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Mechanistic Review of Drug-Induced Steatohepatitis

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Abstract

Drug-induced steatohepatitis is a rare form of liver injury known to be caused by only a handful of compounds. These compounds stimulate the development of steatohepatitis through their toxicity to hepatocyte mitochondria; inhibition of beta-oxidation, mitochondrial respiration, and/or oxidative phosphorylation. Other mechanisms discussed include the disruption of phospholipid metabolism in lysosomes, prevention of lipid egress from hepatocytes, targeting mitochondrial DNA and topoisomerase, decreasing intestinal barrier function, activation of the adenosine pathway, increasing fatty acid synthesis, and sequestration of coenzyme A. It has been found that the majority of compounds that induce steatohepatitis have cationic amphiphilic structures; a lipophilic ring structure with a side chain containing a cationic secondary or tertiary amine. Within the last decade, the ability of many chemotherapeutics to cause steatohepatitis has become more evident coining the term chemotherapy-associated steatohepatitis (CASH). The mechanisms behind drug-induced steatohepatitis are discussed with a focus on cationic amphiphilic drugs and chemotherapeutic agents.

Keywords

Drug-induced steatohepatitis; Chemotherapy-associated steatohepatitis; Cationic amphiphilic drugs; carnitine palmitoyltransferase-I; mitochondrial toxicity

INTRODUCTION

Though many compounds are toxic to the liver, very few are capable of inducing steatohepatitis. The compounds that do cause this toxicity span a variety of therapeutic classes. Steatohepatitis is characterized by intrahepatic accumulation of lipids, metabolic syndrome, and hepatic inflammation. The ability of drugs to lead to the development of steatohepatitis is largely due to off therapeutic target effects which cause mitochondrial damage. The major mechanisms of mitochondrial toxicity involve the inhibition of fatty acid beta-oxidation, oxidative phosphorylation, and mitochondrial respiration, however, the exact mechanisms by which each drug effects these pathways vary. As beta-oxidation is one of the

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primary pathways by which lipids are metabolized, the inhibition of beta-oxidation leads to the observed accumulation of lipids within hepatocytes. Inhibition of oxidative phosphorylation and mitochondrial respiration is toxic to the mitochondria and can lead to the release of reactive oxygen species (ROS). Together, the buildup of intracellular lipids and ROS likely causes the formation of oxidized lipids and subsequent inflammation. The ability to concurrently stimulate the accumulation of lipids and ROS is of importance as drugs that solely cause lipid accumulation induce simple steatosis but rarely steatohepatitis.

Several generalized models exist that attempt to explain the development of steatohepatitis, either drug-induced or through non-exogenous mechanisms. The first is the “two-hit” model, which states that the steatohepatitis develops in response to two sequential events; first the accumulation lipids followed by a second injury that stimulates inflammation (Day and James, 1998). The second model states that steatohepatitis is a systemic disease of inflammation shared with atherosclerosis and obesity. In this second model, in variance with the “two-hit” model, accumulation of lipid and inflammation occur simultaneously (Bieghs *et al.*, 2012). With this in mind, drug-induced steatohepatitis falls into the systemic inflammation model as drugs that induce steatohepatitis simultaneously cause both hits, i.e., fat accumulation and secondary injury with inflammation. The detailed mechanisms by which various xenobiotics induce steatohepatitis will be discussed below. Focuses will be placed on cationic amphiphilic drugs (CAD), chemotherapy agents, and two additional compounds, valproic acid and tetracycline, which cause steatohepatitis by mechanisms not shared by CADs and chemotherapeutic agents.

Before discussing the previously mentioned compounds, it is warranted to give a brief overview of mitochondrial function due to the key involvement of mitochondrial toxicity in drug-induced steatohepatitis (Figure 1). Mitochondria are responsible for ATP synthesis, as well as lipid and carbohydrate metabolism. These three functions are all interconnected. Lipid metabolism via beta-oxidation creates acetyl-Coenzyme A (CoA), which is subsequently used in the tricarboxylic acid (TCA) cycle. The utilization of acetyl-CoA in the TCA cycle then drives further beta-oxidation. Beta-oxidation and the TCA cycle both contribute to the electron gradient necessary for mitochondrial respiration and ATP synthesis. Due to the reliance upon each other, the disruption of any of these processes can have downstream effects on all the others (Pessayre *et al.*, 2012). Finally, an important enzyme to note is carnitine palmitoyltransferase I (CPT-I) as it is involved in the mechanism of toxicity of nearly all steatohepatitis inducing drugs. CPT-I shuttles fatty acid-CoA into the mitochondria for subsequent beta-oxidation. The entry of lipids into the mitochondria by CPT-I is the rate limiting step of beta-oxidation.

CATIONIC AMPHIPHILIC DRUGS

A group of compounds with similar molecular makeup, cationic amphiphilic structures, have been identified to be able to induce steatohepatitis (Figure 2). Cationic amphiphilic drugs (CAD) are characterized by a hydrophobic ring structure with a hydrophilic cationic side chain. Several of these compounds can prompt the development of steatohepatitis via their toxicity to mitochondria. More specifically, many CADs are able to inhibit fatty acid beta-oxidation, uncouple oxidative phosphorylation, and inhibit complexes of the electron

transport chain. The cationic side chain of these compounds, often a secondary or tertiary amine, can become protonated. When the protonated form of the drug enters the mitochondrial matrix, the proton dissociates and uncouples oxidative phosphorylation. The drug kept inside the mitochondria can interact with enzymes involved in beta-oxidation and electron transport (Pessayre *et al.*, 2012). Two cationic amphiphilic molecules well studied for their ability to induce steatohepatitis include amiodarone and perhexiline.

Amiodarone

The iodinated benzofuran compound, amiodarone, has been extensively used for the treatment of various cardiac arrhythmias for nearly three decades. Over this period of time, the ability of amiodarone to induce steatohepatitis has been widely studied. This toxicity has been found to be dependent on cumulative dose and corresponding drug accumulation within hepatocytes. Amiodarone is an extremely lipophilic compound with a half-life ranging between 15–142 days (Pfizer, 2014) and easily accumulates within cells. Once concentrated within hepatocytes, amiodarone affects numerous pathways which lead to the development of steatohepatitis. Specifically, amiodarone induces the buildup of fatty acids through the inhibition of phospholipase A (Shaikh *et al.*, 1987) and inhibition of fatty acid beta-oxidation (Fromenty *et al.*, 1990). The observed inhibitory effect on beta-oxidation is a result of CPT-I inhibition (Kennedy *et al.*, 1996). Amiodarone is also capable of inducing phospholipidosis. The compound concentrates in lysosomal lipid bilayers and interacts with membrane phospholipids subsequently preventing phospholipid degradation (Mesens *et al.*, 2012). Aside from causing the buildup of cellular lipids, amiodarone can simultaneously induce the accrual of ROS and oxidized lipids through its toxicity to mitochondria. This toxicity occurs when amiodarone and several of its metabolites inhibit the electron transport chain complexes I, II, and III and uncouples oxidative phosphorylation (Spaniol *et al.*, 2001).

A structure-activity relationship study found that the amiodarone's benzofuran ring structure is critical for causing mitochondrial, however, the iodine moieties attached to the ring are not necessary (Spaniol *et al.*, 2001). Dronedarone, brand name Multaq®, was approved for the treatment of atrial fibrillation by the FDA in 2009 and is a de-iodinated benzofuran compound. Since its approval, several case reports of dronedarone induced liver injury have already been reported spurring the release of a drug safety communication from the FDA in 2011 (FDA, 2011). This communication stated that among the reported cases two patients developed acute liver injury requiring liver transplantation (Joghetaei *et al.*, 2011). To date, very few mechanistic toxicity studies focusing on hepatotoxicity have been performed on dronedarone. As would be predicted, it appears dronedarone inhibits fatty acid beta-oxidation *in vitro* (Felser *et al.*, 2013) and *in vivo* (Felser *et al.*, 2014). Like other benzofurans, dronedarone also inhibits mitochondrial respiration *in vitro*; uncoupling respiration, decreasing mitochondrial membrane potential, and inhibiting complexes I and II of the electron transport chain (Spaniol *et al.*, 2001). However, these effects on mitochondrial respiration were not present during *in vivo* mouse and rat studies (Serviddio *et al.*, 2011; Felser *et al.*, 2014). It has been proposed that the conflicting *in vitro* and *in vivo* observations may be attributed to the lack of necessary local mitochondrial drug concentration. Dronedarone has an extremely short half-life in comparison to amiodarone;

13–19 hours versus 15–142 days respectively (Pfizer, 2014; Sanofi-Aventis, 2014). Due to its much short half-life, dronedarone may not be able to accumulate intracellularly like amiodarone. This may explain how local concentrations of amiodarone may become large enough to disrupt mitochondrial respiration.

Perhexiline

Another well studied CAD known to induce steatohepatitis is perhexiline. As evidenced by amiodarone, drug accumulation plays a key role in the mechanism of toxicity. Perhexiline induced steatohepatitis requires chronic dosing. Individuals with certain CYP2D6 polymorphisms are at greater risk of steatohepatitis as CYP2D6 is the predominant isoform that metabolizes perhexiline (Barclay *et al.*, 2003). The protonated form of perhexiline enters the mitochondria matrix, releases a proton in mitochondrial matrix, and thereby uncouples of oxidative phosphorylation. The accumulated perhexiline within the mitochondria can also inhibit both complexes I and II of the electron transport chain (Deschamps *et al.*, 1994).

Furthermore, perhexiline is capable of causing lipid buildup within hepatocytes at concentrations much lower than those necessary to affect ATP levels. Increases of palmitic acid and triglycerides by 38% and 98%, respectively, were observed in hepatocytes treated with 5 μ M of perhexiline; a concentration five times lower than the one that leads to ATP depletion (Deschamps *et al.*, 1994). Perhexiline causes the accumulation of lipids by inhibiting the beta-oxidation of short, medium and long chain fatty acids (Deschamps *et al.*, 1994) and inhibiting CPT-I (Kennedy *et al.*, 1996). Similar to amiodarone, perhexiline has been shown to accumulate within lysosome lipid bilayers, interact with phospholipids, and prevent their degradation by phospholipases (Bendirdjian *et al.*, 1982).

Briefly worth mentioning, many CADs can induce steatosis and phospholipidosis but not steatohepatitis. Some CADs known to induce phospholipidosis include propranolol (Leli and Hauser, 1987), chlorpromazine (Leli and Hauser, 1987), tricyclic antidepressants (Fisar, 2005), and chloroquine (Hostetler *et al.*, 1985). Tricyclic antidepressants (Le Dinh *et al.*, 1988) and pirofen (Geneve *et al.*, 1987) have been shown to inhibit beta-oxidation. Atypical antipsychotics are also CADs that have been shown to inhibit complex I of the electron transport chain (Casademont *et al.*, 2007). Other CADs to note that can induced steatohepatitis and will be described later include irinotecan and tamoxifen.

CHEMOTHERAPY-ASSOCIATED STEATOHEPATITIS

Recently it has been found that several chemotherapeutic agents can induce steatohepatitis thereby generating the term chemotherapy-associated steatohepatitis (CASH) in the literature. The agents currently of interest and primarily studied in the literature for CASH include tamoxifen, raloxifene, irinotecan, and methotrexate. The development of CASH can greatly affect patient mortality with one study finding an odds ratio of 10.5 for 90 day mortality rates in patients who develop CASH (Vauthey *et al.*, 2006). For this reason, a mechanistic understanding of how various chemotherapeutics induce CASH may provide valuable and insightful preventative measures to prevent CASH and increase survival rates.

The reported mechanisms by which tamoxifen, raloxifene, irinotecan, and methotrexate induce CASH will be discussed below.

Tamoxifen

The selective estrogen receptor modulator, tamoxifen, used for the treatment of estrogen dependent cancers and has been shown to induce steatohepatitis through its effects on mitochondria. Estrogen receptors ER α and ER β are both present in mitochondria (Chen *et al.*, 2004) although the local role of these receptors is not fully understood. It is known, however, that estrogen can affect the overall beta-oxidation activity in the cell (Maher *et al.*, 2010). An investigation looking at the mRNA levels of enzymes essential to fatty acid beta-oxidation was performed in Sprague-Dawley rats treated with estrogen. The rats dosed with estrogen were found to have increased mRNA levels of peroxisome proliferator-activated receptor alpha and gamma (PPAR α , PPAR γ), beta-3-hydroxyacyl CoA dehydrogenase (HADH β), CPT-I, and pyruvate dehydrogenase kinase 4 (PDK4) compared to control animals. The most dramatic increases in transcription due to estrogen treatment were of CPT-I and PDK4, displaying a seven-fold and twenty-five-fold increase in their mRNA levels, respectively (Campbell *et al.*, 2003). In addition, ER α also interacts with some of these proteins directly in addition to increase their transcription, and a proteomic study identified mitochondrial proteins that interact with local ER α . One of the proteins identified was HADH β . Through the interaction of HADH β and ER α , estrogen can increase the beta-oxidation capacity in the cell. This study was also able to show that tamoxifen can affect the association of HADH β to ER α and in turn decrease beta-oxidation activity (Zhou *et al.*, 2012). Though estrogen increases the expression of CPT-1, tamoxifen treatment of HepG2 cells had no effect on CPT-I mRNA levels (Zhao *et al.*, 2014). Aside from its effects on beta-oxidation, tamoxifen may lead to the accumulation of lipids by increasing fatty acid synthesis. A study performed in HepG2 cells found a threefold increase in sterol regulatory element binding protein 1c (SREBP-1c) expression and subsequent increase in expression of SREBP-1c response genes involved in fatty acid synthesis (Zhao *et al.*, 2014).

Tamoxifen is a CAD and therefore induces steatohepatitis by many mechanisms unrelated to mitochondrial estrogen receptors. Though it did not affect CPT-I mRNA levels, tamoxifen was found to inhibit beta-oxidation by lowering the activity of CPT-I in isolated liver mitochondria (Larosche *et al.*, 2007). This observed effect required extremely high concentrations of tamoxifen of at least 50 μ M. This same study treated mice with tamoxifen for 28 days and found that tamoxifen can intercalate mitochondrial DNA (mtDNA), inhibit mitochondrial topoisomerases, deplete mitochondrial DNA, and lower the synthesis of enzymes involved in mitochondrial respiration (Larosche *et al.*, 2007). The decreased production of the enzymes involved in mitochondrial respiration sensitizes the mitochondria for further injury as tamoxifen directly inhibits complexes III and IV of the electron transport chain (Tuquet *et al.*, 2000). Like other CADs, such as amiodarone and perhexiline, drug accumulation plays an important role in the toxicity of tamoxifen. The described toxicities above require high concentrations of drug to induce toxic effects made possible by the ability of tamoxifen to concentrate in the mitochondria. Isolated liver mitochondria treated *in vitro* with tamoxifen for one minute were found to contain a local concentration of 640 μ M of drug compared to the 16 μ M left in the media (Larosche *et al.*, 2007).

Irinotecan and Oxaliplatin

A subset of patients at particular risk for developing CASH are metastatic colorectal cancer patients as the mainstays of treatment are regimens containing oxaliplatin and/or irinotecan; FOLFOX regimen (5-FU, leucovorin, oxaliplatin), FOLFIRI regimen (5-FU, leucovorin, irinotecan), and FOLFIRINOX regimen (5-FU, leucovorin, irinotecan, and oxaliplatin). The development of CASH in colorectal cancer patients undergoing hepatic metastasis resection was investigated in two studies. The first study found the prevalence of steatohepatitis to be significantly increased in patients treated prior to surgery with irinotecan compared to no chemotherapy (20.2 vs 4.4% respectively, $p = 0.001$), however no significant difference was found in the prevalence of CASH in patients treated with oxaliplatin (6.2% vs 4.4%). Though oxaliplatin did not increase steatohepatitis rates, it did significantly affect the amount of patients with grade 2 or 3 sinusoidal dilation (18.9% vs. 1.9%, $p < 0.001$) (Vauthey *et al.*, 2006). Sinusoid dilation is characterized as the widening of hepatic capillaries often in response a backflow caused by portal or sinusoid obstruction. The second study sought to determine if the severity of CASH developed in patients receiving irinotecan and oxaliplatin prior to hepatic metastasis resection was worse than in controls not receiving chemotherapy before resection. Patients were treated pre-operatively with either: 1) irinotecan and/or oxaliplatin, 2) 5-FU, or 3) no chemotherapy. CASH was graded semi-quantitatively using the Brunt system (Brunt *et al.*, 1999). The irinotecan/oxaliplatin treatment arm was found to have significantly worse scores compared to the no chemotherapy and 5-FU treatment arms (1.9 vs 1.2 vs 1.1 respectively; $p = 0.003$) (Fernandez *et al.*, 2005). The irinotecan/oxaliplatin arm only contained four patients receiving oxaliplatin and therefore predominately consisted of patients on irinotecan.

These two studies together indicate that though oxaliplatin induces liver injury, particularly sinusoidal dilation, its ability to induce steatohepatitis is limited. The primary chemotherapeutic which induces steatohepatitis in colorectal cancer patients is irinotecan, however, the mechanisms by which irinotecan causes steatohepatitis remains unknown. A mouse model for irinotecan associated steatohepatitis has only recently been developed (Costa *et al.*, 2014). Irinotecan is used as a cytotoxic chemotherapeutic agent, which binds to DNA/topoisomerase-1 complexes and prevents the recoiling of DNA. It is known that a related compound, topotecan, is capable of interacting with mtDNA/topoisomerase complexes (Kosovsky and Soslau, 1993). As mitochondrial dysfunction often plays a critical role in the development of drug-induced steatohepatitis, it has therefore been postulated that mitochondrial toxicity plays a key role in the mechanism by which irinotecan causes steatohepatitis.

The mechanism of toxicity for irinotecan induced steatohepatitis may have many similarities to that of tamoxifen. Both irinotecan and tamoxifen may intercalate mtDNA and lead to the decreased synthesis of enzymes involved in electron transport. Irinotecan is also another example of a CAD. For this reason, uncoupling of oxidative phosphorylation or inhibition of mitochondrial respiration maybe potential mechanisms by which irinotecan exerts its toxic effects on mitochondria. A recent study identified a novel inhibitor of oxidative phosphorylation, VLX600, which synergistically increases the cytotoxicity of irinotecan to cultured cells (Zhang *et al.*, 2014). Multicellular spheroids were used rather than monolayer

cultures in order to create metabolic microenvironments that mimic those in solid tumors than monolayer cultures. This study also tested VLX600 against xenografts in NMRI mice. VLX600 is a CAD which inhibits complexes I, II, and IV of electron transport. It is possible that VLX600 synergistically increases the cytotoxicity of irinotecan by potentiating irinotecan's effects on mitochondrial respiration.

Methotrexate

The anti-folate chemotherapeutic agent and immunosuppressant, methotrexate (MTX) has been found to induce steatohepatitis. The development of steatohepatitis due to MTX exposure has been shown to be dependent on cumulative doses with those above four grams putting patients at high risk for the development of steatohepatitis (Arena *et al.*, 2012). Unfortunately, for diseases such as psoriasis and rheumatoid arthritis, many patients receive chronic low dose MTX therapy which over time can lead to very large cumulative doses. In cancer, patients receive large cyclic doses of MTX which can also lead to large cumulative doses. The link of cumulative doses to toxicity is likely due to the buildup of a polyglutamated metabolite of MTX within hepatocytes (Kremer *et al.*, 1986). Reports vary significantly in the ability of MTX to induce liver injury. One study reported that a staggering 26% of patients exposed to a cumulative dose of 1.5 grams of MTX developed cirrhosis (Zachariae *et al.*, 1980), while another study reported only 2.6% and 8.2% of patients developed cirrhosis for cumulative MTX dose exposure of 4.5 grams and 5 grams (Aithal *et al.*, 2004). Within the last decade, it has become apparent that the risk of developing steatohepatitis while treated with MTX is also dependent on the patient's disease state being treated (Aithal *et al.*, 2004). The differences in prevalence of MTX induced cirrhosis stated in the literature can likely be explained by the disease states present in the subset of patients selected for each study (Rosenberg *et al.*, 2007). One patient subset that shows the greatest risk of developing steatohepatitis due to MTX exposure is psoriasis patients. The skin lesions seen on psoriasis patients are only dermal manifestation of the underlying disease. Psoriasis is a disease of systemic inflammation (Hamminga *et al.*, 2006) and metabolic syndrome (Sommer *et al.*, 2006) which combined predisposes patients to steatohepatitis development.

Mechanistically, methotrexate may lead to the formation of steatohepatitis by many ways; mitochondrial dysfunction through the depletion of folate, increased gastrointestinal (GI) permeability, and activation of the adenosine pathway. MTX has been shown to be toxic to mitochondria by depleting mitochondrial folate stores. MTX has been found to have no effect on pre-existing mitochondrial folate levels as it is not transported into the mitochondria. However, MTX limits folate entry into the mitochondria and therefore preventing the replenishment of mitochondrial folate (Kim *et al.*, 1993). MTX stimulated mitochondrial dysfunction has been shown to then lead to the generation of reactive oxygen species (Tabassum *et al.*, 2010), disruption of mitochondrial membrane potential, and induction of caspase-dependent apoptosis (Huang *et al.*, 2005). Aside from its effects on mitochondria, MTX can also lead to steatohepatitis due to its toxicity to the GI tract. MTX damages the GI mucosa, disrupts intestinal barrier functionality, and allows for bacterial translocation from the GI tract to the liver. In one study, Sprague-Dawley rats were treated with MTX for three days and then given *E. coli* expressing green fluorescent protein (GFP)

via oral gavage. Intestinal barrier function was found to be disrupted and GFP expression *E. coli* were found in the liver via microscopy (Song *et al.*, 2006). A leaky GI tract and chronic presence of foreign material or bacteria in the liver can lead to the chronic activation of the immune system in the liver, which subsequently leads to steatohepatitis. Finally, the last mechanism by which MTX can affect steatohepatitis development is through the activation of the adenosine pathway. MTX at a concentration of 100 nM increases the release of adenosine from fibroblasts grown *in vitro* by three-fold (Cronstein *et al.*, 1991). The higher concentration of adenosine can over-activate the adenosine 2A receptor on hepatic stellate cells (Chan *et al.*, 2006) and in turn increases collagen production. Furthermore, it has been shown that adenosine also decreases the activity of stellate cell matrix metalloproteases and thereby decreases collagen degradation (Chan *et al.*, 2006).

ADDITIONAL COMPOUNDS CAUSATIVE OF STEATOHEPATITIS

The mechanisms driving the development of steatohepatitis for two final compounds, valproic acid and tetracyclines, shall be discussed. Though these compounds affect similar pathways as CADs to induce steatohepatitis, valproic acid and tetracyclines are important to discuss as they affect these pathways by different mechanisms.

Valproic Acid

Valproic acid is a compound used as an anticonvulsant and mood stabilizing drug. It is a small branched aliphatic compound containing a carboxylic acid. Inside the cytosol of hepatocytes valproic acid is conjugated to valproyl-CoA which competitively and non-competitively inhibits CPT-I (Aires *et al.*, 2010). It is important to note that CPT-I does not shuttle valproyl-CoA into the mitochondria. The free acid form of valproate enters the mitochondria by diffusion and is then converted to valproyl-CoA (Aires *et al.*, 2007). Valproyl-CoA inside the mitochondria is extensively metabolized via beta-oxidation to the eventual end products of acyl-CoA and propionyl-CoA (Silva *et al.*, 2008). During its metabolism by beta-oxidation, valproyl-CoA sequesters a large quantity of the mitochondria's CoA and greatly decreases the amount of free CoA (Ponchaut *et al.*, 1992). Without free CoA, fatty acids cannot undergo beta-oxidation. The sequestration may be worsened as branched acyl-CoA metabolite esters resist hydrolysis more than straight chain acyl-CoA esters. This has been confirmed experimentally; it has been found that despite CoA levels remaining constant during valproic acid exposure, the amount of free CoA is depleted (Kesterson *et al.*, 1984; Ponchaut *et al.*, 1992). The depletion of free CoA is juxtaposed to a simultaneous increase in the amount of medium chain acyl-CoA. Medium chain acyl-CoA accumulates as valproyl-CoA and its metabolites decreases the rate of beta-oxidation of long-chain and medium-chain acyl-CoA (Silva *et al.*, 2001). Valproyl-CoA also affects beta-oxidation through the competitive and non-competitive inhibition of CPT-I (Aires *et al.*, 2010).

Tetracycline antibiotics

The tetracycline class of antibiotics has been known for decades to induce steatohepatitis in patients treated with large intravenous doses. The prevalence of liver injury in patients treated orally with normal doses is extremely rare (Bjornsson *et al.*, 1997). Tetracycline

induced steatohepatitis is characterized by the marked accumulation of triglycerides within hepatocytes. This accumulation of triglycerides is resultant of several identified mechanisms; tetracyclines prevent the egress of triglycerides out of hepatocytes, down-regulate genes involved in beta-oxidation, potentially up-regulate genes dictating lipogenesis, and lead to the carbonylation and decreased activity of the enzymes involved in beta-oxidation. Tetracyclines and some of the previously described steatosis inducing drugs (amiodarone, pirprofen, and the tricyclic antidepressants amineptine and tianeptine) have been found to inhibit the enzyme microsomal triglyceride transfer protein (MTP). MTP activity leads to the lipidation of apoB with triglycerides and allows for the formation of VLDL particles. By inhibiting MTP, tetracyclines prevent the efflux of VLDL particles out of hepatocytes and subsequently lead to the accumulation of triglycerides within the cell (Letteron *et al.*, 2003). Triglycerides also accumulate due to overall decreased beta-oxidation within the cell (Freneaux *et al.*, 1988). Supporting these findings, a recent transcriptome study performed in rats revealed that tetracycline can decrease the expression of genes involved in beta-oxidation such as PPAR α , CPT-I, and fatty acid binding protein 1 (Szalowska *et al.*, 2014). This same study found no effect of tetracycline on the genes involved in fatty acid synthesis. This conflicts with another study that observed the up-regulation of PPAR γ and SREBP-1c in HepaRG cells (Antherieu *et al.*, 2011). The conflicting observations were explained as a potential result of interspecies variation.

The tetracycline compounds doxycycline and minocycline stimulate oxidative stress in hepatocytes via the activation of activating transcription factor 4 (ATF4) and inhibition of the mammalian target of rapamycin (mTOR) (Bruning *et al.*, 2014). In hepatocytes, ATF4 leads to the generation of ROS via up-regulation of CYP2E1. Treatment of hepatocytes with a CYP2E1 specific inhibitor, diallyl sulphide, prevents ATF4 stimulated ROS (Wang *et al.*, 2014). Hepatocyte deficiency of ATF4 prevents oxidative stress and also was found to mitigate triglyceride accumulation (Wang *et al.*, 2014; Xiao *et al.*, 2013). The protective effect of ATF4 deficiency on triglyceride accumulation may be explained by decreased lipogenesis. Knockout of ATF4 in mice was found to lead to a down-regulation of PPAR γ , SREBP-1c, and fatty acid synthase (Xiao *et al.*, 2013). A proteomic study found that the oxidative stress induced by tetracyclines on hepatocytes has been shown to lead to the carbonylation and decreased activity of key proteins involved in beta-oxidation (Deng *et al.*, 2015). This may pose another explanation how ATF4 deficiency prevents triglyceride accumulation. Some of the proteins found to be carbonylated include acyl-CoA dehydrogenase long chain (ACADL), and electron transfer flavoprotein subunit beta (ETF), ornithine carbamoyltransferase (OCT), malate dehydrogenase (MDH), methylcrotonoyl-CoA carboxylase beta chain (MCC), and methylmalonate semi-aldehyde dehydrogenase (MSAD). ACADL is a key enzyme required for the beta-oxidation of long chain fatty acids. Inhibition of ACADL via oxidative damage has been shown to lead to steatosis (Zhang *et al.*, 2007). ETF is an electron acceptor for dehydrogenases involved in fatty acid beta-oxidation and connects beta-oxidation to the electron transport chain.

Though not investigated by the proteomic study or any other study, it is possible that the inhibition of OCT, MDH, MCC, and MSAD together can lead to the sequestration of CoA and induced steatosis in a mechanism similar to that described for valproate. OCT is the first

step of the urea cycle which can drive the TCA cycle through the aspartate-arginosuccinate shunt. MDH converts malate to oxaloacetate in the TCA cycle. The decreased activity of OCT and MDH together may lead to the shutdown of both the urea and TCA cycles and lead to a buildup of acetyl-CoA. MCC and MSAD are involved in branched amino acid degradation. Their inhibition would lead to the buildup of branched-amino-acid-CoA conjugates. Therefore as seen in valproate toxicity, the CoA levels in the mitochondria would not be affected by tetracyclines, however free CoA would be sequestered away in the forms of acetyl-CoA and branched-CoA derivatives.

CONCLUSION

Mitochondrial dysfunction plays a critical role in the development of drug-induced steatohepatitis. Inhibition of beta-oxidation, mitochondrial respiration, and oxidative phosphorylation can lead to the accumulation of intracellular lipids and ROS. A group of compounds with a cationic amphiphilic structures have been identified to be potentially able to interfere with these processes. These CADs consist of lipophilic ring structures with cationic side chains containing secondary or tertiary amines. Not all CADs induce steatohepatitis; some only have the ability to stimulate simple steatosis or phospholipidosis. Compounds and therapeutic classes with cationic amphiphilic structures include benzofurans, perhexiline, tamoxifen, atypical antipsychotics, tricyclic antidepressants, chloroquine, pirprofen, and propranolol. Some compounds induce steatohepatitis through additional mechanisms besides the inhibition of beta-oxidation, mitochondrial respiration, and oxidative phosphorylation. Other described mechanisms include disruption of phospholipid metabolism in lysosomes, prevention of lipid egress from hepatocytes, targeting mitochondrial DNA and topoisomerase, decreasing intestinal barrier function, activation of the adenosine pathway, increasing fatty acid synthesis, and sequestration of coenzyme A.

As previously described, the majority of drugs that induce steatohepatitis inhibit fatty acid beta-oxidation. As CPT-I is the rate limiting step of beta-oxidation, CPT-I represents a critical enzyme whose inhibition can have great effects on the beta-oxidation activity within the mitochondria. All the compounds, except irinotecan and MTX, described in this paper that induce steatohepatitis alter the functionality of CPT-I. The mechanism by which irinotecan induces steatohepatitis is still mostly unknown, and as irinotecan is a CAD, CPT-I inhibition may be possible. The CADs amiodarone and perhexiline, as well as the non-CAD valproyl-CoA, have been shown to inhibit CPT-I competitively in regards to palmitoyl-CoA and non-competitively in regards to carnitine (Kennedy *et al.*, 1996; Silva *et al.*, 2001). The competitive inhibition of CPT-I by these three compounds was identified as the result of drug interactions with the palmitoyl-CoA binding domain on CPT-I. It has been speculated that these compounds may also inhibit CPT-I activity by modulating the sensitivity of CPT-I to malonyl-CoA, the natural inhibitory ligand of CPT-I. Valproate has also been found to decrease CPT-I expression through the down-regulation of PPAR α (Wang *et al.*, 2012). Similarly, tetracyclines lead to the down-regulation of PPAR α and subsequently decrease CPT-I expression (Szalowska *et al.*, 2014). Tamoxifen was found to decrease CPT-I activity (Larosche *et al.*, 2007) but does not affect CPT-I transcription (Zhao *et al.*, 2014).

Lastly, the cytotoxic properties of various chemotherapeutic agents play a role in the induction of CASH. Through their intended chemotherapeutic properties compounds such as irinotecan, tamoxifen, and MTX can be directly toxic to mitochondria; intercalation of mtDNA, inhibition of mitochondrial topoisomerases, or causing mitochondrial folate deficiency. However, these agents can also lead to CASH via other mechanisms. CADs comprise compounds from many therapeutic classes including chemotherapeutics. The recognized ability of tamoxifen and irinotecan to cause CASH may also be attributable to their cationic amphiphilic structures.

Abbreviations

ACADL	acyl-CoA dehydrogenase long chain
ATF4	activating transcription factor 4
CAD	cationic amphiphilic drug
CASH	chemotherapy-associated steatohepatitis
CPT-I	carnitine palmitoyltransferase I
ETF	electron transfer flavoprotein
FOLFIRI	chemotherapy regimen containing 5-fluorouracil, leucovorin, and irinotecan
FOLFIRINOX	5-fluorouracil, leucovorin, irinotecan, and oxaliplatin
FOLFOX	5-fluorouracil, leucovorin, and oxaliplatin
GI	gastrointestinal
HADHβ	beta-3-hydroxyacyl-CoA dehydrogenase
MCC	methylcrotonyl-CoA carboxylase
MDH	malate dehydrogenase
MSAD	methylmalonate semi-aldehyde dehydrogenase
MTP	microsomal triglyceride transfer protein
MTX	methotrexate
OCT	ornithine carbamoyl transferase
PDK4	pyruvate dehydrogenase kinase 4
SREBP-1c	sterol response element binding protein 1c

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Highlights

- Reviewed the mechanisms underlying drug-induced steatohepatitis for many compounds
- Mitochondrial dysfunction is critical in the development of drug-induced steatohepatitis
- Majority of drugs that induce steatohepatitis are cationic amphiphilic drugs
- Chemotherapeutics that induce CASH are cationic amphiphilic drugs
- Majority of drugs that induce steatohepatitis are carnitine palmitoyltransferase-I inhibitors

Figure 1. Coordination of fuel oxidation and ATP synthesis within the mitochondria

TCA cycle and beta-oxidation occur within the mitochondrial matrix while electron transport complexes are imbedded within the inner mitochondrial membrane. TCA cycle and beta-oxidation are linked to mitochondrial respiration via NADH and FADH₂. Proton gradient driving oxidative phosphorylation located in intermembrane space. LCFA Long-chain fatty acid, PDH Pyruvate dehydrogenase, F-ATPase ATP synthase, ANT Adenosine nucleotide translocator.

Figure 2. Structures of several discussed cationic amphiphilic drugs

Common structural characteristics include lipophilic ring structures with side chains or additional rings which contain secondary or tertiary amines. (A) Amiodarone (B) Perhexiline (C) Amitriptyline (D) Propranolol (E) Pirprofen (F) Clozapine (G) Irinotecan (H) Tamoxifen

Table 1

Mechanisms by which each compound induces the development of steatohepatitis.

Drug	Mechanisms underlying development of steatohepatitis	Reference
Amiodarone	Intracellular drug accumulation important in developing steatohepatitis ($t_{1/2} = 14\text{--}152$ days)	Pfizer, 2014
	Inhibition of phospholipase A	Shaikh <i>et al.</i> , 1987
	Inhibition of fatty acid beta-oxidation	Fromenty <i>et al.</i> , 1990
	Inhibition of CPT-I	Kennedy <i>et al.</i> , 1996
	Drug accumulation in lysosomes prevents phospholipid degradation	Mesens <i>et al.</i> , 2012
Perhexiline	Inhibition of electron transport complexes I, II, III and uncoupling of oxidative phosphorylation	Spaniol <i>et al.</i> , 2001
	Development of steatohepatitis requires chronic dosing and drug accumulation. CYP2D6 polymorphisms increases susceptibility to drug accumulation	Barclay <i>et al.</i> , 2003
	Inhibition of beta-oxidation	Deschamps <i>et al.</i> , 1994
	Inhibition of CPT-I	Kennedy <i>et al.</i> , 1996
	Drug accumulation in lysosomes prevents phospholipid degradation	Bendirjian <i>et al.</i> , 1982
Tamoxifen	Inhibits electron transport complexes I and II, and uncouples oxidative phosphorylation	Deschamps <i>et al.</i> , 1994
	May disrupt estrogens ability to increase a cell's capacity for beta-oxidation. In Sprague-Dawley rats estrogen treatment leads to the upregulation of PPAR α , PPAR γ , HADH β , CPT-I, and PDK4	Campbell <i>et al.</i> , 2003; Maher <i>et al.</i> , 2010
	Inhibition of HADH β interactions with ER α thereby decreasing the amount of beta-oxidation within the cell	Zhou <i>et al.</i> , 2012
	Tamoxifen does not inhibit CPT-I upregulation in HepG2 cells	Zhao <i>et al.</i> , 2014
	Inhibition of CPT-I activity in isolated liver cells at concentrations above 50 μ M	Larosche <i>et al.</i> , 2007
Irinotecan	Accumulates within isolated liver mitochondria	Larosche <i>et al.</i> , 2007
	Intercalates mitochondrial DNA, inhibits mitochondrial topoisomerases, and lowers enzymes involved in mitochondrial respiration	Larosche <i>et al.</i> , 2007
	Increases fatty acid synthesis in HepG2 cells via upregulation of SREBP-1c and SREBP-1c response genes	Zhao <i>et al.</i> , 2014
	Inhibition of electron transport complexes III and IV	Tuquet <i>et al.</i> , 2000
	Clinically shown to induce steatohepatitis though mechanisms still largely unknown	Fernandez <i>et al.</i> , 2005; Vauthey <i>et al.</i> , 2006
Methotrexate	Irinotecan induced steatohepatitis mouse model recently developed	Costa <i>et al.</i> , 2014
	Mitochondrial toxicity induced by intercalation of mitochondrial DNA and inhibition of topoisomerases may play a role	Kosovsky and Soslau, 1993
	CAD structure therefore inhibition of mitochondrial respiration and oxidative phosphorylation may play a role. Co-treatment of cultured cells with irinotecan and an inhibitor of electron transport synergistically increases irinotecan cytotoxicity.	Zhang <i>et al.</i> , 2014
	Clinical risk of developing steatohepatitis increases with large cumulative doses of MTX above 4 grams	Arena <i>et al.</i> , 2012
	Polyglutamated metabolite of MTX accumulates within hepatocytes	Kremer <i>et al.</i> , 1986

Drug	Mechanisms underlying development of steatohepatitis	Reference
	Damages GI tract mucosa thereby disrupting intestinal barrier functionality and allowing for bacterial translocation to the liver	Song <i>et al.</i> , 2006
	Increases adenosine release from fibroblasts threefold in vitro.	Cronstein <i>et al.</i> , 1991; Chan <i>et al.</i> , 2006
	Activation of stellate cells by adenosine increases collagen production and decreases the activity of matrix metalloproteases.	Kim <i>et al.</i> , 1993
	Induces mitochondrial toxicity by preventing replenishment of mitochondrial folate stores	Huang <i>et al.</i> , 2005; Tabassum <i>et al.</i> , 2010
Valproic Acid	Mitochondrial toxicity leads to ROS generation, disruption of the mitochondrial membrane, and caspase-dependent apoptosis	Aires <i>et al.</i> , 2010
	Competitive and non-competitive inhibition of CPT-1	Ponchaut <i>et al.</i> , 1992
Tetracyclines	Sequesters cellular stores of free coenzyme A preventing fatty acids from undergoing beta-oxidation	Bjornsson <i>et al.</i> , 1997
	Large IV doses required to induce steatohepatitis	Letteron <i>et al.</i> , 2003
	Accumulation of hepatic triglycerides via inhibition of MTP	Freneaux <i>et al.</i> , 1988; Szalowska <i>et al.</i> , 2014
	Decreases cellular beta-oxidation in rats through the down-regulation of PPAR α , CPT-1, and fatty acid binding protein	Antherieu <i>et al.</i> , 2011
	Increases fatty acid synthesis in HepaRG cells via upregulation of PPAR γ and SREBP-1c	Bruning <i>et al.</i> , 2014; Wang <i>et al.</i> , 2014
	Doxycycline and minocycline inhibit mTOR and activate ATF-4.	
	Activated ATF4 increases ROS production by up-regulating CYP2E1	
	Leads to the carbonylation and decreased activity of enzymes involved in beta-oxidation including ACADL and ETF	Deng <i>et al.</i> , 2015
	Leads to the potential sequestration of free CoA via the carbonylation of OCT, MDH, MCC, and MSAD	Deng <i>et al.</i> , 2015