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Rapid Report

Cell Membrane Transporters Facilitate the Accumulation of Hepatocellular Flucloxacillin Protein Adducts: Implication in Flucloxacillin-Induced Liver Injury

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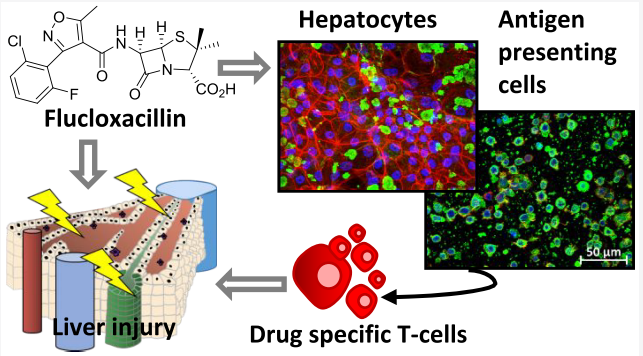
ABSTRACT: Flucloxacillin is a β-lactam antibiotic associated with a high incidence of drug-induced liver reactions. Although expression of HLA-B*57:01 increases susceptibility, little is known about the pathological mechanisms involved in the induction of the clinical phenotype. Irreversible protein modification is suspected to drive the reaction through the presentation of flucloxacillin-modified peptides by the risk allele. In this study, the binding of flucloxacillin to proteins of liver-like cells was characterized. Flucloxacillin was shown to bind to proteins localized in bile canaliculi regions, coinciding with the site of clinical disease. The localization of flucloxacillin was mediated primarily by the membrane transporter multidrug resistance-associated protein 2. Modification of multiple proteins by flucloxacillin in bile canaliculi regions may provide a potential local source of neo-antigens for HLA presentation in the liver.



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.[1,2] On the other hand, the strong association of HLA-B*57:01 with flucloxacillin-induced liver injury[3] and the detection of flucloxacillin-specific T-cells in patient liver[4] and flucloxacillin-specific CD8+ T-cells from peripheral blood mononuclear cells[5] suggest that the adaptive immune system is directly involved in the tissue pathology.

Flucloxacillin has previously been shown to bind covalently to nucleophilic lysine residues on proteins such as human serum albumin, and such adducts were found in the sera of patients receiving the antibiotic.[6] For such reactive drugs, covalent binding to intracellular proteins, peptide-HLA complexes, and the HLA molecule itself is all possible. We have recently shown that flucloxacillin forms protein adducts in immune-like cells (C1R B lymphoblastoid), and flucloxacillin haptenated peptides can be presented by HLA-B*57:01.[7] However, whether flucloxacillin can form hepatic antigens that activate local immune cells remains to be seen. Previously, Burban et al. have shown that flucloxacillin induced direct cholestatic effects in hepatocytes through activation of the JAK/ROCK signaling pathway.[2] However, the events leading up to activation of the pathway are largely unknown and yet to be defined. We hypothesized that transport-dependent cellular accumulation of flucloxacillin can either induce cellular stress or covalent binding to proteins, leading to activation of the JAK/ROCK signaling pathway. This study was therefore performed to investigate the effect of cell membrane transporters on the transportation of flucloxacillin and covalent binding of flucloxacillin to proteins using the liver cell lines HepG2 and HepaRG.

We first assessed the flucloxacillin cholestatic risk using HepaRG cells, which were cultured in growth media for 1.5 weeks, and then differentiated in 50:50 growth/differentiation media for 0.5 weeks and differentiation media for a further 4 weeks (Figure S1). Following differentiation, cells were treated with flucloxacillin (0.1 μM−10 mM) and the bile acid mixture (1%, v/v) for 24 h. The cholestatic risk of a compound is determined by calculating the cholestatic index (CIx), CIx = (EC_{50} − ATP compound and bile acids)/(EC_{50} − ATP

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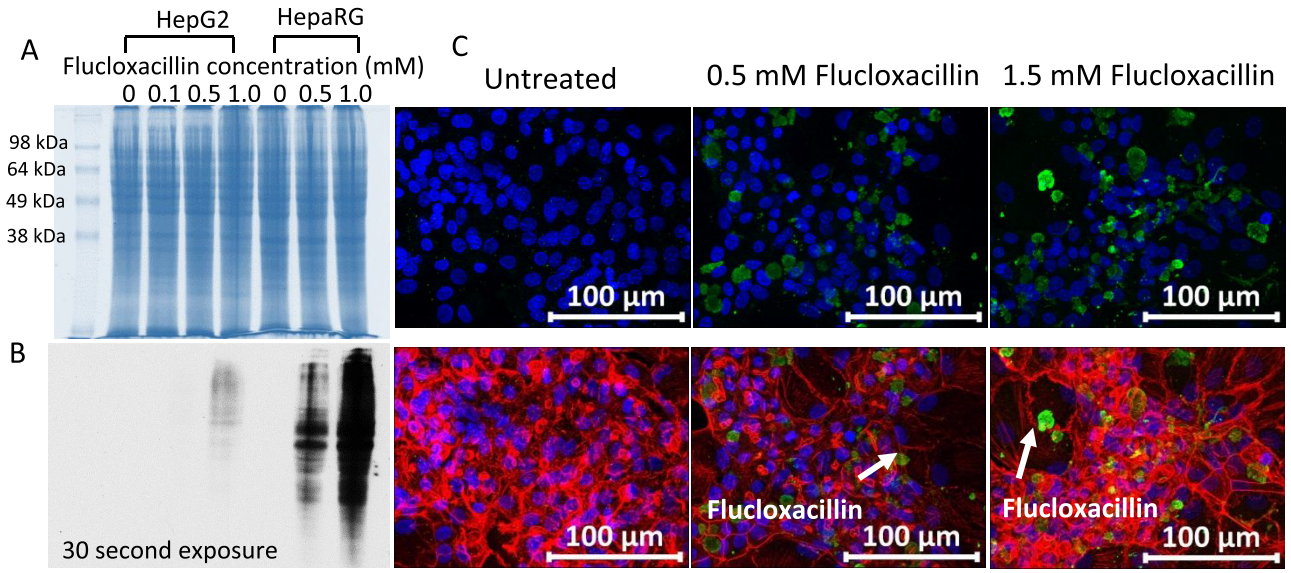

Figure 1. Flucloxacillin forms protein adducts in human hepatocyte-like cells. (A) Coomassie blue SDS-PAGE shows no alteration in protein abundance across flucloxacillin treatments in lysates extracted from HepG2 and HepaRG cell lines. (B) Western blot analysis using antiflucloxacillin antibody reveals covalent protein binding after 24 h of treatment in both cell lines in a dose-dependent manner. Proteins extracted from HepaRG cells are modified by flucloxacillin at lower concentrations compared with HepG2 cells. (C) Covalent binding in HepaRG cells is observed at 0.5 and 1.5 mM concentrations in a dose-dependent manner. Red = F-actin, blue = nuclear staining, and green = flucloxacillin.

compound alone). Cholestatic risk is recorded when CIx $\leq$ 0.80.[8] Based on the CIx value, flucloxacillin was classified as a compound that had cholestatic risk (CIx = 0.56 $\pm$ 0.11). A compound is deemed to possess a mitochondrial liability if EC_{50}-ATP glucose/EC_{50}-ATP galactose $\geq$ 2. There were no significant differences in ATP levels between glucose or galactose media for all treatment regimens, indicating that flucloxacillin does not cause mitochondrial toxicity mediated via electron transport chain dysfunction (Figure S2).

To assess whether covalent binding of flucloxacillin to proteins in the liver cells is responsible for the observed tissue injury, the hepatocyte cell lines HepG2 and HepaRG were treated with flucloxacillin (0.5−1.5 mM) for 24 h. Cell lysates were separated by SDS-PAGE and probed with anti-flucloxacillin antibodies that are highly specific to flucloxacillin (Figure S3) to detect intracellular protein adduct formation. Coomassie blue SDS-PAGE shows no alteration in protein abundance across flucloxacillin treatments in lysates extracted from HepG2 and HepaRG cell lines (Figure 1A). Western blot analysis using anti-flucloxacillin antibody reveals covalent binding after 24 h of treatment in both cell lines in a dose-dependent manner (Figure 1B). It is important to note that flucloxacillin forms protein adducts in HepaRG cells at lower concentrations compared with HepG2 cells, indicating cellular disposition of flucloxacillin is different between these two cell types. It has been shown that the efflux transporter, multidrug resistance-associated protein 2 (MRP2), is expressed equivalently in HepaRG and primary hepatocytes, however, it is not expressed in HepG2 monolayer cell cultures. Another efflux transporter, P-glycoprotein (P-gp), was found to be expressed equivalently in HepG2 cells, while overexpressed in HepaRG cells.[9] The different expression levels of efflux transporters may contribute to the cellular flucloxacillin concentration and consequently the levels of protein adduct. Immunocytochemistry further confirmed that covalent binding in HepaRG cells was dose dependent (Figure 1C).

We next employed immunocytochemistry to determine the localization of flucloxacillin-modified proteins using HepaRG cells. HepaRG cells were selected for this study due to their ability to terminally differentiate into hepatocellular epithelial-like cells and form bile canaliculi (BC). The expression of the efflux membrane transporters MRP2 and P-gp on HepaRG cells was detected using anti-MRP2 and anti-P-gp antibodies. These transporters were localized within the tight actin bundles (Figure S4A). The activity of MRP2 and P-gp was assessed using CMFDA, a membrane-impermeant fluorescent dye. Inhibition of MRP2 and P-gp (using MK571 and valspodar, respectively) resulted in a reduced CMFDA accumulation within the HepaRG BC (Figure S4B). Upon incubation of HepaRG cells with 1.5 mM flucloxacillin, immunofluorescence imaging revealed a localization of protein adducts within the BC, mirroring the localization of MRP2 and P-gp. These data indicate the active transport of flucloxacillin from hepatocytes into the bile epithelia (Figure 2A). We then examined the effect of drug treatment on BC morphology using an anti-MRP2 antibody. Immunofluorescence imaging showed that exposure to flucloxacillin (1.5 mM) led to BC dilatation in a time-dependent manner. Initial BC dilation was visualized upon 24 h treatment, with further dilation occurring after 7 days (Figure 2B).

To further examine the effect of transporters on the covalent binding of flucloxacillin to hepatocellular proteins, MK571 and valspodar were used to block the activity of MRP2 and P-gp, respectively. Cells were cultured in the presence of 1.5 mM flucloxacillin overnight with the addition of one or both of the transporter blockers. Prior to incubation with flucloxacillin, cells were pretreated with their respective blockers for 1 h. In the control (no membrane transporter inhibitors), flucloxacillin can easily be seen localizing within the BC (Figure S5A). Upon the addition of the MRP2 block, the levels of flucloxacillin adducts localized within BC reduced. Instead, covalent binding of flucloxacillin contained within the cytoplasm appeared to be increased (Figure S5B). Interest-

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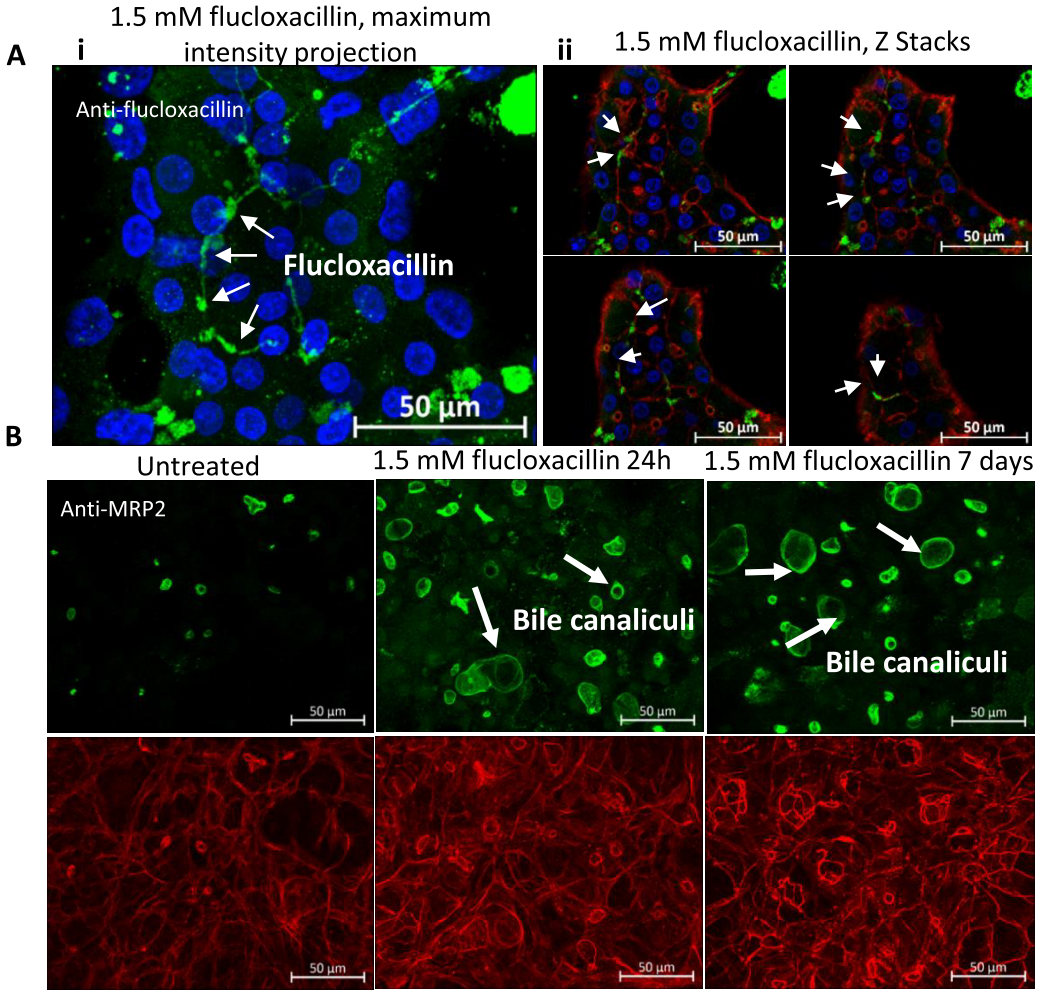

Figure 2. Localization of flucloxacillin protein adducts. (A) Flucloxacillin (1.5 mM) treatment (green) for 16 h resulted in the localization of drug within the bile canaliculi, as observed in the maximum intensity projection (i) and Z-stack images (ii) (white arrows). (B) Anti-MRP2 antibody (green) was used to detect the expression of MRP2 in HepaRG cells to identify biliary cells. Bile canaliculi were shown to become dilated after prolonged flucloxacillin exposure. Red = F-actin and blue = nuclear staining.

ingly, inhibition of P-gp had no observable effects on flucloxacillin adducts formation in BC (Figure S5C). As expected, the levels of intracellular flucloxacillin modification were increased when both MRP2 and P-gp were inhibited (Figure S5D,E).

The flucloxacillin protein adducts located at the BC, the primary site of tissue injury in patients with cholestasis, could potentially activate both immune and non-immune pathways involved in liver injury. We have recently shown that flucloxacillin protein adducts formed in immune cells can be processed and presented by HLA-B*57:01.[7] It is plausible that the protein adducts within BC can be presented by the local antigen presenting cells, e.g. cholangiocytes, leading to activation of residential immune cells.[10] In addition, we have demonstrated that hepatocyte-derived exosomes transport flucloxacillin-modified liver proteins to dendritic cells. Peptides derived from the flucloxacillin-modified liver proteins activated naïve T-cells from healthy human donors.[11] Therefore, flucloxacillin hepatocyte-derived antigenic signals can activate immune cells through either direct or indirect presentation. On the other hand, covalent binding of flucloxacillin to proteins within BC may induce local hepatic stress. As a molecular initiating event, the hepatic stress may promote pathological inflammatory responses via multiple pathways. Flucloxacillin has recently been shown to induce direct cholestatic injury in in vitro cultured human HepaRG cells through PKC/P38 and PI3K/AKT signaling pathways. At high concentrations (>6 mM), cholestatic injury was evidenced by dilation of BC, reduced bile acid efflux, and secretion of hepatocyte pro-inflammatory cytokines such as IL-6 and IL-1β.[1,2,12] The alteration of BC morphology by flucloxacillin was further confirmed by the current study when a lower concentration of drug was used (1.5 mM). 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.[11] Thus, signaling pathways between flucloxacillin-treated hepatocytes and immune cells could potentiate drug- and tissue-specific adaptive immune attack.

Collectively, these data suggest that covalent binding of flucloxacillin to local proteins at the site of tissue injury may activate both immune and non-immune-mediated pathways, leading to liver injury. It is important to note that the levels of covalent binding and the dilation of BC are concentration and time dependent, indicating accumulation of protein adducts at the site of tissue injury could be an important risk factor. Although flucloxacillin-induced liver injury is idiosyncratic and associated with multiple risk factors, the risk of injury was

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indeed increased with the increasing number of prescriptions and prolonged duration of use.[13] Further studies are warranted to establish the quantitative relationship between the covalent binding and toxicological outcome. Further investigation of whether flucloxacillin is a substrate or inhibitor of MRP2 and P-gp will help define the roles of these transporters in flucloxacillin-induced cholestatic liver injury.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00400.

Methods and additional figures (Figure S1−S5) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MRP2, multidrug resistance-associated protein 2; P-gp, P-glycoprotein; BC, bile canaliculi.

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