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Algorithm-Based Liquid Formulation Development Including a DoE Concept Predicts Long-Term Viral Vector Stability

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ABSTRACT

Specifically tailored amino acid–based formulations were previously shown to have a high potential to avoid stress-mediated degradation of complex molecules such as monoclonal antibodies and viral vectors. By using adenovirus 5 (*Ad5*) as a model, we studied whether such formulations may also efficiently protect viral vectors in thermal stress experiments and during long-term liquid storage. Algorithm-based amino acid preselection using an excipient database and subsequent application of design of experiments (*DoE*) in combination with a 37°C challenging model enabled the prediction of long-term storage stability of Ad5. By statistical analysis of the Ad5 infectivity, amino acids with significant influence on Ad5 stability were detected after 2 and 3 weeks of liquid storage at 37°C. Ad5 formulations comprising positively selected amino acids did not reveal any loss of infectivity after 24 months in liquid storage at 5°C. By contrast, a 2 log reduction after 3 months and complete loss of infectivity after 18 months was observed with a standard viral vector formulation. By an optimization round, we designed a simple and well-balanced formulation avoiding $MgCl_2$, previously considered essential in Ad5 formulations. This work demonstrates the efficacy of an algorithm-based development approach in the formulation development for viral vectors.

Introduction

Significant improvements in vector engineering, delivery, and safety have placed replication-deficient recombinant viral vectors at the forefront of modern medicine, representing a rapidly growing field for vaccination[1] and gene therapy.[2] Viral vectors and virus-like particles offer a series of advantages over traditional vaccines. In addition to inducing adequate antibody responses, they elicit cytotoxic T lymphocytes that are crucial for the control of intracellular pathogens and cancer, a feature not observed by protein-based vaccines.[1] Moreover, viral vectors have been used in recent years for the treatment of various diseases such as metabolic, cardiovascular, muscular, hematologic, ophthalmologic, and infectious diseases, as well as different kinds of cancers.[2] Furthermore, preventive and therapeutic approaches have been developed in the area of immunotherapy, whereas preclinical and clinical studies demonstrated therapeutic and prophylactic efficacy of viral vectors.[2] Meanwhile, several vector-based drugs have been globally approved.[2]

As reviewed by Rollier et al.,[1] Lundstrom et al.,[2] and Ura et al.,[3] many viral species have been evaluated as recombinant gene delivery vehicles in the fields of vaccination and gene therapy, including, retroviruses, lentiviruses, flaviviruses, vaccinia viruses (modified vaccinia Ankara virus), adenoviruses (*Ad*), adeno-associated viruses (*AAV*), cytomegaloviruses, Sendai viruses, measles viruses, herpes simplex viruses, rhabdoviruses (vesicular stomatitis virus), and picornaviruses. However, to date the most widely evaluated vectors are adenovirus serotype 5 (*Ad5*), AAV serotypes 2, 3, 5, 6, 8, 9, and members of the poxvirus family, for example, vaccinia virus, particularly modified vaccinia Ankara virus.[1-3]

A drawback associated with manufacturing, storage, and distribution, is that viral vectors are complex supramolecular ensembles of macromolecules which are prone to a variety of chemical and physical degradation pathways.[4,5] One major risk factor for the stability of viral vector formulations is the loss of viral

Conflict of interest: Dr. Kristina F. Kemter, Dr. Eva B. Reinauer, Dr. Stefan R. Henz, Stella S. Grosso, Julia A. Rabas, and Carina Rodenstein are employees of LEUKOCARE AG, Martinsried. Prof. Dr. Martin Scholz and Dr. Jens Altrichter were in the board of directors and currently are external consultants of LEUKOCARE AG.

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particle integrity and infectivity as a result of exposure to elevated temperatures or other kinds of stresses.[6]

During the whole manufacturing process of viral vectors, cross-linking and vector particle interaction is typically caused by host cell proteins and DNA on the viral surface at different stages of production, storage, and application.[7,8] This intrinsic tendency of viral vectors for particle agglomeration within a composition leads to inhomogeneous size distribution associated with increased polydispersity and subsequent loss of infectivity. This in turn results in a significant loss of therapeutic efficacy and can even lead to adverse effects.[7,8] Furthermore, aggregation of the viral vectors is considered to impair biodistribution and to increase nonspecific immunogenicity.[7,8] As high polydispersity is associated with high viscosity, such compositions are also expected to elicit minor syringeability and injectability.[7-9]

To avoid viral particle agglomeration during production and final formulation for preclinical and clinical studies with highly concentrated AAV 2 vectors, the use of high ionic strength solutions (e.g., by addition of multivalent ions such as citrate, sulfate, Mg^{2+} or phosphate, as well as by efficient removal of residual vector surface host cell DNA by treatment with nucleases) was reported to be an effective strategy.[7] In another approach, a favorable polydispersity index of $\leq$0.5 for viral vector formulations was achieved by using at least one sugar and at least 3 different excipients selected from hydrophilic and amphiphilic excipients.[9] Nevertheless, formulations that further improve quality, storage, stability, and effectivity of viral vector–based vaccines and gene therapeutics are still urgently needed.

Currently, most formulations are commercially used as frozen or dry viral vector preparations, but do not sufficiently elicit long-term stability during liquid storage.[10] Liquid storage of viral vectors is highly appreciated because time-consuming and expensive freeze-thaw procedures associated with high molecular stress and degradation can be avoided during manufacturing. Although the effects of freeze-drying or spray-drying on the integrity of viral vectors are well known,[11] systematic efforts to optimize liquid viral vector formulations and stability are rarely reported.[12-17]

For example, the complex stabilizing formulation (A195) for adenovirus serotype 5 as described by Evans et al.,[18] is still in use as "gold standard" for the stabilization of liquid Ad5 formulations. It comprises sucrose as an effective osmolytic excipient, divalent cations for example, Mg^{2+} ($MgCl_2$), polysorbate 80 as nonionic surfactant to prevent Ad5 surface adsorption, a combination of the metal chelator ethylenediaminetetraacetic acid (*EDTA*), and free-radical oxidation inhibitors, such as ethanol and histidine, all well-known as hydroxyl radical scavengers. By means of such standard formulations, 99% infectivity of Ad5 was preserved during liquid storage at 4°C for up to 24 months and 95% during liquid storage for up to 1 day at 37°C.[18] This held true even during liquid storage for up to 1 month at 40°C.[19] However, excipient modifications and simplifications of liquid Ad5 formulations were rather empiric in the past,[6,18,20,21] lacking a rational, methodologically structured, and systematical method of selection.

We recently demonstrated that specifically tailored amino acid–based formulations have a high potential to avoid stress-mediated degradation of complex molecules such as monoclonal antibodies[22-24] and vaccines.[25] This formulation approach has been shown in the past to stabilize a broad spectrum of target molecules during drying and reconstitution as well as during liquid storage, according to the concepts of preferential binding and preferential exclusion,[26-29] respectively. In previous studies with lyophilized Ad5, we demonstrated significantly higher infection rates in cell culture experiments after several freeze-thaw cycles and dry storage with this amino acid–based formulation approach, compared to Ad5 in standard formulations. Lower polydispersity and better viral vector integrity were shown by dynamic light scattering and electron microscopy.[9] Therefore, in this study, we set the goal to investigate whether this amino acid–based formulation approach also enables the maintenance of viral vector functionality during long-term liquid storage.

One of the major difficulties in formulation development is the lack of predictability of long-term stability at the beginning of downstream processing on the basis of accelerated aging results.[30] The failure of formulations during real-time storage, for example, after 2 years, may result in insufficient stability, loss of molecular integrity, and aggregation, all entailing increased risks and safety issues. As a consequence, formulations have to be redesigned and yet again, expensive and time-consuming studies have to be conducted. To date, only little is known about systematic procedures that allow the identification of highly effective excipients and formulations for long-term liquid storage of viral vectors already in the beginning of downstream processing.

Here a novel rational approach was introduced to efficiently and rapidly select the best stabilizing excipients for liquid storage of Ad5 from a database, in combination with a design of experiments (*DoE*) procedure for formulation development and a 37°C challenging model. Ad5 was chosen in this model because of its wide application as gene delivery vector, its well-characterized molecular structure and its intensively studied degradation pathways such as surface adsorption, freeze-thaw damage, and free-radical oxidation, all leading to loss of functionality.[18,19] In general, the adenovirus is a nonenveloped virus particle of 70-100 nm diameter with an icosahedral protein capsid which is an assembly of 252 protein subunits, containing a linear double-stranded DNA genome of 36 kb.[3,6,31-33] The functional and structural integrity of Ad5 viral particles was evaluated by analysis of the infective titers in cell culture experiments based on an immunostaining method (hexon staining)[34] by using a genetically modified replication-deficient Ad5 with deleted E1/E3 gene regions.

The first search level of the used database has been designed to identify amino acids that proved earlier to stabilize similar biomolecules. These selected amino acids form the basis for the design and development of first formulations in combination with short-term challenging experiments to predict long-term effects.

This study showed for the first time, the predictability of long-term liquid storage stability under application of a novel rational approach for formulation development of liquid Ad5 formulations finally resulting in a remarkable simplification of the formulation design compared to the currently accepted best of formulations for Ad5 viruses associated with a consistently good stabilizing efficacy.

Materials and Methods

Algorithm-Based Formulation Development Approach Using DoE

Our applied in-house database (LEUKOCARE AG, Munich, Germany) contains information about particular physicochemical and structural properties of different kinds of biologics, formulations, and >100 excipients previously identified to be effective in stabilizing these biologics together with literature-known characteristic stabilization data. On the basis of this extensive amount of information, we developed search algorithms for the preselection of excipients most suitable for stabilizing the particular biologics against specific kinds of stresses in the desired physical form of formulation (dry or liquid). Under application of characteristic search criteria regarding the selected biologic (e.g., the biologic type, the known main degradation pathways, the physical state of the formulation), the evaluated algorithm identifies the most suitable stabilizing excipients for each specific target. In this study focusing on Ad5 viral vector in liquid as target, this approach

Table 1
Iterative Optimization Round (Round 2) Based on the Two Best of Formulations of Round 1 F1_29 and F1_13

Round 2	Modifications	$MgCl_2$ *6 H_20	Osmolality [mOsmol/kg]	pH Value
F2_1	F1_29	yes	584	7.4
F2_2	F1_29 change of osmolytic amino acids Ala to Gly	yes	580	7.4
F2_3	F1_29 change of basic Lys to Arg	yes	552	7.4
F2_4	F1_29 w/o $MgCl_2$	no	584	7.4
F2_5	F1_29 Change of sugar to sugar alcohol mannitol	yes	575	7.4
F2_6	F1_29 Addition of Glu	yes	649	7.4
F2_7	F1_13	yes	428	7.4
F2_8	F1_13 w/o $MgCl_2$	no	422	7.4
F2_9	F1_13 Addition of Met	yes	441	7.4

F, formulation.

resulted in the preselection of stabilizing excipients including the 8 amino acids Arg, Ala, Gly, Lys, His, Trp, Glu, and Met as most promising candidates.

Based on these selected 8 amino acids, the next step of formulation development included the implementation of a statistical DoE matrix, developed with the package "DoE.wrapper"[35] in R 3.6.1 (R Core Team, 2019).[36] To reduce the amount of experimental effort, time, and costs, as well as to eliminate physically impossible testing design regions, we reduced the full-factorial design space (i.e., the theoretical design space) by the introduction of various constraints under application of a D-optimal design algorithm. The resulting DoE matrix, tailored for the Ad5 viral vector, was composed of 40 formulations containing combinations of the aforementioned 8 different amino acids Arg, Ala, Gly, Lys, His, Trp, Glu, and Met and fixed amounts of sucrose and $MgCl_2$ at a fixed pH value of 7.4. The DoE formulations of this development round (round 1) were labeled as F1_1 to F1_40. The application of the implemented DoE matrix in combination with a 37°C challenging model supports the prediction of long-term stability of the target molecule and the further iterative optimization of the resulting formulations. The iteratively optimized formulations were modified according to Table 1 and are labeled as F2_1 to F2_9 (Table 1).

All components are nontoxic and routinely used in parenteral solutions. All excipients were purchased from Sigma-Aldrich (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), or Merck (Darmstadt, Germany).

Ad5 Preparation

An adenoviral stock solution (Ad5-CMV-EGFP: E1/E3-deleted human adenovirus, serotype 5) stored at −80°C with a concentration of 7.5×10^{10} IFU/mL in the original supplier formulation (*OF*), designed for frozen storage (Sirion Biotech GmbH, Martinsried, Germany) was used.

Ad5 was diluted to 1×10^8 IFU/mL in OF and in the designed formulations according to round 1 and 2 (see the aforementioned). As assay control, the original supplier formulation was depicted as positive control when stored frozen at −80°C. In round 2, a more complex reference formulation (*Ref*) including $MgCl_2$ was included for comparison.[18,37]

Storage Conditions

The resulting Ad5 formulations were initially stored under short-term stress conditions at 37°C. To evaluate the predictive capability of the applied approach using a short-term storage model at 37°C for liquid storage under real-time conditions, these samples were additionally stored for up to 6 months at 25°C and for up to 24 months at 5°C.

In addition, on the basis of the best of formulations and the determined effects of each amino acid used in the DoE matrix on the Ad5 infectivity during short-term storage at 37°C for up to 35 days (round 1), an iterative formulation optimization was performed (round 2). The resulting liquid Ad5 formulations were stored for up to 12 months at 25°C and for up to 24 months at 5°C. At indicated time points during liquid storage, the Ad5 stability was evaluated using an infectivity assay.

Infectivity Assay

To analyze the infective titer of the Ad5 formulations, antibody-based virus titration assays in adherent HEK-293 cell cultures were conducted. Antibody-mediated immunostaining of the adenoviral hexon protein was applied after successful amplification of the Ad5 in the infected cells. Therefore, 2.5×10^5 HEK-293 cells in 500 μL per well were seeded in a 24-well plate and further used when cells started attaching to the surface (after 2-3 h). Serial dilutions of the Ad5 samples were prepared and 50 μL of the resulting dilutions per well were used for infection of the cells. For positive control, aliquots of Ad5 in OF (see the aforementioned) stored at −80°C with a concentration of 1×10^8 IFU/mL were used. Cells were inoculated for 42 ± 2 h at 37°C (+5% CO_2) and subsequently fixed with methanol (Carl Roth GmbH & Co. KG). Immunostaining was performed stepwise by incubation with the primary antihexon protein antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), the secondary horseradish peroxidase−conjugated anti-mouse antibody (Cell Signaling Technology Inc., Beverly, MA), and an horseradish peroxidase enzymatic reaction with diaminobenzidine (Carl Roth GmbH & Co. KG). The number of infected cells was quantified by counting the stained (brown colored) cells under the light microscope. Up to five visual fields per well were counted. Each stained cell was considered as one infective viral particle to calculate the infective units per mL (IFU/mL) according to the standardized calculation procedure.[34] The dynamic range of the assay allows titer determination between 9.87×10^4 IFU/mL and 2.04×10^8 IFU/mL.

Data Analysis

For round 1, Ad5 infectivity data obtained at indicated time points during liquid storage at 37°C, 25°C, and 5°C were preliminary analyzed by full linear regression modeling in R and visualized in GraphPad Prism (Version 7.04). An analysis of variance was performed on log-transformed absolute titer levels (infectivity levels) for estimating the influence of each amino acid on the Ad5 stability at the 2 last time points evaluated. Effects were considered statistically significant, statistically very significant, and statistically highly significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), respectively.

To get deeper insights on the effects of possible quadratic terms and pairwise interactions of relevant amino acids on Ad5 stability, a more comprehensive, explorative statistical evaluation of the Ad5 infectivity data at indicated time points during liquid storage at 37°C, 25°C, and 5°C was performed post hoc. Response data

Table 2
Amino Acids as Factors and Minimum and Maximum Concentration Levels of the Amino Acids Applied in the DoE Calculations

Amino Acid Factors	Concentration Levels [g/L]
Arg	0-30
Ala	0-20
Gly	0-20
Lys	0-30
His	3-30
Trp	0-7
Glu	0-10
Met	0-1.5

(absolute titer levels, Tukey-transformed when normality assumptions were violated) were therefore analyzed by multiple linear regression modeling with least square method in R.[36] Three sets of regression models were built up for each temperature at 2 storage time points to investigate the impact of the 8 selected amino acid predictors and their pure linear, 2-factor interactions, and quadratic terms. Stepwise backward regression was applied to select the model with the lowest Akaike's information criterion for each set. A k-fold cross-validation was applied in case the most significant model expressing the highest fit by R^2 coefficient was overfitting, leading to restrict the selection to the most parsimonious model (i.e., the model where only the main factors that have significant influence on infectivity levels are included) for each set. The final Akaike's information criterion—based and k-fold-reduced models for each time and temperature were tested for statistical validation via analysis of variance, and here the "winning" model (defined by highest R^2 and significant p values, see Tables in Fig. 2 and in text F-statistics) is reported. Data visualization for this analysis was performed in R. Effects were considered statistically significant, statistically very significant, and statistically highly significant at $p < 0.05$ (*), $p < 0.005$ (**), and $p < 0.001$ (***), respectively.

All experiments from iterative round 2 were carried out in triplicates (3 × 5 values). Data are depicted as mean ± SD, except when indicated otherwise (Figs. 4a-4c).

Results

Algorithm-Based Formulation Development Approach Using DoE

By means of the algorithm-based preselection approach including an excipient database screening, we first identified and positively selected 8 amino acids for testing in a short-term as well as in a long-term liquid storage challenging model with Ad5. To create a full-factorial design matrix, the selected amino acids were incorporated as factors with relevant concentration levels into DoE calculation (Table 2). Subsequently, the calculation of a more compact design space was performed by introducing formulation-specific constraints as parameters to define a design space following Quality by Design principles.[38] The result was a D-Optimal DoE matrix of 40 formulations at a fixed pH value of 7.4 (formulation number F1_1 to F1_40) optimized to investigate linear and 2-factor interactions for the 8 preselected amino acids in various amounts, concentration ratios, and concentration levels (Table 2) in combination with the well-known stabilizing substances for adenoviral vectors, sucrose, and $MgCl_2$.[18,37] To account for eventual nonlinearity in the system, second-order terms were also included in the DoE design for 7 predictor amino acids (Arg, Ala, Lys, Glu, Gly, His, and Trp).

Short-Term Liquid Storage at 37°C

A liquid storage study under accelerated aging conditions at 37°C was performed for Ad5 in the 40 DoE formulations given

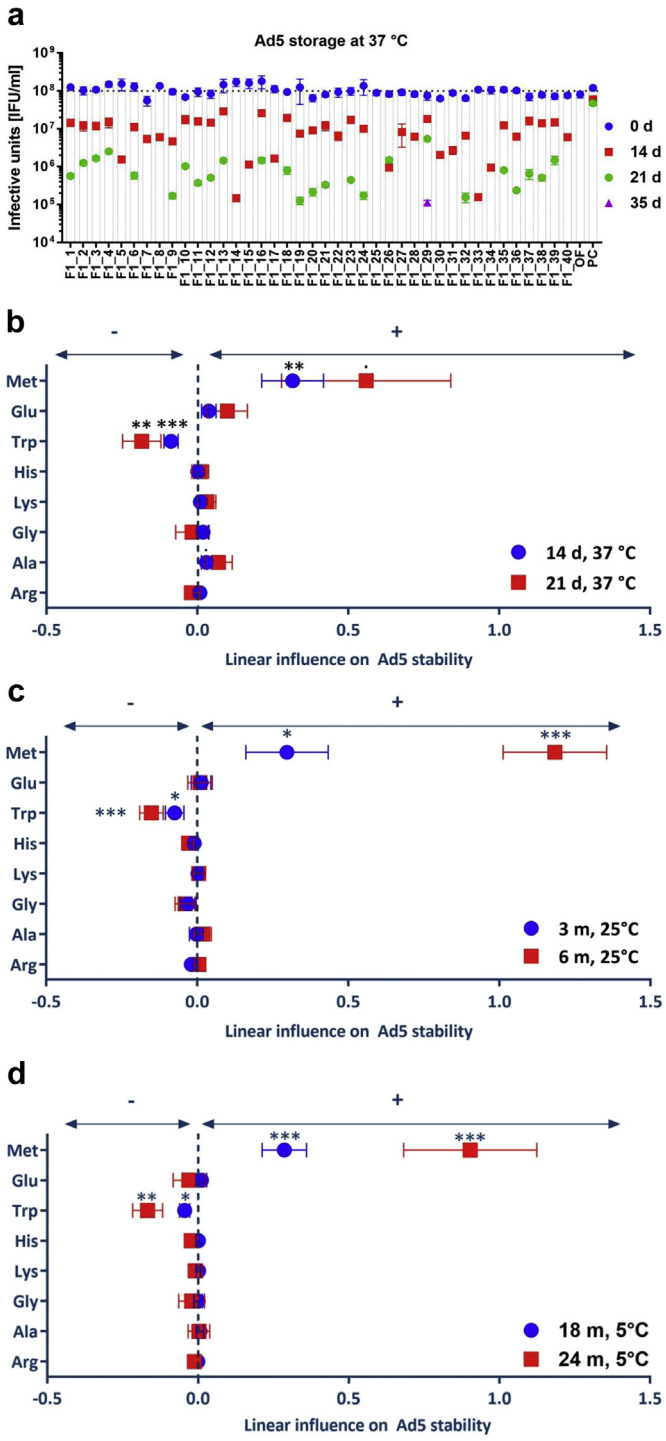
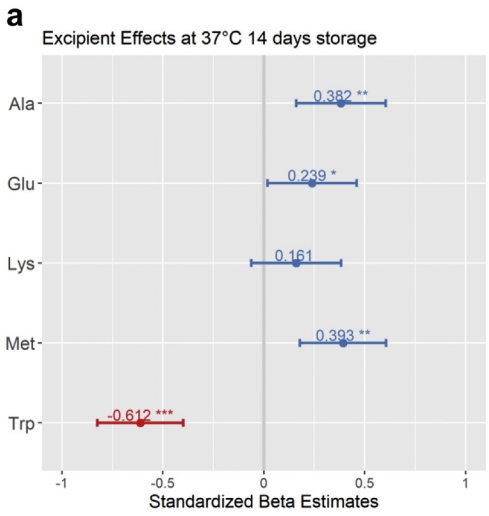
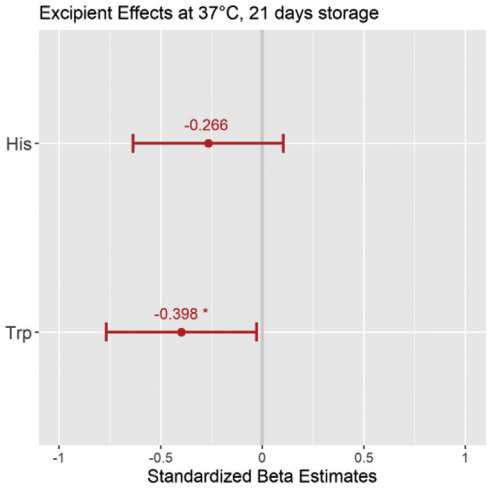


Figure 1. Infectivity of Ad5 in liquid DoE formulations (round 1) after accelerated aging at 37°C and results of the linear regression analysis of the Ad5 infectivity at indicated time points during liquid storage at 37°C, 25°C, and 5°C. (a): infectious units per mL (IFU/mL) from infectivity assay are depicted. The 40 DoE formulations F1_1 to F1_40, the original formulation (OF), and the frozen-stored positive control (PC) were prepared with 1×10^8 IFU/mL Ad5. The PC served as positive control without thermal stress (frozen-stored). Effects of each formulation on Ad5 infectivity is shown after liquid storage at 37°C for 14, 21, and 35 days. The mean ± SD from IFU/mL calculations based on at least 5 countings of hexon-positive cells are shown. (b-d): linear regression coefficients (blue for short storage and red for long storage) of single amino acids on Ad5 stability. Values between −1 and +1 which indicate the linear positive and negative effects of single amino acids on Ad5 stability and functionality calculated as IFU/mL from infectivity assay are depicted. (b) influence at short-term storage for up to 21 days (d) at 37°C. (c) Influence at long-term storage for up to 6 months (m) at 25°C. (d) Influence at long-term storage for up to 24 months (m) at 5°C. (Statistical significance: ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$).





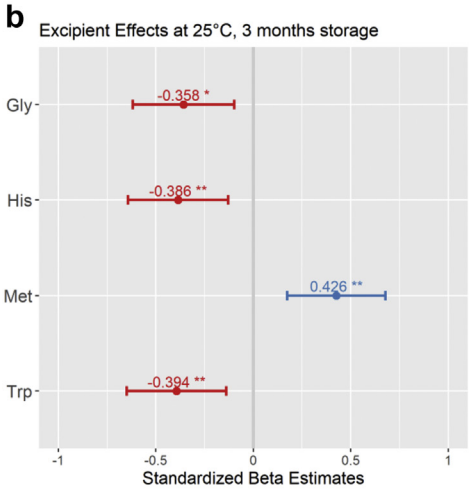
	37°C 14 days			37°C 21 days		
Predictors	*St. Betas*	*t-Stat*	*p-Value*	*St. Betas*	*t-Stat*	*p-Value*
Ala	0.38 (3.37)	3.37	**0.002**			
Lys	0.16 (1.41)	1.41	0.167			
Trp	-0.61 (-5.65)	-5.65	**<0.001**	-0.40 (-2.11)	-2.11	**0.046**
Glu	0.24 (2.12)	2.12	**0.041**			
Met	0.39 (3.61)	3.61	**0.001**			
His				-0.27 (-1.41)	-1.41	0.172
Observations	39			25		
R^2 / R^2 adjusted	0.617 / 0.559			0.219 / 0.148		
AIC	847.936			-113.527		

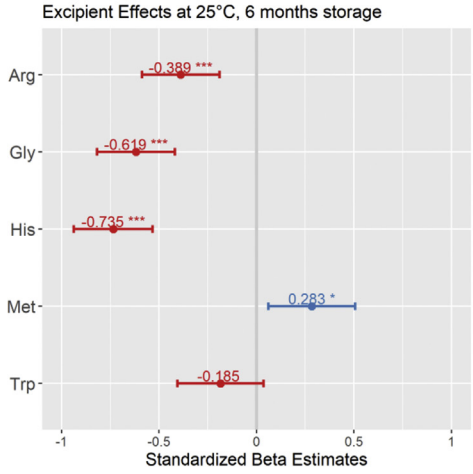
Figure 2. Multiple regression standardized beta coefficients (red for negative and blue for positive effects) of the winning reduced model fitting the effects of relevant amino acids and pairwise interactions on Ad5 stability after short- and long-term storage. (a) Influence at short-term storage for up to 21 days at 37°C. (b) Influence at long-term storage for up to 6 months at 25°C. (c) Influence at long-term storage for up to 24 months at 5°C. (Statistical significance: ***$p < 0.001$, **$p < 0.005$, and *$p < 0.05$).

previously as well as in the original formulation as assay control (Fig. 1a).

The initial statistical evaluation of linear effects of single amino acids using first-order regression during liquid storage at 37°C (Fig. 1b) revealed positive, neutral, and negative influences of individual amino acids on Ad5 infectivity levels after 14 days of storage ($F(8,31) = 3.76$, $p < 0.01$, adjusted $R^2 = 0.36$) and 21 days of storage ($F(8,31) = 3.06$, $p < 0.05$, adjusted $R^2 = 0.29$). The antioxidative effective amino acid Met showed a very significant ($p < 0.01$) positive influence on Ad5 stability after 14 days and a trend toward significance ($p = 0.05$) after 21 days short-term liquid storage at 37°C. A similar trend was found for the osmolytic amino acid Ala, revealing a minor positive effect ($p = 0.08$) on Ad5 stability during liquid storage for up to 21 days at 37°C. In contrast, the radical scavenging amino acid Trp elicited a consistent very significant ($p < 0.01$) negative effect during liquid storage for up to 21 days short-term liquid storage at 37°C (Fig. 1b). Arg (basic amino acid), Lys-HCl (basic amino acid), His (buffering amino acid, radical scavenger, basic, aromatic), Glu and Gly (osmolytic amino acids) did not show any significant influence on response.

To achieve deeper insights into the impact of preselected amino acids on the Ad5 stability during short-term liquid storage at 37°C and particularly their applicability as predictors for long-term liquid storage, an explorative multiple stepwise regression analysis of the resulting Ad5 infectivity at indicated time points was performed post hoc. Regression results revealed that after 14 days of storage at 37°C, 5 amino acids (of which four exhibiting a significant main effect) were useful predictors explaining 56% of the variance in Ad5 infectivity ($F(5,33) = 10.6$, $p < 0.001$, see the Table in Fig. 2a). In line with the results of the linear regression, the antioxidative effective amino acid Met showed a very significant positive effect on Ad5 stability (all p values in Tables), followed by a minor but now detectable positive effect on Ad5 stability of the osmolytic amino acids Ala and Glu (Fig. 2a top). A further





	25°C 3 months			25°C 6 months		
Predictors	*St. Betas*	*t-Stat*	*p-Value*	*St. Betas*	*t-Stat*	*p-Value*
Gly	-0.36 (-2.70)	-2.70	**0.011**	-0.62 (-6.05)	-6.05	**<0.001**
His	-0.39 (-2.94)	-2.94	**0.006**	-0.74 (-7.12)	-7.12	**<0.001**
Trp	-0.39 (-3.03)	-3.03	**0.005**	-0.18 (-1.64)	-1.64	0.114
Met	0.43 (3.30)	3.30	**0.002**	0.28 (2.49)	2.49	**0.020**
Arg				-0.39 (-3.83)	-3.83	**0.001**
Observations	39			32		
R^2 / R^2 adjusted	0.444 / 0.379			0.755 / 0.708		
AIC	1258.081			-25.493		

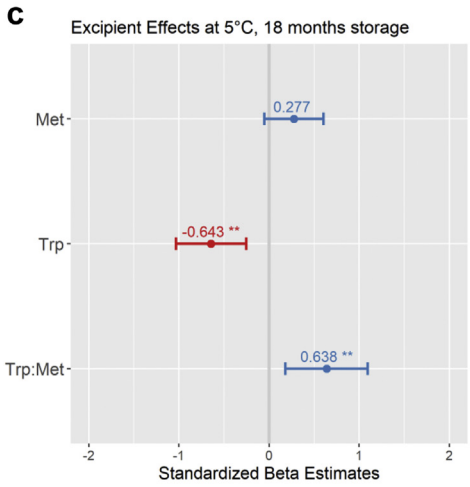
Figure 2. (*continued*).

contribution was given by the already identified radical scavenging amino acid Trp that exerted a highly significant negative effect on the response. After 21 days at 37°C, the total infectivity was considerably reduced, making it more challenging to fit a model with the remaining overdetectable level observations. The best reduced model showing a trend to significance ($F(2,22) = 3.08$, $p = 0.06$, see the Table in Fig. 2a) revealed that the only amino acid exhibiting a significant main effect on Ad5 was Trp (confirming the preliminary linear analysis results), negatively influencing infectivity and explaining, together with a not-detectable effect of His, 15% of the overall variance (Fig. 2a bottom and see Table in Fig. 2a). No interaction or second-order term revealed to be helpful to increase model accuracy, supporting the main relevance of linear effects on Ad5 predictor modeling.

The identified effects of single amino acids by statistical evaluation of the infective titers at indicated time points during storage at 37°C were in line with the determined infective titers of formulations comprising these amino acids as determined in liquid storage experiments for up to 21 days (37°C; Figs. 3a and 3b). The most effective stabilizing formulations (F1_3, F1_4, F1_10, F_13, F1_16, F1_29, F1_39) partially retained Ad5 infectivity on liquid storage at 37°C for up to 21 days (Fig. 3a) with a titer loss of approx. 1 to 2 log levels. By contrast, when the original formulation designed for frozen storage of the virus was used, Ad5 infectivity already dropped below the limit of detection (*LOD*) after 14 days liquid storage at 37°C (Fig. 3a).

After liquid storage for more than 1 month (5 weeks) at 37°C, only Ad5 in formulation F1_29 remained active with a titer loss of approx. 3 log levels, while infectivity dropped below the LOD within all other formulations. All stabilizing formulations contained either 2 or 3 selected amino acids with positive effects (Figs. 1b and 2a) and lacked Trp, which was shown earlier to elicit a significant negative effect on Ad5 stability.

In contrast to the best stabilizing formulations such as F1_29, the weakest stabilizing formulations maintained measurable Ad5 infectivity only during liquid storage for up to 14 days storage at 37°C (Fig. 3b). In line with the results of the preliminary linear and subsequent multiple stepwise regression analysis of the DoE-based infectivity data, these weakly stabilizing formulations contained the amino acid Trp with its significant negative effect on Ad5



	5°C 18 months			5°C 24 months		
Predictors	*St. Betas*	*t-Stat*	*p-Value*	*St. Betas*	*t-Stat*	*p-Value*
Trp	-0.64 (-3.24)	-3.24	**0.003**	-0.70 (-2.78)	-2.78	**0.009**
Met	0.28 (1.65)	1.65	0.108	0.25 (1.42)	1.42	0.165
Trp:Met	0.64 (2.73)	2.73	**0.010**	0.72 (2.49)	2.49	**0.018**
Observations	40			38		
R^2 / R^2 adjusted	0.507 / 0.466			0.419 / 0.368		
AIC	3103.756			4836.528		

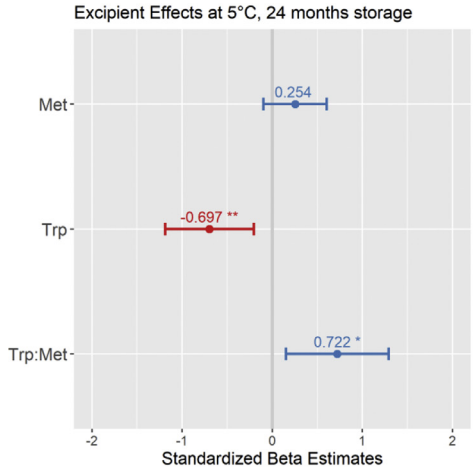


Figure 2. (*continued*).

stability. Accordingly, the best-performing formulations did not comprise Trp.

Based on these results, the addition of the most effective stabilizing amino acids Met, Ala, and Glu, as well as the elimination of Trp, were considered for long-term storage experiments and further iterative optimization of the formulations.

Long-Term Liquid Storage at 25°C and 5°C

The initial linear regression of the DoE-based Ad5 infectivity results analyzed at indicated time points, according to guideline ICH Q1, during long-term liquid storage at 25°C for 3 months ($F(8,31) = 2.16$, $p = 0.06$, adjusted $R^2 = 0.19$) and up to 6 months ($F(8,31) = 8.4$, $p < 0.001$, adjusted $R^2 = 0.6$) and at 5°C for 18 months ($F(8,31) = 2.97$, $p < 0.05$, adjusted $R^2 = 0.29$) and up to 24 months ($F(8,31) = 3.69$, $p < 0.01$, adjusted $R^2 = 0.36$) revealed similar influences of single amino acids on the Ad5 stability compared to liquid storage at 37°C (Fig. 1). Consistently with the results obtained during short-term liquid storage of Ad5 at 37°C, the amino acid Met showed a highly significant stabilizing effect ($p < 0.001$) on Ad5 during liquid storage at 25°C for 6 months and at 5°C for 18 and 24 months. In a similar manner, the amino acid Trp elicited significant negative effects on Ad5 stability during liquid storage after 3 ($p < 0.05$) and 6 months ($p < 0.001$) at 25°C and after 18 ($p < 0.05$) and 24 months at 5°C ($p < 0.01$), thus mimicking the pattern already present in the analysis performed at 37°C (Figs. 1c-1d). The already marginal positive effect observed for Ala during short-term storage at 37°C was slightly reduced resulting in an overall neutral effect of this amino acid during long-term liquid storage at 25°C and 5°C.

Subsequent exploratory multiple stepwise regression analysis of the Ad5 infectivity data at indicated time points, during long-term liquid storage at 25°C (for up to 6 months) and 5°C (for up to 24 months), confirmed similar influences of single amino acids on the Ad5 stability compared to liquid storage at 37°C.

At 25°C, the results of the regression indicated that four predictors (amino acids Gly, His, Met, and Trp) had a main effect in explaining 38% of the variance in Ad5 infectivity ($F(4, 34) = 6.8$, $p < 0.001$, see the Table in Fig. 2b). After 6 months of storage, one further predictor (the basic amino acid Arg) was included to fit the

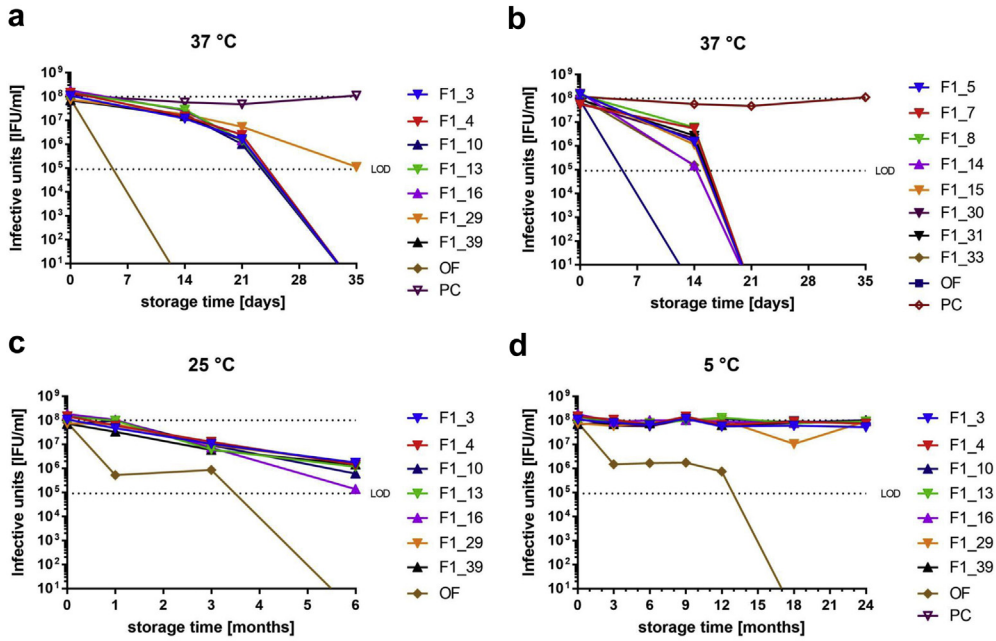


Figure 3. Infectivity of Ad5 in liquid DoE formulations after different storage conditions. The 40 DoE formulations F1_1 to F1_40 and the original formulation (OF) were prepared with 1×10^8 IFU/mL Ad5 (upper dotted line) and stored at different thermal stress conditions: (a) best performing formulations are shown as time kinetics for up to 35 days at 37°C, (b) worst performing formulations are shown as time kinetics for up to 35 days at 37°C, (c) best performing formulations are shown as time kinetics for up to 6 months at 25°C, (d) best performing formulations are shown as time kinetics for up to 24 months at 5°C. The lower dotted line indicates the limit of detection with a value of 9.87×10^4 IFU/mL. The mean ± SD from IFU/mL calculations based on at least 5 countings of hexon-positive cells are shown.

best model, reaching 71% of explained variance in Ad5 infectivity ($F(5, 26) = 16.1$, $p < 0.001$, see the Table in Fig. 2b). The main positive effect of Met on Ad5 stability during liquid storage at 25°C was very significant at 3 months and significant also later at 6 months of storage (see Fig. 2b). The very significant negative effect of Trp on Ad5 stability, which was already detectable in the analyses at 37°C, was visible at 3 months storage but did not reach significance 3 months later at 25°C (6 months) because of high variance fluctuations (see Fig. 2b). Three further amino acids (the osmolytic amino acid Gly, the buffering amino acid, radical scavenger, basic and aromatic His, and the basic amino acid Arg) showed a very significant negative main effect on Ad5 stability after 6 months of storage at 25°C.

Overall, these results demonstrate the higher accuracy of the applied multiple stepwise regression analysis on Ad5 infectivity response at indicated time points during long-term storage for up to 6 months at 25°C over the preliminary linear regression analysis, offering a more detailed picture about the influence of single amino acids on the Ad5 stability beside the 2 main effects of Met and Trp. The post hoc analysis served also to detect interactions over Ad5 infectivity at indicated time points during long-term storage at 5°C. Multiple stepwise regression results indicated that after 18 months at 5°C, 2 predictors (amino acid Met and Trp) and their interaction explained 47% of the variance in Ad5 infectivity ($F(3,36) = 12.3$, $p < 0.001$, see the Table in Fig. 2c). After 24 months, the analysis confirmed the impact of the same 2 predictors (amino acid Met and Trp) and their interaction on Ad5 infectivity, explaining this time 37% of the overall variance ($F(3, 34) = 8.18$, $p < 0.001$, see the Table in Fig. 2c). Although amino acid Met alone did not have an impact on Ad5 stability during liquid storage for 18 and 24 months at 5°C, the interaction of Met and Trp showed a surprising significant stabilizing effect on Ad5 infectivity during liquid storage for 18 months as well as for 24 months at 5°C (see Fig. 2c top and Fig. 2c bottom). By contrast, amino acid Trp alone elicited a very significant negative effect on Ad5 stability during liquid storage for 18 months as well as for 24 months at 5°C in line with the findings for liquid storage for 3 and 6 months at 25°C.

The evaluated results during liquid long-term storage of the seven best of stabilizing liquid formulations (F1_3, F1_4, F1_10, F1_13, F1_16, F1_29, F1_39), already identified during short-term storage at 37°C, are shown in Figure 3. Figure 3c depicts the corresponding results obtained at indicated time points during liquid storage for up to 6 months at 25°C. The loss of only maximal 1 log level of the virus infectivity was observed after 3 months and 2-3 log levels after 6 months storage at 25°C (Fig. 3c). By contrast, after liquid storage for 3 months, the infective titer of the Ad5 virus formulated in original formulation completely dropped below the LOD.

As shown in Figure 3d, the stabilizing effects of the seven best of stabilizing formulations (F1_3, F1_4, F1_10, F1_13, F1_16, F1_29, F1_39) and their impact on Ad5 infectivity during liquid storage at 5°C are shown over a storage time of up to 24 months. A dramatic loss of infectivity was found for Ad5 in original formulation already after 3 months storage at 5°C, which was expected for a formulation designed for frozen storage. Accordingly, after 12 months storage at 5°C, a complete drop of the Ad5 infectivity below the LOD was observed in the original formulation. By contrast, best of stabilizing formulations almost completely maintained Ad5 viral infectivity during long-term storage at 5°C for up to 24 months. These results are in line with the statistical analysis of the linear effects of single amino acids on Ad5 stability. For example, all identified best of formulations lack Trp which was shown to have negative effects on the Ad5 infectivity at all storage temperatures (Figs. 1 and 2). Formulations without the significant positive amino acid Met resulted in a drop of the infective titer below the LOD after 24 months at 5°C or to a loss of infectivity up to 1 to 2 log levels during liquid storage for 24 months at 5°C (data not shown). During long-term liquid storage for up to 6 months at 25°C, the same formulations showed only minor stabilizing effects on functional integrity of the viral vector (data not shown).

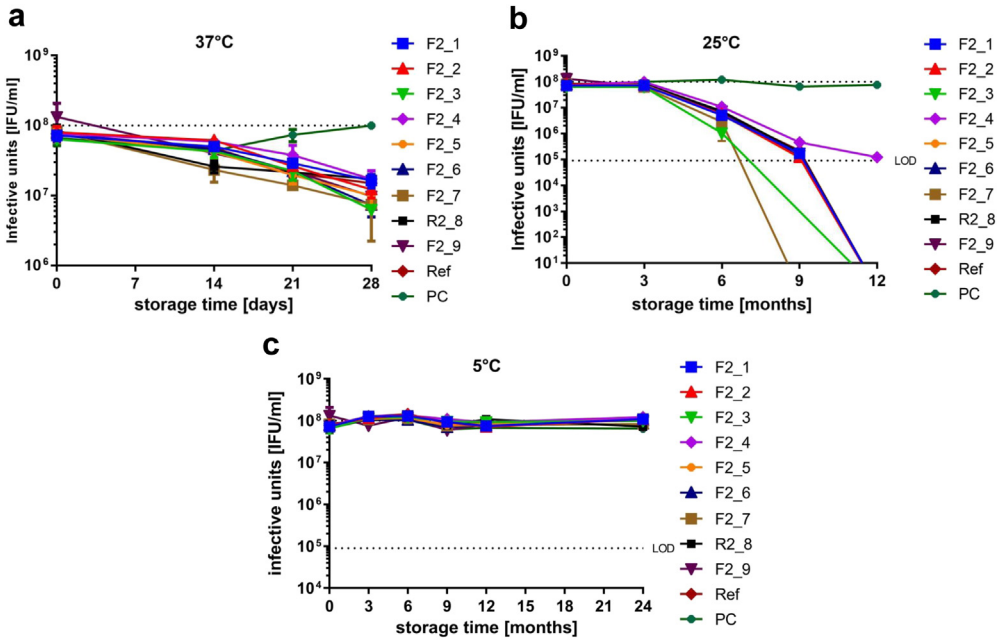


Figure 4. Infectivity of Ad5 in liquid formulations (round 2) after different storage conditions. Ad5 formulated in optimized formulations F2_2 to F2_6 and F2_8 and F2_9 were compared with the selected formulations from round 1 (F1_13 = F2_7 and F1_29 = F2_1) and the positive control (PC) were prepared with 1×10^8 IFU/mL Ad5 (upper dotted line) and stored at different conditions: (a) all formulations are shown as time kinetics for up to 28 days at 37°C, (b) all formulations are shown as time kinetics for up to 12 months at 25°C, (c) all formulations are shown as time kinetics for up to 24 months at 5°C. The lower dotted line indicates the limit of detection with a value of 9.87×10^4 IFU/mL. The values show the mean ± SD generated of at least 15 countings of hexon-positive cells (5 countings from 3 biological replicates).

In line with the aforementioned observations, the best stabilizing formulations during liquid storage for up to 24 months at 5°C all contained the significant positive amino acid Met in combination with the negative influencing amino acid Trp (data not shown). These results indicate that during long-term storage for up to 24 months at 5°C, the presence of the strong significant positive acting amino acid Met counteracts the negative influence of Trp, as shown by the significant interaction effect found in the regression analyses at 5°C.

Iterative Optimization of the Stabilizing Ad5 Formulations

The identified most effective stabilizing amino acids Met, Ala, and Glu, and 2 of the most effective stabilizing amino acid compositions (Fig. 3a; formulations F1_13 and F1_29) of the first round provided the framework for the second iterative stabilization round for the Ad5 vector. Based on learnings from the DoE approach, formulations F1_13 and F1_29 were iteratively modified regarding the amino acid composition with a focus on Met, Ala, Glu, type of sugar and sugar alcohol, and the elimination of $MgCl_2$. Formulation F1_13 contained the osmolytic amino acids Ala and Glu in combination with the buffering amino acid His, sucrose, but also $MgCl_2$. Although formulation F1_13 did not contain Met, this formulation was one of the most effective Ad5 stabilizing formulations in round 1 (Fig. 3a). Therefore, we assumed that formulations containing high amounts of the osmolytic amino acid Ala and intermediate amounts of the osmolytic acidic amino acid Glu may overcome the lack of the antioxidative amino acid Met. Interestingly, the stabilizing effectivity of formulation F1_13 without $MgCl_2$ (labeled in the second round as F2_8) was comparable to the high stabilizing efficacy of formulations F2_1 (previous F1_29) and F2_4 (previous F1_29, but without $MgCl_2$) during liquid storage at 37°C (Table 1).

Formulation F1_29 (labeled as F2_1 in the second round) contained the osmolytic amino acid Ala, the basic amino acid Lys-HCl, His as buffering agent, and the antioxidative effective amino acid Met, in combination with sucrose and $MgCl_2$. To analyze the influence of various amino acids, sucrose, and, in particular, $MgCl_2$, on the stability of Ad5 viral vector during liquid storage, the following modifications were made: exchange of amino acids with similar characteristics, exclusion of $MgCl_2$, substitution of sucrose by mannitol, or addition of the osmolytic amino acid glutamic acid (Glu; Table 1). Again, the elimination of $MgCl_2$ from formulation F1_29 (F2_1) resulting in formulation F2_4 in the second round led to equal results during liquid storage for up to 28 days at 37°C (Fig. 4a).

Liquid storage for 6 months at 25°C led to a loss of titer only between 1.5 and 2 log levels of Ad5 infectivity for most formulations. The most remarkable loss was observed when formulation F1_29 was modified by substituting the basic amino acid Lys-HCl by the other basic amino acid Arg (formulation F2_3; 2.66×10^5 IFU/mL) and in formulation F2_7 (7.19×10^5 IFU/mL). After liquid storage for 9 months at 25°C, the infective Ad5 titer of these 2 formulations dropped below the LOD. The negative effect of Arg (formulation F2_3) was already observed in the DoE approach in round 1 and particularly in the multiple stepwise regression analysis of the infective titers of Ad5 after 6 months storage at 25°C (Fig. 2b; bottom). In line with the results from round 1, formulation F2_7 without the positive effective amino acid Met (labeled as F1_13 from the first round) revealed comparable titers to all other formulations during liquid storage for up to 6 months at 25°C. Only the extension of the storage time up to 9 months at 25°C in round 2 led to the loss of Ad5 titer in F2_7 below the LOD possibly as a result of lack of Met. Interestingly, the elimination of $MgCl_2$ in formulation F2_7 resulting in F2_8 led to the retention of the infective titer after liquid storage for up to 9 months at 25°C comparable to the other formulations.

Ad5 viral vector formulated in the other iterative modified formulations according to round 2 (F2_1; F2_2; F2_4; F2_5; F2_6; F2_8 and F2_9; Table 1) retained the infective titer even after 9 months storage at 25°C. The best stabilizing effect was observed with the formulation F2_4 without $MgCl_2$, which performed best with a titer

of 1.23×10^5 IFU/mL after 12 months at 25°C (Fig. 4b). These results suggest that the addition of $MgCl_2$ to the stabilizing formulation of an adenoviral vector is not unambiguously necessary for the stabilization of the Ad5 virus during liquid storage at elevated temperature. Moreover, during long-term storage for up to 24 months at 5°C, all formulations from round 2 effectively stabilized Ad5 with only minor differences in infective titer between each time point (Fig. 4c).

Discussion

Our study showed for the first time that an algorithm-based preselection of excipients by means of a pre-existing in-house database and by implementation of a DoE formulation approach in conjunction with a tailored 37°C challenging model is highly efficient for the design of viral vector liquid formulations. Here, we used Ad5 which is considered to be one of the main candidates for vaccination and therapeutic gene transfer in medicine.[2,3] Although different formulations for Ad5 vectors have been extensively studied in the past,[6,18,37] the formation of higher-order aggregates and consequently functional loss are still major issues during manufacturing and particularly during downstream processing.[7,9] For example, approaches to avoid agglomeration of viral vectors by using glycerol, divalent cations, for example, Mg^{2+}, nonionic surfactants,[39] or high ionic strength solutions (e.g., by addition of multivalent ions in combination with nuclease treatments[7]) have shown some limited beneficial effects. However, those effects are often associated with other unappreciated regulatory drawbacks during downstream processing. Although in the early phase of downstream processing, the selection of excipients seems to remain rather empiric or based on limited knowledge, the currently known best-performing stabilizing formulations for Ad5 viral vectors are very complex, exhibiting several different constituents, of which some formulation components are even associated with regulatory issues. Taking all into account, high evidence-based predictive approaches for the identification of excipients and formulations that stabilize the target viral vectors during long-term storage are urgently needed. These approaches would avoid costly and time-consuming reformulation rounds during manufacturing.

In this study, we accomplished to preselect relevant amino acids as effective stabilizing excipients for Ad5 viral vector from our in-house database. The search module of this database allowed for a systematic retrieval of excipients that in the past were successfully used in various formulations for similar target molecules, for example, nonenveloped viral vectors containing a protein capsid or proteins at different stress conditions. The combination of a DoE approach with a specifically designed 37°C challenging model helped to further downsize the design space, reducing the impact predictors, the number of preselected amino acids, and the concentration range of other excipients. Thus, the application of this strategy is associated with a remarkable reduction of experimental effort, time, and costs with a concurrent increase in accuracy and methodological evaluation of the results. The most effectively stabilizing formulations from round 1 regarding the retention of Ad5 infective titer during liquid storage for up to 5 weeks at 37°C and the amino acids selected by the results of a linear regression applied to the single amino acids were used in an iterative optimization round 2 for identification of the best formulations.

A major finding was the influence of single amino acids on Ad5 stability evaluated by functional infectivity assays: amino acids that were positively selected in the 37°C challenging model (2 weeks at 37°C) also positively influenced stability and function of Ad5 during intermediate storage conditions (3 and 6 months at 25°C) and long-term storage (18 and 24 months at 5°C). For example, Met had significant positive effects on Ad5 stability and infective functionality in the accelerated aging model after 14 days of storage at 37°C as well as during long-term storage. Similar findings were observed for the other preselected amino acids. A prominent amino acid with significant negative influence on Ad5 stability was found to be Trp in the 37°C challenging model and thus was not considered for further formulation development steps.

A rather unexpected finding in this study was that the amino acid Trp with its significant negative influence on Ad5 stability under 37°C challenging conditions was present in the most effective stabilizing formulations after 24 months liquid storage at 5°C, although all formulations containing Trp in the 37°C challenging model failed to stabilize Ad5 during short-term storage at 37°C. However, these formulations all contained the strong significant positive acting Met, suggesting a partial masking of the negative influence of Trp by the stabilizing effect of Met during long-term liquid storage at 5°C and a cross-over reaction between Trp and Met. These results were substantiated by the significant interaction effect between the amino acids Met and Trp found in the multiple stepwise regression analysis of the infective Ad5 titers at indicated time points during liquid storage at 5°C. This Met-mediated phenomenon to mask Trp-negative effects was not evident in the accelerated aging experiments at 37°C in which the negative Trp effect alone was found to be very prominent, without significant interaction effects. This was different during long-term storage at 5°C where the positive effect of the interaction between Met and Trp was statistically very significant, with Met alone exhibiting also a significant main effect in the 37°C challenging model, statistically very significant only after 14 days storage at 37°C, but not significant after 21 days storage at 37°C.

The significant positive effect of the antioxidative amino acid Met on Ad5 stability under all examined liquid storage conditions implies that oxidation might be the main degradation pathway of the protein capsid of Ad5 viral vectors. In general, the degradation pathways of the capsid proteins of the Ad5 viruses under the influence of various stress conditions are comparable to the well-known main degradation pathways of proteins, for example, aggregation, deamidation, Asp isomerization, and oxidation. By contrast, previous studies have shown that the main cause of damage of Ad5 viral particles during long-term storage for 24 months at 5°C is free-radical oxidation, which was limited by the addition of combinations of metal chelators and hydroxyl radical scavangers.[6,18,19] It is well-known that Met acts as scavenger (but not as radical scavenger) for dissolved oxygen in liquid pharmaceutical formulations of therapeutic proteins competitive with Met side chains in the protein. By contrast, the amino acid Trp is a well-known radical scavenger because of its aromatic ring system in the side chain. In this context, it was surprising that Trp alone showed significant negative effects on the Ad5 stability during short-term storage for 14 days at 37°C, as well as during long-term storage at 5°C and 25°C. It was also unexpected that the combination of the positive acting amino acid Met with Trp showed significant positive effects on the Ad5 stability during long-term storage at 5°C, but not during short-term and intermediate-term storage at elevated temperatures. An explanation might be that the radical scavenging activity of Trp recycles dissolved oxygen that leads to oxidation and subsequent damage of the Ad5 particle in the absence and even in the presence of Met at elevated temperatures, suggesting that the 2 kinds of reactions are not in equilibrium. By contrast, during long-term storage at 5°C, these 2 reactions, the oxygen scavenging activity of Met and the radical scavenging activity of Trp, might elicit additive effects.

Similarly, the effects of Ala and Glu, which elicited a slightly protecting effect in the 37°C challenging model, turned out to be

not relevant during long-term liquid storage at 5°C. Another example for a combination of 2 amino acids which revealed to have negative effects was Trp and His. High concentration of Trp in combination with His showed negative effects on Ad5 stability at 37°C and 3 weeks of storage. Moreover, the multiple stepwise regression analysis of the infective titer of Ad5 revealed that amino acids Gly, His, and Arg showed a significant negative main effect on Ad5 infectivity after liquid storage for 6 months at 25°C. From these findings, we conclude that future rounds of screening and optimization DoE-based experiments should be implemented to determine the individual and interaction effects of amino acid combinations in more detail.

Based on the learnings from the DoE approach of the first round, we were able to optimize stabilizing effectivity of the formulations in a second optimization round. The identified best stabilizing single amino acids Met, Ala, and Glu in combination with the best 2 formulations of the first round provided the framework for the iterative optimization of the stabilizing formulations. By means of this approach, we underlined the strength of an algorithm-based preselection process of amino acid−based stabilizing excipients in combination with a 37°C challenging model. For example, the finally selected formulation F2_4 for liquid storage of Ad5 revealed to be less complex compared to state-of-the-art formulations.[18] Moreover, this formulation did not require $MgCl_2$, which is a standard excipient in most presently existing stabilizing formulations for Ad5,[18,39,40] without loss of stabilizing effectivity.

Overall, these results underline the predictive value of the 37°C challenging model and the importance of balancing amino acid combinations and other excipients to achieve tailored target-specific formulations.

From our findings, we conclude that the combination of the 37°C challenging model with a database driven excipient preselection and a DoE-based formulation design has an important predictive value for long-term storage stability. Moreover, the formulation approach enabled further iterative optimization, resulting in a simplified stabilizing Ad5 formulation with enhanced stabilizing efficacy. Nevertheless, the infectivity assay revealed differences in the total numbers of measured infective virus particles between the first formulation round and the optimization round. This should be harmonized in future experiments, especially when the current approach is intended for regulatory relevant standard operative procedures during downstream processing. It is important to notice that our preselected excipients not only revealed mainly linear stability effects, but interaction terms also came into play, in particular for Met and Trp. Future DoE designs should therefore incorporate interactions between excipients as important predictors to explain combinatory effects in the resulting viral infectivity during long-term storage.

In conclusion, the preselection approach applied with our database platform may enable the generation of best-in-class stability formulations for Ad5 viruses in liquid. However, further studies with other viral vectors ought to be performed accordingly to validate the general applicability of this procedure. Because of its predictive value, this approach could have a significant economic impact when applied early in downstream processing during viral vector-based vaccine or gene transfer vehicle manufacturing, therefore circumventing time-consuming and costly reformulation loops after real-time storage.

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