TROVAFLOXACIN POTENTIATES LIPOPOLYSACCHARIDE-INDUCED TUMOR NECROSIS FACTOR-ALPHA IN A MACROPHAGE CELL-LINE: MECHANISTIC INSIGHTS TO IDIOSYNCRATIC LIABILITY

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology-Environmental Toxicology -- Doctor of Philosophy

2013

ABSTRACT

TROVAFLOXACIN POTENTIATES LIPOPOLYSACCHARIDE-INDUCED TUMOR NECROSIS FACTOR-ALPHA IN A MACROPHAGE CELL-LINE: MECHANISTIC INSIGHTS TO IDIOSYNCRATIC LIABILITY

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Idiosyncratic drug-induced liver injury (IDILI) is an adverse response to many pharmaceuticals representing a significant public health risk associated with significant morbidity and mortality in humans. IDILI is responsible for many of the FDA-imposed restrictions on drug therapies, representing additional regulatory and financial burdens. Despite the extensive efforts put forth to develop safe and effective drugs, the causes of IDILI are not well understood. Models of IDILI in animals have been developed recently in which coadministration of a nontoxic dose of an IDILI-associated drug and an otherwise nontoxic dose of bacterial lipopolysaccharide (LPS) precipitates hepatocellular injury.

A key component to most of these drug-LPS hepatotoxicity models is a drug-mediated increase or prolongation of LPS-induced tumor necrosis factor-alpha (TNF) release in the plasma of animals that precedes the onset of toxicity. The focus of this dissertation was to model a drug-mediated increase in LPS-induced TNF release *in vitro* and study the underlying mechanisms responsible for the increased LPS-induced TNF release.

Trovafloxacin (TVX), an antibiotic with IDILI liability, is hepatotoxic in mice when coadministered with LPS. TVX prolongs LPS-induced plasma TNF in mice, and this prolongation is required for hepatotoxicity. One hypothesis for the prolonged plasma is that TVX increased LPS-induced synthesis and release of TNF from inflammatory cells.

Accordingly, a model of TVX/LPS coexposure was established in RAW 264.7 macrophage-like cells to recapitulate the increased LPS-induced TNF release. TVX increased LPS-induced TNF release in a concentration- and time-dependent manner. Analysis of the changes to upstream inducers of *Tnf* expression revealed that TVX activated mitogen activated protein kinases (MAPKs) ERK and JNK. The increased LPS-induced TNF release from RAW cells required ERK- or JNK-dependent signaling.

The next group of studies tested the hypothesis that TVX increases LPS-induced TNF release due to eukaryotic topoisomerase poisoning, an off-target effect of TVX. An *in silico* analysis indicated that TVX could bind favorably to human topoisomerase, and TVX decreased human topoisomerase activity in a cell-free assay. In RAW cells, TVX induced a marker of DNA damage and activated ataxia telangiectasia-mutated Rad-3-related (ATR) kinase. ATR-dependent signaling was required for the TVX-mediated increase in LPS-induced TNF release. These results indicated that TVX poisoned topoisomerase in RAW cells and the resultant DNA damage led to ATR activation, which was required for the TVX-mediated increase in LPS-induced TNF release.

In summary, the phenomenon of the TVX-mediated increase in LPS-induced TNF release in RAW cells *in vitro* recapitulated observations *in vivo*. The model in RAW cells was used to identify key signaling mechanisms which could increase LPS-induced plasma TNF in animals and identified a possible TVX-specific target in cells which could explain the IDILI liability associated with TVX in humans.

ACKNOWLEDGMENTS

As I spent the last few weeks composing this dissertation, I was forced to look back and consider the entirety of my time in graduate school. Hours and hours of class work, staring at computer screens, yelling at computer screens, screaming at computer screens, working weekends/holidays, making revisions of revisions, running up against deadlines; and I now understand that it was the best series of events I have ever experienced in my life. All together, I have now been trained and prepared for the rest of my life's work, and it is just beginning now. I cannot wait for the challenges of describing the unknown and the virtually limitless paths I can travel in this remarkable field of scientific research. I humbled by the sacrifices and generosity of so many people in my life. Any successes I may enjoy in my career or life would not be possible if not for that selflessness.

I would first like to thank my mentor, Dr. Robert Roth. When I decided to attend MSU for graduate school, Bob's email was one of the first I received to congratulate me. I knew right then I wanted to be a part of Bob's laboratory. Bob believes in his students, even when they do not believe in themselves, and his support in my toughest times over the last 5 years bolstered and pushed me forward. Bob's record of graduate student mentorship and professional reputation are second-to-none, and I am deeply honored to have been one of his trainees. I also enjoy that Bob goes out of his way to engage his students outside of the lab, which makes his lab a family.

I would also like to thank Dr. Patricia Ganey. Without exception, Patti has the sharpest mind of any person I've encountered in my life. She's the type of person who

seems to know the answer before the question has been asked. In addition, I have not met an individual who is better at conveying scientific findings than Patti. I know my writing and presenting skills are better because of her tireless attention to detail. Over the last 5 years, I have learned to absorb everything I can about each technique I use and every rationale for using those techniques. Patti's meticulous nature and mentorship are the reason for that. I am a remarkably more comprehensive scientist and thinker because of her.

I'll never really be able to repay Bob and Patti for what they gave me throughout graduate school. I hope they understand what profound respect I have for them both. I only hope to honor them by being the best scientist I can be and transferring their passion, precision and excellence to the next generation of scientific trainees.

MSU, The Graduate School, Department of Pharmacology & Toxicology and Center for Integrative Toxicology have also provided an infrastructure that allows for trainees such as myself to receive a world-class education and exposure to the very latest developments in Science. I have been so very fortunate to receive various amounts of funding from all of these entities at MSU as well as the NIH training grant administered by the CIT. The funding kept a roof over my head, food in my stomach and gas in my car. I have traveled to various meetings across the country with the funding support I have received. Further thanks to Bob for his NIH grants that funded my research.

My coworkers in the lab cannot go unmentioned, namely: Dr. Erica Sparkenbaugh, Dr. Aaron Fullerton, Dr. Kevin Beggs, Dr. Kazuhisa Miyakawa, Ashley Maiuri, Ryan Albee and Nicole Crisp. The strange organism that is a functioning

laboratory requires a peaceful coexistence with coworkers. Not only did we all get along, but we're all friends and spent a great deal of time outside of the lab together whether it was in winter bowling league, Lansing Lugnuts games, holiday parties, distance running, tailgating or playing Rock Band. They all made the lab a place where I wanted to be, not just a place where I had to be. I would also like to recognize Dr. Nathan Tykocki. We were roommates for 2+ years and although we didn't work in the same lab, we saw each other every day and in the process formed a lifelong friendship.

To my mother and father, Cathy and Kevin, thank you for being exceptionally supportive of me as I wandered through life before choosing this path into research. Not unlike my feelings towards my mentors, I cannot ever repay them for what they gave me over the years of my life. I simply hope to be the best person I can be. To my brother-in-law Michael Fett, I don't think "in-law" is required, brother. You are a fantastic human being, and I am a better man for having you in my life.

Finally, to the love of my life, Jessica Eldridge, you've made me the luckiest guy I know. Every morning, every evening and every time I see you, I feel love. Without that love and your unending support, I would have never completed my graduate education. I cannot wait to spend the rest of our lives together.

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KEY OF SYMBOLS OR ABBREVIATIONS

ActD	actinomycin D
AFT	aflatoxin
ALT	alanine aminotransferase
AP1	activator protein-1
APC	antigen-presenting cell
ARE	A+U-rich elements
ATM	ataxia telangiectasia-mutated
ATR	ataxia telangiectasia-mutated and Rad3-related
CCL4	carbon tetrachloride
CPX	ciprofloxacin
CPZ	chlorpromazine
DAMP	damage-associated molecular pattern
DDR	DNA damage response
DILI	drug-induced liver injury
DSB	DNA double-strand break
ERK	extracellular regulated kinase 1/2
ETAN	etanercept
FAM	famotidine
HSC	hepatic stellate cell
HuR	Hu-antigen R
IDILI	idiosyncratic drug-induced liver injury

IkB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
IRAK	IL-1R-associated kinase
JNK	c-Jun N-terminal kinase
KC	Kupffer cell
LBP	LPS binding protein
LPS	bacterial lipopolysaccharide
LTA	lipoteichoic acid
LVX	levofloxacin
MAPK	mitogen activated protein kinase
MCT	monocrotaline
MOX	moxifloxacin
MyD88	myeloid differentiation factor 88
NK	natural killer
NPC	nonparenchymal cells
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
p38	p38/SAPK2/reactivating kinase
PAMP	pathogen-associated molecular pattern
pH2A.X	phosphorylated histone 2A.X
PI3K	phosphoinositide 3-kinase
PTX	pentoxyfylline
RAN	ranitidine
SAL	saline

SEC	sinusoidal endothelial cell
TLR	Toll-like receptor
TNF	tumor necrosis factor-alpha
TopIIa	eukaryotic topoisomerase II-alpha
TTP	tristetraprolin
TVX	trovafloxacin
WORT	wortmannin

CHAPTER 1

General Introduction and Specific Aims

1.1 Overview of liver function

The liver is an organ that plays an essential role in the homeostasis of humans. The liver receives a large portion of the body's blood supply in a mixture from arterial and venous supply. The venous supply of blood to the liver originates from the hepatic portal vein, responsible for conducting ingested nutrients and drugs as well as pathogens from the intestinal lumen (245). Portal circulation is responsible for delivering the majority of blood supply to the liver, roughly 80%, while arterial delivery constitutes the remaining portion (36). The primary cell-type that carries out the principal functions of the liver is the hepatic parenchymal cell, referred to as the hepatocyte. The liver fulfills several necessary roles that are metabolic, synthetic and detoxifying in nature (207). Although hepatocytes comprise the vast majority of cells in the liver, a complex mixture of nonparenchymal cells (NPC) interact with hepatocytes and are required for the health and proper function of the liver

1.1.1 Liver cell types

Hepatocytes carry out most of the classically defined functions of the liver including extracting and processing nutrients for utilization in the body, synthesizing several protein factors, metabolizing drugs or clearing toxins (245). Hepatocytes help to maintain glucose homeostasis in the body, achieved through glycogen synthesis or breakdown, as well as synthesis of glucose from other sugars or amino acids in gluconeogenesis (245). Hepatocytes synthesize bile acids, necessary for fat emulsification in the small intestine for proper absorption and digestion of lipids (245). Several protein factors in the blood, such as albumin, fibrinogen and complement

proteins are synthesized and released by hepatocytes (245). The secreted proteins from hepatocytes maintain oncotic pressure in the blood and aid in coagulation and in host defense against pathogens. Hepatocytes are also rich in cytochrome P450 enzymes, responsible for detoxifying and aiding in the excretion of drugs and toxins (245).

Although hepatocytes are the most numerous in the liver, the complex functions of the liver require the involvement of many other resident cell populations, the NPCs. NPCs are significantly fewer in number than hepatocytes, especially when considering there are several NPC populations that comprise the ~20% of total cells in the liver. Some of the cell-types which reside in the liver including, but not limited to, the sinusoidal endothelial cells, hepatic stellate cells, natural killer cells, and resident macrophages of the liver, the Kupffer cells (91). All of the NPCs mentioned serve to separate the hepatocytes from circulating blood and allow for dynamic response to the multitude of factors to which the liver is exposed (19, 20).

Sinusoidal endothelial cells (SECs) separate the hepatocytes from the liver's blood supply, forming the sinusoidal lumen (6, 233). SECs represent an incomplete barrier between the blood travelling through the sinusoidal lumen and the hepatocytes, but also serve as immune sentinels and participate in immune responses. SECs remove and internalize antigens, cellular debris and immune complexes from the hepatic circulation (198, 234). SECs also participate in host defense through release of interleukins and eicosanoids, both of which are involved in the inflammatory response to pathogens or organ damage (198, 234), and express adhesion molecules to anchor circulating immune cells in the liver (220). The SECs are separated from the underlying

hepatocytes, leading to the formation of the space of Disse, where many of the remaining NPCs reside (245).

Hepatic stellate cells (HSCs) reside in the space of Disse and play an essential role in vitamin A and lipid storage (222). HSCs demonstrate contractile activity and regulate blood flow through the liver sinusoids (99, 160, 172, 247). In conditions of chronic liver injury and damage, HSCs become activated and are fibrogenic, transforming into myofibroblast-like cells. Transformed HSCs secrete extracellular matrix (ECM) components such as collagens, hyaluronic acid and laminins, among many others (70). Ultimately, the transformed HSCs are critical to the pathogenesis of chronic liver disease, leading to cirrhosis and liver failure (167). HSCs also play a minor role in activation of T cells and the adaptive immune response (21, 231).

Natural killer (NK) cells are also referred to and first identified as pit (pit: "seed of fruit") cells, due to large and dense granules located in the cytoplasm (232). The azurophilic granules are lysosomal in nature and released in response to immune stimuli, and were later identified as liver-associated NK cells (96). NK cells are derived from circulating granular lymphocytes (232) and possess potent cytotoxic activity against tumor cells (22). NK cells also release many factors that induce tumor cell death (200).

The NPCs in the liver participate in many activities essential to liver homeostasis and health of the host. Some of the NPC functions listed so far suggest the liver is also an important immune organ outside of its metabolic and synthetic activities.

1.1.2 Kupffer cells: the resident macrophages of the liver

The Kupffer cells are arguably the most important cells to the various immune functions carried out by the liver. Kupffer cells (KCs) are named for the pathologist C. von Kupffer who first identified this NPC, although it was not until some years later that KCs were accurately described as macrophages (17). KCs only represent about one-third of NPCs in the liver yet represent the largest fixed-population (80-90%) of macrophages in the body and appear to be derived from bone-marrow-derived monocytes in circulation (17). KCs are considered relatively stationary, adherent to liver SECs and are proximally exposed to the contents of the liver's blood supply (57, 91).

KCs remove particulate matter and cells from the portal blood, in addition to foreign substances that are intestinal in origin, such as pathogens or pathogen-derived materials (234). KCs ingest various materials in the liver blood supply by distinct endocytotic mechanisms: bristle-coated micropinocytosis, pinocytosis veriformis, pinocytosis (fuzzy-coated vacuole), and phagocytosis (232, 234). KCs also produce several crucial mediators in host defense against pathogens, including inflammatory cytokines and eicosanoids, as well as reactive oxygen species (42). The released mediators from KCs, although essential to clearing and killing of pathogens, can also lead to damage to the liver and the host (42).

The impact of KCs to the health of the liver and host is highlighted by studies wherein KC depletion leads to complete mortality from an otherwise sublethal dose of *Listeria monocytogenes* (49) and a significant increase in mortality during *Borrelia burgdorferi* infection, normally not toxic to mice (116). Additionally, most microorganisms to which the liver is exposed are bound and eliminated by KCs (69).

These studies enforce the critical contribution of KCs to health of the host and host defense through pathogen clearance (105).

The defense functions of KCs are also attributed to complex interactions with other cell-types, not just autonomous endocytotic activity (65, 185, 231). In response to infection, the interaction between KCs and circulating neutrophils is required for KC-dependent pathogen clearance. For instance, elimination of some *Listeria* requires KCs, but this is not due to KC-mediated phagocytosis (49, 69). The KC-dependent pathogen elimination occurs through extracellular trapping of the pathogen and involves the recruitment of other immune cells (leukocytes and T-lymphocytes) for elimination (49, 69).

KCs can promote the health of their host by other means outside of defense against pathogens. The liver is also responsible for clearance of endogenous macromolecules and senescent cells. As red blood cells reach the end of their lifespan or become dysfunctional, KCs clear these cells from circulation to prevent tissue damage resulting from red blood cell-derived, hemoglobin-mediated oxidative injury (111). KCs also clear activated platelets and immune cells, serving as a critical regulator of the duration and magnitude of coagulation and the immune response, thereby limiting damage to the host (17).

Another function KCs fulfill is that of an antigen-presenting cell (APC). As APCs, they express MHCI and MHCII along with costimulatory surface receptors required for T cell activation and response to pathogens (85). KCs, however, are less potent APCs in the absence of additional stimuli such as pathogen-associated molecular patterns (PAMPs). PAMPs are pathogen-derived materials that activate KCs to overcome

tolerance to normally circulating T cells (244). Tolerance prevents aberrant activation of the immune response, such as T-cell activation, thereby limiting damage to the host. PAMPs are a broad class of macromolecules encompassing lipid, protein, lipoprotein and nucleic acid constituents of pathogens (25). PAMPs interact with the evolutionarily conserved Toll-like receptor (TLR) family. KCs express several other surface receptors in addition to TLRs, all of which are important components in KC function and activation.

Activation of complement receptors on KCs, for instance, stimulates the production of inflammatory eicosanoids and cytokines which lead to uptake of opsonized immune complexes (64). Scavenger receptor activation leads to uptake of bacteria, apoptotic cells and circulating vesicles (64). The mannose receptor family facilitates clearance of pathogenic or endogenous molecules containing mannosyl residues (132). The complement, scavenger and mannose receptors are predominantly activated by materials in the hepatic or systemic circulation, but as noted previously, the liver is selectively exposed to exogenous substances absorbed from the gut.

KCs are one of the first cells exposed to exogenous substances absorbed from the gastrointestinal tract (107). As such, PAMP recognition by TLRs expressed on KCs is quite important to KC function. KCs are some of the first cells exposed to gut microbiota and various bacterial components that translocate from the intestine to the liver. This indicates the importance of KCs as proximal responders to immune stimuli.

1.1.3 Kupffer cells in inflammation

As critical immune regulators, the role of KCs as immune mediators is best described in response to pathogenic stimuli. Bacterial lipopolysaccharide (LPS), or endotoxin, is a macromolecule comprising glyocolipids from the outer membrane of gram-negative bacteria that normally colonize in the gut (203). Accordingly, as the liver and KCs are continuously exposed to LPS and other bacterial products, a swift response to prevent bacteria from entering systemic circulation is required (148). KCs are activated in response to LPS through LPS binding to its cognate receptor, Toll-like receptor 4 (TLR4) (15). KCs clear LPS from the blood and subsequently produce several inflammatory mediators, including cytokines (128). Cytokines are small protein mediators released by cells, including KCs, which mediate activation of the cells of the adaptive and innate immune response (90). Cytokines also affect non-immune tissues, such as altering the cell cycle and inducing cell-death (90, 187). The liver is known to be a significant source of cytokines, such as tumor necrosis factor-alpha (TNF), that are primarily released by KCs (77, 128). The activation of the TLR pathway is a complex and coordinated pathway of intracellular adaptors and signaling kinases that results in the induction of many inflammatory mediators, including cytokines (25).

The primary ligand for TLR4 is LPS, but several endogenous factors have been identified which activate TLR4**.** It is hypothesized that TLR4, for example, acts as a sensor of tissue injury (95). Endogenous molecules that are released from stressed, damaged or dying cellsm called damage-associated molecular patterns (DAMPs), activate TLR4. Some DAMPs that activate TLR4 include heparan sulfate (93), fibrinogen (199), hyaluronan fragments (210) and high-mobility group box-1 (142). It is

therefore possible that TLR4 activation leads to the detection of, response to and resolution of sterile tissue injury, such as injury following ischemia/reperfusion (214) or cardiac transplantation (95), hemorrhagic shock (44) or femur fracture (118).

In addition to being released from damaged or dying cells, these DAMPs can be increased in circulation due to underlying disease states such as atherosclerosis, rheumatoid arthritis, lupus and inflammatory bowel disease (159). The increasing list of endogenous ligands for the TLR family of receptors draws attention to the significant impact of TLR activation and TLR-mediated signaling and how commonplace inflammatory stress can be in people. DAMPs continue to be discovered which can activate or modulate activation of TLR4 and contribute to adverse health problems in people. In consideration of the robust inflammatory responses resulting from TLR activation in inflammatory cells, including KCs, the impact of TLR-mediated effects on the health of the host cannot be underestimated.

KCs also participate in the health of liver and host that does not involve defense against pathogens. KCs are implicated to be protective in models of hepatocellular carcinoma. Isolated KCs are cytotoxic against tumor cells, and their cytotoxic activity is enhanced by cytokine stimulation or TLR activation (80, 173). KC-dependent cytotoxicity can be directly tied to cytokine production; neutralizing antibodies directed against cytokines prevent KC-dependent cytotoxicity (38). Additionally, KC inactivation is known to increase metastasis size in the liver whereas KC activation by microbial components can decrease tumor size (180).

Despite the many KC-dependent protective functions known, KCs can also contribute to the pathogenesis of liver disease (107). Liver fibrosis is a complex disease

process and is typically attributed to HSCs. HSCs transform to cells that increase production of collagen in response to chronic stress (59). KCs are involved in the progression of fibrosis through cytokine and growth factor production that induces HSC transformation (139). KCs produce the cytokine TGF-β, a principal cytokine identified in stimulating HSCs and driving fibrosis in the liver due to chronic alcohol consumption (135) and carbon tetrachloride (CCl4) (40).

The pathogenesis of influenza-mediated hepatitis also involves KCs (162). Cytotoxic T-cells were found to be in contact with KCs in foci associated with hepatocellular necrosis, which was prevented if KCs were depleted, despite cytotoxic T-cells persisting in the liver during infection in the absence of KCs. This suggests that KCs are required for the anchoring of T-cells in the liver to facilitate hepatocellular damage. Another explanation is that KCs directly kill hepatocytes through activation of apoptotic pathways (1) as well as inflammatory cytokine and/or reactive oxygen species production.

In all of the instances mentioned, the protective or pathogenic roles performed by KCs are highly associated with immune functions. Immune functions are mostly usually attributed to defense against pathogens, but the evolving concept is far more comprehensive. A majority of processes in the liver and the body are considered to have a substantial immune component associated with them.

Having established that KCs play various but important roles in the health of the liver, the focus will now turn to an important KC-derived inflammatory cytokine, TNF. TNF is implicated in many modes of liver injury (187). Since KCs are significant producers of TNF, the role of TNF in liver injury will be highlighted. Hepatic injury in

response to ischemia followed by reperfusion is mediated by induction of TNF release from KCs (179). Ischemia/reperfusion is a major health problem that can result from liver transplantation, liver resection and circulatory shock (187). TNF is considered critical to hepatocyte death in response to ischemia/reperfusion, as TNF receptor knockout is protective and prolongs survival in animals (179).

The galactosamine/LPS model of septic shock demonstrates produces pronounced liver injury in animals. The liver injury is due to endogenously produced TNF, as p55 TNF receptor knockout animals are protected from galactosamine/LPS-dependent liver injury (152). TNF is also a mediator of systemic toxicity, including liver injury, in response to pathogens. In response to high-dose LPS models, mammals develop symptoms of shock, that of hypotension, massive coagulation and resultant injury to the kidneys and liver (14). The lethal effect of LPS was later described to be TNF-dependent, as mice immunized against TNF were largely protected against LPS-dependent mortality (14).

Taken together, KCs are crucial mediators in the health and injury of the liver. KCs are producers of several liver-injury mediators, such as TNF. TNF expression is induced by many factors in a robust fashion in response to TLR4 activation. Since KCs are proximally exposed LPS as well as endogenous activators of TLR4, it is important to understand TLR4-mediated signaling and how TLR4 activation induces TNF expression and release.

1.2 TLR4-mediated signaling

1.2.1 Proximal adaptors in TLR-mediated signaling

LPS binding to TLR4 requires two protein mediators to maximize the sensitivity of TLR4 to LPS. LPS binding protein (LBP) is produced by the liver (186, 236) and binds to the lipid portion of LPS with high affinity. The binding of LPS to LBP enhances the transfer of LPS to CD14, the second mediator that maximizes TLR4 sensitivity to LPS (211, 236). CD14 is a membrane-bound protein that facilitates the transfer of LPS to TLR4 and modulates LPS recognition by TLR4 (236). Following LPS recognition by TLR4, activated TLR4 receptors dimerize (or oligomerize) and recruit downstream adaptors to initiate signal transduction (127).

TLR4 activation, and hence induction of TNF expression, is dependent on binding of the proximal signaling adaptor of TLR4, myeloid differentiation factor 88 (MyD88). The MyD88-dependent pathway is critical to TLR-dependent induction of proinflammatory cytokines such as TNF through several downstream signaling mechanisms. After activation and dimerization of TLR4s, protein adaptors interact with the intracellular Toll/interleukin-1 receptor (TIR) domain of TLR4 (138). All TLRs but TLR3 utilize MyD88 to initiate signaling after TLR activation (25). MyD88 is recruited to the TIR domain on TLR4 and recruits MyD88-adaptor-like (Mal) (55) and the IL-1R-associated kinase-4 (IRAK4) (120). This complex recruits another IRAK-4 family kinase, IRAK-1 (145). The IRAK-4/IRAK-1 complex then facilitates the recruitment and ensuing activation of TNF receptor associated factor (TRAF)-6, the penultimate step prior to formation of a signaling complex which includes TGF-β-activated kinase-1

(TAK1) and TAK1 binding proteins (TAB)-1,TAB2 and TAB3 (25). The multimeric TAK/TAB protein complex then activates the effector signaling responsible for proinflammatory cytokine synthesis; that of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) (67) and the mitogen activated protein kinases (MAPK) (25, 66, 72).

1.2.2 TLR4-mediated effector signaling

NF-kB represents a group of structurally related proteins that are activated in response to many stimuli, including LPS, to initiate gene expression (67). NF-kB transcriptional dimers are inactive in the cytoplasm due to interaction with an inhibitory subunit, inhibitor of kappa B (IkB). TRAF6 activation downstream of LPS binding to TLR4 activates a multimeric complex of IkB kinases (IKK) (248). IKK α/β/γ activation induces phosphorylation of IkB, which releases and leads to the degradation of IkB, allowing the newly freed transcriptional dimer to translocate to the nucleus and induce gene expression, including *Tnf* (67, 72).

The canonical MAPKs activated downstream of TLR4 activation are the extracellular regulated kinase 1/2 (ERK) (206), the c-Jun N-terminal kinase (JNK) also known as stress-activated protein kinase (SAPK1) (141), and p38/SAPK2/reactivating kinase (153). These MAPKs are a related group of serine/threonine protein kinases that are activated in response cellular stress and inflammation (72, 92). The MAPKs exert their influence on proinflammatory cytokine expression through activation of other downstream kinases to activate transcription factors, stabilize extant cytokine mRNA

and facilitate translation of cytokine mRNA and the release of cytokines (4, 43, 176, 206).

1.2.3 Control of TNF expression

The signaling of NF-kB and MAPKs induce cytokine expression and fulfill several roles before, during and after transcription. Control of TNF expression is highly regulated downstream of MAPK and NF-kB activation, from transactivation of the *Tnf* gene through shedding of pro-TNF protein from cell membranes. As TNF plays a significant role in many types of liver injury and TNF is robustly induced by TLR activation, the effects of NF-kB and MAPKs on TNF expression are summarized in Table 1.

Table 1. Effect of LPS-activated signaling on TNF expression and release. The roles of NF-kB, ERK, JNK and p38 in TNF expression and release.

TLR4-Activated Signaling Mediator	Effect on TNF Synthesis
NF-kB	• Transactivation of the *Tnf* gene (67, 72)
p38	• Transactivation of the *Tnf* gene (72) • Stability of TNF mRNA (43) • pro-TNF Shedding (241)
ERK	• Transactivation of the *Tnf* gene (66, 72) • Stability of TNF mRNA (43) • pro-TNF Shedding (241)
JNK	• Transactivation of the *Tnf* gene (72) • TNF translation (206)

The locus of *Tnf* on the DNA is bound by multiple transcription factors activated by MAPKs. The activated factors lead to the recruitment of polymerases to transcribe the *Tnf* gene. There are several core elements that regulate transcription of the TNF distal and proximal promoter regions as well as an enhancer downstream of the *Tnf* gene (3'-TNF-enhancer) (193). The regulatory elements are highly conserved across species. The distal TNF promoter contains at least three binding sites for NF-kB (215). The proximal promoter, closer to the transcriptional start site for *Tnf*, contains multiple transcription factor-binding sites required for the coordinated expression of TNF (52). The 3'-TNF-enhancer contains NF-kB binding sites responsible for loop formation on the chromatin for continuous steady-state gene transcription (109).

In the frequently used transformed murine macrophage cell-line RAW 264.7, several transcription factors binding to these regulatory sequences are required for maximal TNF expression in response to LPS (156, 213). These regulatory sequences contain NF-kB, early growth response-1 (EGR1), CRE and activator protein-1 (AP1) binding sites. Transcription factors binding to these promoter regions of the TNF gene exhibit selective time-dependence following LPS exposure (156). In addition, there are some species- and cell type-specific arrangements in the array of transcription factors interacting at the *Tnf* promoter (137, 156), and these should be considered in experimental studies investigating TNF expression.

1.2.4 Post-transcriptional control of TNF expression

After transcription of TNF, the TNF mRNA requires extensive regulation prior to translation. The 3' untranslated region of *Tnf* contains adenylate-uridylate-rich elements, so-called A+U-rich elements (ARE). AREs facilitate the transport of TNF mRNA from the nucleus to the cytoplasm (48) and contribute to translational repression of TNF (108). TNF mRNA is subject to rapid turnover mediated by proteins which bind its ARE: tristetraprolin (TTP), Hu-antigen R (HuR) and ARE RNA-binding factor 1 (88). TTP, for example, binds to TNF mRNA and targets it for degradation, whereas HuR binding promotes stabilization of the mRNA (4). MAPK activation can augment TNF mRNA stability mediated through ARE-binding proteins (43). LPS-induced MAPK signaling through p38 and ERK can stabilize TTP protein yet prevent TTP-mediated TNF mRNA turnover (43). JNK signaling, however, is less associated with transcriptional stability but is required for maximal translation of TNF mRNA to TNF protein in response to LPS (206).

TNF is initially translated as a pro-form that is a membrane-bound precursor (110). The 26 kilodalton pro-TNF is shed to a 17 kilodalton soluble form by a Zn-dependent metalloprotease named TNF-alpha-converting enzyme, or ADAM-17 (18). Shedding of membrane-bound precursors is altered by several stimuli, including bacterial toxins (223), cell-death signaling (30) and MAPKs (241).

1.2.5 Negative regulation of TLR4-mediated signaling

So far, the expression of *Tnf* and release of TNF protein have been described downstream of TLR4 activation. There are several negative regulators that contribute to tight regulation of TLR4 signaling, thus preventing damage to the host from excessive activation (122). LPS induces the expression of an IRAK family member IRAKM (106), and IRAKM suppresses the association of IRAK4 with TRAF6, preventing TLR4-dependent signaling through NF-kB and MAPKs. Another LPS-induced negative regulator of TLR4 signaling is suppressor of cytokine signaling-1 (103). SOCS1 suppresses TLR4 signaling through IRAK1 and has also been shown to degrade a necessary adaptor in the MyD88 pathway, Mal (131). These negative regulators limit TLR4-dependent signaling events that could decrease activation of NF-kB and MAPKs.

MAPKs and NF-kB can induce negative feedback loops also. Dual-specificity phosphatases (DUSPs), for example, are induced by MAPKs, and DUSPs limit the extent and duration of MAPK activation in response to many stimuli, including LPS (113). A20 is another negative regulator of TLR4 signaling that is a de-ubiquitinating enzyme that prevents signaling downstream of TRAF6. A20 expression is driven by NF-kB and prevents excessive TLR4-dependent signaling (239) in addition to preventing NF-kB and MAPK activation (74, 194).

TLR4-dependent signaling is a remarkably coordinated and tightly regulated process. Aberrant or increased cytokine expression is well known in several chronic diseases and acute toxic episodes. The potential for damage caused by increased TNF in the plasma, for instance, might not be solely due to increased activation of

inflammatory receptors such as TLR4, but the result of insufficient negative feedback of the pathways leading to the increased synthesis and release of TNF.

1.3 Models of liver injury with inflammation

1.3.1 LPS augments intrinsic hepatotoxicity

LPS can modulate the sensitivity to intoxication from xenobiotic exposure (62). The effect of LPS in modulating toxicity to xenobiotics is not necessarily limited to TNF expression, but could be due to many LPS-dependent effects. Aside from TNF and cytokine expression in KCs, LPS-dependent effects include production of reactive oxygen species in the liver (208), induced adhesion molecule expression in SECs (240) and the extravasation and activation of neutrophils (89). There are several studies that demonstrate LPS contributing to hepatotoxicity, and many of the varied LPS-dependent effects are implicated in the models of hepatotoxicity.

Carbon tetrachloride (CCl4) is intrinsically hepatotoxic in the absence of other stimuli. CCl4 is metabolized by cytochrome P450 isoforms to a trichloromethyl radical that can bind to cellular maromolecules to impair normal cellular processes. The trichloromethyl radical induces lipid peroxidation and deleterious alterations to calcium homeostasis, both of which contribute to cellular damage (228). Gut-derived, endogenous LPS, however, can play a role in CCL4-mediated toxicity. Rats made tolerant to LPS are less sensitive to CCL4-mediated toxicity (151). An antibody directed against LPS protects from CCl4 injury (41) and polymyxin B, an inactivator of LPS, reduces fibrosis in response to CCl4 (195). Additionally, coadministration of LPS with CCl4 in rats can potentiate acute liver injury (31).

Allyl alcohol is used in the production of food flavorings, and when a nontoxic dose of allyl alcohol is administered to rats, it is rendered hepatotoxic when coadministered with LPS (201). The hepatotoxic response to the combination of allyl alcohol and LPS depends on KCs, as pretreating rats with gadolinium chloride, a KC inactivator, protects rats from injury. Rats are also protected from injury in this model when LPS-induced cycloxygenase-2 (COX2) is inhibited (63) or neutrophils are depleted (103).

Aflatoxin (AFT) and monocrotaline (MCT) are environmental toxicants to which humans are exposed. AFT is a toxic fungal contaminant of stored grains (27) and MCT is a toxic, plant-derived alkaloid used in nutritional supplements an herbal teas (86). Both of these naturally occurring toxicants are acutely hepatotoxic in humans (144, 171). Interestingly, nontoxic doses of AFT or MCT coadministered to rats with an otherwise nontoxic dose of LPS result in hepatotoxicity (9, 242). The hepatotoxicity from rats coexposed to AFT/LPS or MCT/LPS required TNF (10, 243).

Cocaine and ethanol are two substances consumed by humans recreationally. Chronic use of either cocaine (158) or ethanol (146) can result in liver disease in humans. Cocaine (112) or ethanol (134) coadminstered with LPS in rodents results in acute hepatotoxicity. In the case of ethanol, however, acute and chronic ethanol consumption increases LPS in the plasma (146). Since ethanol consumption can increase gut permeability and increase plasma LPS, the liver might be sensitized to ethanol-induced toxicity. In cocaine users, it was suggested that endotoxemia might occur due to increased susceptibility to bacterial infections in drug users (112). The

models of ethanol and cocaine coexposure to LPS require KCs, LPS-induced reactive oxygen species and TNF to precipitate hepatotoxicity (112, 134).

The previously mentioned studies indicate a significant role for LPS mediating xenobiotic-induced hepatotoxicity or altering the susceptibility to intoxication by xenobiotics. In each of these models, KC-dependent effects are common and required for an hepatotoxic interaction between a xenobiotic and LPS. Taken together, the studies suggest that underlying inflammatory stress that activates KCs could increase the susceptibility of an individual to xenobiotic-associated hepatotoxicity.

There are several rationales to explain how LPS concentration could be elevated above basal levels to precipitate an hepatotoxic response with xenobiotics. Ethanol consumption, for example, is well known to increase endotoxin in the plasma from ethanol-increased gut permeability (146). CCl4 exposure can increase bacterial translocation from the gut (56). It is also possible that modest liver injury from a xenobiotic can decrease endotoxin clearance and thereby increase LPS plasma concentration (151). As CCl4, AFT and MCT are all intrinsically hepatotoxic, relatively low doses of these xenobiotics could induce modest hepatic damage. This modest damage to the liver, for instance, could decrease LPS clearance and thereby increase LPS in the plasma. The concentration of DAMPs could also be increased by modest hepatic damage. The synergistic toxicity, therefore, does not have to be due to a xenobiotic only interacting with LPS, but could be explained by increased DAMPs activating TLR4 (25). There are more than 20 DAMPs identified that activate TLR4 that are released due to tissue damage or underlying disease. It is possible, therefore, that

DAMPs are released in response to modest cellular/tissue damage or due to underlying disease and could mediate the effects of TLR4 activation akin to LPS.

1.3.2 Idiosyncratic drug-induced liver injury

Drug-induced liver injury (DILI) is responsible for the majority of acute liver failure cases in the United States (154). DILI is associated with significant morbidity and mortality in patients. DILI is also the most common reason that prevents approval for new drug entities and withdrawal of existing drugs from the market (227). These adverse drug reactions in the liver are therefore a significant public health problem, as well as a significant regulatory and financial burden (192). It is estimated that the cost of developing therapeutic candidates to the market exceeds $1 billion USD (143) with preclinical toxicity assessment representing ~20% this cost (140). Despite the extensive efforts put forth to market safe and effective drugs, the prevalence of adverse drug responses in humans remains a significant public health risk.

Idiosyncratic drug-induced liver injury (IDILI) is a subset of DILI that is responsible for the bulk of post-marketing restrictions on drug therapies in the United States (114). These idiosyncratic responses occur in a minority of patients during drug therapy and can result in acute liver failure or death. In addition to the adverse health risk to patients, IDILI is quite difficult to predict, and the underlying mechanisms remain largely a mystery (192). The problem in predicting a drug with IDILI liability lies in the characteristics associated with idiosyncratic responses. The frequency of IDILI in patients during drug therapy can be quite rare, < 1%, IDILI occurs at doses considered

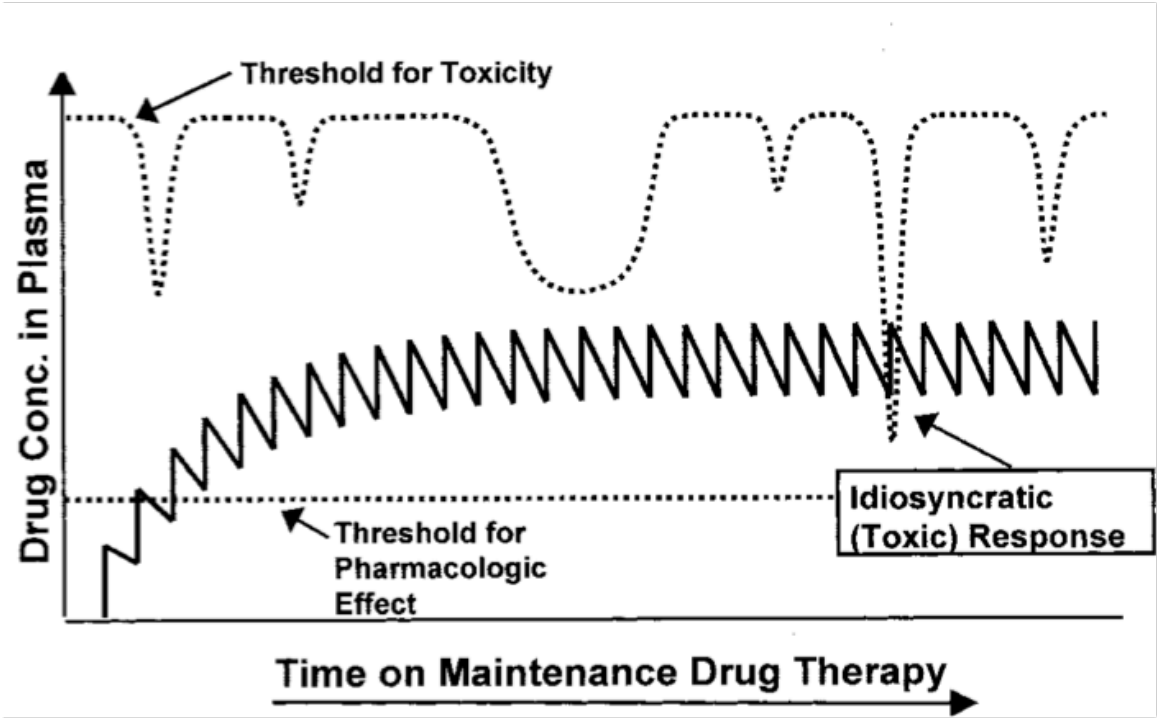
safe in the majority of patients undergoing drug therapy, with no apparent relation to onset of drug exposure (192).

The lack of predictive models of IDILI in animals or *in vitro* is not a surprise. Since episodes of IDILI are so rare in humans, if it is assumed that animals or *in vitro* systems are susceptible to intoxication by IDILI-associated drugs at a similar rate of incidence as in humans, the amount of experimental replicates (from animals or cultured cells) to produce toxicity would be prohibitively expensive and time-consuming (115, 192). Understanding the mechanisms underlying IDILI is certainly a challenge, but several hypotheses have been developed to explain the pathogenesis of IDILI. None of the hypotheses have been proven conclusively to explain how IDILI occurs. In fact, it is likely that a combination of the current hypotheses is required to fully explain IDILI.

1.3.3 Inflammatory stress hypothesis

One of the hypotheses to explain IDILI in humans is the inflammatory stress hypothesis, which states that an acute episode of inflammation that occurs during drug therapy can alter the threshold for toxicity to precipitate IDILI (174, 192). Inflammatory episodes are commonplace in people and occur erratically (62, 192), which could explain why the timing of IDILI is unpredictable. Drugs could amplify an otherwise nontoxic inflammatory episode, rendering a person undergoing drug therapy susceptible to intoxication. Conversely, inflammatory stress from translocated LPS could alter a person's sensitivity to intoxication from xenobiotics (67, 192). If a drug increases the inflammatory burden in the body or an unrelated transient inflammatory episode occurs in humans during drug therapy, either scenario could explain why IDILI occurs at otherwise nontoxic doses of IDILI-associated drugs and why IDILI-associated drugs are nontoxic in the majority of patients (227). In other words, inflammatory stress may decrease the threshold for toxicity in patients below the therapeutic concentration of a drug in the plasma to precipitate hepatotoxicity. The inflammatory stress hypothesis is presented in Figure 1, which demonstrates how an inflammatory stress can augment the toxic threshold for a drug. Figure 1 further illustrates if that inflammatory episode decreases the toxic dose sufficiently below steady-state drug concentrations in the plasma, a toxic response can result (174). Taken together, the inflammatory stress hypothesis represents a promising means to better understand IDILI and uncover the many unexplained mechanisms underlying IDILI in humans.

Figure 1. The inflammatory stress hypothesis (174). Transient episodes of inflammation are commonplace and arise from several stimuli (62, 174). Hypothetical timecourse of plasma-drug concentration in a patient undergoing drug therapy. The toxic threshold could be augmented by inflammatory episodes that increase the susceptibility to intoxication in a patient (62, 174).



1.3.4 Other IDILI hypotheses

There are several other modes of action proposed to explain IDILI outside of the inflammatory stress hypothesis. Some of the other hypotheses address specific mechanisms to explain IDILI, and others are more conceptual explanations. All of the IDILI hypotheses have support from certain studies involving IDILI-associated drugs, but the selective evidence upon which each hypothesis is based limits the power of these other hypotheses. A singular explanation for IDILI is unlikely, but inflammatory stress can play a role in each current hypothesis.

The reactive intermediate hypothesis describes how the metabolism of a drug to a reactive metabolite could disrupt intracellular homeostasis and ultimately lead to cell death (97). Various rare polymorphisms in metabolism genes, for example, could support why IDILI is so infrequent in humans (192). A significant obstacle to the reactive intermediate hypothesis is that many drugs are converted to reactive metabolites but are not associated with IDILI. Interestingly, reactive metabolite formation can happen in the presence of inflammatory stress. Myeloperoxidase, an enzyme released from activated neutrophils, has been demonstrated previously (82) to generate reactive intermediates of xenobiotics, suggesting a role for inflammation in the reactive metabolite hypothesis.

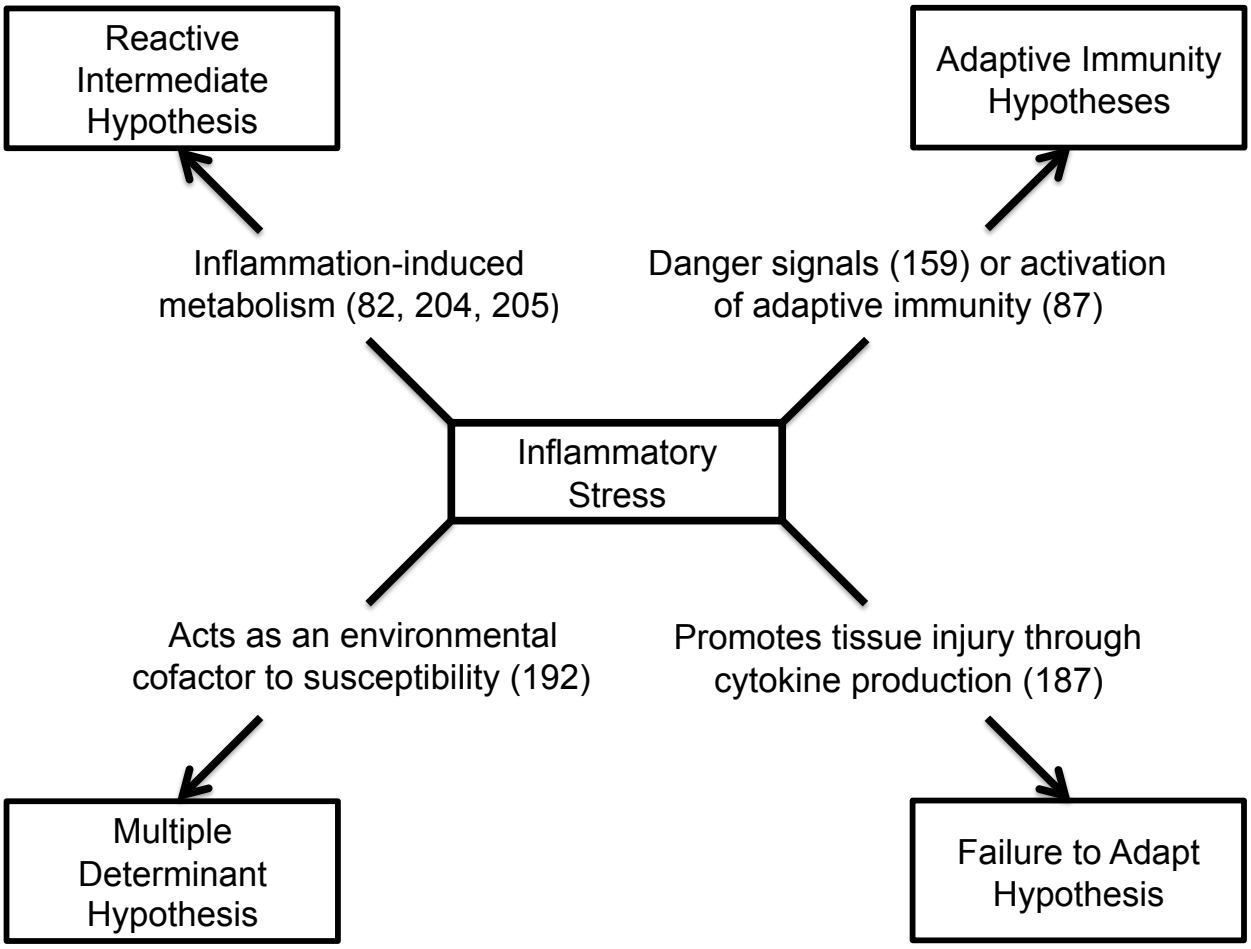
The hapten hypothesis (218) and closely related danger hypothesis (147, 217) both suggest that IDILI develops due to activation of the adaptive immune system. Both of these hypotheses suggest that an IDILI-associated drug or a drug metabolite can form protein adducts that lead to activation of a toxic adaptive immune response. Very limited experimental data exist to support either hypothesis, and acute inflammatory

stress can be involved in activation of the adaptive immune response. The innate and adaptive immune systems are interconnected (87), such that the innate immune system is known to regulate the adaptive immune response. Cytokines, for example, are involved in lymphocyte population expansion, which is required by the hapten and danger hypotheses.

The multiple determinant (119) and failure-to-adapt (227) hypotheses fail to describe specific factors that explain IDILI. They may be useful in considering what is hypothetically required to precipitate toxicity from IDILI-associated drugs, but cannot address how to test and detect drugs with IDILI liability directly. The multiple determinant hypothesis proposes that the combination of several distinct factors are required to observe an adverse response to a drug in an individual (119). These factors can be genetic or environmental, as well as take into account the physicochemical properties of the drug itself (119, 192). One of the environmental factors, for example, could be inflammatory stress. The failure-to-adapt (227) hypothesis suggests that a large portion of patients during drug therapy develop modest injury to which they adapt. Individuals susceptible to intoxication by this hypothesis would therefore be those which "fail to adapt". In consideration for the models wherein LPS precipitates hepatotoxicity with otherwise nontoxic doses of intrinsic hepatotoxicants, it is conceivable that inflammatory stress could exacerbate modest but reversible injury from a drug.

Figure 2 summarizes the role inflammatory stress can play in current IDILI pathogenesis hypotheses. The inflammatory stress hypothesis is therefore an attractive and testable means to understand IDILI and the mechanisms underlying these hepatotoxic responses.

Figure 2. Role of inflammatory stress in other IDILI hypotheses. Inflammatory stress can play various roles in other IDILI hypotheses. Inflammatory stress is linked to other hypotheses attempting to explain IDILI (Adapted from 192)



1.3.5 IDILI models in rodents

Several models of hepatotoxicity have been developed in rodents recently with IDILI-associated drugs including trovafloxacin (TVX), ranitidine (RAN), halothane, sulindac, chlorpromazine, amiodarone and others (28, 47, 60, 61, 126, 129, 189, 216, 249). In each of these models, when rodents were coexposed to a nontoxic dose of the IDILI-associated drug and an otherwise nontoxic dose of LPS, hepatotoxicity was observed. In some instance, drugs in the same pharmacological class as an IDILI-associated drug with a lesser or no association with IDILI were used as negative comparators. Levofloxacin (LVX) and famotidine (FAM), for example, have dramatically lower IDILI liabilities than TVX and RAN, respectively. Neither LVX nor FAM precipitated hepatotoxicity in combination with LPS, indicating a selective role for inflammatory coexposure in these IDILI models (129, 189, 216).

Many of these rodent models of IDILI represent the first animal models to demonstrate acute hepatotoxicity with IDILI-associated drugs. In several of these models, some common factors emerged after the hepatotoxic interactions were further characterized. In the models of TVX (189), RAN (216), amiodarone (126), sulindac (249) and chlorpromazine (61), the combination of drug and LPS increased or prolonged the LPS-induced TNF release prior to toxicity, suggesting a causal role for TNF in models of IDILI. Aside from IDILI-associated drugs, rodent models wherein LPS augmented susceptibility to xenobiotic toxicity demonstrated similar, early increases in LPS-induced TNF release that preceded toxicity. AFT (10) and MCT (243), for example, increased LPS-induced TNF release that preceded toxicity. It is intriguing how commonly an increase in TNF is observed in models wherein LPS acts as a

contributing factor to hepatotoxicity with intrinsically toxic xenobiotics as well as IDILI-associated drugs. Xenobiotic-mediated increases in LPS-induced TNF release, therefore, might represent a sensitive and testable marker of an IDILI-associated drug.

In the models of TVX, RAN, amiodarone, and sulindac, TNF was determined to be required in the hepatotoxic interaction between the drug and LPS. This was also true of the models of AFT and MCT coexposure with LPS. If TNF synthesis was prevented pharmacologically by pentoxifylline (PTX) (10, 43, 189, 216) or neutralized by administration of the soluble TNF receptor fusion protein etanercept (ETAN) (126, 189, 216, 249), toxicity was reduced or even prevented. This strengthened the causal link between the increase in TNF release and hepatotoxicity. The link between the xenobiotic-mediated increase in LPS-induced TNF release and hepatotoxicity is further enhanced when considering that LPS alone robustly increased TNF in all of these models but did not result in hepatotoxicity without an IDILI-associated drug present. Collectively, the relatively brief and modest xenobiotic-mediated increases in LPS-induced TNF release are common and required for toxicity in models of xenobiotic/LPS coexposure.

As previously mentioned, the selectivity and validity of the inflammatory stress hypothesis is further reinforced by the use of negative comparators. LVX is in the same pharmacological class as TVX with a markedly lesser association to IDILI than TVX (117). Indeed, LVX/LPS coexposure did not precipitate hepatotoxicity in mice and did not augment LPS-induced TNF release (189). The same is true of the RAN/LPS model. Although FAM is in the same pharmacological class as RAN, FAM has a lesser association to IDILI than RAN (129, 216). FAM/LPS was not hepatotoxic in animals and

did not augment LPS-induced TNF release (129, 216). Interestingly, both TVX/LPS and RAN/LPS coexposure led to prolongations of TNF in the plasma of mice, not just an increase in plasma TNF concentration, whereas LVX and FAM did not. These studies add to the evidence that a drug-mediated increase in LPS-induced TNF release could represent a promising predictive marker of a drug with IDILI liability.

1.3.6 TVX: Model of hepatotoxicity in mice and TNF prolongation

TNF plays a significant role in TVX/LPS-dependent hepatotoxicity, but it is necessary to first focus on the onset of injury relative to LPS exposure and the prolongation in plasma TNF. Mice were dosed with TVX by oral gavage 3h prior to injection of LPS (189). This timing between TVX and LPS administration was used as it maximized the toxic response to TVX and LPS (189). Plasma alanine aminotransferase (ALT) activity was used as the biomarker for hepatocellular injury, and ALT activity was significantly increased as early as 4.5 hours after LPS injection and peaked between 15 and 21 hours after LPS (Figure 3A). The TVX/LPS-dependent hepatocellular injury was confirmed by histopathological examination, as TVX/LPS coexposure induced necrotic lesions. It is important to note that neither TVX nor LPS alone increased ALT activity or induced necrotic lesions.

The role of TNF in TVX/LPS-dependent hepatotoxicity was evaluated by several approaches. PTX administered one hour prior to LPS to prevent LPS-induced TNF production markedly decreased TNF in the plasma of mice. The decrease in LPS-induced TNF release was associated with attenuated liver injury as measured by plasma ALT activity and histopathology. TVX/LPS-coexposed mice administered the

soluble TNF receptor ETAN one hour prior to LPS were also protected from injury. The TVX/LPS-dependent hepatotoxic interaction required TNF, yet the peak of TNF in the plasma was not significantly increased by TVX (189). TVX, however, prolonged the plasma concentration of TNF (Figure 3B), and the relevance of this prolongation was tested next. ETAN was administered at the peak of LPS-induced TNF in the plasma of mice, thereby preventing the plasma TNF prolongation, and significantly reduced liver injury (189). This suggested that the TVX-mediated plasma TNF prolongation was required for hepatotoxicity.

The requirement of TNF to precipitate TVX-mediated hepatotoxicity in mice was confirmed when mice were cotreated with TNF rather than LPS. The combination of TVX/TNF was hepatotoxic (191), and plasma TNF was prolonged by TVX/TNF coexposure in mice. The prolongation was quite similar to TVX/LPS-coexposed mice. If ETAN was administered either one hour before TNF or at the peak of plasma TNF (191), animals were protected as previously observed in TVX/LPS coexposure (189). In contrast, ETAN administered after the prolongation did not protect mice from TVX/TNF-mediated injury, once again suggesting that the prolongation of plasma TNF is necessary to precipitate TVX-mediated hepatotoxicity (191). The study did demonstrate that TVX reduced the clearance of plasma TNF. Decreased clearance of TNF is a possible explanation for the TVX-induced TNF prolongation, but a role remained for increased synthesis of LPS-induced TNF (191). The findings relevant to the involvement of TNF in the model of TVX/LPS coexposure in mice are summarized in Figure 3 and serve as the basis for this dissertation. In brief,

In summary, significant evidence has been generated to suggest that TNF plays a pivotal role in many of the drug-LPS coexposure models of hepatotoxicity in rodents. In addition to TNF being necessary to precipitate toxicity, increased or prolonged plasma TNF is yet another aspect of the drug-LPS interactions *in vivo* that could be modeled and studied *in vitro*. Development of an *in vitro* system that attempts to recapitulate increased LPS-induced TNF production during drug coexposure is a novel approach to study the toxic liability of IDILI-associated drugs.

Figure 3. TVX/LPS hepatotoxicity requires prolonged plasma TNF in mice. A) TVX/LPS coexposure results in significant hepatocellular injury 4.5 hours after LPS administration (189). **B)** LPS-induced TNF release is prolonged by TVX but not LVX before hepatocellular injury (189). ∗ - Significantly different from the same treatment group at 0 h. # - Significantly different from Veh/LPS-treated mice at the same time.

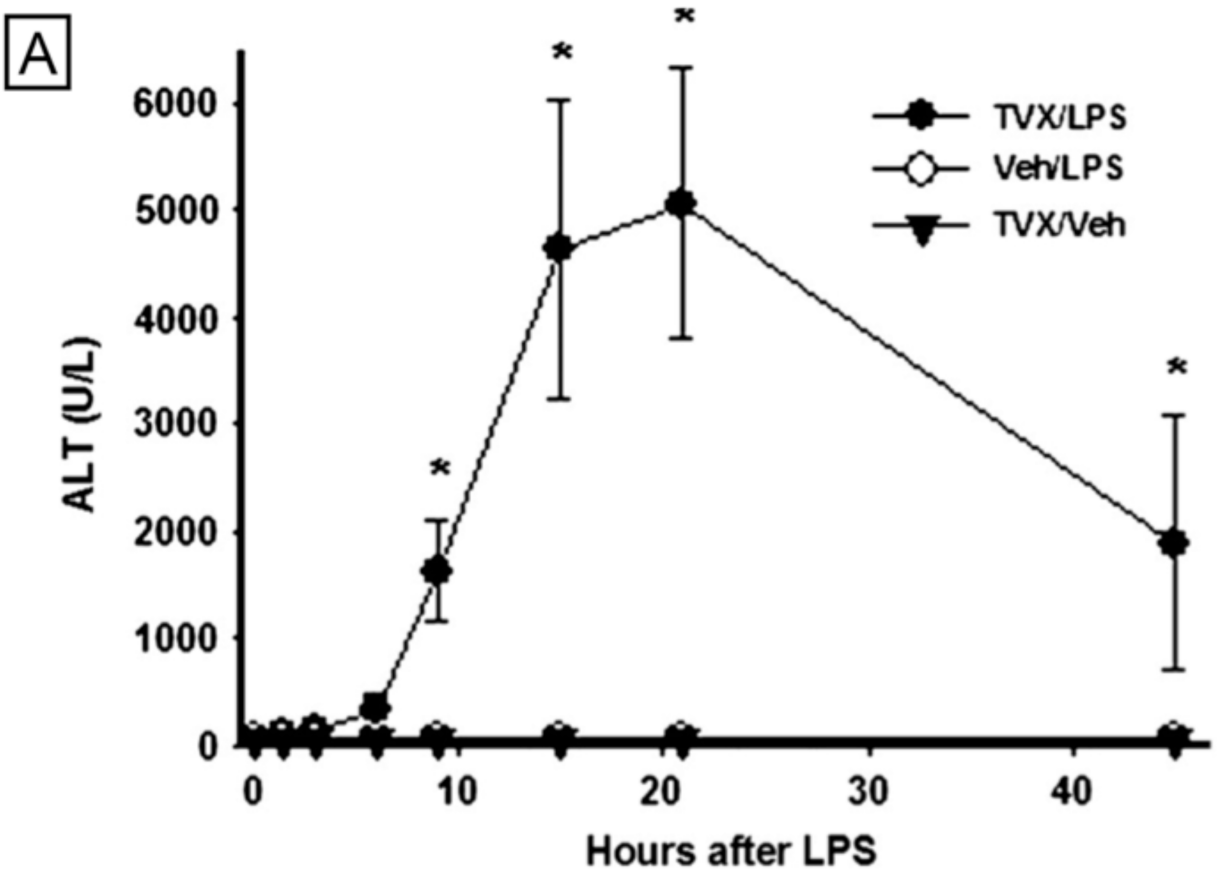
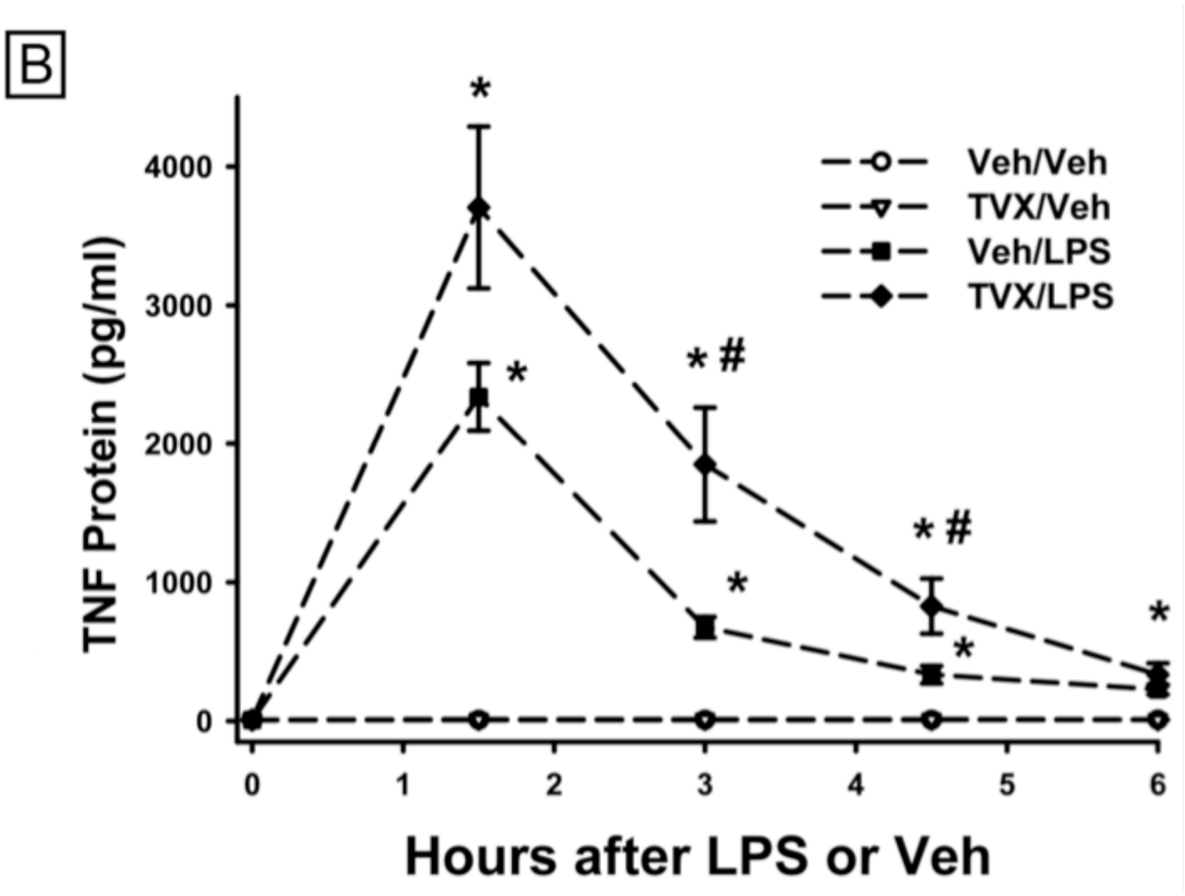


Figure 3 (cont'd)



1.4 Development of a drug-LPS coexposure model *in vitro*

1.4.1 Purpose of model development

Many of the animal models of hepatotoxicity with IDILI-associated drugs developed using the inflammatory stress hypothesis are the first demonstrations of reproducible hepatotoxic responses to these drugs. As such, the application of the inflammatory stress hypothesis in preclinical toxicity testing for drug candidates is intriguing and attractive. Implementing whole-animal models of drug-inflammagen coexposure with drug candidates during preclinical toxicity testing would be a difficult undertaking. The substantial cost of preclinical toxicity testing during drug development would be increased by the inclusion of yet another battery of animal tests. The heterogeneity of drug-LPS coexposure models with respect to proper dose-combinations in animals further complicates the issue.

The inflammatory stress hypothesis-derived models demonstrate unique temporal dependences with respect to drug and inflammagen exposure. In rats, for example, TVX/LPS is hepatotoxic when LPS administration precedes TVX (123). In mice, however, TVX administration must precede LPS to precipitate hepatotoxicity (189). In just the example above, the temporal relationship between TVX and LPS exposure needed to precipitate hepatotoxicity represents a significant obstacle to implementing these whole-animal models for preclinical toxicity screening. Finding the proper dose and temporal relationship between drug and inflammagen could be cost- and time-prohibitive; with standard animal testing being relatively expensive and low-throughput. Finally, animal toxicity models are considered "under-powered" (71), i.e., the smallest group of animals possible are used to prevent unnecessary animal

suffering and to limit cost. One of the most significant challenges in animal testing is the ethical concern related to animal welfare (75). Developing *in vitro* systems to assist in predicting toxic responses could avoid some of the hardships associated with animal testing.

1.4.2 Considerations for developing *in vitro* models

As the noted statistician George E.P. Box once wrote, " Remember that all models are wrong; the practical question is how wrong do they have to be to not be useful". This quote is useful in addressing some of the deficits that have been attributed to *in vitro* modeling. That is to say, *in vitro* testing and the results generated *in vitro* are quite useful if the appropriate context and cautions are applied to interpretation. Interpreting the translational impact of any findings *in vitro* to human health must be thoroughly scrutinized.

Most *in vitro* systems utilize monolayers of primary cells or transformed cell-lines. The monolayer cell cultures lack the unique architecture of the parent organ in which the cells normally reside. The phenotype of isolated primary cells grown out of the context of the parent organ, e.g. hepatocytes from a liver, can differ significantly from the phenotype *in vivo* (76). The other cells grown *in vitro* are transformed cell-lines. Transformed cells are advantageous given their nearly limitless replication potential and fairly consistent phenotype in culture. That being said, transformed cells are cancerous in origin, possessing a potential litany of mutations (58) as well as potentially containing variable cell types in a given line (29). Perhaps most importantly, the integrated response of an organ or animal to a drug is nearly impossible to replicate *in vitro* due to

the complex and heterogeneous cellular milieu in tissues as well as critical metabolic activation that occurs with many drugs (76). There are several advantages to *in vitro* models, however, especially if the findings are addressed and confirmed in subsequent experiments *in vivo* or if the *in vitro* model recapitulates what has been previously done *in vivo.*

Cells grown *in vitro* are both amenable to various culture conditions and high-throughput configurations. Studies performed *in vitro* have also come to be indispensible for studying changes in intracellular signaling. Many of the established *in vitro* culture systems and cells used are relatively reliable, thoroughly characterized and yield reproducible results. These attributes therefore allow for automation, an enticing characteristic to a high throughput model (76). Taken together, an *in vitro* model that screens for drug-mediated increases in LPS-induced TNF release could represent a preclinical test to identify drugs with IDILI liability.

Several cell-lines for macrophage-like cells as well as other inflammatory cells are commercially available, are well characterized and produce TNF in response to LPS. Multiple combinations of drug and LPS concentrations can be tested simultaneously for TNF release. Furthermore, if a drug is found to increase LPS-induced TNF release, mechanistic studies can follow to assess drug-mediated changes in intracellular signaling or identify selective targets of the drug that lead to increased TNF release.

The findings from any *in vitro* system, such as those that would stem from studying a drug-mediated increase in LPS-induced TNF release, would lack merit if not verified in animal studies. The findings from the TVX/LPS model in mice serve as a

convenient foundation for developing an *in vitro* system to predict the IDILI liability of a drug. Until recently, TVX was a drug associated with hepatotoxicity of unknown etiology in humans. The model of TVX/LPS coexposure in mice represented a significant step towards understanding TVX-mediated hepatotoxicity in humans. Establishing an *in vitro* model to assess TVX-mediated changes in LPS-induced coexposure could increase the understanding IDILI liability associated with TVX. Furthermore, the evidence supporting IDILI drug-mediated increases in LPS-induced TNF release in animal models of hepatotoxicity strengthens the relevance of an *in vitro* model used to assess the IDILI liability associated with drug candidates.

1.4.3 Discovery of mechanisms underlying IDILI liability of TVX

The model of TVX/LPS coexposure in mice is highlighted due to the substantial amount of evidence generated that describes TVX-mediated hepatotoxicity in mice. Despite the extensive characterization of TVX/LPS coexposure in mice, the specific mechanism underlying the hepatotoxic liability of TVX remains elusive. Work from others suggested that a cyclopropylamine moiety of the TVX parent molecule might be metabolized to a reactive intermediate (204, 205). This reactive intermediate (204) could covalently bind to intracellular hepatic proteins and result in liver damage (205). These studies were performed strictly *in vitro* in cell-free reaction mixtures, but suggested a role for inflammatory stress in TVX-dependent hepatotoxicity. The TVX-derived cyclopropylamine moiety was incubated in the presence of myeloperoxidase, an enzyme released by activated neutrophils (82, 204, 192).

Another study implicated inflammatory stress as a determinant in TVX-mediated hepatotoxicity and suggested a potential target in cells to explain the hepatotoxic liability of TVX (83). TVX was found to enhance mitochondrial stress in mice with underlying mitochondrial dysfunction (83). The TVX-mediated hepatotoxicity in the mitochondrial dysfunction model was modest in comparison to TVX/LPS coexposure in mice, yet the authors suggested the TVX-mediated increase in oxidative stress in the liver could be macrophage-derived (83). Another explanation the authors in (83) posited to explain the toxic mode of action associated with TVX was off-target eukaryotic topoisomerase inhibition.

1.4.4 TVX as a topoisomerase inhibitor

Others have suggested that TVX might act as a eukaryotic topoisomerase inhibitor previously. Global gene expression analysis of TVX-treated rat livers and TVX-treated hepatocytes suggested that topoisomerase inhibition could explain several of the TVX-mediated effects on gene expression (124, 226). Another study indirectly showed that TVX acted as a eukaryotic topoisomerase inhibitor. Potent eukaryotic topoisomerase inhibitors etoposide and doxorubicin were responsible for significant alterations in chromosomal expression patterns and colocalization of regulated genes attributed to topoisomerase inhibition (170). TVX was also found to influence the chromosomal expression patterns in a similar fashion as etoposide and doxorubicin (170). Taken together, several lines of evidence implicate TVX acting as a eukaryotic topoisomerase inhibitor.

TVX is a broad-spectrum fluoroquinolone antibiotic that has an expanded bactericidal spectrum and relatively higher bioavailability than other fluoroquinolones (54). The attributes of TVX allowed for once daily dosage that could enhance patient compliance, making it therapeutically advantageous in humans. Fluoroquinolone-mediated bactericidal activity is due to poisoning of the type-II bacterial topoisomerase enzymes, DNA gyrase and topoisomerase IV (23). Roughly 2.5 million TVX prescriptions were filled and a total of 140 reports of hepatotoxicity led to severe restriction on the use of TVX (202). Among the 140 reports of hepatotoxicity, 14 resulted in liver failure, 5 required liver transplantation and 5 deaths were associated with TVX (7). Other fluoroquinolones ciprofloxacin (CPX) and moxifloxacin (MOX) have associated with IDILI (117), suggesting that the IDILI liability associated with some fluoroquinolones might be attributed to their pharmacological class.

DNA topoisomerases are ubiquitously expressed enzymes that relieve torsional stress on DNA manifest through processes such as transcription, replication and genomic maintenance (163). Topoisomerases relax the twisted DNA by cutting the phosphodiester backbone of the DNA helix and religating it after the torsional stress is relieved (163). The prokaryotic targets of fluoroquinolones share sequence homology to eukaryotic, but fluoroquinolones are designed to selectively target prokaryotic topoisomerases. Fluoroquinolones, however, have been shown to inhibit the activity of eukaryotic forms (2, 8, 24, 79, 169). The phenomenon of topoisomerase inhibition can be driven by several different mechanisms and is commonly referred to as "topoisomerase poisoning" (133).

Fluoroquinolone-mediated eukaryotic topoisomerase poisoning would not be as potent as drugs developed to target eukaryotic isoforms. Off-target eukaryotic topoisomerase poisoning by fluoroquinolones would require relatively higher drug concentrations in eukaryotes as compared to selective eukaryotic topoisomerase poisons (197). Despite the relatively weak poisoning of eukaryotic topoisomerases by fluoroquinolones, genotoxic effects by fluoroquinolones in mammalian cells have been described (2, 51, 79, 169).

The connection between fluoroquinolone poisoning of eukaryotic topoisomerase and hepatotoxicity is therefore plausible, save that TVX, CPX and MOX, for example, are predominantly nontoxic in humans (117). Furthermore, the genotoxic stress mediated by fluoroquinolones is mild compared to drugs specifically designed to poison eukaryotic topoisomerase, i.e., etoposide (5, 197). A susceptibility factor, such as inflammatory stress, might be required to transform an otherwise nontoxic dose of a fluoroquinolone to a dose that is hepatotoxic. If that rationale is applied to the TVX/LPS model of hepatotoxicity, there is a need to address how topoisomerase poisoning can lead to increased LPS-induced TNF release.

The relationship between increased TNF expression and release and fluoroquinolone-mediated topoisomerase poisoning is supported by several other studies involving topoisomerase poisons and DNA damaging agents. Topoisomerase poisoning stabilizes the enzyme-DNA complex, and this stabilized complex ultimately leads to DNA damage (121, 125, 181). Doxorubicin and etoposide, both topoisomerase poisons, as well as the anti-metabolite 5-fluorouracil increased cytokine expression in murine macrophages in vitro and mice in vivo (50, 235). In some studies, the DNA

damaging drug induced cytokine expression that was linked to cytokine-mediated toxicity. Cisplatin-induced nephrotoxicity in mice is associated with increased (166) TNF production, and anti-mitotic paclitaxel can induce TNF expression and release in murine and human lymphocytes (230, 246). In addition, etoposide exposure in cells demonstrated a DNA damage signaling-dependent activation of NF-kB (237, 238). Furthermore, MAPK activation in response to DNA damage is also well known (45). Taken together, DNA damaging agents can induced cytokine synthesis and activate the pathways and effectors in Table 1 likely responsible for increased TNF synthesis and/or release.

1.5 Summary

The liver is an organ of complex physiology requiring several different cell-types to carry out its various physiological functions. The liver is also an organ of immunity that is critical to the health of the host. The KCs are a key mediator in the immune responses in the liver, and KCs play a dichotomous role in the health of the liver and host. The animal models of hepatotoxicity employing xenobiotics or IDILI-associated drugs in combination with LPS point to a significant role for KCs and KC-derived TNF in precipitating hepatotoxicity. Establishing an *in vitro* model to recapitulate and investigate a drug-mediated increase in LPS-induced TNF release in a macrophage cell-line could help to identify drugs with IDILI liability and uncover the molecular targets of IDILI-associated drugs. The experimental work described in this dissertation is establishes a model of increased LPS-induced TNF release in TVX/LPS-coexposed macrophage-like cells. This model could then be used to detect and/or test potential TVX-selective targets that mediate this phenomenon of increased LPS-induced TNF release, such as the off-target poisoning of topoisomerase by TVX.

1.6 Hypothesis and Aims

The overall hypothesis to be tested is that trovafloxacin poisons eukaryotic topoisomerase, which increases LPS-induced tumor necrosis factor-alpha release in vitro. This hypothesis was created because increased LPS-induced TNF release by TVX is critical to TVX-dependent hepatotoxicity in vivo and because TVX-mediated topoisomerase poisoning in eukaryotic cells has been suggested many times but remains untested.

Aim 1 Hypothesis: TVX increases LPS-induced TNF release through activation of NF-kB or MAPK signaling (Chapter 2).

Aim 2 Hypothesis: TVX-mediated increase in LPS-induced TNF release is due to TVX poisoning of eukaryotic topoisomerase (Chapter 3).

CHAPTER 2

Poulsen KL, Albee RP, Ganey PE and Roth RA. Trovafloxacin Potentiation of Lipopolysaccharide-Induced Tumor Necrosis Factor Release from RAW 264.7 cells requires ERK and JNK. Submitted to J Pharmacol Exp Ther 11-2013

2.1 Abstract

Trovafloxacin (TVX) is a fluoroquinolone antibiotic known to cause idiosyncratic, drug-induced liver injury (IDILI) in humans. The mechanism underlying this toxicity remains unknown. An animal model of IDILI in mice revealed that TVX synergizes with inflammatory stress from bacterial lipopolysaccharide (LPS) to produce an hepatotoxic interaction that requires prolongation of the appearance of tumor necrosis factor-alpha (TNF) in the plasma. A model of TVX/LPS interaction *in vitro* was established in RAW 264.7 cells acting as a surrogate for TNF-releasing cells *in vivo*. Pretreating cells with TVX for 2h before LPS addition led to increased TNF protein release into culture medium in a concentration- and time-dependent manner relative to cells treated with LPS or TVX alone. During the pretreatment period, TVX increased TNF mRNA, but this was less apparent after LPS addition, suggesting that the pivotal signaling events that increase TNF expression occurred during the TVX pretreatment period. Indeed, TVX exposure increased activation of ERK, JNK and p38 MAPKs. Inhibition of either ERK or JNK decreased the TVX-mediated increase in TNF mRNA and LPS-induced TNF protein release, but p38 inhibition did not. These results demonstrated that the increased TNF appearance from TVX-LPS interaction in vivo can be reproduced in vitro and occurs in an ERK- and JNK-dependent manner.

2.2 Introduction

Drug-induced liver injury (DILI) is responsible for more than half of acute liver failure cases in the United States (154). DILI is associated with significant morbidity and mortality. It is the most common adverse effect that prevents market approval for new drug entities, and it prompts removal of existing drugs from the market (227). An important subset of DILI is idiosyncratic, drug-induced liver injury (IDILI), which accounts for 13% of all cases of acute liver failure (154). Although this represents a fraction of all instances of DILI, the bulk of FDA-imposed restrictions on the use of drugs is due to idiosyncratic adverse drug reactions (114).

Causes of IDILI are not well understood. Among several hypotheses put forth to explain IDILI is the inflammatory stress hypothesis, which states that a mild inflammatory episode interacts with a drug resulting in hepatotoxicity (192). Animal models based on this hypothesis have been developed for several drugs that have caused IDILI in humans, including chlorpromazine, ranitidine, amiodarone, doxorubicin, sulindac and trovafloxacin (TVX) (28, 78, 126, 129, 189, 249). In each of these models, bacterial lipopolysaccharide (LPS) was used to cause a modest, nontoxic, acute inflammatory episode.

Binding of LPS to toll-like receptor 4 (TLR4) on inflammatory cells leads to activation of proximal intracellular signaling factors in the MyD88-dependent pathway (33). The result is TLR4-dependent signaling that activates canonical mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (168, 182, 206). Activated MAPKs induce the

transactivation of genes that encode tumor-necrosis factor alpha (TNF) and other mediators of acute inflammation as well as increase the stability of TNF mRNA (43, 224). TNF has been implicated as a critical mediator of liver injury in drug-inflammagen cotreatment models employing several of the aforementioned IDILI-associated drugs.

TVX is a broad-spectrum, quinolone antibiotic that acts by inhibiting bacterial DNA gyrase and topoisomerase IV (23). Soon after its introduction into the market, TVX use was severely restricted due to 14 cases of acute liver failure, of which 5 were fatal (7). An animal model of TVX IDILI was established in which mice coexposed to nontoxic doses of TVX and LPS developed pronounced hepatocellular injury (189). A defining characteristic of this model was that the liver injury depended upon TNF. Pharmacological intervention with pentoxifylline to inhibit TNF transcription or with etanercept to neutralize released TNF prevented the injury (189). Studies in TNF receptor knockout mice supported a key role for TNF in TVX/LPS hepatotoxicity (190). Importantly, the LPS-induced increase in plasma concentration of TNF was significantly prolonged by TVX, and this prolongation proved to be critical in the pathogenesis of liver injury. In animals treated with TVX and TNF rather than LPS, liver injury also occurred, and TVX caused the appearance of TNF in plasma to be prolonged due in part to enhanced TNF synthesis (191). Whether this resulted from a direct effect on TNF-producing cells was unknown. Accordingly, the purpose of the present study was to test the hypothesis that TVX pretreatment directly increases LPS-induced TNF synthesis and release by cells *in vitro* and to explore the underlying intracellular signaling involved in the response.

2.3 Material and Methods

2.3.1 Chemicals and inhibitors

All chemicals and reagents in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Antibiotic/antimycotic and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Life Technologies (Grand Island, NY).

2.3.2 Cell culture

The RAW 264.7 macrophage cell line (American Type Culture Collection, Manassas, VA) was maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic at 37°C in 5% CO_2. Cells were harvested by detachment with a sterile spatula and plated at a density of 4 X 10^4 cells per well in 24-well plates (Costar, Lowell, MA) for cytokine release and RNA isolation or 1 X 10^6 cells per well in 10 cm plates (Costar) for protein isolation. Medium was replaced after 24h with DMEM (0.5% FBS) for cell synchronization prior to drug exposure 16h later.

2.3.3 TNF release

Synchronized RAW 264.7 cells, at 80-90% confluency, were pretreated for 2h with various concentrations of TVX (1-100 μM) in 0.5% fetal bovine serum-containing medium to the same medium. TVX was dissolved in 0.1N potassium hydroxide (VEH) at a stock concentration of 50 mM and diluted to the final concentration in 0.5% fetal bovine serum -containing medium. This 2-hour incubation was followed by a medium change to medium containing LPS from Escherichia coli serotype O55:B5 (Lot

075K4038) at various concentrations (0.1-100 ng/ml) or saline vehicle (SAL) as control. The LPS had an activity of 3.3×10^6 EU/mg as determined using a colorimetric, *Limulus* amebocyte lysate assay (Cambrex Corp., Kit 50-650U; East Rutherford, NJ). For determination of TNF release, an enzyme-linked immunosorbent assay (ELISA) was performed (BD Biosciences, San Jose, CA). Cell culture medium was withdrawn at various times and stored at -20°C until the time of analysis. Ninety-six well plates were coated with an anti-TNF capture antibody in a coating buffer overnight at 4°C. Medium was diluted to remain within standard curve concentrations.

2.3.4 Protein isolation

RAW 264.7 cells seeded in 10 cm plates as previously stated were exposed to TVX or an equivalent volume of VEH. At indicated times, plates were washed twice with cold phosphate-buffered saline (PBS) and scraped with 1 ml of cold PBS. The cells were pelleted (400 *g* x 2 minutes) then resuspended in cold RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with serine protease inhibitor PMSF and HALT protease and phosphatase inhibitor cocktails (Thermo Scientific). After a 10-minute incubation on ice, suspended pellets were sonicated twice with 5-second pulses. Suspensions were centrifuged at 22,000 *g* for 30 minutes, and supernatants were withdrawn and stored at -80° C until analysis occurred.

2.3.5 Western blot analysis

Protein concentration in extracts was determined by bicinchoninic acid assay (Thermo Scientific). All western analyses were performed by loading 20 µg of protein

on precast NuPAGE® SDS-PAGE gels (Life Technologies) using all NuPAGE® reagents. For phospho-MAPK, samples were separated on precast 12% gels. After separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) for 1 hour at 4° C. Membranes were blocked in 5% BSA dissolved in tris-buffered saline plus 0.1% Tween20 (TBST) for 1 hour prior to incubation with primary antibody. They were probed with phospho-ERK1/2 (Thr202/Tyr204**)**, phospho-p38 (Thr180/Tyr182), phospho-MAPKAPK2 (Thr 334) or phospho-ATF2 (Thr71) rabbit polyclonal antibodies (Cell Signaling Technology). For subsequent probes, membranes were stripped with Restore western blot stripping agent (Thermo Scientific), washed for 30 minutes in TBST and blocked prior to incubation with antibodies to total ERK, ATF, p38 or Lamin B1.

2.3.6 RNA isolation and RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) per manufacturer's instructions. RNA quantity and quality were assessed using Nanodrop 2000 (Thermo Scientific). cDNA was prepared using 1 µg of RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The expression of specific genes was analyzed using SYBR Green (Applied Biosystems, Foster City, CA). Expression level was normalized to β-actin. PCR primers used were: mouse TNF [5' - TCTCATGCACCACCATCAAGGACT- 3' (forward) and 5' - ACCACTCTCCCTTTGCAGAACTCA- 3' (reverse)] and mouse β-actin [5' – TGTGATGGTGGGAATGGGTCAGAA- 3' (forward) and 5' – TGTGGTGCCAGATCTTCTCCATGT- 3' (reverse)].

2.3.7 Statistical analysis

A one- or two-way Analysis of Variance (ANOVA) was performed on data sets with Tukey's post-hoc test applied for multiple comparisons between groups. The criterion for significance was $p < 0.05$.

2.4 Results

2.4.1 Trovafloxacin pretreatment potentiates LPS-induced TNF release in a dose- and time-dependent manner

Raw cells were treated using the regimen depicted in Figure 4. It is important to note that the TVX-LPS interaction described below required pretreatment with TVX for 2h followed by removing the TVX-containing medium and replacing it with medium containing LPS or its vehicle, saline (SAL).

Exposure of cells for 6h to LPS (0.1-100 ng/ml) resulted in a concentration-dependent increase in TNF concentration in the culture medium (Figure 5A). Pretreatment with TVX (1, 10 or 100 uM) significantly increased LPS-induced TNF release in a concentration-dependent manner. The maximal difference between VEH- and TVX-pretreated cells was detected with a combination of 100 μM TVX and 10 ng/ml LPS, so further studies were conducted using this combination. Levofloxacin (LVX), a quinolone antibiotic used as a negative comparator due to its lesser association with IDILI, did not significantly increase TNF release 6h after LPS exposure (Figure 5B). This is consistent with previous results in mice treated with LVX/LPS (189).

TVX pretreatment increased LPS-induced TNF release into cell culture medium at all times (1-10h) after LPS exposure (Figure 6). TVX pretreatment also increased TNF release into the medium in SAL-treated cells at all times observed, although the increase was small. In cells treated only with LPS, the rate of TNF release (ie, slope of lines in Figure 6) was highest from 1-3h and decreased thereafter. In comparison, the release rate in LPS/TVX-cotreated cells was greater from 1-3h and remained constant

through 6h (Table 2). TVX pretreatment also increased the rate of release in the absence of LPS.

Figure 4. Protocol for treatment of RAW cells with TVX/LPS. After an overnight synchronization in 0.5% FBS-containing medium, VEH or TVX was added to RAW cells for a two-hour pretreatment. The medium was then withdrawn and replaced with SAL or LPS, and samples were collected at various times 1-10 hours thereafter. For some studies, cultures were examined immediately after the TVX treatment (ie, 0 h).

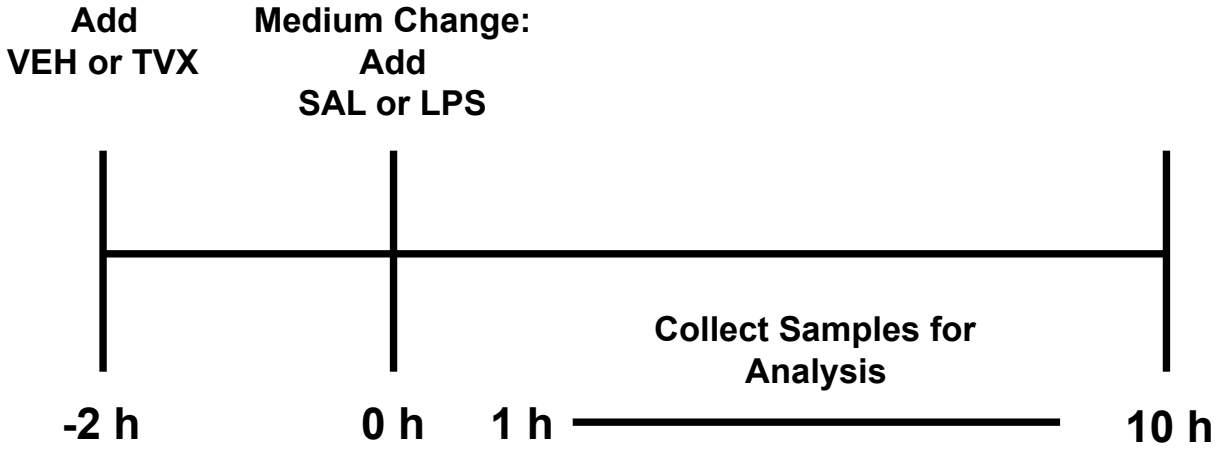
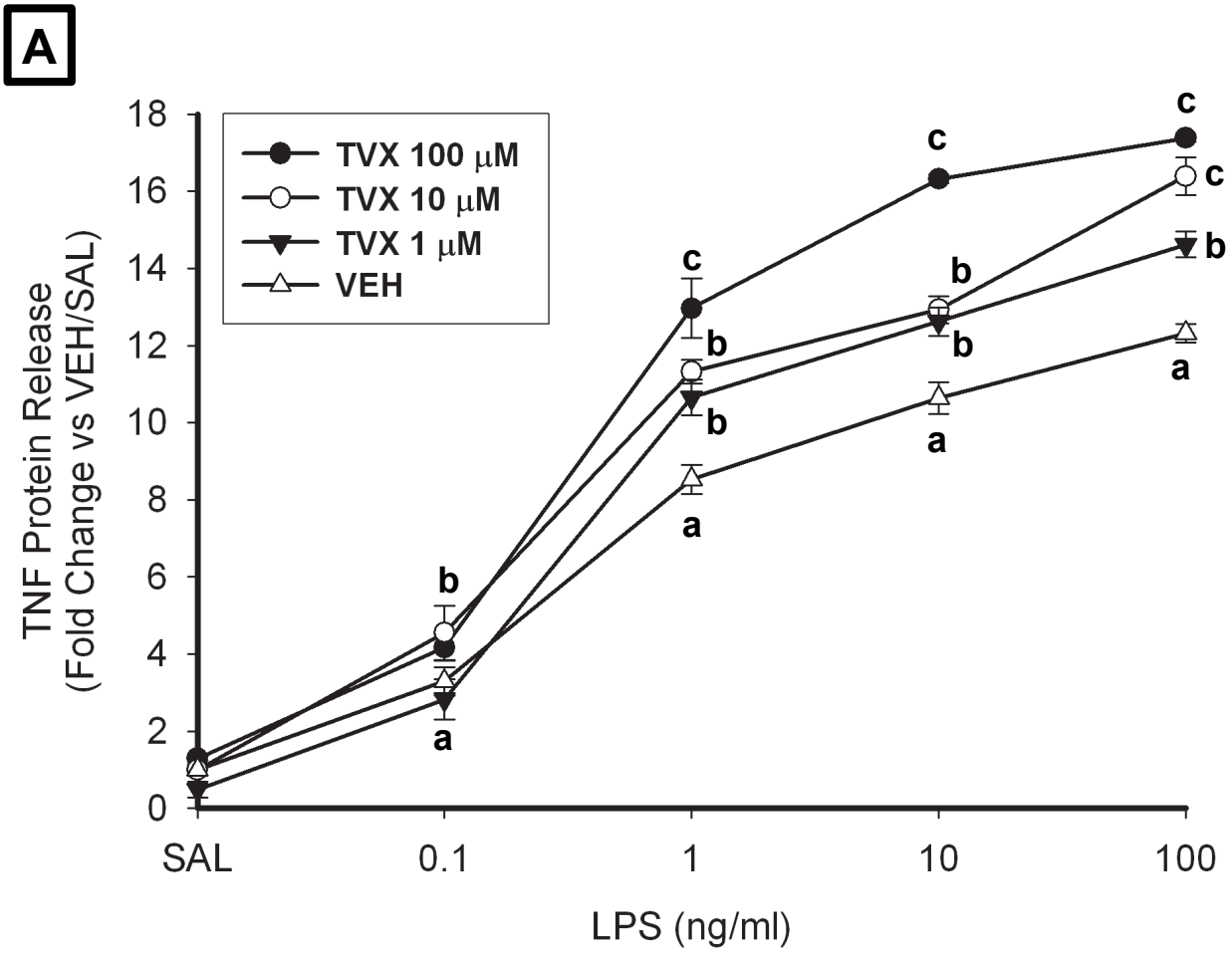


Figure 5. TNF production in TVX/LPS-treated RAW cells: concentration-response. A) RAW cells were pretreated with VEH or TVX (1, 10 or 100 μM) for 2 hours before exposure to SAL or various concentrations of LPS (0.1 – 100 ng/ml), and TNF concentration was assessed by ELISA 6 hours after LPS. Values are means ± SEM for fold change of TNF release relative to VEH/SAL-treated cells, n=3. **B)** RAW cells were pretreated with VEH or LVX (30 or 100 μM) for 2 hours before LPS addition (10 ng/ml) and TNF concentration was assessed by ELISA 6 hours after LPS. Values are means ± SEM fold change of TNF release relative to VEH/SAL-treated cells, n=3. Values with different letters differ significantly within LPS concentration, $p < 0.05$



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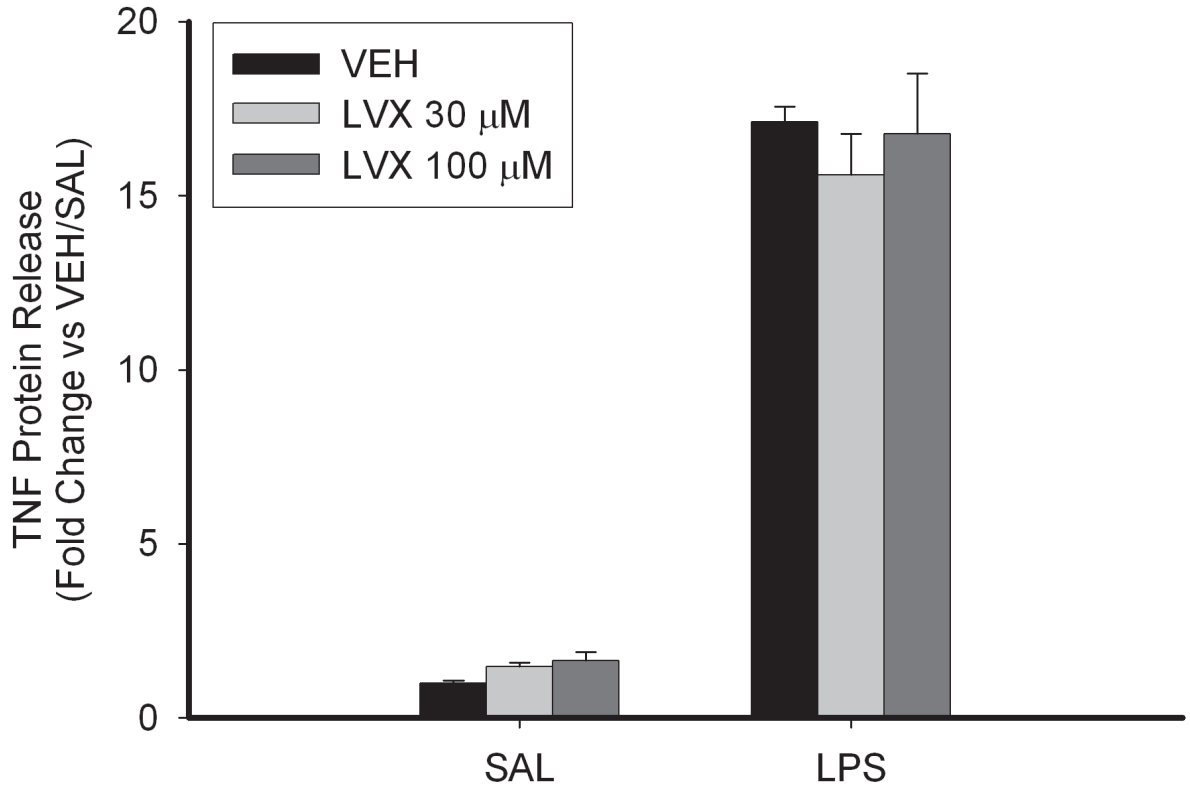


Figure 6. Timecourse of TNF release in TVX/LPS-treated RAW cells. A) RAW cells were pretreated with VEH or TVX (100 μM) then with SAL or LPS (10 ng/ml) as shown in Figure 4, and TNF concentration was assessed by ELISA. Values are mean concentration ± SEM, n=3. a – $p < 0.05$ vs. VEH/SAL within a timepoint, b - $p < 0.05$ vs. VEH/LPS within a timepoint

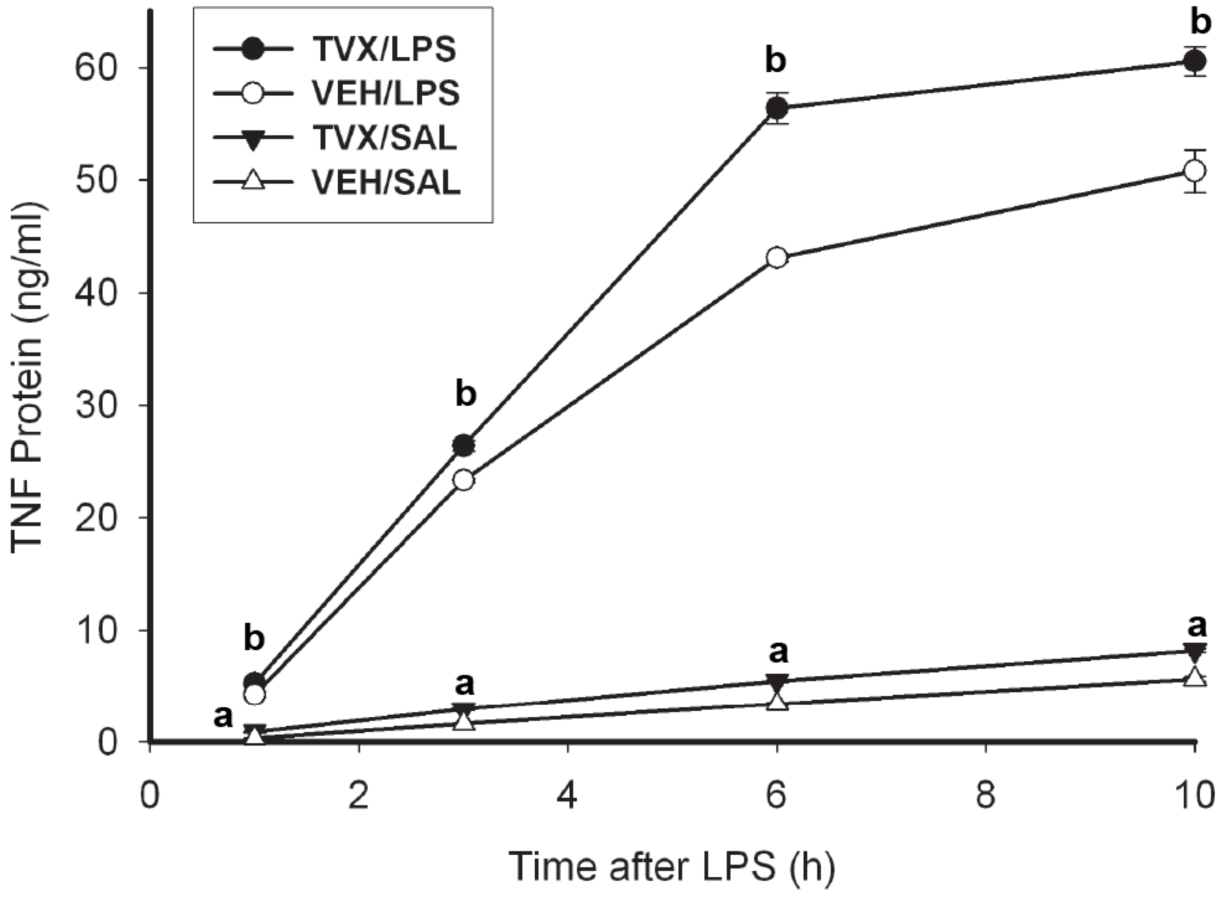


Table 2. Calculated rate of change in TNF release from RAW cells. Rate of TNF release from RAW cells was calculated from data in Figure 6 as the (Δ[TNF]/ΔTime) ± SEM. a – $p < 0.05$ vs. VEH/SAL within a timepoint, b – $p < 0.05$ vs. VEH/LPS within a timepoint

Treatment	Rate of change in medium TNF concentration (ng/ml/hr)		
	1–3 h	3-6 h	6-10 h
VEH/SAL	0.66 ± 0.01	0.59 ± 0.02	0.57 ± 0.07
TVX/SAL	1.01 ± 0.02[a]	0.85 ± 0.04[a]	0.69 ± 0.07[a]
VEH/LPS	9.51 ± 0.14	6.59 ± 0.18	1.93 ± 0.55
TVX/LPS	10.55 ± 0.22[b]	10.00 ± 0.32[b]	1.04 ± 0.07[b]

2.4.2 TVX, but not LVX, significantly increases TNF mRNA prior to LPS addition

TVX pretreatment of RAW cells did not increase TNF mRNA from 1-3h after SAL or LPS exposure despite the consistent increases in TNF protein release, although a trend was observed (Figure 7). Accordingly, the effect of TVX on TNF mRNA prior to LPS addition was assessed. Exposure of RAW cells to TVX for 2h increased TNF mRNA (Figure 8). In contrast, exposure to LVX for 2h failed to affect TNF mRNA (Figure 9).

Figure 7. Effect of TVX treatment on TNF mRNA expression in RAW cells after LPS exposure. RNA was isolated from RAW cells after SAL- or LPS-exposure at the indicated times. The RNA was converted to cDNA, and TNF mRNA was quantified by RT-PCR and normalized to beta actin mRNA. Values are means of fold change of VEH/SAL ± SEM, n=6. No significant differences were observed.

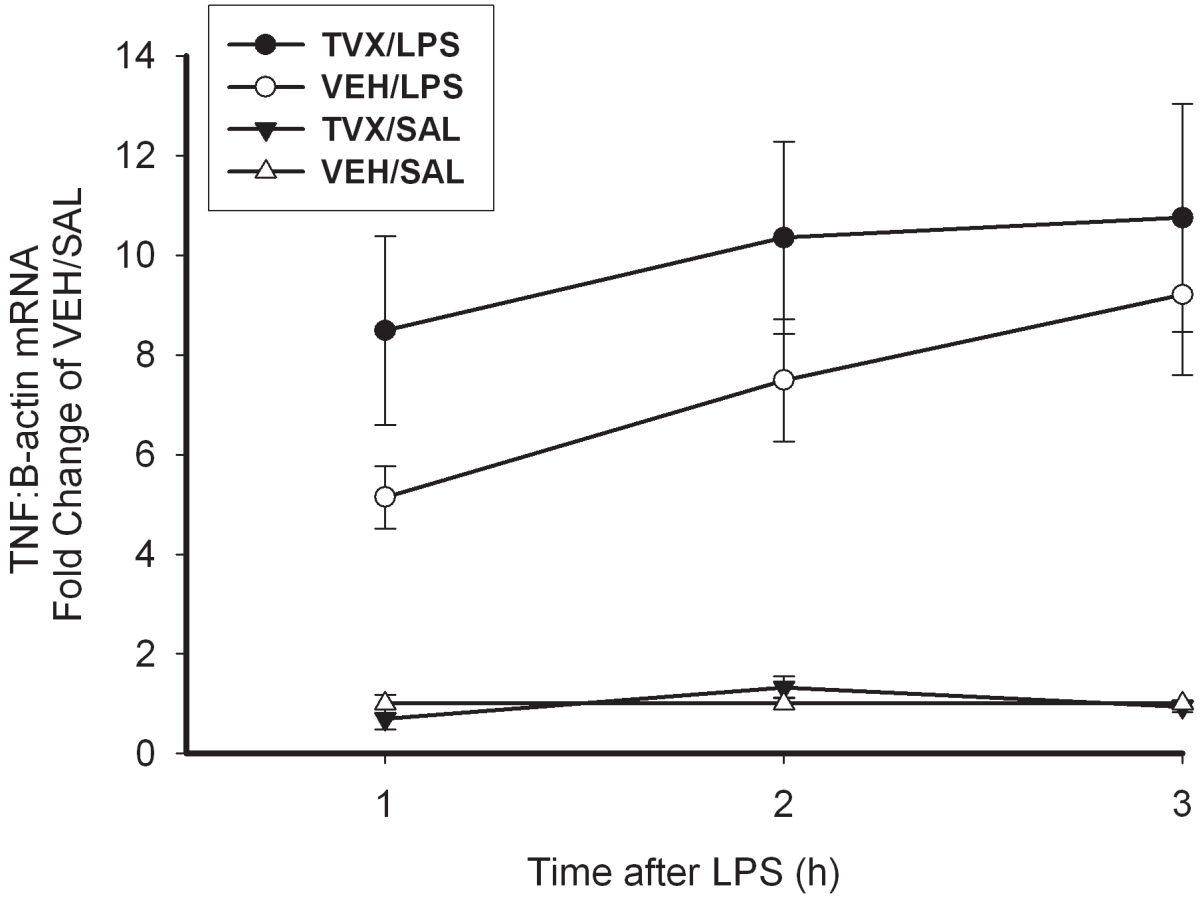


Figure 8. Effect of TVX treatment on TNF mRNA in RAW cells. RNA was isolated from RAW cells after TVX treatment, converted to cDNA, and TNF mRNA was quantified by RT-PCR and normalized to beta actin mRNA. Values are means ± SEM, n=3. a - $p < 0.05$ vs. VEH within a timepoint.

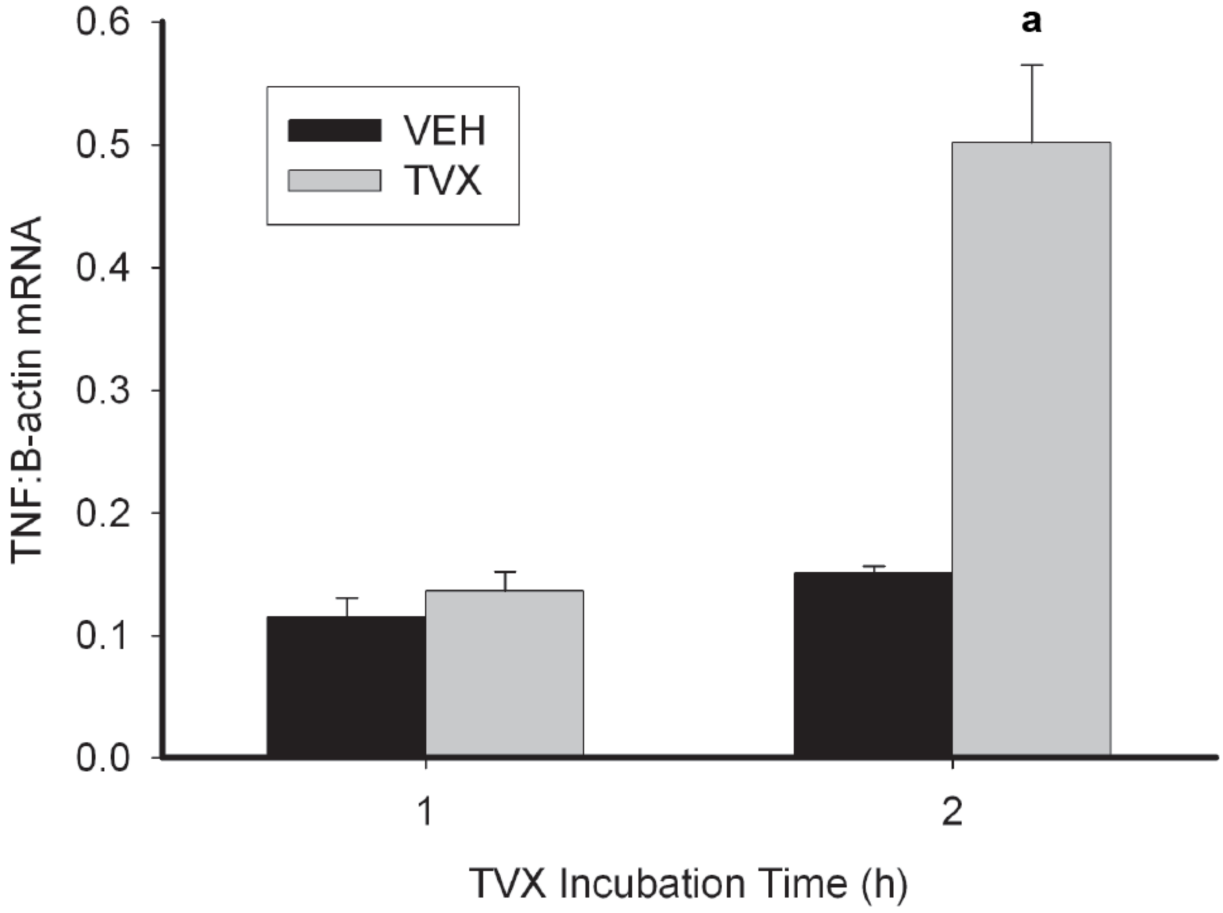
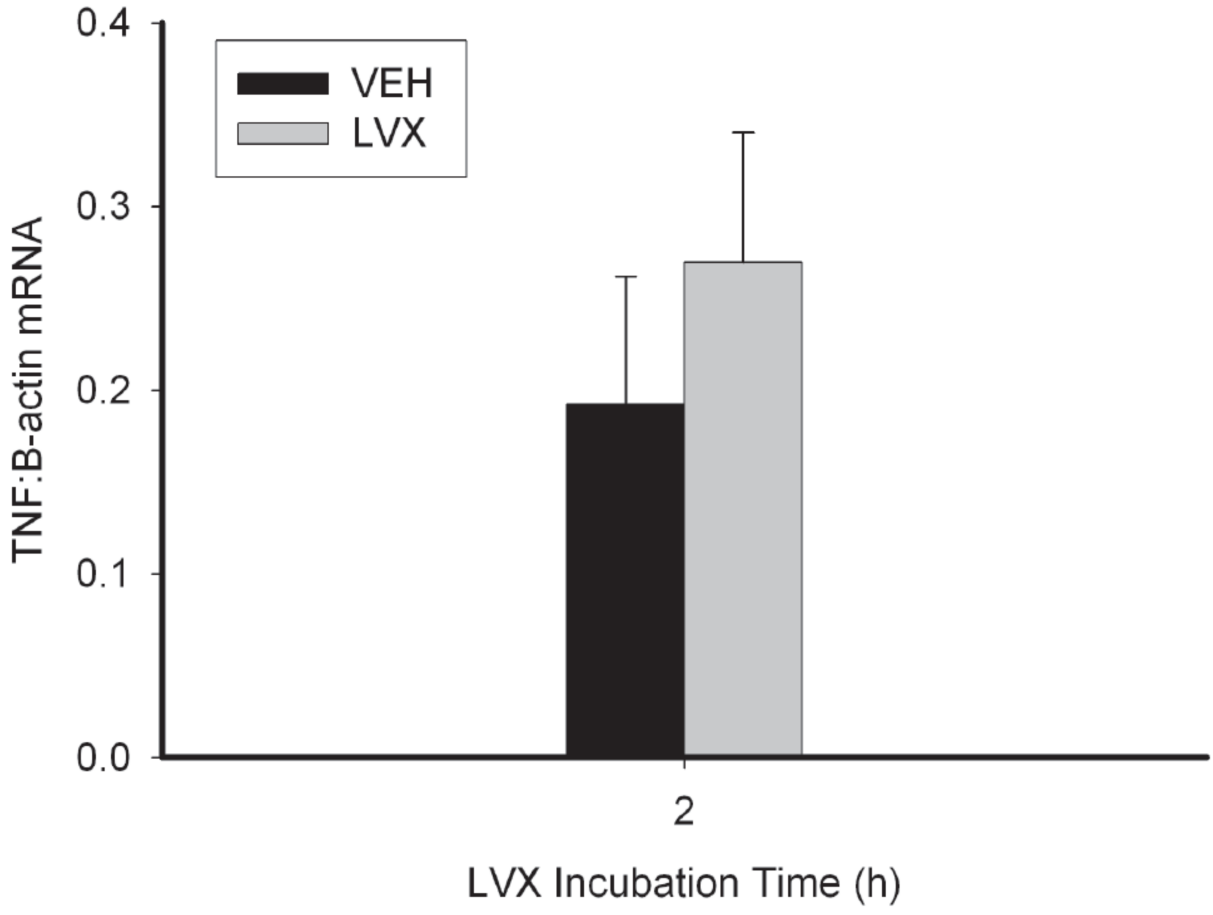


Figure 9. Lack of effect of LVX treatment on TNF mRNA expression in RAW cells. RNA was isolated from RAW cells after VEH or LVX (300 uM) exposure for 2 hours, converted to cDNA, and TNF mRNA was quantified by RT-PCR and normalized to beta actin mRNA. Values are means ± SEM, n=3. No significant difference was observed.



2.4.3 TVX treatment increases MAPK phosphorylation prior to LPS addition, and ERK and JNK signaling are required for TVX-mediated increases in LPS-induced TNF release

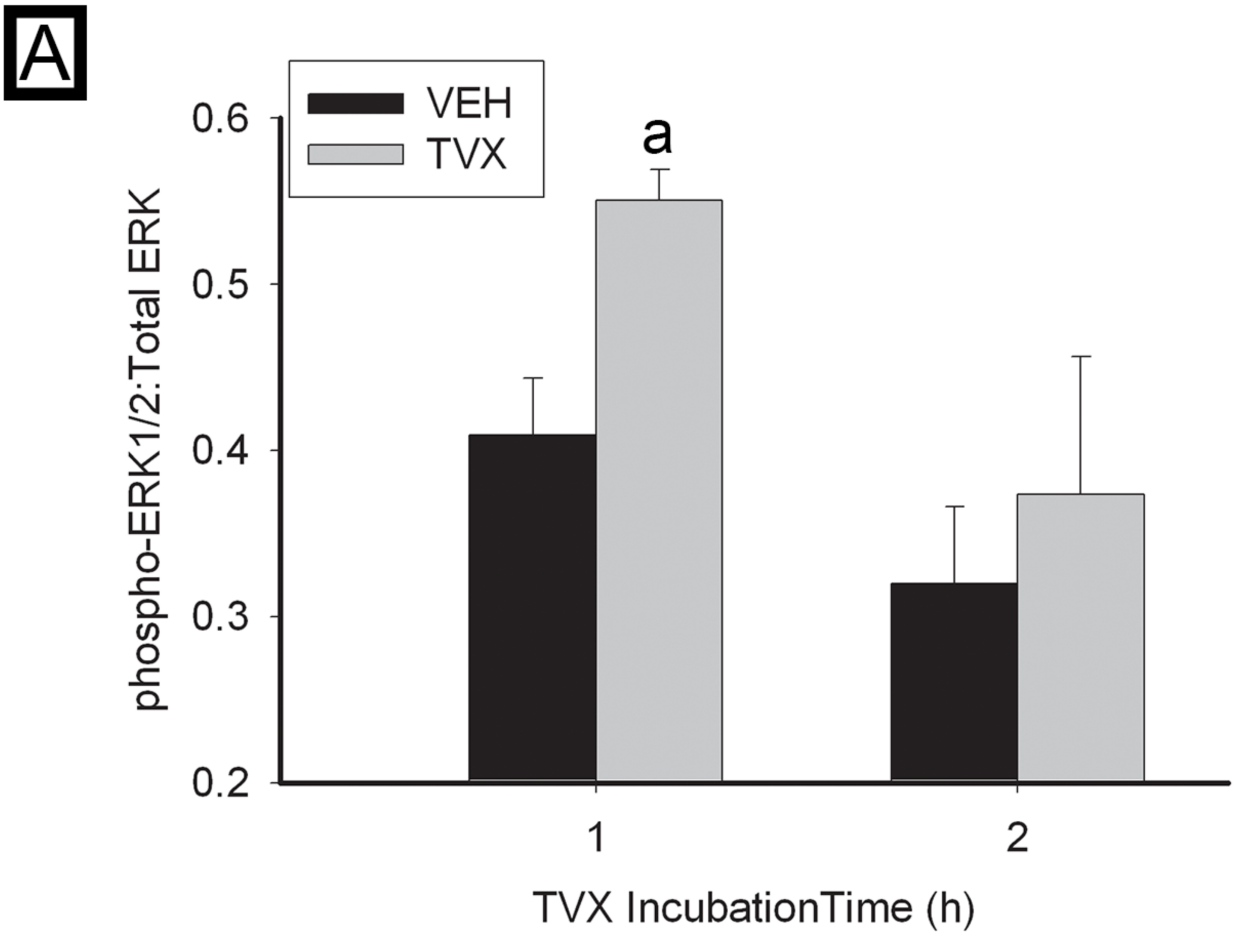
These results indicated that pivotal changes in signaling that led to increased TNF mRNA occurred during the TVX pretreatment, so the next studies investigated NF-kB and MAPK activation during this period. MAPK activation can increase TNF mRNA in inflammatory cells (72). Accordingly, the role of ERK, JNK and p38 in TNF mRNA induction was assessed in TVX-treated RAW cells.

ERK phosphorylation was increased after 1h but not after 2h of TVX exposure (Figure 10A). The selective MEK1 inhibitor and ERK-activation inhibitor, U0126, abolished all phospho-ERK signal, indicating a complete inhibition of ERK 1/2-mediated signaling. As expected, TVX caused an increase in TNF mRNA, and U0126 prevented this increase (Figure 10B). TNF mRNA was also decreased VEH-pretreated cells treated with U0126. The TVX-mediated increase in LPS-induced TNF release at 3h and 6h was completely prevented by U0126 (Figure 13).

JNK phosphorylation was not readily detectable by western blotting, but a known downstream target of JNK signaling, ATF2, was evaluated (Figure 11A). TVX treatment increased phospho-ATF2 at 1h and 2h. The selective JNK inhibitor, SP600125, markedly reduced JNK-dependent signaling (Figure 11A) and decreased the TVX-mediated increase in TNF mRNA (Figure 11B). SP600125 completely prevented TVX-mediated enhancement of LPS-induced increase in TNF protein release 3h and 6h after LPS exposure (Figure 13).

p38 phosphorylation was increased 1h and 2h after TVX exposure (Figure 12A). SB203580 is a selective inhibitor of p38 activity, but it does not prevent its phosphorylation. Accordingly, the phosphorylation of MAPKAPK-2, a direct downstream target of activated p38 (16) was evaluated, and SB203580 markedly reduced MAPKAPK-2 phosphorylation. Despite the TVX-mediated increase in p38 phosphorylation (Figure 12A), p38 inhibition did not reduce the TVX-induced increase in TNF mRNA (Figure 12B). SB203580 also did not affect the TVX-induced increase in TNF protein release in the absence or presence of LPS (Figure 13). Interestingly, SB203580 exposure during the TVX pretreatment period decreased LPS-induced TNF release in VEH-pretreated controls at 3h and 6h after LPS (Figure 13).

Figure 10. ERK activation during TVX exposure and ERK involvement in TVX-induced TNF mRNA expression. RAW cells were treated with VEH or TVX and with U0126 (500 nM) or DMSO (0.005%) to assess **(A)** TVX-mediated ERK activation and **(B)** ERK-dependence in TNF mRNA expression. **A)** RAW cells were lysed at the indicated times after TVX exposure, and ERK phosphorylation was evaluated in protein extracts. **B)** RNA was isolated from RAW cells after a 2-hour incubation with VEH or TVX and with DMSO or U0126, and TNF mRNA was assessed by RT-PCR. a - $p < 0.05$ vs. VEH within a timepoint or VEH/DMSO, b - $p<0.05$ vs. TVX/DMSO, c - $p<0.05$ vs. VEH/U0126.



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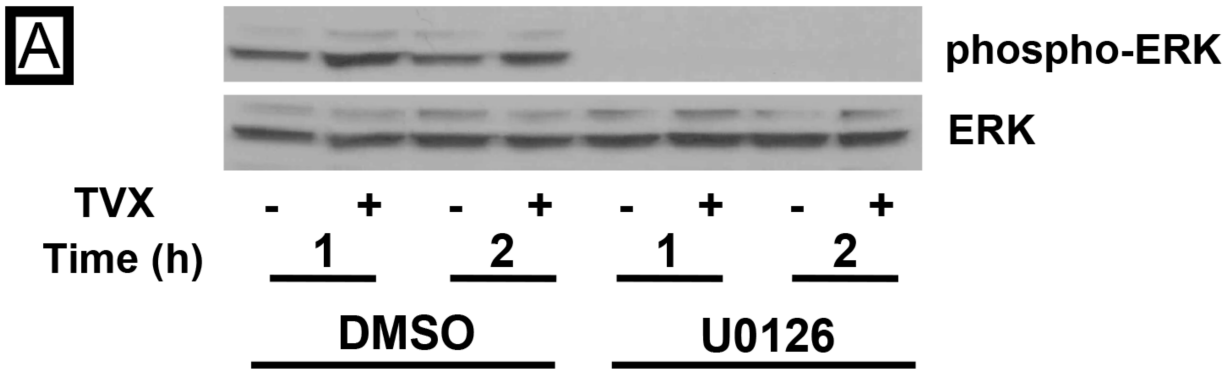


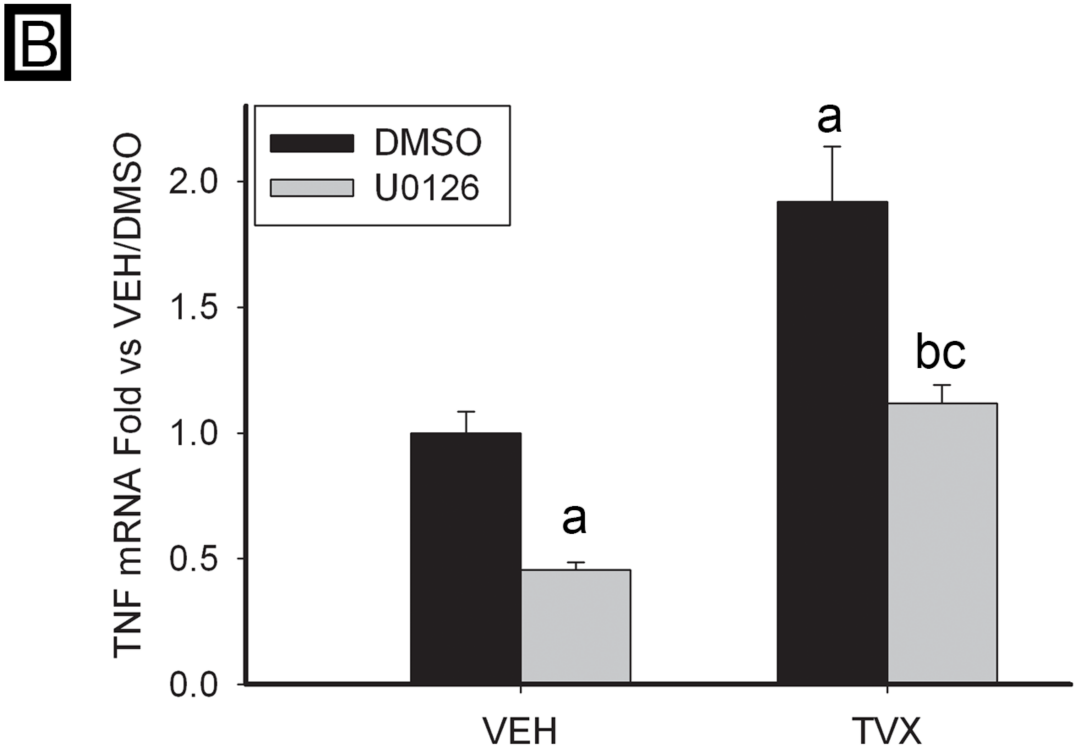
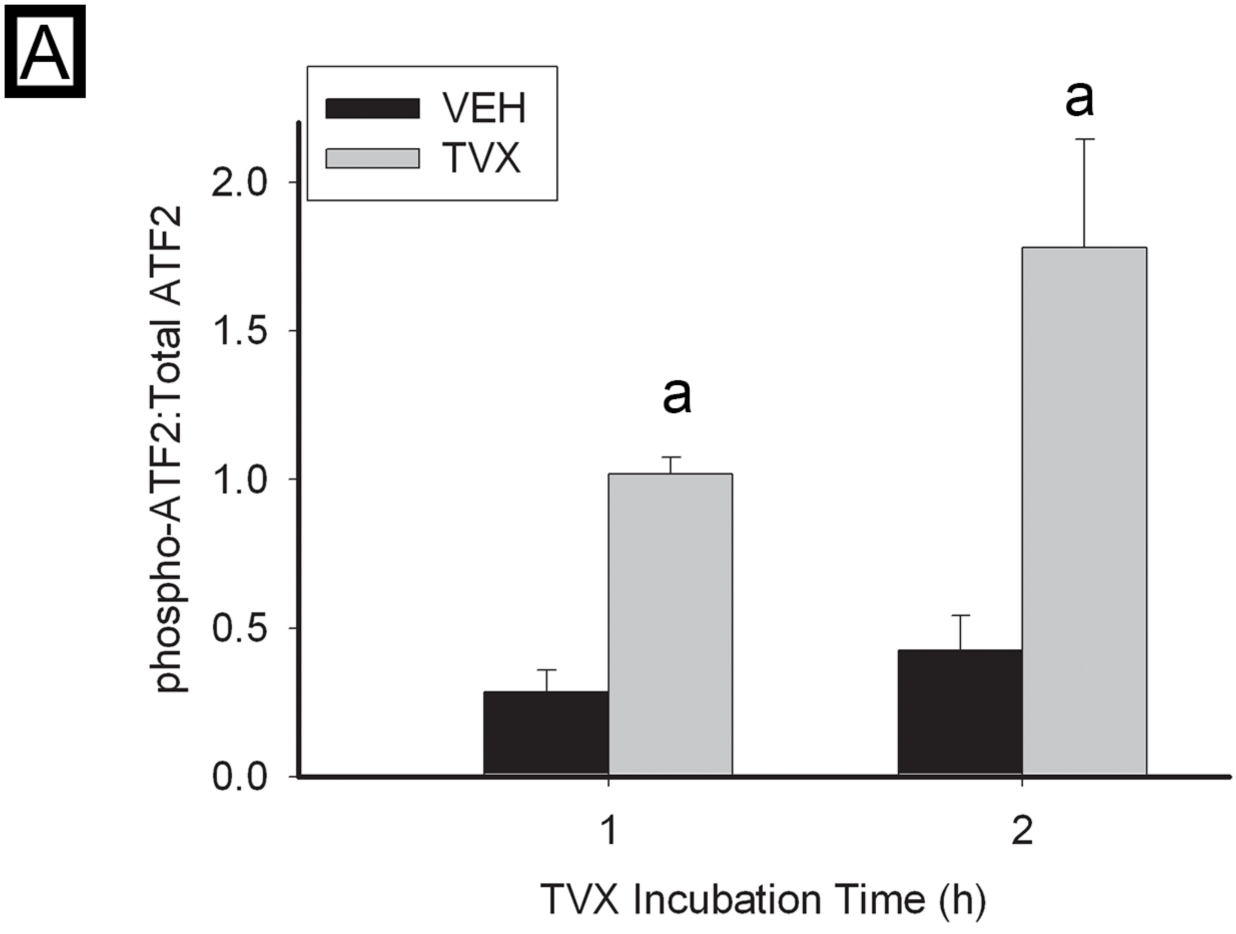


Figure 11. JNK activation during TVX exposure and JNK involvement in TVX-induced TNF mRNA expression. RAW cells were treated with VEH or TVX and with SP600125 (10 uM) or DMSO (0.1%) to assess **(A)** TVX-mediated JNK activation and **(B)** JNK-dependence in TNF mRNA expression. **A)** RAW cells were lysed at the indicated times after TVX exposure, and protein extracts were probed for phospho-ATF2 and total ATF2. **B)** RNA was isolated from RAW cells after a 2-hour incubation with VEH or TVX and with DMSO or SP600125, and TNF mRNA was assessed by RT-PCR. a - $p < 0.05$ vs. VEH within a timepoint or VEH/DMSO, b - $p<0.05$ vs. TVX/DMSO, c - $p<0.05$ vs. VEH/SP600125



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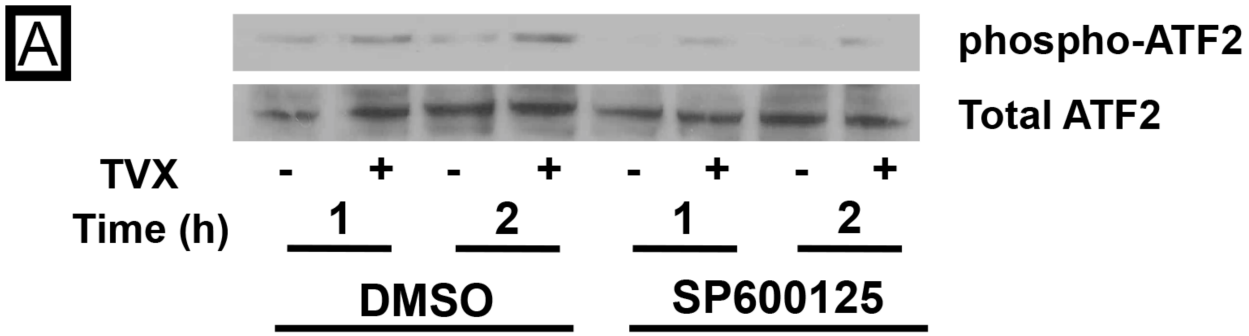


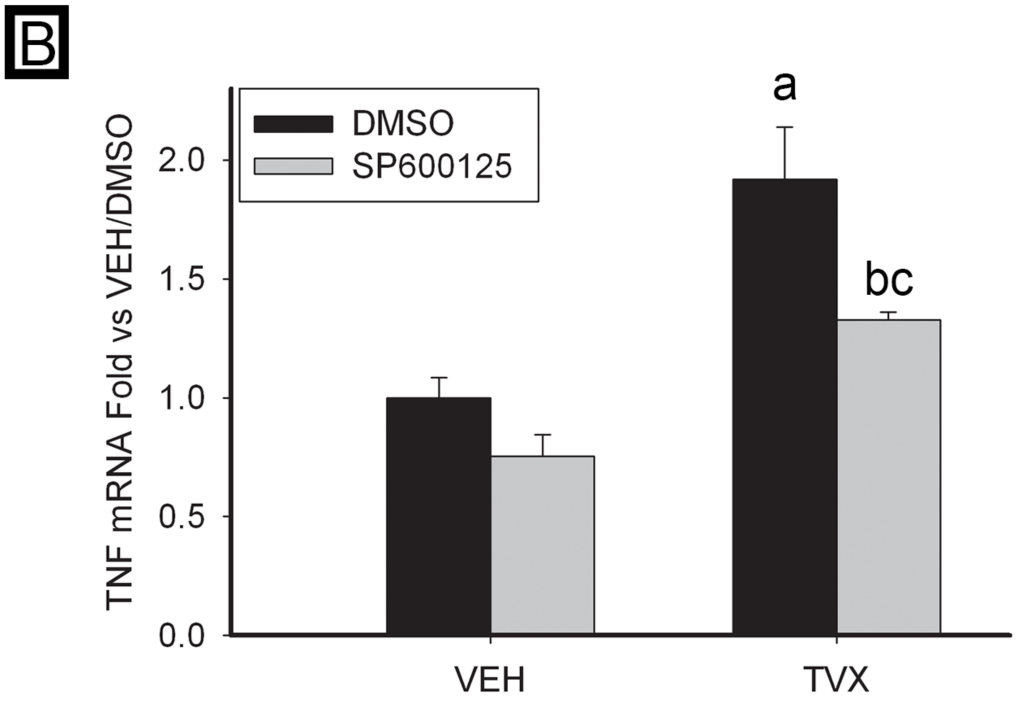
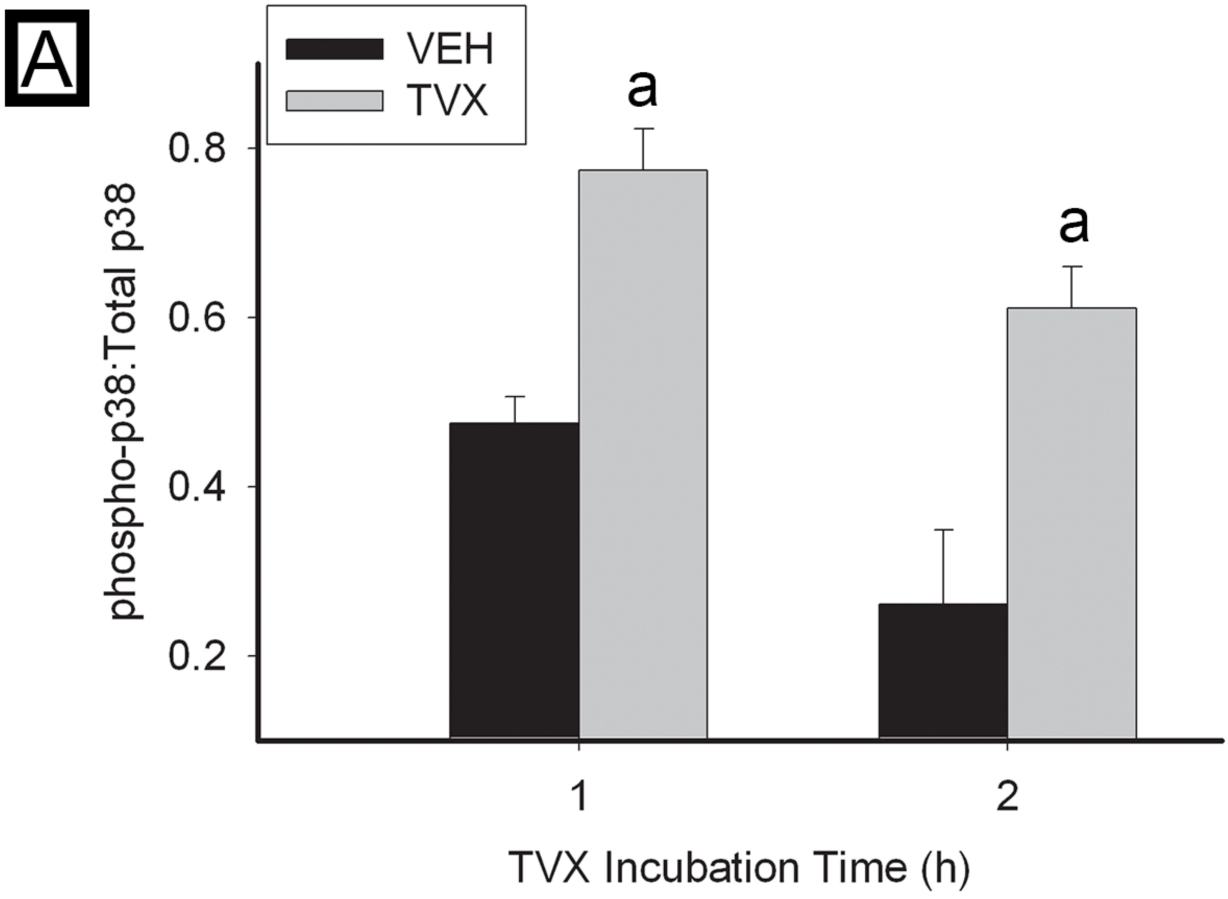


Figure 12. p38 activation during TVX exposure and p38 involvement in TVX-induced TNF mRNA expression. RAW cells were treated with VEH or TVX and with SB203580 (10 uM) or DMSO (0.1%) to assess **(A)** TVX-mediated p38 activation and **(B)** p38-dependence in TNF mRNA expression. **A)** RAW cells were lysed at the indicated times after TVX exposure, and protein extracts were probed for phosphorylated p38, MAPKAPK-2, ATF-2 and total p38. **B)** RNA was isolated from RAW cells after a 2-hour incubation with VEH or TVX and with DMSO or SB203580, and TNF mRNA was assessed by RT-PCR. a - $p < 0.05$ vs. VEH within a timepoint or VEH/DMSO.



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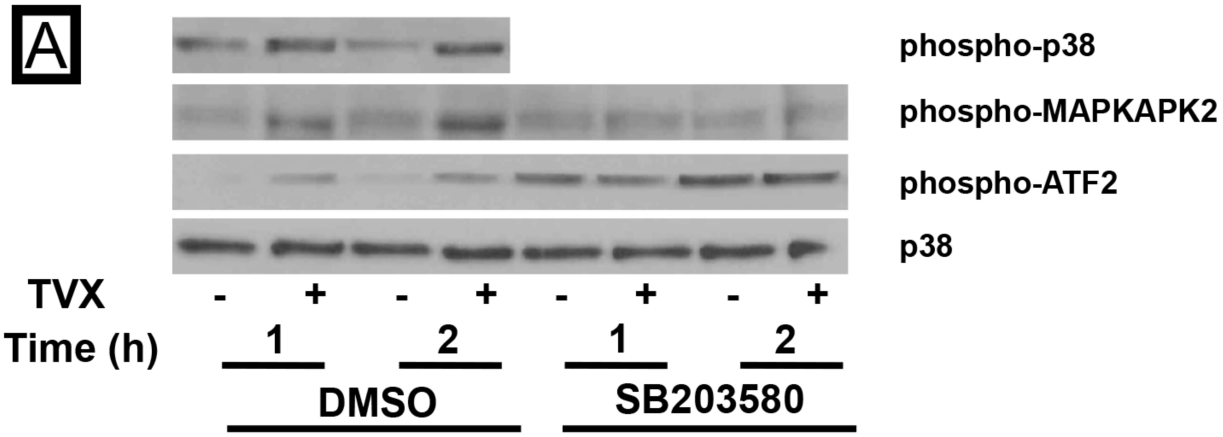


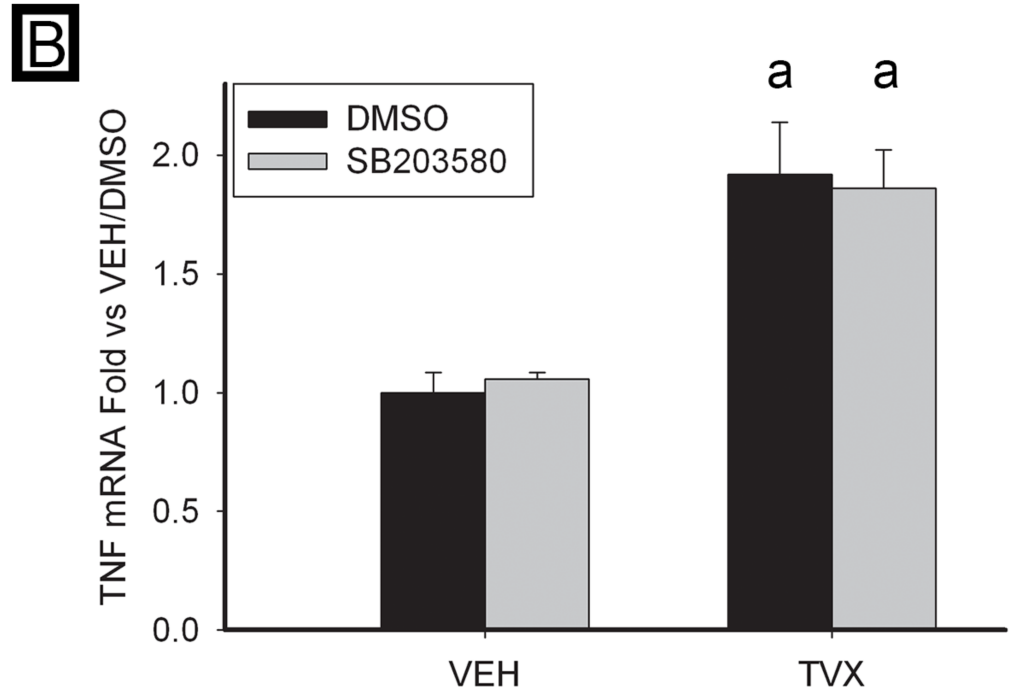
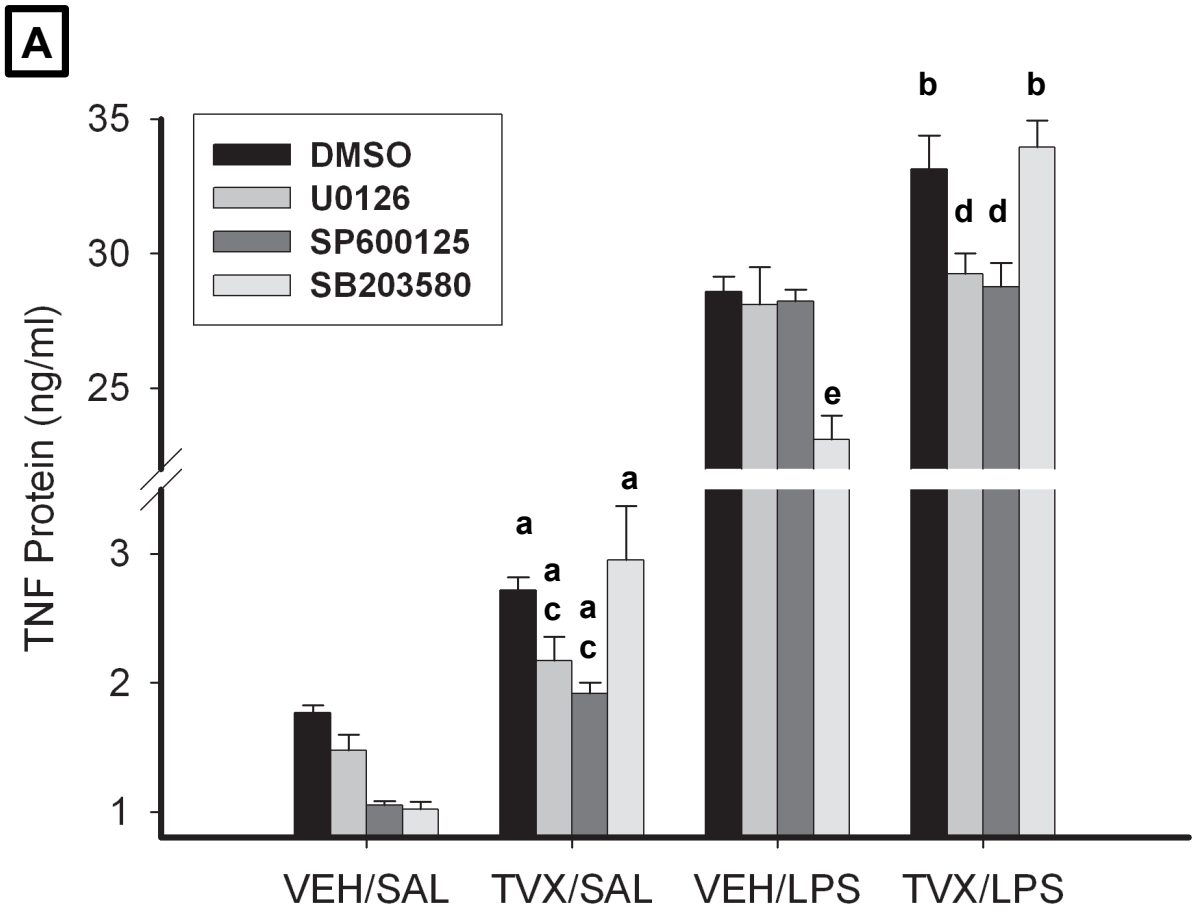


Figure 13. Effect of MAPK inhibition on TVX-mediated changes in LPS-induced TNF protein release. RAW cells were treated with MAPK inhibitors (U0126, SP600125 or SB203580) or 0.05% DMSO during a two-hour TVX incubation, after which time medium was replaced with one containing SAL or LPS (without inhibitors). TNF protein release was measured **(A)** 3h or **(B)** 6h after LPS addition. Values are means ± SEM, n=6. a - $p<0.05$ vs. respective VEH/SAL group, b - $p<0.05$ vs. respective VEH/LPS group, c - $p<0.05$ vs. TVX/SAL/DMSO, d - $p<0.05$ vs. TVX/LPS/DMSO, e - $p<0.05$ vs. VEH/LPS/DMSO



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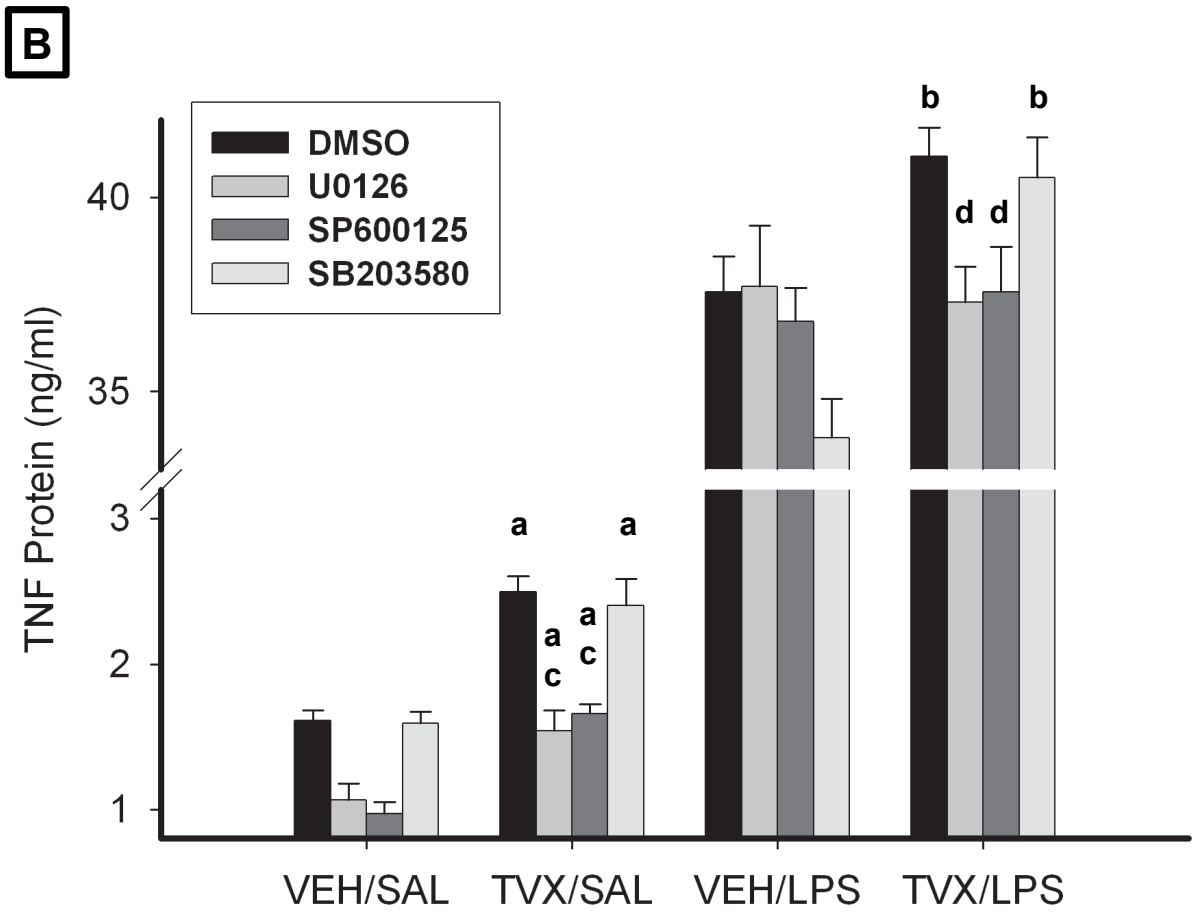


2.5 Discussion

TNF is a critical factor in several models of liver injury, and it has been demonstrated that it is a necessary, proximal mediator in several animal models of IDILI that are based on the inflammatory stress hypothesis. In each of the models in which the role of TNF has been examined, a drug-induced increase and/or prolongation in LPS-induced plasma TNF concentration occurred (126, 189, 216, 249). Developing a model *in vitro* that reproduces the increased TNF production is important to understand these drug-LPS interactions, inasmuch as such a model could be used to discover critical molecular targets of IDILI-associated drugs in humans.

In TVX/LPS-treated mice, the drug-induced increase in LPS-stimulated TNF appearance is relatively small in magnitude, and the increased duration of plasma TNF is relatively short (189). However, this relatively small prolongation in plasma TNF proved to be essential to the hepatotoxic TVX-LPS interaction. Etanercept given at the time of peak TNF appearance in LPS-treated mice eliminated the prolongation of TNF appearance in TVX/LPS-cotreated mice and protected them from liver injury. In contrast, etanercept administered after the TVX-mediated prolongation of plasma TNF concentration had ended failed to protect against the hepatocellular necrosis. Thus, the brief prolongation of TNF appearance was required for the pathogenesis of liver injury in TVX/LPS-cotreated mice (191). Accordingly, we sought to determine if a similar interaction between TVX and LPS could be reproduced in TNF-producing cells in vitro.

Kupffer cells (KC) are the largest fixed population of macrophages in the body and a likely source of TNF in response to LPS (203). RAW cells were chosen as a KC surrogate because of their ease of use, well-characterized LPS-induced TNF

production, and suitability for high-throughput screening (13). TVX pretreatment increased LPS-induced TNF release in RAW cells (Figures 5A and 6), whereas LVX did not (Figure 5B), consistent with the failure of LVX to enhance LPS-mediated TNF release in mice (189). Accordingly, the drug-induced changes in TNF production observed in TVX/LPS-cotreated mice were reproduced in cultured RAW cells.

In the timecourse study (Figure 6), analysis of the rate of TNF release (Table 2) indicated that TVX-pretreated cells released TNF at a greater rate than VEH-pretreated controls through 6h after the addition of LPS but not thereafter. The results suggested that the capacity to release TNF in RAW cells was nearly exhausted by 6h after LPS, which has been observed previously in RAW cells exposed to LPS (177, 225). TVX pretreatment increased TNF release after removal of TVX-containing medium and replacement with LPS- or SAL-containing medium even though TNF mRNA did not change after medium replacement (Figure 7). The data strongly suggest that relevant TVX-induced signaling that contributed to increased LPS-induced TNF mRNA occurred before the cells were exposed to LPS.

TVX treatment did increase TNF mRNA during the two-hour drug exposure (Figure 8). The increase in TNF mRNA occurred in a relatively short time between 1h and 2h. LVX did not significantly increase TNF mRNA at this time (Figure 9), providing an explanation for the lack of effect of LVX on LPS-stimulated TNF appearance in vitro and in vivo (Figure 5B and 189). LPS-induced TNF gene expression involves activation of NF-kB and/or MAPKs (53, 66, 206), so these signaling proteins became a focus for investigation. Although NF-kB is a well-characterized inducer of TNF expression, TVX did not induce p65 binding to DNA before or after LPS exposure (data

not shown), suggesting that MAPKs are largely responsible for the TVX-mediated increase in TNF mRNA.

All three MAPKs assessed were activated during TVX pretreatment (Figures 10-12). ERK activation occurred early (1h), whereas JNK and p38 activation were increased at both times assayed (1h and 2h). It therefore became important to assess which of these MAPKs contributed to increased TNF release in RAW cells. TNF biosynthesis can involve p38 at several levels: activation of trans-acting factors, stabilization of TNF mRNA and shedding of membrane-bound TNF (43, 178, 188). Surprisingly, p38 inhibition failed to alter the TVX-mediated increase in TNF mRNA (Figure 12B). Inhibition of ERK, however, prevented the TVX-mediated increase in TNF mRNA and also depressed basal levels of TNF mRNA (Figure 10B). ERK phosphorylation was detected in VEH-pretreated cells (Figure 10A), suggesting that basal TNF mRNA expression is ERK-mediated in RAW cells. The TVX-mediated increase in TNF mRNA was reduced but not totally eliminated when JNK signaling was inhibited (Figure 11B). The marker of JNK activation, ATF2, is also a target of p38 signaling in inflammatory cells (26, 81), but as shown in Figure 12A, SB203580 did not reduce phosphorylation of ATF2, arguing for ATF2 as a selective target for JNK in this model. Together, these results suggest that both ERK and JNK are involved causally in the increase in TNF mRNA caused by TVX.

U0126 and SP600125 are reversible inhibitors of ERK and JNK, respectively. They were present only during the TVX pretreatment period, not during exposure to LPS. If the increase in TNF mRNA during the TVX pretreatment period was linked to TNF protein release prompted by the later exposure to LPS, then these MAPK inhibitors

should reduce the TVX-mediated increase in TNF release, as observed in Figure 13. Despite an incomplete reduction in the TVX-mediated increase in TNF mRNA prior to LPS exposure, SP600125 completely eliminated the TVX interaction with LPS. It is possible that JNK contributes to this interaction not only through increased TNF mRNA, but also through a post-transcriptional mechanism, such as increased TNF translation (206). It is also possible that ERK and JNK both contribute to the TVX-mediated increase in TNF expression through a similar mechanism, such as increased AP-1-dependent transactivation of the TNF gene (32, 39) but this remains a topic for future investigation.

ERK has been shown to mediate increases in TNF mRNA and protein in other models. For example, chronic ethanol treatment increases LPS-induced TNF mRNA expression and TNF protein release by macrophages in an ERK-dependent manner (104, 164). In addition, both pro- and anti-inflammatory effects of adiponectin on LPS-induced TNF release in RAW cells are mediated through ERK-dependent signaling (84, 155). ERK signaling plays a significant role in experimental models of inflammatory liver injury from alcohol or bile acid exposure (3, 130). These results attest to the dynamic and important role ERK signaling plays in TNF biosynthesis in macrophages in models of hepatotoxicity involving inflammatory stress.

JNK has been implicated in cell-death signaling, and examples of JNK dependence in drug-inflammation interaction models of hepatocellular injury have been described (12, 61). Chlorpromazine (CPZ) is a phenothiazine antipsychotic drug associated with IDILI in humans, and coexposure of rats to CPZ and LPS precipitates liver injury (28). Exposure of mice or primary murine hepatocytes to CPZ combination

with LPS or the toll-like receptor 2 agonist, lipoteichoic acid (LTA), resulted in hepatocellular injury that was associated with prolonged JNK activation (60). Toxicity was preceded by an increase and prolongation of TNF in the plasma of CPZ-LPS or CPZ-LTA coexposed animals, raising the possibility of a link between prolonged plasma TNF and hepatotoxicity, similar to what is observed during TVX-LPS coexposure. Another recent study described JNK-dependent cytotoxicity resulting from TVX/TNF coexposure in an hepatocyte cell line (12). It is therefore possible that a common upstream stimulus in hepatocytes and macrophages activates JNK in response to TVX and that this results in cell death in hepatocytes and increased cytokine release in macrophages. Both of these effects could contribute to liver injury from TVX and, more generally, from drugs associated with IDILI.

The knowledge generated in this model of TVX/LPS-treated RAW cells enhances our understanding of TVX-LPS interaction in the murine model of IDILI. The degree to which our findings apply to IDILI models with other drugs remains to be determined. Moreover, the specific molecular targets of TVX that activate MAPKs remain unknown and are the subject of current investigation. Nevertheless, since several drug-inflammation interaction models in animals are also associated with increases in TNF and require TNF for hepatotoxicity, the results of this study raise the possibility that common MAPK signaling mechanisms are at play.

CHAPTER 3

Poulsen KL, Olivero J, Beggs KM, Ganey PE and Roth RA. LPS-trovafloxacin interaction in macrophages: role of the DNA damage response. Manuscript in preparation for submission.

3.1 Abstract

Trovafloxacin (TVX) is a drug with idiosyncratic hepatotoxic (IDILI) liability in humans. In a murine model of IDILI, otherwise nontoxic doses of TVX and the inflammagen, lipopolysaccharide (LPS), interacted to produce pronounced hepatocellular injury. The liver injury depended on a small but significant prolongation of TNF appearance in the plasma by TVX coexposure (189). In vitro, the enhancement of TNF expression by TVX was reproduced in RAW 264.7 murine macrophages (RAW cells) stimulated with LPS. The current study was designed to identify the molecular target of TVX responsible for this response in RAW cells. An *in silico* analysis revealed a favorable binding profile of TVX to eukaryotic topoisomerase II-alpha (TopIIa), and a cell-free assay revealed that TVX inhibited eukaryotic TopIIa activity. Topoisomerase inhibition is known to lead to DNA damage, and TVX increased the DNA damage marker phosphorylated H2A.X in RAW cells. Moreover, TVX induced activation of the DNA damage sensor kinases, ataxia telangiectasia mutated (ATM) and Rad3-related (ATR). The ATR inhibitor NU6027 prevented the TVX-mediated increases in LPS-induced TNF mRNA and protein release, whereas a selective ATM inhibitor (KU55933) was without effect. These results suggest that TVX can inhibit eukaryotic topoisomerase, leading to activation of ATR and potentiation of TNF release by macrophages. This off-target effect might contribute to the ability of TVX to precipitate IDILI in humans.

3.2 Introduction

Idiosyncratic, drug-induced liver injury (IDILI) is an adverse response to numerous pharmaceuticals. IDILI is responsible for approximately 13% of all cases of acute liver failure (154) and for many of the FDA-imposed restrictions on drug use (114). Although typically rare, these reactions cause significant morbidity and mortality and pose a financial burden to pharmaceutical companies when offending drugs must be withdrawn from the market (192). Although drugs from several pharmaceutical classes have been associated with human IDILI, many are antibiotics. For example, in the class of broad-spectrum, fluoroquinolone antibiotics, trovafloxacin (TVX), ciprofloxacin (CPX) and moxifloxacin (MOX) have caused IDILI in human patients, whereas levofloxacin (LVX) has shown little to no such liability (117).

Several hypotheses have emerged to explain IDILI, yet none have been proven conclusively (192). One hypothesis states that a transient inflammatory episode can interact with a normally nontoxic dose of a drug to bring about liver injury. Rodent models of IDILI based on this inflammatory stress hypothesis have been developed for several drugs, including trovafloxacin (TVX), sulindac, amiodarone and others (175). In these models, drugs associated with IDILI in humans synergize with an inflammagen such as lipopolysaccharide (LPS) to precipitate hepatotoxicity. At the doses used in these models, LPS exposure prompts an early increase in tumor necrosis factor-alpha (TNF) in the plasma but no liver necrosis. IDILI-associated drugs do not by themselves cause TNF expression, but coadministration of drug with LPS causes a small prolongation of the LPS-stimulated TNF appearance that is critical to the pathogenesis of liver injury (126, 189, 191, 249).

An example is a murine model involving TVX/LPS coexposure. TVX is not hepatotoxic in mice even when given at large doses. However, when mice were cotreated with TVX and an otherwise nontoxic dose of LPS, pronounced hepatocellular necrosis occurred. Interestingly, this hepatotoxic interaction with LPS did not occur upon cotreatment with LVX. The liver injury from LPS/TVX cotreatment was absent in TNF receptor knockout mice or when TNF was neutralized by etanercept treatment (189, 190). Importantly, when etanercept was administered at the peak of LPS-stimulated TNF appearance to prevent the prolongation of TNF appearance in TNF/LPS-cotreated mice, liver injury was prevented. Thus, although the prolongation was relatively brief and the increase was minor in magnitude compared to that which occurred from LPS alone, it was required for hepatotoxicity (189, 191).

Examination of the TVX-LPS interaction in the murine model in vivo did not reveal a specific molecular target of TVX. The enhancement of LPS-stimulated TNF release by TVX could arise from a direct effect of the drug on TNF-producing cells in the liver. Indeed, pretreatment of murine RAW 264.7 cells (RAW cells) with TVX potentiated LPS-induced TNF release (Chapter 2). Thus, the influence of TVX on LPS-stimulated TNF appearance that occurs in vivo was recapitulated in a macrophage cell line, thereby providing an in vitro system that can be employed to evaluate mechanisms of the LPS-drug interaction.

The antibiotic activity of the fluoroquinolones derives from their ability to inhibit bacterial topoisomerases and gyrases (23). Interestingly, in addition to their ability to inhibit prokaryotic topoisomerases, fluoroquinolones TVX, CPX and MOX have weak inhibitory activity against eukaryotic topoisomerase II-alpha (TopIIa) (2, 8, 79, 169). It is

well recognized that inhibiting (“poisoning”) topoisomerases can lead to DNA damage (46, 181). DNA damage prompts intracellular signaling involving activation of kinases that might enhance TNF expression. Accordingly, we tested the hypothesis that potentiation of LPS-induced TNF production in RAW cells by TVX results from topoisomerase inhibition and the consequent DNA damage response.

3.3 Material and Methods

3.3.1 Chemicals and inhibitors

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Antibiotic/antimycotic and DMEM were purchased from Life Technologies (Grand Island, NY). KU55933 was purchased from Tocris Bioscience (Bristol, United Kingdom).

3.3.2 *In silico* docking of TVX and LVX to topoisomerase II-alpha (TopIIa)

The geometries of TVX and LVX were optimized using Density Functional Theory at the B3LYP/6-31G level, and calculations were carried out with the Gaussian 03 software package (221). Open Babel was used to transform geometries to Mol2 format for subsequent processing (73). Molecular docking was used to model the ability of TVX and LVX structures to form complexes with TopIIa. MGL tool 1.5.0 (183) was employed to prepare protein structures for molecular docking, and protein-ligand docking calculations were performed with the AutoDock Vina 1.0 program (212). The binding site for the ligands on TopoIIa (PDB ID: 1ZXN, chains A and B) was defined by forming a cube with the dimensions 86 × 70 × 90 Å, engulfing the whole protein structure, using a grid point spacing of 1.0 Å and center grid boxes of 63.249, 3.440 and 58.618, in X, Y and Z dimensions, respectively. All calculations with AutoDock Vina included 20 number modes, an energy range of 1.5, and exhaustiveness equal to 25. Five hundred docking runs were executed for each ligand, saving the best-obtained pose for each one. The average affinity for best poses was computed as the affinity

value for a given complex. *In silico* binding affinities, measured as Kcal/mol, were averaged for each theoretical binding site detected by AutoDock Vina.

3.3.3 Topoisomerase decatenation assay

TopIIa isoform activity was analyzed in the presence of VEH or TVX at various concentrations using etoposide as a positive control with the Human Toposiomerase II Assay Kit (TopoGEN Inc, Port Orange, FL). Briefly, 1 unit of human TopIIa was incubated with 200 ng kinetoplastid DNA (kDNA) in the presence of VEH or TVX in complete assay buffer at 37°C for 30 minutes. 1 unit of topoisomerase is defined as the amount of enzyme required to separate the highly catenated kDNA substrate at 37°C for 30 minutes. The reaction was stopped using the stop buffer provided, and the reaction products were loaded onto a 1% agarose gel for analysis of topoisomerase activity.

3.3.4 Cell culture

RAW 264.7 macrophage-like cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic (Life Technologies) at 37°C in 5% CO_2. Cells were harvested by detachment with a sterile spatula and plated at a density of 4×10^4 cells per well in 24-well plates (Costar, Lowell, MA) for cytokine release and RNA isolation or 1.5×10^5 cells per well in 6-well plates (Costar). 24 hours after plating, cells were synchronized

by replacing medium with 0.5% FBS-containing medium. After overnight incubation, cells were exposed to drug.

3.3.5 RNA isolation, RT-PCR and mRNA stability

Total RNA was isolated from RAW cells using TRIzol reagent (Life Technologies). cDNA was prepared with the iScript cDNA synthesis kit using 1 µg of isolated RNA (Bio-Rad Laboratories, Hercules, CA). The expression level of TNF was analyzed using the StepOne Real-Time PCR machine and SYBR Green reagents for amplicon detection (Applied Biosystems, Foster City, CA). Expression level was normalized to beta actin (β-actin). TNF mRNA stability was assessed by treating cells with TVX or an equal volume of 0.1N KOH vehicle (VEH) for 1 hour before adding 5 µg/ml actinomycin D (ActD) to stop transcription. RNA was isolated at 15 minute-intervals after the addition of ActD and converted to cDNA, and TNF mRNA was measured and normalized to β-actin.

PCR primers used were: mouse TNF [5' -TCTCATGCACCACCATCAAGGACT-3' (forward) and 5' - ACCACTCTCCCTTTGCAGAACTCA- 3' (reverse)] and mouse β-actin [5' –TGTGATGGTGGGAATGGGTCAGAA- 3' (forward) and 5' –TGTGGTGCCAGATCTTCTCCATGT- 3' (reverse)].

3.3.6 Western blot analysis

Cells were lysed in radioimmunopreciptation assay (RIPA) buffer supplemented with HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration in cell isolates was determined by the bicinchonic assay (BCA). Western

analyses were performed by loading 20 µg of protein on precast NuPAGE® SDS-PAGE gels (Life Technologies) using all NuPAGE® reagents. Samples were separated on precast 12% gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) for 1 hour at 4° C. Membranes were blocked in 5% BSA dissolved in tris-buffered saline plus 0.1% Tween20 (TBST) and then probed for phospho-(Ser/Thr) ATM/ATR substrate, phospho-histone H2A.X (Ser139) (Cell Signaling Technology, Boston, MA). Membranes were then stripped with Restore western blot stripping agent (Thermo Scientific) and reprobed for Lamin B1 (Abcam, Cambridge, MA).

3.3.7 Measurement of TNF concentration

For determination of TNF protein in culture medium, an enzyme-linked immunosorbent assay (ELISA) was performed (BD Biosciences, San Jose, CA). Cell culture medium was withdrawn at various times and stored at -20°C until the time of analysis. Ninety-six-well plates were coated with an anti-TNF capture antibody in a coating buffer overnight at 4°C. Medium was diluted to remain within standard curve concentrations.

3.3.8 Studies with inhibitors

Inhibitors KU55933, NU6027 and wortmannin were dissolved in DMSO at a stock concentration of 10 mM and diluted to final concentrations in 0.5% FBS-containing medium. Inhibitors or an equivalent volume of DMSO vehicle were added at the moment RAW cells were exposed to VEH or TVX, unless noted otherwise.

3.3.9 Statistical analysis

A one- or two-way Analysis of Variance (ANOVA) was performed on data sets with Tukey's post-hoc test applied for comparisons among groups. The criterion for significance was $p < 0.05$.

3.4 Results

3.4.1 TVX interaction with TopIIa: *in silico* analysis

Human TopIIa was selected for analysis as it is the eukaryotic homolog to prokaryotic DNA gyrase and topoisomerase IV (11, 46). TVX binding to eukaryotic TopIIa occurred at two binding sites (Figure 14A), the most frequently occupied of which (99.4%) was the one with the greatest predicted affinity (9.3±0.0 Kcal/mol) (Fig 14B). In contrast, LVX was predicted to bind to TopIIa at three sites (Fig 14C). The site most frequently occupied by LVX (95%) (Figure 14D) differed from that to which TVX bound most frequently. In addition, the absolute affinity for LVX binding to TopIIa (8.5±0.0 Kcal/mol) was smaller than that observed for TVX. These results indicate that TVX is predicted to bind to eukaryotic TopIIa and does so at a different site and with greater affinity than LVX.

Figure 14. *In silico* analysis of TVX binding to TopIIa. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation. **A)** Theoretical binding sites for TVX on TopIIa molecule are shown with binding affinities displayed in Kcal/mol. **B)** Theoretical frequency of occupation (BF) for TVX in sites 1 and 2. **C)** Theoretical binding sites for LVX on TopIIa molecule are shown with binding affinities displayed in Kcal/mol. **D)** Theoretical frequency of occupation for LVX in sites 1-3. For explanation of analysis and calculations, see Methods.

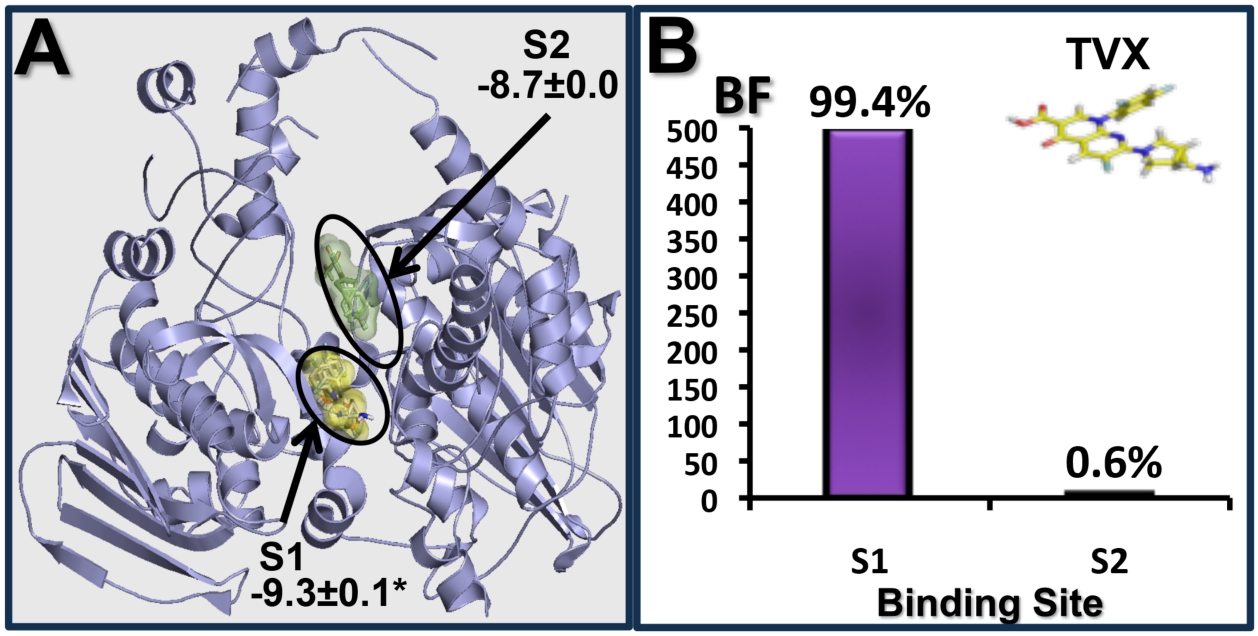
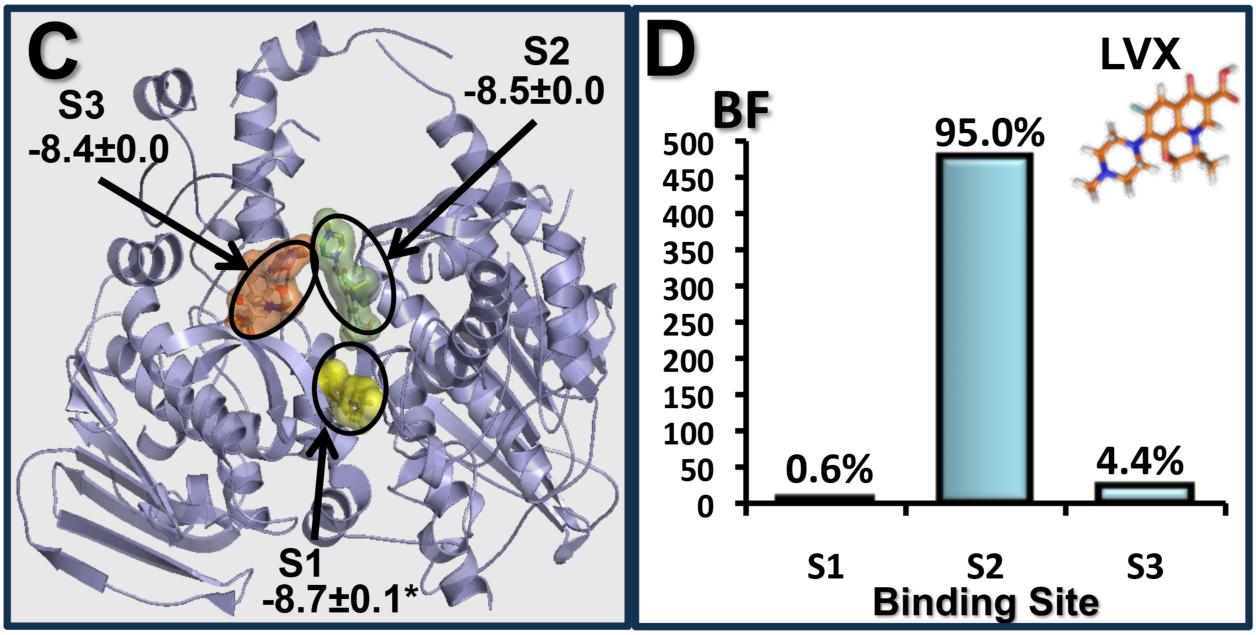


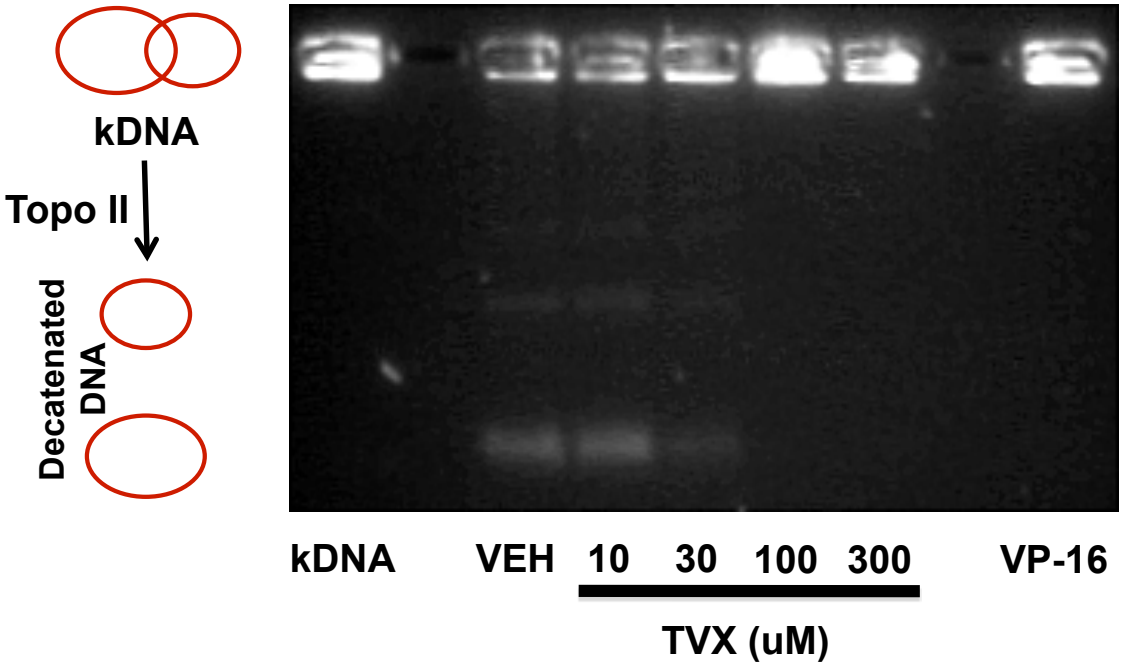
Figure 14 (cont'd)



3.4.2 TVX inhibits TopIIa-dependent decatenation of kDNA

The ability of TVX to inhibit TopIIa-dependent decatentaion of kDNA was evaluated in a cell-free assay (Figure 15). In this assay, decatenation of kDNA by TopIIa results in two distinct DNA catenates of different molecular weights that migrate through the agarose gel, whereas kDNA remains in the loading wells. In the absence of TopIIa (lane labeled kDNA) the kDNA does not migrate. As a positive control, VP-16, a potent inhibitor of human TopIIa, completely prevented kDNA decatenation. The presence of TVX (30 – 300 μM) decreased decatenated DNA products and increased kDNA retention as compared to VEH control containing only TopIIa. This indicated that TVX could inhibit eukaryotic topoisomerase at concentrations near those attained in the plasma during TVX therapy (209).

Figure 15. Effect of TVX on TopIIa activity. A reaction mixture containing kinetoplastid DNA (kDNA) in the absence (1st lane on the left; kDNA) or presence of TopIIa (all other lanes) was incubated with 0 (VEH), 10, 30, 100 or 300 μM TVX or 10 μM VP-16. After 30 minutes, the reaction was quenched and samples were separated on a 1% agarose gel and stained with ethidium bromide to visualize DNA decatenate migration.



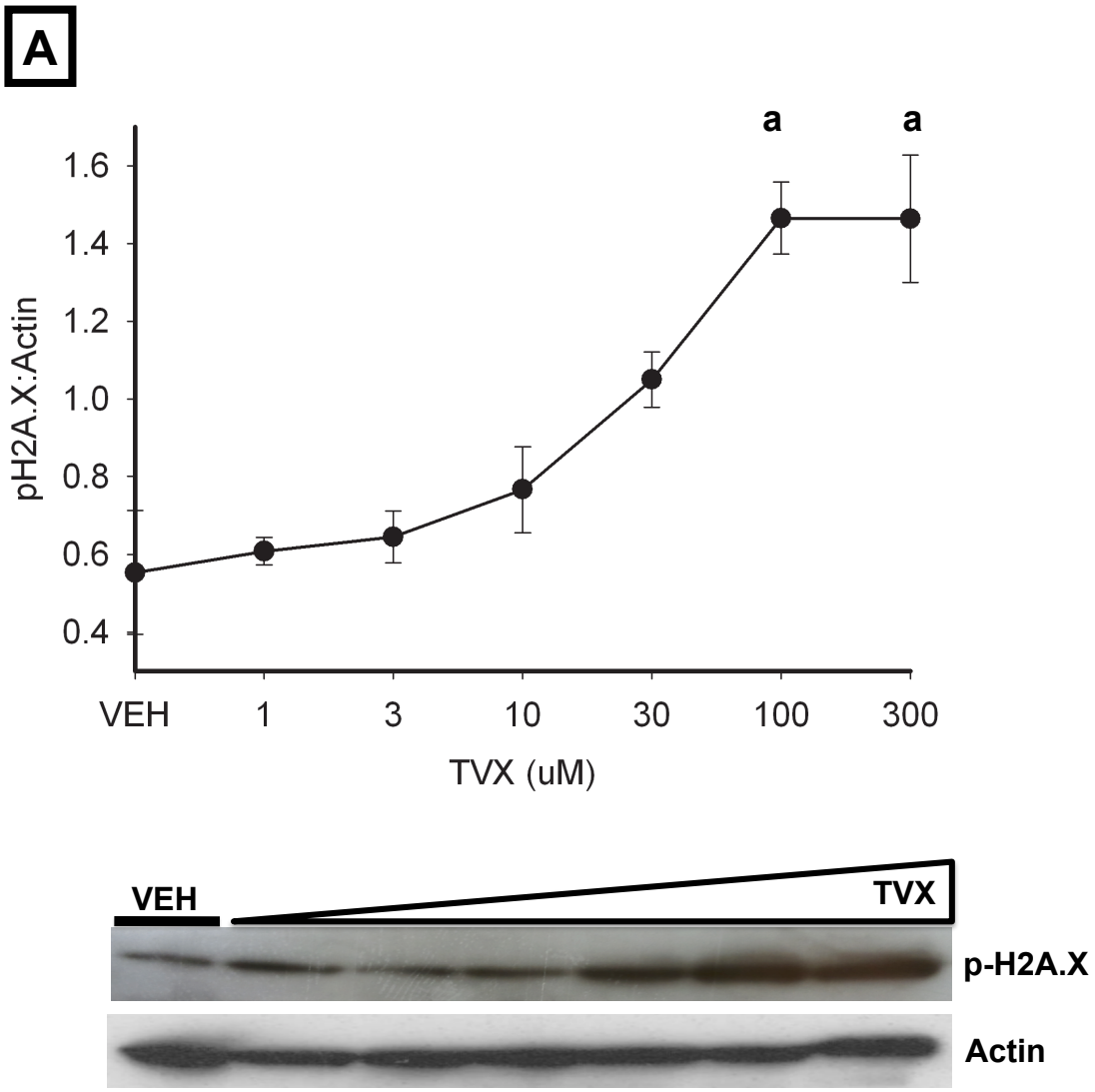
3.4.3 TVX increases DNA lesions in RAW 264.7 cells

Poisoning of topoisomerase activity in cells leads to several outcomes, one of which is the formation of double-stranded lesions in DNA (46, 181). Phosphorylated histone 2A.X (pH2A.X) is a sensitive marker of DNA lesions and is induced rapidly after the onset of a lesion by the damage-sensing kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated and Rad3-related (ATR) (98). After a 2-hour incubation of RAW cells with TVX, pH2A.X increased in a concentration-dependent manner (Figure 16A). LVX, however, did not increase pH2A.X in RAW cells over the same duration of exposure (Figure 16B).

3.4.4 TVX activates ATM/ATR-dependent signaling

ATM and ATR are phosphoinositide 3-kinases (PI3Ks) that share a common minimal phosphorylation motif on protein substrates; i.e., a serine or threonine residue is phosphorylated if the amino acid occurs between leucine and glutamine (101). Incubation of RAW cells with TVX for 1 hour increased phosphorylation of a substrate containing the minimal ATM/ATR phosphorylation motif (Figure 17A). This increase was absent after a 2-hour exposure to TVX. KU55933, a selective ATM inhibitor, and NU6027, an ATR-signaling inhibitor (157), each prevented phosphorylation of this ATM/ATR-substrate motif in VEH- or TVX-exposed RAW cells (Figure 17B), indicating that ATM- and ATR-dependent signaling was activated by TVX.

Figure 16. TVX-induced DNA damage in RAW cells. A) RAW cells were exposed to TVX (1-300 μM) for 2 hours. phosphorylated H2A.X (pH2A.X) was assessed in protein extracts by western blot. Signals for pH2A.X were densitized and normalized to actin. **B)** RAW cells were exposed to TVX (100 μM) or LVX (300 μM) for 2 hours. pH2A.X induction was assessed in protein extracts. Signals for pH2A.X were densitized and normalized to actin. Blots are representative from a minimum of 3. a - Significantly different from VEH, $p<0.05$.



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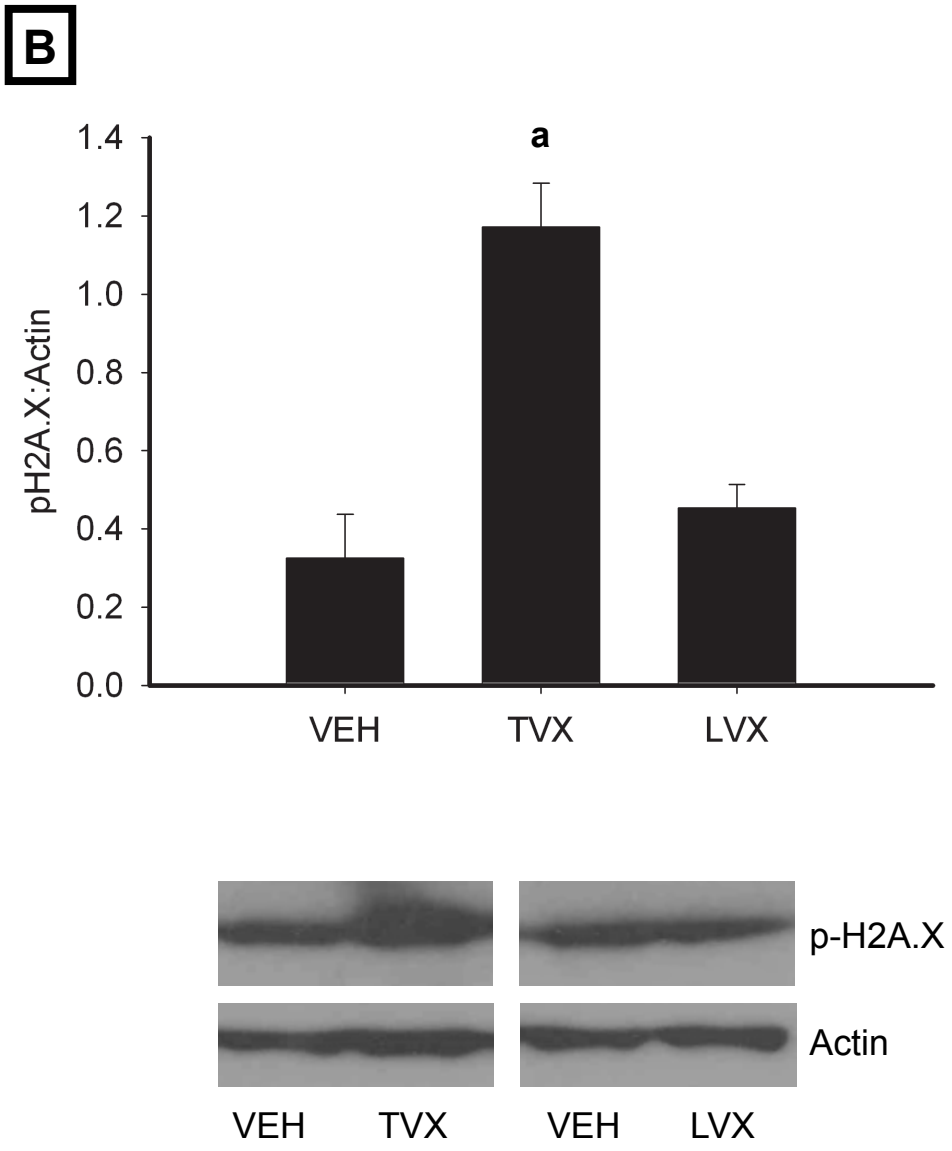
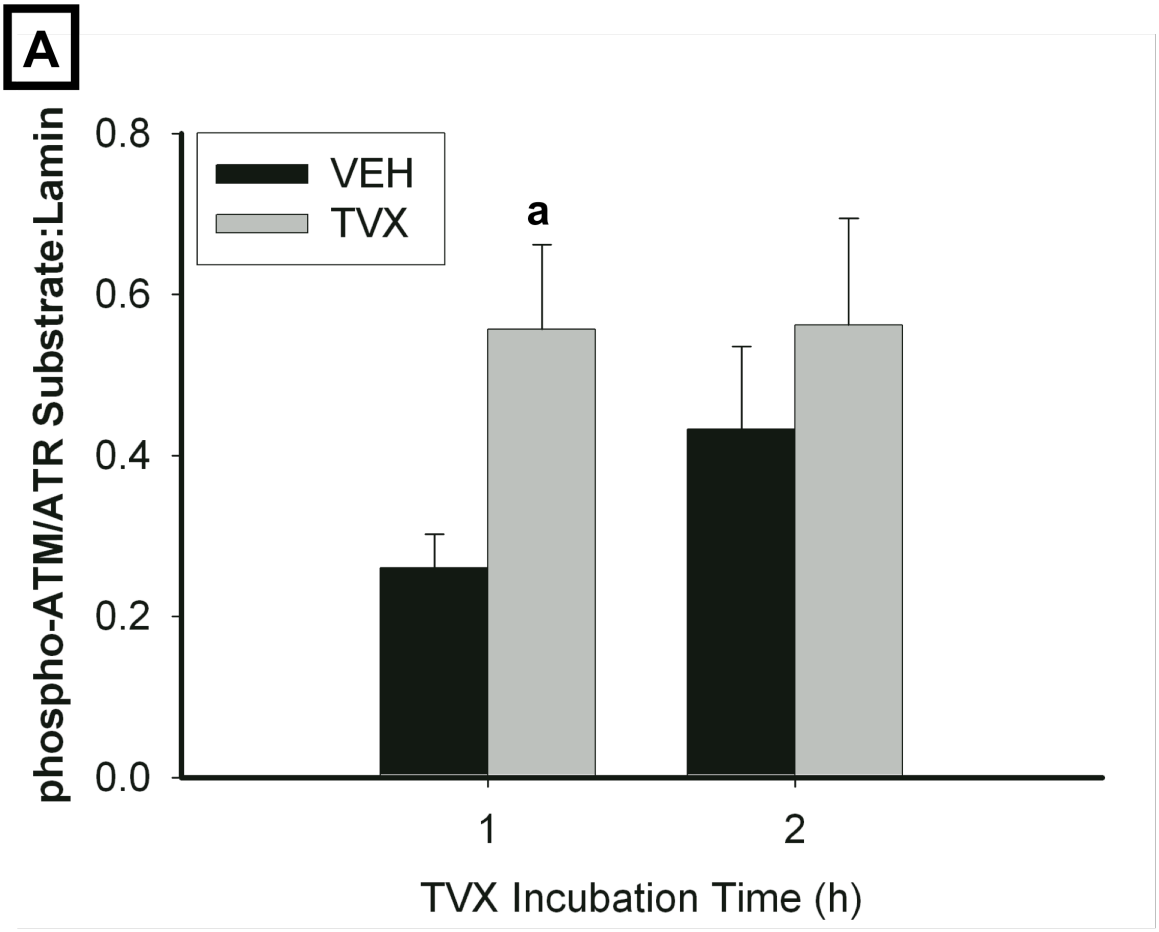


Figure 17. ATM and ATR activation by TVX in RAW cells. A) RAW cells were exposed to VEH or TVX (100 μM) for 1 or 2 hours. Phospho-Ser/Thr ATM/ATR substrate motif was assessed in isolated protein extracts by western analysis densitized and normalized to lamin B1. **B)** RAW cells were exposed to VEH or TVX (100 μM) and to ATM inhibitor KU55933 (1 μM), ATR inhibitor NU6027 (10 μM) or their DMSO (0.05%) vehicle for 1 hour. Phospho-Ser/Thr ATM/ATR substrate motif was assessed in isolated protein extracts by western analysis and normalized to lamin b1. Blots are representative from a minimum of 3. a - Significantly different from VEH, $p<0.05$.



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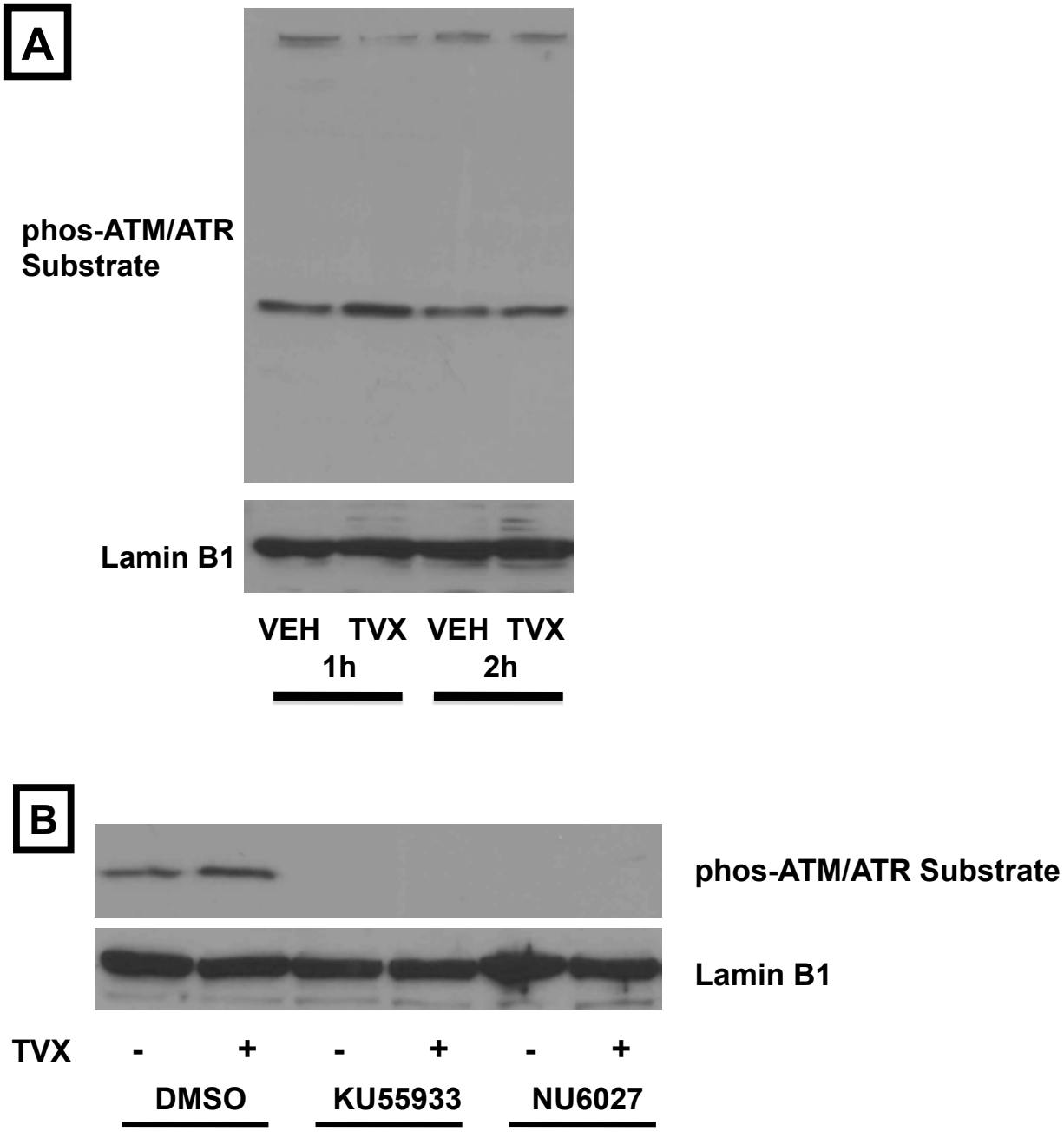


3.4.5 TVX increases TNF mRNA in an ATR-selective manner

As noted above, TNF is a critical factor in the pathogenesis of liver injury in TVX/LPS cotreated mice, and TVX increases TNF expression in LPS-stimulated RAW cells in vitro (Chapter 2, 189). The influence of ATM and ATR activation on TVX-dependent TNF expression in RAW cells was assessed. TVX increased TNF mRNA after a two-hour exposure to the drug (Figure 18A). The increase in TNF mRNA was reduced by NU6027, but not by KU55933 or by the nonselective PI3K inhibitor, wortmannin (WORT).

One way that increases in mRNA can occur is by stabilization of the transcript. To address this possibility, RAW cells were exposed to TVX for one hour before adding ActD to prevent RNA synthesis. This time of ActD addition was chosen because it coincides with the TVX-mediated increase in ATR signaling (Figure 17A) but precedes the increase in TNF mRNA (seen at 2h in Figure 18A). TVX markedly increased the stability of TNF mRNA (Figure 18B), and NU6027 markedly reduced this increase. This result suggested that the increase in TNF mRNA depicted in Figure 18A was due to at least, in part, an ATR-dependent stabilization of TNF transcripts.

Figure 18. ATR-dependent expression of TNF mRNA in response to TVX. A) RAW cells were coexposed to VEH or TVX (100 μM) and to WORT (1 μM), KU55933 (1 μM), NU6027 (10 μM) or their DMSO (0.05%) vehicle for 2 hours. TNF mRNA was assessed by RT-PCR. Values are expressed as fold of VEH/DMSO ± SEM or VEH/Inhibitor ± SEM, n=3-6. a - $p<0.05$ vs. VEH group with same inhibitor, b - $p<0.05$ vs. TVX/DMSO. **B)** RAW cells were exposed to TVX (100 μM) or its VEH and to NU6027 (10 μM) or its DMSO (0.05%) vehicle for 1 hour before addition of ActD (5 μg/ml), and RNA was isolated at indicated times after ActD. TNF mRNA was normalized to the t = 0 value for each group. Values are expressed as %TNF remaining ± SEM, n=6. a - $p<0.05$ vs. VEH/DMSO at the same time, b - $p<0.05$ vs. TVX/DMSO at the same time

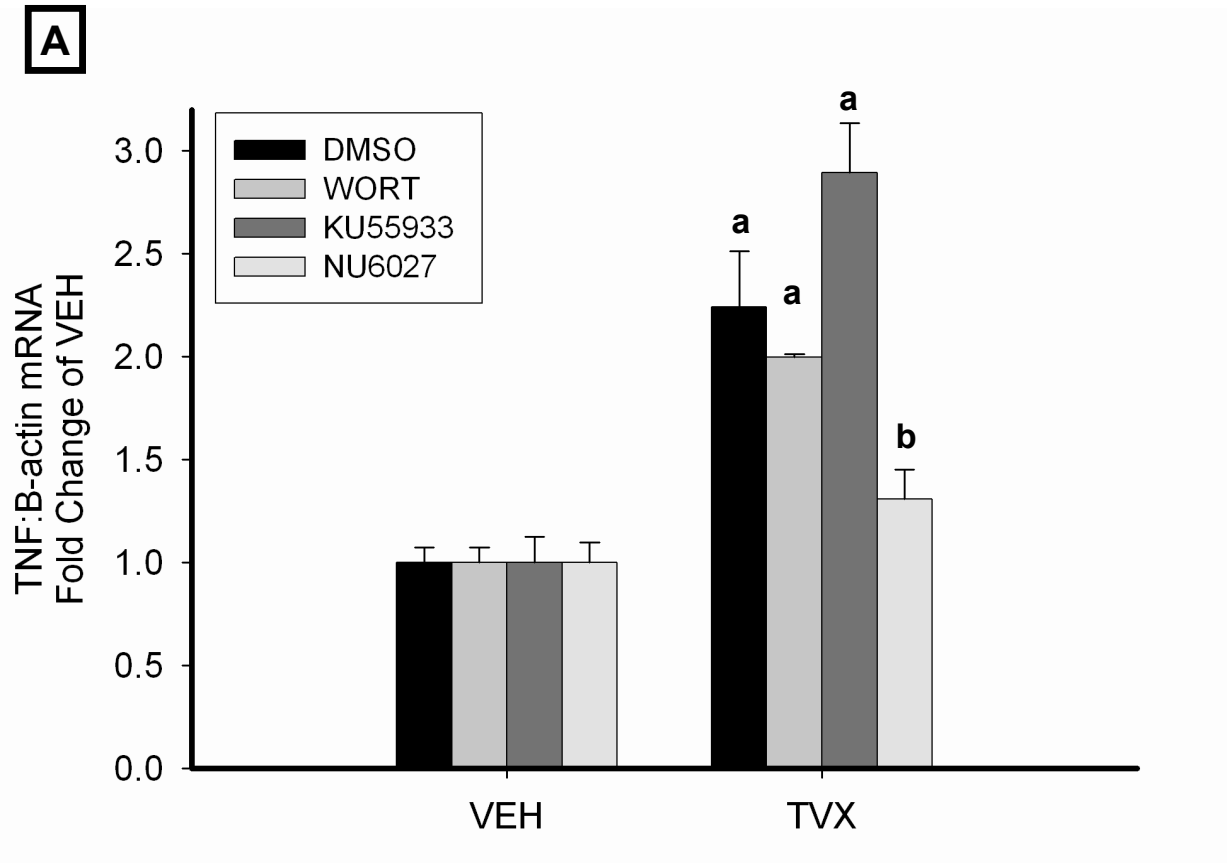
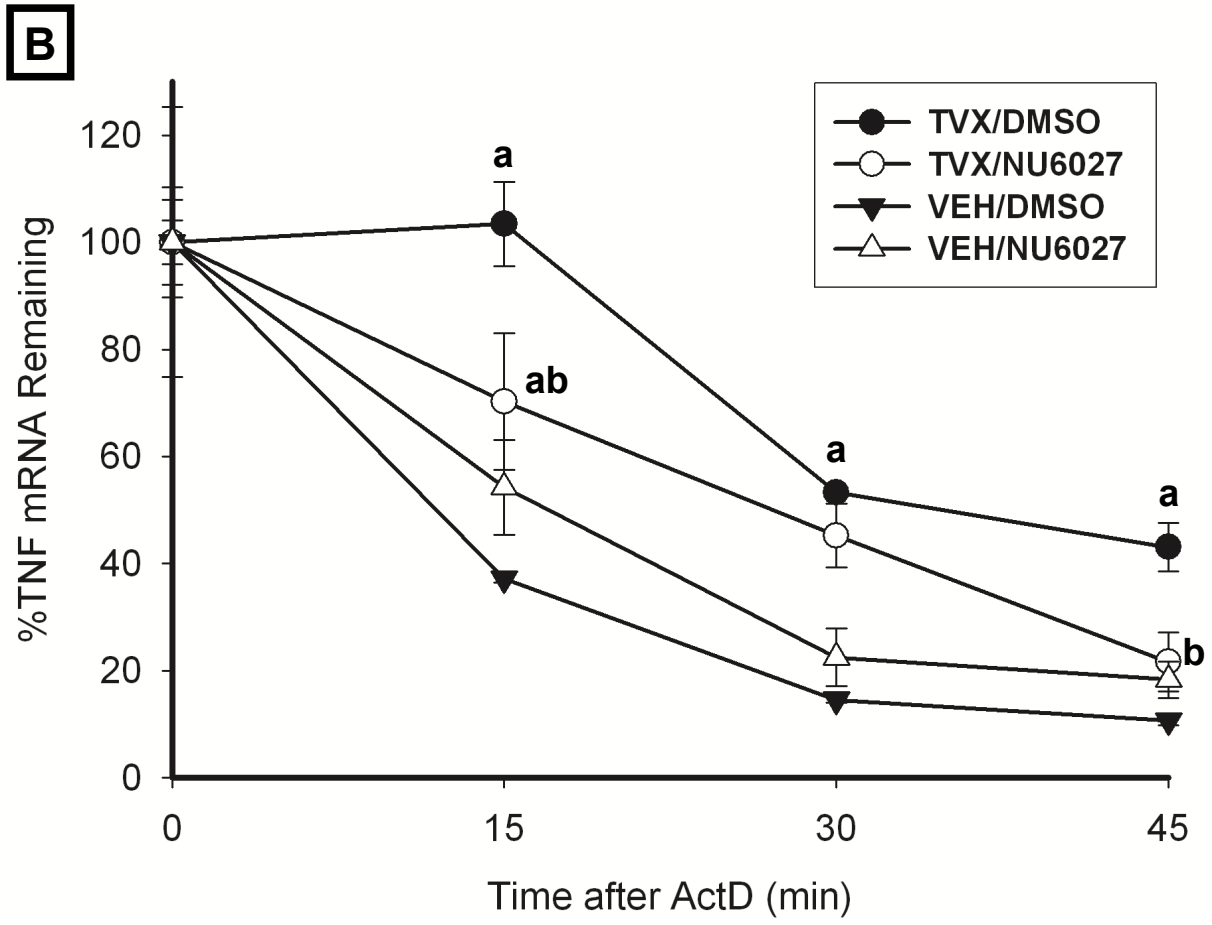


Figure 18 (cont'd)



3.4.6 TVX increases LPS-induced TNF protein release in an ATR-dependent manner

The role of ATR in the TVX-mediated increase in TNF release from RAW cells was assessed next. As expected, LPS stimulated the release of TNF from RAW cells (Figure 19). TVX-pretreatment increased TNF release 3h after SAL or LPS exposure (Figure 19A, black bars). As found with TNF mRNA (Figure 18), the increase in TNF release was insensitive to KU55933 or WORT but reduced by NU6027. In cells cotreated with TVX and LPS, NU6027 reduced TNF release to the level stimulated by LPS alone. Unlike the results after 3h, TNF release 6h after LPS exposure was largely unaffected by NU6027 (Figure 19B). At this time, NU6027 reduced the increase in TNF due to exposure to TVX alone but did not prevent the increase in LPS-induced TNF release caused by TVX pretreatment. In addition, if NU6027 was added only at the moment of LPS addition, the TVX-mediated increase in LPS-induced TNF release 3h after LPS (Figure 20). The results in Figure 20 indicate that the TVX-mediated ATR activation must only occur during TVX pretreatment, consistent with the results in Figures 17-18. Taken together, the results suggested that the TVX-mediated increase in LPS-induced TNF release depended on ATR at 3h but not 6h after LPS exposure.

Figure 19. Effect of ATM and ATR inhibition on TVX-mediated increases in LPS-induced TNF release from RAW cells. RAW cells were pretreated with VEH or TVX (100 μM) and with NU6027 (10 μM), WORT (1 μM), KU55933 (1 μM), or their DMSO (0.05%) vehicle for 2 hours, after which time medium was replaced with one containing SAL or LPS (10 ng/ml) without inhibitors. TNF protein release was assessed at **A)** 3h **B)** 6h after LPS exposure. Values are means ± SEM from 3-6 separate experiments, each performed in triplicate. a - Significantly different from VEH/SAL with same inhibitor treatment, $p<0.05$, b – Significantly different from VEH/LPS with same inhibitor treatment, $p<0.05$, c – Significantly different from TVX/SAL/DMSO group, $p<0.05$, d – Significantly different from TVX/LPS/DMSO group, $p<0.05$.

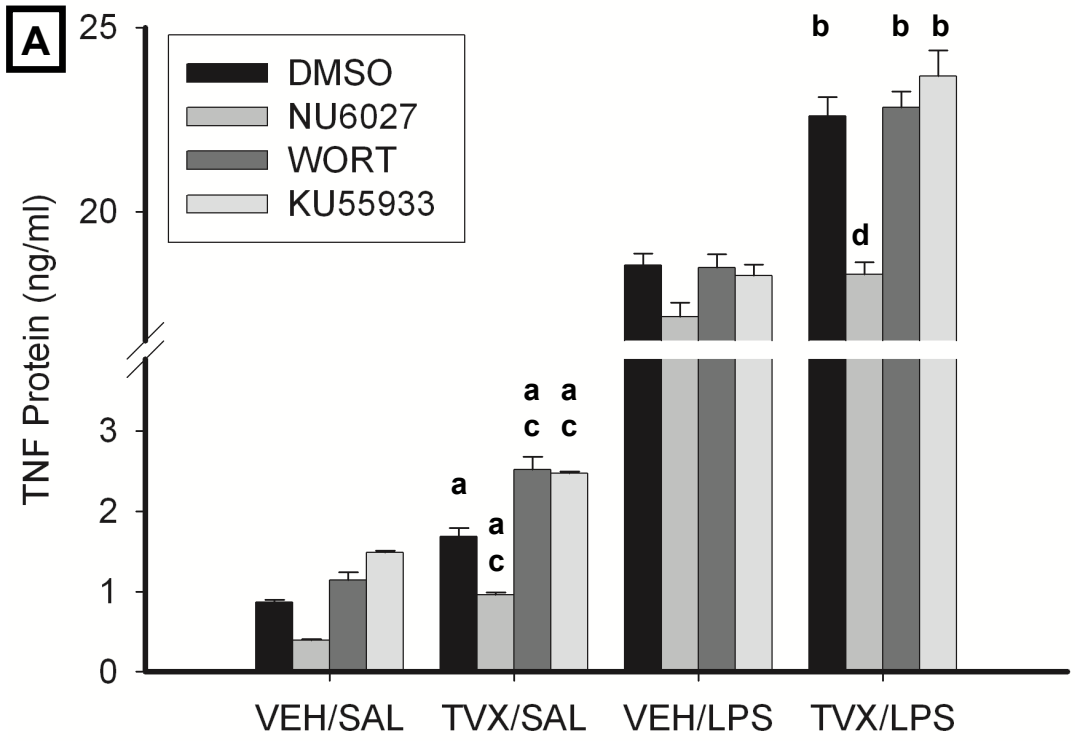


Figure 19 (cont'd)

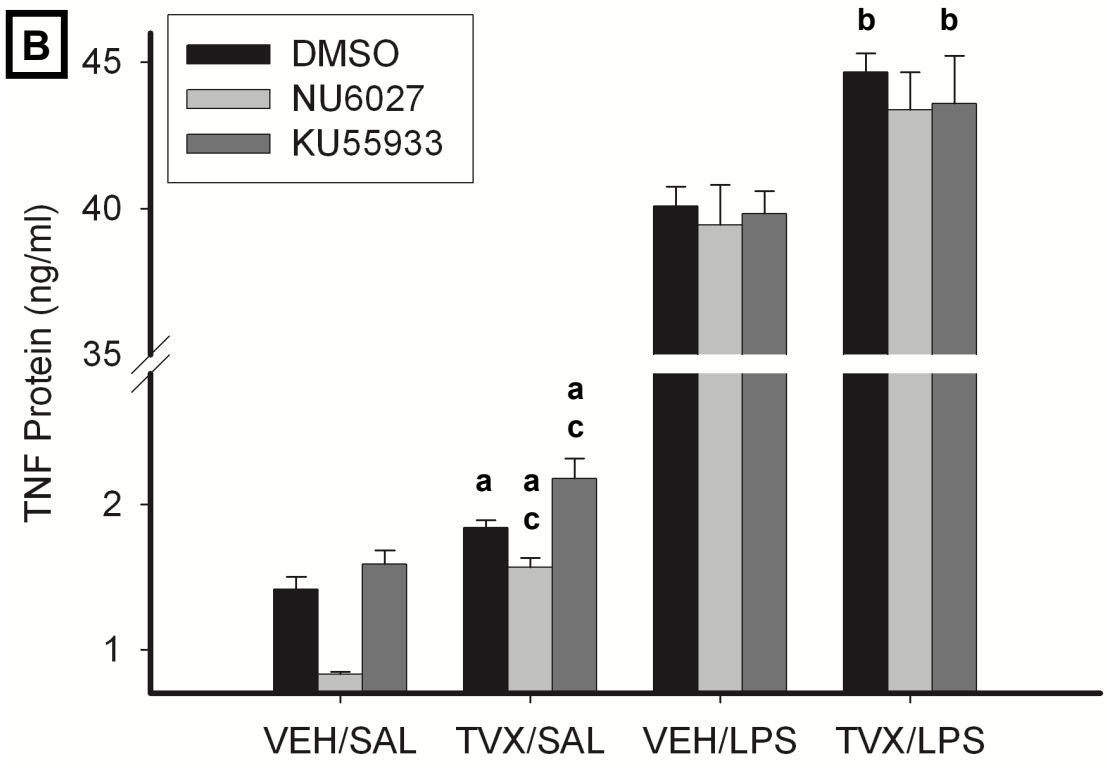
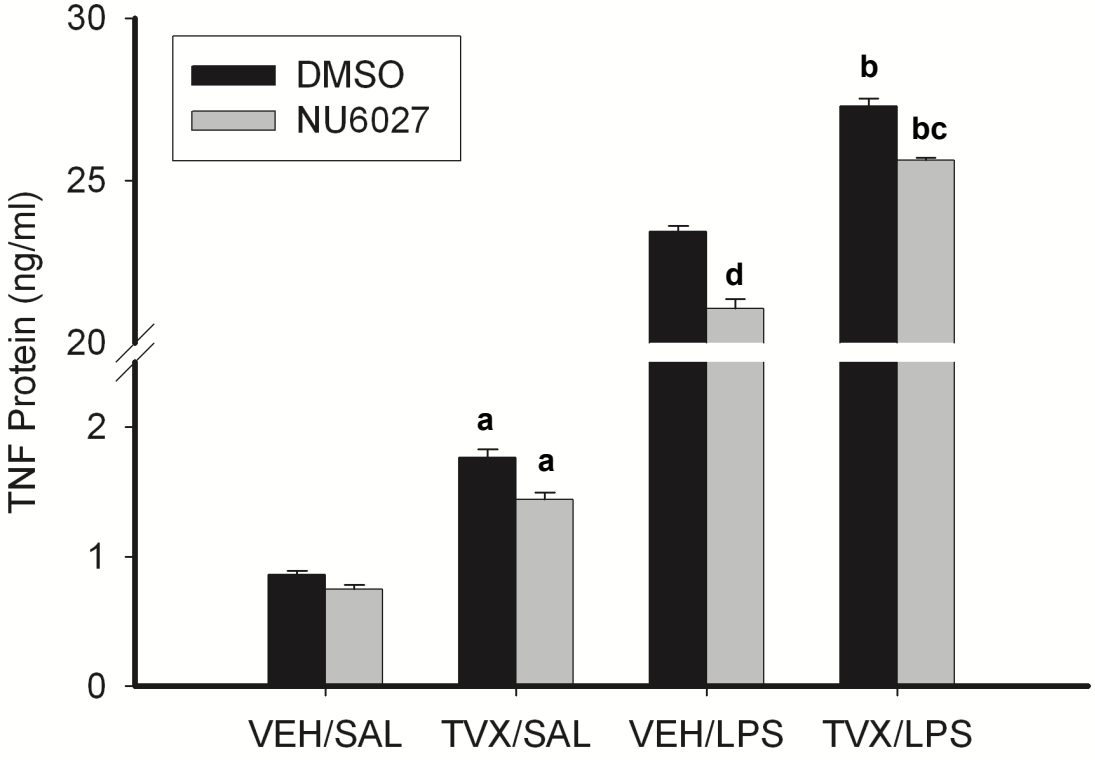


Figure 20. Effect of ATR inhibitor NU6027 added at the time of LPS addition on TNF release. RAW cells were pretreated with VEH or TVX (100 μM) for 2 hours, after which time medium was replaced with one containing SAL or LPS (10 ng/ml) and NU6027 (10 μM) or its DMSO (0.05%) vehicle. Values are means ± SEM, n=3 a – Significantly different from VEH/SAL, $p<0.05$, b – Significantly different from VEH/LPS with same inhibitor treatment, $p<0.05$, c – Significantly different from TVX/LPS/DMSO group, $p<0.05$, d – Significantly different from VEH/LPS/DMSO group, $p<0.05$.



3.5 Discussion

Fluoroquinolone antibiotics are designed to target prokaryotic topoisomerase selectively, but mounting evidence describes off-target effects due to structural homology between eukaryotic and prokaryotic type-II topoisomerases (2, 5, 197). The off-target effect of fluoroquinolones requires drug concentrations a few orders of magnitude greater in eukaryotes than in prokaryotes, yet genotoxic effects in mammalian cells have been described at therapeutically relevant doses. For example, CPX induced DNA damage and genotoxicity in cell-free assays and in cultured cells (8, 79). Although demonstrating little genotoxicity by itself, MOX enhanced apoptosis caused by etoposide (51). Furthermore, CPX and MOX each enhanced cytotoxicity in HT-29 cells from exposure to camptothecin, a type-I topoisomerase poison (169). Consistent with these observations, fluoroquinolones are being evaluated as adjunctive therapy to enhance efficacy of cancer chemotherapeutic drugs (5, 196, 197). Fluoroquinolone-mediated cases of IDILI, therefore, could be associated with topoisomerase poisoning in humans.

TVX is associated with IDILI in humans, whereas LVX is not (117). In mice, TVX/LPS coexposure precipitated hepatotoxicity, but LVX/LPS did not (189). Global gene expression analysis of livers from rats or primary hepatocytes treated with TVX or LVX suggested that TVX, but not LVX, selectively targets eukaryotic topoisomerases (124, 226). Additionally, TVX affected chromosomal expression patterns in a manner similar to the known topoisomerase poisons, etoposide and doxorubicin, suggesting further that TVX might act as a topoisomerase poison (170). *In silico* binding analysis

(Figure 14) also suggested that TVX binds favorably to eukaryotic TopIIa. The results suggested TVX has the capacity to bind to TopIIa in a distinct manner compared to LVX. Furthermore, TVX prevented human TopIIa-dependent decatenation of kinetoplastid DNA in a concentration-dependent manner (Figure 15). Taken together, these results raise the possibility that the IDILI liability associated with TVX might be attributed to off-target poisoning of eukaryotic topoisomerase.

These findings in cell-free models were extended to RAW cells. Topoisomerase inhibition in cells can lead to double-strand DNA breaks (DSBs) generated from topoisomerase-DNA covalent complexes (46, 121, 125, 181). pH2A.X was chosen as a sensitive marker of DSBs. TVX increased pH2A.X in a concentration-dependent manner in RAW cells after a 2-hour incubation (Figure 16A). LVX, however, did not increase pH2A.X (Fig 16B). These differences in ability of the two drugs to damage DNA are consistent with the difference in binding affinity and/or preferred binding site for Top IIa predicted *in silico* (Figure 14).

A recent screen of novel bacterial type II topoisomerase inhibitors in murine L5178Y lymphoma cells used pH2A.X as an indicator of topoisomerase inhibition (197). A substantial proportion of the novel inhibitors, 22/63 of inhibitors tested, as well as CPX and MOX modestly increased pH2A.X in mammalian cells, and this increase coincided with a >6-fold increase in mutation frequency, suggesting that many bacterial topoisomerase inhibitors including fluoroquinolones can induce genotoxic effects in eukaryotic cells (197). The phosphorylation of H2A.X that we observed with TVX was modest compared to the effect of potent eukaryotic DNA damaging agents (5, 197). Additionally, TVX was nontoxic at large doses in mice in the absence of a concurrent

inflammatory stress (189). These observations are consistent with the possibility that TVX causes modest genotoxic insult to the liver, which only rises to overt liver damage in the presence of a secondary stress, such as inflammation.

Taken together, the results in Figures 14-16 suggest that TVX poisons topoisomerase in RAW cells and that this leads to DNA damage which does not result in cytotoxicity (data not shown). DNA lesions activate several mediators and intracellular signaling pathways in a coordinated and dynamic manner that is referred to as the DNA damage response (DDR) (34). ATM and ATR are rapidly activated kinases that are critical to the DDR (98). Treatment with TVX caused an increase in phosphorylation of an epitope in proteins (Figure 17A) that is a target for both ATM and ATR (101). This occurred prior to pH2A.X generation (Figure 16), and ATM and ATR inhibitors prevented TVX-induced phosphorylation of this epitope (Figure 17B), suggesting that TVX activated ATM and ATR. Although each kinase is thought to be activated by a distinct type of lesion, i.e., DSBs for ATM and single-strand DNA breaks for ATR, significant crosstalk can occur between these pathways in response to DNA damage (35, 37). The results in Figures 14 - 17 support a scenario in which TVX is poisoning eukaryotic topoisomerase, damaging DNA and activating DDR kinases.

A large amount of evidence supports a link between induction of DNA damage and upregulation of cytokine expression. Potent eukaryotic topoisomerase poisons doxorubicin and etoposide as well as the anti-metabolite 5-fluorouracil can increase cytokine expression in murine macrophages *in vitro* and in mice *in vivo* (50, 235). In mice treated with TVX and LPS, hepatotoxicity requires a TVX-mediated prolongation of the LPS-induced increase in TNF concentration in the plasma (189). Since ATM and

ATR signaling was activated in RAW cells (Figure 17), the roles of these kinases were investigated with respect to TVX-mediated induction of TNF mRNA. LVX does not increase TNF mRNA in RAW cells (Chapter 2). The increase in TNF mRNA caused by TVX was insensitive to ATM inhibition and to nonselective PI3K inhibition (Figure 18A). In addition, WORT is a nonselective PI3K inhibitor, but ATR is far less sensitive to inhibition by WORT than ATM or DNA-PK (184). Only ATR inhibition prevented the increase in mRNA in TVX treated cells. Thus, although several PI3Ks, including ATM, ATR and DNA-PK, are activated in response to DNA lesions (34), only ATR was implicated in the enhancement of TNF expression by TVX.

TNF mRNA rapidly degrades in the absence of an inflammatory stimulus (43). Interestingly, treatment with TVX stabilized TNF mRNA prior to LPS exposure (Figure 18B), and NU6027 reduced this effect, suggesting that TVX-dependent ATR activation contributes to stabilization of TNF mRNA. The increased stability of TNF mRNA by ATR is a novel observation, as only ATM has been identified previously as an inducer of TNF transcription in response to genotoxic stress (161, 237, 238). The increase in TNF mRNA might be due to ATR-mediated activation of HuR, a protein that binds to the TNF mRNA 3'-ARE. ATM/ATR are known to activate HuR and induce binding of HuR to mRNAs in response to genotoxic stress (250, 251). The TVX-mediated TNF mRNA stability could be the result of ATR- but not ATM-dependent HuR activation and this selective role for ATR would be the subject of further investigation.

Since ATR was implicated in the TVX-induced increase in TNF mRNA (Figure 18), the involvement of ATR in LPS-induced TNF protein release was examined. KU55933 and NU6027 were included only during the period of exposure to TVX and

were removed before addition of LPS or SAL. This was to determine if the critical TVX-induced signaling changes occurred prior to LPS exposure. TVX pretreatment enhanced LPS-induced TNF release within 3h after LPS addition, and this increase was prevented by NU6027 (Figure 19A). When NU6027 was added after LPS addition, the TVX-mediated potentiation of TNF release was not prevented (Figure 20). Accordingly, the critical ATR activation must have occurred during the TVX pretreatment period, not after LPS addition. Elimination of the LPS-TVX interaction by NU6027 was evident 3h after LPS addition, but not at 6h. That is, TVX-mediated potentiation of LPS-induced TNF release was reestablished 6h after LPS despite the presence of NU6027 during TVX pretreatment (Figure 19B). ATR could be activated after withdrawal of NU6027-containing medium if the DNA damage in RAW cells has not been resolved prior to LPS exposure. Another explanation is that TVX-mediated ATR activation mediated the early phase (3h) of the increased LPS-induced TNF release but other signaling is required to explain later phase (6h) of the TVX-mediated increase in LPS-induced TNF release, such as MAPKs (Chapter 2 and Section 4.1.3).

The effect of TVX on cytokine expression has been addressed in two other studies (100, 165). In both of these studies, TVX decreased TNF expression in LPS-pretreated cells (100, 165), contrasting with the increase identified in this study. A key difference in those studies is that TVX was added to monocytes or PBMCs previously stimulated with LPS, whereas in our study TVX was present only before LPS addition. In both studies wherein TVX decreased TNF mRNA and protein release, the results were attributed to TVX acting as a topoisomerase inhibitor in eukaryotic cells (100, 165). Accordingly, the difference between the results could be due to temporal differences in

TVX exposure relative to LPS. When cells are synthesizing TNF in response to LPS, the addition of TVX and subsequent topoisomerase poisoning might prevent maximal transcription of TNF. As the results from the current study indicate, the TVX-mediated DDR and consequent activation of ATR before LPS exposure appears to be critical for the TVX-mediated increase in LPS-induced TNF release.

Taken together, the results of *in silico,* cell-free and cultured cell approaches indicate that TVX, but not LVX, can decrease topoisomerase activity and induce DNA damage at concentrations that approach those occurring in patients treated with TVX (209). TVX activated ATM/ATR-dependent signaling, and ATR played a critical role in mediating increased TNF mRNA stability and LPS-induced TNF protein release from macrophages. The results from this study uncovered a previously unknown role for the DDR and specifically ATR in increasing TNF expression by macrophages exposed to modest genotoxic stimuli. This suggested that topoisomerase inhibition might contribute to IDILI caused by TVX and perhaps other fluoroquinolone antibiotics.

CHAPTER 4

Summary and Conclusions

4.1 Summary of research

4.1.1 Role of TVX-mediated MAPK activation in a model of increased LPS-induced TNF release.

The hypothesis to be tested in this dissertation was that trovafloxacin poisons eukaryotic topoisomerase which increases LPS-induced tumor necrosis factor-alpha release. The findings presented in Chapter 2 describe the establishment of a model of TVX pretreatment in LPS-exposed RAW cells and the changes in intracellular signaling that resulted from TVX pretreatment. TVX increased LPS-induced TNF release only when RAW cells were pretreated with TVX and the TVX-containing medium was replaced with LPS-containing medium (Figure 4). TVX pretreatment increased LPS-induced TNF release in a concentration- and time-dependent manner (Figures 5 and 6) and LVX did not increase LPS-mediated TNF release, consistent with observations *in vivo* (189). In addition, TVX alone increased TNF concentration in the medium at all times after medium replacement.

A potential explanation for the TVX-mediated increase in LPS-induced TNF release was increased TNF mRNA after LPS exposure (Figure 7). There was a trend towards an increase in TNF mRNA after LPS exposure from TVX pretreatment, but no significant difference was detected from LPS alone. This was a surprising finding, since TVX increased LPS-induced TNF release at all times after LPS (Figure 6) as well as the rate of TNF increase (Table 2) through 6h after LPS exposure. The focus on TVX-mediated changes in TNF expression therefore turned to the TVX pretreatment period.

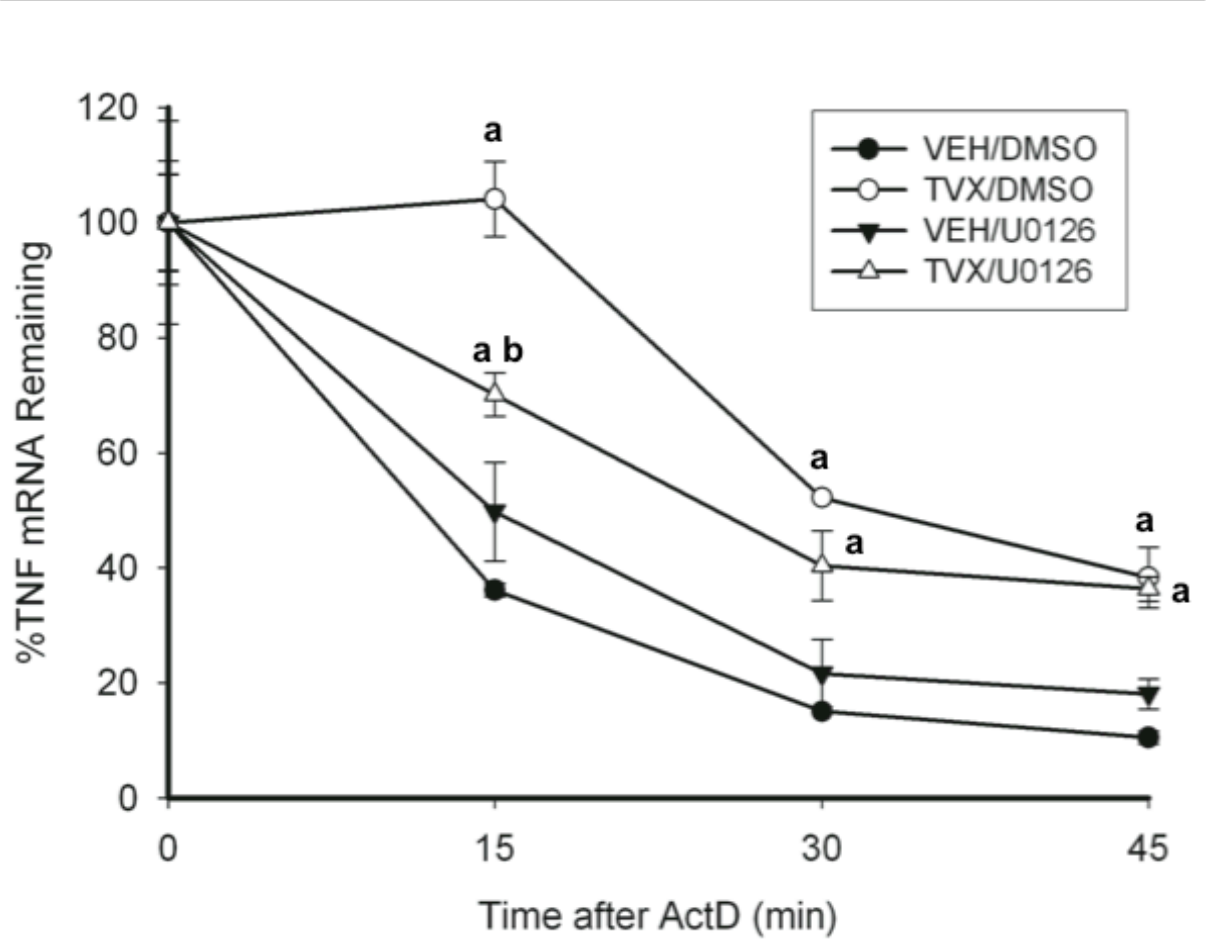
Indeed, TVX increased TNF mRNA prior to LPS (Figure 8), whereas LVX did not (Figure 9).

Since NF-kB and MAPKs are required for many steps in the regulation of TNF expression and release (Table 1), the activation of NF-kB and MAPKs were assessed during the TVX pretreatment period. It was surprising to find that NF-kB activation (data not shown) was not increased by TVX despite the various regulatory sequences bound by NF-kB in the *Tnf* gene that increase *Tnf expression* (Chapters 1.2.1-1.2.2 and Table 1). Since NF-kB was not activated, MAPKs were likely driving the TVX-mediated increase in TNF expression. TVX pretreatment increased activation of ERK (Figure 10A), JNK (Figure 11A) and p38 (Figure 12A). TVX-mediated TNF mRNA induction was decreased by ERK (Figure 10B) and JNK (Figure 11B) inhibition, but not by p38 inhibition (Figure 12B). This finding was surprising, as p38 is critical to induction of TNF expression and release in many models (Table 1).

Reversible MAPK inhibitors were included during the TVX incubation period only, since ERK, JNK and p38 are all required for LPS-induced TNF expression and release. This allowed us to determine if the early TVX-mediated MAPK activation was required for the TVX-mediated increase in LPS-induced TNF release. ERK and JNK inhibition prevented the TVX-mediated increase in LPS-induced TNF release 3h and 6h after LPS exposure, whereas p38 inhibition did not (Figure 13). Another intriguing aspect from the p38 inhibition was that it decreased LPS-induced TNF release in the absence of TVX. Furthermore, the magnitude of LPS-induced TNF release following TVX pretreatment was unchanged by p38 inhibition, emphasizing the selective roles of ERK and JNK in the TVX/LPS interaction in RAW cells.

We assessed if the increase in TNF mRNA prior to LPS was due to an increase in TNF mRNA stability. In this experiment, cells were incubated for 1h with TVX prior to addition of ActD (5 μg/ml) to stop all *Tnf* transcription and measured TNF mRNA stability. The time of ActD addition was chosen to allow for activation of all MAPKs and preceded the increase in TNF mRNA detected after a 2h TVX incubation (Figure 8). TVX stabilized TNF mRNA at all times measured after ActD (Figure 21). ERK played a role in the TVX-mediated stabilization of TNF mRNA: ERK inhibition decreased the early TVX-mediated protection of TNF mRNA but did not change TVX-mediated stability of TNF mRNA at any other time. JNK is not typically associated with TNF mRNA stabilization so it was not tested (43) but p38 is a known mediator of TNF mRNA stabilization. As p38 inhibition did not change TVX-induced TNF mRNA after 2h (Figure 12B), it was not deemed necessary to test its involvement in TVX-induced TNF mRNA stability.

Figure 21. TVX-mediated TNF mRNA stabilization involves ERK. RAW cells were exposed to TVX (100 μM) or its VEH and to U0126 (500 nM) or its DMSO (0.005%) for 1 h before addition of ActD (5 μg/ml), and RNA was isolated at indicated times after ActD. TNF mRNA was normalized to the t = 0 value for each group. Values are expressed as %TNF remaining ± SEM, n=6. a - $p < 0.05$ vs VEH/DMSO, b- $p < 0.05$ vs. TVX/DMSO



ERK or JNK is required for the TVX-mediated increase in TNF mRNA and LPS-induced TNF protein release (Figure 5 and Figure 6). ERK inhibition prevented the TVX-induced increase in TNF mRNA (Figure 10B); therefore ERK-mediated stabilization of TNF (Figure 21) is not the sole mechanism by which ERK contributes to the TVX-induced TNF mRNA. It is possible that ERK acts to increase TNF mRNA transcription through an undetermined mechanism in TVX-exposed RAW cells. The data suggest, however, that the TVX-mediated increases in TNF mRNA and LPS-induced TNF release are ERK-dependent. ERK, therefore, appears necessary and sufficient for the TVX potentiation of LPS-induced TNF release.

JNK, on the other hand, could contribute to TVX-mediated TNF mRNA and protein release through distinct but possibly interdependent mechanisms. JNK can activate AP-1 (39) to induce transcription of *Tnf* in RAW cells, a possible explanation for the JNK-dependent increase in TNF mRNA (156). JNK is also required for LPS-induced TNF mRNA translation (206). JNK inhibition did not completely decrease the TVX-induced TNF mRNA but prevented the TVX-mediated increase in LPS-induced TNF release, suggesting that TVX-mediated JNK activation plays a role in both TNF transcription and translation in this TVX/LPS model in RAW cells.

In summary, the TVX-mediated increase in TNF mRNA and TNF release required ERK or JNK. ERK and JNK can each increase transcription of *Tnf* (67, 72) or act at other downstream points during TNF biosynthesis (43, 176, 206, 241). The observations in Figure 5 and 6 suggested a possibility that ERK is largely responsible for the increased TNF mRNA whereas JNK is required for TNF translation. That is to say that the ERK-mediated increase in TNF mRNA might require JNK-mediated

translation to fully explain the TVX-dependent effects on TNF expression and the increase in LPS-induced TNF release. This cooperative activity between ERK and JNK that could contribute to the TVX/LPS model in RAW cells requires further study.

4.1.2 Role of the DNA damage response in TVX-increased LPS-induced TNF release.

There are few hypotheses to explain the IDILI liability associated with TVX with respect to a TVX-specific intracellular target. As discussed in Section 1.4.3, TVX is implicated as a potential eukaryotic topoisomerase poison. The connection to topoisomerase poisoning and increased cytokine synthesis is described in several models (Section 1.4.3). The purpose of the studies in Chapter 3 was to test the hypothesis that the TVX-mediated changes in intracellular signaling that increase LPS-induced TNF release are due to TVX poisoning eukaryotic topoisomerase.

Human TopIIa is the eukaryotic homolog to the TVX-targeted bacterial type-II topoisomerases. The *in silico* binding analysis (performed by Dr. Jesus Olivero-Verbel) suggested that TVX could bind to the human TopIIa (Figure 14A). TVX was predicted to have the highest affinity (Figure 14A) and binding frequency (Figure 14B) for a particular site on TopIIa. LVX was predicted to have lesser affinity toward TopIIa (Figure 14C) and to bind to a different site on TopIIa than TVX (Figure 14D). This did not exclude LVX as a topoisomerase poison, but it was consistent with the two drugs acting differently on the enzyme.

TVX decreased human TopIIa activity in a concentration-dependent manner in a cell-free assay (Figure 15). The *in silico* binding analysis and TVX-mediated decrease

in TopIIa activity indicated that TVX could act as a topoisomerase poison in eukaryotic cells. Since the hypothesis to be tested in Chapter 3 was that TVX-mediated topoisomerase poisoning increased LPS-induced TNF release in RAW cells, the next experiments were performed to assess if TVX acted as a topoisomerase poison in RAW cells.

Topoisomerase poisoning induces DNA lesions and DNA double-strand breaks in cells (46, 121, 125), so an accepted marker of DNA lesions, pH2A.X, was evaluated in RAW cell protein isolates following a 2 h TVX incubation. TVX increased pH2A.X in a concentration dependent manner (Figure 16A), whereas LVX did not increase pH2A.X. The TVX-dependent increase in DNA damage was consistent with the observations from Figures 14-15 that indicated TVX acted as a topoisomerase poison. The TVX-mediated increase in DNA damage was not cytotoxic to RAW cells (unpublished observation) consistent with the acutely nontoxic induction of pH2A.X observed with other fluoroquinolones in mouse lymphoma cells (197). In addition, fluoroquinolone-mediated DNA damage is modest as compared to potent topoisomerase poisons etoposide and doxorubicin. The modest induction of DNA damage by TVX might explain why TVX is largely nontoxic in rodent models and in humans by itself and requires a second stress, i.e., inflammation, to precipitate an hepatotoxic response.

The TVX-mediated induction of pH2A.X suggested that the DDR was activated in RAW cells. H2A.X is phosphorylated by the DDR kinases ATM, ATR and DNA-PK (34). The ATM/ATR activation marker that was detected in RAW cell protein isolates was an epitope selectively targeted for phosphorylation by ATM and ATR (101). ATM and ATR inhibitors prevented detection of this phosphorylated epitope in TVX-treated RAW cell

protein isolates (Figure 17B), and this suggested that TVX activated ATM and ATR in RAW cells.

The connection between TVX-induced DNA damage and TVX-mediated TNF expression was first investigated with the DDR kinase, ATM. ATM is known to increase NF-kB activation in response to DNA damage (238). ATM inhibition did not decrease the TVX-induced TNF mRNA (Figure 18A) and since NF-kB was not activated by TVX, the role of ATM in TVX-induced TNF expression in RAW cells was further diminished. ATR signaling has not been described to increase cytokine expression previously, yet ATR inhibition decreased TVX-induced TNF mRNA (Figure 18A). Since WORT did not decrease TVX-induced TNF mRNA (Figure 18A), it was likely that TVX-activated ATR mediated the induction of TNF mRNA by TVX.

TVX-increased TNF mRNA stability was studied since TVX increased TNF mRNA (Figure 18A). ATR inhibition attenuated TVX-mediated TNF mRNA stability (Figure 18B). This observation suggested that TVX-induced TNF mRNA is due to ATR-mediated stabilization of TNF mRNA. This is a novel role for ATR, as no reports exist for ATR inducing cytokine expression. However, ATR has been reported to regulate HuR activation (250, 251). HuR binds to the ARE on TNF mRNA and stabilizes the transcript to decrease its turnover (4). Accordingly, ATR-activated HuR might bind to the ARE (Discussed in Section 1.2.2) on TNF mRNA, thus promoting TNF mRNA stability, but this requires further study.

The presence of an ATR inhibitor only during the TVX pretreatment period prevented the TVX-mediated increase in TNF release 3h after LPS exposure. However, ATR inhibition did not prevent the TVX-mediated increase in TNF release 6h after LPS

exposure (Figure 19B). In addition, if the ATR inhibitor was added at the time of LPS exposure, the TVX-mediated increase in LPS-induced TNF release was not prevented (Figure 20). Taken together, the observations suggested that TVX-mediated ATR activation that increased LPS-induced TNF release in RAW cells was restricted to the TVX pretreatment period.

In summary, the observations from Chapter 3 suggested that TVX could act as a topoisomerase poison and activate the DDR. Inhibition of the DDR kinase ATR prevented TVX-induced TNF mRNA. ATR inhibition also prevented the TVX-mediated increase in LPS-induced TNF release at an early time after LPS, but not later. Since ATR inhibition did not prevent the TVX-mediated increase in LPS-induced TNF release 6h after LPS, TVX-dependent changes in signaling independent of ATR activation are likely responsible for the later (6h) increase in LPS-induced TNF release. It is possible that TVX-dependent MAPK (ERK and/or JNK) activation is involved in the activation of ATR.

4.1.3 MAPKs and ATR: is there a connection?

The observations presented in Chapters 2 and 3 suggest that TVX increased LPS-induced TNF release in RAW cells by mechanisms that require ERK, JNK or ATR. I have generated limited experimental evidence that the ATR inhibitor does not change MAPK, i.e., ERK, phosphorylation after 1h or 2h incubations with TVX, but the replicates are not sufficient to conclude if ATR signaling influences MAPK activation. However, a recent review of ATR activation suggests an alternative explanation, indicating that ERK is critical in modulating ATM/ATR activation even if DNA damage likely activates ATM, ATR and ERK simultaneously (229).

Activation of ERK is observed in several cell-types in response to many genotoxic agents: topoisomerase poisons, ultraviolet radiation, ionizing radiation and anti-mitotic agents (Reviewed in 229). The ERK activation from genotoxic agents is relatively rapid (15 - 60 min) and regulated by DDR components. The collective evidence also suggests that ERK activation in response to DNA damage requires the upstream MAPK-kinase, MEK1. Furthermore, although ATM, ATR and ERK all appear to be activated rapidly in response to DNA damage, ERK signaling might influence ATM and ATR activation (Figure 22A).

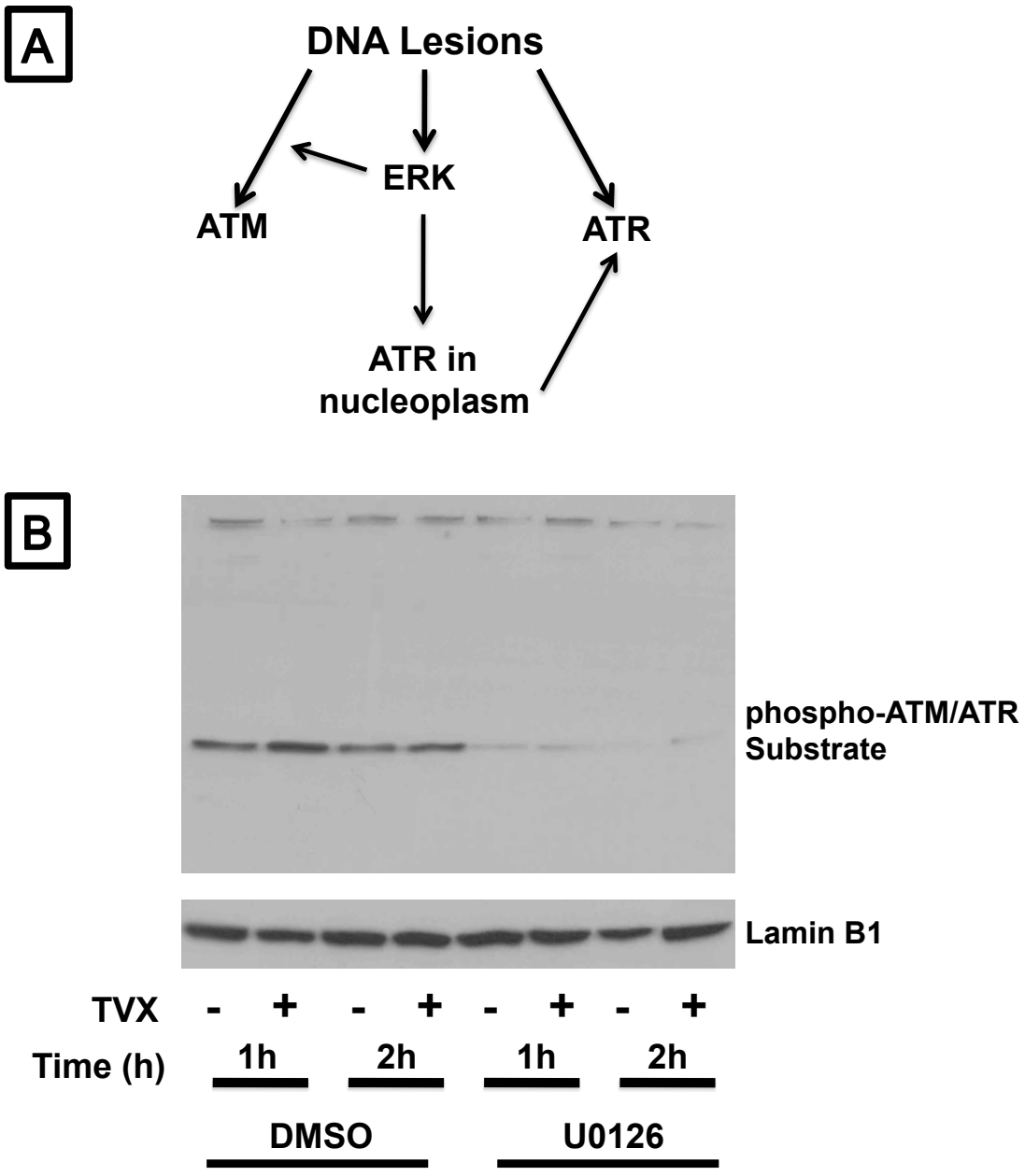
In a preliminary experiment (n=1), the influence of ERK on TVX-induced ATM and ATR activation was tested in RAW cells. Using the selective MEK1 inhibitor, U0126, the TVX-mediated increase in the phosphorylated ATM/ATR substrate signal in RAW cell protein isolates was markedly reduced. This result (Figure 22B) suggests that the TVX-induced activation of ATM and ATR in RAW cells requires ERK. It is therefore possible that a connection exists between the TVX-mediated increases in MAPK and

ATR activation. The observations from Chapters 2 and 3 might be connected, if the TVX-induced DNA damage directly activates ERK and ATR, while activated ERK, in turn, enhances ATR activation in response to TVX.

The influence of ERK on ATR activation (Figure 22B) could explain the observations concerning TVX-induced TNF mRNA stability (Figures 18B and 21). The TVX-induced stabilization of TNF mRNA was sensitive to ERK or ATR inhibition. ERK inhibition decreased ATM/ATR substrate phosphorylation (Figure 22B), but did not completely prevent it. ERK might be required for the full extent of ATR activation, but even when ERK is inhibited in RAW cells some ATR-dependent signaling still remained. This could explain why ATR inhibition destabilized TVX-induced TNF mRNA stability to a greater extent than ERK inhibition. The decrease in TVX-induced TNF mRNA stability when ERK is inhibited, therefore, might be due to decreased ERK-dependent ATR activation. This hypothesis requires further investigation.

An ERK-mediated enhancement of ATR activation could also explain why ATR inhibition failed to prevent the TVX-mediated increase in LPS-induced TNF release 6h after LPS (Figure 19B). ERK inhibition prevented the TVX-mediated increase in LPS-induced TNF release 3h and 6h after LPS (Figure 13), whereas ATR inhibition only prevented the increase in LPS-induced TNF release 3h after LPS. The early ERK-dependent phase (3h) of the TVX-mediated increase in LPS-induced TNF release could be due to ERK enhancing ATR activation (Figure 22B). The later phase (6h) of the TVX/LPS interaction would remain ERK-dependent (Figure 13B) but independent of downstream ATR activation.

Figure 22. **Evidence for ERK-dependent ATR activation in TVX-treated RAW cells. A)** Activation of the DDR and ERK might occur simultaneously and ERK can modulate DDR kinase activation/activity (229). **B)** RAW cells were exposed to VEH or TVX (100 μM) and DMSO (0.005%) or U0126 (500 nM) for 1 or 2 hours. Phospho-Ser/Thr ATM/ATR substrate motif was assessed in isolated protein extracts and lamin b1 was probed as a loading control, n=1.



4.2 Thoughts on *in vitro* experimentation

The primary focus in developing this model of TVX pretreatment in LPS-exposed cells was to recreate a significant and potentially necessary phenomenon observed in the majority of IDILI models, that of IDILI-associated drugs increasing LPS-induced plasma TNF. The preliminary experiments generated a significant amount of data in a short span of time that suggested the model TVX-enhancement of LPS-induced TNF in RAW cells was reliable and reproducible. The mechanistic studies performed to elucidate why TVX increased LPS-induced TNF release took significantly longer, largely due to complications quite common to *in vitro* experiments.

For nearly a year, our cultures in incubators as well were contaminated with what was presumed to be *mycoplasma*, a persistent bacterial contaminant in many, if not all cell cultures. As a result, I could not generate reproducible data. The impact of bacterial contamination in models using inflammatory stress is pernicious and unpredictable. During the period we think our cultures were contaminated, almost all experimental results were inconsistent and therefore unreliable and unusable, especially with RAW 264.7 cells that are very sensitive to TLR agonists. Some minor contamination, in my experience, seems unavoidable, even when aseptic technique is applied and culture medium contained antibiotics. The key is to detect contamination through routine surveillance by several means: cytospinning cell culture flask contents and simple staining to detect bacterial presence (a 30-minute procedure), weekly sterilization of incubators (a 1-hour procedure) and intense scrutiny when thawing cells from frozen stocks. The time and resources saved by carefully maintaining cultures is invaluable.

The other factor I found to be critical to reliable and reproducible results from RAW 264.7 cells was the source and storage of the fetal bovine serum (FBS) supplement. FBS can be sourced from several different countries, have varying concentrations of endotoxin and varying levels of purity. FBS stored over several months in freezers is known to degrade FBS protein factors necessary for cell culture growth and viability. I observed the variation stemming from FBS stored long-term several times. FBS stocks from the same lots, but stored for varying amounts of time, drastically altered the proliferation of RAW cells and led to confounding results. After considering how many variables in FBS contribute to experimental variability, I decided on a few keys to successfully growing cells: 1) Buy small allotments of FBS, no more than 2-3 months of supply at one time. 2) If possible, obtain certificates of analysis for FBS lots, and compare when purchasing more FBS to an FBS lot that was reliable. 3) The first time a given lot of FBS is used, do not experiment on those cells until consistent growth of cells is observed and it is determined that the cellular morphology is the same as with the last. If one works with the same cells for months or years, one should be familiar with the cellular morphology. In my experience, any deviation in the appearance of cells is the first sign of a problem.

4.3 Major findings and implications

1. TVX increases LPS-induced TNF release in RAW 264.7 cells. LVX did not change LPS-induced TNF release in RAW cells. These observations are consistent with TVX/LPS coexposure in mice (189) inasmuch as TVX, but not LVX prolonged LPS-induced TNF appearance in the plasma of mice. This suggests that TVX-mediated increase in LPS-induced TNF release is due to a direct effect of TVX on TNF-producing cells.

2. TVX increased TNF mRNA prior to LPS, and this appeared to be necessary for the increased LPS-induced TNF release. LVX did not affect TNF mRNA expression. Further study indicated that TVX-induced TNF mRNA is likely due to TVX-mediated stabilization of TNF mRNA.

3. MAPK signaling is activated by TVX in the absence of LPS exposure. ERK- or JNK-dependent signaling is required for the TVX-mediated increase in LPS-induced TNF. ERK appears to be a key mediator in the interaction between TVX and LPS in RAW cells. ERK could play a pivotal role in the model of TVX/LPS hepatotoxicity in mice.

4. TVX decreased TopIIa activity in a cell-free assay, and this was a likely cause of the TVX-mediated increase in DNA damage in RAW cells. LVX did not increase DNA damage, a possible explanation for the lesser IDILI liability associated with LVX. TVX activated the DDR kinases ATM and ATR. The TVX-mediated increases in TNF mRNA

and LPS-induced TNF release are likely ATR-dependent. ATR is the least characterized DDR kinase, and its ability to increase cytokine expression is a novel finding. The TVX-dependent ATR activation in RAW cells adds to the knowledge about ATR-mediated effects in cells responding to a genotoxic stimulus.

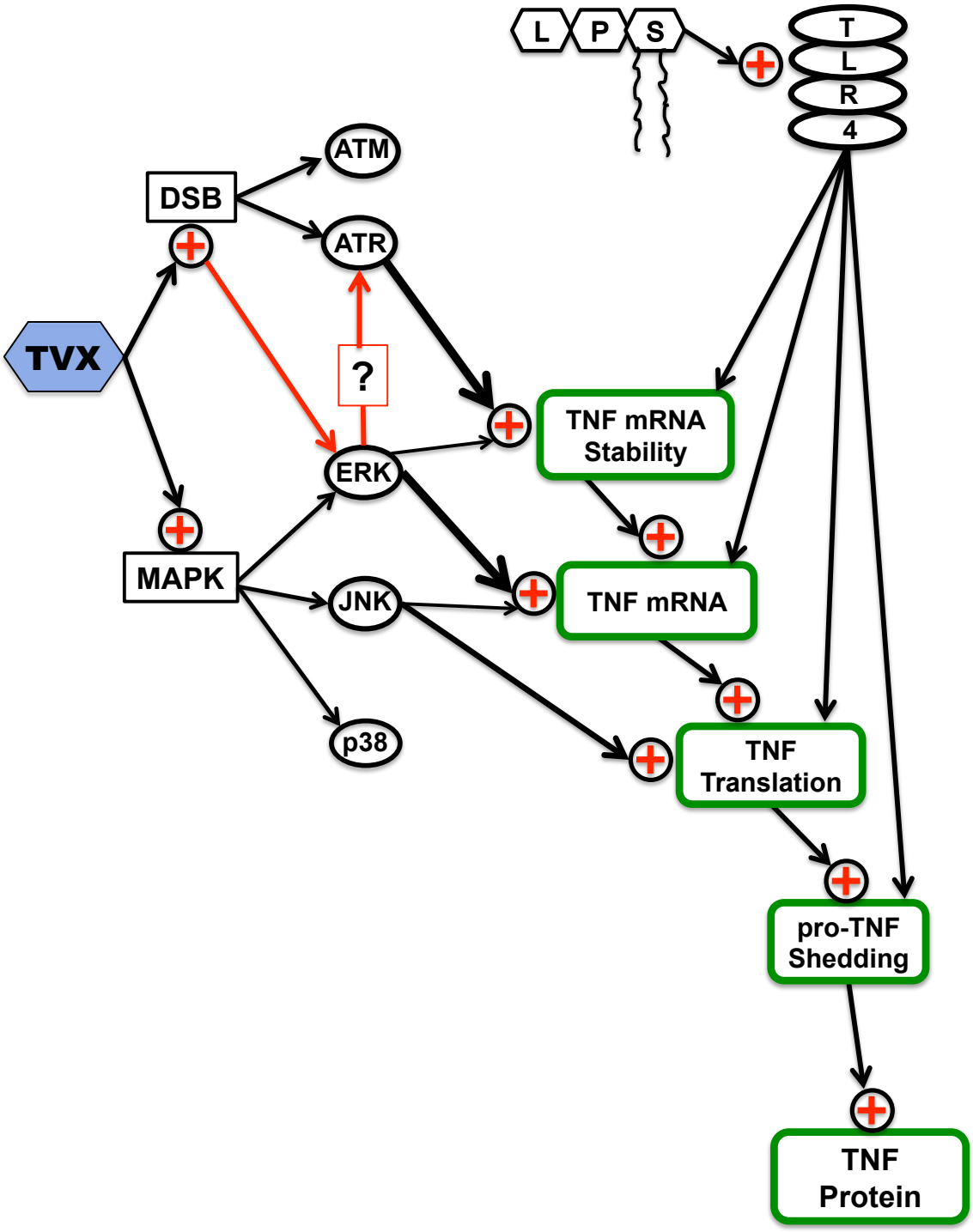
5. The IDILI liability associated with TVX might be due to off-target eukaryotic topoisomerase poisoning. Topoisomerase poisoning might also explain the IDILI liability associated with other fluoroquinolones CPX and MOX. The evidence for TVX acting as a eukaryotic topoisomerase poison adds further knowledge to the off-target effects of fluoroquinolones in eukaryotic cells.

TVX effects on intracellular signaling in RAW cells that augment TNF expression and/or LPS-induced TNF release are summarized in Table 3 and depicted as a signaling pathway in Figure 23.

Table 3. Summary table of TVX-mediated kinase activation from observations in Chapters 2 and 3.

Kinase activated by TVX	TVX-induced effect on TNF in RAW cells	Experimental support
ERK	• Increased transcription • Increased mRNA stability • Increased LPS-induced TNF release	• Figure 10 • Figure 13 • Figure 21
JNK	• Increased transcription • Increased translation (untested) • Increased LPS-induced TNF release	• Figure 11 • Figure 13
p38	• None	• Figure 12 • Figure 13
ATM	• None	• Figure 18A • Figure 19
ATR	• Increased transcription • Increased mRNA stability • Increased LPS-induced TNF release (early)	• Figure 18A • Figure 18B • Figure 19

Figure 23. Proposed pathway of TVX-dependent effects that increase LPS-induced TNF release. See section 4.1.1, 4.1.2 and 4.1.3 for a detailed description of the TVX-dependent effects on pathways leading to increased LPS-induced TNF release. Table 3 summarizes where to find experimental support.



4.4 Knowledge gaps and future studies

The findings described in Chapters 2 and 3 describe an *in vitro* model based upon a critical observation from the hepatotoxicity observed in TVX/LPS coexposed mice. The observations represent some of the first mechanistic studies that describe TVX-mediated changes in intracellular signaling that could underlie the IDILI liability of TVX. RAW cells are a transformed murine cell line, so it would useful to apply the model of TVX pretreatment in LPS-exposed primary macrophages such as isolated murine peritoneal macrophages or Kupffer cells. The ERK- and JNK-dependent aspects of TVX pretreatment in RAW cells could be tested in primary cells.

As the models of IDILI in animals attempt to describe the IDILI liability of drugs in humans, it would be of further use to apply the model of TVX pretreatment prior to LPS exposure in the human THP-1 monocytic cell line or isolated human peripheral blood mononuclear cells (PBMCs). The observation that ERK or JNK signaling is required for TVX/LPS interaction would be important to validate in human THP-1 cell line or PBMCs. The findings generated in transformed-murine cells have limited translational impact if not verified in human cells. In addition, the experiments in human cells could indicate if the TVX-mediated effects in RAW cells are species-specific.

The observations made about TVX-mediated effects in RAW cells would be strengthened if observed in mice. For example, it would be useful to assess MAPK or DDR activation by immunological detection or by flow cytometry in dissociated liver cells. As indicated in Section 1.4, *in vitro* studies are useful if confirmed through follow-up animal studies. The studies in RAW cells were based upon *in vivo* observations and

the aspects of the results in Chapters 2 and 3 were consistent with TVX/LPS coexposure in mice, but the complex physiology *in vivo* cannot be recapitulated *in vitro*.

The working hypothesis in Figure 23 suggests that TVX increases MAPK and DDR activation, which leads to increased TNF expression and release. LVX did not increase the DNA damage marker pH2A.X in Figure 16B. Since LVX did not increase TNF mRNA or LPS-induced TNF release, and if DNA damage is responsible for activation of MAPK or ATR signaling, MAPK or ATR activation in the presence of LVX should be evaluated. If LVX does not activate MAPK or ATR, the conclusion that TVX-mediated MAPK or ATR activation is responsible for increased TNF expression or release would be strengthened.

Results presented in Chapter 3 suggested that TVX acts as a topoisomerase poison in eukaryotic cells. Topoisomerase poisoning was not evaluated in cells, but it was suggested by *in silico* prediction and observed in a cell-free assay. The “band depletion assay” is one method that can demonstrate topoisomerase inhibition in cells. This assay detects "free" topoisomerase molecules that are not covalently bound to the DNA in cellular lysates, since covalent topoisomerase-DNA complexes result from topoisomerase poisoning. Another assay to detect topoisomerase inhibition in cells is also available commercially (Topogen *in vivo* link kit). Cells would be treated with TVX and rapidly lysed with strong ionic detergents. The strong detergent stabilizes the topoisomerase-DNA complex; the lysate is separated by density, with free topoisomerase being separated from topoisomerase-DNA complexes by mass. Results from these approaches could strengthen results from Chapter 3 and evaluate the extent of TVX-mediated poisoning as compared to potent eukaryotic topoisomerase poisons.

Like TVX, CPX and MOX are weak eukaryotic topoisomerase poisons (8, 79, 169), which supported the hypothesis that TVX could act as a eukaryotic topoisomerase poison. It would be very useful to assess whether CPX or MOX pretreatment in RAW cells could increase TNF mRNA and/or LPS-induced TNF release. Since CPX, MOX and TVX all have similar IDILI liability in humans, such a result would strengthen the conclusions Chapter 3. In addition, the role of CPX- or MOX-induced ATM/ATR activation could be assessed. Studies with CPX or MOX in combination with LPS would support many of the conclusions about the hepatotoxic liability of TVX *in vivo*. Furthermore, if an increase or prolongation in plasma TNF was detected in CPX/LPS or MOX/LPS coexposed animals, the conclusions about TVX-mediating increase LPS-induced TNF release in animals would be strengthened.

Chapters 2 and 3 presented evidence for the roles of the MAPK and DDR in response to TVX, but did so largely through small-molecule pharmacological inhibition of the kinases. Pharmacological inhibitors are quite useful, but are notorious for off-target inhibition. The use of siRNA knockdown is becoming nearly indispensible in identifying critical signaling factors in biological systems/models. Knockdown of ERK or JNK would likely decrease LPS-induced TNF release in the absence of TVX, so it is likely to confound any observations about the role or ERK or JNK in the TVX-mediated increase in LPS-induced TNF release. ERK or JNK knockdown could serve as strong confirmation of the conclusions from Chapter 2 in the absence of LPS. In addition, ectopic overexpression of kinase-dead ERK or JNK mutants would be further confirmation of ERK- or JNK-dependence in TVX-exposed RAW cells. Since siRNA directed against ERK or JNK would likely remove most of the protein translated from the

gene's mRNA, the phenotype of a cell can be altered outside of the loss of ERK- or JNK-dependent signaling.

Since ATR appeared to play a large role in the model of TVX/LPS in RAW cells, ATR knockdown would be of interest also. The ATR inhibitor, NU6027, used in studies in Chapter 3, was first designed as an inhibitor of cyclin-dependent kinases, so it might not solely inhibit ATR signaling. ATR knockdown would be necessary to selectively implicate TVX-mediated activation of ATR as critical to TVX mediating an increase in LPS-induced TNF release. Finally, hydroxyurea is an accepted ATR activator, and studies involving hydroxyurea pretreatment of RAW cells would strengthen the role of ATR activation increasing LPS-induced TNF release. Various combinations of hydroxyurea doses and durations of hydroxyurea incubation in RAW cells prior to LPS would be essential to experimental design to optimize the chances of detecting any increase in LPS-induced TNF release.

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