Role of Cytotoxicity Experiments in Pharmaceutical Development

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Abstract

Through the twentieth century, the road from synthesizing a new drug molecule to become an actual product got longer than ever before. Cytotoxicity assays are a quick way to assess a certain chemical compound's effects on a given human cell line. The most well-known techniques are the MTT- and the LDH-assays. These tests are cheap, easy to execute, but not very precise and dependent on various environmental factors and also, they show no detail about the time-dependency of the toxic effect. Cytotoxicity experiments are a crucial part of a modern pharmaceutical development process. They are a cheap and safe way to get vital information about a new molecule's biological attributes focusing on its basic tolerability. These studies not only save human lives and test animals, but they save the time and resources to be spared on a test molecule which is a complete failure having no *in vitro* safety.

Keywords: cytotoxicity, pharmaceutical development, preformulation, cell cultures, *in vitro* toxicity

1. Role of cytotoxicity tests in the pharmaceutical development

Pharmaceutical safety is essential factor in the development of every medicament [1]. The early stage of the formulation development must include wide toxicity screening of the applied components; not exclusively the API, but all the incorporated excipients as well. When a formulation of a new chemical with interesting biological properties enters this process, it will undergo extensive testing designed to address and solve many complex issues [2]. However, the pharmaceutical effectiveness is essential, but might seem as an insufficient factor of a

successful medication [3]. The formulated delivery system must support the administration of the API to improve patient compliance [4]. Moreover excipients are able to enhance the API's effectiveness in many cases [5]. Besides their role in solubility or stability issues many excipients are used as penetration enhancers, since their effect on biological membranes [6]. It has been concluded that this advantageous property of excipients must be considered before the formulation but toxicity aspect shows great impact as well [7]. To optimize the procedure cost of this part of preclinical phase, simple but reliable methods must be performed [8]. Traditional drug toxicity tests are great possibilities for the pharmaceutical developers, not only because of the associated loss of human life or health, but also because of immense financial loss of investment [9]. Early application of appropriate cell-based assays in drug development offers the single-most impactful solution to the challenge of human toxicity [10]. *In vitro* cell line models for evaluating toxicity should predict human specific toxicity. This may be due in part to the success that *in vitro* screening for certain absorption, distribution, metabolism, and elimination (ADME) endpoints has seen over the past 10 years. *In vitro* systems designed to evaluate permeability, interaction with membrane transporter systems, and metabolic stability in cell models with human relevance reduced this failure rate to less than 10% [11]. To ensure success in toxicology evaluation methods, comprehensive and tiered screening methods must be employed. To avoid adverse results or conclusions, potential liabilities and limitations of the experiments must be investigated [12]. It is unlikely that a single *in vitro* viability test or cell line model would be sufficient as a final decision point for toxicity [13]. In a well-built toxicity screening procedure, different test types on different cell cultures must be performed.

In tiered approach, *in vitro* toxicity screening models are based on the cell viability alteration of different human cell lines. These determinations must be well characterized and predictive of *in vivo* effects with a low incidence of false positive or negative results. The methods must have the capacity to evaluate various molecules in a short period of time with a minimum amount of compound. The results should provide information on potential mechanisms of toxicity, and subcellular targets as well. These results might ensure useful data for the inventors to modify structures of API or alter the amount or types of excipients that are applied in the delivery system development. The modified compositions can be re-screened for toxicity without large cost. The first decisive step is the cell line selection. According to the desired administration route, developers are able to select the most appropriate cell cultures for the *in vitro* assessments [7]. Nowadays, a wide range of different immortalized or primary cell and tissue models are available from safe sources for *in vitro* toxicity evaluation. However a well selected cell type ensures valuable information about the developed dosage form, the investigators often faced difficulties; since the main strengths and weakness of the models must be considered wisely. Routine toxicity screening procedures require a robust cell culture model that can be maintained easily in flasks and 96-well culture plates or inserts. The cells must be genetically stable and provide reproducible results in each assessment. The cells should be well characterized in terms of their doubling time, optimal growth conditions, and biochemistry. Various human cell lines are used routinely to evaluate toxicity. For example, Caco-2, HaCaT, CaLu, HeLa, 3 T3, HEK293, and many more. During the cell line selection process, it is important to totally characterize the morphology and understand the relevant biochemistry of the cells. These data are required to understand the background of potential mechanisms of toxicity [14]. *In vitro* cytotoxicity assays offer special and early identification of potential

cytotoxicity of compounds, the property that might leads to irritancy during the application. *In vitro* toxicity tests are found to be advantageous in preclinical studies because of their eligibility, cost effectiveness, and reproducibility. Cell viability assays are developed to measure activities attributable to cellular maintenance and survival. Beside metabolic biomarkers, such as mitochondrial reductase, ATP reductase etc., homeostatic enzyme activities can be monitored as well [15]. During these measurements, various compounds can be routinely investigated with a relatively short incubation. In case of cytotoxicity assays, the test focuses on to detect loss of membrane integrity associated with cell death [16].

Lactate dehydrogenase (LDH) is one of the most preferred marker for cell death. LDH assay is a robust and most cost-effective method for cell cytotoxicity measurements. Real time cell condition monitoring is a relatively new possibility in toxicity screening. During these evaluation methods, a special instrument measures impedance-based signals in both cellular and cell invasion assays without any exogenous label use [17]. These systems are able to detect cell responses continuously and non-invasively without disrupting the natural cell environment. Moreover, not exclusively cell-mediated cytotoxicity and cell adhesion assays, but even receptor-mediated signaling and virus-mediated cytopathogenicity tests can be performed [18]. These experiments allow more flexibility, quick results, and illustrious precision although relatively high expenses must be considered during the experimental design. High variety of assays provide an excellent possibility in ranking compounds for consideration in drug discovery. As it was shown, the highest expenses of pharmaceutical development occur in the preclinical and clinical studies. By the application of these *in vitro* tests, the inventors are able to reduce risk significantly. Assays, performed on cell culture models, are able to unequivocally support the process of drug development by perfecting efficiency and improving the probability of success.

2. Use of cytotoxicity tests

2.1. Application of cytotoxicity tests

Nowadays the development of a new drug molecule costs around 1 billion dollars and out of 10,000–30,000 possible candidates, only 1 will find its way to the drug market [19]. This means that the number of companies, which have actual financial background for such a research, is decreasing and the whole process of drug development is slowing down. This is the consequence of ICH's GCP protocol, which in one hand, not only created a worldwide secure standard for clinical trials of new drug molecules, but also radically increased the expanses. On the other hand, non-drug related medical researches, such as medical devices (insulin pumps, implants, etc.), also appeared on the market and the requirements of GCP was too complicated and usually unnecessary.

Such circumstances lead to the increased popularity and development of cell culture model systems. Cell lines are a cheap way to investigate the effect of any questioned molecule or device on a given cell type. They can primarily be purchased from cell banks such as European Collection of Authenticated Cell Cultures (ECACC) and the American Type Culture Collection (ATCC). Cell lines can either be primary as they are directly isolated from a tissue or organ. Their structure, protein expression patterns, metabolism, and genetical code are identical with

the *in vivo* cells. Also, they are very sensitive to any effect during cultivation and they have a determined lifespan, meaning that they can only endure a limited number of passages. Secondary cell lines are immortalized by some method, which means that they have a hypothetically infinite lifespan. However, we can say from our own experience that for example Caco-2 cells are best suited for transport experiments (where they have to create a monolayer on an insert) between the 20th and the 30th passages. After 50 passages, the cells are hardly able to reproduce their own number, thus, they are no longer sufficient for cell viability tests.

Also, it is crucial to choose the right cell line for the given experiment. If the question is the biocompatibility of a chemical compound, which is about to be used on humans, then a human cell line must be chosen for the given experiment and even, an appropriate organ should be selected. A good summarizing table was created by Amelian et al. [20] (**Table 1**).

As it can be seen, there are multiple available cell lines with the same origin. The selection and the test system must be based on the later application of the device/compound, as each cell line has a different medium requirement and cultivation method as they can act differently in cell viability tests.

Also, because anti-proliferative drugs main attribute is their cytotoxicity, the following methods are ideal for testing these substances on cell lines. Because the secondary, immortalized cells can be seen as cancer cells (because their apoptotic or growth stop signals are suppressed by mutations), they are capable to react to certain promising anti-cancer molecules like their *in vivo* counterparts.

2.2. Advantages of cytotoxicity tests

As scientific and medical studies are getting more expensive over the recent decades, most of the universities, research institutes are underfinanced, the importance of a certain method's price is greater than ever before. Animal experiments are expensive and the administrative burden is overwhelming, so they are only used when no other test is suitable. Also, every year new plants and their respective metabolites are described and through the various methods of

Cell line	Origin	Application
HeLa, A431	Epithelial cervical cancer	Very rapid growth, cells commonly used in cancer research
Caco-2, HT29, HCT-116	Colon adenocarcinoma	Studies of absorption <i>via</i> intestinal epithelium, toxicity tests
U2OS	Osteosarcoma cells	Studies of transport and absorption of drugs
SkBr3, MCF-7, MDA-MB231, ZR-75-1	Breast cancer	Studies of transport and absorption of drugs, screening anticancer compounds
Calu-3	Serous cells of submucosal gland	Studies of absorption <i>via</i> bronchial epithelium, metabolic and transport model to study drug delivery to the respiratory epithelium
HaCaT	Adult human skin	Penetration of drug through the skin
16HBE140	Bronchial epithelium	Studies of absorption and excretion through the bronchial epithelium

Table 1. Most common cell lines used in cytotoxicity studies [20].

chemistry, new molecules are synthesized, and even an older compound might be re-evaluated for a new indication. Secondary cell lines are ideal for the screening of the enormous amount of test subjects. If the right cell line is chosen, not just the cytotoxicity, but the biological activity of the chosen material can be measured. In short time, multiple experiments can be carried out, with high reproducibility in the same test system. If we use more than one method, with different signaling mechanisms and different cell lines, we will have a more complex view on the *in vitro* toxicity data. This means more information when planning the *in vivo* experiments. Generally, it can be said, that if a compound proves to be non-toxic, then *in vivo* it will be tolerable. If it is moderately toxic, there is a chance that the 3D structure and the different cell types of an organ or the human body can effectively recover from the cytotoxic damage or the damage will only be minimal.

2.3. Disadvantages of cytotoxicity tests

The results of the cytotoxicity tests require additional consideration. They—even if the right cell lines were used—are not an automatic green light for *in vivo* application. If a given chemical proves to be non-toxic, it only means that we do not necessarily have to start our next experiment in an animal with the smallest dosage, but from a medium or a high concentration, to determine the maximum single dose, maximum daily dose and the LD50 value. Also, if we do not use the appropriate cell line—like testing an ointment preservative on enterocytes—then the scientific value of our study will be questioned. The cytotoxicity tests usually end up in a specific IC50 value. These values usually cannot be compared, because these tests are highly dependent on the parameters of the test system. Such parameters can be: cell line, passage number of the cells, number of cells/well, volume of medium/well, growth time of a plate, concentration/volume of reagent, manufacturer of the reagent, length of incubation, reaction time, solubilization solution (if needed) even the performance of the spectrophotometer. We suggest that instead of using static IC50 values, trends, comparisons should be seen. Stating that compound A has an IC50 value of 0.5 mg/ml and B has 0.25 mg/ml should be changed that A is about twice as tolerable, than B. This fact does not decrease the significance of the cytotoxicity tests, but prevents to deny of a scientifically accurate study, because the written IC50 value cannot be reproduced. Another issue is that a specific compound can interact with the reagents or the mechanism of the method, thus a false positive or negative result can be detected. Such interaction can only be found if we use more than one method or in the scientific literature. If we only use one type of cytotoxicity test, the scientific value will be low and the chance of detecting false results will be high. This is not necessarily caused by a specific interaction, but because of the given test system, the whole method will over- or underestimate cytotoxicity. Thus, it is advisable to use different types of tests, so link MTT with LDH or RT-CES, but not with XTT, because they are both tetrazolium based assays and have the same limitations.

3. Description of different cytotoxicity tests

The cellular damage caused by different chemical compounds can be various and thus, the methods to measure this effect are numerous. To select the proper test, we must know: number

of treated cells, number of treatments, what kind of treatment the cells got, do we need these cells later, or the chosen method can terminate them? Also, do we want to know the kinetics of the post-treatment population's changes or simply what happens with the cells after a short incubation with the selected chemical? The price, the reliability, the user-friendliness of the kit is an important question too. The mechanism of cytotoxicity can be various so a single method only gives a simple view on a chosen material. Multiple tests and methods must be used before anyone can make a solid point about the biocompatibility of a chemical compound. Also, it must be noted, that a compound can interfere with the detection mechanism of a certain assay, resulting in a false positive or negative result; thus, multiple methods are needed to avoid such cases.

3.1. Assays

Cell viability assays are usually cheap, easy-to-perform methods, where after a given incubation with the selected chemical compound, the number of the surviving cells is measured by some method. They use no antibodies or radioactive chemicals. Usually, these assays are carried out on 96- or 384-well plates, making them ideal for screening experiments. It must be noted, that in some special cases, multiple measurements can be made, but these methods are not suited for long-term, kinetical or time-dependent killing studies. We must be aware of the fact, that most methods use some kind of "signal molecule" which—in normal cases—is directly proportional with the number of cells. If there is an uncontrolled factor that increases or decreases the strength of the signal, we can get false positive or false negative results. Such factor is usually increased uptake into the cell or increased activity of the specific enzyme, responsible for the creation of the signaling molecule. Usually not a single enzyme, but multiple proteins catalyze the reaction, so the overall metabolic state of the cell must be considered. It can be said that additional filters for background measuring can greatly increase the sensitivity. Nearly all eukaryotic cells can perform these biochemical reactions, but previous research can avoid incompatibility with certain cell lines. In the following table, the most well-known cell viability assays are listed. Prices are approximating, and they mean the price of 1000 tests of the kit, according to the manufacturer (Table 2).

Name	Mechanism	How to detect	Price (€)
XTT	Enzymatic activity	Spectrophotometer	235
MTT	Enzymatic activity	Spectrophotometer	129
WST-1	Enzymatic activity	Spectrophotometer	235
WST-8	Enzymatic activity	Spectrophotometer	420
MTS	Enzymatic activity	Spectrophotometer	172
LDH	Enzymatic activity	Spectrophotometer	315
Resazurin	Enzymatic activity	Spectrofluorometer	110
Neutral Red	Lysosomal uptake	Spectrophotometer	339

Table 2. Most common cytotoxicity assay methods.

MTT assay is a cheap, popular way, to measure cell death [21]. The reduction of the tetrazolium structure in the MTT dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide leads to a colored formazan product—this is the basic chemical reaction in every tetrazolium based assay. The MTT dye has a positive charge, thus it is taken up by the cells and intracellular oxidoreductases catalyze the mentioned reaction. The oxidative state of the cell and the mitochondrial respiratory chain are essential in the conversion. Basically, the concentration of NADH limits the process and any chemical compound that modifies the oxidative potential of the cell can possibly decrease or increase the signal of the assay [22]. If this effect is not linked with direct cytotoxic activity, then, it may lead to a false positive or negative result. Various reports have already indicated that certain test compounds have radically different results in tetrazolium assay, than in other methods because of the possible antioxidant capabilities [23]. The reaction starts immediately, but 1–4 h should pass before measuring the absorbance of the test system—the exact time must be setup according to the parameters of the current test system. A too short amount of time might result in low signal strength; a too long may mask the difference between different treatments/concentrations. Various organic solvents might be used to dissolve final product, the insoluble, purple formazan crystals, but we suppose an isopropanol:hydrochloride acid (25:1) solution, because it is safe and cheap to use. The acidification of the system is required, to reduce the amount of the original yellow dye, thus, give us a stronger main signal. The absorbance must be measured at 570 nm (**Figures 1 and 2**).

XTT, MTS, WST-1, and WST-8 assays are the improved versions of the old MTT. The final product of the reaction is soluble in water/cell culture medium; yet, the solubility of the original dye is greatly reduced, they must be used at 1–2 mg/ml concentrations instead of the 0.2–0.5 mg/ml of the original MTT dye [24]. The XTT and the WST-1/8 compounds have a negative charge, so they cannot penetrate the cell membrane; their reduction takes place in the extracellular space [22]. To enhance the effectiveness of transmembrane oxidoreductases, an

Figure 1. MTT dye reaction, the tetrazolium ring opens as a result of the reduction.

Figure 2. Simplified mechanisms of MTT and MTS assays.

intermediate electron acceptor (IEA in figure) is required, which gets reduced by NADH and intensifies the final signal of the assay. The specific IEA (such as phenazine ethyl sulfate) is usually part of the assay kits and its concentration must be determined according to the given test system. The MTS is at least partially reduced in the intracellular compartment, thus an IEA is not essentially needed [22]. Because, the final formazan product is soluble in water, the solubilization step is unnecessary; these assays are more flexible to combine with additional methods and easier to carry out (Figures 3 and 4).

Resazurin assay (or Alamar Blue) is based on the enzymatic conversion of the cell permeable blue resazurin to the pink resorufin [25]. Both chemicals are soluble in water and the assay provides higher sensitivity than the previous methods, because the detection is based on fluorescence, not spectrophotometry. Also, it must be noted that the reagent is toxic, so the appropriate reaction time must be based on the sensitivity of the given cell line so the toxicity of the reagent can be distinguished from the tested chemical. The fluorescence should be measured with 560 nm excitation/590 nm emission filters. Also, it can be combined with other techniques such as caspase activity assay because of the different detection method and the non-interference of the respective mechanisms [26] (Figure 5).

LDH assay is based on the activity of a cytoplasmic enzyme, the lactate dehydrogenase which reduces the NAD to NADH. NADH then reduces a tetrazolium dye (or other reagent) whose concentration can be measured spectrophotometrically [27]. The assay is mainly used for the detection of membrane leakage which correlates with the cell damage. The most common reagent is the 2-p-iodophenyl-3-p-nitrophenyl tetrazolium chloride (INT) which forms a red formazan product. The LDH is quite stable for the duration of the assay in the extracellular space and its amount is dependent on the cell size and oxidative activity, but similar among the cells of the same cell line. It is excellent when compared with other tetrazolium based methods because it can measure the damaged cells as well, not just the dead ones. Also in special cases, it can be used to detect the intracellular LDH concentration. A limitation of this method is that serum has a high LDH activity on its own, so serum-supplemented mediums are not ideal for

Figure 3. Chemical formulae of the new generation tetrazolium assays from left to right: MTS, XTT, and WST-1/8.

Figure 4. Simplified mechanism of the XTT and WST-1/8 assay.

Figure 5. Mechanism of the Resazurin assay.

the LDH assay or multiple wash steps are needed before applying the assay reagent on the cells. Another problem is that if the cells are dead (or growth is inhibited), but the specific cytotoxic chemical does not distort the cell membrane, then the actual number of dead cells is underestimated. A solution of it can be the usage of control compounds which have a similar way-of-killing, than the tested chemical [28] (**Figure 6**).

Neutral red assay is not based on a directly enzymatic biochemical reaction, but the dye is taken up by the cells and it stains the lysosomes of the cells [29]. A weak cationic compound, the neutral red is taken up by micropinocytosis or by non-ionic diffusion and is accumulated into the lysosomes [30]. After the cytotoxic treatment with the possibly cytotoxic chemical, the cells should be washed, then the staining solution must should be added to the test system. After an appropriate time of incubation, the dye must be removed and the cells are washed again. The incubation with a solubilization solution forces the cells to excrete the neutral red dye and thus, the concentration of it can be measured at 540 nm. As damaged cells can only take up and store neutral red at a decreased rate and dead cells are not stained at all, it is a sensitive assay, but, the cells are washed, disturbed multiple times which—if not carried out smoothly enough—can decrease the cell number, destroy the monolayer or other kind of collateral damage can happen to the cells (**Figure 7**).

3.2. RT-CES

The simple cytotoxicity assays are limited in case of kinetical or time-dependent killing experiments. Simply, multiple measurements cannot be executed, because the test system is disturbed, some xenobiotics (tetrazolium dyes, other cofactors, etc.) are added to the cells or in case of some methods, the cells are solubilized. The reagents used in the assays can be directly

Figure 6. Mechanism of LDH membrane leakage assay.

cytotoxic or at least they modify the cells biochemical equilibrium and activity in a way that the results are no longer relevant for the latter *in vivo* experiments. This means that after a certain treatment with the probably cytotoxic compound, we can only make an end-point measurement because the test system is irreversibly changed after the addition of the given signaling molecule(s). Also, if the tested chemical can absorb light or is capable of fluorescence, it can interfere with the detection system. Because of these limitations the need for a non-invasive, yet precise method resulted in the invention of real-time cell electronic sensor.

This technique is based on the impedance changes of the cell populations [31]. The cells are seeded into special E-plates, which are available in multiple size (4-, 8-, 16-, 96-wells, etc.). These special devices have a positive and a negative electrode in every well, and a low voltage alternate current flows through the well. As the cells grow, they have a higher impedance (resistance in AC circuits); and as they die, the impedance value lowers. This effect has literally no impact on the cells, so it is a non-invasive technique. The length of the experiment is theoretically unlimited, as there is no end-point of AC current flow. For this reason, the cell growth can be measured during multiple treatments of the cells, and not just the cytotoxic or non-toxic effects, but the possible recovery of the cells can be studied as well. It is important, that first, a part of the cell medium and the solution of the screened chemical must be placed into the wells of the E-plate, thus the connected software can detect it as a background, with zero impedance, so chemicals with ionic charges do not interfere with the measured signal. The cells should be added to the wells after the background detection in a high-density suspension. Also, the whole experiment can be stopped at any point, to remove the test solution or to add a new compound to test system (Figure 8).

However, this system is ideal for cell viability studies, it has some disadvantages as well. The devices and the E-plates have a high price and a limited number of slots to use. The E-plates can be used multiple times after a specific cleaning protocol, but the sensitive, microelectronic sensing arrays are easily damaged by washing and organic solvents. This means, that it is not suited for high performance screening experiments, because multiple assays can be done during the same amount of time.

3.3. Other methods

Sulforhodamin B is a dye which stains the total protein amount of the cells [33]. The reagent is an aminoxanthene dye which binds stoichiometrically with the amino-acids under acidic pH. First, the cells must be fixed with trichloroacetic acid, then washed and dried and the wells respective optical density measured for background detection. The sulforhodamin B must be added after this, and it should stain the cells for 20–30 min. After a wash step, the stained cells must be solubilized and the absorbance measured at 565 nm. The protocol is quite long and

Figure 7. Neutral red dye.

Figure 8. A typical RT-CES diagram showing a time and dose dependent cytotoxicity of 2,6-dichloro-(1,4)-benzoquinone [32].

requires an experienced crew to execute perfectly. Also, the total protein only works, if the cells grew in the presence of the cytotoxic chemical, otherwise, the dead cells cannot be distinguished from the viable ones. However, there are several studies indicating that the sulforhodamin B results correlate well with the MTT results [34]. A slight advantage of this method is that because of the multiple wash steps, the tested chemicals can hardly interact with the dye, unlike other enzyme-based methods. The optimization with the specific cell line is also much easier because the lack of dependence on metabolic activity (**Figure 9**).

Calcein-AM/Hoechst 33342 and propidium iodide are dyes that stain viable and dead cells [35]. In appropriate concentration, Calcein-AM, a lipophilic derivative of calcein is capable pass through cell membranes and stains the cell, as intracellular enzymes cleave the lipophilic carbon chain from the dye [36]. Hoechst 33342 binds the A-T rich regions of the DNA. Propidium iodide stains the nucleus of the cell, but cannot penetrate the cell membrane, thus it only binds to the dead cells. As the two reagents can be detected at different wavelengths, multiple emission and excitation filters are needed. Also, every cell line has a different binding rate and the ideal concentration must be found through testing, as the cytosol of the cells can be stained by each dye and instead of spectrophotometric detection, manual counting is needed with a microscope with the specific filters/lamps (**Figure 10**).

Figure 9. Sulforhodamin B.

Figure 10. Chemical formula of the Calcein-AM (upper image), Hoechst 33342 (left image) and the propidium iodide (right image).

3.4. Comparison between *in vitro* cytotoxicity data and *in vivo* data

As the whole, medical science and industry is based on the modification, repair of damaged or badly functioning cells and tissues in human or animal body, the correlation between *in vitro* and *in vivo* data is crucial. Do these artificial test systems, cell lines truly replicate how a real tissue would react to a certain treatment or compound? The answer is based on the application of multiple *in vitro* methods and the careful planning of the *in vivo* experiments. A good example of the practice is the study of Yu et al. [37]. *Xanthii fructus* is a traditional Chinese herbal drug and clinical reports indicated its renal toxicity. The study was based on MTT and LDH assays of the main components of the herbal drug on a renal cell line, as well as acute and chronic toxicity experiments in rats. While the main component of the drug, the atractyloside potassium salt showed no cytotoxicity on the cell lines, the water extract of the fruit had an inhibitory effect in case of high concentrations on the MTT assay, but no membrane damage on the LDH assay. These results indicate that the secondary components of the water extract have cytotoxic capabilities and the exact mechanism of killing might involve the suppressed metabolic activity of the cells, but not the damage of the cell membrane. The acute *in vivo* toxicity showed that only high concentrations could terminate the rats and cause abnormalities in the organs and the chronic toxicity showed only minor changes in the highest concentration group. Overall, this complex study created a much more accurate, scientific point of view about the toxicity of *Xanthii fructus*, what chemicals are responsible for its toxicity, what are the exact dosages, and what are the side effects that are caused by the herbal drug. It could only be made by the co-application of various *in vivo* and *in vitro* methods.

4. Conclusion

The importance of cytotoxicity assays in early drug development is unquestionable although it must be concluded that no assay technology for detecting cytotoxicity *in vitro* is perfect. Strong arguments can be made for and against using cell viability or cytotoxicity assays as a reliable model of human medication. Depending upon the objectives of the investigation, either

viability or cytotoxicity assays can be performed. Cytotoxicity assays based on membrane integrity changes are positive-readout assay which are most typically indicated for shorter-term exposure models (48 h or less). These assays may not properly determine the absolute degree of early or late stage cytotoxicity since the kinetics of biomarker emergence or degradation. Viability assays measure the level of biomarker activity inversely correlated with cytotoxicity, and therefore may be used at any endpoint during a compound/cell incubation period. Each biomarker of viability and cytotoxicity has advantages and disadvantages. Moreover in early drug discovery, *in vitro* evaluations of new drug candidates is often met with skepticism since their reliability and *in vivo* correlatability. It can be concluded that however there is some validity to this argument, it is important to put *in vitro* toxicity data into consideration during pharmaceutical development.

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