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Abstract

Around one hundred drugs of our modern pharmacopeia are efficacious and usable as antibiotics in medicine; they are used to kill or block growth of bacteria. Bactericidal antibiotics can induce a common oxidative damage pathway, leading to the production of reactive oxygen species and cell death. Antibiotics can also damage various mammalian cell types and tissues but mechanisms of action remain relatively unclear. Both bactericidal and bacteriostatic antibiotics can target mitochondria but only the former usually induce mitochondrial dysfunction and oxidative stress at clinically relevant doses. Human liver is a major target of antibiotics of which toxicity is mostly idiosyncratic. Interestingly, β-lactam penicillinase-resistant antibiotics, which are known to cause mostly immune reactions in patients, induce an early endoplasmic reticulum stress in *in vitro* human hepatocytes at low concentrations; this stress is inhibited by activation of the HSP27 protein which acts as a protective response associated with occurrence of cholestatic features. In this review, we analyze the importance of oxidative and endoplasmic reticulum stress in cellular damage induced by antibiotics, especially in hepatocytes and highlight specific cellular protection mechanisms associated with penicillinase-resistant antibiotic treatments.

1. Introduction

Despite the identification of thousands of antibiotics (ATBs), only around one hundred compounds are used for treatment of infected patients. They comprise several classes; the most important being β-lactams (penicillins, cephalosporins), quinolones, aminosides and macrolides. They are used to kill (bactericide) or block growth (bacteriostatic) of bacteria. ATB targets and modes of action have been widely studied and relatively well characterized in bacteria. Irrespective of their drug-target interactions, bactericidal ATBs can induce a common oxidative damage in bacteria, leading to the production of reactive oxygen species (ROS) and to DNA, protein, and lipid damage which results in cell death [1-5]. As an example, interaction of aminoglycosides with ribosomes can cause mistranslation and abnormal folding of membrane proteins, leading to production of toxic peptides and subsequent activation of the bacterial envelope stress response, oxidative stress generation and finally cell death [2]. However, generation of oxidative stress as a common mechanism involved in killing of bacteria has been debated [6]. Conflicting results are likely related to differences in specificity and sensitivity of the methods employed for the detection of ROS and the use of several methods has been recommended [7].

All bactericidal drug classes can utilize internal iron released from iron-sulfur clusters to promote Fenton-mediated hydroxyl radical formation and these events appear to be mediated by the tricarboxylic acid cycle and transient depletion of NADH [1]. Hydroxyl radicals can directly damage DNA, proteins and lipids. Bactericidal ATB effects can be alleviated by administration of the antioxidant *N*-acetylcysteine or prevented by preferential use of bacteriostatic ATBs [8]. Indeed, bacteriostatic drugs such as tetracycline and the macrolide erythromycin (ERY) do not stimulate hydroxyl radical production [1].

A large set of ATBs can also damage mammalian tissues and cells; however their modes of action are not as well characterized as in bacteria [8]. Deleterious effects are usually produced by clinically relevant doses of bactericidal ATBs in only few treated patients. Regardless of their molecular targets, the major classes of bactericidal ATBs quinolones, β-lactams and aminoglycosides can induce mitochondrial dysfunction in various mammalian cell types [8]. Studies in *in vitro* cell models and in mice have revealed parallel ATB-target

interactions in mammalian mitochondria and bacteria [9-11]. Thus, quinolones target bacterial gyrases [12] and mitochondrial DNA topoisomerase 2 [13], β-lactams disturb bacterial cell wall functioning [14] and mitochondrial carnitine/acylcarnitine translocase [15], and aminoglycosides target both bacterial [16] and mitochondrial ribosomes [17]. The present review focuses on the induction of oxidative and endoplasmic reticulum (ER) stress by ATBs in mammalian cells, especially hepatocytes, and highlights protective molecular mechanisms involved in response to stress induced by the penicillinase-resistant antibiotic (PRA) family.

2. **The liver is a major target organ for adverse side reactions induced by ATBs**.

ATBs can induce adverse side effects in various tissues and cells, e.g. ototoxicity [17, 18], nephrotoxicity [19], and tendinopathy [11]. A major target is the liver. ATBs represent the most common causes of drug-induced liver injury (DILI) [20] and have been associated with high rate of morbidity as well as many cases of liver transplantation and death resulting from acute liver failure [21]. ATB-induced hepatotoxicity is mostly idiosyncratic, occurring in rare treated patients and possibly through an immunological reaction or in response to generation of reactive metabolites and/or formation of protein adducts. Its frequency depends on the ATB. Liver injury induced by the major ATB classes frequently includes hepatocellular damage and cholestasis but can be restricted to cholestasis (bland cholestasis).

The β-lactam PRA family, including amoxicillin/clavulanic acid, cloxacillin, nafcillin, and most notably flucloxacillin (FLX), is known to cause severe liver injury. Several susceptible factors such as female sex, age, high daily doses and especially HLA-B*57:01 allele have been shown to be associated with higher risk of liver injury due to FLX. This ATB is a highly prescribed semi-synthetic β-lactam PRA for staphylococcal infections. It is estimated to cause cholestasis liver injury in about 8/100,000 patients [22], making it a significant medical problem [23]. FLX is the most common reason of idiosyncratic liver injury in Sweden and the second most common cause of drug-induced cholestasis in the United Kingdom, respectively

[24, 25]. It was assumed that liver injury under FLX treatment occurred through an immunological mediated reaction; however, these have not been observed in a substantial proportion of patients, suggesting that non-immune mechanisms may also be operative in human liver [26]. In a recent study, using the metabolically competent HepaRG cell line [27] and primary human hepatocytes we demonstrated that PRAs induced early cholestatic features typified by impaired dynamics and dilatation of bile canaliculi, and reduced bile acid efflux in absence of immune response and in non-cytotoxic conditions. Cytotoxic damage was observed only with high PRA concentrations [28].

Quinolones are generally safe and effective with few examples of liver toxicity. One exception is trovafloxacin (TVX), the use of which can cause idiosyncratic hepatocellular injury and cholestasis [29]. This drug has been reported to have an incidence rate of adverse hepatic effects of 5.6/100000 prescriptions with 10% fatal cases, leading to withdrawal from market [30]. Macrolides (particularly ERY, more rarely clarithromycin and azithromycin) may also cause cholestatic hepatitis [31]. Several other ATBs have been reported to induce liver injury, including tetracyclines, nitrofurantoin, sulfonamides, fusidic acid and isoniazid [32]. This later drug, an antituberculostatic agent, induces acute idiosyncratic hepatitis with hepatocellular necrosis [33].

3. **Antibiotics induce oxidative stress in human hepatocytes**

ROS comprise various species, such as superoxide anion, hydrogen peroxide and hydroxyl radical that can interact with different physiological and pathological targets. An excess of ROS causes mitochondrial dysfunction and enhanced permeability. Reactive aldehydes, e.g. 4-hydroxynonenal, can be released by ROS that inactivate the mitochondrial respiratory chain by impeding electron transport chain and activating oxidative stress [34]. Oxidative stress is associated with oxidative tissue damage and up-regulated expression of key genes involved in antioxidant defense mechanisms, such as heme oxygenase 1, manganese superoxide dismutase and NF-E2-related factor 2.

Both bactericidal and bacteriostatic ATBs can target mitochondria. However, at clinically relevant doses only the former, including β-lactams, quinolones and aminoglycosides, impair mitochondrial function. In an extensive study Kalghatgi and co-workers [8] have shown that contrary to bacteriostatic ATBs such as tetracycline, bactericidal ATBs can cause various mitochondrial morphological and functional alterations *in vivo* and in primary cultures or cell lines of diverse cell types, leading to enhanced ROS production and cellular damage. These drugs can induce ROS in mitochondria by various mechanisms. In particular, they may act via disruption of the tricarboxylic acid cycle and inhibition of electron transport chain, primarily complexes I and III, which have been associated to decrease in mitochondrial membrane potential, ATP levels and respiratory capacity; these disturbances represent major sources of ROS formation. They can also cause oxidative stress by increasing NADPH oxidase activity and reducing antioxidant defenses [35] and by inducing relatively high levels of nuclear DNA damage [8].

Hepatocytes are characterized by high drug-metabolism and detoxifying capacity. ATBs act either directly or indirectly after their biotransformation into one or several toxic reactive metabolites by cellular enzymes such as cytochromes P450. Many hepatotoxicants produce reactive metabolites that covalently bind to liver proteins [36, 37]. Circulating albumin-conjugated adducts have been identified in PRA-treated patients [37, 38].

In some cases toxicant-adducted proteins can stimulate specific members of the heat shock protein (HSP) family that are thought to chaperone these non-native proteins leading to protection against cell death [39]. Among them the multifunctional protein HSP27 plays a critical role. Its abnormal phosphorylation has been closely linked to major diseases [40, 41]. HSP27 phosphorylation is essential in actin cytoskeleton organization and actin-dependent events in response to growth factors and stress (41, 42). Our recent data support this point of view in showing that early cholestatic features induced by PRAs in human hepatocytes, characterized by F-actin fiber relaxation and dilatation of bile canaliculi, are associated with inhibition of the Rho-kinase signaling pathway and that these effects are induced through activation of HSP27 [28].

As other HSPs, mainly 70 and 90, HSP27 exerts a crucial cytoprotective role in conditions such as oxidative and ER stress, chemical stress, immune reactions and abnormal protein folding [42]. In agreement, caspase 3 activity and ROS levels were found increased only after 6h with high FLX concentrations. In parallel, transcripts of heme oxygenase-1 (ROS marker), and ATF4 (activating transcription factor 4) and CHOP (CCAAT-enhancer-binding protein homologous protein) (ER stress markers) were upregulated [28]. Noteworthy, the protective effect of phosphorylated HSP27 observed with PRAs was associated with activation of the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signaling pathway, resulting in caspase 3 inhibition [28] (**Figure 1**). This pathway was not activated with other penicillin derivatives such as penicillin G or V, amoxicillin and ampicillin which only rarely cause DILI [28], suggesting that major differences in stress response can be observed depending on the ATB and its ability to activate the PI3K/AKT pathway. Moreover, the lower sensitivity of hepatocytes to PRA-induced injury, compared to poorly differentiated biliary cells and probably many other non-hepatic cells, could be explained by their high anti-oxidant capacity [28]. In addition, the data support the conclusion that in the clinic, besides immune reactions, PRAs can cause non-immune-mediated cholestasis which is not restricted to patients possessing certain genetic determinants.

Fluoroquinolones have also been reported to cause oxidative stress and mitochondrial membrane damage in various cell types. The most toxic drug TVX can cause oxidative stress in human tendon cells [9] and up-regulate related genes in cultured hepatocytes [43]. It has been shown to induce peroxynitrite stress in liver mitochondria of mice exhibiting heterozygous deficiency in mitochondrial superoxide dismutase 2 (Sod2), suggesting that a genetic defect in this enzyme might aggravate TVX-induced mitochondrial adverse effects [44]. These effects were associated with increasing cytosolic free calcium which likely resulted in activation of mitochondrial nitric oxide synthase activity, leading to increased formation of nitric oxide radical. Peroxynitrite was formed by the reaction between nitric oxide and superoxide; it is a very reactive oxygen species which is known to react with various mitochondrial targets including aconitase-2 and superoxide dismutase [44]. *In vitro*, TVX does not cause death of hepatocytes; however, interestingly if these cells are exposed to the drug in an inflammatory context created by co-addition of lipopolysaccharide or

tumor necrosis factor alpha they become sensitized to injury which is associated with sustained activation of c-Jun N-terminal kinase and increased caspase 3 activity [45]. Mechanisms involved in early interactions between TVX and tumor necrosis factor alpha deserve further investigations.

4. Early endoplasmic reticulum stress is induced by β-lactam penicillinase-resistant antibiotics

ER stress can be evidenced following drug treatment. Mechanisms involved remain largely unknown. ER stress and impaired protein folding can lead to significant production of ROS; it can also be a late event occurring after ROS overproduction [46]. Recent studies were performed in order to determine whether ER stress is associated with cholestatic and hepatocellular injury induced by ATBs with diverse chemical structures and therapeutic use [47]. A set of six ATBs comprising three cholestatic PRAs (FLX, cloxacillin and nafcillin), two fluoroquinolones (TVX and levofloxacin) and the macrolide ERY were investigated using differentiated HepaRG cells. Early accumulation of misfolded proteins was revealed by enhanced fluorescence of the small molecule thioflavin-T [48]; its association with phosphorylation of the unfolded protein response sensors, eukaryotic initiation factor 2 (eIF2α) and/or inositol requiring enzyme-1α (IRE1α), was evidenced with all tested hepatotoxic ATBs [47]. However, major differences were observed in fluorescence intensity between the tested ATBs. The highest pattern of fluorescence was observed with PRAs and ERY; indeed, ER stress was detected with low cholestatic non cytotoxic concentrations and strongly increased with cytotoxic concentrations while it was generated by the two fluoroquinolones only at concentrations that were cytotoxic after 24h treatment. The elevated ER stress observed with ERY could be related to the ability of this drug to be metabolized by and to covalently bind to cytochrome P4503A4 proteins that are located in ER membranes [31]. Interestingly, inhibition of early ER stress markedly restored bile acid efflux and prevented bile canaliculi dilatation in PRA-treated HepaRG hepatocytes [47]. Oxidative stress was observed later and with higher ATBs concentrations [47], supporting the clinical reports showing that PRAs induce mainly cholestasis and only rarely hepatocellular injury [49]. Accordingly, the protective HSP27-PI3K-AKT signaling pathway

was activated only in PRA-treated cells **(Figure 1**) and its inhibition by KRIBB3 and LY294002 inhibitors, resulted in significant enhanced ROS generation and caspase-3 activity [47]. Of note, ER stress preceding oxidative stress has also been observed in HepaRG cells [50] and renal proximal tubule cells [51] treated with cyclosporine A, another well-known cholestatic drug. Compared to PRAs the three other tested ATBs, i.e. TVX, levofloxacin and ERY, caused lower BC dilatation and tendency to constriction which is associated with major irreversible liver injury, by increasing their concentrations.Noteworthy, it would be of great interest to evaluate the clinical relevance of ER stress as well as protective potential of the HSP27-PI3K-AKT signaling pathway which were found to be activated by PRAs in *in vitro* treated human hepatocytes.

5. Conclusions and future directions

ATBs with different chemical structures can cause adverse side effects in various human tissues and cells, especially the liver. ATBs frequently cause cholestasis which most often is associated with hepatocellular injury typified by mitochondrial dysfunction and overexpression of ROS, leading to oxidative stress and cell death. Various ATBs can also induce early ER stress. However, only PRA family was found to counteract this stress by activating the HSP27 protein which acted as a protective response against cell death in cultured human hepatocytes at non-cytotoxic concentrations. Whether similar PRA effects can occur in *in vivo* rodent models and treated patients has to be determined. Noticeably, epidemiological studies would be very useful to better evaluate clinical consequences of oxidative damage caused by ATB treatments.

Up to now, most experimental studies on ATB-induced toxicity to mammalian tissues and cells have been performed using *in vivo* rodent models and 2-D human and animal cell cultures. More sophisticated approaches/models would be desirable. *In vitro* 3-D cell models containing several cell types should allow identifying the influence of cell-cell interactions. For instance, in addition to hepatocytes other liver cells, i.e. Kupffer, endothelial and stellate cells, also produce ROS and likely modulate the response of liver parenchymal cells [52]. Co-cultures associating immune cells could be more appropriate to estimate the influence of innate immunity in ATB-induced liver injury. Cholangiocytes also represent an important cell

target for ATBs such as amoxicillin/clavulanic acid and FLX of which induced injury mainly involves immune-mediated reactions [53]. Potential generation of ER and/or oxidative stress by these cells deserves further studies.

Various ATBs can induce cholestatic features *in vitro* that include intracellular accumulation of bile acids when hepatocytes are cultured in the presence of a mixture of exogenous bile acids [54]. The levels and profile of accumulated bile acids vary with composition and concentrations of bile acids added in the mixture and the duration of exposure. The influence of extracellular bile acid environment on ATB-induced ER and/or oxidative stress remains largely unknown.

In conclusion, obviously if major progress has been made in the characterization of mitochondrial targets and mitochondrial ROS sources molecular mechanisms underlying cell injury and particularly oxidative stress overexpression caused by ATBs in mammalian tissues still remain poorly understood. As examples, generation of ER stress, identification of protein adducts and protective cellular responses deserve further research. Further investigations should lead to the development of new therapeutic strategies for prevention of ATB-induced side effects in treated patients.

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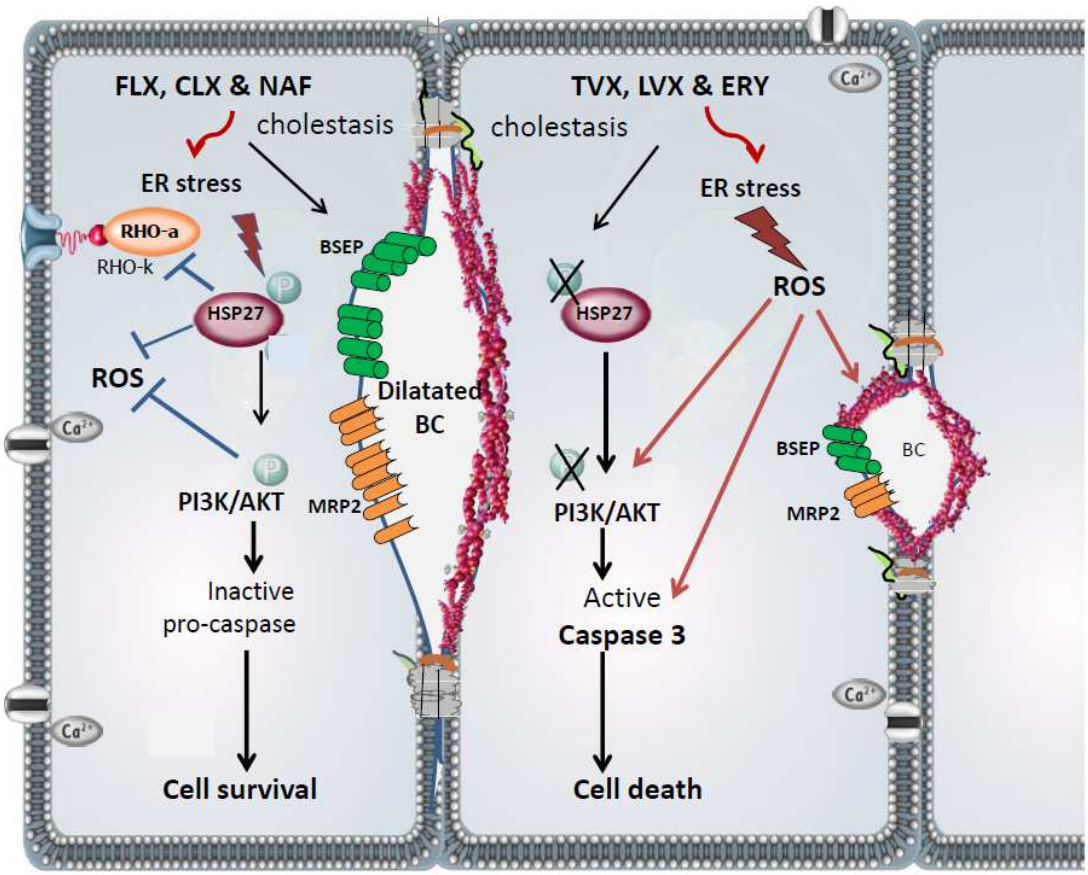
Figure 1



Legend to Figure 1

Proposed molecular mechanisms involved in ATB-induced cholestasis and hepatotoxicity. **(Left)** Penicillinase-resistant antibiotics (PRAs), i.e. flucloxacillin (FLX), cloxacillin (CLX), nafcillin (NAF), induce endoplasmic reticulum (ER) stress leading to pure cholestasis characterized by impaired dynamics and dilatation of bile canaliculi (BC) associated with inhibition of the Rho-kinase (Rho-k) signaling pathway and the bile acid transporters, bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2). PRA-induced ER stress activates HSP27 and the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway, preventing generation of reactive oxygen species (ROS) and activation of caspase-3, thus leading to cell survival. **(Right)** the two fluoroquinolones trovafloxacin (TVX) and levofloxacin (LVX) and the macrolide (ERY) induce ER stress leading to mixed cholestatic and cytotoxic effects. ER stress results in ROS generation and induction of caspase-3 activity. The protective HSP27-PI3K/AKT pathway is not activated in TVX-, LVX- and ERY-treated hepatocytes. BC dilatation induced by these three antibiotics is much lower than that observed with PRAs.

Annotated references

° REF. 1. This study demonstrates that bactericidal antibiotics induce a common mechanism of cell death in bacteria

°° REF. 8. An excellent description of mitochondrial dysfunction and oxidative damage induced in mammalian cells by bactericidal antibiotics

° REF. 45. This paper discusses possible molecular mechanisms of hepatocellular apoptosis induced by trovafloxacin-tumor necrosis factor-alpha interaction

° REF 47. This paper describes mechanisms involved in endoplasmic reticulum stress and oxidative stress induced by penicillinase-resistant and other antibiotics