trenzyme
life science services

Testing of hepatotoxic compounds for cytotoxicity and lipid accumulation

Hannes Veihelmann, Laura-Marie Palitzsch, Janina Brückner & Tanja Waldmann

trenzyme GmbH | Konstanz | Germany | August 2023

Introduction

Causes for the development of liver steatosis are complex and potential risk factors and predispositions for non-alcoholic fatty liver disease (NAFLD) are numerous. Among them, there are alcohol abuse, obesity and medication. If the disease is not due to excessive alcoholic consumption, the disease is called NAFLD. NAFLD is an umbrella term for a continuum of liver abnormities. It includes non-alcoholic fatty liver (NAFL) and more advanced stages such as non-alcoholic steatohepatitis (NASH) (Friedman et al., 2018). The course of NAFLD can often be asymptomatic for years, although with progressing it can lead to liver cirrhosis and hepatocellular carcinomas requiring liver transplantation (Mittal et al., 2016). Numerous medical drugs are on the market that increase the lipid content in the liver and eventually can lead to NASH. Beside nutrition and life style numerous drugs are known to induce the first steps of NASH, namely accumulation of lipid droplets in hepatocytes. Therefore, it is important to test drugs for their ability to damage the liver by inducing lipid droplet accumulation in hepatocytes.

The most prominent advantage of a cell-based toxicological test method over an animal-based test is that no species differences have to be considered. The use of human cells avoids the extrapolation between species. However, on the other hand a cell line and even when a primary cell can be used, the major disadvantage is that the metabolism of a specific cell type in culture may not be the same than in a living organism. Therefore, several aspects must be taken into account during development of such a test method: (i) the endpoint must be toxicological and biological relevant; (ii) the endpoint must be measurable and quantifiable; (iii) a training set of compounds must be defined to validate the test and (iv) the cellular system must be reliable and well characterized. As described above the accumulation of lipid droplets in liver cells is the first step for many different fatty-liver associated diseases, therefore we have chosen to implement this as an endpoint. Lipid droplets can be easily measured by staining with a specific dye, Nile Red, which binds to intracellular lipids. However, this measurement is image-based and represents a challenge for quantification. Here, we used the freeware CellProfiler to quantify the images. The definition of a training set of compounds are usually done stepwise from only a few compounds to over 20 compounds. This set must consist of compounds known to have an influence on the endpoint (i.e. in this study lipid droplet accumulation) in vivo. Furthermore, compounds that have no effect on the endpoint, i.e. negative controls must be included in the training set. At the end, this enables to calculate the false negative (sensitivity) and false positive (specificity) rate for the test method and represents an important quality measure. Finally, here we aimed to develop a very simple test method and therefore we used the well-established and characterized HepG2 cell line.

Training set of compounds

We have exemplified here a set of 8 compounds containing 3 drugs known to induce steatosis in vivo and 3 compounds of which is known that they do not have any influence on the fat content of the human liver. Furthermore, 2 hepatotoxic compounds are included known to induce general cytotoxicity but not steatosis in humans in vivo (Table 1). All compounds are listed as gold standard compound for liver toxicity in ToxBank (https://wiki.toxbank.net/wiki/Main Page).

Tamoxifen is a selective estrogen receptor modulator that is predominantly used for the treatment of breast cancer (Brauch et al., 2009). As one major side effect of long-term administration of tamoxifen, the development of fatty liver was observed in patients (Farrell, 2002). The steatotic effects of tamoxifen have been investigated in vitro before and are referable to its modulatory actions on the lipid metabolism (Zhao et al., 2014).

Valproic acid (VPA) is an antiepileptic drug (Perucca, 2002), which when used in long-term administration causes side effects such as weight gain (Biton et al., 2001), insulin resistance (Verrotti et al., 2002) and hepatic steatosis.

Compound	Substance Background	Substance Type
Saccharin	artifcial sweetener	negative control
Ascorbic acid	essential vitamin	negative control
Penicillin	antibiotic	negative control
Tamoxifen	drug against breast cancer	positive control
Valproic acid	anti-epileptic drug	positive control
Rifampicin	antibiotic	positive control
Aflatoxin B1	potent natural toxin	cytotoxicity control
Ketoconazole	antimycotic	cytotoxicity control

Table 1: Set of compounds tested for cytotoxicity and lipid droplet accumulation.

Rifampicin is one of the main drugs used for anti-tuberculosis-therapies and shows a strong general hepatotoxicity (J.-H. Kim et al., 2017), such as hepatocellular dysfunction, centrilobular necrosis and hepatic lesions (Westphal, Vetter and Brogard, 1994). Besides this general hepatotoxicity, rifampicin was also found to induce lipid accumulation in primary hepatocytes, which might be a pre-requisite for the above mentioned general hepatotoxicity (Li et al., 2015).

Aflatoxin B1 is a very potent liver toxin produced by Aspergillus flavus. It is a well-known toxicant responsible for manifold liver injuries and was used here as a general cytotoxicity control. Ketoconazol is an anti-fungal drug and shows strong side effects affecting the liver due to cytotoxicity and was therefore included as general hepatotoxic drug.

Negative controls are one of the most underestimated task to be done during testing and especially during the development of a test method. In principal, negative controls are defined as compounds known not to have an effect on the measured endpoint in vivo (Bal-Price et al., 2018). In this study, we used the artificial sweetener saccharin, the vitamin ascorbic acid and the antibiotic drug penicillin. All three negative control compounds are known to neither have any effect on hepatotoxicity nor on the accumulation of fat in the human liver.

Cytotoxicity testing using viability assay by resazurin reduction

The purpose of the test is to follow the accumulation of lipid droplets in HepG2 cells. However, in order to test this, the appropriate test concentrations have to be determined. These concentrations should not yet completely kill the cells but should be high enough to see an effect in the functional assay. Therefore, a common strategy is to set the more relevant functional assay in relation to cytotoxic concentrations. The functional test concentrations are then set around the so-called highest non-cytotoxic concentration or concentrations around the IC80 concentration of viability.

In order to determine the IC80 concentration the compounds were tested with 6 - 10 concentrations as indicated in Figure 1B and a resazurin reduction assay was performed. 24 hours after seeding, the cells were treated with increasing concentrations of compounds and incubated for another 48 hours. On the day of analysis medium was change to medium containing 10 µg/ml non-fluorescent resazurin and incubated for 1 hour (Figure 1A). If cells with intact mitochondria are present in the wells, resazurin gets reduced to fluorescent resorufin, which can be measured by 590 nm using a multilabel reader (Wallac VICTOR3V multilabel plate reader (PerkinElmer)).

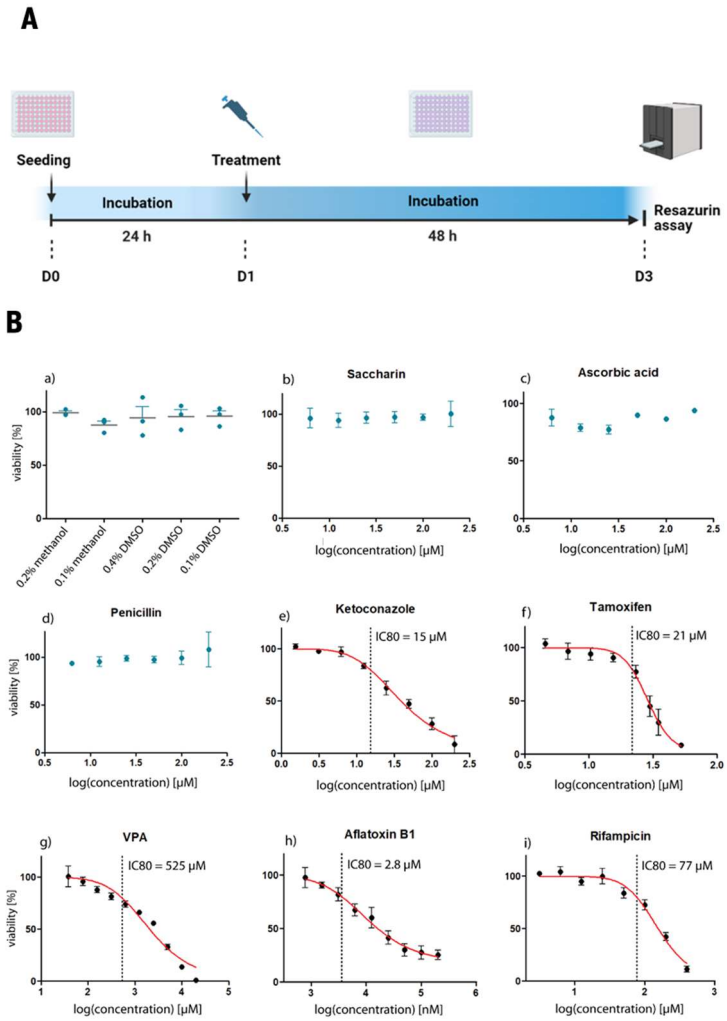

Figure 1: Resazurin assay to determine viability.

A: Experimental setup of toxicity testing for compounds in the training set. Cells are seeded to 96-well plates and then incubated for 24 hours. The cells are treated with compounds and after an incubation period of 48 hours a resazurin toxicity assay is conducted. Mitochondrial metabolism is measured via detecting resorufin in the supernatant.

B: Cytotoxicity curves for compounds of the training set. HepG2 cells were treated with the different compounds for 48 hours. Then, a resazurin cytotoxicity assay was conducted. Error bars depict SEM of three biological replicates a) Cytotoxicity of solvent controls in appropriate concentrations. b)-d) Cytotoxicity of negative control compounds in the training set. Used concentrations: two-fold dilutions from 12.5-200 µM. e) Cytotoxicity curve of ketoconazole. IC80 = 15 µM. Concentration range: 12.5-200 µM, 2-fold dilution. f) Cytotoxicity curve of tamoxifen. IC80 = 21 µM. Concentration range: 4.6-52 µM, 1.5 fold dilution. g) Cytotoxicity curve of VPA. IC80 = 525 µM. Concentration range: 39-20000 µM, 2-fold dilution. h) Cytotoxicity curve of aatoxin B1. IC80 = 2.8 µM. Concentration range: 0.8-200 µM, 2-fold dilution. i) Cytotoxicity curve of rifampicin. IC80 = 77 µM. Concentration range: 3.1-400 µM, 2-fold dilution.

The solvent controls (Fig. 1B(a)) and the three negative controls (Fig. 1B(b – d)) did not show any cytotoxicity as expected even not in the highest used concentrations of 200 µM. In contrast, for all positive controls as well as the cytotoxicity controls an IC80 could be determined.

Ketoconazole served as cytotoxicity control and was tested using concentrations of 1.6 µM to 200 µM in a 2-fold dilution series. The evaluation of three biological replicates revealed an IC80 of 15 µM with a 95% CI of 11 to 21 µM (Fig. 1B(e)). The coefficient of determination for the fit of the hill curve to the data was R^2 = 0.95.

Tamoxifen was chosen as positive control for the training set. The cytotoxicity of tamoxifen was evaluated with concentrations of 4.6 µM to 52 µM in a 1.5-fold dilution series. For better representation of the watershed, 30 µM was added as test concentration. The evaluation of three biological replicates revealed an IC80 of 21 µM with a 95% CI of 18.5 to 24.9 µM (Fig. 1B(f)). The regression line showed a good fit with R^2= 0.91.

VPA served as a second positive control for NAFLD in the training set. The cytotoxicity of VPA was evaluated with concentrations of 38 µM to 20 mM in a 2-fold dilution series. The analysis of three biological replicates revealed an IC80 of 525 µM with a 95% CI of 360 to 767

µM (Fig. 1B(g)). The regression curve had a goodness of fit of R^2= 0.94.
The second cytotoxicity control in the training set is Aflatoxin B1. The cytotoxicity of aflatoxin B1 was evaluated with concentrations of 0.8 µM to 200 µM in a 2-fold dilution series. The evaluation of three biological replicates revealed an IC80 of 2.8 µM with a 95% CI of 1.7 to 4.5 µM (Fig. 1B(h)). The R^2 for the regression line was 0.85.
Finally, Rifampicin, the third positive control, was evaluated with concentrations of 3.1 µM to 400 µM in a 2-fold dilution series. The evaluation of three biological replicates revealed an IC80 of 77 µM with a 95% CI of 57 to 102 µM (Fig. 1B(i)). The fit of the hill curve regression showed an R^2 of 0.93.

In summary, we could determine IC80 concentrations for all positive and cytotoxic controls, whereas the chosen negative controls were all non-cytotoxic.

Lipid droplet accumulation assay (LDAA)

In the next step, the steatotic effects of the known NAFLD inducing agents VPA, rifampicin and tamoxifen were investigated by staining the treated cells with Nile Red. Nile Red is a fluorescent dye that specifically stains cellular lipids. Also, the negative control ascorbic acid was included in the tests. The experiments were carried out analogously to the cytotoxicity testings described before. Instead of a resazurin assay, the cells were fixed with PFA and stained with Hoechst (to detect the nuclei) and Nile Red (to detect lipid droplets) after 48h of treatment.
The quantitative analysis of the stainings was done via the open source software CellProfiler, which detects and counts the nuclei via Hoechst staining and the lipid droplets via Nile Red stainings. For each tested concentration three wells were seeded and from each well three images were captured and analysed. The highest tested concentrations did not exceed the specific IC50 values so that cytotoxicity would not majorly influence the image analysis. The tested concentration range for each compound is shown in Table 2.

Compound	IC80	tested conc in LDAA
Tamoxifen	21 µM	6.9 µM – 30 µM
Rifampicin	77 µM	12.5 µM – 100 µM
VPA	525 µM	156 µM – 2.5 mM
Ascorbic acid	n.a.	6.25 µM – 200 µM

Table 2: Tested concentration range in the lipid droplet assay.

For quantitative evaluation of lipid accumulation, lipid droplets in the images were counted and divided by the corresponding amount of cells. The steatoic effects of the positive controls could be clearly detected by the quantification of the stainings. The highest tested concentrations for each compound showed a clear increase of lipid droplets compared to control. Tamoxifen increased the amount of lipid droplets 5.7-fold, rifampicin showed a 5-fold increase and finally VPA increased the lipid droplets by 3-fold (Fig. 2). Importantly, the negative control ascorbic acid showed no detectable lipid accumulation even not with the highest concentration of 200 µM.
In summary, this small study demonstrates the first steps of the development of an in vitro cell-based test method for lipid accumulation in hepatocytes. With all the drawbacks of the cell type (the hepatocellular cancer cell line HepG2), these are promising results as the so far tested positive controls induce lipid droplet accumulation in the cells as it was expected and is known from in vivo testings. The next steps in further validating the test now will be to expand the training set in order to calculate first measures for sensitivity, specificity and accuracy of the test method.

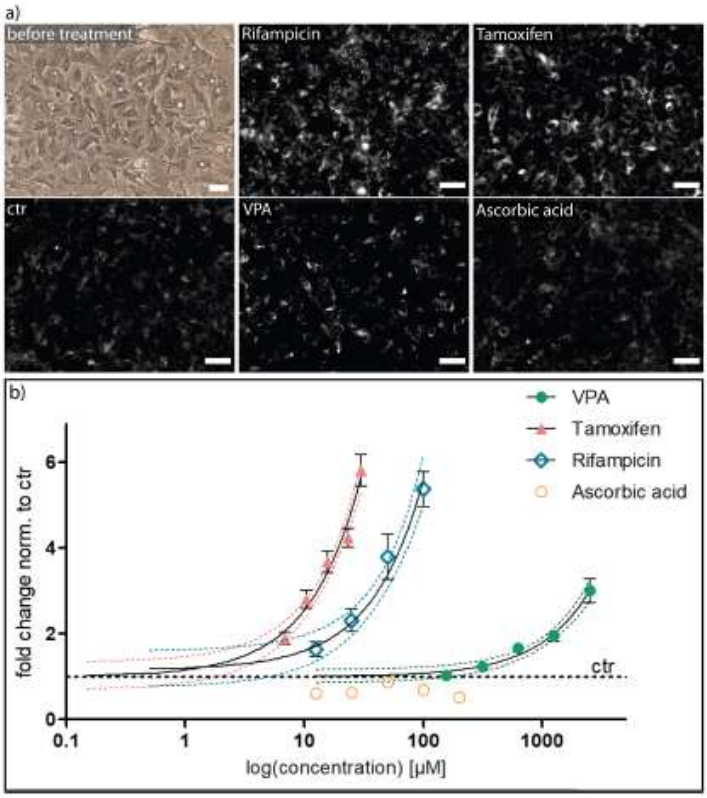

Figure 2: Steatotic effects of positive and negative controls of the training set in HepG2 cells.

Experiments were done analogously to the cytotoxicity tests with the training set. Instead of a resazurin assay, cells were fixed with PFA after 48 h of treatment and stained with Hoechst and Nile Red. a) upper left: phase contrast image of HepG2 cells before treatment. Remaining images: Representative images of Nile Red staining in HepG2 cells upon treatment with compounds. Scale bars = 50 µm. b) Image based quantifcation of lipid accumulation. Each tested concentration was seeded in triplicates and three images were captured from each well. The nine images per concentration were analyzed via Cell-Profiler for the number of lipid droplets per cell and the results were normalized to the untreated control. The shown data points are means +/- SEM from three biological replicates (i.e. 3x9 analyzed images per concentration). The black dotted line indicates the base line of the untreated control. The black continuous line for each treatment indicates the linear regression of the steatotic effects. Note, that the x-axis indicating the compound concentrations is on logarithmic scale. Colored dashed lines indicate the borders of the 95% CIs for the calculated regressions. For ascorbic acid, no regression was computed, as no consistent trend was observed.

References

Bal-Price A, Hogberg HT, Crofton KM, Daneshian M, FitzGerald RE, Fritsche E, Heinonen T, Hougaard Bennekou S, Klima S, Piersma AH, Sachana M, Shafer TJ, Terron A, Monnet-Tschudi F, Viviani B, Waldmann T, Westerink RHS, Wilks MF, Witters H, Zurich MG, Leist M. Recommendation on test readiness criteria for new approach methods in toxicology: Exemplified for developmental neurotoxicity. ALTEX. 2018;35(3):306-352. doi: 10.14573/altex.1712081. Epub 2018 Feb 23. Erratum in: ALTEX. 2019;36(3):506. PMID: 29485663; PMCID: PMC6545888.

Biton V, Mirza W, Montouris G, Vuong A, Hammer AE, Barrett PS. Weight change associated with valproate and lamotrigine monotherapy in patients with epilepsy. Neurology. 2001 Jan 23;56(2):172-7. doi: 10.1212/wnl.56.2.172. PMID: 11160951.

Brauch H, Mürdter TE, Eichelbaum M, Schwab M. Pharmacogenomics of tamoxifen therapy. Clin Chem. 2009 Oct;55(10):1770-82. doi: 10.1373/clinchem.2008.121756. Epub 2009 Jul 2. PMID: 19574470.

Farrell GC. Drugs and steatohepatitis. Semin Liver Dis. 2002;22(2):185-94. doi: 10.1055/s-2002-30106. PMID: 12016549.

Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med. 2018 Jul;24(7):908-922. doi: 10.1038/s41591-018-0104-9. Epub 2018 Jul 2. PMID: 29967350; PMCID: PMC6553468.

Kim JH, Nam WS, Kim SJ, Kwon OK, Seung EJ, Jo JJ, Shresha R, Lee TH, Jeon TW, Ki SH, Lee HS, Lee S. Mechanism Investigation of Rifampicin-Induced Liver Injury Using Comparative Toxicoproteomics in Mice. Int J Mol Sci. 2017 Jul 2;18(7):1417. doi: 10.3390/ijms18071417. PMID: 28671602; PMCID: PMC5535909.

Li L, Li H, Garzel B, Yang H, Sueyoshi T, Li Q, Shu Y, Zhang J, Hu B, Heyward S, Moeller T, Xie W, Negishi M, Wang H. SLC13A5 is a novel transcriptional target of the pregnane X receptor and sensitizes drug-induced steatosis in human liver. Mol Pharmacol. 2015 Apr;87(4):674-82. doi: 10.1124/mol.114.097287. Epub 2015 Jan 27. PMID: 25628225; PMCID: PMC4366797.

Mittal S, El-Serag HB, Sada YH, Kanwal F, Duan Z, Temple S, May SB, Kramer JR, Richardson PA, Davila JA. Hepatocellular Carcinoma in the Absence of Cirrhosis in United States Veterans is Associated With Nonalcoholic Fatty Liver Disease. Clin Gastroenterol Hepatol. 2016 Jan;14(1):124-31.e1. doi: 10.1016/j.cgh.2015.07.019. Epub 2015 Jul 18. PMID: 26196445; PMCID: PMC4690789.

Perucca E. Pharmacological and therapeutic properties of valproate: a summary after 35 years of clinical experience. CNS Drugs. 2002;16(10):695-714. doi: 10.2165/00023210-200216100-00004. PMID: 12269862.

Verrotti A, Basciani F, De Simone M, Trotta D, Morgese G, Chiarelli F. Insulin resistance in epileptic girls who gain weight after therapy with valproic acid. J Child Neurol. 2002 Apr;17(4):265-8. doi: 10.1177/088307380201700405. PMID: 12088081.

Westphal JF, Vetter D, Brogard JM. Hepatic side-effects of antibiotics. J Antimicrob Chemother. 1994 Mar;33(3):387-401. doi: 10.1093/jac/33.3.387. PMID: 8040106.

Zhao F, Xie P, Jiang J, Zhang L, An W, Zhan Y. The effect and mechanism of tamoxifen-induced hepatocyte steatosis in vitro. Int J Mol Sci. 2014 Mar 5;15(3):4019-30. doi: 10.3390/ijms15034019. PMID: 24603540; PMCID: PMC3975381.

trenzyme GmbH | Phone +49 (0) 7531 12290 0 | Fax +49 (0) 7531 12290 11 | contact@trenzyme.com | www.trenzyme.com

trenzyme offers as research service provider a wide range of highly customized services in cell line development, iPSC differentiation and characterization as well as recombinant protein production. Visit www.trenzyme.com to learn more about all our services and products.