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Role of Cytotoxicity Experiments in Pharmaceutical Development

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139
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139
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 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.
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 8. A typical RT-CES diagram showing a time and dose dependent cytotoxicity of 2,6-dichloro-(1,4)-benzoquinone [32].

Figure 9. Sulforhodamin B.
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]. *Xantii 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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