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Crizotinib and Ceritinib Induce Apoptosis and Necrosis in Primary Rat Hepatocytes with Distinct Capacity

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Abstract

Drug development makes up a major portion of biomedical engineering research interests. The FDA oversees the introduction, experimentation, and implementation of all drugs before market approval is granted. Even after market approval is granted, the FDA continues to monitor the safety of all drugs. Crizotinib and ceritinib are two anaplastic lymphoma kinase (ALK) inhibitors recently approved by the FDA. Both drugs are indicated for treatment of non-small cell lung cancers (NSCLC) with abnormal ALK gene, and they are approved with a companion diagnostic test that determines ALK abnormality. Clinical trial data suggest that crizotinib and ceritinib can cause liver injury, and this information has been included in the "Warnings and Precautions" section of their labeling. The mechanism of ALK inhibitor induced hepatotoxicity is unknown. This study aimed to observe if crizotinib and ceritinib are directly toxic to liver cells. Primary cultured rat hepatocytes were treated with crizotinib and ceritinib at clinically relevant concentrations for 4, 8 and 24 h, and apoptosis and necrosis were measured. A ~125% to ~150% increase in caspase 3/7 activity was observed at 8 h for ceritinib treated hepatocytes, and significant necrosis (~40%) occurred at 24 h. Ceritinib treated hepatocytes also showed remarkable cytochrome c release at 4 h, the time point when no cell death was detectable. Crizotinib showed no toxicity at 10-fold the maximal blood concentration (Cmax), while ceritinib became toxic at 3-fold Cmax and caused ~40% cell death at 6-fold Cmax, indicating that ceritinib, the second-generation ALK inhibitor, is significantly more toxic than the first-generation drug crizotinib. These data provide novel insights into the mechanisms of ALK inhibitors associated hepatotoxicity.

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Introduction

Kinases play a major role in the facilitation of many cell processes in the human body. Kinases act to phosphorylate certain proteins in the body, which in turn progresses a signal transduction pathway, with the end result being the activation of a certain cellular process.[1] There are 500 unique kinases, 150 of which are involved in the onset or progression of human disease including cancer. While there is a variety of protein kinases in the human body, the target of much drug development research to date is tyrosine kinase. Tyrosine kinase, much like any other form of kinase, catalyze the phosphorylation of tyrosine residues by transferring a phosphate group from ATP. Tyrosine kinase is known to play a major role in biological processes like cell proliferation, differentiation, and survival, as well as disease development including diabetes and cancer.[2] Due to its role in the development and progression of some forms of cancer, tyrosine kinase is a protein of interest in the cancer therapy drug industry. Since the discovery of tyrosine kinase's role in cancer progression, many drugs have gained FDA approval for the treatment of cancer through tyrosine kinase inhibition. There are a variety of FDA approved tyrosine kinase inhibitors, a novel cancer therapy drug class, to date that treat a spectrum of cancer types. The FDA approved the first tyrosine kinase inhibitor imatinib (Gleevec) in May of 2001. Since imatinib's approval, up to the date of this research, the FDA has approved and/or investigated 23 tyrosine kinase inhibitors (FDA.gov). Many of these approved tyrosine kinase inhibitors are still of great interest to the FDA due to their unknown nature when concerning off-target toxicity. Most tyrosine kinase inhibitors work through competitive inhibition of ATP, resulting in the loss of tyrosine kinase's phosphorylation abilities, and halting the pathway in which the tyrosine kinase lies.[3] Since a major function of tyrosine kinase is cellular proliferation, a root cause of tumor formation, it is clear to see why the inhibition of the protein may aid in cancer therapy. However, due to the broad spectrum nature of the drug, the concerns of off-target toxicity of these drugs is still present. 16 out of the 23 approved tyrosine kinase inhibitors have warnings for hepatotoxicity. Five of the 16 warnings are black box warnings, the strongest FDA safety warning (FDA.gov). Since there is a clear concern for hepatotoxicity of these drugs, the FDA and affiliates are still investigating the severity of the toxicity.

Crizotinib (trade name Xalkori) is the first generation anaplastic lymphoma kinase (ALK)-positive non-small cell lung cancer drug. Crizotinib is a kinase inhibitor that blocks the ATP binding site of ALK, halting the progression of cancer.[4] ALK-Positive Non-Small Cell Lung Cancer tumors are highly dependent of ALK, and as a consequence, are very sensitive to ALK inhibition. This ALK inhibition is known to lead to an increase in progression-free survival of cancer patients.[5] One shortcoming of the drug is its labels warning of hepatotoxicity, claiming 0.2% of patients developed fatal hepatotoxicity following drug administration.[6] The label suggests dose reduction, temporary suspension, or permanent drug discontinuation if liver tests indicate drug related hepatotoxicity. Continuous administration of crizotinib can result in develop resistance of the drug. Mutations, including changes in the solvent exposed region of the ATP-binding pocket or amplification of the ALK fusion gene, lead to inefficacy of the drug; for this reason, a seconded generation ALK-Positive Non-Small Cell Lung Cancer drug was formed. Ceritinib (trade name Zykadia) acts in a similar way as crizotinib to suppress the progression of ALK-Positive Non-Small Cell Lung Cancer.[7] Ceritinib's label also warns of hepatotoxicity.[8]

Drug development constitutes a large portion of biomedical engineering related research. One very important step in the drug development process is checking for efficacy and safety of the drug. The FDA closely monitors developing drugs before market approval can be granted. There are many reasons the FDA will deny approval of a drug, including adverse side effects. Drug induced side effects come in many

forms, some of which can be very serious. A major side effect seen in a variety of drugs is hepatotoxicity. The liver plays a major role in the metabolism of drugs, and therefore is susceptible to drug-induced damage. Many drug types can take a toll on the liver, resulting in drug-induced liver injury (DILI).[9] DILI is the leading cause of acute liver failure and one of the most common reasons for drug removal from the market.[10] The liver has an impressive ability to regenerate upon cell loss, however, many drugs can induce cell death at a rate in which the regenerative properties of hepatocytes cannot keep up. When concerning cell death, it is important to differentiate the two different forms of cell death; apoptosis and necrosis. Apoptosis, commonly referred to as programmed cell death, is a pathway mediated form of cell death in which, for the most part, unwanted or unneeded cells are shed to free up space for new cells. Apoptosis is essential to healthy tissue formation and upkeep in the human body. In contrast, necrotic cell death is non-programmed and accidental in nature which can lead to necrosis.[11] While it may seem that necrotic cell death is the only harmful form of cell death in the body, over activation of apoptosis inducing molecules can lead to unwanted cell death as well. When testing for the mechanism behind DILI, it is important to test for both necrosis and apoptosis induction.

While there is cause for concern for hepatotoxicity with a large portion of tyrosine kinase inhibitors, this research primary focuses on first and second generation ALK-Positive Non-Small Cell Lung Cancer drugs crizotinib and ceritinib. The hepatotoxicity of supplements and drugs can be gaged using a number of assays and accompanying mathematical formulas. Crizotinib and ceritinib are known to induce some form of hepatotoxicity, however, the mechanism in which it induces this is unclear. In this research, primary rat hepatocytes were incubated in varying dosages of the drugs and three assays/protocols were used to gain insight on their role in apoptosis and necrosis; Cytochrome C release for apoptosis, Caspase 3/7 production for apoptosis, and LDH release for necrosis.

Cytochrome C is a key protein in the apoptosis cascade. Apoptosis is characterized by cell shrinkage, membrane blebbing, and DNA fragmentation. Release of cytochrome c from the mitochondrial inner membrane space to the cytosol is a known indicator of apoptosis.[12] Using western blot, it is possible to test for cytochrome c release from the hepatocyte's mitochondrial inner membrane space. Downstream from cytochrome c are a family of Cysteine-dependent proteases (termed caspases) that also participate in the apoptosis cascade. Caspases act to cleave substrates, propagating the cell death signal until apoptosis is initiated. There are many different forms of caspase, however, executioner caspase 3 and 7 are located near the end of the apoptosis cascade; Increase in their activity is a strong indicator of apoptosis.[13] In this study, cytochrome c detection with western blot and caspase 3/7 assay were used to measure apoptosis of hepatocytes treated with crizotinib and ceritinib.

The nonprogrammer form of cell death, necrosis, is usually caused by a series of unwanted chemicals or conditions that lead to cell swelling and eventually membrane rupturing.[14] Necrosis is rarely a wanted result of any biological process and due to its fast spreading nature can lead to large scale tissue damage. When concerning hepatotoxicity, necrosis can be very dangerous in nature. Cytotoxicity and necrosis alike are commonly associated with damage to the cellular membrane. Lactate dehydrogenase (LDH)-release assays are common place in the toxicology field as they are accurate when gaging a drug's cytotoxicity level.[15] Necrosis levels can be estimated by measuring the amount of LDH release caused by the drugs and comparing that to the total LDH in the cell. In this study, LDH-release assay was used to measure cell viability loss and necrosis in primary rat hepatocytes treated with crizotinib and ceritinib.

In theory, results indicating crizotinib and ceritinib induced apoptosis and necrosis in primary hepatocytes at relevant levels would back up the claim that these drugs do in fact have hepatotoxic properties.

Materials and Methods

Hepatocyte Isolation

Primary rat hepatocytes were isolated following the NCTR Center for Hepatotoxicology SOP, "Isolation of Primary Rodent Hepatocytes for Cell Culture Using Collagenase Perfusion" (Version 02/14/2011). The use of rats for hepatocyte isolation was approved by the NCTR Institutional Animal Care and Use Committee. The cells were cultured as monolayers for 18 h before drug treatment. All experiments were completed within 48 h after isolation to ensure that hepatocytes maintain similar functions as under in vivo conditions.

Cytochrome C Release - Western Blot

Primary rat hepatocytes were cultured in 6 well plates for 18 h before drug testing began. Following the 18 h culture, culture medium was discarded and cells were carefully washed with PBS twice. The medium was replaced with a drug and culture medium mixture. Cells subjected to crizotinib treatment were cultured in medium containing 10, 15, 20, 25, and 30 µM concentrations of the drug. One well was cultured in DMSO as a control (0 µM crizotinib). Cells subjected to ceritinib treatment were cultured in medium containing 4, 6, 8, 10, and 12 µM concentrations of the drug. One well in this test group was also cultured in medium containing DMSO as a control (0 µM ceritinib). Hepatocytes in both drug test groups were cultured for 4, 8, and 24 h. Following incubation, the drug containing medium was discarded and the cells were washed thoroughly with PBS twice (3 ml/ 1 min each time). 200 µl/well 0.002% digitonin was added to the 6 well plates. Each plate was rocked gently in order to ensure even distribution of the digitonin solution. After 5 min, the solution was extracted from each well and placed in individual 0.5 ml centrifuge tubes. The samples were labeled and centrifuged at 1000 rpm for 3 min to remove floating cells. 150 µl of each of the remaining samples was transferred to a new 0.5 ml tube and mixed with 50 µl 4x reduced loading buffer. The result of the sample preparation protocol was a set of 36 samples; one sample for each drug concentration at each time point. 10 µl samples were loaded into gels and ran for about 1 h. A variety of sample sizes and gel sizes were tested during the experimentation process, however, it was concluded that the 4-20% precast polyacrylamide, 15 well, 15 µl gels resulted in the best outcome. Following protein separation by gel electrophoresis, gel to membrane transfer was performed. The transfer process was performed using a Bio-Rad Trans-Blot Turbo transfer system with recommended settings for Mini-PROTEAN TGX gels. Membranes were rinsed in TBS. Blotting-Grade Blocker #1706404 in TBS was added to a tray in which the membranes were placed. The blocking process was completed on a rocker for 1 h at room temperature. The membranes were rinsed 3 times in TBS following blocking to remove any residual blocking agent. In order to observe cytochrome c, membrane integrity WB antibody cocktail (ab110414) was used as the primary antibody. The primary antibody was added to TBS and poured into the tray containing the membrane. The membranes were incubated with the primary antibody overnight (approximately 18 h) on a rocker in the fridge. Membranes were rinsed with TBS to remove residual primary antibodies. The secondary antibody (Anti-Mouse CY5) was added to TBS and poured into the tray containing the membrane. The

membranes were incubated with the secondary antibody on a rocker at room temperature for 1 h. The membranes were lightly rinsed with TBS and left to dry. Sufficiently dry membranes were imaged using a Amersham Imager 600.

Caspase-Glo® 3/7 Assay

Promega's Caspase-Glo© 3/7 Assay was used for caspase 3/7 detection. Primary rat hepatocytes were cultured in 96-well plates for 18 h before crizotinib and ceritinib were introduced to the cells. Cells were then cultured in drug containing medium at varying times and concentrations as follows: 6, 8, and 10 µM ceritinib for 4 h; 6, 8, and 10 µM ceritinib for 8 h; 15, 20, and 25 µM crizotinib for 4 h; 15, 20, and 25 µM crizotinib for 8 h. Caspase-Glo© Reagent was prepared by transferring contents of Caspase-Glo© Buffer bottle to the Caspase-Glo© Substrate bottle immediately preceding experimentation. Drug containing medium was discarded from the 96-well plates and replaced with 100 µl of culture medium. 100 µl Caspase-Glo© Reagent was added to each well. 96-well plates were gently mixed for 30 seconds before returning to the incubator. The plates were left in the incubator for 3 h. Luminescence of the plates were read using a Turner Biosystems Modulus Microplate Reader.

Lactate dehydrogenase (LDH) Leakage

Primary rat hepatocytes were cultured in 96-well plates for 18 h before crizotinib and ceritinib were introduced to the cells. Cells were then cultured in drug containing medium at varying times and concentrations as follows: 0, 2, 4, 6, 8, 10, and 12 µM ceritinib for 8 h; 0, 2, 4, 6, 8, 10, and 12 µM ceritinib for 24 h; 0, 5, 10, 15, 20, 25, and 30 µM crizotinib for 8 h; 0, 5, 10, 15, 20, 25, and 30 µM crizotinib for 24 h. The following reagents were prepared for the LDH release assay:

1. buffer (stored at room temperature)
    a. 81.3 mM Tris: 9.83 g/1000 ml
    b. 203.3 mM NaCl: 11.9 g/1000 ml
    c. HCl to adjust pH to 7.2
2. LDH-1 (stored at -80 C: 1 ml/5 ml tube)
    a. 10 mM: 709.4 mg in 100 ml buffer NADH
3. LDH-2 (stored at -80 C: 1 ml/5 ml tube)
    a. 85 mM: 935.3 mg in 100 ml buffer monosodium pyruvate

LDH release assay reaction buffer was prepared by mixing 1 ml LDH-1, 1 ml LDH-2, and 48 ml buffer. 10 µl supernatant per well was taken from the original 96-well plate and added to a new 96-well plate (Plate S). 90 µl of the Caspase-Glo© buffer was added to the original 96-well plate and left to incubate for 3 h. 10 µl supernatant was removed from the Caspase-Glo© buffer containing plate and placed in a new 96-well plate (Plate T). 220 µl LDH release assay reaction buffer was added to the plates. The plates were read with using a Biotek Synergy™ 4 Hybrid Microplate Reader reading at 60 s intervals over 3 min. The following equations were used to calculate LDH release:

$$\left(\frac{\Delta A_{plate\ s}}{\Delta A_{plate\ t}}\right) * 100\% \quad (1)$$

Where;

$$\Delta A = A_{1\,min} - A_{2\,min} \quad (2)$$

Data Analysis

All data analysis was performed on Microsoft Excel 2016.

Results

Cytochrome C Release – Western Blot Results

Western blot is a reproducible and accurate way to identify broad concentrations of certain proteins in a cell. The results pictured in Figure 1 represent the cytochrome c release values of hepatocytes treated with crizotinib and ceritinib. It is important to note that the drugs were administered at multiples of Cmax. Cmax is the peak drug concentration found in the body following administration. This value is important in the toxicology field as it is a way to correlate cell culture toxicity to in vivo toxicity. The Cmax of ceritinib and crizotinib are 2 µM and 1 µM respectively. All drug concentrations at both time points showed some level of cytochrome c translocation. The DMSO control showed a trace amount of cytochrome c translocation as well. In the 4 h treatment results, crizotinib induced an increasing amount of cytochrome c release from 15 to 20 to 25 *Cmax (Figure 1). There was a drop in cytochrome c release at 30 *Cmax. Ceritinib induced an increase in cytochrome c translocation from 3 to 6 *Cmax (Figure 1). There was no observable decrease in cytochrome c release at the peak concentration. Following the 8 h treatment, crizotinib treated hepatocytes released an increasing amount of cytochrome c from 15 to 20 to 25 *Cmax that dropped off almost completely in the 30 *Cmax lane (Figure 1). 8 h ceritinib treated hepatocytes expressed an increase in cytochrome c release from 3 to 4 to 5 *Cmax that dropped off slightly at 6 *Cmax.

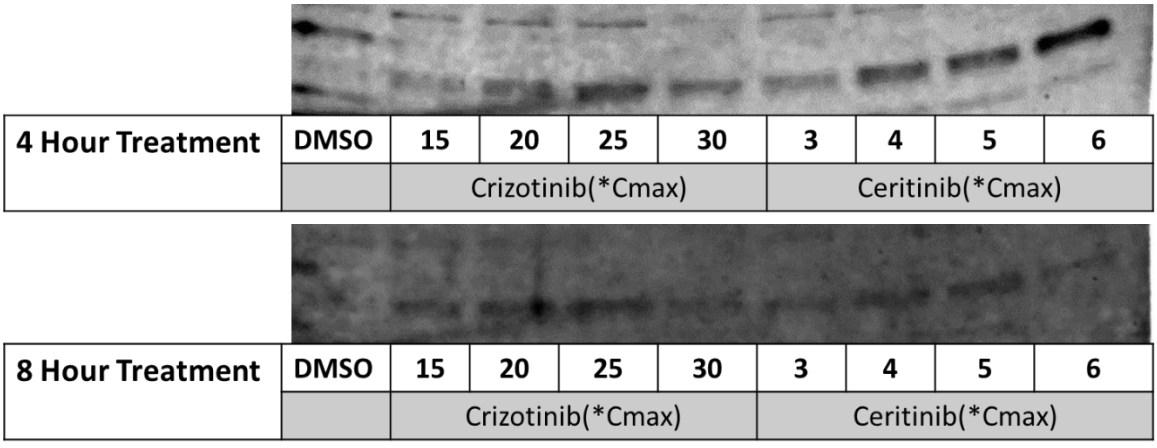
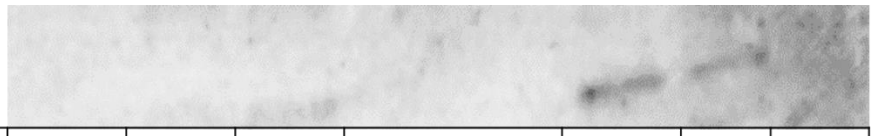
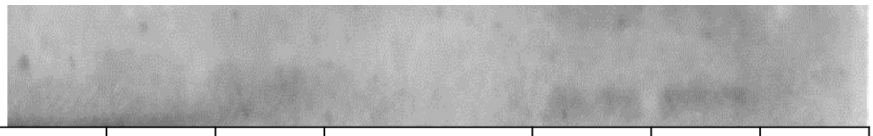


Figure 1. Western blot was ran using hepatocyte culture supernatant following crizotinib and ceritinib treatment. Treatment consisted of 4 and 8 h incubation times at varying Cmax as labeled. Membrane integrity WB antibody cocktail (ab110414) was used to mark cytochrome c present in the samples. Anti-Mouse CY5 was used to mark the primary antibody for imagining. Cytochrome c was identified as bands located around the 12 kDa range as marked by the western blot protein ladder. The images seen were taken using a Amersham Imager 600.

Figure 2 presents another western blot ran to identify cytochrome c release in crizotinib and ceritinib treated hepatocytes. Drug administration concentrations are shown in µM in this figure as opposed to Cmax as seen in Figure 1. The 0 µM control lanes for crizotinib treated hepatocytes showed basically no cytochrome c release after 4 and 8 h treatments, but did show trace amounts in the 24 h lane (Figure 2). At peak concentration (30 µM), crizotinib treated hepatocytes showed a decreasing trend of cytochrome c release as time of treatment increased. All control lanes for the ceritinib treated hepatocytes showed no cytochrome c translocation. The peak concentration (12 µM) lanes for ceritinib showed a decreasing trend of cytochrome c release to the point where there is almost no detectable cytochrome c after 24 h treatment (Figure 2).



Crizotinib (µM)	0	0	0	Blank	30	30	30
Time	4h	8h	24h		4h	8h	24h



Ceritinib (µM)	0	0	0	Blank	12	12	12
Time	4h	8h	24h		4h	8h	24h

Figure 2. Western blot was ran using hepatocyte culture supernatant following crizotinib and ceritinib treatment. Treatment consisted of 4, 8, and 24 h incubation times at varying µM as labeled. Membrane integrity WB antibody cocktail (ab110414) was used to mark cytochrome c present in the samples. Anti-Mouse CY5 was used to mark the primary antibody for imagining. Cytochrome c was identified as bands located around the 12 kDa range as marked by the western blot protein ladder. The images seen were taken using a Amersham Imager 600.

Caspase-Glo© 3/7 Assay Results

Promega's Caspase-Glo© 3/7 assay is used to estimate caspase activity in a cell. Figures 3 and 4 present the assay results for hepatocytes treated with either crizotinib or ceritinib. Crizotinib induced very little increase in caspase activity after 4 h treatment. Hepatocytes treated with 15 µM crizotinib for 4 hours showed an increase in caspase activity of about 120% (Figure 3). This number decreased to ~115% with 20 µM of crizotinib and ~110% with 25 µM. The 8 h treated hepatocytes showed a significantly larger increase in caspase activity. Hepatocytes treated with 15 µM crizotinib for 8 h showed a ~145% increase in caspase activity that increased to about ~175% with 20 µM crizotinib and decreased slightly to around ~165% with 25 µM crizotinib (Figure 3).

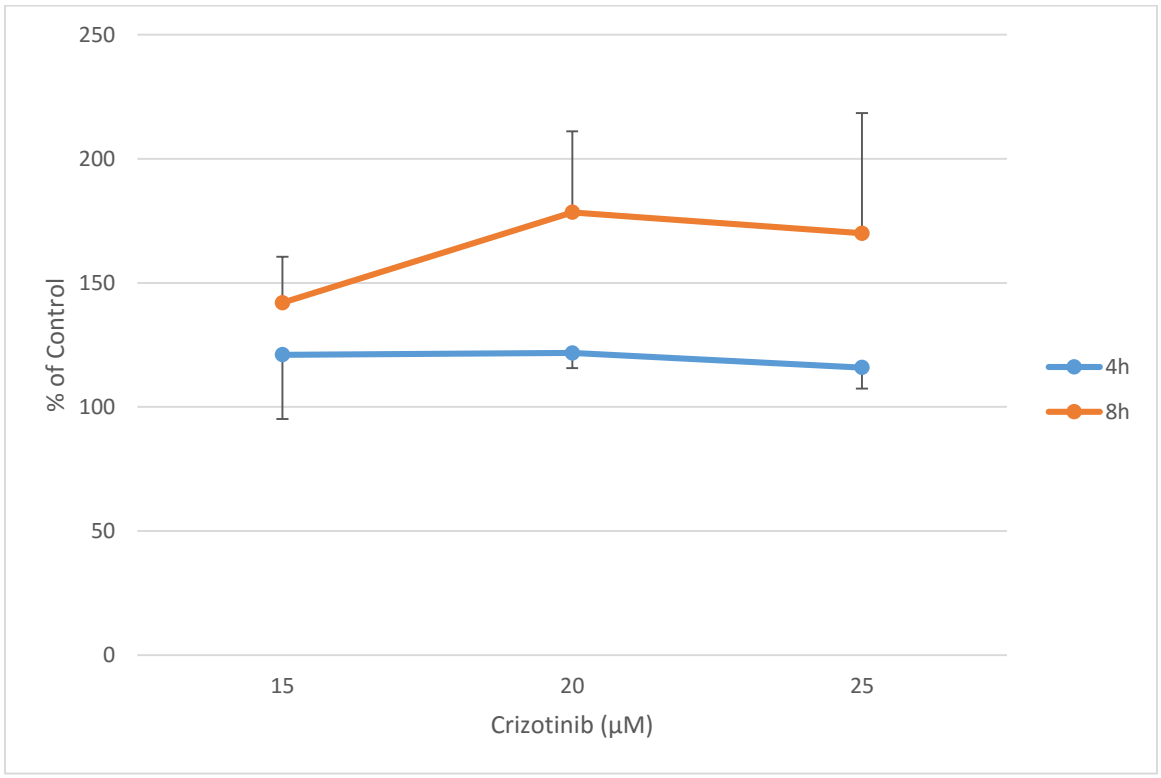


Figure 3. Promega's Caspase-Glo© 3/7 assay was used to estimate caspase activity in crizotinib treated hepatocytes. Hepatocytes were incubated with crizotinib for 4 and 8 h at varying concentrations as labeled. Positive standard deviation is shown for the 8 h line and negative standard deviation is shown for the 4 h line to minimize data overlap. All data values graphed are shown in Tables A2 and A4 located in the appendix.

After 4 h treatment, ceritinib induced an initially slightly higher amount of caspase activity in hepatocytes when compared to crizotinib. Hepatocytes treated with 6 µM ceritinib for 4 hours expressed a ~130% increase in caspase activity that raised to ~135% with 8 µM ceritinib and remained steady at ~135% with 10 µM ceritinib (Figure 4). Hepatocytes treated with 6 µM ceritinib for 8 h showed a ~120% increase in caspase activity when compared to the control. This number raised to ~140% with 8 µM ceritinib and ~160% with 10 µM ceritinib (Figure 4).

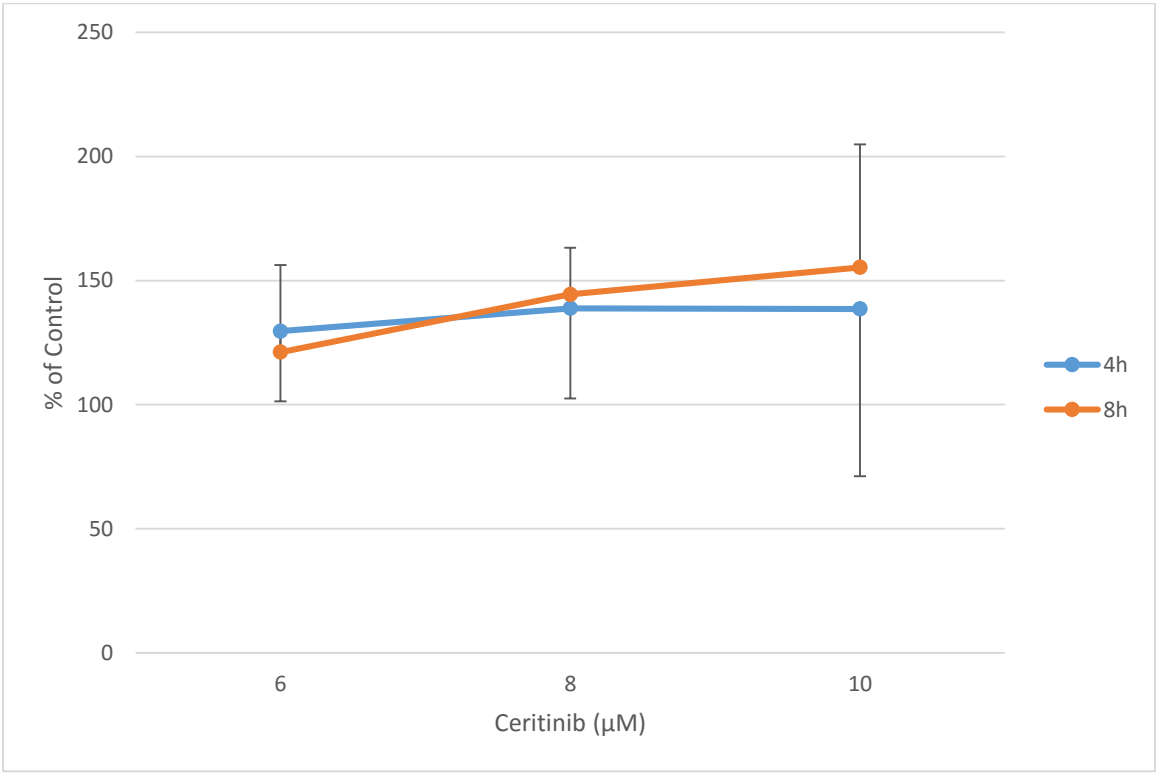


Figure 4. Caspase-Glo© 3/7 assay was used to estimate caspase activity in ceritinib treated hepatocytes. Hepatocytes were treated with ceritinib for 4 and 8 h at varying concentrations as labeled. Negative standard deviation is shown for the 4 h line and positive standard deviation is shown for the 8 h line to minimize data overlap. Data values graphed can be found in Tables A1 and A3 located in the appendix.

Lactate dehydrogenase (LDH) Leakage Results

LDH leakage is a known indicator of necrosis and cell viability loss. LDH leakage assays are used to quantify the extent in which cell viability is lost across a cell population. The LDH leakage assay results for hepatocytes treated with either crizotinib or ceritinib are located in Figures 5 and 6. Hepatocytes treated with crizotinib for 8 h released about 35% of their LDH contents at the maximum concentrations tested (30 µM). LDH released from hepatocytes treated with crizotinib concentrations lower than 30 µM for 8 h started out at around ~9% (0 µM) and steadily increased to ~18% release at 25 µM (Figure 5). Following 24 h of treatment, crizotinib induced a significantly higher amount of LDH release when compared to the 8 h treatments. 10 µM crizotinib induced ~30% LDH release after 24 h treatment that increased to ~70% at 30 µM (Figure 5).

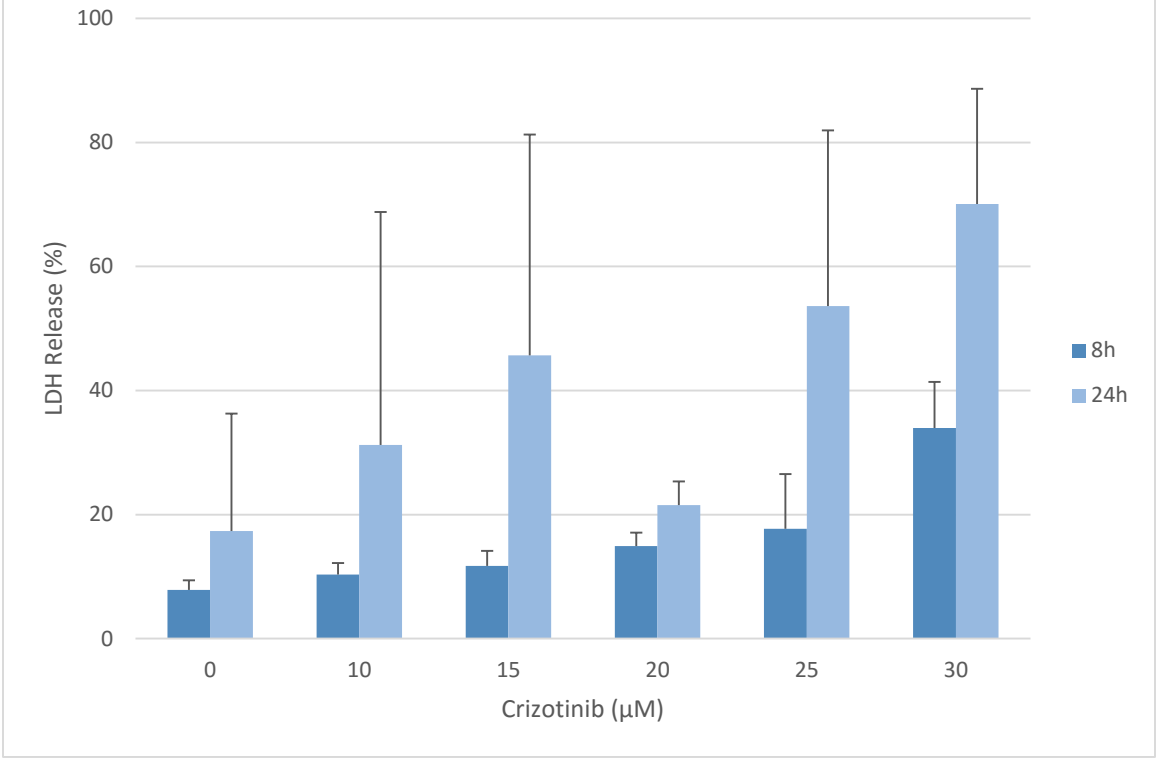


Figure 5. LDH release assay was performed on crizotinib treated hepatocytes as outlined in the materials and methods section. Hepatocytes were incubated with crizotinib for 8 and 24 h at varying concentrations as labeled. Both data sets show positive standard deviation. Data values can be found in Tables A6 and A8 located in the appendix.

After 8 h treatment, ceritinib induced no significant increase in LDH release at any of the concentrations tested compared to the control (Figure 6). At the maximum concentration tested (12 µM), ceritinib induced ~40% LDH release following 24 h treatment. The lower concentrations did not show any significant increase in LDH release after 24 h treatment when compared to the control.

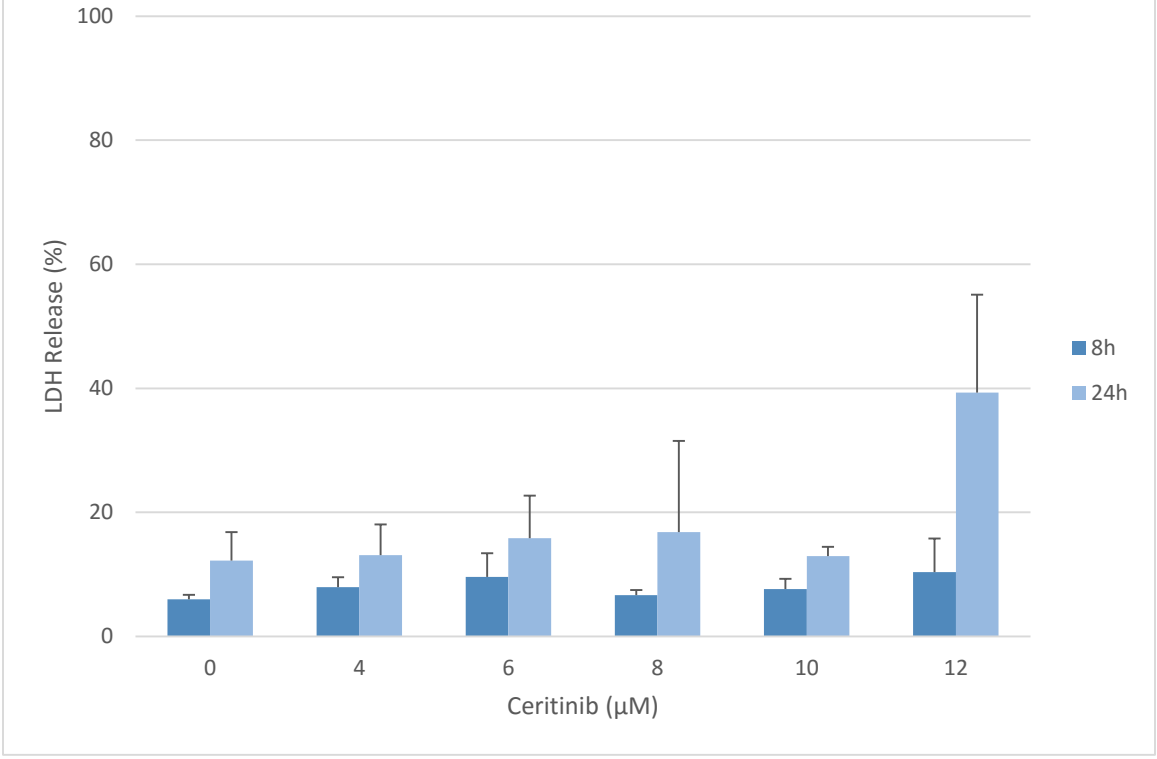


Figure 6. LDH release assay was performed on ceritinib treated hepatocytes as outlined in the materials and methods section. Hepatocytes were incubated with ceritinib for 8 and 24 h at varying concentrations as labeled. Both data sets show positive standard deviation. Data values can be found in Tables A5 and A7 located in the appendix.

Discussion

When assessing the cytotoxicity of any drug, it is important to narrow down the possible mechanism in which the toxicity occurs as well as the drug's level of toxicity itself. Ceritinib and crizotinib have clear warnings for hepatotoxicity on their FDA labels, however, the mechanism and threat level of this claim is unknown at the moment. It is also necessary to work in clinically relevant dosages when testing toxicity in vitro. To an extent, almost any drug will kill cells in culture at high concentrations; for this reason, the Cmax's of ceritinib and crizotinib were calculated before this study began. Ceritinib has a Cmax of about 2 µM and crizotinib has a Cmax of about 1 µM. Using these values, it is possible to infer from the in vitro results what level of toxicity these drugs might cause in vivo.

One mechanism of hepatotoxicity is the translocation of apoptosis inducing proteins such as cytochrome c. Apoptosis is a form of cell death that is believed to be programmed and occurs in all healthy tissues in the body, however, over activation of the apoptosis cascade can lead to unwanted cell death and toxicity. The translocation of cytochrome c from mitochondrial inner membrane space to cytosol is a known initiating event of apoptosis. Western blot was used to observe any possible changes in cytochrome c levels in the cytosol. Figure 1 shows the western blot results for both crizotinib and ceritinib treated hepatocytes at varying times and concentrations. For this protocol, *Cmax was used as a measure of concentration to show clinically relevant levels of drug treatment. In Figure 1, crizotinib treated hepatocytes showed an increase in cytochrome c leaves from 15 to 25 *Cmax; this is true for both the 4 and 8 h treatments. This is especially clear when comparing the bands to that of the control (DMSO) which shows little to no cytochrome c. At the 30 *Cmax concentration band, there is a drop in cytochrome c levels; this is again true for both the 4 and 8 h treatments. The likely reason for this is that necrosis occurred and cytochrome c is released into the cell culture medium and subsequently discarded prior to western blot detection. For the hepatocytes treated with ceritinib for 4 h, there is a steady increase in cytochrome c levels from 3 to 6 *Cmax. Since there is this steady increase in cytochrome c levels, the cells in all concentration columns are still alive, but in the pre-apoptosis state. The hepatocytes treated with ceritinib for 8 h show an increase in cytochrome c levels from 3 to 5 *Cmax and a decrease at 6 *Cmax. This decrease is again indicative of cell death. When comparing these two drugs, it is important to note that while crizotinib did induce more cell death at its highest concentration (30 *Cmax), Ceritinib induced cell death at a more clinically relevant concentration (6 *Cmax).

In Figure 2, a faint increase in cytochrome c release is seen from 4 to 24 h when cells are treated with the DMSO control, however, a low level of apoptosis is common in any cell culture, so seeing some cytochrome c is not unexpected. Hepatocytes treated with 30 µM crizotinib showed a relatively high level of cytochrome c release at the 4 h time point, which declined over the 8 and 24 h time points. The results for hepatocytes treated with 12 µM ceritinib showed a similar pattern.

An increase in caspase activity is another known marker of apoptosis. Caspase is in fact the executor of apoptosis. In this study, Promega's Caspase-Glo© 3/7 assay was used to estimate caspase activity in hepatocytes treated with ceritinib and crizotinib. Caspase assay results for crizotinib treated hepatocytes are shown in Figure 3. Hepatocytes treated with crizotinib for 4 h showed little to no increase in caspase activity (compared to the control) with increase in drug concentration. Hepatocytes treated with crizotinib for 8 h showed an initial increase in caspase activity at the 15 µM concentration (compared to the control) that increased further when the drug concentration was raised to 20 µM. However, the caspase activity differed very little from the 20 µM concentration to the 25 µM concentration. These results imply that crizotinib did not induce an increase in caspase activity at any concentration with the 4 h treatment, thus there was no increase in apoptosis activity. The hepatocytes treated with the drug for 8 h, however, did show a 2.5 to 2.75-fold increase in caspase activity, indicating that there was indeed an increase in apoptosis activity in the cell population. Caspase activity assay results for ceritinib treated hepatocytes are seen in Figure 4. Much like the crizotinib results, the 4 h ceritinib treatment did not seem to increase caspase activity in hepatocytes. The 8 h treated hepatocytes saw a 2.25 to 2.5-fold increase in caspase activity compared to the control, indicating that apoptosis was induced at all concentrations tested.

Another mechanism for cytotoxicity is the initiation of necrosis. Necrosis is generally believed to be a non-programed form of cell death that can lead to catastrophic tissue damage. Due to its dangerous

nature, necrosis is a strong indicator of cytotoxicity. In this study, necrosis was estimated using a Lactate Dehydrogenase (LDH) leakage assay. LDH leakage is a known indicator of cell viability loss and necrosis. LDH leakage assay results for hepatocytes treated with crizotinib are shown in Figure 5. After 8 hours of crizotinib treatment, the hepatocytes showed a slight increase in LDH leakage at the 10 μM concentration that increased to about 37% LDH leakage at the highest concentration tested (30 μM). Hepatocytes treated with crizotinib for 24 hours showed a much more dramatic increase in cell viability loss, starting out at 30% for the 10 μM concentration and ending up around 70% for the 30 μM concentration. Considering these values are 10 to 30 *Cmax, the induction of necrosis by crizotinib is not unexpected and is not a cause for great concern. Hepatocytes treated with ceritinib for 8 hours showed only a slight increase in LDH leakage with increase in drug concentration (Figure 6). After 24 hours of ceritinib treatment, hepatocytes showed significant increase in LDH leakage at the lowest concentration and over 40% cell viability loss at the highest concentration. Unlike crizotinib, ceritinib was tested at clinically relevant levels, therefore seeing hepatotoxicity at just 2 *Cmax is of clinical concern.

When addressing all the results presented in the study, it is shown that both drugs do induce an observable level of apoptosis and necrosis in primary rat hepatocytes. While these three cytotoxicity assays are separate from each other, comparing all results side-by-side provides some insight into the mechanism in which these drugs induce hepatotoxic effects. The hepatocytes treated with ceritinib saw little to no necrotic cell death at lower concentrations after the 8 h treatment (Figure 5), however, these same concentration levels and treatment times showed an increase in caspase activity (Figure 3) and an increase in cytochrome c translocation (Figure 1). This observation leads to the conclusion that even though crizotinib did not induce large scale necrosis at lower concentrations and treatment times, the drug did initiate apoptosis. Similarly, ceritinib treated hepatocytes showed basically no necrotic cell death following 8 h treatment (Figure 6). Once again, however, ceritinib did induce caspase activity (Figure 4) and translocation of cytochrome c (Figure 1), indicating the initiation of apoptosis prior to large scale necrosis. As previously addressed, the promotion of apoptosis is still harmful to healthy tissue, and is indicative of hepatotoxicity. When comparing the two drugs, ceritinib showed hepatotoxicity at concentrations as low as 2 *Cmax, while crizotinib did not show signs of hepatotoxicity until concentrations upwards of 10 *Cmax were administered. This leads to the conclusion that ceritinib is more harmful in clinical situations than crizotinib. Overall, these drugs are used for a type of cancer (Non-Small Cell Lung Cancer) that can be very aggressive, therefore the hepatotoxicity measurement presented in this study should be weighed against the therapeutic benefits of these two drugs prior to discontinuation. It should also be noted that data presented here are preliminary in nature. Further tests with hepatocytes from more animals will be needed to confirm the major findings.

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Disclaimer

This research was completed in part for completion of Alec Salminen's honors degree. The information in these materials is not a formal dissemination of information by FDA and does not represent agency position or policy.

References

[1]Hunter, T.; 1995. Protein Kinases and Phosphatases: The Yin and Yang of Protein Phosphorylation and Signaling. Cell. 80: 225-236.

[2]Encyclopedic Reference of Molecular Pharmacology. Tyrosine Kinases. 935-941.

[3]Hartmann, J. T.; Happ, M.; Kopp, H.; Lipp, H.; 2009. Tyrosine Kinase Inhibitors – A Review On Pharmacology, Metabolism and Side Effects. Current Drug Metabolism. 10: 470-481.

[4]2012. Crizotinib. AHFS Consumer Medication Information. 1-1.

[5]Pearson, R.; Kolesar, J. M.; 2011. Targeted therapy for NSCLC: ALK inhibition. Journal of Oncology Pharmacy Practice. 18(2): 271-274.

[6]http://www.fda.gov/safety/medwatch/safetyinformation/ucm295722.htm

[7]Li, S.; Qi, X.; Huang, Y.; Liu, D.; Zhou, F.; Zhou, C.; 2015. Ceritinib (LDK378): A Potent Alternative to Crizotinib for ALK-Rearranged Non-Small-Cell Lung Cancer. Clinical Lung Cancer. 16(2): 86-91.

[8]http://www.fda.gov/safety/medwatch/safetyinformation/ucm458065.htm

[9]Björnsson, E. S.; 2015. Drug-induced liver injury: an overview over the most critical compounds. Arch Toxicol. 89: 327-334.

[10]Kiang, T. K. L.; Teng, X. W.; Karagiozov, S.; et al.; 2010. Role of Oxidative Metabolism in the Effect of Valproic Acid on Markers of Cell Viability, Necrosis, and Oxidative Stress in Sandwich-Cultured Rat Hepatocytes. Toxicology Sciences. 118(2): 501-509.

[11]Lematers, J. J.; Nieminen, A.; Qian, T.; et al.; 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. BBA – Bioenergetics. 1366(1-2): 177-196.

[12]Luo, X.; Budihardjo, I.; Zou, H.; Slaughter, C.; Wang, X.; 1998. Bid, a Bcl2 Interacting Protein, Mediates Cytochrome C Release from Mitochondria in Response to Activation of Cell Surface Death Receptors. Cell. 94: 481-490.

[13]Cardona, M.; Lopez, J.A.; Serafin, A.; Rongvaux, A.; et al.; 2015. Executioner Caspase-3 and 7 Deficiency Reduces Myocyte Number in the Developing Mouse Heart. PLoS ONE. 10(6): 1-21.

[14]Hsieh, S.; Li, Y.; Chang, W.; et al.; Induction of Necrosis in Human Liver Tumor Cells by α-Phellandrene. Nutrition and Cancer. 66(6): 970-979.

[15]Zou, Y.; Kim, D.; Yagi, M.; et al.; 2013. Application of LDH-Release Assay to Cellular-Level Evaluation of Toxic Potential of Harmful Algal Species. Biosci Bitechnol Biochem. 77(2): 345-352.

Appendix

Caspase-Glo© 3/7 Assay Raw Data

Table A1. Sample of complete data collection and interpretation for a single Caspase-Glo© 3/7 Assay run. Hepatocytes were treated with Ceritinib (Drug 51) for 4 h prior to Caspase assay. All data interpretation was completed using Microsoft Excel 2016. Data collected and formulated was used in part to create Figure 4.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	51
Time	4h
Caspase 3/7	

	Raw Data Readings					
	1	2	3	4	5	6
	218164	202533	229096	277522	226874	269973
	203087	167948	215347	219667	210465	209550
	188968	240443	195042	276729	230772	261709
	186947	230380	249536	355736	242666	240457
	254666	247782	214500	294477	199577	212613
	227429	247962	222964	235754	189902	238168
	210007	323971	195310	282332	188281	228480
	243681	272886	226169	368942	270629	278299
Average	216618.625	241738.125	218495.5	288894.875	219895.75	242406.125
µM	0	4	6	8	10	12
% Control		111.5961866	100.8664421	133.3656674	101.5128547	111.9045627

7	8	9	10	11	12
244682	226736	289768	232294	259162	293748
220095	228737	220436	316974	192765	256128
215500	195530	436466	214310	195455	304574
244292	218645	346314	213947	263242	293471
203268	269711	207980	198271	183386	358285
225300	215590	241242	234911	247987	281113
215239	197827	276710	214601	212335	266855
239518	232951	338936	281703	213079	290193

Average	225986.75	223215.875	294731.5	238376.375	220926.375	293045.875
µM	0	4	6	8	10	12
% Control		98.77387723	130.4198144	105.4824564	97.76076474	129.6739189

13	14	15	16	17	18
35098.1	95386.7	63470.8	82373.5	139969	181033
77370.5	103437	34532.4	51032.3	143954	198995
81827.6	121658	61093.8	107466	142860	144442
117034	123012	115534	118703	139311	147870
98432.5	131614	126595	136754	136681	143247
25927.5	134169	123243	134945	126328	153216
42469.2	125564	128670	138971	144783	173241
28868.6	133894	145579	130167	123738	196283

Average	63378.5	121091.8375	99839.75	112551.475	137203	167290.875
µM	0	4	6	8	10	12
% Control		191.0613812	157.5293672	177.5862083	216.48193	263.9552451

Average % of control	133.8104817	129.6052079	138.811444	138.5851831	168.5112422
Concentration (MM)	4	6	8	10	12
STDEV	49.99351986	28.34024449	36.35904455	67.48664255	83.1330606

Table A2. Key data collected for Caspase-Glo© 3/7 assay on Crizotinib (Drug 25) treated hepatocytes. Hepatocytes were treated in Crizotinib for 4 h prior to Caspase assay. Data collected and formulated was used in part to create Figure 3.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	25
Time	4h
Caspase 3/7	

Average % of control	114.6144063	120.9937006	121.7584142	115.8751086	112.579917
Concentration (uM)	10	15	20	25	30
STDEV	29.88424329	25.82083019	6.127731756	8.49012424	25.72372623

Table A3. Key data collected for Caspase-Glo© 3/7 assay on Ceritinib (Drug 51) treated hepatocytes. Hepatocytes were treated in Ceritinib for 8 h prior to Caspase assay. Data collected and formulated was used in part to create Figure 4.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	51
Time	8h
Caspase 3/7	

Average % of control	112.2934356	121.1526027	144.4357085	155.3062186	161.5224722
Concentration (uM)	4	6	8	10	12
STDEV	20.63367204	35.17921751	18.80838098	49.62077058	46.23077041

Table A4. Key data collected for Caspase-Glo© 3/7 assay on Crizotinib (Drug 25) treated hepatocytes. Hepatocytes were treated in Crizotinib for 8 h prior to Caspase assay. Data collected and formulated was used in part to create Figure 3.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	51
Time	8h
Caspase 3/7	

Average % of control	112.2934356	121.1526027	144.4357085	155.3062186	161.5224722
Concentration (uM)	4	6	8	10	12
STDEV	20.63367204	35.17921751	18.80838098	49.62077058	46.23077041

LDH Release Assay Raw Data

Table A5. Sample of complete raw data collection and interpretation of LDH release for Ceritinib (Drug 51) treated hepatocytes. Hepatocytes were treated with Ceritinib for 8 h prior to LDH release assay. All data interpretation was done using Microsoft Excel 2016. Data collected and formulated was used in part to create Figure 6.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	51
LDH Release Assay	
Time	8h

Replicate 1

Raw Data - Plate S

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.829	0.828	0.843	0.83	0.825	0.844	0.842	0.825	0.832	0.83	0.833	0.839	340 Read#1
B	0.841	0.836	0.827	0.825	0.829	0.833	0.832	0.833	0.826	0.833	0.829	0.816	340 Read#1
C	0.843	0.825	0.83	0.821	0.831	0.835	0.825	0.834	0.815	0.834	0.827	0.839	340 Read#1
D	0.815	0.819	0.823	0.83	0.826	0.838	0.835	0.826	0.831	0.836	0.845	0.843	340 Read#1
E	0.835	0.827	0.83	0.838	0.827	0.852	0.833	0.843	0.831	0.83	0.836	0.835	340 Read#1
F	0.819	0.823	0.834	0.821	0.833	0.843	0.833	0.832	0.826	0.834	0.828	0.835	340 Read#1
G	0.829	0.828	0.837	0.829	0.831	0.832	0.83	0.841	0.838	0.836	0.84	0.841	340 Read#1
H	0.839	0.825	0.831	0.828	0.821	0.832	0.84	0.83	0.838	0.837	0.84	0.848	340 Read#1
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.792	0.793	0.805	0.796	0.786	0.803	0.804	0.787	0.794	0.79	0.792	0.759	340 Read#6
B	0.806	0.806	0.792	0.784	0.795	0.796	0.801	0.801	0.79	0.796	0.791	0.714	340 Read#6
C	0.805	0.795	0.797	0.792	0.796	0.804	0.797	0.802	0.779	0.799	0.788	0.774	340 Read#6
D	0.78	0.788	0.793	0.798	0.792	0.806	0.802	0.786	0.791	0.797	0.807	0.77	340 Read#6
E	0.8	0.792	0.798	0.802	0.793	0.821	0.801	0.807	0.795	0.791	0.801	0.746	340 Read#6
F	0.793	0.792	0.795	0.792	0.805	0.806	0.804	0.803	0.795	0.799	0.792	0.754	340 Read#6
G	0.805	0.801	0.804	0.792	0.799	0.789	0.797	0.807	0.802	0.801	0.801	0.767	340 Read#6
H	0.805	0.79	0.796	0.793	0.772	0.774	0.795	0.79	0.798	0.799	0.806	0.792	340 Read#6

Read 6 - Read 1												
0.037	0.035	0.038	0.034	0.039	0.041	0.038	0.038	0.038	0.04	0.041	0.08
0.035	0.03	0.035	0.041	0.034	0.037	0.031	0.032	0.036	0.037	0.038	0.102
0.038	0.03	0.033	0.029	0.035	0.031	0.028	0.032	0.036	0.035	0.039	0.065
0.035	0.031	0.03	0.032	0.034	0.032	0.033	0.04	0.04	0.039	0.038	0.073
0.035	0.035	0.032	0.036	0.034	0.031	0.032	0.036	0.036	0.039	0.035	0.089
0.026	0.031	0.039	0.029	0.028	0.037	0.029	0.029	0.031	0.035	0.036	0.081
0.024	0.027	0.033	0.037	0.032	0.043	0.033	0.034	0.036	0.035	0.039	0.074
0.034	0.035	0.035	0.035	0.049	0.058	0.045	0.04	0.04	0.038	0.034	0.056

Averages:	0.033	0.03175	0.034375	0.034125	0.035625	0.03875	0.033625	0.035125	0.036625	0.03725	0.0375	0.0775
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

Raw Data - Plate T

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.97	1.064	1.026	1.014	1.029	1.047	1.024	1.045	1.054	1.041	1.057	1.056	340 Read#1
B	0.976	1.02	1.027	1.019	1.027	1.03	1.032	1.054	1.08	1.046	1.046	1.06	340 Read#1
C	1.001	1.009	1.022	1.013	1.017	1.025	1.018	1.038	1.042	1.032	1.04	1.057	340 Read#1
D	0.996	0.992	1.002	0.996	1.01	1.015	1.008	1.028	1.04	1.025	1.038	1.06	340 Read#1
E	1.002	0.998	1.006	0.999	0.993	1.011	1.005	1.02	1.029	1.006	1.03	1.045	340 Read#1
F	0.998	0.994	1.004	0.999	0.994	1.009	1.008	1.019	1.032	1.012	1.039	1.053	340 Read#1
G	0.992	0.989	0.998	1.004	0.997	1.008	1.005	1.02	1.029	1.021	1.031	1.045	340 Read#1
H	0.956	0.975	0.976	0.984	1.004	0.995	0.999	1.006	1.028	1.014	1.032	1.021	340 Read#1
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.628	0.869	0.834	0.751	0.818	0.793	0.712	0.853	0.842	0.802	0.849	0.843	340 Read#6
B	0.672	0.84	0.865	0.781	0.838	0.766	0.776	0.898	0.885	0.797	0.832	0.848	340 Read#6
C	0.758	0.817	0.843	0.8	0.813	0.763	0.746	0.86	0.839	0.743	0.831	0.85	340 Read#6
D	0.753	0.795	0.809	0.744	0.805	0.775	0.746	0.843	0.83	0.741	0.817	0.853	340 Read#6
E	0.779	0.796	0.805	0.787	0.767	0.76	0.755	0.823	0.802	0.709	0.823	0.836	340 Read#6
F	0.767	0.8	0.802	0.79	0.775	0.775	0.772	0.832	0.806	0.722	0.829	0.844	340 Read#6
G	0.767	0.781	0.794	0.804	0.783	0.789	0.775	0.826	0.822	0.782	0.829	0.842	340 Read#6
H	0.722	0.761	0.766	0.77	0.787	0.772	0.758	0.803	0.816	0.809	0.83	0.797	340 Read#6

Read 6 - Read 1

0.342	0.195	0.192	0.263	0.211	0.254	0.312	0.192	0.212	0.239	0.208	0.213
0.304	0.18	0.162	0.238	0.189	0.264	0.256	0.156	0.195	0.249	0.214	0.212
0.243	0.192	0.179	0.213	0.204	0.262	0.272	0.178	0.203	0.289	0.209	0.207
0.243	0.197	0.193	0.252	0.205	0.24	0.262	0.185	0.21	0.284	0.221	0.207
0.223	0.202	0.201	0.212	0.226	0.251	0.25	0.197	0.227	0.297	0.207	0.209
0.231	0.194	0.202	0.209	0.219	0.234	0.236	0.187	0.226	0.29	0.21	0.209
0.225	0.208	0.204	0.2	0.214	0.219	0.23	0.194	0.207	0.239	0.202	0.203
0.234	0.214	0.21	0.214	0.217	0.223	0.241	0.203	0.212	0.205	0.202	0.224

Averages:	0.256	0.198	0.193	0.225	0.211	0.243	0.257	0.187	0.212	0.262	0.209	0.211
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

	Key Results											
LDH Release %	6.4547677	8.027813	8.911212	7.579123	8.456973	7.960966	6.532297	9.41689	8.658392	7.122371	8.965929	18.40855
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

Replicate 2

Raw Data - Plate S

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.92	0.898	0.916	0.901	0.894	0.912	0.902	0.903	0.91	0.905	0.89	0.911	340 Read#1
B	0.88	0.901	0.896	0.894	0.899	0.902	0.902	0.908	0.901	0.914	0.901	0.9	340 Read#1
C	0.9	0.889	0.888	0.891	0.894	0.903	0.878	0.898	0.881	0.899	0.89	0.909	340 Read#1
D	0.889	0.889	0.883	0.905	0.897	0.9	0.899	0.908	0.9	0.907	0.903	0.897	340 Read#1
E	0.892	0.89	0.889	0.895	0.896	0.899	0.904	0.907	0.896	0.895	0.894	0.899	340 Read#1
F	0.886	0.87	0.886	0.881	0.905	0.886	0.917	0.908	0.885	0.905	0.893	0.889	340 Read#1
G	0.897	0.892	0.894	0.887	0.895	0.891	0.899	0.896	0.893	0.898	0.895	0.888	340 Read#1
H	0.891	0.882	0.887	0.893	0.887	0.888	0.892	0.89	0.897	0.899	0.887	0.892	340 Read#1
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.884	0.874	0.864	0.873	0.864	0.87	0.875	0.875	0.879	0.869	0.847	0.849	340 Read#6
B	0.817	0.876	0.84	0.844	0.869	0.865	0.874	0.881	0.872	0.882	0.865	0.859	340 Read#6
C	0.861	0.861	0.842	0.863	0.863	0.868	0.854	0.866	0.849	0.869	0.859	0.877	340 Read#6
D	0.852	0.857	0.841	0.877	0.865	0.866	0.869	0.874	0.871	0.877	0.872	0.849	340 Read#6
E	0.86	0.858	0.853	0.862	0.862	0.869	0.874	0.874	0.865	0.862	0.869	0.858	340 Read#6
F	0.852	0.834	0.851	0.852	0.879	0.853	0.888	0.872	0.853	0.877	0.869	0.836	340 Read#6
G	0.865	0.865	0.863	0.855	0.868	0.867	0.87	0.87	0.868	0.86	0.858	0.833	340 Read#6
H	0.853	0.849	0.861	0.858	0.841	0.852	0.863	0.86	0.87	0.873	0.846	0.841	340 Read#6

Read 6 - Read 1												
0.036	0.024	0.052	0.028	0.03	0.042	0.027	0.028	0.031	0.036	0.043	0.062
0.063	0.025	0.056	0.05	0.03	0.037	0.028	0.027	0.029	0.032	0.036	0.041
0.039	0.028	0.046	0.028	0.031	0.035	0.024	0.032	0.032	0.03	0.031	0.032
0.037	0.032	0.042	0.028	0.032	0.034	0.03	0.034	0.029	0.03	0.031	0.048
0.032	0.032	0.036	0.033	0.034	0.03	0.03	0.033	0.031	0.033	0.025	0.041
0.034	0.036	0.035	0.029	0.026	0.033	0.029	0.036	0.032	0.028	0.024	0.053
0.032	0.027	0.031	0.032	0.027	0.024	0.029	0.026	0.025	0.038	0.037	0.055
0.038	0.033	0.026	0.035	0.046	0.036	0.029	0.03	0.027	0.026	0.041	0.051

Averages:	0.039	0.030	0.041	0.033	0.032	0.034	0.028	0.031	0.030	0.032	0.034	0.048
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

Raw Data - Plate T

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.775	0.825	0.844	0.819	0.804	0.817	0.821	0.821	0.838	0.849	0.845	0.87	340 Read#1
B	0.784	0.827	0.844	0.812	0.807	0.815	0.832	0.833	0.836	0.853	0.861	0.853	340 Read#1
C	0.774	0.816	0.845	0.814	0.825	0.828	0.815	0.825	0.823	0.839	0.84	0.851	340 Read#1
D	0.765	0.825	0.847	0.806	0.805	0.809	0.813	0.824	0.833	0.841	0.854	0.847	340 Read#1
E	0.763	0.829	0.849	0.809	0.799	0.817	0.811	0.819	0.836	0.835	0.851	0.843	340 Read#1
F	0.738	0.813	0.844	0.785	0.782	0.803	0.807	0.832	0.822	0.832	0.844	0.84	340 Read#1
G	0.745	0.825	0.843	0.794	0.799	0.823	0.805	0.819	0.842	0.825	0.854	0.843	340 Read#1
H	0.744	0.808	0.818	0.776	0.78	0.822	0.806	0.811	0.838	0.833	0.85	0.861	340 Read#1
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
A	0.414	0.631	0.653	0.527	0.446	0.478	0.466	0.485	0.495	0.548	0.536	0.523	340 Read#6
B	0.492	0.643	0.709	0.54	0.479	0.52	0.534	0.529	0.501	0.565	0.61	0.519	340 Read#6
C	0.454	0.622	0.712	0.569	0.538	0.579	0.543	0.546	0.538	0.529	0.611	0.521	340 Read#6
D	0.45	0.654	0.723	0.525	0.473	0.541	0.523	0.552	0.572	0.567	0.642	0.501	340 Read#6
E	0.454	0.665	0.741	0.551	0.501	0.566	0.543	0.562	0.604	0.551	0.648	0.563	340 Read#6
F	0.402	0.668	0.73	0.518	0.476	0.567	0.533	0.597	0.596	0.568	0.656	0.547	340 Read#6
G	0.409	0.67	0.738	0.532	0.546	0.617	0.527	0.593	0.669	0.577	0.691	0.622	340 Read#6
H	0.438	0.642	0.645	0.492	0.525	0.645	0.56	0.586	0.661	0.624	0.697	0.722	340 Read#6

Read 6 - Read 1												
0.361	0.194	0.191	0.292	0.358	0.339	0.355	0.336	0.343	0.301	0.309	0.347
0.292	0.184	0.135	0.272	0.328	0.295	0.298	0.304	0.335	0.288	0.251	0.334
0.32	0.194	0.133	0.245	0.287	0.249	0.272	0.279	0.285	0.31	0.229	0.33
0.315	0.171	0.124	0.281	0.332	0.268	0.29	0.272	0.261	0.274	0.212	0.346
0.309	0.164	0.108	0.258	0.298	0.251	0.268	0.257	0.232	0.284	0.203	0.28
0.336	0.145	0.114	0.267	0.306	0.236	0.274	0.235	0.226	0.264	0.188	0.293
0.336	0.155	0.105	0.262	0.253	0.206	0.278	0.226	0.173	0.248	0.163	0.221
0.306	0.166	0.173	0.284	0.255	0.177	0.246	0.225	0.177	0.209	0.153	0.139

Averages:	0.322	0.172	0.135	0.270	0.302	0.253	0.285	0.267	0.254	0.272	0.214	0.286
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

	Key Results											
LDH Release %	6.038835	8.630736	14.95845	6.085146	5.295821	6.704602	4.953968	5.763824	5.807087	5.808081	7.845433	8.362445
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

Final Results						
Average LDH Release %	5.9949669	7.959816	9.583785	6.64868	7.641039	10.35914
STDEV	0.7270362	1.570584	3.849639	0.839501	1.629203	5.412538
Concentration (uM)	0	4	6	8	10	12

Table A6. Key results collected from LDH release assay on Crizotinib (Drug 25) treated hepatocytes. Hepatocytes were treated is Crizotinib for 8 h prior to LDH release assay. Data collected and formulated was used in part to create Figure 5.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	25
LDH Release Assay	
Time	8h

Key Results - Replicate 1												
LDH Release %	6.1601151	9.625323	9.82641	15.81832	13.39564	29.43677	7.26884	12.02866	10.46718	12.09877	17.57493	45.08561
Drug Concentration (uM)	0	10	15	20	25	30	0	10	15	20	25	30

Key Results - Replicate 2												
LDH Release %	9.9350649	8.010563	11.42749	17.16141	30.06024	30.48259	7.990379	11.67532	15.20241	14.63981	9.801678	30.91174
Drug Concentration (uM)	0	10	15	20	25	30	0	10	15	20	25	30

Final Results						
Average LDH Release %	7.8385999	10.33496	11.73087	14.92958	17.70812	33.97918
STDEV	1.5874655	1.877194	2.40607	2.15007	8.826134	7.430153
Concentration (uM)	0	10	15	20	25	30

Table A7. Key results collected from LDH release assay on Ceritinib (Drug 51) treated hepatocytes. Hepatocytes were treated is Ceritinib for 24 h prior to LDH release assay. Data collected and formulated was used in part to create Figure 6. Red cell denotes outlier removed.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	51
LDH Release Assay	
Time	24h

Key Results - Replicate 1												
LDH Release %	9.624697337	18.45372	13.46877	9.73172	15.09669	48.36538	8.852868	8.691309	7.94347	8.219822	11.61593	54.25466
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

Key Results												
LDH Release %	18.91891892	12.22642	24.19106	10.40222	12.73458	18.51201	11.6092	70.94595	17.70701	38.86256	12.40132	36.08826
Drug Concentration (uM)	0	4	6	8	10	12	0	4	6	8	10	12

Final Results						
Average LDH Release %	12.25141987	13.12382	15.82758	16.80408	12.96213	39.30508
STDEV	4.594120266	4.94269	6.860594	14.73395	1.498323	15.79323
Concentration (uM)	0	4	6	8	10	12

Table A8. Key results collected from LDH release assay on Crizotinib (Drug 25) treated hepatocytes. Hepatocytes were treated is Crizotinib for 24 h prior to LDH release assay. Data collected and formulated was used in part to create Figure 5.

Date	6/22/2015
Rat Sex	Female
Rat DOB	3/28/2014
Rat BW	490
Perfusion Results	Good
Drug	25
LDH Release Assay	
Time	24h

Key Results - Replicate 1												
LDH Release %	9.601936525	10.05155	26.0631	20.31767	37.14722	53.12316	7.933923	6.667986	7.883495	17.19506	23.50092	65.727
Drug Concentration (uM)	0	10	15	20	25	30	0	10	15	20	25	30

Key Results - Replicate 2												
LDH Release %	45.65217391	86.73469	87.43017	22.22696	85.08772	64.90066	6.215084	21.50706	61.30268	26.35359	68.82353	96.54822
Drug Concentration (uM)	0	10	15	20	25	30	0	10	15	20	25	30

Final Results						
Average LDH Release %	17.35077925	31.24032	45.66986	21.52332	53.63985	70.07476
STDEV	18.91819585	37.53721	35.5928	3.830322	28.28353	18.56408
Concentration (uM)	0	10	15	20	25	30