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The 6-chromanol derivate SUL-109 enables prolonged hypothermic storage of adipose tissue-derived stem cells

Ghazaleh Hajmousa [a], Pieter Vogelaar [b], Linda A. Brouwer [a], Adrianus C. van der Graaf [b], Robert H. Henning [c], Guido Krenning [a, b, *]

[a] Cardiovascular Regenerative Medicine, Dept. Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1 (EA11), 9713GZ, Groningen, The Netherlands
[b] Sulfateq B.V., Admiraal de Ruyterlaan 5, 9726GN, Groningen, The Netherlands
[c] Dept. Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Hanzeplein 1 (EB71), 9713GZ, Groningen, The Netherlands

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ABSTRACT

Encouraging advances in cell therapy research with adipose derived stem cells (ASC) require an effective short-term preservation method that provides time for quality control and transport of cells from their manufacturing facility to their clinical destination. Hypothermic storage of cells in their specific growth media offers an alternative and simple preservation method to liquid nitrogen cryopreservation or commercial preservation fluids for short-term storage and transport. However, accumulation of cell damage during hypothermia may result in cell injury and death upon rewarming through the production of excess reactive oxygen species (ROS). Here, the ability of the cell culture medium additive SUL-109, a modified 6-chromanol, to protect ASC from hypothermia and rewarming damage is examined. SUL-109 conveys protective effects against cold-induced damage in ASC as is observed by preservation of cell viability, adhesion properties and growth potential. SUL-109 does not reduce the multilineage differentiation capacity of ASC. SUL-109 conveys its protection against hypothermic damage by the preservation of the mitochondrial membrane potential through the activation of mitochondrial membrane complexes I and IV, and increases maximal oxygen consumption in FCCP uncoupled mitochondria. Consequently, SUL-109 alleviates mitochondrial ROS production and preserves ATP production. In summary, here we describe the generation of a single molecule cell preservation agent that protects ASC from hypothermic damage associated with short-term cell preservation that does not affect the differentiation capacity of ASC.

1. Introduction

Adipose derived Stem Cells (ASC) hold great promise in regenerative medicine. ASC are multipotent mesenchymal-like stem cells that reside in the perivascular niche as pericytes or periadventitial cells [1] in white adipose tissue throughout the body [2–4]. ASC secrete a plethora of trophic factors [5] that suppress inflammation and apoptosis, yet promote angiogenesis and mitosis of parenchymal cells [6]. Moreover, ASC have the ability to differentiate into several cell types, including adipocytes, chondrocytes, osteoblasts and muscle cells under lineage-specific culture conditions [2–4].

Encouraging advances in cell therapy research using ASC or ASC-derived tissue engineered constructs demonstrated their ability in the regeneration of bone and cartilage defects [7,8], ischemic limb disease [9], skin wound healing [5], and myocardial infarction [10]. However, the clinical application of ASC requires an effective short-term preservation method, thus providing time for quality control and transport of cells from their manufacturing facility to their clinical destination.

Hypothermic storage of cells in their normal culture media offers an accessible, logistically easy and cost-effective alternative to cryopreservation, vitrification and protocols using preservation fluids (*e.g.* ViaSpan or HypoThermosol) for short-term storage and transport [11]. In contrast to cryopreservation, which is a complicated cell type-specific process hampered by cell loss and mutagenesis [12,13], hypothermic storage should be a simplified process

* Corresponding author. Cardiovascular Regenerative Medicine, Dept. Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Hanzeplein 1 (EA11), 9713GZ, Groningen, The Netherlands.
E-mail address: g.krenning@umcg.nl (G. Krenning).

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that voids these complications at low costs. Hypothermia slows metabolic activity and cell cycle progression, thereby maintaining the ASC in their current state. However, hypothermia may produce significant cell injury and death, particularly upon rewarming when excess reactive oxygen species (ROS) are produced [14,15]. Current hypothermic preservation fluids suffer from this rewarming damage; whilst effective at low temperature, they do not prevent severe cell damage upon rewarming [16]. Additionally, the chemical composition of commercially available hypothermic preservation fluids differs vastly from the chemical composition of culture media, which might induce undesirable dedifferentiation of cells or failure of a tissue engineered construct [17,18]. Consequently, hypothermic storage warrants strategies to alleviate these deleterious effects of hypothermia and rewarming, while reliably and consistently maintaining the key characteristics of cells, including viability, phenotype, and, in the case of ASC, differentiation potential.

Substituted 6-chromanols represent a novel class of pharmacological compounds that preserve cell viability under a number of conditions [19–21]. Here, we describe the identification of a 6-chromanol derivate (SUL-109), a single molecule cell culture additive, as a preservation agent that protects human ASC from hypothermia and rewarming damage without affecting their subsequent differentiation capacity.

2. Materials & methods

2.1. ASC isolation and culture

Human subcutaneous adipose tissue samples from healthy human subjects with (BMI < 30) were obtained after liposuction surgery (Bergman Clinics, The Netherlands). All donors provided informed consent and all procedures were performed in accordance to national and institutional guidelines as well as with the ethical rules for human experimentation as stated in the Declaration of Helsinki.

Lipoaspirates were enzymatically digested with 0.1% Collagenase A (Roche Diagnostic, Mannheim, Germany) in PBS, containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MI) at 37 °C for 90 min. Centrifugation (300*g*, 4 °C, 20 min) separated adipocytes and lipid content from the stromal cell fraction. The stromal cell fraction was subjected to Lymphoprep (Axis-Shield PoC, Oslo, Norway) density gradient centrifugation (300*g*, 20 °C, 30 min). The cells from the interface were collected and cultured in DMEM (Lonza #707F, Breda, The Netherlands) containing 10% Fetal Bovine Serum (FBS, GE LifeSciences #SH30071, Pittsburg, PA), 1% Penicillin/Streptomycin (Sigma-Aldrich #P4333, St. Louis, MI) and 2 mM L-glutamine (Life Technologies #25030, Carlsbad, CA). ASC at passage 1 were routinely checked for the expression of pericyte and mesenchymal cell markers according to [22] and had the following phenotype: $CD10^+$, $CD13^+$, $CD31^-$, $CD34^-$, $CD44^+$, $CD45^-$, $CD73^+$, $CD90^+$ and $CD105^+$. At 80% confluence, ASC were dissociated using 0.12% Trypsin, 0.02% EDTA solution in PBS (#59430, Sigma-Aldrich, St. Louis, MI) pelleted by centrifugation (300*g*, 4 °C, 5 min), and reseeded at a density of 10.000 cells/cm^2. ASC at passage 3 were used for experiments described below.

2.2. Cell viability, adherence and proliferation

Alpha-Tocopherol (10 μM, #T1539 Sigma-Aldrich, St. Louis, MI), TROLOX (10 μM, # 10011659, Cayman Chemical, Ann Arbor, MI) or SUL-109 (10 μM; #Rokepie-01, Sulfateq BV, Groningen, The Netherlands) were added to the ASC culture medium. After 2 h culturing at 37 °C, ASC cultures were placed under hypothermic conditions (4 °C) in a standard laboratory refrigerator for 48 h. Thereafter, ASC were re-warmed for 2 h under normal cell culture conditions (37 °C and 5% CO_2).

Viability was assessed using the Apoptosis & Necrosis Kit (Promokine #PK-CA707-30018, Heidelberg, Germany) as by manufacturer's instructions. In short, ASC were dissociated using Trypsin-EDTA solution and suspended in 1× AnnexinV-binding buffer at a concentration of $2 \cdot 10^6$ cells/ml. Next, ASC were incubated with 5 μl fluorescein-conjugated AnnexinV and 5 μl Ethidium Homeodimer III in the dark at room temperature for 15 min. Fluorescence was recorded on a BD FACSCalibur (BD Bioscience, Franklin Lakes, NJ) by a skilled operator at the UMCG Flow Cytometry Core Facility within 1 h of staining.

To assess the adherence capacity of ASC, hypothermic preserved ASC were dissociated using Trypsin-EDTA in 0.9% NaCl and fluorescently labeled with the CFDA SE Cell Tracer (ThermoFisher #V12883, Waltham, MA) according to manufacturer's instructions. Next, fluorescently-labeled ASC were plated at a density of $5 \cdot 10^3$ cells/cm^2 in standard 96-well culture plates (Corning #CLS3596, St. Louis, MI) and allowed to adhere under standard culture conditions (37 °C and 5% CO_2) for 15–240 min. Following extensive washing with PBS to remove non-adhered cells, fluorescence of adherent cells was recorded on a Varioskan spectrofluorometer at Ex/Em 492/520 nm.

To assess cell proliferation, ASC were fixed using 2% paraformaldehyde (Sigma-Aldrich #P6148, St. Louis, MI) in PBS for 20 min. Next, samples were permeabilized using 0.5% Triton ×100 (Life Technologies #85112, Carlsbad, CA) in PBS and incubated in 5% rabbit normal goat serum (Jackson Immunoresearch #005-000-121, Suffolk, UK) at room temperature to block non-specific antibody binding for 10 min. Samples were incubated with polyclonal antibodies to Ki67 (Monosan #PSX1028, San Diego, CA) at a concentration of 2.5 μg/ml. After extensive washing in PBS/Tween-20 (0.1%), samples were incubated with AlexaFluor555-conjugated antibodies to rabbit IgG (Life Technologies #A-21428, Carlsbad, CA) at a dilution of 1:300 in PBS containing 5 μM DAPI (4′,6-Diamidino-2-Phenylindole, Life Technologies #D1306, Carlsbad, CA) at room temperature for 30 min. Samples were mounted in citifluor (Citifluor Ltd #AP-1, London, UK) and visualized on a Zeiss AxioObserver Z1 microscope. Total cell number and Ki67-positive cells were quantified using automated fluorescence microscopy software (TissueFaxs, TissueGnostics, Vienna, Austria).

2.3. Multipotency of ASC

ASC were subjected to hypothermia and rewarming as described above, after which the culture media was changed for differentiation media. To induce multi-lineage differentiation of ASC, cells were cultured in adipogenic medium (DMEM containing 10% FBS, 1% Penicillin/Streptomycin, 2 mM L-glutamine, Dexamethasone (0.1 μM, Sigma-Aldrich #D4903, St. Louis, MI), Insulin (1 nM, Sigma-Aldrich #I2643, St. Louis, MI) and IBMX (0.5 mM, Sigma-Aldrich #I7018, St. Louis, MI)), osteogenic medium (DMEM containing 10% FBS, 1% Penicillin/Streptomycin and 2 mM L-glutamine, Dexamethasone (0.1 μM), β-glycerophosphate (10 mM, Sigma-Aldrich #G9422, St. Louis, MI) and L-ascorbic acid (0.5 mM, Sigma-Aldrich #A5960, St. Louis, MI)), or myogenic medium (DMEM containing 10% FBS, 1% Penicillin/Streptomycin and 2 mM L-glutamine and TGF-β1 (10 ng/ml, Peprotech #100-21, Rocky Hill, NJ)) for 21 days.

To assess differentiation, ASC were fixed using 2% paraformaldehyde (Sigma-Aldrich, #P6148) in PBS for 20 min and phenotyped using (immuno)histological stains. For adipogenesis, ASC were rinsed with 60% isopropanol and stained with Oil Red O (0.5%, Sigma-Aldrich #O0625, St. Louis, MI) at room temperature for 15 min. For osteogenesis, samples were stained in Alizarin Red

(0.5%, Sigma-Aldrich #A5533, St. Louis, MI) at room temperature for 10 min. Samples were counterstained using hematoxylin and mounted in Kaisers Glycerin. To assess myogenic differentiation, ASC were permeabilized with 0.5% Triton ×100 (Life Technologies #85112, Carlsbad, CA) in PBS and incubated with polyclonal antibodies to SM22 alpha (2.5 μg/ml, #ab14106, Abcam, Cambridge, UK) for 60 min. After extensive washing in PBS/Tween-20 (0.1%), samples were incubated with AlexaFluor555-conjugated antibodies to rabbit IgG (2.0 μg/ml, Life Technologies #A-21428, Carlsbad, CA) in PBS containing 5 μM DAPI (4′,6-Diamidino-2-Phenylindole, Life Technologies #D1306, Carlsbad, CA) at room temperature for 30 min. Samples were mounted in citifluor (Citifluor Ltd #AP-1, London, UK) and visualized on a Zeiss AxioObserver Z1 microscope in fluorescence mode.

2.4. Antioxidant assays

5 μM 2′7′-dichlorodihydrofluorescein diacetate (DCF, Sigma-Aldrich #D6883, St. Louis, MI) was dissolved in Hanks Balanced Salt Solution (#H1641, Sigma-Aldrich, St. Louis, MI) and supplemented with α-tocopherol, TROLOX, or SUL-109 at concentrations ranging from 10^{-9} to 10^{-3} M. 3 μM hydrogen peroxide (H_2O_2, MerckMillipore #107209, Darmstadt, Germany) was added as reactive oxygen donor and samples were incubated at room temperature for 1 h. Fluorescence was recorded on a Varioskan spectrofluorometer (ThermoScientific, Waltham, MA) at 488/525 nm (Ex/Em).

2.5. Metabolic state, mitochondrial structure and mitochondrial membrane potential analyses

The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of hypothermically preserved ASC were determined after rewarming in the presence or absence of SUL-109 for 2 h, using the Seahorse Bioscience Extracellular Flux analyzer (Seahorse Bioscience, Billerica, MA). In short, changes in extracellular oxygen tension and pH were determined on the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). After steady state oxygen consumption and basal extracellular acidification rates were obtained, 2 μM Oligomycin (ATP Synthase inhibitor, Seahorse Bioscience #9634398, Billerica, MA) and 5 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, mitochondrial uncoupler, Seahorse Bioscience #9634398, Billerica, MA) were sequentially added through the reagent delivery chambers of the flux analyzer to measure the maximum oxygen consumption rates and maximum acidification rates. Finally, a mixture of 2 μM Rotenone and 2 μM Antimycin A was used to block mitochondrial electron transport. As OCR and ECAR reflect the metabolic activity of cells and are influenced by cell number, OCR and ECAR values were normalized to total protein content in a sample.

Mitochondrial morphology was assessed using a mitochondrial membrane potential-independent dye (*i.e.* MitoTracker-Green, ThermoFisher Scientific #M7514, Waltham, MA). In short, ASC were incubated with 120 nM MitoTracker-Green at 37 °C in a 5% CO2 humidified chamber for 45 min. Next, cells were analyzed by live cell imaging on a confocal microscope (Leica TCS SP2 Confocal Microscope, Wetzlar, Germany) with a 63× oil immersion objective using an excitation wavelength of 488 nm and emission was recorded through a band pass 500–550 nm filters. ASC were kept at 37 °C in a 5% CO_2 humidified microscope stage chamber throughout the analysis.

The mitochondrial membrane potential was assessed using the JC-1 MitoProbe (ThermoFisher Scientific #M34152, Waltham, MA). ASC were dissociated using Trypsin-EDTA in 0.9% NaCl and suspended at $1{\cdot}10^6$ cells/mL in warm medium containing 2 μM JC-1. Membrane potential-dependent JC-1 accumulation in the mitochondria (indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm)) was recorded by flow cytometry on a FACS Calibur (BD Biosciences, Franklin Lakes, NJ). To depolarize the mitochondrial membrane, 50 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added for 15 min prior to JC-1 loading.

2.6. Mitochondrial complex I-V activity measurements

ASC-derived mitochondria were isolated using density gradient centrifugation using the MitoCheck® Mitochondrial Isolation Kit (Cayman Chemical #701010, Ann Arbor, MI) according to manufacturer's instructions and assessed for the activities of mitochondrial complex I-V. In all activity measurements, isolated ASC-derived mitochondria were pre-incubated with a concentration series of SUL-109 (10^{-9}–10^{-4} M) at room temperature for 15 min. Mitochondrial complex I (NADH oxidase/co-enzyme Q reductase) activity was determined by measuring the decrease in NADH oxidation, which is reflected by a decreased in absorbance at 340 nm (Cayman Chemical #700930, Ann Arbor, MI) in the presence of 2 mM potassium cyanide (KCN) to prevent the oxidation of Q. Activity of mitochondrial complex II (Succinate dehydrogenase/co-enzyme Q reductase) was assessed by the rate of reduction of DCPIP, which is protonated by reduced co-enzyme Q (Cayman Chemical #700940, Ann Arbor, MI), and is reflected by a decrease in absorbance at 600 nm. To prevent interference of mitochondrial complexes I, III and IV, complex II activity measurements were performed in the presence of 1 μM Rotenone, 10 μM Antimycin A and 2 mM KCN (all Sigma-Aldrich (#R8875, #A8674, #60178), St. Louis, MI), respectively. Mitochondrial Complex III (Co-enzyme Q cytochrome *c* oxidoreductase) activity was determined by the rate of cytochrome c reduction, which is reflected by increased absorbance at 550 nm (Cayman Chemical #700950, Ann Arbor, MI). To prevent backflow of electrons through complex I and the reduction of cytochrome c by complex IV, activity measurements were performed in the presence of 1 μM Rotenone and 2 mM KCN. The activity of Mitochondrial Complex IV (cytochrome *c* oxidase) was determined by measuring the rate of oxidation of cytochrome *c*, which is reflected by a decrease in absorbance at 550 nm (Cayman Chemical #700990, Ann Arbor, MI). The activity of Mitochondrial Complex V (F_1F_0 ATP Synthase) was determined by the rate of NADH oxidation, which can be monitored at 340 nm (Cayman Chemical #701000, Ann Arbor, MI). Under physiologic conditions, mitochondrial complex V uses the proton gradient generated by complexes I-IV to generate ATP from ADP in the presence of P_i. However, complex V can also run in reverse [23]. In the determination of complex V activity, ATP is converted in ADP by complex V, which is used for the conversion of phosphoenolpyruvate into pyruvate by pyruvate kinase. Pyruvate is subsequently reduced to lactate in the presence of lactate dehydrogenase and NADH. Hence, complex V activity is reflected by the rate of NADH oxidation, which can be measured by a change in absorption at 340 nm. To prevent interference of mitochondrial complex I, which also oxidizes NADH, all complex V activity measurements were performed in the presence of 1 μM Rotenone.

2.7. Measurement of ATP and ROS production

To assess the ATP production of human ASC, we used the ATP Determination Kit (ThermoFisher Scientific #A22066, Carlsbad, CA). In short, ASC were treated with SUL-109 (10^{-9}-10^{-4} M), 0.1 μM Rotenone, 1 μM KCN or a combination of SUL-109 and the Mitochondrial Complex I or IV inhibitors for 1 h. Next, cells were lysed in a Tris/Glycine buffer (25 mM, pH 7.8, 5 mM $MgSO_4$, 0.1 mM EDTA

and 0.1 mM sodium azide) that contained 0.5 mM D-luciferin, 1.25 μg/ml firefly luciferase and 1 mM DTT. ATP production was assessed as luminescence measured on a Luminoskan (ThermoFisher Scientific, Carlsbad, CA) set to an integration time of 500 ms at 1200 mV.

To assess ROS production, ASC were treated with SUL-109 (10^{-9}–10^{-4} M), 0.1 μM Rotenone, 1 μM KCN or a combination of SUL-109 and the Mitochondrial Complex I or IV inhibitors for 1 h. Next, cells were incubated with the fluorescent ROS-indicator DHE (1 μM, Life technologies #D11347, Carlsbad, CA) for 30 min. Fluorescence was recorded on a Varioskan spectrofluorometer (TheroScientific, Waltham, MA) at Ex/Em 518/605.

2.8. Statistical analysis

Data are expressed as average ± SEM and relative to vehicle controls of at least 3 independent experiments. Statistical evaluation was performed using ANOVA followed by Bonferroni *post hoc* analysis. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. (Modified) 6-chromanols are water soluble α-tocopherol derivatives

Alpha-tocopherol, TROLOX and SUL-109 (Fig. 1) belong to chemical class of 6-chromanols. SUL-109 and TROLOX differ from α-tocopherol by the absence of the large hydrophobic side chain. In contrast to the hydrophobic α-tocopherol (logP = 9.98), TROLOX and SUL-109 are hydrophilic compounds (logP = 3.19 and 2.25, respectively). Consequently, the water solubility at room temperature ranges from α-tocopherol (S = 63.1 nM) < TROLOX (S = 2.75 mM) < SUL-109 (S = 4.90 mM).

3.2. SUL-109 improves ASC viability, adhesion and proliferation after hypothermic damage

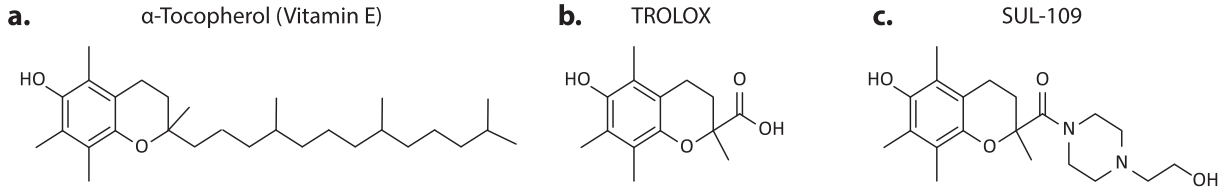
A hypothermic challenge (4 °C, 48 h) reduced ASC viability to ~7% ($p < 0.001$, Fig. 2a). Pretreatment of ASC with α-tocopherol marginally increased cell viability (viability ~16%, $p < 0.05$) whereas SUL-109 completely rescued cell viability (viability ~84%, $p < 0.001$, Fig. 2a). Moreover, viability of ASC that received a hypothermic challenge in the presence of SUL-109 did not differ from ASC that were maintained at 37 °C under standard cell culture conditions or ASC that were preserved using cryopreservation (Fig. 2a). Cell death resulting from hypothermia was primarily induced by necrosis (~66%, $p < 0.001$ compared to ASC maintained at 37 °C, Fig. 2c) and to a lesser extent by apoptosis (~27%, $p < 0.001$, Fig. 2b). Consistent with the improved cell viability, SUL-109 completely blocked the initiation of apoptosis and necrosis following the hypothermia (Fig. 2b and c), whereas α-tocopherol did not reduce apoptosis and necrosis (maximum ~10% reduction, $p = 0.10$, Fig. 2b and c). Further, hypothermic preservation of ASC using SUL-109 was effective with full preservation of viability (~88%, Fig. 2d) over a period of four days.

As tissue engineering and regenerative medicine strategies require ASC to readily adhere and grow at the treatment site following injection or implantation, we assessed the effects of 6-chromanols on their adhesion capacity and growth potential after hypothermic treatment (Fig. 2e–g). Hypothermia challenged ASC had a decreased adherence capacity (~4-fold, $p < 0.001$) compared to ASC that were maintained under standard culture conditions over a period of 4 h post-seeding (Fig. 2d). Pretreatment with the 6-chromanols α-tocopherol and TROLOX did not attenuate the loss in adherence capacity (Fig. 2d). In contrast, ASC that were treated with SUL-109 prior to the hypothermic preservation, adhered to the same extent as ASC maintained at 37 °C throughout the experiment (Fig. 2d).

The growth potential of ASC that underwent hypothermic preservation was investigated by Ki67 expression. ASC that were exposed to hypothermia were devoid of Ki67 expression (Fig. 2f) for as long as 48 h after rewarming (Fig. 2f), indicating prolonged growth arrest. Pretreatment with the 6-chromanols increased ASC proliferation to 25% (α-tocopherol, $p < 0.05$), 40% (TROLOX, $p < 0.001$) and 62% (SUL-109, $p < 0.001$, Fig. 2g). Notably, ASC pretreated with SUL-109 had a growth potential equal to cryopreserved and normothermic ASC (Fig. 2f, g). Thus, pretreatment of ASC with SUL-109 fully preserves cell viability, adherence capacity and proliferative capacity following hypothermic storage.

3.3. ASC maintain their multipotent differentiation capacity after hypothermic damage

The ability of ASC to differentiate into multiple cell types under lineage-specific culture conditions holds promise for tissue engineering and regenerative medicine. Hence, preservation protocols



	α-Tocopherol (Vitamin E)	TROLOX	SUL-109
IUPAC name:	(2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol	(6-hydroxy-2,5,7,8-tetramethylchroman)-2-carboxylic acid	(6-hydroxyl-2,5,7,8-tetramethylchroman-2-yl)(4-(2-hydroxyethyl)piperazin-1-yl)methanone
MW:	430.71	250.29	399.09
log P:	9.98	3.19	2.25
log S:	-7.20	-2.56	-2.31
Topological Polar Surface Area:	29.46	66.67	73.24
TEAC:	0.97	1.00	0.53

Fig. 1. (Modified) 6-chromanols are water soluble α-tocopherol derivatives. Structural and chemical properties of the compounds under investigation; (a) α-tocopherol (vitamin E), (b) TROLOX, and (c) SUL-109.

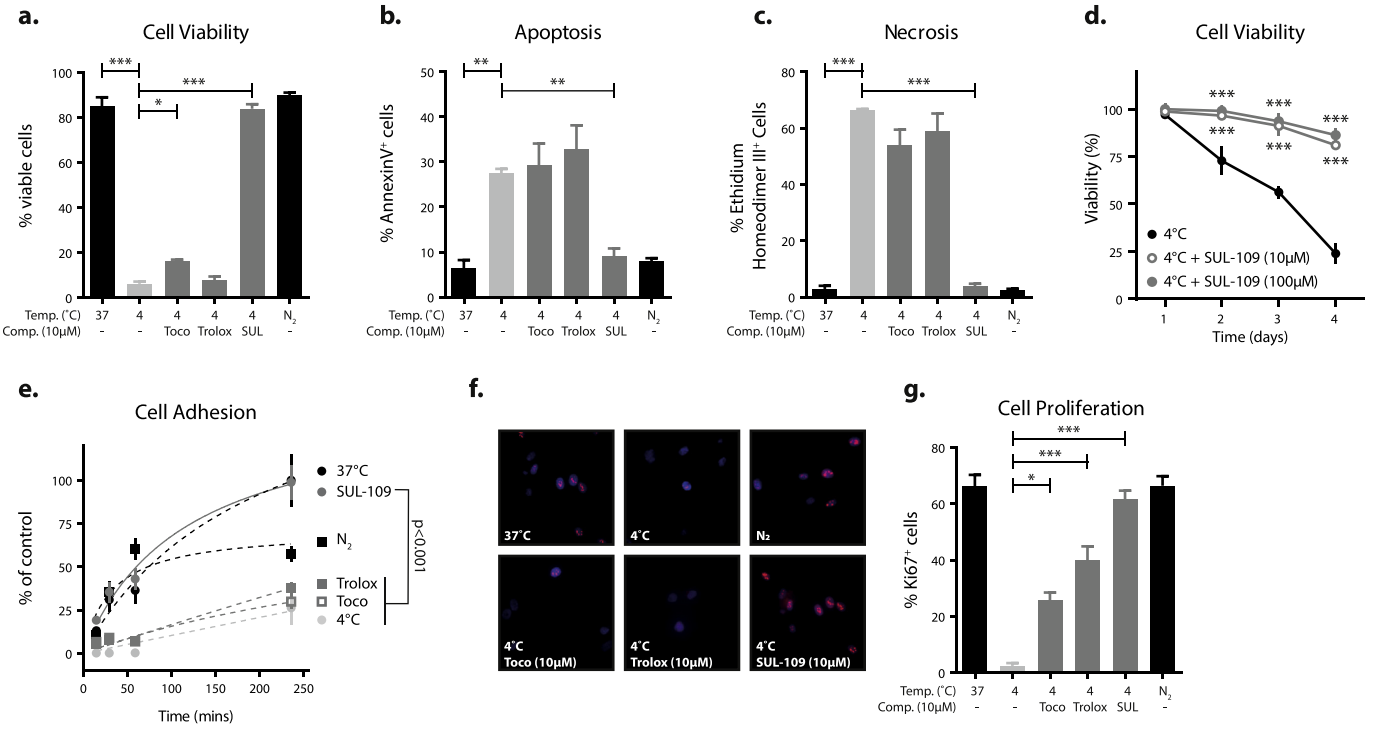


Fig. 2. SUL-109 protects ASC from hypothermic damage. Hypothermia strongly reduces cell viability (a). Hypothermia-induced cell death was both caused by apoptosis (~27% Annexin V-positive cells; b) and necrosis (~66% Ethidium Homeodimer III-positive cells; c). Alpha-tocopherol and TROLOX do not inhibit hypothermia-induced cell death, but SUL-109 abrogates both the apoptotic and necrotic cell death responses of ASC during hypothermic preservation (b,c). SUL-109 maintains ASC viability during hypothermic preservation for 4 days (d). Cells that encountered hypothermic damage for 48 h followed by 2 h re-warming show poor adherence during 240 min post-seeding (e), whereas ASC treated with SUL-109 prior to hypothermia adhered at a similar rate as ASC that were maintained at 37 °C (e). Hypothermia slows metabolic activity and cell cycle progression resulting in failure to undergo the G2/M transition after cooling from 37 °C to 4 °C (g). ASC show cell cycle arrest (absence of Ki67; f) after hypothermic preservation despite 48 h of rewarming (f). In contrast, hypothermically stored ASC that were treated with α-tocopherol, TROLOX or SUL-109 (e,f) maintained their cycling activity and SUL-109-treated ASC proliferated at a similar rate to control cells (g) that were maintained at 37 °C. TOCO = α-tocopherol, N_2 = liquid nitrogen cryopreservation, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, N_2 = liquid nitrogen cryopreservation.

should have minimal interference with these characteristics and must maintain the multipotency of ASC. Hypothermic preserved ASC readily differentiated along the adipogenic (Oil Red O), osteogenic (Alizarin Red) or myogenic (SM22α) lineage (Fig. 3). Although hypothermia causes a large decrease in available cell numbers, ASC exposed to hypothermia did not display any signs of decreased multilineage specialization (Fig. 3). Moreover, the adipogenic, osteogenic and myogenic differentiation capacity of ASC treated

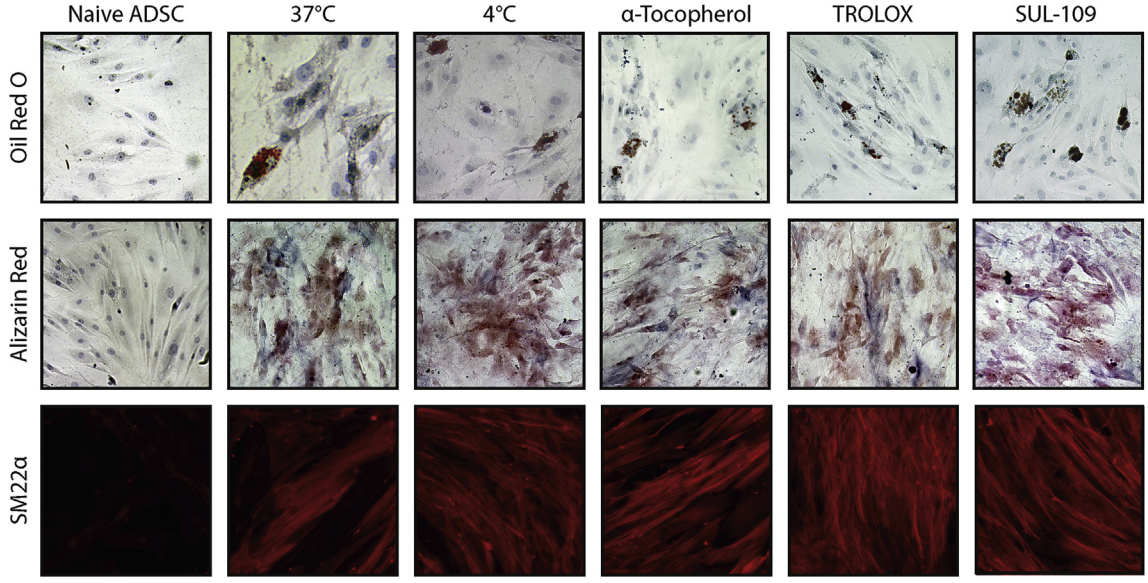


Fig. 3. Hypothermia does not reduce the multipotency of ASC. ASC differentiation into the adipogenic (Oil Red O-positive cells), osteogenic (Alizarin Red-positive cells) and myogenic (SM22α-positive cells) lineage. Naive ASC differentiate efficiently into the three lineages when treated with the appropriate stimuli. ASC that survived the hypothermic challenge maintained their differentiation capacity and treatment with the 6-chromanols did not affect multilineage differentiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with α-tocopherol, TROLOX or SUL-109 prior to hypothermia did not differ from ASC that were maintained under standard culture conditions (Fig. 3).

3.4. Cell survival is not associated with the antioxidant capacity of (modified) 6-chromanols

Hypothermic damage is associated with a drastic increase in reactive oxygen species (ROS) and can be reduced by antioxidants [24]. Therefore we investigated in a test-tube setting if the 6-chromanol derivate SUL-109 (Fig. 1a) possessed antioxidant properties [19,20] using the fluorescent ROS sensor 2′7′-dichlorodihydrofluorecein diacetate (DCF). The oxidation of DCF by H_2O_2 was strongly inhibited by the co-incubation with α-tocopherol (IC_{50} = 0.40 μM, TROLOX-equivalent antioxidant capacity (TEAC) = 0.98 ± 0.05) and TROLOX (IC_{50} = 0.32 μM, TEAC = 1.0 ± 0.11), whereas SUL-109 only inhibited DCF oxidation at concentration above 10^{-5} M (IC_{50} = 229 μM, TEAC = 0.53 ± 0.06, Fig. 4a). ASC survival during hypothermia increased dose-dependently with the addition of (modified) 6-chromanols (Fig. 4b). Alpha-tocopherol provided protection against hypothermia at concentrations above 10^{-3} M (EC_{50} = 5.40 mM), whereas the modified 6-chromanols TROLOX (EC_{50} = 306.20 μM) and SUL-109 (EC_{50} = 2.65 μM) showed a higher potency (Fig. 4b) with effective concentrations in the micromole range. These data imply that the protective effects of 6-chromanols are not derived from their antioxidant capacity, but from their interference with specific cellular processes. Indeed, the TEAC of α-tocopherol, TROLOX and SUL-109 shows a positive correlation with ASC viability, whereas a negative correlation would be expected if antioxidant capacity was the driving force (r^2 = 0.952,p = 0.003, Fig. 4c).

3.5. SUL-109 maintains mitochondrial integrity, mitochondrial membrane potential and ATP production by activation of mitochondrial complexes I and IV

As cell death and the induction of growth arrest are both highly influenced by cellular redox state and mitochondrial function [25,26], we investigated if 6-chromanols influenced the mitochondrial function of hypothermically preserved ASC (Fig. 5). Hypothermia induces a drastic decrease in mitochondrial respiration through oxidative phosphorylation as observed by the decrease in basal oxygen consumption rate (OCR, ~2.6-fold, p < 0.001, Fig. 5a,c), without changing the extracellular acidification rate (ECAR, Fig. 5b and c). TROLOX and SUL-109 pretreatment maintained basal mitochondrial respiration, normalizing the OCR to the level of normothermic controls (Fig. 5a,c). Further, in contrast to α-tocopherol and TROLOX, SUL-109 fully prevented the drop in FCCP-induced maximal OCR of hypothermically preserved ASC (Fig. 5a, d), indicative of increased mitochondrial reserve capacity and increased mitochondrial coupling [27] compared to hypothermia-treated ASC and ASC pretreated with α-tocopherol or TROLOX (Fig. 5a, d). Notably, the SUL-109-induced increase in mitochondrial respiration was only observed for oxidative phosphorylation (OCR, Fig. 5d) and not for glycolysis (ECAR), indicating that SUL-109 preserves mitochondrial coupling. Indeed, the dense network of fused mitochondria observed in normothermic ASC became fragmented following hypothermia (Fig. 5e). Pretreatment of ASC with α-tocopherol did not prevent mitochondrial fragmentation, whereas TROLOX only partially inhibited the fission of the mitochondrial networks. SUL-109 pretreatment fully blocked the hypothermia-induced fragmentation, as ASC maintained a dense network of fused mitochondria (Fig. 5e). Corroboratively, hypothermic storage for 48 h induced a complete loss of the mitochondrial membrane potential, whereas α-tocopherol and TROLOX only partially prevented the collapse in mitochondrial membrane potential (Fig. 5f). ASC that received SUL-109 treatment prior to hypothermic preservation maintained their mitochondrial membrane potential at the level of normothermic ASC (Fig. 5f). Concurrent with the loss in mitochondrial membrane potential, hypothermic ASC, as well as α-tocopherol and TROLOX-treated ASC showed an increase in mitochondrial superoxide production (Fig. 5g), which is associated with cell death [28]. SUL-109-treated ASC did not show excessive mitochondrial superoxide formation (Fig. 5g).

As SUL-109 maintained mitochondrial spare capacity in ASC following hypothermia/rewarming, we questioned at what level SUL-109 influenced mitochondrial respiration and performed activity measurements for mitochondrial complexes I-V on isolated ASC-derived mitochondria (Fig. 6). SUL-109 dose-dependently increased the activity of mitochondrial complexes I and IV (1.1-fold and 2.1-fold (p < 0.05), respectively, Fig. 6a,e) but did not affect the activity of mitochondrial complexes II, III and V (suppl.Fig. 1). Concurrently, SUL-109 at a concentration of 1 μM inhibited the rotenone-induced reduction of complex I activity (Fig. 6b) and the potassium cyanide (KCN)-induced reduction of complex IV activity (Fig. 6f).

Given the maintenance of mitochondrial function by SUL-109, we next investigated whether it also preserved ATP production and inhibited mitochondrial ROS production in ASC following damage. To this end we treated mitochondria isolated from ASC with either rotenone or KCN to inhibit ATP production and induce ROS generation. Indeed, SUL-109 treatment almost completely restored ATP production in rotenone-treated ASC (Fig. 6c) and

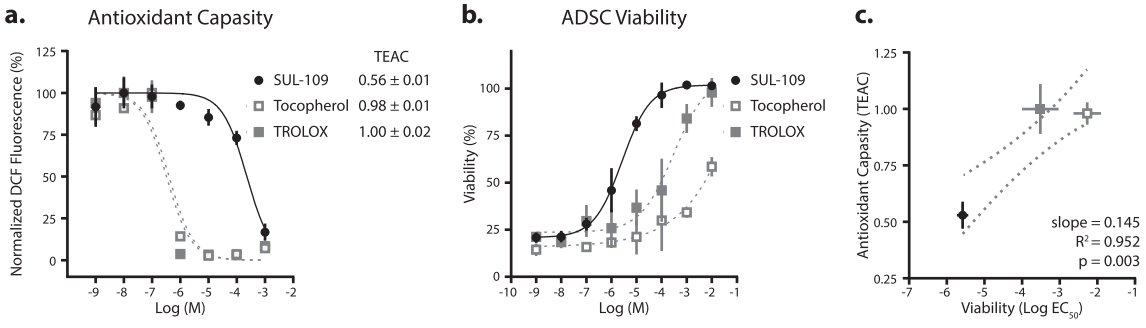


Fig. 4. ROS-scavenging by 6-chromanols does not associate with cell survival. Dose-response of the antioxidant capacities of α-tocopherol, TROLOX and SUL-109 (a). Alpha-tocopherol and TROLOX are strong antioxidants with and IC_{50} for H_2O_2-induced DCF fluorescence of 0.40 μM and 0.32 μM, respectively. SUL-109 is a weak antioxidant (TEAC 0.56) with an IC_{50} of 229 μM for H_2O_2-induced DCF fluorescence. Dose-response of 6-chromanols on the viability of hypothermia-challenged ASC (b). Alpha-tocopherol and TROLOX increase cell viability after hypothermia only at high concentrations. Alpha-tocopherol had an EC_{50} of 2.2 mM, TROLOX had an EC_{50} of 0.3 mM. In comparison, SUL-109 readily increases cell viability with an EC_{50} of 2.6 μM. Association between antioxidant capacity and cell viability (c). TOCO = α-tocopherol, TEAC = TROLOX-equivalent antioxidant capacity.

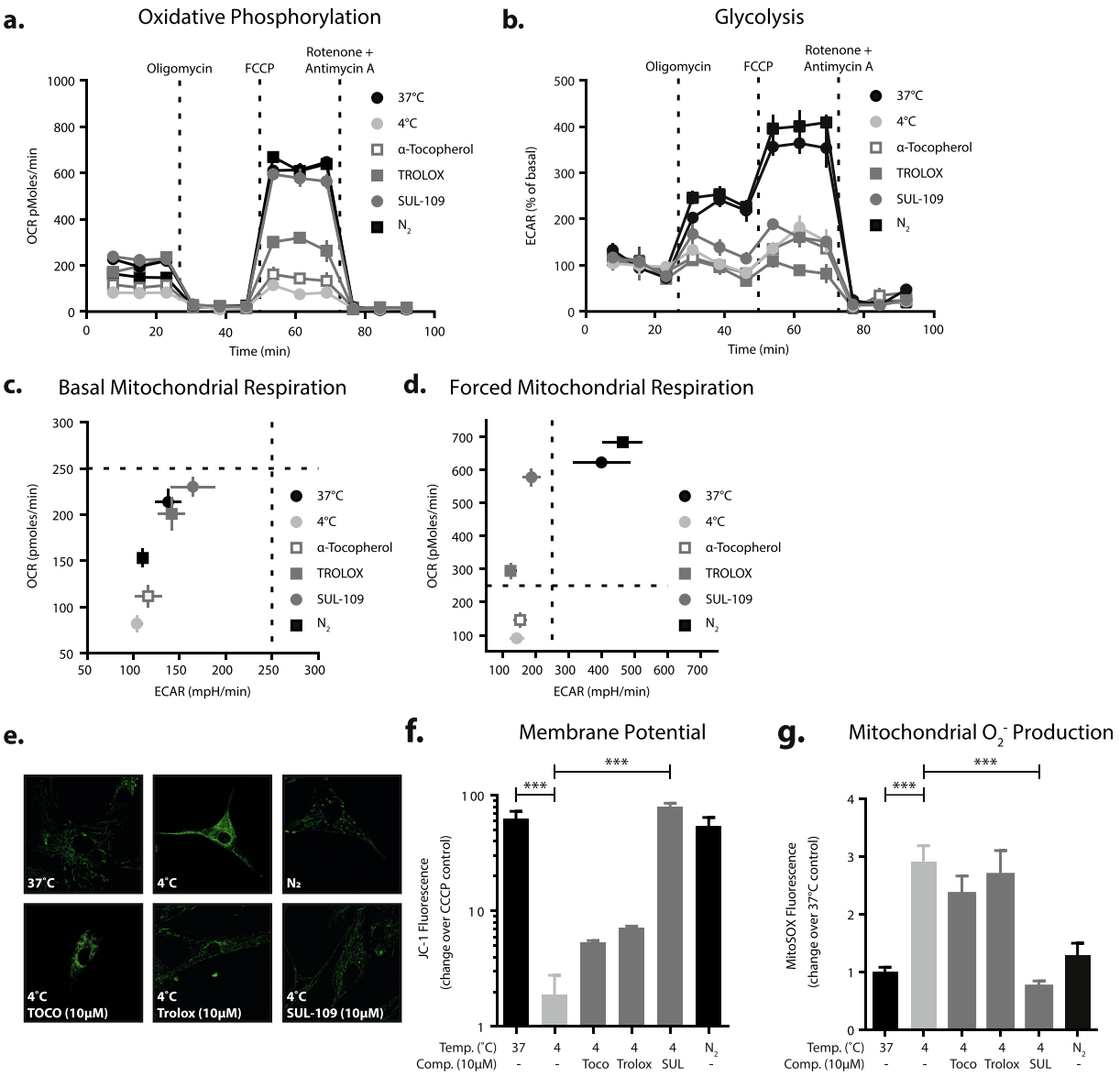


Fig. 5. SUL-109 maintains mitochondrial function and integrity and function during hypothermic damage. Assessment of mitochondrial oxidative phosphorylation (a) and glycolysis (b) in hypothermia challenged ASC. Hypothermia reduces the basal oxygen consumption rate (OCR) of ASC, which is normalized by treatment with TROLOX or SUL-109 (c). During forced respiration, SUL-109 maintains the mitochondrial OCR, but does not affect glycolysis (d). 6-chromanols protect against hypothermia-induced fission of mitochondria with different efficacies (e) and maintain the mitochondrial membrane potential (f). Concurrently, mitochondrial Superoxide production is reduced (g). TOCO = α-tocopherol, OCR = Oxygen Consumption Rate, ECAR = Extracellular Acidification Rate, FCCP = Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, CCCP = Carbonyl cyanide *m*-chlorophenyl hydrazone, N_2 = liquid nitrogen cryopreservation, ***$p < 0.001$.

normalized their mitochondrial ROS production (Fig. 6d) to baseline levels. Likewise, SUL-109 treatment dose-dependently restored ATP production (Fig. 6g) and inhibited ROS generation (Fig. 6h) in KCN-treated ASC. These data imply that SUL-109 protects cells from oxidative stress by maintenance of the mitochondrial function through the specific activation of mitochondrial complexes I and IV.

4. Discussion

Here we describe the efficacy of the novel pharmacologic compound, (6-hydroxyl-2,5,7,8-tetramethylchroman-2-yl)(4-(2-hydroxyethyl)piperazin-1-yl)methanone (SUL-109), that protects ASC from hypothermic damage and cell death during cell preservation without affecting their multipotency. SUL-109 conveys its protective effect by preserving the mitochondrial network structure and activating mitochondrial complexes I and IV, therefore maintaining ATP production and preventing ROS formation. SUL-109 protects ASC from damage associated with hypothermic cell preservation and simplifies the application of ASC in cell therapy and tissue engineering by removing the major limitations of cell death and dysfunction encountered during the manufacturing process.

Despite the encouraging progress in experimental regenerative medicine applications of stem cells for the treatment of chronic diseases [3,4,8,9], clinically acceptable methods of cell preservation are poorly developed [11,29–31]. Current advances in cell therapy and tissue engineering thus create the need for standardized procedures for short term cell preservation that void the necessity of using expensive cryopreservation or vitrification equipment and laborious protocols [11] or the usage of cell preservation reagents

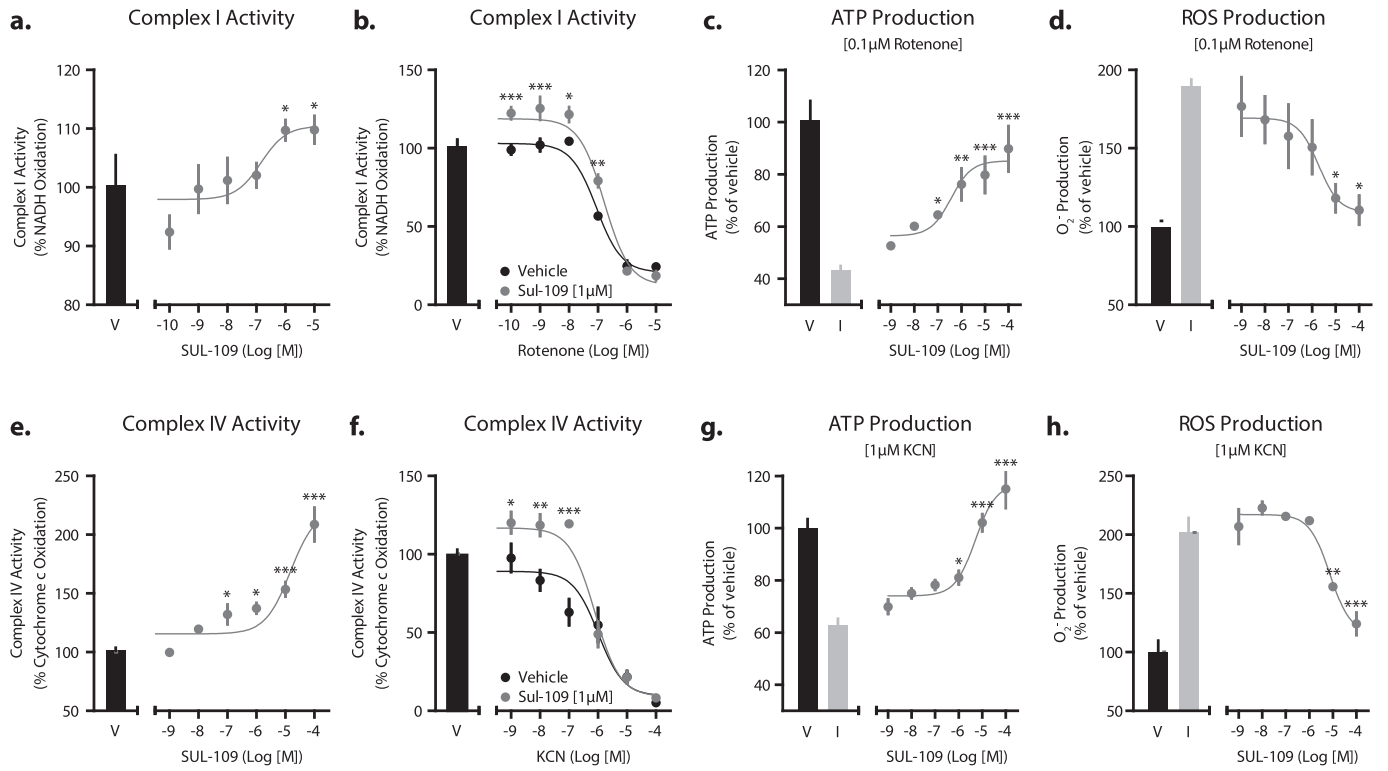


Fig. 6. SUL-109 activates mitochondrial complex I and IV to maintain ATP production and inhibit ROS formation. SUL-109 activates mitochondrial complex I (a) and dose-dependently limits the rotenone-induced inhibition of complex I activity (b) in isolated ASC-derived mitochondria. Resulting from its effects on complex I, SUL-109 dose-dependently increases ATP production in rotenone-treated ASC (c) and reduces mitochondrial ROS formation (d). Besides, SUL-109 activates mitochondrial complex IV (e) and dose-dependently limits the KCN-induced inhibition of complex IV activity (f) in isolated ASC-derived mitochondria. Resulting from its effects on complex IV, SUL-109 dose-dependently increases ATP production in KCN-treated ASC (g) and reduces mitochondrial ROS formation (h). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, KCN = potassium cyanide.

that are toxic to cells at ambient temperatures (*e.g.* dimethyl sulfoxide) [32–34], to allow for proper quality control and transport of cells from their manufacturing facility to their clinical destination. Low-temperature cell pausing, or hypothermic preservation of cells in their specialized culture media, holds great potential as short-term cell storage in these respects [11,35].

Low temperatures have a wide range of beneficial effects in the context of cell preservation, *i.e.* a reduction of energy-dependent processes such as cell cycle progression and protein synthesis [36–39], but may also induce cell damage. The hypothermia-induced reduction in ATP production causes an influx of sodium ions that is no longer counteracted by the Na^{+}-K^{+}-ATPase, which is followed by an influx of water and the concomitant cell swelling results in necrotic cell damage [40,41]. Additionally, the generation of ROS during the rewarming phase causes a significant apoptotic response [42–44], wherein only slight changes in temperature (~5 °C) can have detrimental effects on stem cell survival [45]. The addition of antioxidants to cell preservation media is reported to reduce the hypothermia-induced apoptosis [14,46], but fails to limit the necrosis induction as ATP production is limited during hypothermia. Moreover, some cell preservation solutions are reported to be toxic to ASC [REF], stressing the urgency of developing a cell culture additive that can void the hypothermic damage. Notably, SUL-109 maintained ATP production under hypothermic conditions and inhibited the generation of ROS upon rewarming. Concurrently, SUL-109 inhibited both the hypothermia-induced apoptosis and necrosis.

Hypothermic cell preservation induces cell cycle arrest due to the reduction of available ATP in the cold phase and the accumulation of ROS-induced DNA damage during the rewarming phase [25,36,37]. We show hypothermia to induce growth arrest which is maintained in ASC 48 h post-rewarming. Alpha-tocopherol, TROLOX and SUL-109 maintained cell cycle activity, whereas proliferation of SUL-109-treaded cells was similar to ASC that had not encountered a hypothermia challenge.

Hypothermia and cryopreservation protocols can also limit the multipotency or induce differentiation of stem cells [47,48]. Therefore, we investigated the multilineage differentiation of ASC that were subjected to a hypothermic challenge for 48 h. ASC that survived the hypothermic challenge maintained their differentiation capacity towards adipocytes, osteoblasts and muscle cells regardless of their treatment.

We explored the mechanisms-of-action by which SUL-109 could protect the ASC from damage encountered during hypothermic preservation and rewarming and found that the protective effects were not derived from the ROS-scavenging capability of 6-chromanols [19,20,49], as antioxidant capacity did not associate with cell survival. Rather, SUL-109 increases the activity of mitochondrial complexes I and IV, thereby maintaining the mitochondrial membrane potential, ATP production and minimalizing ROS generation. 6-chromanols might form Quinones [49,50], the electron acceptor substrate for mitochondrial complexes I and II, upon oxidation. Therefore, it seems conceivable that such mechanism underlies the increase in mitochondrial complex I activity by SUL-109 following hypothermia [51–53]. Notably, short-chain Quinones are able to bypass a deficiency in complex I by shuttling electrons directly from the cytoplasm to complex III of the mitochondrial respiratory chain to maintain ATP production [54].

Cytochrome *c*-mediated lipid peroxidation, resulting in a decreased mitochondrial membrane potential and decreased ATP production, is highly influenced by ROS and hypothermia [55–57]. Cytochrome *c* acts as an electron shuttle between mitochondrial

complexes III and IV [58], which facilitates oxidative phosphorylation and thus ATP production. ROS or hypothermia-induced conformational changes in cytochrome *c*, facilitate its binding to cardiolipin and the resulting partial unfolding of cytochrome *c* [59], enable its lipid peroxidation activity while decreasing the amount of cytochrome *c* available for oxidative phosphorylation [60–62]. Hence, this conformational change in cytochrome *c* might contribute to the mitochondrial dysfunction observed during hypothermia. Pentamethyl-6-chromanol (PMC), another vitamin E analogue with structural similarity to SUL-109, reduces cytochrome *c*-mediated lipid peroxidation [62] by a mechanism that is currently not elucidated. Therefore, we postulate that SUL-109 also preserves the conformational state of cytochrome *c*, which thereby remains available for oxidative phosphorylation and ATP production. Although this mechanism might explain how SUL-109 maintains the mitochondrial membrane potential and ATP production by complex IV following hypothermia, it does not clarify why SUL-109 raises the activity of mitochondrial complex IV in normothermic cells. Shuttling of electrons from the cytoplasm to mitochondrial complex III [54] and the concomitant increase in oxidized cytochrome *c* represent potential mechanisms, but this effect of SUL-109 needs further biochemical exploration.

In conclusion, here we describe the efficacy of SUL-109, a 6-chromanol derivate that protects ASC against hypothermic cell damage during preservation. The protective effects of SUL-109 against hypothermia are twofold; (1) SUL-109 has an antioxidant capacity, and (2) SUL-109 maintains the integrity of the mitochondria and activates mitochondrial complexes I and IV, which conserved ATP production and mitigates ROS production. Therefore, SUL-109 represents a single molecule cell preservation agent that protects ASC from hypothermic damage associated with short-term cell preservation that does not affect the stemness of ASC.

Conflicts of interest

A.C.G. is stock holder and the chief executive officer, G.K. is chief scientific officer and P.V. is chief of operations at Sulfateq B.V. (Groningen, the Netherlands), a company that owns patents on SUL-109, and produces and markets similar compounds.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.12.008.

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