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Assessing cell lines for cell growth assays as alternative for existing cytotoxicity assays

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Assessing a new Cytotoxicity Test for Material Characterization of Single-Use Products

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BACKGROUND

In recent years, reduction of mammalian cell growth in single-use (SU) bioreactors and Erlenmeyer shake flasks have been observed, despite the fact that these bioreactors and the respective raw materials to manufacture those SU containers have been extensively tested according to existing cytotoxicity standards (e.g. USP<87> and DIN ISO 10993-5). For example, *bis*(2,4-di-*tert*-butylphenyl)phosphate (bDtBPP) has been identified in irradiated bioreactor film extracts by *Hammond et al.* and its cytotoxicity has been correlated to cell growth reduction [1, 2]. This enabled suppliers to adjust their manufacturing process and increase the performance of the films, in the case of Sartorius the performance of the new Flexsafe® film [3].

While impact of bDtBPP was resolved, the question still remains how this could have been missed and how suppliers can ensure that such incidents will not occur in the future again. To gain a better understanding of the necessary criteria for a suitable cell growth standard in biopharma applications we compared the influence of three known cytotoxins on the growth of both L-929 cells, a cell line which is recommended in the USP<87>, and a suspension CHO-DG44 cell line. Assuming suppliers of raw material, in particular resins suppliers, have good control on the main ingredients, a suitable growth test would need to identify impacts of minuscule amounts of cytotoxins.

EXPERIMENTAL APPROACH

Cytotoxins

In this study, three cytotoxins with different modes of cell interactions where used.

Mitomycin C (Fig. 1) inhibits DNA synthesis. It reacts covalently with DNA, *in vivo* and *in vitro*, forming crosslinks between the complementary strands of DNA. This prevents the separation of the complementary DNA strands, thus inhibiting DNA replication.

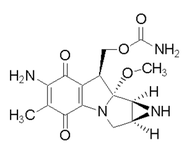

Figure 1. Structure of Mitomycin C

Cycloheximide (Fig. 2) binds to the ribosome and blocks translational elongation, thus inhibiting protein biosynthesis of the cells.

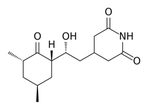

Figure 2. Structure of Cycloheximide

bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP, Fig. 3) induces a decrease of the mitochondrial membrane potential of CHO cells.

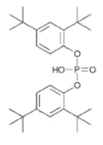

Figure 3. Structure of bDtBPP

Cell based assay

Two cell lines were used to compare the effect of the cytotoxins: the adherent L-929 cell line as recommended in the USP <87>, and a suspension CHO clone (Cellca, D). Cells were expanded for one passage before transferring them into multi-well plates (MWP). The cytotoxins where dissolved either in DMSO or in phosphate buffer based on their dissolubility and added to the medium as a single addition.

Cells were grown for 1 or 3 days, respectively and afterwards counted with the NucleoCounter (CHO) or used for an XTT-Test (L-929). Culture conditions are listed in Table 1 for pre-cultivation and Table 2 for the cytotoxicity.

Table 1. Pre-culture conditions

Parameter	Setpoint CHO DG44	Setpoint L-929
Shaking Rate	120 rpm	Static
Orbital Diameter	50 mm	Static
Temperature	36.8 °C	37 °C
pCO_2	7.5 %	5 %
Humidity	85 %	100 %
Initial Cell Density	0.2 · 10^6 cells/mL	2 · 10^6 cells/flask ~17,000 cells/cm^2
Working volume	150 ml	50 mL
Cultivation time	3 days	3 days
Base medium	SMD-6 (ActiCHO)	MEM
Serum content	0 %	10 % FBS
Container	Erlenmeyer flask (500 ml)	T-175 flask

Table 2. Test conditions

Parameter	Setpoint CHO DG44	Setpoint L-929
Shaking Rate	160 rpm	Static
Orbital Diameter	50 mm	Static
Temperature	36.8 °C	37 °C
pCO_2	7.5 %	5 %
Humidity	85 %	100 %
Initial Cell Density	0.2 · 10^6 cells/mL 2 · 10^6 cells/well	1 · 10^5 cells/mL 1 · 10^4 cells/well
Working volume	10 mL/well	100 µL/well
Cultivation time	3 days (cytotoxin)	1 day (expansion) +1 day (+cytotoxin)
Base medium	ActiCHO	MEM
Serum content	0 %	5 %
Container	ThinCert 6WP	96WP

RESULTS AND DISCUSSION

Sensitivity to bDtBPP

The sensitivity of CHO-DG44 growth test to bDtBPP was compared to the L-929 test sensitivity (Fig. 4). Due to the dissolubility, bDtBPP was dissolved in DMSO, followed by a dilution in cell culture medium.

CHO cell growth was significantly impacted at concentrations >0.21 mg/L, and was reduced to 21% normalized growth (compared to the reference) at 0.42 mg/L. According to this results an EC_{50} concentration of approx. 0.3 mg/L was determined for CHO-DG44.

However, L-929 cell growth was only moderately affected by 0.21 – 3.36 mg/L bDtBPP. While it requires more data to calculate the EC_{50} it is obvious that it is higher than 16.8 mg/L. Separate cytotoxicity tests with DMSO confirmed that the solvent does not impact the bDtBPP test under these conditions (data not shown).

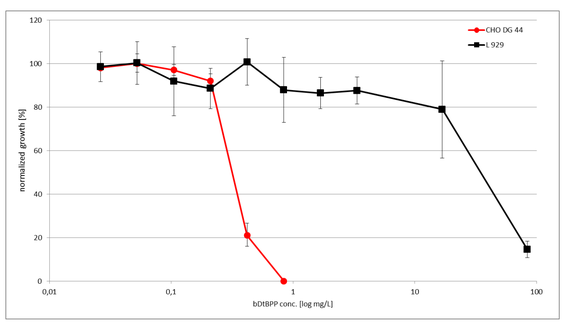

Figure 4. Dose dependency of bDtBPP-cytotoxicity on CHO-DG44 and L-929 cell growth.

Sensitivity to Cycloheximide

Again, DMSO is required as solvent for Cycloheximide. Cell proliferation of both cell lines is strongly dose-dependent (Fig. 5). In the presence of 1 mg/L L-929 cell growth was reduced to 60% compared to the reference. For concentrations above 1 mg/L the cell growth-interfering influence of DMSO has to be examined in more detail, e.g. by interference tests. Thus, the EC_{50} could not be calculated from the available data.

In contrast, the proliferation of the CHO-DG44 cell line was impacted more strongly by Cycloheximide with an EC_{50} of approx. 0.07 mg/L. At this concentrations DMSO does not impact the cytotoxicity test on Cycloheximide (data not shown).

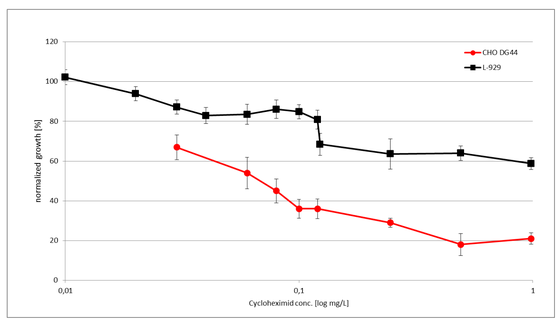

Figure 5. Dose dependency of Cycloheximide-cytotoxicity on CHO DG44 and L-929 cell growth.

Sensitivity to Mitomycin C

As opposed to the bDtBPP and Cycloheximide, Mitomycin C is highly soluble in water and was dissolved in PBS buffer. Therefore, an influence of a solvent can be ruled out for this test.

As shown with bDtBPP, Mitomycin C (Fig. 6) was more toxic to CHO-DG44 than to L-929 cells. With an EC_{50} of 0.05 mg/L Mitomycin C is the strongest cytotoxin of the three toxins assessed. For the L-929 cells the EC_{50} is approx. 29 mg/L. Until now there is no data available on the other toxin to conclude which of the three toxins is most toxic for the L-929 cells.

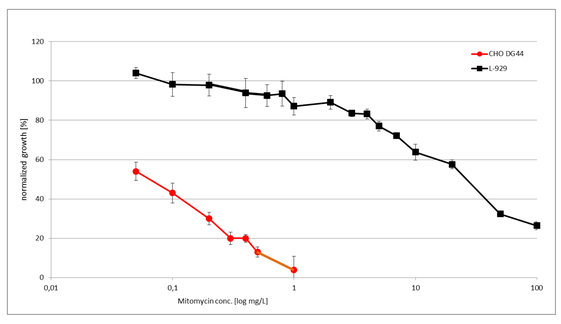

Figure 6. Dose dependency of Mitomycin C-cytotoxicity on CHO-DG44 and L-929 cell growth.

Impact of serum on cytotoxicity

Due to the different cell line specific toxin sensitivity the impact of the test conditions need to be assessed. One major difference is the use of a chemically defined medium for the CHO cells and the use of a serum-containing medium for the L-929 cells. Serum albumin is known for its extraordinary ligand binding capacity [4]. Therefore, we assessed the impact of serum on the CHO test with bDtBPP in the absence and presence of different serum concentrations (Fig. 7). The result clearly show that the cytotoxic effect of bDtBPP can be masked at least up to 0.84 mg/L. It is likely that the outcome of the cytotoxicity test with the recommended L-929 test is dominated by the ligand binding capacity of serum as well.

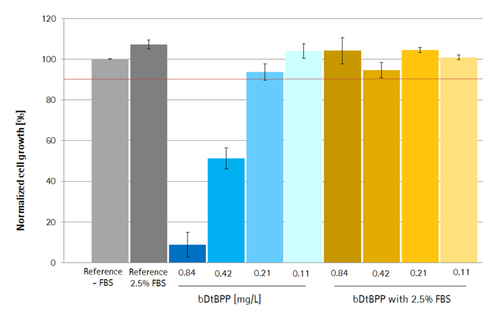

Figure 7. Effect of FBS on the cytotoxicity of bDtBPP during the cell growth test.

CONCLUSION

All three cytotoxins showed increased cytotoxicities for CHO cells compared to L-929 cells. CHO cells seem to be more suitable for testing of raw materials for biopharma processes. These results indicate that serum albumin is impacting the test outcome of the cytotoxicity tests by its extraordinary ligand binding capacity. For a more detailed analysis it would be helpful to harmonize both growth tests in equipment involved. Further trials should focus on additional process parameters, in particular cultivation time.

Acknowledgement

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References

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