**TITLE PAGE**

**Identification of flucloxacillin-haptenated HLA-B\*57:01 ligands: evidence of antigen processing and presentation**

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Running title: Naturally processed flucloxacillin haptenated HLA ligands

**Abstract**

Flucloxacillin is a β-lactam antibiotic associated with a high incidence of drug-induced liver reactions. Although expression of HLA-B\*57:01 increases susceptibility, little is known of the pathological mechanisms involved in the induction of the clinical phenotype. Irreversible protein modification is suspected to drive the reaction through the modification of peptides that are presented by the risk allele. In this study, the binding of flucloxacillin to immune cells was characterized and the nature of the peptides presented by human leukocyte antigen HLA-B\*57:01 was analyzed using mass spectrometric based immunopeptidomics methods. Flucloxacillin modification of multiple proteins was observed, providing a potential source of neo-antigens for HLA presentation. Of the peptides eluted from flucloxacillin-treated C1R-B\*57:01 cells, 6 putative peptides were annotated as flucloxacillin-modified HLA-B\*57:01 peptide ligands (Data are available via ProteomeXchange with identifier PXD020137). To conclude, we have characterized naturally processed drug-haptenated HLA ligands presented on the surface of antigen presenting cells that may drive drug-specific CD8+ T-cell responses.

**Keywords:** haptenated HLA ligands, immune-mediated liver injury, intracellular covalent binding

**Introduction**

Flucloxacillin is a β-lactam antibiotic associated with a high incidence of drug-induced cholestatic liver injury. Although the pathological mechanisms involved in the induction of the clinical phenotype remain to be defined, both non-immune- and immune-mediated mechanisms may be involved. A non-immune-mediated mechanism that involves activation of HSP27 and the sequential molecular events was reported for flucloxacillin-induced cholestasis (Burban et al. 2018; Burban et al. 2017). Moreover, oral exposure of mice to flucloxacillin resulted in mild elevations in alanine aminotransferases and a marked swelling of the gall bladder (Nattrass et al. 2015). On the other hand, the strong association of the Human Leukocyte Antigen allele HLA-B\*57:01 with flucloxacillin-induced liver injury (Daly et al. 2009), the detection of flucloxacillin-specific T-cells in patient liver (Wuillemin et al. 2014) and flucloxacillin-specific CD8+ T-cells from peripheral blood mononuclear cells (Monshi et al. 2013) suggest that the adaptive immune system is directly involved in the tissue pathology. Furthermore, naïve CD8+ T-cells from healthy donors are activated by flucloxacillin in a HLA-B\*57:01-restricted manner, indicating an essential role for HLA-B\*57:01 in the activation of drug-responsive CD8+ T-cells (Monshi et al. 2013; Wuillemin et al. 2013; Yaseen et al. 2015).

HLA class I molecules are responsible for presenting peptides derived from the breakdown of intracellular proteins to circulating and tissue resident T-cells. Self-peptides derived from endogenous proteins are constitutively presented on the cell surface of nucleated cells. When novel peptides appear, such as those from proteins of intracellular pathogens, immune activation may follow. A number of drugs have been shown to elicit off-target T cell responses, yet for most drugs precisely how this occurs remains to be fully elucidated. Three models have been proposed for the interaction between drugs and immune receptors (HLA and T-cell receptors [TCRs]) to induce T-cell activation. 1) The hapten hypothesis states that reactive drugs or metabolites (haptens) bind covalently to proteins to initiate immune reactions. 2) Alternatively, the pharmacological interaction hypothesis states that a direct, reversible interaction of drugs with HLA/TCR proteins will activate T-cells (Meng et al. 2018; Pichler et al. 2011), whilst 3) stable, non-covalent interactions within the antigen binding cleft of the HLA can alter the repertoire of peptides bound to the HLA leading to T-cell activation by novel HLA-drug-self-peptide complexes (Illing et al. 2016). However, for many drugs it remains controversial as to whether drugs interact preferentially with HLA proteins or HLA bound peptides. Functional studies challenging T-cell lines and clones from hypersensitive patients with parent drugs, metabolites, synthetic drug-modified proteins or designer HLA binding peptides, support both covalent and non-covalent interactions (Elsheikh et al. 2010; Ogese et al. 2019; Zhao et al. 2019). Nevertheless, knowledge of the structures generated naturally and displayed by HLA molecules remains sparse.

In recent years, mass spectrometric analysis of HLA-peptide complexes has identified thousands of peptides naturally presented on the cell surface by HLA molecules. The peptide sequence information derived from this method, in conjunction with X-ray crystallographic analysis of HLA peptide complexes, has provided new insights into the mechanisms of immune-mediated disease. In terms of drug hypersensitivity, research from three teams found that abacavir, an antiretroviral which causes hypersensitivity in HLA-B\*57:01+ individuals, interacts with the peptide binding cleft of HLA-B\*57:01, altering the array of peptides that bind (Illing et al. 2012; Norcross et al. 2012; Ostrov et al. 2012). Structural elucidation of drug-peptide-HLA complexes revealed that abacavir bound non-covalently in the vicinity of the F pocket, reducing the space available to the C-terminal anchor residue of the bound peptide and explaining the shift towards presentation of peptides with smaller C-terminal residues (Trp/Phe to Ile/Leu/Val). Although hundreds of peptides were identified uniquely within the abacavir-induced peptide repertoire, their contribution to the CD8+ T-cell response seen in abacavir hypersensitive patients is yet to be defined. Importantly, drug-induced changes in the HLA-binding motif have not yet been observed with other drugs.

Flucloxacillin is intrinsically more reactive than abacavir. Flucloxacillin was previously shown to bind covalently to nucleophilic lysine residues on proteins such as human serum albumin and such adducts found in the sera of patients receiving the antibiotic (Jenkins et al. 2009). Whilst we and others have shown that T cells in healthy HLA-B\*57:01+ donors appear to respond to soluble drug (Wuillemin et al. 2013), those from allergic patients are driven by stably bound drug consistent with presentation of covalently modified peptides (Monshi et al. 2013). For reactive drugs such as flucloxacillin, covalent binding to intracellular proteins, peptide-HLA complexes, and the HLA molecule itself are all possible sources of covalently modified epitopes stimulating responses in allergic patients. Yet, intracellular protein targets of covalent modification by flucloxacillin are largely unknown, and to date no naturally processed and presented flucloxacillin modified HLA ligands have been identified. Therefore, the objective of this study was to investigate intracellular flucloxacillin protein adduct formation within immune cells and discover whether flucloxacillin-conjugated peptides can be displayed on the surface of antigen presenting cells for surveillance by CD8+ T-cells.

**Materials and methods**

***Chemicals.*** Flucloxacillin (Wockharat) was gifted from collaborators. All other chemicals were purchased from Sigma.

***Anti-flucloxacillin antibody production.*** Ovalbumin-flucloxacillin conjugates used as antigens for antibody production were prepared in a mixed buffer (carbonate buffer, 0.1M Na2CO3, 0.1M NaHCO3, pH 11 and phosphate buffer, 13.08 mM KH2PO4, 62.27 mM K2HPO4, pH 7.4 at a 1:1 volume ratio). Antibody production was performed by Kaneka Eurogentec S. A. (Belgium) using a speedy 28-polyclonal package. Detailed information is available online (eurogentec.com).

***Cell culture****.* The lymphoblastoid C1R cell line (Storkus et al. 1989; Zemmour et al. 1992), that expresses low levels of endogenous Major histocompatibility complex (MHC)-class I (predominantly HLA-C\*04:01) and transfected to express high levels of HLA-B\*57:01, was used as a source of peptides (Chessman et al. 2008). C1R-B\*57:01 cells were maintained in F1 media (RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 100 mM L-glutamine, 100 µg/mL penicillin, 100 U/mL streptomycin) and 0.5 mg/mL geneticin (37°C, 5% CO2). C1R-B\*57:01 cells were grown in multiple T175 culture flasks (Nunc) until a cell number of 1x109 cells was achieved. Treatment with 1.5 mM flucloxacillin sodium salt was for 24h for the identification of modified proteins or 48h for immunopeptidome analysis (to ensure a steady state of maximum presentation of neoepitopes was reached).

W6/32 (Sigma Aldrich, Dorset, UK) mouse hybridoma cells lines, used for the production of anti-pan class I antibody, were maintained in F1 media (37°C, 5% CO2). W6/32 hybridomas were grown to confluence before the supernatant was recovered after centrifugation and stored at 4°C until required.

***Detection of intracellular flucloxacillin-modified proteins by Western blotting.*** C1R-B\*57:01 cells were harvested and washed with HBSS (Sigma Poole, UK) prior to being pelleted and snap frozen. Cell pellets were lysed (7.0M urea, 2.0M thiourea, 4% CHAPS, 40mM Tris base and 1% DTT) and soluble lysates were collected for protein quantification. Proteins were denatured and reduced by heating to 100°C for 10 minutes in Laemmli sample buffer (Sigma, Poole, UK). One hundred micrograms of protein from C1R-B\*57:01 cell lysates were separated in two dimensions on a 10% SDS-polyacrylamide gel for both Coomassie stained gels and Western blots. The first dimension was performed by rehydrating IPG strips (pH 4-7) with sample in rehydration solution and separating based on pH using the Multiphor Electrophoresis System (GE Healthcare, MA, USA). (Kitteringham et al. 2003). For Western blotting, gels were transferred onto nitrocellulose membrane by electroblotting. The nitrocellulose membrane was washed in deionized water and blocked in tris/saline/tween (TST) buffer (150 mM NaCl, 10 mM Tris-HCl, 0.05% Tween 20, pH 8.0) containing 2% nonfat dry milk (Bio-Rad) for 1h at room temperature. The nitrocellulose was washed in TST and incubated in primary polyclonal rabbit-anti-flucloxacillin antibody (custom order, Eurogentec, Belgium) in TST containing 2% nonfat dry milk for 1h. The nitrocellulose membrane was washed in TST repeatedly and incubated with horseradish peroxidase conjugated goat-anti-rabbit secondary antibody (Dako, Agilent, CA, USA) for 1 hour. After further washing with TST, signal was detected using enhanced chemiluminescence (Western Lightning, PerkinElmer Life and Analytical Sciences, Waltham, MA).

***Detection of flucloxacillin modified proteins by immuno-cytochemistry.*** C1R-B\*57:01 cells were cultured in the presence of flucloxacillin (1.5 mM) and adhered to glass cover slips using Cell-Tak (Corning, MA, USA). Cells were washed with PBS (pH 8.0) and fixed using 4% paraformaldehyde. Cells were permeabilized (0.004% Tween 20, 0.025% Triton-X-100, PBS) for 30 minutes and blocked with bovine serum albumin (5% in permeabilization buffer) for 1 hour at room temperature. Subsequently blocking buffer containing polyclonal rabbit-anti-flucloxacillin antibody at 4°C was added overnight. After washing with permeabilization buffer, goat anti-Rabbit IgG secondary antibody (Alexa Fluor 488, ThermoScientific, MA, USA) was applied for one hour. After further washes, the cells were incubated in Hoechst 33342 (ThermoScientific, MA, USA) and Alexa-Fluor-568-phalloidin (ThermoScientific, MA, USA) for nuclear and f-actin staining respectively. Cover slips were mounted onto glass slides with Pro-Long Gold (ThermoScientific, MA, USA) and sealed. Images were taken using a Carl Zeiss Axio Observer microscope with Apoptome using 40x oil objective.

***LC-MS/MS analysis of HLA-B\*57:01 peptide complexes.*** HLA-bound peptides from pellets of 1x109 cells were isolated and analyzed by LC-MS/MS as described previously(Purcell et al. 2019) Briefly, cell pellets were lysed in 0.5% IGEPAL, 50 mM Tris pH 8.0, 150 mM NaCl and protease inhibitors (complete protease inhibitor cocktail, Roche). The lysate was cleared by ultracentrifugation (180,000 x g) and MHC class I complexes captured through solid-phase-bound W6/32 antibody. Bound complexes were eluted in 10% acetic acid. The mixture of MHC-bound peptides, MHC heavy chain and β2-microglobulin was fractionated using by reversed phase-HPLC over a 100 x 4.6 mm monolithic C18 column (Chromolith, Merck) using mobile phases containing 0.1% trifluoroacetic acid (A) and 80% acetonitrile/0.1% trifluoroacetic acid (B).

Peptide containing HPLC fractions were pooled and concentrated in a vacuum centrifuge (Speedvac, Eppendorf) at 30°C. Reconstituted fraction pools were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) either as previously described (Thomson et al. 2020) or using a Triple TOF 5600 mass spectrometer (Sciex) where peptide fractions were delivered into the instrument using a Eksigent NanoLC Ultra HPLC system. Samples were loaded onto a nanoACQUITY UPLC Symmetry C18 Trap Column (P/N 186007496, Waters, MA, USA) for 10 min at 2 µL/min with 0.1% formic acid (FA). Peptides were separated over a Peptide BEH C18 nanoACQUITY Column (P/N 186003815, Waters, MA, USA) with a gradient from 1.7% ACN/0.1% FA to 64% ACN/0.1% FA was applied over 79 min at a flow rate of 300 nL/min). MS was operated in positive ion mode with survey scans of 200 ms, and an MS/MS accumulation time of 150 ms for the 20 most intense ions (total cycle time 3.2 seconds). A threshold for triggering MS/MS of 40 counts per second was used with an exclusion of former target ions for 30 seconds. Rolling collision energy was applied. Data dependent acquisition of ions in the mass range of 200-1,800 amu (MS) and 60-1,800 amu (MS/MS) was collected using Analyst TF 1.6. The instrument was automatically calibrated using a β-galactosidase digest every 3rd sample.

***Analysis of HLA-B\*57:01 peptide ligands.*** LC-MS/MS data were searched against the reviewed human proteome (UniProt/SwissProt accessed October 2018), including a list of common contaminating proteins for LC/MS experiments, using PEAKS Studio 8.5 (Bioinformatics Solution Inc). Data were refined using default parameters, and PEAKS DB searches performed with the following parameters: Parent Mass Error Tolerance 50 ppm, Fragment Mass Error Tolerance 0.1 Da, no enzymatic restriction, Variable modifications – methionine oxidation (+15.99), asparagine and glutamine deamidation (+0.98), cysteine oxidation to cysteic acid (+47.98), flucloxacillin modification of lysine, arginine and cysteine (+453.06, incorporating a neutral loss of 159.04 on fragmentation). The maximum number of variable posttranslational modifications per peptide was 3. False discovery rate (FDR) was estimated with decoy-fusion. Sequences annotated with a flucloxacillin modification were considered valid if they contained the diagnostic 160.04 ion. Flucloxacillin-modified HLA peptides were also manually identified using characteristic fragment ions of m/z at 160.04, 295.03, and 454.06; diagnostic ions indicative of the presence of a covalently linked flucloxacillin molecule. Manual annotation of MS/MS spectra was performed to identify the peptides and subsequent NCBI Blast searches were used to identify the native protein sources.

For HLA-B\*57:01 peptide repertoire analysis, identifications to the target database at a 2% peptide spectrum match FDR were exported from PEAKS Studio for further processing. Peptides mapping to the contaminant database were removed, as were peptides previously isolated from CIR parental cells (lacking HLA-B\*57:01 expression) via similar procedures ((Schittenhelm et al. 2014) and list provided by Shutao Mei). The cell treatment and HLA isolation followed by ligand analysis process was performed 4 times for flucloxacillin treated cells, and once for untreated cells. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium ([http://proteomecentral.proteomexchange.org](http://proteomecentral.proteomexchange.org/)) via the PRIDE partner repository (Perez-Riverol et al. 2019) with the dataset identifier PXD020137. An additional 3 untreated CIR.B\*57:01 data sets were generated via searching 3 previously published data sets (Thomson et al. 2020) publicly available in the PRIDE Archive PXD015398 (Perez-Riverol et al. 2019) as above using PEAKS 8.5. Peptide sequence lists derived from each treatment group were combined, and duplicated sequences were removed. Processed peptide sequences were used for both length distribution and amino acid motif analysis. Seq2logo was used to generate sequence motifs using default settings (Thomsen and Nielsen 2012). Predicted binding affinities were calculated by NetMHC4.0 (Andreatta and Nielsen 2016; Nielsen et al. 2003).

***Purification and analysis of HLA-B\*57:01 heavy chain.*** Fractions from RP-HPLC HLA/peptides purification containing the dissociated HLA heavy chain were pooled and concentrated in a vacuum centrifuge (Speedvac, Eppendorf) at 30°C. HLA heavy chain was suspended in 50 µL 50 mM ammonium bicarbonate, reduced and alkylated, and digested overnight with trypsin (Promega). The tryptic digests were purified with a C18 ZipTip (Merck) following manufacturer’s instructions and analyzed on a Triple TOF 5600 mass spectrometer (Sciex) using previous methods (Meng et al. 2016).

***Immuno-detection of flucloxacillin adducts in RP-HPLC fractions of purified MHC.*** Nitrocellulose membrane was divided into a grid and 3 x 10 µL spots corresponding to each HPLC fraction were spotted into each square, allowing for drying between each spot. A positive control (flucloxacillin bound to ovalbumin, the immunogen used for antibody generation) was applied to two corners of the membrane. Spots were probed for flucloxacillin with anti-flucloxacillin antibody, using the methods described for Western blot analysis.

***Detection of flucloxacillin modified proteins via mass-spectrometry.*** C1R-B\*57:01 cells treated with 1.5 mM flucloxacillin for 24h were pelleted and lysed using the methods described for the detection of flucloxacillin modified proteins by Western blotting. Cell lysates were cleared using centrifugation, reduced (10 mM DTT, room temperature for 20 minutes) and alkylated (55 mM IAA, room temperature in the dark, for 20 minutes). Digestion was performed using sequencing grade modified trypsin (Promega) overnight at 37°C. Samples were purified using C18 ZipTips (Millipore) and dried in a centrifugal concentrator (Eppendorf speedvac). Samples were reconstituted in 2% ACN, 0.1% FA (v/v) prior to analysis using a Triple TOF 5600 mass spectrometer (Sciex) delivered into the instrument using a Eksigent NanoLC Ultra HPLC system. Samples were injected onto a nanoACQUITY UPLC Symmetry C18 Trap Column (P/N Waters, MA, USA) and washed for 10 min at 2 µL/min with 0.1% FA. A gradient from 1.6% ACN/0.1% FA to 95% ACN/0.1% FA was applied over 95 minutes at a flow rate of 300 nL/min through a Peptide BEH C18 nanoACQUITY Column (Waters, MA, USA). MS was operated as described in previous methods (Meng et al. 2016).

***Analysis of tryptic peptides.*** LC-MS/MS data were searched as for HLA peptide ligands with the following changes: incorporating enzymatic cleavage restriction for Trypsin (maximum 3 missed cleavages, allowing for non-specific cleavage), fixed modification carbamidomethylation of cysteine, Variable modifications – methionine oxidation (+15.99), asparagine and glutamine deamidation (+0.98), flucloxacillin modification of lysine and arginine (+453.06, incorporating a neutral loss of 159.04 on fragmentation). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium ([http://proteomecentral.proteomexchange.org](http://proteomecentral.proteomexchange.org/)) via the PRIDE partner repository (Perez-Riverol et al. 2019) with the dataset identifier PXD020137. For searches of tryptic digestion of the heavy chain of HLA-B\*57:01, the search database was altered to incorporate the mature length HLA-B\*57:01 (signal peptide removed), and protein N-terminal flucloxacillin (+453.06) incorporated as a variable modification.

***14-3-3 modelling.*** Crystal structure of 14-3-3 gamma (PDB 4E2E) was used to generate models. For covalent docking to lys50, the corresponding side chain was removed from the protein and the ligand modified to contain the side chain to allow flexibility. The site of covalent attachment was at the lysine Cα. A generic algorithm with ChemPLP as the fitness function was used to generate 10 binding modes per ligand. Default settings were retained for the “ligand flexibility”, “fitness and search options”, and “GA settings.

***HLA-B\*57:01 modelling.*** Crystal structure of HLA-B\* 57:01 (PDB code 5vuf) (Illing et al. 2018) was used to generate models by removal of the peptide using Pymol (2.0, Schrodinger). GOLD 5.2 (CCDC software) (Naisbitt et al. 2015) was used to dock the HTAHIAC[O3]KFA peptide and its flucloxacillin-modified counterpart within the binding groove, with the binding site defined as 15 Å around the binding point. The binding point was further refined with key amino acid residues within B pocket (Tyr9, Ala24, Met45, Ala46, Glu63, Asn66, Met67, and Tyr99) and F pocket (Asn77, Ile80, Ala81, Ile94. Ile95, Val97, Asp114, Ser116). A generic algorithm with ChemPLP as the fitness function was used to generate 10 binding modes per ligand. Default settings were retained for the “ligand flexibility”, “fitness and search options”, and “GA settings.

**Results**

**Flucloxacillin covalently binds to proteins within immune cells.** C1R-B\*57:01 have been used previously to characterize the HLA-B\*57:01 immunopeptidome. We therefore sought to determine if intracellular protein modification by flucloxacillin might provide a source of modified peptide ligands. Treatment of C1R-B\*57:01 cells with 1.5 mM flucloxacillin for 24 hours did not overtly change the intracellular protein profile compared to that of the control (Figure 1A, i & ii). However, western blot analysis revealed a huge diversity of proteins irreversibly bound by flucloxacillin (Figure 1A, iii & iv). Immunocytochemical analysis showed flucloxacillin modification of a range of cytoplasmic proteins while no detection of flucloxacillin (green) was be observed in the untreated cells (Figure 1A, v-viii).

Mass spectrometric analysis of the tryptic digests of flucloxacillin treated C1R-B\*57:01 cell lysates revealed several flucloxacillin-modified peptides (Table 1). Figure 1B shows a representative MS/MS spectrum for a triply charged ion of m/z 652.9838, which corresponds to the tryptic peptide 43NLLSVAYKNVVGAR56 with a mass addition of 453.1054 Da, indicating the presence of flucloxacillin. The presence of protonated flucloxacillin (m/z 454.0686) and a characteristic fragment ion (m/z 160.0433, thiazolidine ring fragment) derived from flucloxacillin fragmentation during CID provided further evidence of modification. This peptide is derived from proteins 14-3-3 gamma (P61981|1433G\_HUMAN) or beta (P31946|1433B\_HUMAN), members of a family of phosphoprotein-binding proteins which regulate major cellular functions including cell cycle progression and apoptosis. The modification site (Lys50) was confirmed by the presence of y ions from y\*7 - y\*12, all with the adduction of 294 Da, which is the mass of the adduct after cleavage of the thiazolidine ring fragment (Figure 1B & Supplementary Table 1A). Two more flucloxacillin-modified peptides from 14-3-3 family NLLSVAYK\*NVIGAR (P62258|1433E\_HUMAN) and NLLSVAYK\*NVVGGR (P27348|1433T\_HUMAN) were also detected, all modified at Lysine. Of note, Lys50 is one of 3 positively charged residues (Lys50, Arg57, and Arg132) important for binding to phosphorylated proteins. Modelling of the modification of Lys50 on 14-3-3 by flucloxacillin predicted a clash between flucloxacillin and Arg57, which could block its binding to signaling proteins (Figure 1C). In addition to 14-3-3 proteins, flucloxacillin modification was also detected on human elongation factor 1-alpha (P68104|EF1A1\_HUMAN) and actin (P60709|ACTB\_HUMAN) (Table 1).

**Characterization of HLA-B\*57:01 ligands presented by antigen presenting cells treated with flucloxacillin.** To explore the impact of flucloxacillin on peptide presentation by HLA-B\*57:01, C1R-B\*57:01 cells were incubated in the presence of 1.5 mM flucloxacillin. Subsequently, HLA-peptide complexes were purified using W6/32 (anti-pan class I antibody) and the bound peptides were extracted and analyzed by mass spectrometry. HLA-B\*57:01 peptide ligands eluted from C1R-B\*57:01 cells were analyzed using PEAKS Studio 8.5 (Bioinformatics Solution Inc) as described in the methods. 7,551 non-redundant peptide sequences were identified as HLA-B\*57:01 ligands from untreated data sets collected here and previously (Thomson et al. 2020) and allowed characterization of the constitutive HLA-B\*57:01 immunopeptidome. 8,973 HLA-B\*57:01 ligands (non-redundant by sequence) were similarly obtained from analysis of CIR-B\*57:01 cells after 48hrs treatment with flucloxacillin. 28.9% (3,640 peptides) of the total peptide sequences were exclusively identified in samples from untreated cells, whereas 40.1% (5,062 peptides) were only identified in samples from the flucloxacillin treated cells. The remaining 31% (3,911 peptides) were identified in both datasets (Figure 2A). For both treatment groups, identified peptides were predominately 9-11 amino acids in length, consistent with MHC class I presentation (Figure 2B). Although a greater proportion of 9mer ligands terminating in tryptophan were identified in the combined flucloxacillin data set, no significant differences in primary anchor residue prevalence at P2 or P9 were observed between the control and flucloxacillin data sets. Furthermore, the sequence motifs were consistent with published HLA-B\*57:01 ligandomes (Abelin et al. 2017; Illing et al. 2012; Norcross et al. 2012; Ostrov et al. 2012) and 9-mer HLA-B\*57:01 ligands reported in the Immune Epitope Database ([www.iedb.org](http://www.iedb.org/)) (Vita et al. 2019) (Figure 2C & Supplementary Figure 1).

Within the peptides isolated from flucloxacillin-treated CIR-B\*57:01 cells, over 70 spectra (Supplementary Table 2) were identified containing the diagnostic ions of flucloxacillin modification in high abundance (*m/*z 160.04 intensity >200). From these spectra, six putative flucloxacillin-modified peptides were annotated by PEAKS Studio 8.5 (Bioinformatics Solution Inc.) and/or manual *de novo* sequencing (Table 2).Consistent with the flucloxacillin-modified tryptic 14-3-3 peptide, the presence of characteristic flucloxacillin fragment ions and a series of flucloxacillin-adducted *b* and *y* ions provided substantial evidence of modification. As shown in Figure 3A & Supplementary Table 1B, the observed triply charged ion of m/z 533.8612 could be assigned as peptide HTAHIAC(O3)K\*FA with a mass increment of 453.0516 amu. Peptide HTAHIAC(O3)K\*FA was derived from human elongation factor 1-alpha, an abundant and multifunctional protein, also identified as modified in the proteomic analysis, albeit at a different site. Three flucloxacillin-haptenated peptides, VSDHEATLR\*C(O3)WA, ISDHEATLR\*C(O3)WA, and TAAQITQRK\*W appeared to originate from the MHC alpha chain itself, whereby they were likely modified, processed by the proteasome and presented by a second MHC class I (Figure 3B & Supplementary Table 1B, Figure S2, Table 2). Notably, two peptides contain a flucloxacillin-modified arginine residue, which was not detected in previous *in vivo* studies (Jenkins et al. 2009). However, modification of arginine residues is highly possible through an S-N-acyl transfer due to the presence of the adjacent cysteine residue. All six peptides were predicted to have higher affinity for HLA-B\*57:01 in their native form than the co-expressed HLA-C\*04:01 of the CIR-B\*57:01 cell line (Table 2).

Flucloxacillin modified HLA peptides were also identified from C1R-B\*57:01 cells incubated with flucloxacillin (1.5 mM) for only 10 minutes, indicating direct modification of ligands already displayed at the cell surface (Figure S3A). Immuno-detection of flucloxacillin adducts in fractions generated by RP-HPLC fractionation of MHC isolated from flucloxacillin treated C1R-B\*57:01 cells revealed modification in the last three fractions, indicative of modified HLA heavy chain protein (Figure S3B). No staining was observed in heavy chain fractions from untreated CIR-B\*57:01 (data not shown). Further mass spectrometric analysis of the HLA heavy chain fractions revealed flucloxacillin bound to the HLA molecule at the N-terminal glycine ([Flucloxacillin]-GSHSMR) (Figure S3C). Thus, the reactive nature of flucloxacillin and its ability to covalently modify proteins was seen on multiple levels – at the cellular protein level (the source of HLA-bound peptides), at the level of HLA-bound peptides, and at the level of the HLA-B\*57:01 heavy chain itself.

To explore how flucloxacillin might be displayed for T cell recognition, computational modelling of native and flucloxacillin modified HTAHIAC(O3)KFA with HLA-B\*57:01 (PDB code 5vuf) was performed. Docking of the native HTAHIAC(O3)KFA peptide with HLA-B\*57:01 shows consistent conformation in the binding groove, with P2 (Thr) and P9 (Phe) located in the B and F pocket, respectively (Figure 3C). In contrast, the predicted conformation of flucloxacillin modified-HTAHIAC(O3)K(Flucloxacillin)FA peptide is different from the native one, with flucloxacillin pointing out of the binding groove for T-cell recognition (Figure 3D).

**Discussion**

β-lactam antibiotics are characterized by their ability to covalently modify essential cell wall forming proteins in bacteria. This same mechanism elicited by the chemical warhead on these molecules also appears to be associated with unwanted immune reactions to these drugs. Herein, we have studied flucloxacillin modification of immune cells and detected natural HLA class I peptides presented on the surface of antigen presenting cells treated with flucloxacillin. For the first time, we demonstrate that flucloxacillin-haptenated peptides can be naturally processed and presented by HLA-B\*57:01 through multiple pathways.

Previous studies demonstrated a strong association between HLA-B\*57:01 and susceptibility to flucloxacillin-induced liver injury (Daly et al. 2009). Furthermore, activation of T-cells from patients with liver injury by flucloxacillin has been detected and shown to be HLA-B\*57:01-restricted (Monshi et al. 2013; Wuillemin et al. 2014). Thus, a key objective of this study was to identify the flucloxacillin-associated structures displayed by HLA-B\*57:01 on the surface of antigen presenting cells. Almost 9,000 HLA-B\*57:01 peptide ligands were identified from flucloxacillin treated cells, of these, >5000 peptides were not identified in the control data sets, accounting for 40% of the overall identified ligandome. Although it should be noted that more than 2000 of these peptides have previously been reported as HLA-B\*57:01 ligands in the Immune Epitope Database (<http://www.iedb.org>) (Vita et al. 2019), suggesting the true number of unique ligands to flucloxacillin treatment is lower (data not shown). Whether flucloxacillin modifications diversify the peptide repertoire through altering protease cleavage sites, resulting in the presentation of novel peptide sequences, remains to be explored. Both the distribution of peptide lengths and amino acid anchor residues remained the similar between untreated and flucloxacillin treated conditions.

Over 70 spectra were found to contain high intensity flucloxacillin-adduct diagnostic ions (Supplementary table 2). Analysis with PEAKS Studio 8.5 and manual *de novo* sequencing allowed us to annotate six of the flucloxacillin-haptenated peptides in the HLA-B\*57:01 ligandome. We anticipate that flucloxacillin-haptenated peptides can be presented through processing-dependent and -independent pathways (Figure 4). Through the processing-dependent pathway, flucloxacillin-haptenated proteins undergo intracellular processing to produce haptenated peptides that are loaded onto the HLA prior to presentation on the cell surface (Figure 4, ii). Three flucloxacillin-haptenated peptides (RTKK\*VGIVGKY, KTK\*KSLESI, and HTAHIACK\*FA) derived from intracellular proteins were displayed by HLA-B\*57:01, although the precise localization of the modification was ambiguous for RTKK\*VGIVGKY and KTK\*KSLESI due to multiple possible target residues. Although a huge diversity of flucloxacillin-haptenated intracellular proteins were detected by western blot analysis of cell lysates, it is not surprising that only a handful of flucloxacillin modified peptides were identified in the naturally processed and presented immunopeptidome as generally less than 0.1% peptides derived from the human proteome are known to be presented (Neefjes and Ovaa 2013). Furthermore, proteins with posttranslational modifications have been shown to be more resistant to proteolysis, and cleavage patterns are influenced by the sites of modification posing a further barrier to presentation (Ninkovic and Hanisch 2007; Purcell et al. 2008). Notably, although multiple isoforms of the human 14-3-3 proteins were modified by flucloxacillin, none of these modification sites were detected in the HLA-B\*57:01 ligandome. In addition, the protein EF1A is the second most abundant protein (1-3% of total protein content) expressed in many cells, including immune cells and liver cells (Abbas et al. 2015). Although K146 on EF1A was also modified by flucloxacillin, only a peptide containing modified K371 (HTAHIACK\*FA) was identified as a ligand of HLA-B\*57:01. Neither of the flucloxacillin-modified lysine residues are a known site of epigenetic modification involved in its canonical function of translation elongation (Andersen et al. 2003; Hamey and Wilkins 2018). However, these modifications may play a role in non-canonical functions including regulation of the cytoskeleton through AKT and PI3K signaling pathways (Abbas et al. 2015; Amiri et al. 2007).

Flucloxacillin could also directly bind to peptides already presented on the cell surface within the HLA peptide binding cleft (Figure 4, i). In this respect, flucloxacillin-haptenated peptides and unmodified counterparts were detected (Table 2), indicating direct cell surface peptide modification is possible. Furthermore, flucloxacillin-modified peptides were detected after C1R-B\*57:01 cells were treated with flucloxacillin for only 10 minutes, a period too short to allow for marked *de novo* HLA-peptide complex formation and egress, providing further evidence for direct, cell surface haptenation. This direct haptenation could lead to immediate activation of T-cells upon flucloxacillin treatment without the need for antigen processing. Indeed, Wuillemin *et al.* demonstrated that stimulation of flucloxacillin-specific T-cells could occur independent of proteasomal processing (Wuillemin et al. 2013). Although we have identified flucloxacillin modified HLA-B\*57:01 ligands, whether the association of DILI is explained by selective modification of HLA-B\*57:01 binding peptides is yet to be determined. One possible mechanism is selective, non-covalent binding of flucloxacillin to HLA-B\*57:01-peptide complexes that contain nucleophilic residues pointing out of the peptide-binding cleft, and that this selective non-covalent interaction could position the drug in favorable orientations to facilitate covalent binding.

Flucloxacillin has recently been shown to induce features of direct cholestatic injury in *in vitro* cultured human HepaRG cells through PKC/P38 and PI3K/AKT signaling pathways. Cholestatic injury was exemplified by dilation of bile canaliculi, reduced bile acid efflux and secretion of hepatocyte pro-inflammatory cytokines such as IL-6 and IL-1β (Burban et al. 2018; Burban et al. 2017; Sharanek et al. 2019). Flucloxacillin-treatment of primary human hepatocytes was also associated with the release of high-mobility group box 1 protein and dendritic cells exposed to flucloxacillin-treated hepatocyte supernatant secreted TNF-α, IL-6 and IL-1β (Ogese et al. 2019). Thus, signaling pathways between flucloxacillin-treated hepatocytes and immune cells could potentiate drug- and tissue-specific adaptive immune attack. Most recently, we have demonstrated that hepatocyte-derived exosomes transport flucloxacillin-modified liver proteins to dendritic cells silently through a process of phagocytosis. Peptides derived from the liver proteins activated naïve T-cells from healthy human donors (Ogese et al. 2019). Hence, flucloxacillin hepatocyte-derived stress and antigenic signals are transported to immune cells. It is plausible that an orchestration of immune and non-immune-mediated pathways may induce local tissue injury through formation of covalent adducts with proteins such as EF1A and 14-3-3.

Collectively, we show that drug-haptenated peptides bind to HLA-B\*57:01 and can be presented at the cell surface. We propose that these peptides originate from either direct haptenation of peptides already in complex with the HLA or following processing of drug-modified intracellular proteins. These data indicate that drug-associated antigens displayed by HLA-B\*57:01 are available for immune surveillance and have the potential to trigger drug-specific T-cell responses. Further research to establish the immunogenicity of these naturally presented flucloxacillin modified HLA-B\*57:01 peptides in patients with a history flucloxacillin induced DILI is ongoing. Assessing the functionality of these peptides will be pivotal to our understanding of the immunopathology of flucloxacillin induced liver injury.

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**Conflicts of Interest Statement**

The authors declare no conflicts of interest.

**List of abbreviations:**

TCRs, T-cell receptors. HLA, human leukocyte antigen, TST, tris/saline/tween, LC-MS/MS, liquid chromatography-tandem mass spectrometry, FDR, false discovery rate

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Table 1. Flucloxacllin-modified proteins identified from flucloxacillin treated C1R cells1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **m/z observied** | **Retention time** | **Amino acid** | **Peptide sequence2** | **Accession3** |
| 873.7822 | 81.51 | Lys146 | EHALLAYTLGVK\*QLIVGVNK | P68104|EF1A1\_HUMAN |
| 607.9854 | 70.25 | Lys146 | TLGVK\*QLIVGVNK |
| 574.2839 | 69.05 | Lys146 | LGVK\*QLIVGVNK |
| 652.9764 | 70.66 | Lys50/51 | NLLSVAYK\*NVVGAR | P61981|1433G\_HUMAN P31946|1433B\_HUMAN |
| 657.6615 | 70.71 | Lys50 | NLLSVAYK\*NVIGAR | P62258|1433E\_HUMAN |
| 648.2938 | 68.19 | Lys49 | NLLSVAYK\*NVVGGR | P27348|1433T\_HUMAN |
| 497.2252 | 60.47 | Lys328 | IK\*IIAPPER | P60709|ACTB\_HUMAN |

1. C1R-B\*57:01 cells were treated with 1 mM flucloxacillin for 24 hours prior to lysis; cell lysate was digested with trypsin and analyzed by LC-MS/MS. 2. Peptide sequences were determined by both manual de novo sequencing and PEAKS Studio 8.5 (Bioinformatics Solution Inc), \* indicates the flucloxacillin modified residue (dmass +453.06). 3. Additional source protein information can be found in Supplementary Table 3.

**Table 2**. Flucloxacillin-haptenated peptides eluted from C1R cells expressing HLA-B\* 57:01

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Entry** | **m/z observed** | **Peptide sequence3** | **Predicted HLA-B\*57:01 nM affinity**  **(% Rank, binding level)4** | **Predicted HLA-C\*04:01 nM affinity**  **(% Rank, binding level)4** | **Accession5** |
| 1 | 426.21371 | RTKK\*VGIVGKY¥ | ---- | ---- | P61513|RL37A\_HUMAN |
| 416.92951 | RTKKVGIVGKY | 5074  (4.5, NB) | 38956  (55, NB) |
| 2 | 496.23171 | KTK\*KSLESI¥ | ---- | ---- | P62888|RL30\_HUMAN |
| 517.31371 | KTKKSLESI | 1846  (2, WB) | 30283  (25, NB) |
| 3 | 533.86121 | HTAHIAC(O3)K\*FA | ---- | ---- | P68104|EF1A1\_HUMAN |
| 573.75971 | HTAHIAC(O3)KFA | 1211  (1.5, WB) | 40209  (60, NB) |
| 538.25171 | HTAHIAC(O3)KF | 75  (0.25, SB) | 13,951  (5.5, NB) |
| 4 | 630.22671 | VSDHEATLR\*C(O3)WA | ---- | ---- | P30504|1C04\_HUMAN |
| 479.20841,2 | VSDHEATLRC(O3)WA | 2659  (3, NB) | 41228  (70, NB) |
| 682.79721,2 | VSDHEATLRC(O3)W | 1173  (1.5, WB) | 40791  (65, NB) |
| 5 | 634.89691 | ISDHEATLR\*C(O3)WA | ---- | ---- | P18465|1B57\_HUMAN |
| 483.88021,2 | ISDHEATLRC(O3)WA | 1017  (1.4, WB) | 41011  (70, NB) |
| 689.80871,2 | ISDHEATLRC(O3)W | 414  (0.8, WB) | 40531  (65, NB) |
| 6 | 552.58171 | TAAQITQRK\*W | ---- | ---- | P18465|1B57\_HUMAN & P30504|1C04\_HUMAN |
| 401.56031,2 | TAAQITQRKW | 47  (0.15, SB) | 43709  (90, NB) |

1. Peptides were detected in flucloxacillin treated C1R-B\*57:01 cells; 2. Peptides were detected in untreated C1R-B\*57:01 cells; 3. Peptide sequences were determined by manual de novo sequencing and/or PEAKS Studio 8.5 (Bioinformatics Solution Inc), \* indicates the flucloxacillin modified residue (dmass +453.06). Cys modification: (O3) trioxidation (dmass +47.98). ¥ indicates location of modification is ambiguous. 4. Predicted affinity of unmodified peptides to HLA allomorphs was determined by using NetMHC 4.0 (Andreatta and Nielsen 2016), SB-strong binder (<0.5%)), NB-non-binder (>2%), WB-weak binder (between 0.5% and 2%). 5. Additional source protein information can be found in Supplementary Table 3.