peptides from the MHC binding cleft. Following high-performance liquid chromatography (HPLC) fractionation, the peptide repertoire is identified using LC-MS/MS. Prior incubation of cell lines with drug will allow for the identification of novel drug-associated HLA ligands.
As mentioned previously HLA-B*57:01 peptides have been identified in the presence of abacavir using this HLA peptide elution method. Similar studies have been attempted for CBZ. Initial studies reported that similar peptides are bound to HLA-B*15:02 in the presence and absence of CBZ and concluded that the p-i concept was most appropriate to explain this DHR [101]. This has now been challenged by the report of a shift in the peptide repertoire due to CBZ, favouring smaller residues at P4 and P6 and increase of hydrophobic residues at several positions [59]. Unlike abacavir, CBZ did not alter the anchor residues and the shifts were more subtle, nevertheless the same pathway of T-cell activation could apply. More recently, it has been discovered that flucloxacinil-modified peptides are naturally processed and presented by HLA-B*57:01 on immune cells. This seminal work introduced multiple pathways by which flucloxacinil is presented including the intracellular processing of haptenated proteins that are subsequently loaded onto the HLA as hapten modified peptides, or the direct binding of flucloxacinil to the presented HLA ligand [102]. Despite the challenges in detecting drug-modified HLA ligands including the detection of low abundance drug modification, peptide identification and complicated pathways involved in presenting drug modified peptide, novel HLA-B*57:01 peptides were successfully identified in the presence of flucloxacinil. This has provided a platform to assess the immunogenicity of flucloxacinil-haptenated peptides and their role in flucloxacinil-induced liver injury.

3.2 Designer HLA ligands

Alternatively, reverse engineering has been used to design and synthesise drug-modified peptides with HLA-binding motifs for use in T-cell models. ‘Designer’ peptide studies have successfully identified immunogenic T-cell epitopes, initially reported by the Weltzien group using synthetic BP-modified HLA-DRB1*04:01 binding peptides. Such T-cell responses revealed that activation was dependent on the site-specific modification of the peptide backbone [103]. Expanding on this, drug-haptenated candidate peptides have been generated from drug-protein conjugates including BP-haptenated peptides synthesised from BP-HSA conjugates [44] and an amoxicillin-haptenated peptide synthesised from an exosomal protein, SRY-box 30, which covalently bound to amoxicillin in hepatocytes [104]. Both peptides were designed around HLA-binding predictions and shown to stimulate naïve T-cells in healthy donors. More recently, amoxicillin-modified HLA-DRB1*15:01 binding peptides were incorporated into a DILI-patient T-cell model, which resulted in successful T-cell activation [46].

4 STRATEGIES FOR THE DESIGNER PEPTIDE MODEL

The prediction of drug hypersensitivity remains the principal challenge in the field. Since the exact antigenic determinant has not been defined as discussed, they cannot be used in T-cell assays as a predictive tool. While T-cell models for parent drugs exist, they do not define drug hypersensitivity at the peptide level thus the functional epitopes remain unspecific. Rather than focusing on the unknowns, our knowledge of HLA associations of DHRs and the pivotal role of peptides in T-cell activation can be used to generate and investigate potential antigens in the designer peptide T-cell model.

4.1 HLA ligand design rationale

Peptides are designed with the aim of binding to HLA risk alleles, reacting with culprit drug and stimulating T-cells. Firstly, computational methods are used to design the chemical and biological characteristics of the peptide. The antigenicity of a peptide is somewhat linked to the stability and affinity of that peptide binding to the MHC [105]. Peptide binding studies have defined the peptide binding motif of many HLA alleles, revealing that certain amino acids are favourable at certain positions of the peptide, which dock into binding groove pockets, especially P2 and P4 for MHC class I ligands and P1, P4, P6 and P9 for MHC class II ligands [106, 107]. Incorporating these anchor residues enhances the binding affinity of a peptide to the HLA, which can be predicted and scored using peptide binding databases such as The Immune Epitope Database (IEDB), www.iedb.org/, or NetMHCpan, www.cbs.dtu.dk/services/NetMHCpan/. It is important to note that differences in peptide binding motifs are not restricted to MHC class I or MHC class II, rather structurally similar MHC molecules of the same class possess distinct structures of individual pockets thus accommodate different amino acids. Considering the length of the peptide, MHC class I molecules generally accommodate 8-12mer peptides with the optimal binding of 9mer peptides whereas MHC class II molecules can accommodate much longer peptides, typically 12–25 amino acids by virtue of an open-ended binding groove. Indeed, it is possible for non-canonical peptides (>11mer) to bind MHC class I [108], where they can acquire a bulging conformation as the peptide backbone bends and/or extrudes from the pockets. For drug modification, a reactive residue is incorporated into the peptide sequence depending on the reactivity of the drug. Lysine residues are used for β-lactam binding due to the known reactivity of lysine with the β-lactam ring, whereas cysteine residues are used for the binding of SMX-NO. As mentioned previously, the position of a drug modification influences a T-cell response therefore its placement must be deliberate and TCR binding preferences conferred [46]. Structural immunogenicity studies have revealed that positions P4–6 of MHC I peptides are most important in the interaction between TCR and pMHC. Moreover, TCR contact sites have a preference for bulky, aromatic residues [109]. Based on this an MHC I peptide would be designed with a central lysine residue for optimal TCR contact. Chemically synthesised peptides have a free N-terminal that is more reactive than the ε-amino groups of lysine residues. Consequently, peptides are designed with an N-terminal protection group such as FMOC to ensure site-specific modification. Lastly, the physical chemistry of a peptide is considered to assess its solubility for cellular assays as hydrophobic peptides are inclined to aggregate in solution, thus hydrophilic residues may be used as a replacement.
4.2 | Synthesis and drug modification

In the last decade, the discovery of protein targets and peptide epitopes in drug development has massively increased due to advances in omics technologies [110], thus there has been immense demand for peptide synthesis in the pharmaceutical industry for the development of therapeutic peptides. The innovative work of Bruce Merrifield introduced solid-phase peptide synthesis (SPPS) and the automation of peptide synthesis [111], and while multiple approaches to chemical peptide synthesis exist such as solution-phase synthesis, this is the most commonly used method for peptide production today [112]. The principle of SPPS is the anchoring of an amino acid at the C-terminus to an insoluble polymer such as resin that acts as a solid ‘platform’ for the addition of the following amino acids. A linker is attached to the resin for the reversible connection between the solid platform and the amino acid, which the crude peptide is then cleaved from when the sequence is complete. Contrary to protein biosynthesis, synthetic peptides are traditionally assembled in the C-N direction whereby the carboxylic acid group of the next residue is coupled with the amino group of the previously attached residue. N-terminal protecting groups are added to incoming amino acids to ensure this C-N synthesis, which can be removed once the peptide reaction is complete. Moreover, protecting groups are added to reactive amino acid side chains to prevent side reactions.

Herein we review the methodology used in our laboratory to generate drug-modified HLA-binding peptides for functional T-cell assays (Figure 3). The incubation of a synthetic drug with lyophilised peptide allows the chemical reaction to occur between the targeted residue and the reactive moiety of the drug from which the products are separated and purified using HPLC. The production of drug-modified peptides is not standardised for all drugs or peptides due to the differences in their chemical properties therefore the purification process must be uniquely optimised. A succession of HPLC experiments are performed using a gradient method under different conditions (e.g. molar ratios and incubation times) to assess how the compound behaves in solution and to maximise the yield of drug-modified peptide. Using an HPLC time-course is an efficient way to assess if a chemical reacts or degrades over time. Distinct profiles are obtained for the drug and peptide separately, as well as the drug-peptide mixture to identify the drug-modified peptide peaks for fractionation.

Following desalting and concentration, fractionated peptide samples are analysed by LC-MS/MS to confirm successful drug modification and the peptide sequence. Drug-modified peptides are identified by de novo sequencing using characteristic drug fragmentation ions with a defined m/z. Peptide fragmentation ions with a mass addition are indicative of a covalently bound drug. During the first purification stage, the N-terminus is protected with FMOC to prevent non-specific binding that is identified by a fragment ion of m/z at 179. Therefore, following drug-modified ‘protected’ peptide confirmation, a further HPLC purification is required to remove the FMOC to yield the final drug-modified ‘deprotected’ peptide, confirmed in repeated LC-MS/MS data acquisition and analysis.

4.3 | Translation to in vitro T-cell assays

Drug-modified peptides are used to explore the immunogenicity of a specific epitope as well as a predictive tool for HLA susceptibility. PBMC are isolated from hypersensitive patients and healthy donors expressing the HLA of interest and used for naïve T-cell priming and/or T-cell cloning whereby a proliferative T-cell response to a drug-modified peptide is indicative of an antigenic epitope. Drug-modified peptide specific T-cell clones can be characterised to define the T-cell response including cytokine and chemokine profiles, HLA restrictions and the TCR repertoires, to name a few.

4.4 | Challenges in drug-modified peptide synthesis

While drug-modified HLA peptides have been successfully generated for penicillin [44] amoxicillin [46], flucloxacillin and DDS-NO (unpublished data), challenges have arisen. Firstly, chemistry is conducted at a microscale level therefore the ideal method of using SPPS to generate drug-modified peptides by incorporating drug-modified-lysine monomers residues into the sequence production, as executed by the Pallardy group [44], is not cost-effective. Consequently, yielding sufficient peptide in batches for T-cell assays is extremely laborious. Difficulties significantly revolve around peptide stability and side reactions. Without solubility, peptides are not modifiable nor are they of any use for in vitro assays. For some HLA-binding peptides it is tolerable to replace hydrophobic residues for more hydrophilic residues to increase solubility. However, in some cases, this can reduce the binding affinity of the peptide to the MHC, therefore is counterproductive for a model based on HLA binding. While reconstituting the sample with solvents such as acetonitrile for HPLC analysis could overcome this problem, the sample must dissolve in the mobile phase. If the composition of the mobile phase in a gradient system is lower than the diluent needed to dissolve the sample, peak shape and retention time are affected. DMSO is often used to dissolve peptides with poor solubility; however, this can have considerable implications on cellular behaviour as it is toxic to cells at low concentrations and will have to be controlled in cellular models. Importantly, side reactions occur with both drugs and residues; β-lactams can modify the N-terminus of a peptide and although an N-terminal protection group can prevent this as discussed, it must be removed via an extra HPLC purification step that increases labour time and decreases the yield. Moreover, β-lactams yield an array of degradation products therefore a more complex peak profile is formed making the drug-modified peptide peak less distinguishable. Drug dimer- or trimerisation also adds to the complexity of identifying drug-modified products that occur extensively with amoxicillin. Some residues are more prone to side reactions in peptide synthesis than others based on their reactivity. Cysteine residues are known to form disulphide bonds causing aggregation in solution. Usually in post-translational modification studies, this is overcome using cysteine capping; however, this is not feasible in some drug designer peptide synthesis where cysteine residues represent the main target...
The process of peptide synthesis and characterisation. The generation of synthetic drug-modified peptides is described in four steps: (1) The FMOC-protected peptide is incubated with drug for covalent binding. A drug like amoxicillin is bound to a lysine residue in a peptide through an amide linkage. (2) The drug-peptide mixture is purified using a succession of HPLC experiments, and the suspected drug-modified peptide is fractionated. (3) The collected sample is analysed using LC-MS/MS to confirm successful drug modification. Peptides are identified using de novo sequencing thus drug-characteristic fragments are used to identify successful drug modification. The N-terminal FMOC-protection group is confirmed by a peak with \( m/z \) at 179. (4) The peptide is deprotected using reagents like piperidine that removes the N-terminal FMOC protection group. HPLC purification and LC-MS/MS confirmation steps are repeated to confirm the removal of FMOC and yield a final deprotected drug-modified peptide.

Additionally, it is difficult to control oxidation status as cysteine can undergo mono-, di- and tri-oxidation. Tryptophan and methionine also form oxidation products. Challenges are not limited to experimental procedures but also data analysis. A limitation of current peptide identification software such as PEAKS or Protein Pilot to analyse drug-modified peptides is that they do not recognise the neutral loss of drugs, which is common for drugs like β-lactams, therefore modifications must be identified manually, which is timely and has higher error rates than computational methods.
CONCLUSION AND FUTURE WORK FOR THE FIELD

DHRs cannot be predicted therefore remain a significant burden for patients and healthcare providers and a challenge for the pharmaceutical industry. Over the last decade significant progress has been made to define the critical antigen responsible for T-cell activation in the development of a reaction, including the identification and characterisation of antigenic drug-protein conjugates and natural drug-modified HLA-binding peptide. Moreover, HLA association studies have confirmed the importance of the pMHC-TCR interaction in DHRs. Despite this, the exact peptide repertoire presented and the binding interactions between the TCR and MHC remain to be fully defined. The interaction between the TCR and pMHC is considered weak therefore it dissociates rapidly making it difficult to detect T-cell responses in vitro [113]. Between the TCR and pMHC-TCR interaction in DHRs. The interaction between the TCR and MHC remain to be fully defined. The interaction between the TCR and pMHC is considered weak therefore it dissociates rapidly making it difficult to detect T-cell responses in vitro [113].

With that being said, epitope identification facilitates the potential use for tetramers in drug hypersensitivity. A panel of drug-modified HLA-binding peptides can be used to generate multimeric peptide-MHC complexes to enhance a monomeric response, as the avidity of multimerisation significantly prolongs this interaction. Tetramers can be used to screen the diversity of drug-specific T-cells, including their frequency and phenotype. This will identify immunodominant epitopes in the patient population and the extent of cross reactivity. The dominant epitope tetramers may then be used as a screening tool in healthy individuals. Furthermore, the molecular docking approach and X-ray crystallography studies will be used to model the interaction between the MHC and TCR and successfully define the critical epitope.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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