Published in final edited form as:

Toxicology. 2015 May 4; 331: 35–46. doi:10.1016/j.tox.2015.03.002.

Trovafloxacin-induced Replication Stress Sensitizes HepG2 Cells to Tumor Necrosis Factor- α -induced Cytotoxicity Mediated by Extracellular Signal-regulated Kinase and Ataxia Telangiectasia and Rad3-related

Kevin M. Beggs^a, Ashley R. Maiuri^a, Aaron M. Fullerton^a, Kyle L. Poulsen^a, Anna B. Breier^a, Patricia E. Ganey^a, and Robert A. Roth^a

^aMichigan State University, Department of Pharmacology & Toxicology, Center for Integrative Toxicology, 1129 Farm Lane, East Lansing, MI 48824, United States of America

Abstract

Use of the fluoroquinolone antibiotic trovafloxacin (TVX) was restricted due to idiosyncratic, drug-induced liver injury (IDILI). Previous studies demonstrated that tumor necrosis factor- α (TNF) and TVX interact to cause death of hepatocytes in vitro that was associated with prolonged activation of c-Jun N-terminal kinase (JNK), activation of caspases 9 and 3, and DNA damage. The purpose of this study was to explore further the mechanism by which TVX interacts with TNF to cause cytotoxicity. Treatment with TVX caused cell cycle arrest, enhanced expression of p21 and impaired proliferation, but cell death only occurred after cotreatment with TVX and TNF. Cell death involved activation of extracellular signal-related kinase (ERK), which in turn activated caspase 3 and ataxia telangiectasia and Rad3-related (ATR), both of which contributed to cytotoxicity. Cotreatment of HepG2 cells with TVX and TNF caused double-strand breaks in DNA, and ERK contributed to this effect. Inhibition of caspase activity abolished the DNA strand breaks. The data suggest a complex interaction of TVX and TNF in which TVX causes replication stress, and the downstream effects are exacerbated by TNF, leading to hepatocellular death. These results raise the possibility that IDILI from TVX results from MAPK and ATR activation in hepatocytes initiated by interaction of cytokine signaling with drug-induced replication stress.

Keywords

Idiosyncratic drug-induced liver injury; hepatotoxicity; trovafloxacin; TNF; ERK; ATR

© 2015 Published by Elsevier Ireland Ltd.

To whom correspondence should be addressed: Robert A. Roth, PhD, Michigan State University, Department of Pharmacology and Toxicology, 1129 Farm Lane, Room 221, East Lansing, MI 48824, Tel: 1 (517) 353-9841, Fax: 1 (517) 432-2310, rothr@msu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Idiosyncratic, drug-induced liver injury (IDILI) is a typically rare and currently unpredictable adverse response that accounts for as much as 17% of all cases of acute liver failure (Hussaini and Farrington 2007). Due to a lack of understanding of mechanisms of toxicity, host susceptibility, and factors that dictate outcome, IDILI is presently not preventable. One hypothesis to explain IDILI etiology is that an otherwise nontoxic dose of a drug interacts with a concurrent inflammatory stress to precipitate liver injury (Roth et al. 2003). Several animal models have been developed based on this hypothesis (Roth and Ganey 2011). Exploration of these models has allowed the identification of some common factors, including the proinflammatory cytokine tumor necrosis factor- α (TNF), as important in triggering liver injury from drugs with idiosyncratic liability (Shaw et al. 2007; Tukov et al. 2007; Zou et al. 2009). Consistent with this finding in vivo, cotreatment of primary hepatocytes and hepatocyte cell lines with TNF and drugs with IDILI liability caused synergistic cytotoxicity (Beggs et al. 2014; Cosgrove et al. 2009; Fredriksson et al. 2011; Shaw et al. 2009a; Zou et al. 2009). These observations suggest that such drugs sensitize hepatocytes to the cytotoxic effects of TNF.

Trovafloxacin (TVX) is a fluoroquinolone antibiotic that exerts its bactericidal activity by inhibiting prokaryotic topoisomerase enzymes that are critically involved in bacterial cell division (Brighty and Gootz 1997; Gootz et al. 1996). TVX received a black box warning in 1999 after it was associated with life-threatening IDILI in people (Nightingale 1999). In vitro, TVX did not cause death of hepatocytes; however, TVX synergized with TNF to cause cytotoxicity (Beggs et al. 2014; Cosgrove et al. 2009; Shaw et al. 2009a).

Levofloxacin is an antibiotic in the same class as TVX but does not share the propensity to cause IDILI in people, and it did not synergize with TNF to induce cytotoxicity in vitro. The cell death from coexposure to TVX and TNF was apoptotic. Similarly, apoptosis of hepatocytes was observed in a mouse model of TVX-TNF interaction (Shaw et al. 2009a). Cell death in vitro depended on prolonged activation of c-Jun N-terminal kinase (JNK) as well as upon caspase activation, and it was associated with DNA damage (Beggs et al. 2014).

Prolonged activation of JNK can occur from genotoxic stress (Roos and Kaina 2006; Seok et al. 2008) including DNA replication stress (Damrot et al. 2009; Llopis et al. 2012) which can also activate a multitude of other signaling events. These events include induction of the cyclin-dependent kinase inhibitor p21 (Cazzalini et al. 2010), cell cycle arrest, decreased cell proliferation (Houtgraaf et al. 2006) and activation of extracellular signal-regulated kinase (ERK) (Cagnol and Chambard, 2010). Other events associated with genotoxic stress include activation of the DNA damage sensor kinases ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase (DNA-PK) (Yang, et al., 2003). Together, these signaling events can arrest cells to allow for repair of DNA damage or, in cases of severe genotoxic stress, promote apoptotic signaling leading to cell elimination (Cagnol and Chambard 2010; Houtgraaf et al. 2006; Roos and Kaina 2006; Yang et al. 2003). The purpose of this study was to explore further the mechanism by which TVX interacts with TNF to cause cytotoxicity, including a potential contribution from DNA replication stress. Identification of critical, drug-induced signaling events that render

hepatocytes sensitive to cell death from cytokines could facilitate the development of predictive preclinical screening assays to identify drug candidates with idiosyncratic liability.

2. Materials and methods

2.1. Materials

Unless otherwise noted, all materials were purchased from Sigma-Aldrich (St. Louis, MO). Cayman Chemical (Ann Arbor, MI) synthesized the TVX. Recombinant human TNF, z-VAD-fmk (ZVAD) and caspase 3 fluorometric assay kit were purchased from R&D Systems (Minneapolis, MN). Phosphate-buffered saline (PBS), high glucose Dulbecco's Modified Eagles Medium (DMEM), Antibiotic-Antimycotic (ABAM), L-glutamine, and 0.25% trypsin-EDTA were purchased from Life Technologies (Carlsbad, CA). For flow cytometry experiments, Cell Staining Buffer was purchased from Biolegend (San Diego, CA), Perm/Wash Buffer from BD Biosciences (San Jose, CA), and Propidium Iodide/RNase Staining Solution from Cell Signaling Technology (Beverly, MA). U0126 was purchased from Calbiochem (San Diego, CA). KU55933 and Tempol were purchased from Tocris Bioscience (Minneapolis, MN). Cellular reactive oxygen species assay kit was purchased from Abcam (Cambridge, MA).

2.2. Cell Culture

HepG2 human hepatoblastoma cells (American Type Culture Collection, Manassas, VA) were used for these studies. We and others have reported that HepG2 cells and primary murine hepatocytes respond similarly to TVX in the presence of cytokines with respect to caspase-dependent cytotoxicity (Beggs et al., 2014; Cosgrove et al., 2009). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% ABAM in 25-cm² tissue culture treated flasks. Cells were cultured in a humidified atmosphere composed of 95% air and 5% CO₂ and a temperature of 37°C. Cells were passaged twice each week. 0.25% Trypsin-EDTA was used to detach confluent HepG2 cells from the flask. After plating, cells were allowed 7 hours to adhere before treatment. TVX was reconstituted to a stock solution of 200 mM in dimethyl sulfoxide (DMSO): when added to culture wells the maximal final concentration of DMSO was 0.01%. Vehicle controls for TVX are represented as "Veh" throughout. TNF was reconstituted to a stock solution of 100 µg/mL in PBS.

2.3. Protein Isolation

HepG2 cells were plated at 1.2×10^6 cells per well in 6-well tissue culture plates. Cells were treated for various times before being washed with ice-cold PBS. After washing, they were treated with radio-immunoprecipitation assay (RIPA) buffer containing HALT protease and phosphatase inhibitors (Thermo Scientific, Pittsburgh, PA). Cells were scraped, collected in tubes and kept on ice. They were incubated in RIPA buffer for 10 minutes before each sample was sonicated with one 5-second pulse. Lysates were centrifuged at 20,000×g for 20 minutes, and the supernatant was collected for analysis. The bicinchoninic acid (BCA) assay (Thermo Scientific) was used to determine protein concentration.

2.4. Western Blot Analysis

Phospho-H2AX (γ H2AX), p53, p21, phospho-ERK (p-ERK), phospho-(Ser/Thr) ATM/ATR substrate motif (p-ATM/ATR substrate), α -Tubulin and Lamin B1 (Lamin) were detected by loading 15 μ g of protein on NuPAGE 10% Bis-Tris gels (Life Technologies). The proteins were then separated by electrophoresis. Proteins were transferred from gels onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After transfer, the membranes were blocked for 1 hour in a solution of 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST). Membranes were then probed with primary antibodies (Cell Signaling Technology, Beverly, MA). Antibodies were diluted in 5% BSA in TBST to 1:2000 for γ H2AX, α -Tubulin and p21, 1:1000 for p53, 1:10,000 for p-ERK, 1:1000 p-ATR/ATR substrate and 1:10,000 for Lamin. Membranes were incubated with primary antibodies at 4°C for at least 18 hours. PVDF membranes were then washed with TBST and probed with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for one hour at room temperature. Secondary antibodies were diluted in 5% BSA in TBST to 1:2500 for γ H2AX, 1:5000 for p53, p21, α -Tubulin and pATM/ATR substrate, and 1:10,000 for p-ERK and Lamin. HRP was visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA). Membranes were developed on HyBlot CL Film (Denville Scientific, Metuchen, NJ), and densitometry was performed on the developed films using Image J software.

2.5. Flow Cytometry

Cells were plated at 5×10^5 cells per well in 12-well tissue culture plates. After 12 hours of exposure, cells and supernatant were collected in 12mm $\times$ 75mm round-bottomed tubes (BD Biosciences) on ice. Cells were pelleted by centrifugation at 4°C for 5 minutes at $70 \times g$. Culture medium was aspirated before cells were washed with cold Cell Staining Buffer. This cell suspension was added drop wise to an ice-cold solution of 70% ethanol. The suspension was kept at 4°C for at least 12 hours to allow for fixation. After the cells were fixed, they were spun down, and the ethanol solution was aspirated. Cells were washed once with Cell Staining Buffer and pelleted, and the buffer was aspirated. The cells were resuspended in Perm/Wash Solution and kept on ice for 5 minutes. After centrifugation and aspiration of the Perm/Wash Solution, cells were resuspended in PI/RNase Staining Solution at room temperature for 30 minutes. After incubation, cell cycle analysis was performed using a BD FACS Canto II flow cytometer. Data were analyzed using Kaluza software (Beckman Coulter, Brea, CA). Unstained cells were also analyzed to account for auto fluorescence. Cells were first gated by forward scatter height versus forward scatter area to discriminate cellular aggregates. Then the population of interest was gated by forward scatter versus side scatter and analyzed for DNA content based on propidium iodide staining.

2.6. HepG2 Proliferation Studies

For the manual cell count study, cells were plated at 1×10^6 cells per well in 6- well tissue culture plates. After treatment time (0, 24, or 48 hours), culture medium supernatant was collected, and cells were detached with trypsin. The collected supernatant was returned to detached cells to neutralize trypsin, and cell concentration was determined using a hemocytometer. Each sample was counted 6 times (technical replicates), and the average

cell concentration was recorded as $n=1$ when calculating the group mean. For the fluorescent probe study, cells were plated at 1×10^4 cells per well in black-walled, 96-well tissue culture plates. For each experiment, 4 wells were plated for each treatment group. Relative DNA content was determined using the CyQUANT NF Cell Proliferation Assay (Life Technologies) following the manufacturer's instructions. Briefly, at time of measurement culture medium was gently aspirated and replaced with an equal volume of dye solution. Cells were incubated at 37°C for 1 hour to allow for intercalation of the proprietary fluorescent dye into DNA. After incubation, the plates were read on a fluorescent microplate reader with filters for excitation at 485 nm and emission at 530 nm.

2.7. RNA Isolation and RT-PCR

Cells were plated at 1.2×10^6 cells per well in 6-well tissue culture plates. After 6 hours of treatment, RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Thermo Scientific) was used to assess the quantity and quality of the collected RNA. Complementary DNA (cDNA) was prepared from 1 μg of RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories). The expression of TP53, CDKN1A and ACTB genes was determined using a StepOne Real-Time PCR system using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Copy number was determined by comparison with standard curves of the respective genes generated from pooled cDNA of all treatment groups. TP53 and CDKN1A expression was normalized to the expression of ACTB (β -Actin). PCR primers were used as follows: human TP53, 5'-gagcgtgctttccacgac-3' (forward) and 5'-tgtttctgactcagagggg-3' (reverse), human CDKN1A, 5'-accgaggcactcagaggag-3' (forward) and 5'-gccattagcgcatcacagt-3' (reverse), human ACTB, 5'-gcacagagcctcgctt-3' (forward) and 5'-gtgtcgacgacgagcg-3' (reverse).

2.8. HepG2 Cytotoxicity Assessment

HepG2 cells were plated at 4×10^4 cells per well in white-walled, 96-well tissue culture plates. After 24 hours of treatment, cytotoxicity (defined as increased plasma membrane leakage) was measured using the CytoTox-Glo Cytotoxicity Assay (Promega, Madison, WI) following the manufacturer's instructions. All inhibitors were reconstituted in DMSO (represented as "Vehicle" in each study). Cells were exposed to 25 μM pifithrin- α (PFT) or 0.05% DMSO, 10 μM U0126 or 0.05% DMSO, 12.5 μM NU6027 or 0.0625% DMSO, 20 μM KU55933 or 0.1% DMSO, or 10 μM wortmannin or 0.1% DMSO. The concentrations chosen have been shown to inhibit their respective target proteins (Hickson et al. 2004; Komarov et al. 1999; Okayasu et al. 1998; Peasland et al. 2011).

2.9. Caspase 3 Activity Assay

Caspase 3 activity was determined using a fluorometric caspase activity assay kit from R&D Systems. Cells were plated at 1.2×10^6 cells per well in 6-well tissue culture plates. Cells were exposed to TVX or Veh and TNF or PBS in the presence of either U0126 or NU6027 or their vehicle controls. After 12 hours of exposure, caspase 3 activity was measured as described previously (Beggs et al. 2014).

2.10. ATR Measurement

Labeling for phospho-ATR (p-ATR) was conducted using a polyclonal antibody against phosphorylated threonine 1989 on ATR (Genetex, Irvine, CA). Cells were plated in 8-chamber culture slides (BD Biosciences) at 9×10^4 cells per chamber. After 6 hours of treatment, culture medium was gently aspirated. Cells were air-dried and submerged in acetone chilled to -20°C for fixation and permeabilization. Cells were then rinsed with PBS and incubated with a blocking buffer containing 10% goat serum in PBS. Primary p-ATR antibody was diluted to 1:100 in blocking buffer, and cells were incubated with primary antibody solution in a humidified chamber overnight at 4°C . Cells were rinsed with cold PBS and incubated with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (Life Technologies) diluted 1:500 in blocking buffer for 2 hours at room temperature. They were then washed, and an anti-fade mounting medium containing DAPI was applied (Vector Laboratories, Burlingame, CA). Slides were imaged using an Olympus IX71 inverted fluorescence microscope and appropriate filters. Images were taken with an Olympus F-View II digital monochrome camera and were processed using Image J software. Three to six images were taken for each chamber. Cells exposed to only the secondary antibody were used as a negative control, and no significant signal was detected in this group.

2.11. Reactive Oxygen Species Assessment

HepG2 cells were plated at 4×10^4 cells per well in black-walled, 96-well tissue culture plates. Reactive oxygen species (ROS) were measured following the manufacturer's instructions. Briefly, cells were incubated at 37°C with buffer containing dichlorofluorescein diacetate for 45 minutes. Cells were then washed and treatment medium was added. Some cells were treated with medium containing the prooxidant tert-butyl hydroperoxide (TBHP), used as a positive control. Fluorescence of dichlorofluorescein (DCF) was measured at 6 hours. To assess the role of ROS in cell death, cells were exposed to the antioxidants tempol (1 mM), N-acetyl cysteine (NAC; 1 mM), or alpha-tocopherol (200 μM) simultaneously with TVX or vehicle and TNF or PBS, and cell death was measured at 24 hours.

2.12. Statistical Analysis

Results are expressed as mean + S.E.M. Percentile data were subjected to arcsine transformation. Analysis of data was performed using one-way or two-way analysis of variance (ANOVA) followed by pairwise multiple comparisons using the Holm Sidak or Tukey's method. Nonparametric data were analyzed using Kruskal-Wallis test followed by pairwise multiple comparisons using Tukey's or Dunn's method as appropriate. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Cell Cycle Analysis and Proliferation Time Course

In response to DNA damage, many cell types undergo cell cycle arrest (Houtgraaf et al. 2006). Distribution of cells in the various stages of the cell cycle was analyzed by flow cytometry after 12 hours of treatment. The distribution of gated singlet cells in G0/G1, S,

and G2/M after vehicle treatment was 49%, 27%, and 19%, respectively (Figure 1). Treatment with TVX caused an increase in the percentage of G0/G1 cells (71%) and a decrease in the percentage of cells in S phase (6%) but did not change the percentage of cells in G2/M (21%). Results were similar after treatment with TNF alone, although the magnitude of the change was smaller. Treatment of cells with TVX/TNF resulted in a cell cycle distribution almost identical to treatment with TVX alone; that is, TVX/TNF caused an increase in the percentage of cells in G0/G1 as well as a decrease in the percentage in S phase.

Effects on HepG2 cell proliferation were investigated by manual counting using a hemocytometer as well as by measuring DNA content with an intercalating fluorescent probe. Measurements were made at the time of treatment (0 hour), as well as 24 and 48 hours later. Proliferation was apparent in cells treated with vehicle but was halted completely by addition of TVX (Figure 2A). No significant cytotoxicity, as marked by increased plasma membrane leakage, was observed in cells exposed to TVX alone at 24 or 48 hours (data not shown). Similarly, DNA content relative to vehicle controls decreased progressively as a result of TVX treatment in the absence or presence of TNF (Figure 2B). TNF by itself was without significant effect. By 48 hours, cotreatment with TVX/TNF caused a more pronounced decrease in DNA content than treatment with either TNF or TVX alone.

3.2. p53 Expression and Contribution to Cytotoxicity

DNA strand breaks can lead to upregulation of the transcription factor and tumor suppressor protein, p53 (Lakin and Jackson 1999). Though many transformed cell lines have a mutated p53 gene, HepG2 cells express a functional, wild type p53 (Hosono et al. 1991). Expression of TP53 mRNA was not altered by treatment with TVX, TNF or the combination after 4 or 6 hours of exposure (Figure 3A). Similarly, expression of p53 protein was not altered after 6 or 12 hours (Figure 3B). In response to DNA damage, p53 becomes phosphorylated at serine 15 (Shieh et al. 1997). Protein expression of phospho-p53 was also unchanged at 6 and 12 hours (data not shown). The contribution of p53 to cytotoxicity was examined using an inhibitor of p53-mediated transcription, PFT (Komarov et al. 1999). Simultaneous treatment of cells with 25 μ M PFT did not alter the cytotoxicity caused by TVX/TNF after 24 hours (Figure 3C). Similar results were observed after treatment with 50 or 100 μ M PFT (data not shown).

3.3. p21 Transcription and Protein Expression

Transcription of the CDKN1A gene and expression of its protein product p21 were examined. After 6 hours of treatment, both TVX alone and TVX/TNF caused an increase in CDKN1A mRNA expression compared to cells treated with vehicle or TNF (Figure 4A). None of the treatments changed p21 protein expression at 6 hours (data not shown); however, TVX caused an increase in p21 expression compared to vehicle controls by 12 hours after treatment (Figure 4B).

3.4. ERK Activation and Its Role in Cell Cycle and Cytotoxicity

A common response to DNA damage is activation of the ERK MAP kinase. ERK signaling can be involved in promoting either progression or arrest of the cell cycle (Cagnol and Chambard 2010). For these reasons, the activation of ERK was investigated. Although none of the treatments affected ERK phosphorylation after 1 hour (data not shown), by 6 hours both TVX and TVX/TNF treatments led to an increase in ERK activation compared to cells treated with vehicles or TNF (Figure 5). This increase in ERK activation was maintained through 24 hours (Supplemental Figure 1). Total ERK expression was not affected by any treatment (data not shown). U0126 is a selective inhibitor of the MEK1/2 kinases upstream of ERK (Favata et al. 1998). Treatment with U0126 completely prevented ERK phosphorylation in all treatment groups (Figure 5). To evaluate the role of ERK in treatment-induced changes in cell cycle, cell cycle analysis was performed on cells treated with U0126 or its vehicle. In cells treated with either vehicle or TNF, U0126 caused an increased percentage of cells in G0/G1, as well as a decreased percentage of cells in S phase (Figure 6A). Similar to results presented in Figure 1, treatment with TVX or TVX/TNF also caused an increase in the percentage of cells in G0/G1 and a decrease in the percentage of cells in S phase. U0126 did not affect this distribution in TVX- or TVX/TNF-treated cells. The percentage of cells in G2/M was not affected by any treatment in the absence or presence of U0126.

To examine the role that ERK signaling plays in promoting cell death, HepG2 cells were treated with TVX and/or TNF in the presence and absence of U0126. Cytotoxicity was measured 24 hours after treatment. Neither TVX alone nor TNF alone caused cell death in the presence or absence of U0126 (Figure 6B). The TVX/TNF combination was cytotoxic, and U0126 markedly reduced the cytotoxicity. We have reported previously that cell death from TVX/TNF is dependent on caspase 3 (Beggs et al 2014). Exposure to U0126 significantly decreased caspase 3 activity in TVX/TNF-treated cells at 12 hours (Figure 6C).

3.5. Timecourse of Double-strand DNA Breaks

Phosphorylated histone H2AX (γ H2AX), a sensitive marker of DNA double-strand breaks (Rogakou et al. 1998), was measured 6, 12, and 24 hours after treatment. In an earlier study, exposure of HepG2 cells for up to 24 hours to 20 μ M TVX alone caused no cytotoxicity as measured by enzyme release and trypan blue exclusion, whereas exposure to 20 μ M TVX plus 4 ng/mL TNF caused cytotoxicity within 20 hours (Beggs et al. 2014). This concentration of TVX is near that observed in the plasma of patients undergoing therapy (Teng et al. 1996), and the concentration of TNF is in the range of concentrations observed in people during inflammatory stress (Copeland et al. 2005; Taudorf et al. 2007). In the current study, cells were treated simultaneously with these concentrations of TVX and TNF or their vehicles. After 6 hours, none of the treatments had caused a change in the γ H2AX signal (Figure 7A). Treatment with vehicles or TNF did not alter γ H2AX levels at 12 or 24 hours either. In contrast, at 12 hours TVX/TNF treatment caused a nine-fold increase in γ H2AX compared to Veh/PBS-treated cells, and γ H2AX was increased further to fifteen-fold at 24 hours. TVX by itself caused a smaller increase in γ H2AX (4-fold) at 24 hours. Coexposure of cells to the pan-caspase inhibitor ZVAD prevented γ H2AX formation in cells treated TVX alone or in combination with TNF (Figure 7B).

3.6. ATR Activation

ATR is a kinase involved in sensing DNA damage and promoting cell cycle arrest. It is activated by phosphorylation of threonine 1989 (Nam et al. 2011). The activation of ATR was determined by immunofluorescent detection of phosphorylated ATR (p-ATR) in cell nuclei 6 hours after treatment. Cells treated with vehicle or TNF displayed minimal p-ATR foci in the nuclei (Figure 8). Treatment with either TVX or TVX/TNF caused a significant increase in nuclear p-ATR foci. Treatment with NU6027, an inhibitor of ATR (Peasland et al. 2011), significantly reduced the p-ATR detected in the nuclei of TVX- or TVX/TNF-treated cells (Figure 8B). The contribution of ERK to activation of ATR was also evaluated. Treatment with TVX alone increased pATM/ATR substrate signal at 6 hours (Figure 8C). Coexposure to U0126 significantly decreased the signal of pATM/ATR substrate in all groups compared to vehicle control-treated cells.

3.7. Pharmacological Inhibition of DNA Damage-Sensing Kinases

Along with ATR, both ATM and DNA-PK can detect DNA damage. Together, these three make up a family of PI3K-like kinases involved in detecting and responding to DNA damage (Houtgraaf et al. 2006; Yang et al. 2003). To investigate whether these three kinases contribute to TVX/TNF-induced cytotoxicity, a pharmacological inhibitor for each kinase was administered, and cell death was measured after 24 hours. Treatment of cells with NU6027 to inhibit ATR attenuated the TVX/TNF-induced cytotoxicity (Figure 9A). In contrast, treatment with either the ATM-selective inhibitor KU55933 (Hickson et al. 2004) or wortmannin to inhibit DNA-PK (Okayasu et al. 1998) failed to affect the cytotoxicity caused by TVX/TNF (Figure 9B and C).

4. Discussion

We reported previously that treatment of HepG2 cells *in vitro* with 20 μ M TVX, a concentration near that observed in the plasma of patients undergoing therapy (Teng et al. 1996), plus a physiologically relevant concentration of TNF (4 ng/mL) (Copeland et al. 2005; Taudorf et al. 2007) caused cell death that was dependent on caspases and prolonged activation of JNK (Beggs et al. 2014). In studies presented here, there were two distinct cellular outcomes of exposure to TVX in the presence of TNF: disruption of proliferation and cell death. The former appears to be driven largely by TVX, whereas the latter requires both TVX and TNF.

In a cell-free system, TVX inhibited eukaryotic topoisomerase-II α (Poulsen et al. 2014), which is involved in DNA replication and cell cycle regulation (Larsen et al. 1996). This can create a replication stress that initiates events involved in cell death and inhibition of proliferation. TVX decreased the rate of cell proliferation and caused cell cycle arrest (Figures 1 and 2), as has been reported for TVX treatment of several cell types *in vitro* (Holtom et al. 2000; Thadepalli et al. 2005; Zakeri et al. 2000). Interestingly, several other drugs that cause IDILI inhibit cell proliferation *in vitro* as well (Basta-Kaim et al. 2006; Chennamaneni et al. 2012; Francavilla et al. 1989; Rajabalian et al. 2009).

Key factors involved in halting progression through the cell cycle include p21, which inhibits cyclin-dependent kinases and favors cell cycle arrest, and p53, which enables expression of CDKN1A, the gene that encodes p21. There was no evidence of activation of p53 after any treatment, and inhibition of p53 did not affect cytotoxicity (Figure 3). Despite a lack of involvement of p53, CDKN1A gene expression was increased by TVX/TNF treatment, and treatment with TVX led to increased p21 protein (Figure 4). There are other examples in which cell cycle arrest and p21 upregulation resulting from replication stress are p53-independent (Jeong et al. 2010; Macleod et al. 1995). The upregulation of CDKN1A expression was also observed in an animal model of TVX/LPS-induced liver injury (Shaw et al. 2009b).

Although treatment with either TVX or TVX/TNF led to enhanced expression of CDKN1A mRNA in HepG2 cells, only treatment with TVX alone increased p21 protein. One explanation for this difference is that caspase 3 can cleave p21; such cleavage promotes apoptosis during DNA damage (Chai et al. 2000; Gartel and Tyner 2002; Zhang et al. 1999). We have reported that caspase 3 is activated 8 hours after cotreatment with TVX/TNF but not with TVX alone (Beggs et al 2014), and increases in p21 protein were observed 12 hours after treatment with TVX (Figure 5). It could be that activation of caspase 3 in TVX/TNF-cotreated cells led to cleavage of p21 in that treatment group.

The other outcome of treatment with TVX/TNF was cell death, which occurred only in the presence of both TVX and TNF and involved signaling through ERK. Treatment with TVX led to ERK activation by 6 hours that persisted through 24 hours (Figure 5, Supplemental Figure 1). Although ROS have been reported to activate the MEK/ERK signaling pathway (Cagnol and Chambard 2010; Lin et al. 2013), this was apparently not the case in TVX/TNF-treated cells, since ROS scavengers afforded no protection (Supplemental Figure 3). One alternative possibility is a replication stress-induced reduction in expression of MAPK phosphatases that reduce ERK translocation to the nucleus where it activates gene transcription (Masuda et al. 2010). Activation of the MEK/ERK pathway occurs in response to a multitude of genotoxic stressors and can play a role in altering the cell cycle and promoting apoptosis (Cagnol and Chambard 2010).

We have reported previously that cytotoxicity from TVX/TNF in HepG2 cells is caspase-dependent (Beggs et al 2014). Inhibition of ERK signaling reduced activation of caspase 3 and cytotoxicity (Figure 6C). ERK signaling can induce the intrinsic pathway of apoptosis and transcription of proapoptotic Bcl-2 family members Bax, Bak, and PUMA. This disrupts the mitochondrial membrane to facilitate cytochrome c release that triggers sequential activation of caspases 9 and 3 and ultimately apoptosis (Cagnol and Chambard 2010; Tamura et al. 2004). Caspase 9 is also activated in TVX/TNF-treated cells (Beggs et al 2014), lending support to this scenario.

Signaling from ERK led to caspase activation, which in turn led to induction of double-strand breaks in DNA (Figure 7A). In TVX/TNF-treated cells, DNA double-strand breaks occurred at 12 hours, a time before the onset of cell death, which occurs between 16 and 20 hours (Beggs et al. 2014). TVX alone also caused DNA double-strand break formation at 24 hours, but this was not associated with cell death. We had anticipated that formation of

γ H2AX would be mediated by ATR in response to replication stress from inhibition of topoisomerase II α by TVX (Ward and Chen 2001). Instead, formation of double-strand breaks in DNA was mediated by entirely by caspases (Figure 7B), which in turn were activated by ERK signaling (Figure 6C).

ERK was also involved in the activation of ATM/ATR (Figure 8C), which has been observed in various instances of genotoxic stress (Lin et al. 2013; Wei et al. 2011; Wu et al. 2006). Activation of ATR is associated with replication stress in the presence of stalled replication forks, which can occur when topoisomerases are inhibited (Pommier et al. 2010; Zeman and Cimprich 2014). Once activated, ATR contributed to cell death (Figure 9). Activation of ATR is often associated with protection of cells and the resolution of genotoxic stress (Myers et al. 2009); however, in certain circumstances ATR signaling can promote apoptosis (Joe et al. 2006; Roos and Kaina 2013; Sidi et al. 2008; Yim et al. 2006). The exact mechanism by which ATR promoted cytotoxicity in TVX/TNF-treated cells remains undetermined; however, caspase 3 does not appear to be involved because inhibition of ATR did not alter TVX/TNF-induced caspase 3 activity (Supplemental Figure 2). ATR is known to activate caspase 2, which can promote DNA damage and mediate apoptosis independently of caspase 3 (Dahal et al. 2007; Sidi et al. 2008).

Do these two cellular outcomes, i.e., inhibition of cell proliferation and cell death, represent separate, parallel pathways, or is there crosstalk between them? It is unlikely that ERK signaling contributes to impaired cell proliferation because ERK inhibition failed to modify the pronounced TVX- or TVX/TNF-induced decrease in the percentage of cells in S-phase (Figure 6). On the other hand, replication stress and cell cycle arrest can sensitize cells to the cytotoxic effects of TNF (Gera et al. 1993; Rodriguez et al. 2007; Shih and Stutman 1996). Furthermore, replicative repair is an essential determinant of severity of toxic responses: in the absence of replication and repair, liver injury from a wide variety of hepatotoxic agents is greater (Chanda and Mehendale 1996; Mehendale 2005).

TVX caused activation of ERK and ATR, and both of these contributed to cytotoxicity only when TNF was present. This suggests that another component of the TNF signaling pathway is required to evoke cell death through ATR and ERK. JNK can lead to apoptotic cell death (Cagnol and Chambard 2010; Win et al. 2011). Accordingly, this additional component might be JNK, since TVX/TNF coexposure caused a prolonged activation of JNK that contributed to cytotoxicity (Beggs et al. 2014). In cells treated with TVX/TNF, both JNK and ERK might be required to initiate apoptotic signaling. A working hypothesis based on the results reported in this study and from Beggs et al., 2014 is depicted in Figure 10.

Although these results indicate that a drug associated with human IDILI can cause replication stress that activates cell death signaling pathways, the relationship of these results to liver injury in vivo from exposure to TVX or other IDILI-associated drugs remains to be proven. It is of interest, however that several other drugs associated with human IDILI induce cell cycle arrest in vitro (Basta-Kaim et al. 2006; Chennamaneni et al. 2012; Francavilla et al. 1989; Rajabalian et al. 2009). Our results suggest that interference of DNA replication by certain drugs might act as a first insult that sensitizes cells to a secondary insult from cytokines such as TNF that are produced by an activated immune system.

Importantly, this confluence of events is required for cell death. Knowledge of the mechanism(s) involved in the interaction between the DNA damage response and death receptor ligand signaling might enhance understanding of IDILI pathogenesis and susceptibility factors in human patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Kazuhisa Miyakawa and Ryan Albee for technical assistance.

Funding: This work was supported by the National Institutes of Health grant number RO1DK061315. The National Institutes of Health had no involvement in the collection, analysis or interpretation of data, study design or writing of this report.

Abbreviations

ATR	ataxia telangiectasia and Rad3 related
CDC25	cell division cycle 25 phosphatase
Cdk	cyclin-dependent kinase
Chk1	checkpoint kinase 1
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
IDILI	idiosyncratic drug-induced liver injury
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
PBS	phosphate-buffered saline
pATM/ATR substrate	phospho-(Ser/Thr) ataxia telangiectasia mutated/ATM and Rad3 related substrate motif
PI	propidium iodide
ROS	reactive oxygen species
TNF	tumor necrosis factor-alpha
TVX	trovafloxacin.

References

Basta-Kaim A, Budziszewska B, Jagla G, Nowak W, Kubera M, Lason W. Inhibitory effect of antipsychotic drugs on the Con A- and LPS-induced proliferative activity of mouse splenocytes: a possible mechanism of action. *J Physiol Pharmacol.* 2006; 57:247–264. [PubMed: 16845229]

- Beggs KM, Fullerton AM, Miyakawa K, Ganey PE, Roth RA. Molecular Mechanisms of Hepatocellular Apoptosis Induced by Trovafloxacin-Tumor Necrosis Factor- α Interaction. *Toxicol Sci.* 2014; 137:91–101. [PubMed: 24097668]
- Brighty KE, Gootz TD. The chemistry and biological profile of trovafloxacin. *J Antimicrob Chemother.* 1997; 39(Suppl B):1–14. [PubMed: 9222064]
- Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. *FEBS J.* 2010; 277:2–21. [PubMed: 19843174]
- Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E. Multiple roles of the cell cycle inhibitor p21(CDKN1A) in the DNA damage response. *Mutat Res.* 2010; 704:12–20. [PubMed: 20096807]
- Chai F, Evdokiou A, Young GP, Zalewski PD. Involvement of p21(Waf1/Cip1) and its cleavage by DEVD-caspase during apoptosis of colorectal cancer cells induced by butyrate. *Carcinogenesis.* 2000; 21:7–14. [PubMed: 10607727]
- Chanda S, Mehendale HM. Nutritional modulation of the final outcome of hepatotoxic injury by energy substrates: an hypothesis for the mechanism. *Med Hypotheses.* 1996; 46:261–268. [PubMed: 8676764]
- Chennamaneni S, Zhong B, Lama R, Su B. COX inhibitors Indomethacin and Sulindac derivatives as antiproliferative agents: synthesis, biological evaluation, and mechanism investigation. *Eur J Med Chem.* 2012; 56:17–29. [PubMed: 22940705]
- Copeland S, Warren HS, Lowry SF, Calvano SE, Remick D. Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol.* 2005; 12:60–67. [PubMed: 15642986]
- Cosgrove BD, King BM, Hasan MA, Alexopoulos LG, Farazi PA, Hendriks BS, Griffith LG, Sorger PK, Tidor B, Xu JJ, Lauffenburger DA. Synergistic drug-cytokine induction of hepatocellular death as an in vitro approach for the study of inflammation-associated idiosyncratic drug hepatotoxicity. *Toxicol Appl Pharmacol.* 2009; 237:317–330. [PubMed: 19362101]
- Dahal GR, Karki P, Thapa A, Shahnawaz M, Shin SY, Lee JS, Cho B, Park IS. Caspase-2 cleaves DNA fragmentation factor (DFF45)/inhibitor of caspase-activated DNase (ICAD). *Arch Biochem Biophys.* 2007; 468:134–139. [PubMed: 17945178]
- Damrot J, Helbig L, Roos WP, Barrantes SQ, Kaina B, Fritz G. DNA replication arrest in response to genotoxic stress provokes early activation of stressactivated protein kinases (SAPK/JNK). *J Mol Biol.* 2009; 385:1409–1421. [PubMed: 19109974]
- Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem.* 1998; 273:18623–18632. [PubMed: 9660836]
- Francavilla A, Panella C, Polimeno L, Di Leo A, Makowka L, Barone M, Amoruso A, Ingrosso M, Starzl TE. Effect of cimetidine, ranitidine, famotidine and omeprazole on hepatocyte proliferation in vitro. *J Hepatol.* 1989; 8:32–41. [PubMed: 2564010]
- Fredriksson L, Herpers B, Benedetti G, Matadin Q, Puigvert JC, de Bont H, Dragovic S, Vermeulen NP, Commandeur JN, Danen E, de Graauw M, van de Water B. Diclofenac inhibits tumor necrosis factor- α -induced nuclear factor- κ B activation causing synergistic hepatocyte apoptosis. *Hepatology.* 2011; 53:2027–2041. [PubMed: 21433042]
- Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther.* 2002; 1:639–649. [PubMed: 12479224]
- Gera JF, Fady C, Gardner A, Jacoby FJ, Briskin KB, Lichtenstein A. Inhibition of DNA repair with aphidicolin enhances sensitivity of targets to tumor necrosis factor. *J Immunol.* 1993; 151:3746–3757. [PubMed: 8376804]
- Gootz TD, Zaniewski R, Haskell S, Schmieder B, Tankovic J, Girard D, Courvalin P, Polzer RJ. Activity of the new fluoroquinolone trovafloxacin (CP-99,219) against DNA gyrase and topoisomerase IV mutants of *Streptococcus pneumoniae* selected in vitro. *Antimicrob Agents Chemother.* 1996; 40:2691–2697. [PubMed: 9124824]
- Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ, Smith GC. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res.* 2004; 64:9152–9159. [PubMed: 15604286]

- Holtom PD, Pavkovic SA, Bravos PD, Patzakis MJ, Shepherd LE, Frenkel B. Inhibitory effects of the quinolone antibiotics trovafloxacin, ciprofloxacin, and levofloxacin on osteoblastic cells in vitro. *J Orthop Res*. 2000; 18:721–727. [PubMed: 11117292]
- Hosono S, Lee CS, Chou MJ, Yang CS, Shih CH. Molecular analysis of the p53 alleles in primary hepatocellular carcinomas and cell lines. *Oncogene*. 1991; 6:237–243. [PubMed: 1847999]
- Houtgraaf JH, Versmissen J, van der Giessen WJ. A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc Revasc Med*. 2006; 7:165–172. [PubMed: 16945824]
- Hussaini SH, Farrington EA. Idiosyncratic drug-induced liver injury: an overview. *Expert Opin Drug Saf*. 2007; 6:673–684. [PubMed: 17967156]
- Jeong JH, Kang SS, Park KK, Chang HW, Magae J, Chang YC. p53-independent induction of G1 arrest and p21WAF1/CIP1 expression by ascofuranone, an isoprenoid antibiotic, through downregulation of c-Myc. *Mol Cancer Ther*. 2010; 9:2102–2113. [PubMed: 20587660]
- Joe Y, Jeong JH, Yang S, Kang H, Motoyama N, Pandolfi PP, Chung JH, Kim MK. ATR, PML, and CHK2 play a role in arsenic trioxide-induced apoptosis. *J Biol Chem*. 2006; 281:28764–28771. [PubMed: 16891316]
- Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, Gudkov AV. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science*. 1999; 285:1733–1737. [PubMed: 10481009]
- Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. *Oncogene*. 1999; 18:7644–7655. [PubMed: 10618704]
- Larsen AK, Skladanowski A, Bojanowski K. The roles of DNA topoisomerase II during the cell cycle. *Prog Cell Cycle Res*. 1996; 2:229–239. [PubMed: 9552399]
- Lin X, Yan J, Tang D. ERK kinases modulate the activation of PI3 kinase related kinases (PIKKs) in DNA damage response. *Histol Histopathol*. 2013; 28:1547–1554. [PubMed: 23832672]
- Llopis A, Salvador N, Ercilla A, Guaita-Esteruelas S, Barrantes Idel B, Gupta J, Gaestel M, Davis RJ, Nebreda AR, Agell N. The stress-activated protein kinases p38alpha/beta and JNK1/2 cooperate with Chk1 to inhibit mitotic entry upon DNA replication arrest. *Cell Cycle*. 2012; 11:3627–3637. [PubMed: 22935704]
- Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev*. 1995; 9:935–944. [PubMed: 7774811]
- Masuda K, Katagiri C, Nomura M, Sato M, Kakumoto K, Akagi T, Kikuchi K, Tanuma N, Shima H. MKP-7, a JNK phosphatase, blocks ERK-dependent gene activation by anchoring phosphorylated ERK in the cytoplasm. *Biochem Biophys Res Commun*. 2010; 393:201–206. [PubMed: 20122898]
- Mehendale HM. Tissue repair: an important determinant of final outcome of toxicant-induced injury. *Toxicol Pathol*. 2005; 33:41–51. [PubMed: 15805055]
- Myers K, Gagou ME, Zuazua-Villar P, Rodriguez R, Meuth M. ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. *PLoS Genet*. 2009; 5:e1000324. [PubMed: 19119425]
- Nam EA, Zhao R, Glick GG, Bansbach CE, Friedman DB, Cortez D. Thr-1989 phosphorylation is a marker of active ataxia telangiectasia-mutated and Rad3- related (ATR) kinase. *J Biol Chem*. 2011; 286:28707–28714. [PubMed: 21705319]
- Nightingale SL. From the Food and Drug Administration. *JAMA*. 1999; 282:19. [PubMed: 10404896]
- Okayasu R, Suetomi K, Ullrich RL. Wortmannin inhibits repair of DNA double-strand breaks in irradiated normal human cells. *Radiat Res*. 1998; 149:440–445. [PubMed: 9588354]
- Peasland A, Wang LZ, Rowling E, Kyle S, Chen T, Hopkins A, Cliby WA, Sarkaria J, Beale G, Edmondson RJ, Curtin NJ. Identification and evaluation of a potent novel ATR inhibitor, NU6027, in breast and ovarian cancer cell lines. *Br J Cancer*. 2011; 105:372–381. [PubMed: 21730979]
- Pommier Y, Leo E, Zhang H, Marchand C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem Biol*. 2010; 17:421–433. [PubMed: 20534341]
- Poulsen KL, Olivero-Verbel J, Beggs KM, Ganey PE, Roth RA. Trovafloxacin enhances lipopolysaccharide-stimulated production of tumor necrosis factor-alpha by macrophages: role of the DNA damage response. *J Pharmacol Exp Ther*. 2014; 350:164–170. [PubMed: 24817034]

- Rajabalian S, Meimandi MS, Badinloo M. Diclofenac inhibits proliferation but not NGF-induced differentiation of PC12 cells. *Pak J Pharm Sci.* 2009; 22:259–262. [PubMed: 19553170]
- Rodriguez R, Campa VM, Riera J, Carcedo MT, Ucker DS, Ramos S, Lazo PS. TNF triggers mitogenic signals in NIH 3T3 cells but induces apoptosis when the cell cycle is blocked. *Eur Cytokine Netw.* 2007; 18:172–180. [PubMed: 17993451]
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998; 273:5858–5868. [PubMed: 9488723]
- Roos WP, Kaina B. DNA damage-induced cell death by apoptosis. *Trends Mol Med.* 2006; 12:440–450. [PubMed: 16899408]
- Roos WP, Kaina B. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett.* 2013; 332:237–248. [PubMed: 22261329]
- Roth RA, Ganey PE. Animal models of idiosyncratic drug-induced liver injury--current status. *Crit Rev Toxicol.* 2011; 41:723–739. [PubMed: 21726137]
- Roth RA, Luyendyk JP, Maddox JF, Ganey PE. Inflammation and drug idiosyncrasy--is there a connection? *J Pharmacol Exp Ther.* 2003; 307:1–8. [PubMed: 12954806]
- Seok JH, Park KA, Byun HS, Won M, Shin S, Choi BL, Lee H, Kim YR, Hong JH, Park J, Hur GM. Long-term Activation of c-Jun N-terminal Kinase through Receptor Interacting Protein is Associated with DNA Damage-induced Cell Death. *Korean J Physiol Pharmacol.* 2008; 12:185–191. [PubMed: 19967054]
- Shaw PJ, Beggs KM, Sparkenbaugh EM, Dugan CM, Ganey PE, Roth RA. Trovafloxacin enhances TNF-induced inflammatory stress and cell death signaling and reduces TNF clearance in a murine model of idiosyncratic hepatotoxicity. *Toxicol Sci.* 2009a; 111:288–301. [PubMed: 19638433]
- Shaw PJ, Ditewig AC, Waring JF, Liguori MJ, Blomme EA, Ganey PE, Roth RA. Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. *Toxicol Sci.* 2009b; 107:270–280. [PubMed: 18930950]
- Shaw PJ, Hopfensperger MJ, Ganey PE, Roth RA. Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. *Toxicol Sci.* 2007; 100:259–266. [PubMed: 17709330]
- Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell.* 1997; 91:325–334. [PubMed: 9363941]
- Shih SC, Stutman O. Cell cycle-dependent tumor necrosis factor apoptosis. *Cancer Res.* 1996; 56:1591–1598. [PubMed: 8603407]
- Sidi S, Sanda T, Kennedy RD, Hagen AT, Jette CA, Hoffmans R, Pascual J, Imamura S, Kishi S, Amatruda JF, Kanki JP, Green DR, D'Andrea AA, Look AT. Chk1 suppresses a caspase-2 apoptotic response to DNA damage that bypasses p53, Bcl-2, and caspase-3. *Cell.* 2008; 133:864–877. [PubMed: 18510930]
- Tamura Y, Simizu S, Osada H. The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. *FEBS Lett.* 2004; 569:249–255. [PubMed: 15225643]
- Taudorf S, Krabbe KS, Berg RM, Pedersen BK, Moller K. Human models of low-grade inflammation: bolus versus continuous infusion of endotoxin. *Clin Vaccine Immunol.* 2007; 14:250–255. [PubMed: 17267590]
- Teng R, Liston TE, Harris SC. Multiple-dose pharmacokinetics and safety of trovafloxacin in healthy volunteers. *J Antimicrob Chemother.* 1996; 37:955–963. [PubMed: 8737145]
- Thadepalli H, Salem F, Chuah SK, Gollapudi S. Antitumor activity of trovafloxacin in an animal model. *In Vivo.* 2005; 19:269–276. [PubMed: 15796186]
- Tukov FF, Luyendyk JP, Ganey PE, Roth RA. The role of tumor necrosis factor alpha in lipopolysaccharide/ranitidine-induced inflammatory liver injury. *Toxicol Sci.* 2007; 100:267–280. [PubMed: 17698507]
- Ward IM, Chen J. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem.* 2001; 276:47759–47762. [PubMed: 11673449]

- Wei F, Xie Y, He L, Tao L, Tang D. ERK1 and ERK2 kinases activate hydroxyurea-induced S-phase checkpoint in MCF7 cells by mediating ATR activation. *Cell Signal*. 2011; 23:259–268. [PubMed: 20840867]
- Win S, Than TA, Han D, Petrovic LM, Kaplowitz N. c-Jun N-terminal kinase (JNK)-dependent acute liver injury from acetaminophen or tumor necrosis factor (TNF) requires mitochondrial Sab protein expression in mice. *J Biol Chem*. 2011; 286:35071–35078. [PubMed: 21844199]
- Wu D, Chen B, Parihar K, He L, Fan C, Zhang J, Liu L, Gillis A, Bruce A, Kapoor A, Tang D. ERK activity facilitates activation of the S-phase DNA damage checkpoint by modulating ATR function. *Oncogene*. 2006; 25:1153–1164. [PubMed: 16186792]
- Yang J, Yu Y, Hamrick HE, Duerksen-Hughes PJ. ATM, ATR and DNA-PK: initiators of the cellular genotoxic stress responses. *Carcinogenesis*. 2003; 24:1571–1580. [PubMed: 12919958]
- Yim H, Hwang IS, Choi JS, Chun KH, Jin YH, Ham YM, Lee KY, Lee SK. Cleavage of Cdc6 by caspase-3 promotes ATM/ATR kinase-mediated apoptosis of HeLa cells. *J Cell Biol*. 2006; 174:77–88. [PubMed: 16801388]
- Zakeri SM, Meyer H, Meinhardt G, Reinisch W, Schrattbauer K, Knoefler M, Block LH. Effects of trovafloxacin on the IL-1-dependent activation of E-selectin in human endothelial cells in vitro. *Immunopharmacology*. 2000; 48:27–34. [PubMed: 10822086]
- Zeman MK, Cimprich KA. Causes and consequences of replication stress. *Nat Cell Biol*. 2014; 16:2–9. [PubMed: 24366029]
- Zhang Y, Fujita N, Tsuruo T. Caspase-mediated cleavage of p21Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. *Oncogene*. 1999; 18:1131–1138. [PubMed: 10022118]
- Zou W, Beggs KM, Sparkenbaugh EM, Jones AD, Younis HS, Roth RA, Ganey PE. Sulindac metabolism and synergy with tumor necrosis factor-alpha in a drug-inflammation interaction model of idiosyncratic liver injury. *J Pharmacol Exp Ther*. 2009; 331:114–121. [PubMed: 19638570]

Figure 1. TVX causes cell cycle arrest

Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS. After 12 hours, cell cycle distribution was assessed using flow cytometry. (A) Representative histograms for each treatment. (B) Percentage of the total cells gated in the various stages of the cell cycle. **a** Significantly different from Veh/PBS-treated group within same phase of cell cycle. **b** Significantly different from Veh/TNF-treated group within same phase of cell cycle. Data represent the mean $\pm$ SEM of 5 separate experiments. 100,000 cells were sampled from each experiment.

Figure 2. TVX decreases HepG2 cell proliferation

(A) Cells were treated with 20 μ M TVX or its vehicle and counted 0, 24 and 48 hours later as described in Methods. Data represent the mean $\pm$ SEM of 3 separate experiments and are expressed as number of cells per mL of culture medium. **a** Significantly different from TVX-treated group at same time. (B) Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS. Fluorescence of the DNA probe was measured 0, 24 and 48 hours after treatment as described in Methods. Data represent the mean $\pm$ SEM of 4 separate experiments and are expressed as fluorescence relative to fluorescence in the Veh/PBS group at each time. **b** Significantly different from same treatment at 0 hour. **c** Significantly different from same treatment at 24 hours. **d** Significantly different from Veh/PBS-treated group at the same time. **e** Significantly different from all other treatment groups at same time. Data represent the mean $\pm$ SEM of 4 separate experiments.

Figure 3. p53 expression is unchanged and does not play a role in TVX/TNF-induced cytotoxicity

Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS. After 4 or 6 hours of exposure, mRNA was collected, and after 6 or 12 hours of treatment protein was isolated as described in Materials and Methods. TP53 mRNA expression was determined by qRT-PCR and p53 protein expression by western analysis. For p53 protein, densitometry was performed on p53 and Lamin bands, and the ratio of p53 to Lamin is presented. (A) TP53 mRNA expression relative to ACTB mRNA at 4 and 6 hours. Data represent the mean $\pm$ SEM of 4 separate experiments. (B) Representative blots of p53 and Lamin 6 and 12 hours after treatment, and quantification of p53 protein expression. Data represent the mean $\pm$ SEM of 4-6 separate experiments. (C) Cells were treated simultaneously with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS in the presence of 25 μ M PFT or its vehicle.

Cytotoxicity was measured after 24 hours. **a** Significantly different from all other treatment groups in the absence of PFT. **b** Significantly different from all other treatment groups in the presence of PFT. Data represent the mean $\pm$ SEM of 4 separate experiments.

Figure 4. TVX treatment led to upregulation of p21

Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS. After 6 hours of treatment mRNA was collected, and after 12 hours of treatment protein was isolated as described in Materials and Methods. CDKN1A mRNA expression was determined by qRT-PCR, and p21 protein expression by western analysis. For p21 protein, densitometry was performed on p21 and Lamin bands, and the ratio of p21 to Lamin is represented. (A) CDKN1A mRNA expression relative to ACTB mRNA. **a** Significantly different from respective group in the absence of TVX. Data represent the mean $\pm$ SEM of 4 separate

experiments. (B) Representative blots of p21 and Lamin 12 hours after treatment, and quantification of p21 protein expression. **b** Significantly different from Veh/PBS-treated group. Data represent the mean $\pm$ SEM of 3 separate experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5. TVX exposure activates ERK

Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS in the presence of 10 μ M of the MEK-1/2 inhibitor U0126 or its vehicle. Phosphorylation of ERK was determined by western analysis at 6 hours. Representative blots are shown. Densitometry was performed for cells not treated with U0126, and the ratio of p-ERK to Lamin is shown. **a** Significantly different from respective group in the absence of TVX. Data represent the mean $\pm$ SEM of 4 separate experiments.

Figure 6. ERK contributes to TVX/TNF-induced cytotoxicity but does not mediate TVX-induced cell cycle arrest

Cells were treated simultaneously with 20 μ M TVX or vehicle and with 4 ng/mL TNF or PBS in the presence of 10 μ M U0126 or its vehicle. After 12 hours of treatment, cell cycle distribution was analyzed by flow cytometry and caspase 3 activity was measured. Cytotoxicity was assessed 24 hours after treatment. (A) Percent distribution of cells gated in the G0/G1 and S phases of the cell cycle. **a** Significantly different from respective Veh/PBS-treated group. **b** Significantly different from respective Veh/TNF-treated group. **c** Significantly different from same treatment without U0126. Data represent the mean $\pm$ SEM of 3 separate experiments. 100,000 cells were counted for each sample. (B) Cytotoxicity 24 hours after treatment. **d** Significantly different from respective groups treated with TVX alone or TNF alone. **e** Significantly different from TVX/TNF-treated group in the absence of U0126. (C) Caspase 3 activity 12 hours after treatment. **d** Significantly different from respective groups treated with TVX alone or TNF alone. **e** Significantly different from TVX/TNF-treated group in the absence of U0126. Data represent the mean $\pm$ SEM of 3-4 separate experiments.

Figure 7. TVX-induced DNA double-strand breaks are mediated by caspases and exacerbated by TNF

Cells were treated simultaneously with 20 μ M TVX or vehicle and with 4 ng/mL TNF or PBS for 6, 12 or 24 hours. (A) Phosphorylation of H2AX was determined by western analysis. Representative blots are shown. Densitometry was performed on phospho-H2AX (γ H2AX) and Lamin bands, and the ratio of γ H2AX to Lamin for each treatment group relative to the ratio for Veh/PBS treatment is shown for each time. **a** Significantly different from all other treatment groups at the same time. **b** Significantly different from Veh/PBS

treatment group at the same time. (B) Cells were treated similarly with TVX and TNF in the presence of 40 μ M ZVAD or 0.2% DMSO vehicle control for 24 hours. Densitometry was performed on γ H2AX and α -Tubulin bands, and the ratio of γ H2AX to α -Tubulin for each treatment group is shown. **c** Significantly different from Veh/PBS-treated group in the absence of ZVAD. **d** Significantly different from TVX/PBS-treated group in the absence of ZVAD. **e** Significantly different from respective treatment in the absence of ZVAD. Data represent the mean $\pm$ SEM of 3–6 separate experiments.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8. TVX treatment causes ATR activation

Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS in the presence of 12.5 μ M of the ATR inhibitor NU6027 or its vehicle. 6 hours after treatment, cells were subjected to western analysis for pATM/ATR substrate and immunolabeling for phospho-ATR (p-ATR) as described in Materials and Methods, and imaged using fluorescence microscopy. (A) Representative images from each treatment without NU6027. Gray areas represent a positive DAPI signal, indicating cellular nuclei. Black areas represent colocalized PI and DAPI signals, indicating p-ATR positive signal located in nuclei of cells.

Insets in TVX/PBS- and TVX/TNF-treated groups depict punctate p-ATR foci within individual nuclei. (B) Quantification of the percentage of nuclei colocalizing with p-ATR foci in response to treatment. **a** Significantly different from respective group without TVX. **b** Significantly different from same treatment without NU6027. (C) Representative blots of pATM/ATR substrate and α -tubulin in the presence of U0126 or its vehicle, and quantification of protein expression 6 hours after treatment. **a** Significantly different from respective group without TVX. **c** Significantly different from the same treatment without U0126. Data represent the mean $\pm$ SEM of 3- 4 separate experiments.

Figure 9. An inhibitor of ATR, but not of ATM or DNA-PK, attenuates TVX/TNF-induced cytotoxicity

Cells were treated with 20 μ M TVX or Veh and with 4 ng/mL TNF or PBS in the presence of (A) 12.5 μ M NU6027, (B) 20 μ M KU55933, and (C) 10 μ M wortmannin, or their respective vehicles. Cytotoxicity was assessed after 24 hours. **a** Significantly different from all other treatment groups in the absence of an inhibitor. **b** Significantly different from all other treatment groups in the presence of an inhibitor. **c** Significantly different from same treatment without inhibitor. Data represent the mean $\pm$ SEM of 3-7 separate experiments.

Exposure of hepatocytes to TVX causes inhibition of topoisomerase-II α . Polymerases such as DNA polymerase synthesizing DNA can collide with inhibited topoisomerase-II α on the DNA, resulting in DNA replication stress. Hepatocytes respond with upregulation of p21 and consequent inhibition of Cdk, as well as activation of ATR. Both of these prevent cell proliferation, which can reduce tissue repair and thereby exacerbate liver injury (dashed line). DNA replication stress also activates the JNK and ERK MAP kinases. Upon exposure only to TNF, JNK is activated early and transiently. However, in hepatocytes exposed to both TVX and TNF an enhanced activation of JNK occurs early, and JNK remains activated

for a prolonged period. The combined action of prolonged ERK and JNK signaling promotes mitochondrial dysfunction, resulting in the activation of caspase 9 and caspase 3. Activation of these caspases mediates the death of hepatocytes from TVX/TNF-coexposure. ATR also contributes to TVX/TNF-mediated cell death by a mechanism that is currently not understood but is independent of caspase 3. (See Discussion and Beggs et al. 2014 for supporting data.)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript