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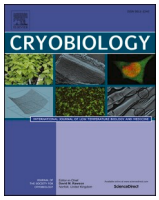

Reducing dimethyl sulfoxide content in Jurkat cell formulations suitable for cryopreservation

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ABSTRACT

Cell-based medicinal products (CBMPs) are usually cryopreserved in formulations containing up to 10 % dimethyl sulfoxide (Me_2SO) at temperatures below −145 °C. Although Me_2SO effectively protects cells during the freezing process, it can be damaging to cells at ambient temperatures and lead to side effects in patients. The aim of this study was to reduce the amount of Me_2SO in cryopreservation formulations for an immortalized T cell line (Jurkat cells). A design of experiment (DoE) approach was applied for formulation development using seven different excipients, i.e., Me_2SO, trehalose, sorbitol, proline, ectoine, poloxamer 188 (P188) and poly vinyl pyrrolidone 40 (PVP). A DoE model was generated to predict optimal formulations resulting in a high post-thaw viability and a high glass transition temperature of the formulation to allow for frozen storage without the use of liquid nitrogen. Subsequently a stability study was performed with promising lead candidates over three months at storage temperatures of −145 °C, −80 °C, −40 °C. Three benchmark solutions were used, i.e., Cryostor CS10, CryoSOfree as well as 10 % Me_2SO in Roswell Park Memorial Institute Medium (RPMI). The excipient affecting the post-thaw viability of Jurkat cells the most was, as expected, Me_2SO, which led to increased viabilities at higher concentrations. Most formulations resulted in similar viabilities for cells stored at −145 °C and −80 °C, whereas samples stored at −40 °C did not survive. In general, benchmark formulations resulted in slightly higher viabilities than the tested formulations. Furthermore, cell samples stored at −80 °C were recultivated in cell culture and the viability was assessed after 24h. The cell viability after 24h was much lower compared to the cells analyzed directly post-thaw, indicating that freeze-thaw damages continue to unfold after thawing. In summary, several promising excipients and combinations thereof, e.g., trehalose and PVP, were identified for the cryopreservation of Jurkat cells with reduced concentrations of Me_2SO or Me_2SO-free cryopreservation. Additionally, storage at −80 °C is possible for the developed formulations.

1. Introduction

Cell-based medicinal products (CBMPs) and gene therapy products are a rapidly growing field within the biopharmaceutical industry offering treatment options for severe illnesses such as cancer or genetic diseases [1]. A prominent class of CBMPs are chimeric antigen receptor (CAR) T cells, with six products currently approved by the European Medicines Agency [2]. CBMPs can be either stored frozen or liquid at ambient or refrigerated temperatures [3], whereas liquid storage of CBMPs is limiting their shelf-life to several hours to days [4,5,6]. Therefore, to enable long-term storage of CBMPs cryopreservation is commonly used. The most standard cryoprotective agent (CPA) is dimethyl sulfoxide (Me_2SO), which has been introduced by Lovelock and Bishop in the 1950s [7]. Me_2SO is a cell membrane permeable CPA and protects against freezing damage by reducing intracellular ice formation [8,9]. Commonly used cryopreservation media for CBMPs in clinical trials contain 5–10 % Me_2SO [10]. Despite being used successfully in academia and in approved CBMPs, alternatives to Me_2SO are

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needed as Me_2SO is damaging to cells at ambient temperatures, which limits the handling and processing time of cells during manufacturing [11]. Furthermore, attention must be given to the choice of primary packaging as well as infusion tubing as Me_2SO can promote the release of leachables and extractables from plastic materials [12,13], which requires additional testing for in-use stability [14,15]. Patients may also experience adverse effects such as gastrointestinal, cardiovascular and neurological complications following infusion of Me_2SO-containing products [16,17,18]. Cryopreservation storage is commonly done at temperatures below −140 °C, which is below the glass transition temperature of water [19]. Also, 10 % Me_2SO in phosphate buffered saline (PBS) has a very low glass transition temperature (T_g') with an onset of T_g' at ~ −120 °C [20]. For storage of cryopreserved samples below −140 °C either liquid nitrogen cooling or ultra-low freezers are required. Elevating the storage temperature to, e.g., −80 °C by reducing the concentration of Me_2SO and adding alternative excipients with a higher T_g' would be logistically beneficial. Storage at −140 °C requires a constant supply of liquid nitrogen or ultra-low freezers, which are very energy intensive. In addition, it would be easier to secure the supply chain for CBMP if higher storage temperatures were possible.

Various alternatives to Me_2SO as CPA from small molecules to polymers have been evaluated over the last decades [21,22]. Usually, combinations of different CPAs are needed to successfully cryopreserve cells with reduced Me_2SO concentrations or in Me_2SO-free formulations [23]. Disaccharides such as trehalose are cell-membrane impermeable CPAs, which are thought to be cell membrane stabilizing [24], as well as increasing the glass transition temperature [25]. Similarly, sugar alcohols such as mannitol or sorbitol were previously evaluated for the cryopreservation of cells in combination with sugars and amino acids [23,26]. A commonly used amino acid is proline, which is cell membrane stabilizing similar to trehalose [24]. Furthermore, substances or mechanisms occurring in nature, i.e., in organisms or plants surviving extreme conditions, can be used as a promising approach for cryopreservation [27]. Ectoine, which was isolated from a halophilic bacterium (*Ectothiorhodospira)*, is an example of a natural occurring osmolyte, protecting the organism against high salt concentration [28]. It has been shown that combinations of ectoine with poly-l-lysine and dextran can cryoprotect natural killer cells equally well as 10 % Me_2SO [29]. Lastly, polymers such as polyvinylpyrrolidone 40 (PVP) or poloxamer 188 (P188) can be used for the cryopreservation of cells [21]. From the above mentioned excipients seven compounds were selected, i.e., Me_2SO, trehalose, sorbitol, proline, ectoine, P188 and PVP, for a formulation development study. Ideally, synergistic effects can be achieved by combining excipients from several categories and with different cryoprotective mechanisms. To minimize the number of experiments a design of experiment (DoE) approach was used. A DoE is a statistical approach to model the design space for a formulation [30]. Within a DoE, the effect of the excipients on selected responses (quality attributes) is used to evaluate suitable formulation compositions [30]. The chosen responses depend upon the intended purpose of the formulation development study. For instance, an optimal cryopreservation formulation should result in a high-post thaw viability. Within this study also the T_g' of the formulations was considered with the aim to increase the storage temperature from −140 °C to −80 °C and potentially even to −40 °C.

Two aims were defined for this study: (i) the reduction or elimination of Me_2SO within cryopreservation formulations as well as (ii) evaluating the storage of cells at elevated subzero temperatures such as −80 °C. Jurkat cells, an immortalized T cell line, were used as a model for CAR T cells [31,32]. A design of experiment (DoE) approach was used to screen different formulations followed by one-factor-at-a-time (OFAT) optimization. For the DoE approach we selected appropriate concentration ranges based on literature values [33,34,35] and considering the overall osmolality of the formulations. The DoE software generated the first 17 formulations based on the initial input parameters, which built the base for the formulation screening. Subsequently, a fold-over was performed within the DoE software to evaluate possible excipient interactions. Additionally, we performed a OFAT formulation optimization by selecting promising excipients from the previous DoE studies and changing only one excipient. All tested formulations were used to build a DoE model and to predict the optimal formulation composition. Finally, the lead formulation candidates were further tested in a three-month stability study at different storage temperatures. As benchmark formulations Cryostor CS10 (CS10), containing 10 % Me_2SO, as well as CryoSOfree, a Me_2SO-free cryopreservation medium, were used as commercially available cryopreservation solutions. Additionally, 10 % Me_2SO in Roswell Park Memorial Institute 1640 (RPMI 1640) was used. Post-thaw viabilities were measured either directly after thawing or after 24h of cell recultivation. Several promising formulation candidates were identified. However, further process optimization is needed to improve post-thaw viability as well as the recovery of cells after 24h.

2. Material & methods

2.1. Cell culture

Jurkat cells, an immortalized cell line as model for T cells, were purchased from CLS GmbH (Eppelheim, Germany). Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 % fetal calf serum (FCS, Bio&Sell GmbH) was used as culture medium and cell densities were kept between 0.5 x 10^6 and 3 x 10^6 cells/ml.

2.2. Materials

Trehalose dihydrate and poloxamer 188 (P188) were obtained from Merck. Ectoine, polyvinyl pyrrolidone 40 (PVP40), CryoSOfree Me_2SO free cryopreservation medium, Cryostor CS10, CalceinAM, Me_2SO Hybri-Max and RPMI 1640 were purchased from Sigma Aldrich. Sorbitol and L-proline were purchased from PanReac AppliChem, ITW Reagents. PBS pH 7.4, RPMI 1640 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal calf serum (FCS), Annexin V-FITC dead cell apoptosis kit with Annexin V-FITC and propidium iodide (PI), 0.5 M HEPES with pH 7.4 as well as cryovials (Nunc) were obtained from ThermoFisher Scientific. Ringer's acetate was obtained from Deltamedica.

2.3. Formulation prescreening based on DoE

A design of experiment (DoE) approach with the software Modde Pro 13 (Sartorius) was used for an initial formulation development of cryopreservation media. A DoE based on seven factors, i.e., excipients, at two concentrations (0 and 'high') and two responses was generated. The high concentration values (upper limits) were based on reported literature ranges [33,34,35]. An overview of the initial concentration range for each excipient can be found in Table 1. As multiple excipients were combined, the concentration range for each individual excipient was kept relatively low to avoid high osmolalities, i.e., very hypertonic conditions, in the final formulations. A fractional factorial design with a resolution of IV was used. As responses a high post-thaw viability as well as a high T_g' of the formulation were selected. Ringer's acetate

Table 1
Concentration range of excipients used in the initial design of experiment using a fractional factorial design (Resolution IV).

Excipient	Low concentration	High concentration
Me_2SO [%]	0	5
Trehalose [mM]	0	150
Sorbitol [mM]	0	150
Proline [mM]	0	100
Ectoine [mM]	0	100
P188 [%]	0	1
PVP40 [%]	0	5

supplemented with 20 mM HEPES was used as base medium (RiAc-HEPES) for all subsequent formulation preparation after preparation from 0.5 M HEPES buffer pH 7.0 (AlfaAesar, ThermoFisher Scientific) and pH adjustment to pH 7.4 with a 1 M sodium hydroxide (NaOH) solution (VWR chemicals).

Based on the results of the initial fractional factorial design a fold-over was performed using the Modde software. Subsequently, additional formulations were tested based on promising formulations to evaluate the possibility to reduce Me_2SO by increasing the concentration of the other excipients. For these additional formulations the upper concentration limit for trehalose and sorbitol was changed to 300 mM and the limits for proline and ectoine were increased to 200 mM, whereas Me_2SO was reduced to 2.5 % or 0. For each excipient, except Me_2SO, stock solutions were prepared by dissolving in RiAc-HEPES. Formulation preparation was performed by mixing the required amount of stock solutions with RiAc-HEPES to into conical tubes to reach 125 % of the intended concentration for each excipient. Subsequently, the 1.25-fold concentrated formulations were used for cryopreservation of Jurkat cells by diluting the buffer with cell suspension to 1x.

Cryopreservation for the prescreening was performed by preparing cells in formulation within cryovials, i.e., cryovials with 200 µl of cells at a concentration of $5x10^6$ cells/ml were prepared and 800 µl of the respective 1.25-fold formulation were added dropwise to the cells to reach the final formulation concentration and a cell concentration of $1x10^6$ cells/ml. Duplicate samples were prepared and frozen as described below.

2.4. Stability study of the lead candidates

Subsequently, a stability study was conducted with Jurkat cells over a period of three months. Seven promising formulations from the prescreening were included. The generated DoE model was used to optimize the formulations, whereas the upper limit of Me_2SO was limited to a concentration of 2.5 %. The predicted optimum was 2.5 % Me_2SO, 300 mM trehalose and 5 % PVP, which correlates to the already tested DF61 from the prescreening. Additionally, five new formulations were included based on a rational design of what was previously observed throughout the prescreening, i.e., combinations of trehalose and PVP were tested with or without additional promising excipients. All formulations were prepared by dissolving the excipients in the base medium RiAc-HEPES at a 1.25-fold concentration. The pH was adjusted to pH 7.4, where required with 1 M NaOH (VWR chemicals). For comparison three different benchmark media were included, namely Cryostor CS10 and CryoSOfree, both commercially available cryopreservation solutions, as well as 10 % Me_2SO in RPMI. An overview of the formulations and media included in the stability study can be found in Table 2. The pH was measured for all cryopreservation formulations using a pH-meter (Mettler Toledo). Additionally, the osmolality was determined by freezing-point depression using a Gonotec Osmomat 3000 (Gonotec). Jurkat cells were formulated and stored for three months at −145 °C, −80 °C and −40 °C until thawing and subsequent analysis. For each formulation three biological replicates were frozen.

Cell samples for the stability study were prepared in 50-ml conical tubes. In total 15 ml of cell solution were prepared for each formulation. First, a cell stock was prepared by centrifugation of freshly harvested Jurkat cells (400 g, 5 min) followed by resuspension in RiAc-HEPES to a cell concentration of $5x10^6$ cells/ml. A volume of 3 ml cell stock was aliquoted into 50-ml conical tubes and subsequently 12 ml of the respective cryopreservation formulation were slowly added. Similarly, cells were prepared with 12.5 % Me_2SO in RPMI resulting in a final concentration of 10 % Me_2SO. Benchmark samples were prepared by centrifugation of freshly harvested cells (400 g, 5 min) and direct resuspension in Cryostor CS10 and CryoSOfree, respectively. All cell samples were aliquoted into 1.8-ml cryovials to a sample volume of 1 ml cell suspension and frozen as described below.

2.5. Cryopreservation process

The prepared samples were frozen in an isopropanol filled Mr. Frosty device (18 cryovials with 1–2 ml fill volume, ThermoFisher Scientific) resulting in a freezing rate of approx. 1 °C/min in a −80 °C freezer. Samples for the prescreening were transferred to a −145 °C freezer after 5h and stored overnight. Cell stability samples were either left at −80 °C or transferred to −145 °C or −40 °C after 5h. Stability samples were stored for at least three months prior thawing.

Following storage cryovials were thawed by putting them into a water bath at 37 °C for approximately 3 min. To avoid long-standing times at room temperature only two samples were thawed in parallel. Thawed cell samples were diluted by slowly adding 1 ml of RPMI 1640 and subsequently transferred into a 15-ml conical tube. RPMI 1640 was slowly pipetted into the conical tubes until a final volume of 10 ml was reached. The diluted cells were centrifuged (400g, 5 min) and resuspended in 1 ml of RPMI 1640. Cell samples intended for recultivation were only resuspended in 900 µl of RPMI 1640 and 100 µl of FCS was added to complete the cell growth medium. After resuspension cells were either directly analyzed (T-FT) or transferred into a 24-well plate (Costar, Corning) for recultivation for 24h (T-Rec).

2.6. Viable cell counting and viability determination based on trypan blue staining

Trypan blue staining (Carl Roth) was used to count viable cells

Table 2
Overview of formulations tested within a three-month stability study. DF: design of experiment formulations; F: additional new formulations based on rational design. CS10: Cryostor CS10; CSO: CryoSOfree; 10 %: 10 % Me_2SO in RPMI; Me_2SO: dimethyl sulfoxide; P188: poloxamer 188; PVP40: polyvinyl pyrrolidone 40.

Formulation		Me_2SO [v/v %]	Trehalose [mM]	Proline [mM]	Ectoine [mM]	P188 [w/v %]	PVP40 [w/v %]
Prescreening	DF_4	5	150	0	0	1	5
	DF_56	0	300	100	0	0	5
	DF_60	2.5	0	0	100	0	5
	DF_61	2.5	300	0	0	0	5
	DF_67	0	300	50	50	0	5
	DF_72	2.5	300	50	50	0	0
	DF_73	2.5	300	50	50	0	5
New formulations	F1	0	300	0	0	0	5
	F2	2.5	300	0	100	0	5
	F3	0	300	25	100	0	5
	F4	2.5	300	25	100	0	5
	F5	2.5	200	0	0	0	5
Benchmarks	CS10	10	not disclosed				
	CSO	0	not disclosed				
	10 %	10	0	0	0	0	0

within a sample by preparing 10-fold dilutions for fresh cells and 2-fold dilutions following cryopreservation. A hemocytometry Neubauer counting chamber (Karl Hecht GmbH & Co KG) was used and the number of viable and dead cells within 4 quadrants was counted. Viable cell recovery was calculated as follows:

$$viable\ cell\ recovery\ [\%] = \frac{viable\ cells\ (T-X)}{viable\ cells\ (T0)},$$

where T-X is referring to measurement time points after cryopreservation and T0 is referring to viable cell numbers after the final preparation in cryopreservation formulations. For benchmark formulations a theoretical cell number of $1x10^6$ cells was assumed at T0. Viability was calculated based on:

$$viability\ [\%] = \frac{viable\ cells\ (T-X)}{all\ cells\ (live\ and\ dead\ )\ (T-X)},$$

where T-X is referring to the respective measurement time point, comparing the viability of cells within a sample.

2.7. Imaging flow cytometry for viability assessment

An Amnis FlowSight instrument (Luminex) was used to assess cell viability with two different fluorescence assays. To differentiate between viable and dead cells CalceinAM, which is hydrolyzed to fluorescent Calcein by viable cells, and PI, which only permeates into dead cells, were used. Cell samples were centrifuged and resuspended in PBS prior addition of CalceinAM and PI. The cells were stained for 20 min in the dark and measured with a 488-nm fluorescence excitation laser. Additionally, a double staining to differentiate dead and apoptotic cells from viable cells were used as well: Annexin V-FITC is staining apoptotic cells and PI is staining dead cells. Viable cells were unstained in this assay. Cells were prepared by two washing steps, first into PBS and secondly into Annexin V binding buffer. The cells were stained for 15 min in the dark and subsequently measured with a 488-nm fluorescence excitation laser. The software IDEAS 6.2.183 was used to analyze the data.

2.8. Quantification of cell-sized particles by flow imaging microscopy (FIM)

To determine the total number of cell-sized particles as well as for cell sizing a FlowCam 8100 (Fluid Imaging Technologies) system was used. The instrument is equipped with a high-resolution CMOS camera (1920×1200 pixels) and an objective resulting in a 20× magnification was used. Imaging was performed with 27 frames per second and intensity thresholds of 12 for light and dark pixels were applied to detect particles within the sample. A 1 % [w/v] Terg-a-zyme enzyme detergent solution and highly purified water was used to clean the instrument between measurements. Samples were measured after 5-fold dilution with PBS. The dilution factor was taken into account for all subsequent particle/ml concentrations. Particles with a diameter between 8 and 25 μm were considered a cell. Absolute cell-sized particle recovery was calculated by the following equation:

$$cell-sized\ particle\ recovery\ [\%] = \frac{cells\ (T-X)}{cells\ (T0)}\ x\ 100,$$

whereas T-X is referring to the measurement time point and T0 refers to the number for cells measured from cell stock solution in RiAc-HEPES after 5-fold dilution to the target concentration (same sample preparation as cryopreservation formulations).

2.9. Glass transition temperature determination by differential scanning calorimetry (DSC)

To determine the glass transition temperature of the maximally freeze-concentrated solution (T_g') differential scanning calorimetry (DSC) was used. A Mettler Toledo DSC 1 instrument was used to measure T_g'. For each measurement, 30 μl of the final formulation were pipetted into aluminum crucibles (Mettler Toledo) and crimped. Subsequently, the measurements were performed by cooling to −95 °C, holding for 10 min at −95 °C and rewarming to 20 °C. The cooling and warming rate was 10 °C/min. During the heating scan the T_g' can be determined as the midpoint of the endothermic shift of the baseline. Furthermore, selected formulations containing Me_2SO at 5 % or 2.5 % were additionally measured by using a Netzsch instrument with a liquid nitrogen cooler. Similarly, formulations were analyzed using a crimped aluminum crucible. The samples were cooled to −150 °C and reheated to 20 °C with a scanning rate of 10 °C/min. Formulations containing 5 % Me_2SO or 2.5 % Me_2SO with low concentrations of trehalose or no trehalose were measured with the Netzsch instrument to a cooling temperature of −150 °C, whereas formulations containing 2.5 % Me_2SO with high concentrations of trehalose or Me_2SO-free formulations were measured with the Mettler Toledo instrument with a minimum temperature of −95 °C. The T_g' for samples measured with the Netzsch instrument were similarly determined to the Mettler Toledo measurements.

2.10. Statistical analysis

Statistical analysis was performed by using Graphpad Prism 9.5. Results are reported as mean values ± standard deviation. To compare mean values of all formulations one-way ANOVA followed by Tukey's test was performed. For the comparison of selected formulations, e.g., Me_2SO-containing and corresponding Me_2SO-free formulation, one-way ANOVA followed by Bonferroni multiple comparison test was used. Results were considered significant, when $p < 0.05$.

3. Results

3.1. Formulation prescreening based on DoE

An initial fractional factorial design of experiment with a resolution of IV was used based on 7 excipients at two concentrations (Table 1), resulting in 17 different formulations. Two response factors, i.e., post-thaw viability based on CalceinAM and T_g', were measured. The composition of the formulations within the initial DoE can be found in Table 3. An overview of the obtained viabilities and T_g' can be found in Fig. 1.

A clear correlation between post-thaw viability and presence of Me_2SO can be found with formulations containing Me_2SO resulting in viabilities ranging from approximately 40 to 80 %, whereas formulations without Me_2SO did not result in viable cells. On the other hand, formulations containing Me_2SO showed lower T_g' values as determined by DSC measurements with values ranging from −95 °C to −79 °C. Subsequently, the Modde software was used to complement the DoE with additional experiments to further understand the impact of the excipients. The composition of the additional formulations including the resulting viabilities can be found in Table S1 (DF18 to DF45). Similar to the previous experiments the effect of Me_2SO on the post-thaw viability of Jurkat cells was predominant. To further test the protective effect of single excipients at higher concentrations additional experiments were performed, in which the concentrations of trehalose and sorbitol were increased to 300 mM and the concentration of proline as well as ectoine was increased to 200 mM (DF46 to DF49). Compared to the low concentration single excipients (DF19, DF21, DF25, DF32) a very small increase of 1–4 % in viability was observed, indicating that the optimal concentration range of excipients was not yet included. In a last set of experiments, additional formulations were tested, based on promising

Table 3
Overview of the formulation composition within the initial DoE experiment based on a fractional factorial design (resolution IV) and their resulting post-thaw viability for Jurkat cells. Me_2SO: dimethyl sulfoxide; P188: poloxamer 188; PVP40: polyvinyl pyrrolidone 40.

	Me_2SO [v/v %]	Trehalose [mM]	Sorbitol [mM]	Proline [mM]	Ectoine [mM]	P188 [w/v %]	PVP40 [w/v %]	Viability [%]
DF_1	0	0	0	0	0	0	0	0 ± 0
DF_2	5	0	0	0	100	0	5	76 ± 6
DF_3	0	150	0	0	100	1	0	1 ± 1
DF_4	5	150	0	0	0	1	5	74 ± 7
DF_5	0	0	150	0	100	1	5	1 ± 0
DF_6	5	0	150	0	0	1	0	58 ± 4
DF_7	0	150	150	0	0	0	5	12 ± 7
DF_8	5	150	150	0	100	0	0	33 ± 20
DF_9	0	0	0	100	0	1	5	0 ± 0
DF_10	5	0	0	100	100	1	0	43 ± 3
DF_11	0	150	0	100	100	0	5	3 ± 1
DF_12	5	150	0	100	0	0	0	70 ± 10
DF_13	0	0	150	100	100	0	0	1 ± 1
DF_14	5	0	150	100	0	0	5	60 ± 3
DF_15	0	150	150	100	0	1	0	0 ± 0
DF_16	5	150	150	100	100	1	5	1 ± 1
DF_17	2.5	75	75	50	50	0.5	2.5	1 ± 0

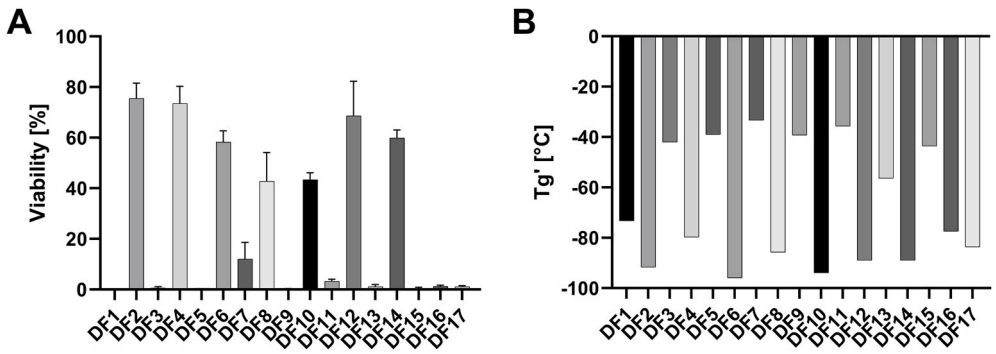

Fig. 1. Viabilities of Jurkat cells after overnight storage at −145 °C based on CalceinAM staining (A) and T_g' of the respective formulations (B). DoE formulations were used based on a fractional factorial design with 7 different factors, i.e., excipients. Even numbered formulations contain 5 % Me_2SO, whereas odd numbered formulations do not contain Me_2SO. For the viability the average of duplicate measurements and standard deviation is shown.

formulations tested so far by reducing the amount of Me_2SO to 0 or 2.5 % and increasing the concentration of the other excipients. The composition of these formulations and their resulting viabilities can be found in Table S1 (DF51 to DF80).

Subsequently, a DoE model based on all tested formulations (DF1 to DF80) was generated. Fig. 2 shows the resulting sweet spot plot highlighting the design space, where both response criteria were met, i.e., post-thaw viability above 45 % and a T_g' above −65 °C. Regarding the post-thaw viability the most dominant factor was Me_2SO, which is further highlighted in a response contour plot in Fig. S1. However, Me_2SO is also the excipient lowering T_g' the most, which can be seen in the response contour plot for T_g' in Fig. S2. Due to the positive dominant effect of Me_2SO on the post-thaw viability as well as the negative dominant effect of Me_2SO on the T_g', the design space for formulation development is rather limited and additional excipients are needed to compensate the negative T_g' effect of Me_2SO or to improve post-thaw viability similar to Me_2SO. The combination of trehalose and at least 5 % PVP40 seems to be crucial to meet both response criteria as indicated by Fig. 2. In summary, a reasonable model was generated for T_g' with a high R^2 (0.89) and Q^2 (0.87) as well as a good model validity (0.85) as shown in the supplementary (Fig. S3). However, the model for viability showed a lower R^2 (0.70) and Q^2 (0.67) and very low model validity (−0.2) (Fig. S3).

3.2. Stability study of the lead candidates

Subsequently, a three-month stability study was conducted at either −145 °C, −80 °C or −40 °C storage temperature. Seven promising formulations from the prescreening were included as well as five new formulations based on learnings obtained throughout the previous formulation screenings. As benchmark formulations commercially available Cryostor CS10, containing 10 % Me_2SO, and CryoSOfree, a Me_2SO-free medium, were included. Additionally, 10 % Me_2SO in RPMI was used as a benchmark. An overview of all tested formulations including their corresponding osmolality, pH and T_g' can be seen in Table 4. In general, formulations containing Me_2SO showed higher osmolalities (above 1000 mOsm/kg) in comparison to Me_2SO-free formulations. The T_g' for formulation DF4 and DF60 were around −80 °C, whereas the other formulations were in the range between −29 °C and −53 °C.

In the following results obtained for samples stored at −80 °C are described. After thawing (T-FT) cells were directly analyzed based on Trypan blue, CalceinAM and Annexin V staining. An additional vial was thawed to recultivate the cells for 24h in cell culture and analyzed subsequently (T-Rec) with all three viability assays as mentioned above. FIM was also used to measure the total concentration of cell-sized particles within the samples. The cell-sized particle recovery based on FIM measurements considers all particles between 8 and 25 μm as cells and is normalized to the number of cells within the cell stock prior freezing. In addition, viable cell recovery was determined by Trypan blue staining. Fig. 3 shows the cell-sized particle recovery as well as the viable cell recovery for samples stored at −80 °C after T-FT (panel A and C) and after T-Rec (panel B and D). At T-FT most formulations led to cell-sized particle recoveries between 30 and 60 % with no significant differences between the formulations (Fig. 3 A). An exception is DF4, which resulted in significantly higher cell-sized particle recovery compared to the other

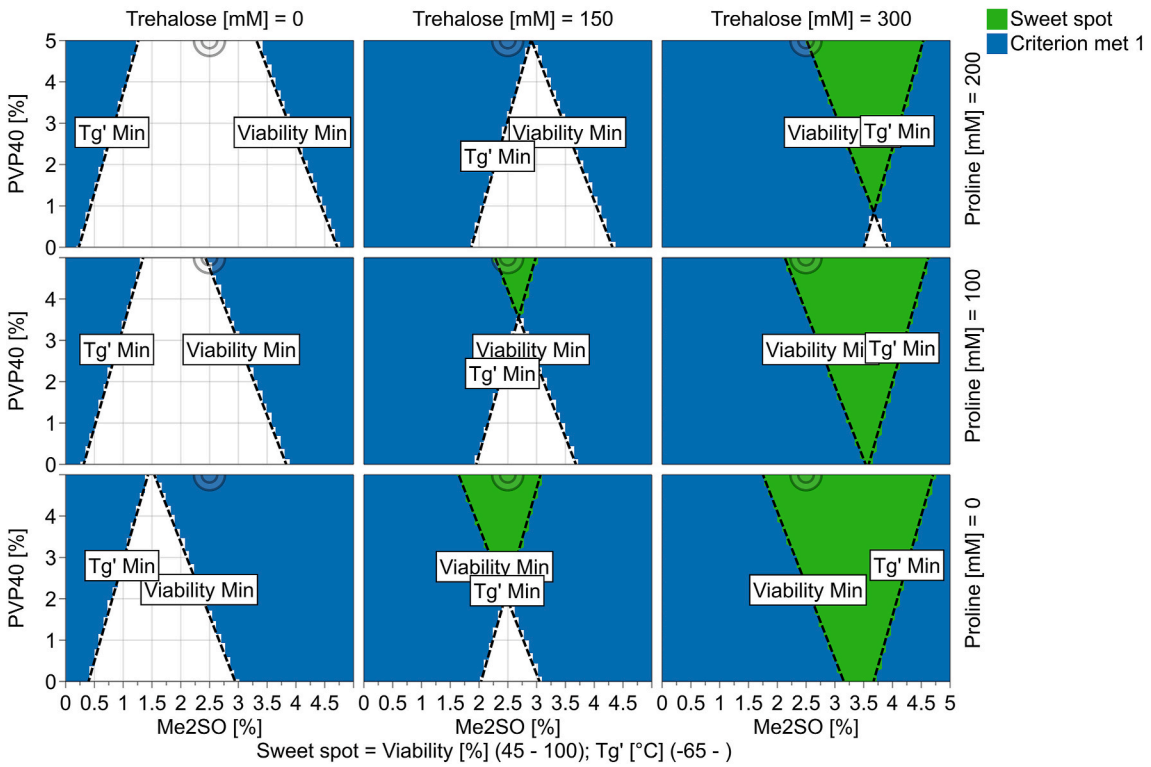

Fig. 2. Four-dimensional sweet spot plot highlighting the design space, where both response criteria, i.e., viability and Tg', are met in green. Blue: one criterion is met. White: no criterion is met. Ectoine, proline and poloxamer 188 (P188) did show little effect and were therefore not part of the predicted optimal design space (concentration was set to approximately 0). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Characterization of the used formulations and benchmarks for the cryopreservation of Jurkat cells including osmolality, pH and the glass transition temperature of the maximally freeze-concentrated solution (T_g'). 10 %: 10 % Me_2SO in RPMI; Me_2SO: dimethyl sulfoxide; P188: poloxamer 188; PVP40: polyvinyl pyrrolidone 40.

	Me_2SO [%]	Trehalose [mM]	Proline [mM]	Ectoine [mM]	P188 [%]	PVP40 [%]	Osmolality [mOsm/kg]	pH	T_g' [°C]
DF_4	5	150	0	0	1	5	1637 ± 16	7.36	−80
DF_56	0	300	100	0	0	5	857 ± 1	7.30	−30
DF_60	2.5	0	0	100	0	5	883 ± 2	7.38	−84
DF_61	2.5	300	0	0	0	5	1284 ± 9	7.31	−44
DF_67	0	300	50	50	0	5	856 ± 6	7.31	−31
DF_72	2.5	300	50	50	0	0	1254 ± 4	7.35	−53
DF_73	2.5	300	50	50	0	5	1475 ± 1	7.34	−45
F1	0	300	0	0	0	5	713 ± 1	7.35	−29
F2	2.5	300	0	100	0	5	1485 ± 3	7.33	−44
F3	0	300	25	100	0	5	896 ± 5	7.35	−31
F4	2.5	300	25	100	0	5	1520 ± 8	7.36	−44
F5	2.5	200	0	0	0	5	1064 ± 3	7.35	−48
CS10	10	not disclosed					3282 ± 11	7.57	−111
CSO	0	not disclosed					2425 ± 20	6.65	−51
10 %	10	0	0	0	0	0	2350 ± 20	7.64	−115

samples with approximately 100 % (one-way ANOVA). Cell-sized particle recoveries at T-Rec are slightly higher than at T-FT ranging from 40 to 75 %. Similar to T-FT, cell-sized particle recovery for DF4 at T-Rec is significantly higher than most samples except for F2, F4, F5 and DF56 (Fig. 3 B). Additionally, viable cell recovery was determined, which is lower than the total cell-sized recovery indicating that many of the recovered cell-sized particles are dead cells. DF4 resulted in a significantly higher viable cell recovery of around 30 % (except for CS10, 10 % Me_2SO and F2), whereas for the other samples values ranging from 4 to 17 % were obtained. However, after 24h of recultivation all samples led to a comparable low viable cell recovery between 4 and 16 %.

Moreover, Fig. 4 shows the size distribution of exemplary cell samples measured with FIM prior freezing (T0), after T-FT and T-Rec. An overview of the size distribution for all samples can be found in the supplementary (Fig. S4). Based on the size distribution a cell loss between T0 and T-FT can be detected for most samples except DF4. In DF4 only a small increase in cell debris could be detected and the particles/ml for a diameter of 10–20 μm are comparable. However, a size shift can be observed towards smaller particles as well as increase in cell debris after T-Rec, indicating that the cells are dying over time in cell culture. For the other formulations a cell loss can be observed from T0 to T-FT as the particle concentration decreased in the diameter range from 10 to 20 μm. Furthermore, a size shift towards smaller particles, i.e., cell debris can be seen for the tested formulations and CSO, but not for CS10 and only to a smaller extent for 10 % Me_2SO in RPMI.

Additionally, the viability of samples stored at −80 °C was determined by using three different assays, namely, Trypan blue, CalceinAM and Annexin V staining. Fig. 5 shows a comparison of all three viability

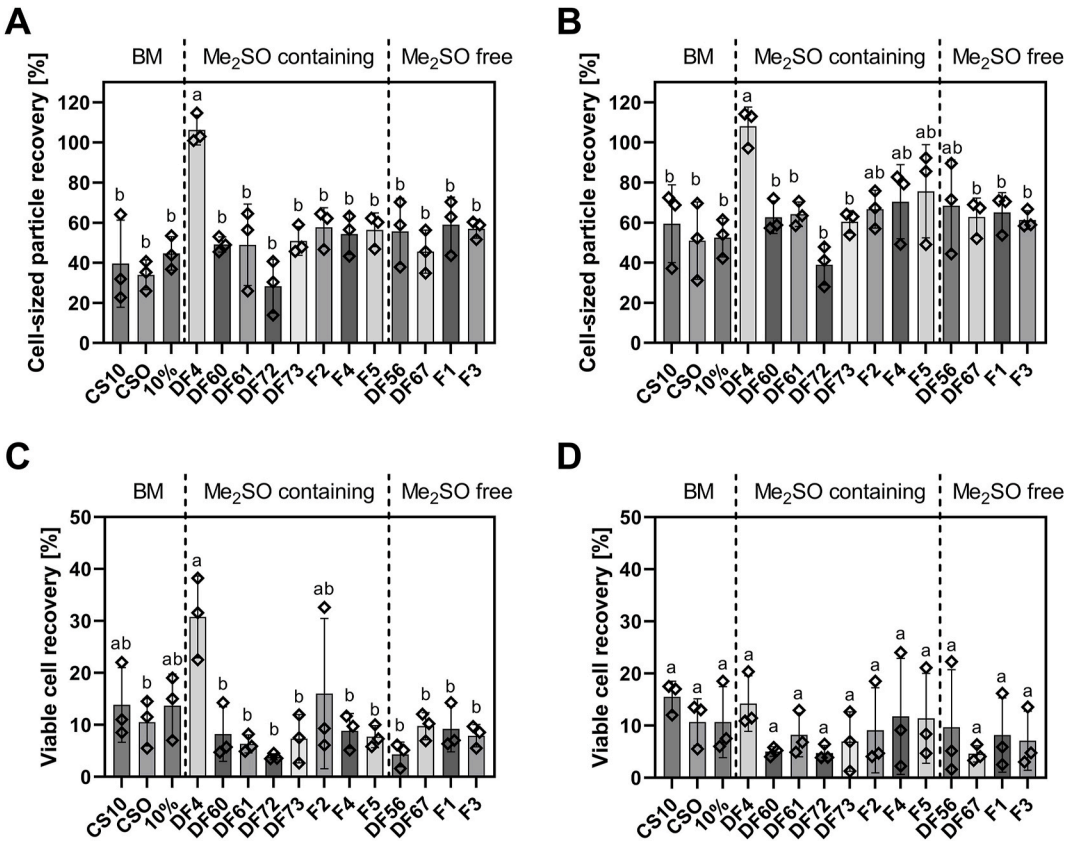

Fig. 3. Cell-sized particle recovery for Jurkat cells after three-month storage at −80 °C determined by FIM (A, B). All particles between 8 and 25 μm are considered cell-sized and numbers were normalized to the cell numbers prior freezing. Additionally, viable cell recovery was determined based on Trypan blue staining (C, D), where only viable cells were counted and normalized with the number of viable cells at T0. The left panel (A, C) shows the recoveries for cells directly after thawing (T-FT) and the right panel (B, D) shows the recoveries after 24h of recultivation of cells (T-Rec). Statistically significant differences ($p \leq 0.5$) based on one-way ANOVA followed by Tukey's test are indicated by different letters.

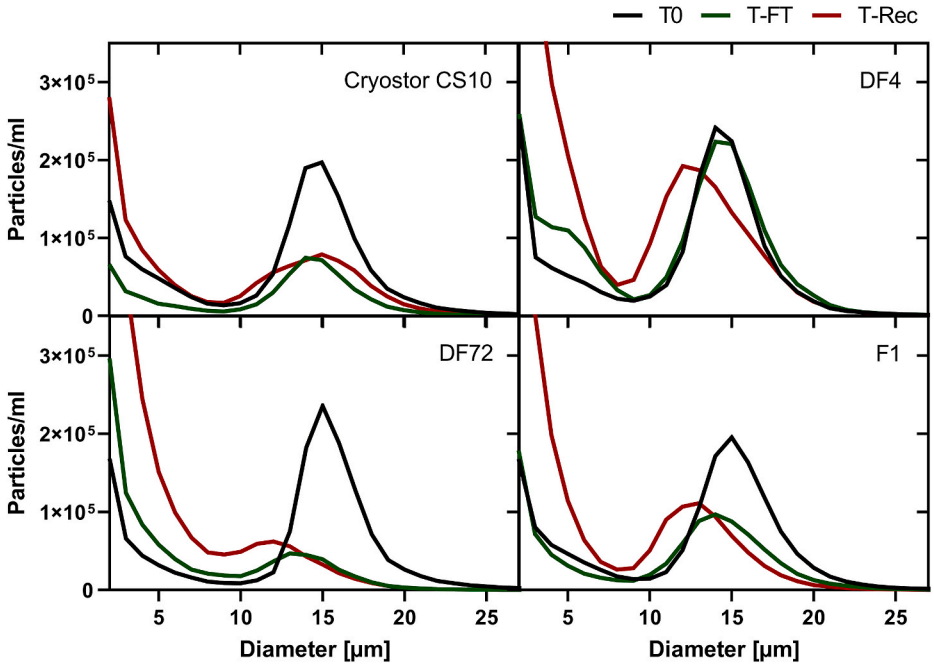

Fig. 4. Comparison of the size distribution of cell samples after harvest in RiAc-HEPES (black line), directly after thawing (T-FT) and after 24h of recultivation (T-Rec). Exemplary size distributions are shown for CS10, DF4, DF72 and F1.

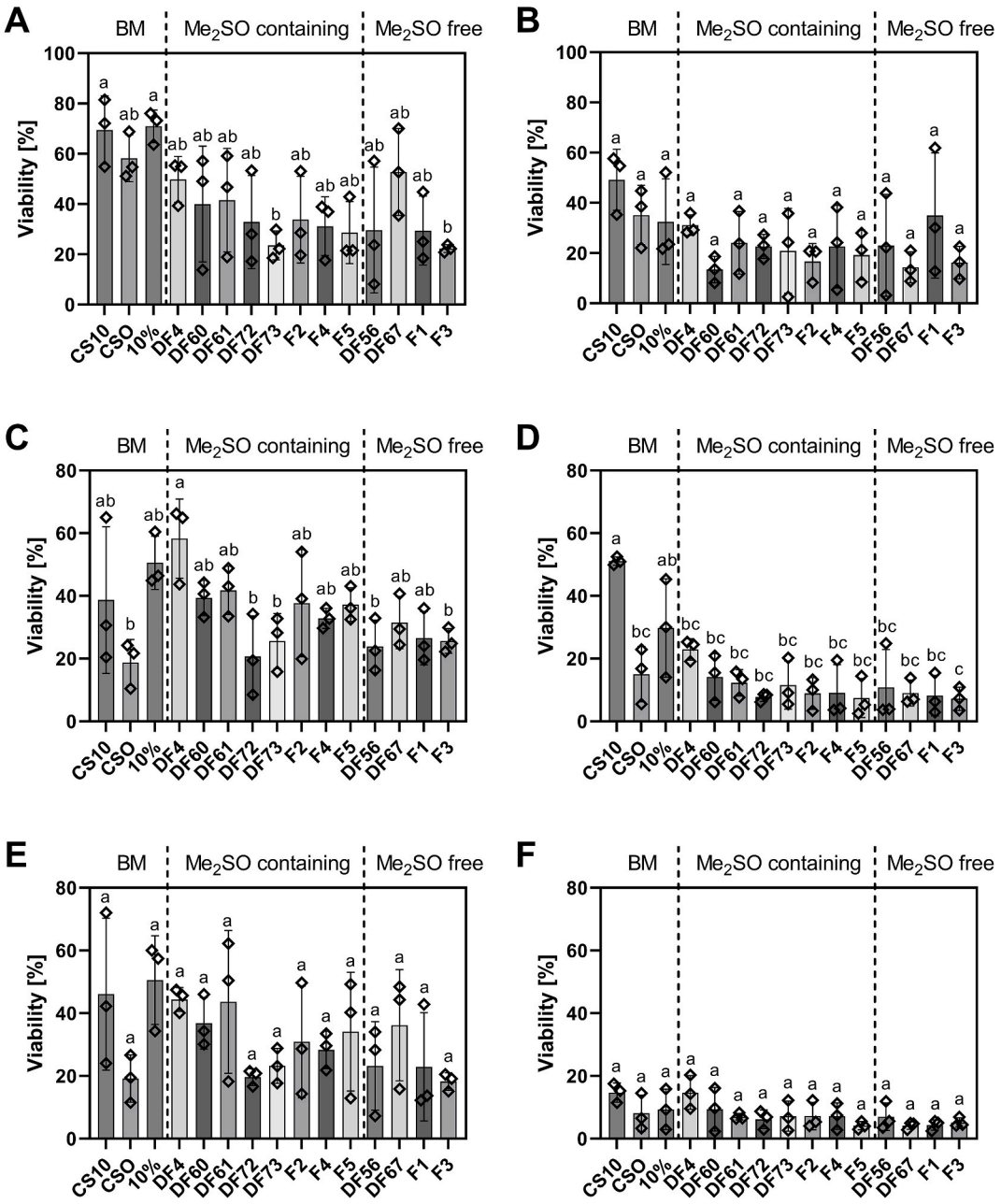

Fig. 5. Comparison of three different viability assays for cells stored at −80 °C for three months at T-FT (left column) and T-Rec (right column). Trypan blue viability staining (A, B), CalceinAM & propidium iodide (C, D) and Annexin V-FITC & propidium iodide (E, F) staining were used to determine cell viability. Trypan blue viability was based on the ratio of viable cells to all cells within each sample. Statistically significant differences ($p \leq 0.5$) based on one-way ANOVA followed by Tukey's test are indicated by different letters. BM: benchmark formulations; Me_2SO: dimethyl sulfoxide. 10 %: 10 % Me_2SO in RPMI.

results. All assays led to different viability results especially for higher viabilities as the assays are based on different measurement principles. The benchmark formulations showed high viabilities of 60–70 % based on Trypan blue staining. However, in both fluorescence assays, CalceinAM and Annexin V, the viability values were lower with 40–50 % for CS10 and 10 % Me_2SO and only around 20 % for CSO. The tested formulations showed minor differences between the viability assays. Based on Trypan blue staining CS10 and 10 % Me_2SO led to slightly higher viability values with approximately 70 % compared to the formulation samples, which were between 20 % and 53 % viability. Among the tested formulations Me_2SO-free DF67 (53 % ± 17 %) and DF4 (50 % ± 9 %) had the highest post-thaw viability using Trypan blue staining. Using the CalceinAM assay DF4 showed the best results with 58 % ± 13 % followed by 10 % Me_2SO in RPMI (51 % ± 9 %). Based on Annexin V staining no significant differences between the viability of the samples were found.

For samples (−80 °C storage temperature) measured after 24h in cell culture the obtained cell viability was in general lower compared to the samples measured directly after thawing. This shows that damages throughout cryopreservation continue to unfold after thawing. For Trypan blue and Annexin V staining no significant differences were found between the samples, whereas CS10 resulted in a significantly higher viability of 51 % ± 1 % based on CalceinAM compared to the other samples. The overall viability range for Trypan blue staining was between 13 % and 50 %, whereas Annexin V was only between 4 % and 15 %. CalceinAM led to intermediate viability values (7–50 %) compared to the other two assays. In summary, no clear trend could be observed regarding Me_2SO concentration for samples stored at −80 °C

directly post-thaw and after recultivation.

Furthermore, samples stored at −145 °C were analyzed regarding cell recovery and cell viability. Fig. 6 shows the viable cell recovery as well as the results for all three viability assays. In general, the results are comparable to the samples stored at −80 °C with an overall low viable cell recovery between 6 and 20 % for most samples and around 40 % for DF4. This again indicates the protective effect of P188 during the processing of cells. The benchmark formulations resulted in slightly higher viabilities (65–74 %) compared to the tested formulations (21–72 %) for Trypan blue staining. Based on CalceinAM and Annexin V staining the obtained viabilities for the benchmark formulations were lower compared to Trypan blue staining. This was also observed for the samples stored at −80 °C. Although the differences between the viabilities of the tested formulations are relatively small, the Me_2SO-containing formulations seem to perform slightly better than Me_2SO-free formulations for samples stored at −145 °C. The effect of Me_2SO was not visible for samples stored at −80 °C, which indicates the importance of ultralow storage temperature during cryopreservation. The measured T_g' for samples without Me_2SO were around −30 °C enabling a storage at −80 °C, whereas Me_2SO-containing formulations at 2.5–5 % showed a T_g' ranging from −44 °C to −84 °C (Table 4). By comparing F3 (Me_2SO-free) and F4 (2.5 % Me_2SO) the positive effect of Me_2SO for samples stored at −145 °C can be seen with approximately 3-fold higher viabilities for F4 in all assays (Fig. 6). However, Me_2SO-free formulation DF67 resulted in a viability comparable to DF73 which contains 2.5 % Me_2SO, highlighting the possible positive effect of the combination of 50 mM proline and 50 mM ectoine. Among the Me_2SO-containing formulations DF72 (without PVP40) resulted in the lowest viability by CalceinAM (31 % ± 16) and Annexin V (29 % ± 15 %) assays and second lowest viability by Trypan blue staining (39 % ± 22 %) indicating the potential beneficial effect of PVP40 on the cryopreservation outcome.

In addition, viability was assessed for samples stored at −40 °C. The benchmark formulations resulted in viabilities of 26–81 %, 20–29 % and 19–42 % for Trypan blue, CalceinAM and Annexin V, respectively. In general, CS10 resulted in the highest viability values for all three assays. On the other hand, obtained viability values for the tested formulations ranged between 0 and 20 % indicating that storage at −40 °C is not suitable for these formulations. A comparison of all three storage temperatures can be found in the supplementary information for cell recovery (Fig. S5) and cell viability (Fig. S6).

4. Discussion

Cryopreservation of cells is essential for the storage of CBMPs and is usually achieved by a standard procedure using 10 % Me_2SO with a freezing rate of 1 °C/min [10]. In this study we explored novel cryopreservation media using a DoE approach followed by subsequent OFAT optimization for formulation development. By reducing the amount of Me_2SO as well as using excipients, which increase the T_g', we aimed to test the storage of cells at elevated subzero temperatures such as −80 °C. The developed formulations were further evaluated in a three-month stability study, where the samples were stored at −145 °C, −80 °C and −40 °C.

A DoE based on a fractional factorial design with a resolution of IV was used. A fractional factorial design is a linear model, where a balanced set of experiments is used to build a linear model. The main factors contributing to the outcome can be identified by this. Generating a proper prediction model for post-thaw viability was not possible as Me_2SO was the predominant factor regarding post-thaw viability. Me_2SO had a strong positive impact on the post-thaw viability, whereas trehalose and PVP40 had only a minor positive effect and the remaining

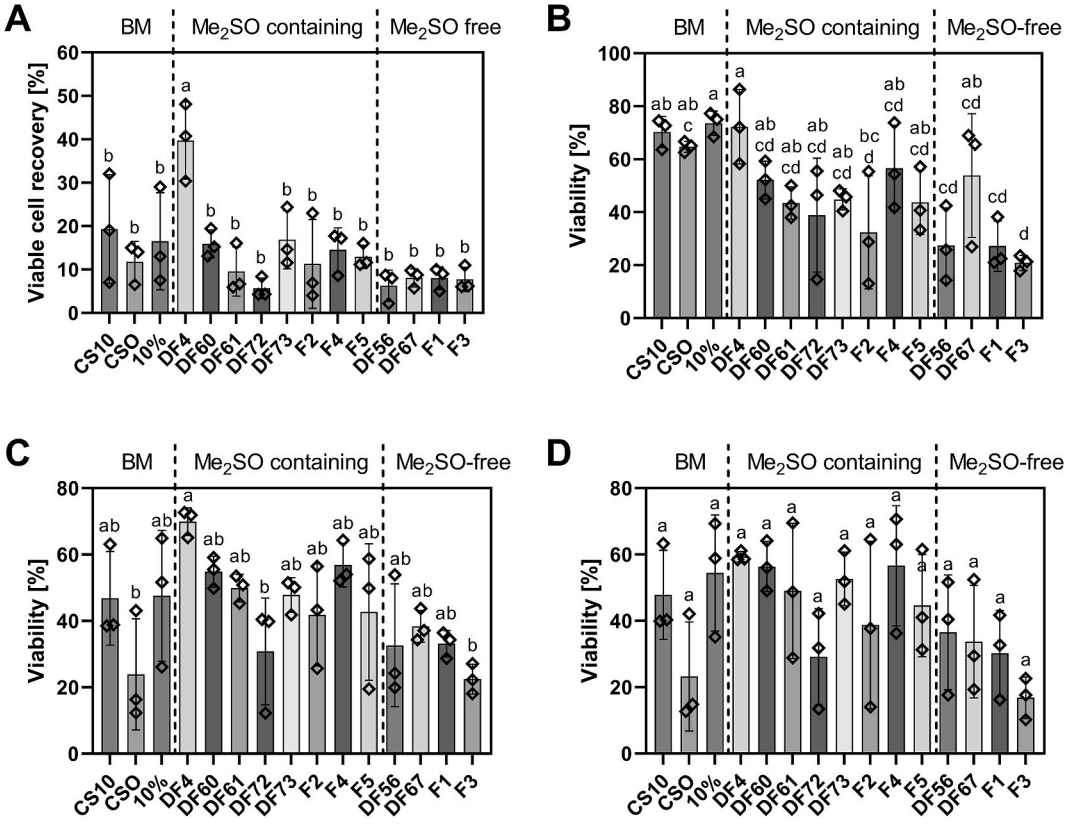

Fig. 6. Analysis of cells stored at −145 °C after 3 months. (A) viable cell recovery based on Trypan blue staining, (B) viability based on trypan blue staining, (C) viability based on CalceinAM & propidium iodide, (D) viability based on Annexin V-FITC & propidium iodide. Statistically significant differences ($p \leq 0.5$) based on one-way ANOVA followed by Tukey's test are indicated by different letters.

excipients seemed to have a negative effect on the post-thaw viability. The combination of trehalose and proline as well as trehalose and ectoine had a very small positive impact on the viability, however as a fractional factorial design was chosen two-factor interactions are confounded.

Considering both response factors (cell viability and T_g') the resulting optimum was 2.5 % Me_2SO, 300 mM trehalose and 5 % PVP40, which was one of the formulations already included in the prescreening (DF61). The model predicted a viability of 47 % and a T_g' −46 °C for this formulation. In the stability study the actual measured viability for cells stored at −145 °C was 49.9 % ± 4.2 % based on CalceinAM staining and the obtained T_g' of the formulation was −43.7 °C. The DoE model was based on viability values obtained by CalceinAM staining, therefore only the CalceinAM assay can be used to compare the predicted viability to the obtained values of the stability study. Both measured values are comparable to the predicted values, although the model for viability has a low validity. The established DoE approach focused on formulation development and process parameters were not included. However, as the formulation composition influences the cryopreservation process, process parameter should be considered as well to improve the DoE predictions. Within this study a standard cryopreservation process was used with a freezing rate of 1 °C/min. The freezing process most likely needs to be adapted depending on the composition of the formulation. Pollock et al. previously reported different optimal freezing rates for Jurkat cells and mesenchymal stem cells depending upon the used cryopreservation medium [36]. The study found that increasing the freezing rate to 10 °C/min, when using a formulation consisting of 300 mM trehalose, 10 % glycerol and 0.01 % ectoine, resulted in a higher viability for Jurkat cells compared to 10 % Me_2SO at a freezing rate of 1 °C/min [36]. In pharmaceutical development DoE approaches are valuable to minimize the number of experiments in a formulation development study. However, during cryopreservation not only the composition of the formulation, but also the interactions between the excipients as well as the process impact the cryopreservation outcome. Therefore, the freezing rate could be included in the DoE as an additional parameter. Additionally, models could be used, which can determine non-linear relationships between factors as interactions between excipients can occur.

After the DoE, a stability study was conducted, where samples were analyzed directly after thawing as well as after recultivation. The cell recovery, including total cell-sized particle recovery as well as viable cell recovery, and cell viability were evaluated for cryopreserved samples. Both parameters are important to understand the cell damages that might occur during the cryopreservation process.

In general, a low viable cell recovery of 4 %–30 % was obtained for samples stored at −80 °C as cells are damaged throughout the cryopreservation process. A viable cell recovery of 16 % was previously reported for 10 % Me_2SO for Jurkat cells at freezing rate of 1 °C/min [36], which is comparable to the viable cell recovery for 10 % Me_2SO in RPMI within this study. The comparison of cell-sized particle recovery to viable cell recovery showed that different factors can negatively affect Jurkat cells throughout cryopreservation. Although a very high cell-sized particle recovery of ~106 % (normalized to cell stock, therefore dilution and measurement deviations can lead to values slightly higher than 100 %) was obtained for cells in DF4, which contains 1 % P188, the amount of viable cells recovered was only 30 % for samples stored at −80 °C. P188 is a non-ionic surfactant, which has been previously shown to be a very effective shear protectant for cells [37,38]. This protecting effect is also visible in our study as all cells remain particulate throughout the processing steps. The cells do not disintegrate. However, the low viable cell recovery indicates, that other stress factors than mechanical damage occurring during freezing and thawing are not prevented by DF4. Each processing step can negatively impact the stability of cells starting with the sample preparation. Benchmark samples CS10 and CSO were prepared by direct resuspension in the respective solution after centrifugation and removal of cell culture medium. The osmolalities of CS10 (3282 ± 11 mOsm/kg) and CSO (2425 ± 20 mOsm/kg) are very high and the changes in cell size due to fast changes in osmolality may have led to cell damages [39]. These damages throughout sample preparation may be prevented by slow addition of the cryopreservation medium as performed for the formulation samples in our study. The total cell-sized particle recovery for the tested formulations were slightly higher than the benchmark formulations. To formulate the cell formulations samples a stock solution of cells in RiAc-HEPES was prepared. Subsequently, the 1.25-fold concentration was slowly added to the cell stock and thereby preventing a high osmotic gradient. Additionally, the osmolality of the tested formulations was lower in comparison to the benchmarks reducing the cell volume excursion during formulation addition as well as removal.

As expected, a positive impact of Me_2SO on the viability of Jurkat cells could be observed upon storage for three months. This effect was more pronounced for samples stored at −145 °C in comparison to −80 °C, where there was no difference between formulations with or without Me_2SO (Fig. 5). These results further highlight the need for ultra-low storage for Me_2SO-containing samples. To enable storage at −80 °C a reduction of Me_2SO to 2.5 % as well as addition of PVP40 and trehalose is needed. In this study, several important formulation aspects with regard to the possibility to reduce Me_2SO as well as storage at −80 °C were identified. The addition of 5 % PVP40 is beneficial for the cryopreservation outcome at both −145 °C and −80 °C storage. PVP40, at 10 % in DMEM, has been previously shown to result in comparable cryopreservation results to 10 % Me_2SO and FCS for human adipose-tissue derived stem cells stored in liquid nitrogen [40]. Furthermore, the combination of 2.3 % PVP40 and 10 % Me_2SO led to a higher recovery for hepatocytes in comparison to cryopreservation with 10 % Me_2SO after storage in liquid nitrogen [41]. Additionally, the combination of 50 mM proline and 50 mM ectoine did positively impact the post-thaw viability of Jurkat cells in our study. The effect of proline and ectoine as cryopreservation excipients were previously evaluated on human endothelial cells stored at −150 °C and a beneficial effect on the recultivation of cells could be observed, when using 50 mM ectoine and 2.5 % Me_2SO [42]. Additionally, the combination of 20 mM proline, 100 mM ectoine and 2.5 % Me_2SO led to recultivation rates comparable to 10 % Me_2SO (storage temperature −150 °C) [42]. Another approach to improve the cryopreservation outcome is to already culture cells with suitable excipients such as proline and/or ectoine prior cryopreservation, which can increase post-thaw cell recovery depending upon the used conditions [43,42].

However, cell samples analyzed after 24h recultivation further decreased in cell viability, showing that damages described as cryopreservation induced delayed-onset of cell death [44] takes place. Therefore, the analysis directly after thawing can lead to an overestimation of cell viability and a combination of different parameters should be analyzed to fully understand the damaging effect of the cryopreservation process including formulation of cells prior freezing and processing of cells after thawing. Cell viability as measured in this paper does not necessarily correlate with the ability to proliferate as we have shown for human T cells (manuscript submitted). Apart from measuring cell viability directly post-thaw a recultivation time point should be included. To avoid false positive results based on viability measurements, the cell viability and viable cell recovery should be measured to evaluate the cryopreservation outcome [45]. Additionally, measuring the viability and recovery after recultivation of cells can detect delayed cryopreservation damage [45]. Furthermore, in addition to viability also functionality, i.e., cell activity such as cytotoxicity for T cells, should be assessed, as viability assays measure specific cell parameters such as membrane integrity (Trypan blue and PI). It has been previously shown that a high post-thaw viability can be obtained based on membrane integrity staining human umbilical vein endothelial cells, although their functionality is not comparable to fresh cells as shown by a tube formation assay, which mimics angiogenesis [46]. This further highlights the need to establish a set of analytical tools to evaluate the

cryopreservation outcome at several time points post-thaw.

In this study a commonly used standard procedure for cryopreservation was performed based on a passive cooling device with a 1 °C/min freezing rate [19]. However, as different formulation compositions and Me_2SO concentrations were tested the standard procedure might not be applicable. The cryopreservation damage in relation to the freezing rate is usually described by the "Two-factor hypothesis" by Mazur et al. [47]. A fast cooling rate can lead to intracellular ice formation, whereas a slow cooling rate induces osmotic stress throughout freezing. Therefore, an intermediate cooling rate has to be chosen to balance both factors. The cooling rate depends on the chosen CPAs and their concentration [48,49]. In this study Me_2SO-free formulations contained 300 mM trehalose, which resulted in an osmolality of ~800 mOsm/kg, depending on the complete formulation composition. As no cell membrane permeating excipients were included in these formulations the cells directly shrink upon resuspension in the respective formulations due to the osmotic pressure. This directly lowers the risk of intracellular ice formation because the water content in the cell is decreasing. Therefore, an increased cooling rate in comparison to 1 °C/min should further be evaluated to decrease the solute effect (osmotic stress) during freezing.

5. Conclusion

In this study formulations for the cryopreservation of Jurkat cells were screened. A special focus was put on the elimination or reduction of Me_2SO to minimize the potential risks and side effects associated with Me_2SO. Additionally, the storage of Jurkat cells at elevated subzero temperatures, i.e., −80 °C and −40 °C, were evaluated. A design of experiment approach was used, however due to the predominant effect of Me_2SO on post-thaw viability the prediction of a suitable formulation was more difficult. Several promising excipients and combinations thereof were identified for the cryopreservation of Jurkat cells with reduced concentrations of Me_2SO or Me_2SO-free formulations. However, the viabilities of formulations containing 2.5 % Me_2SO were lower compared to the evaluated benchmarks containing 10 % Me_2SO after three-month storage. Furthermore, the additional analysis of cells after recultivation showed that post-thaw cell viability further decreases over time highlighting the need to evaluate cells not only directly after thawing.

CRediT authorship contribution statement

Alexandra Roesch: Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Roland Windisch:** Resources. **Christian Wichmann:** Resources. **Willem F. Wolkers:** Conceptualization. **Gideon Kersten:** Writing – review & editing, Supervision, Conceptualization. **Tim Menzen:** Writing – review & editing, Supervision, Conceptualization.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2025.105238.

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