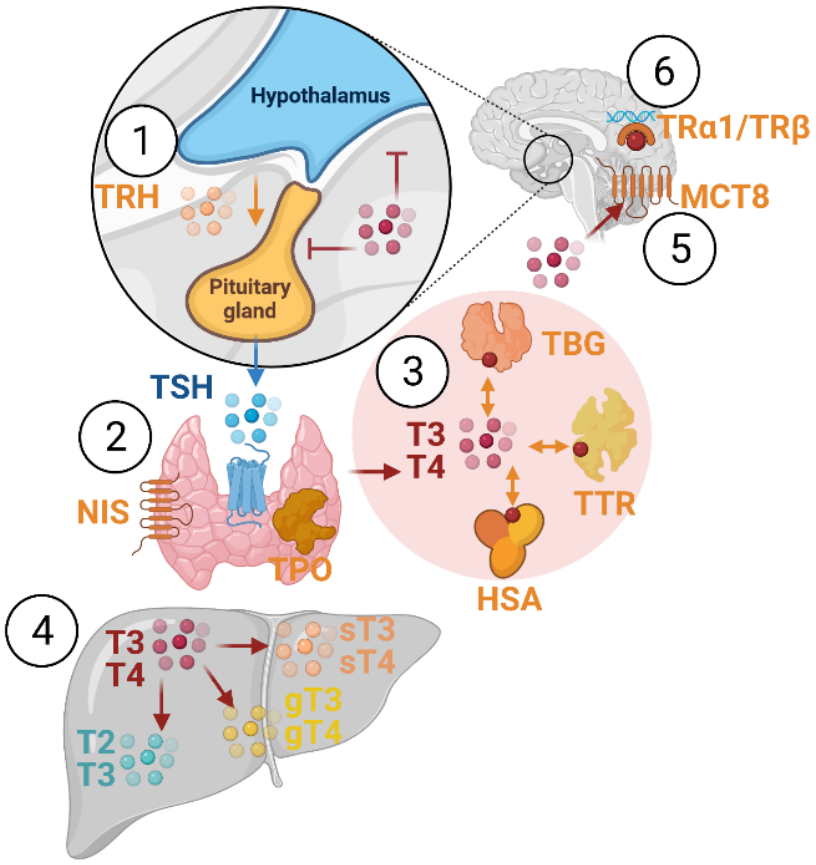
STUDY REPORT

for the validation of the vasculogenesis / angiogenesis in vitro method – Part 1

EURL ECVAM validation study of a battery of mechanistic methods relevant for the detection of chemicals that can disrupt the thyroid hormone system



2022

This study report has been prepared within the context of a collaboration agreement signed in 2021 with the Joint Research Centre (JRC) Directorate for Health, Consumers and Reference Materials (Chemicals Safety and Alternative Methods Unit F3 / EURL ECVAM), for the validation of mechanistic methods to identify potential modulators of thyroid hormone signalling. For information on the methodology and quality underlying the data presented in this report, users should contact the referenced source.

This study report describes the experimental design and includes data generated in Part 1 of the validation study. The method was developed and experimentally assessed by EU-NETVAL laboratory FHAIVE, Finland.

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VALIDATION OF VASCULOGENESIS/ANGIOGENESIS ASSAY

VALIDATION STUDY REPORT

A JOINT EURL ECVAM -COORDINATED VALIDATION OF IN VITRO METHODS FOR DETECTING OF MODULATORS OF THYROID HORMONE SIGNALLING 8c
(PART 1)
AND
FHAIVE's IN-HOUSE FOLLOW-UP VALIDATION OF THE TEST METHOD

Validation study number: VM0019

Date: 20.12.2022

Total number of pages: 26

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Key Words: Vasculogenesis/angiogenesis assay, NRU cytotoxicity assay, test method follow-up validation, HUVEC and HASC cells, Suramin, D-Mannitol

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Written by: 20.12.2022
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Reviewed for GLP compliance by:

20.12.2022
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Other responsible personnel	Sanna Kavén
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LIST OF ABBREVIATIONS

ABS_{540}	absorbance at wavelength 540 nm
hASC	human adipose stromal (stem) cells
HUVEC	human umbilical vein endothelial cells
IC_{50}	inhibitory concentration 50, the concentration that causes 50 % inhibition of tubule formation/cell death compared to unexposed vehicle control
LTC	low tubule control
MAN	D-Mannitol, negative test item in the validation
NRU	neutral red uptake cell viability/cytotoxicity assay
PC	positive control
Stdev	standard deviation
SUR	Suramin, positive control (PC), also used as positive test item in the validation
VA assay	vasculogenesis/angiogenesis assay
VAM	Vasculogenesis/Angiogenesis Model, test system used in VA assay, consisting of hASC and HUVEC co-cultures
VC	vehicle control in cytotoxicity assay, vehicle control (aka tubule control) in VA assay that causes maximal tubule formation
TF	Tubule formation

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1. INTRODUCTION

Vasculogenesis/angiogenesis (VA) assay has been widely used in FHAIVE and its predecessor FICAM. It was initially validated in FICAM in 2015 (VM0010, Toimela et al, 2017). Thereafter the method has been modified as follows: 1) the in-house isolated hASC and HUVEC cells used previously in the method were replaced with commercially available hASC and HUVEC, 2) the concentrations of inductive growth factors of tubule formation, VEGF and FGF-β, were reduced to 20% to enable better distinction of dense tubule networks 3) cytotoxicity assay was changed from WST-1 to NRU assay 4) the analysis software for quantifying tubules was changed from Cell IQ analyser to AngioTool, and 5) calculation templates were added for calculation of cytotoxicity and tubule formation results. The changes required a follow-up validation of the assay.

Simultaneously, FHAIVE had agreed with EURL ECVAM to participate in the EU Validation study for *in vitro* methods to detect modulators of thyroid hormone signalling. EURL ECVAM has identified 18 *in vitro* methods as candidates for a validation study that will be carried out in collaboration with the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL). Methods that perform well could eventually be used in a regulatory context for the identification of endocrine disruptors. FHAIVE's VA assay, "In vitro human adipose stromal cell—human umbilical vein endothelial cell (hASC-HUVEC) vasculogenesis/angiogenesis method (inhibition and induction)" is one of the methods selected by EURL ECVAM for the thyroid validation study, where it is part of the block of integrative cellular assays. The method is thought to be relevant, as one or more modes of action that lead to the disruption of tubule formation are thought to be thyroid related (Liu et al, 2014; Luidens et al, 2010, Davis et al, 2015).

2. AIM OF THE VALIDATION

This was a joint EURL ECVAM -coordinated validation of in vitro methods for detecting thyroid hormone disruptors 8c Part 1 and FHAIVE's in-house follow-up validation of the test method.

The aim of this validation was to show that the vasculogenesis/angiogenesis assay (originally validated in 2015, see FICAM VM0010) is suitable and reliable for its intended use, i.e., detecting potential inhibitors of vasculogenesis and angiogenesis in human. This validation study evaluated the robustness and reliability of the vasculogenesis/angiogenesis assay and its potential use as a marker of thyroid disruption.

Suramin was used as a positive control and as a test item[1] known to inhibit (inhibitor) vasculogenesis/ angiogenesis, and D-Mannitol was used as a test item known not to inhibit (non-inhibitor) vasculogenesis/angiogenesis.

The validation included software validation of the analysis program AngioTool and the MicroSoft Excel calculation templates used for calculating cytotoxicity and angiogenesis/vasculogenesis (tubule formation) results.

This validation study was based on the test method protocol that is presented in FHAIVE's SOP/M/0072-6.0.

[1]The designation test item used in this validation study deviates from FHAIVE´s SOP/S/0001 (Validation of test methods), according to which proficiency chemicals should be called reference items instead of test items in validations. Suramin is also a positive control in the validation study, so it is treated in the same way as the positive controls, while D-mannitol is treated like a reference item.

3. SCIENTIFIC BASIS OF THE METHOD

3.1. The purpose of the test method

The vasculogenesis/angiogenesis *in vitro* test method can be used to test inhibitors of blood vessel formation to detect the human anti-angiogenic response to chemical substances (industrial chemicals, pharmaceuticals).

The method is a tool for evaluating both pharmacological and toxicological effects of chemicals on vasculogenesis/angiogenesis. Information on the developing tubules, such as length and branching, can also be obtained, although not any minute morphological features will be detected.

Inhibition of blood vessel formation may be a potential marker of dysfunction of specific tissues, such as thyroid. Thyroid hormones are strong proangiogenic factors (Luidens et al 2010; Davis et al, 2015). Disbalance of thyroxine and VEGF signaling is known to have profound effects on blood vessel formation (Rajabi et al, 2019; Balzan et al, 2013).

3.2. The mechanistic basis of the test method

The test method is based on a co-culture of human adipose stromal cells (hASC) and human umbilical vein endothelial cells (HUVEC). The co-culture is called Vasculogenesis/Angiogenesis Model (VAM) and it is the test system in VA assay.

In the co-culture, endothelial cells form tubular structures and connect to form tubular networks. Adipose stromal cells provide endothelial cells with specific matrix proteins that make the tubulogenesis possible (Bishop et al. 1999; Donovan et al 2001; Friis et al. 2003) in the presence of specific exogenous growth factors. Also endothelial precursor cells are present in adipose stromal cell fraction to mimic vasculogenesis. The tubular network is formed within six days in the co-culture and can be visualized by immunostaining.

The test is run with tubule/vehicle and low tubule controls. Tubule/vehicle controls contain vasculogenic/angiogenic factors (2 ng/ml VEGF combined with 0.2 ng/ml

FGF-2) that are used to mimic the angiogenesis in human (Ai et al. 2007; Bishop et al. 1999; Donovan et al. 2001; Friis et al. 2003, Nakatsu and Hughes 2008, Sarkanen et al, 2012, Huttala et al, 2015, Toimela et al, 2016). Low tubule control does not contain these angiogenic factors. The test is designed so that in six days the tubule/vehicle control cultures develop tubular network, whereas low tubule controls show no or minor tubule formation.

During the tubule formation assay, tubule formation is chemically induced and simultaneously, eight concentrations of test chemical are present in the cell culture medium. After 6 days of exposure, the tubules are visualized by immunocytochemical staining with anti-von Willebrand factor and/or Collagen IV. Inhibition of tubule formation by chemicals (assessed by measuring total tubule length) is compared to tubule/vehicle controls and toxicity data to classify them as inhibitors or non-inhibitors.

3.3. The relevance of the test method

In vitro Vasculogenesis/angiogenesis model (VAM) mimics all the different phases of natural blood vessel formation in human. Vasculogenesis/angiogenesis assay may replace or supplement several animal tests: zebrafish angiogenesis model, rodent ischemia model, chicken chorion allantoic membrane model and Matrigel plug assay.

4. GUIDELINE COMPLIANCE

The validation was based on "OECD Guidance document No. 34 on the validation and international acceptance of new or updated test methods for hazard assessment (2005)".

This validation was performed in compliance with Good Laboratory Practice Regulations as set forth in OECD [ENV/MC/CHEM(98)17] and the Standard Operating Procedures of FHAIVE.

5. STUDY ORGANIZATION

5.1. Study location

FHAIVE
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5.2. Responsible personnel

Test Facility Management:
Dario Greco, PhD, professor

Validation Study Responsible:
Tarja Toimela (TT), PhD
TT was responsible for the overall conduct of the validation, writing of the validation plan and report, calculation and analysis of results, and validation of Excel

templates and AngioTool software. TT performed thawing of cells for Test2 and performed the exposures for Test 5.

Technicians:
Sanna Kavén (SKa)
SKa made cell cultures, prepared chemical stocks and dilutions, exposed the cell cultures, performed NRU assays, stained tubule structures and performed microscopic imaging of stained tubules (exception, see TT above)

5.3. Time table

Validation plan: 1st September, 2022
Start of validation procedure: 1st September 2022
Completion of experimental procedure: 14th November 2022
Validation report: 20th December 2022

5.4. Quality Assurance

The validation plan and report were audited for GLP compliancy by QA auditor. QA auditor carried out inspections on critical phases in the execution of the validation. Routine facility inspections have been performed annually.

5.5. Archiving and record maintenance

The validation plan, along with the corresponding validation report and data generated in support of this validation study will be archived in FHAIVE according to SOP/O/0002-11.0.

6. MATERIALS

6.1. Test systems

Human adipose stromal cells (hASC) obtained from Promocell, hMSC, cat # C-12977, FHAIVE´s lot 136-hASC/2-p4/1 at passage 5 were used. The certificates of analysis of hASCs are included as Appendix 1.

Human umbilical vein endothelial cells (HUVEC) obtained from Promocell, cat # C-12203, FHAIVE´s lot 135-HUVEC/2-p4/2 at passage 5 were used. The certificates of analysis of HUVECs are included as Appendix 2.

At the end of the validation, 4 million HUVEC and HASC cells will be sent to EURL ECVAM for quality control.

All the procedures in the handling, eg. receipt, storage, creation of master banks, and quality control, complied with the Standard Operation Procedures at FHAIVE.

6.2. Reagents and solutions

- Trypan blue (Sigma #T8154, lot RNBK1828)
- Anti-von Willebrand Factor, antibody produced in rabbit, primary antibody (Dako #A0082, lot 41276634) *
- Immunofluorescence secondary antibody, Polyclonal Antibody to Rabbit IgG Alexa Fluor 568 (Invitrogen #A11011, lot 2192277) *
- Triton X-100 (Molecular Biology #9002-93-1, lot 8J011996)

- Dulbecco's Phosphate Buffered Saline (DPBS) (with calcium and magnesium) (Lonza # BE17-513F, lot 21MB023)
- Distilled H2O (Gibco #15230, lot 2401865)
- Animal-Free Blocker® (Vector laboratories #SP-5030-250, lot 2H0226)
- Neutral red solution (3,3 g/l in DPBS) (Sigma #N2889, lot RNBL0143)
- Glacial acetic acid (Supelco #1.00063.1000, lot K53960263150)
- Ethanol (Altia #12110124, lot 21063
- 70 % ethanol for fixing of cells (prepared according to SOP/M/0020-3.0) lot 70% ethanol/Ska/20/130623
- hASC cell culture medium (prepared according to SOP/M/0037-5.1) lots: hASC/VM0019Test1/Ska/1/011022, hASC/VM0019Test2/TT/1/151022, hASC/VM0019Test3/Ska/1/291022, hASC/VM0019Test4/Ska/1/131122, hASC/VM0019Test5/Ska/1/271122
- HUVEC cell culture medium (prepared according to SOP/M/0002-3.0), lots: HUVEC/VM0019Test1/Ska/1/011022, HUVEC/VM0019Test2/TT/1/ 151022, HUVEC/VM0019Test3/Ska/1/291022, HUVEC/VM0019Test4/Ska/1/131122, HUVEC/VM0019Test5/Ska/1/271122
- VAM medium (prepared according to SOP/M/0068-2.0) lots:
  VAM solution/VM0019Test1/Ska/1/061022,
  VAM solution/VM0019Test2/Ska/1/201022,
  VAM solution/VM0019Test3/Ska/1/041122,
  VAM solution/VM0019Test4/Ska/1/181122,
  VAM solution/VM0019Test5/Ska/1/011222
- VAM stimulation medium (prepared according to SOP/M/0070-2.0) lots:
  VAM stimulation solution/VM0019Test1/Ska/1/060922,
  VAM stimulation solution/VM0019Test1/Ska/2/090922,
  VAM stimulation solution/VM0019Test2/Ska/1/200922,
  VAM stimulation solution/VM0019Test2/Ska/2/230922,
  VAM stimulation solution/VM0019Test3/Ska/1/041022,
  VAM stimulation solution/VM0019Test3/Ska/2/071022,
  VAM stimulation solution/VM0019Test4/Ska/1/181022,
  VAM stimulation solution/VM0019Test4/Ska/2/211022,
  VAM stimulation solution/VM0019Test5/Ska/1/011122,
  VAM stimulation solution/VM0019Test5/TT/2/041122

*There are recombinant antibodies available, but they did not work equally well in the imaging system used

6.3 Test items

The test items are listed in Table I. Note! Suramin served also as the positive control in the vasculogenesis/ angiogenesis assay in each well plate (see Chapter 6.4).

Table I. Test items.

Reference chemical	Abbreviation	CAS	Supplier	*Cat #*	Lot	Physical state	Solvent
Suramin	SUR	129-46-4	Cayman Chemical	11126	0609411-4	solid	VAM stimulation medium

D-Mannitol	MAN	69-65-8	Sigma Aldrich	M4125	WXBD1141V	solid	VAM stimulation medium

The certificates of analysis of the test items are included as Appendix 3.

6.4. Positive control

0.2 mM Suramin (CAS 3380-34-5, Cayman chemical #11126, lot 0609411-4) was used as positive control.

6.5. Low tubule control

VAM solution was used as low tubule control.

6.6. Vehicle /Tubule control

VAM stimulation medium was used as vehicle control (and tubule control).

6.7. Equipment

- Tecan Spark Multiplate reader for absorbance measurement at 540 nm
- Nikon Ti-S fluorescence phase-contrast microscope capable of taking images at 568 nm emission wavelength
- Sterile 48-well flat bottom cell culture microtiter plates (Nunc # 150687)

7. Validation procedure

The validation was performed according to SOP/M/0072-6.0 and according to specifications in the validation study plan. The vasculogenesis/angiogenesis (VA) assay consisted of a tubule formation assay and a parallel cytotoxicity assay (neutral red uptake, NRU) to evaluate cytotoxicity and specificity of the results. Calculation templates (see Chapter 7.7.) and AngioTool analysis software (see Chapter 7.8.) were also validated during the study.

The effective concentration ranges of test items Suramin and D-Mannitol are known from previous tests, therefore no dose range finder tests were performed. Using the two test items Suramin and D-Mannitol VA assay (both cytotoxicity and tubule formation assays) was repeated until five valid runs were obtained. Each repeat (run) was independent and started by thawing of new cells. In documentation, the runs were indicated as Test1, Test2, Test3, Test4 and Test5 supplemented to validation study number. Table II shows the abbreviations of test items and identifiers used for NRU and tubule formation (TF) assays.

Table II. Identifiers for the runs

Test item	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**
Suramin NRU assay	VM0019-Test1-SUR-NRU	VM0019-Test2-SUR-NRU	VM0019-Test3-SUR-NRU	VM0019-Test4-SUR-NRU	VM0019-Test5-SUR -NRU
Suramin tubule formation assay	VM0019-Test1-SUR-TF	VM0019-Test2-SUR-TF	VM0019-Test3-SUR-TF	VM0019-SUR-Test4-TF	VM0019-Test5-SUR -TF

D-Mannitol NRU assay	VM0019-Test1-MAN-NRU	VM0019-MAN-Test2-NRU	VM0019-Test3-MAN-NRU	VM0019-Test4-MAN-NRU	VM0019-Test5-MANNRU
D-Mannitol tubule formation assay	VM0019-Test1-MAN-TF	VM0019-Test2-MAN-TF	VM0019-Test3-MAN-TF	VM0019-Test4-MAN-TF	VM0019-MAN-Test5-TF

Schedule of individual VA assay was the following:
Day -5 Thawing of hASC cells
Day -4 Thawing of HUVEC cells
Day -1 Preparation of hASC/HUVEC cocultures
Day 0 Exposure 1
Day 3 Exposure 2
Day 6 Exposure stops. Cytotoxicity (NRU) measurement. Immunofluorescence tubule staining starts.
Day 7 Immufluorescence staining continues (can also be performed in day 8 or 9)
Day 8 on Microscopic imaging and analysis of results

The original documentation of the performance of this validation study can be found in Folder VM0019 as follows:
Section 2, Test1
Section 3, Test2
Section 4, Test3
Section 5, Test4
Section 6, Test5
Section 7, Validation of calculation templates and AngioTool
Section 8, AngioTool result files
Section 9, Excel calculations (Tubule formation and NRU)
Section 10 GraphPad calculations

7.1 Cell culturing

7.1.1. Media

hASC cell culture medium was prepared according to SOP/M/0037-5.1, and HUVEC cell culture medium according to SOP/M/0002-3.0. hASC cell culture medium contained DMEM/F12 supplemented with 10% Human Serum and 1% L-glutamine. HUVEC cell culture medium contained Lonza´s EGM-2 medium (EBM-2 Basal medium, #CC-3156) and EGM-2 SingleQuots supplements (#CC-4176): 2% Human Serum (replaced FBS), 0.04% Hydrocortisone, 0.4% FGF, 0.1% VEGF, 0.1% IGF, 0.1% Ascorbic acid, 0.1% EGF, 0.1% Heparin.

Exposure medium for low tubule control was VAM solution (SOP/M/0068-2.0). VAM solution contained DMEM/F12 medium supplemented with 2,56 mM L-glutamine, 0,1 nM 3,3',5-Triiodo-L-thyronine, ITS™ Premix: 1,15 µM: 6,65 µg/ml insulin, 6,65 µg/ml Transferrin, 6,65 ng/ml selenious acid, 1 % Bovine serum albumin* and 2.8 mM Sodium puryvate. *BSA replacements do not work (pretest result, not reported here).

Exposure medium for test items (Suramin and D-Mannitol) and VC/tubule control was VAM stimulation medium (SOP/M/0070-2.0). VAM stimulation medium contained VAM solution (see previous chapter) supplemented with 200 µg/ml Ascorbic acid, 0,5 µg/ml Heparin, 2 µg/ml Hydrocortisone, 2 ng/ml VEGF and 0.2 ng/ml FGF-β)

7.1.2. Culture of hASC and HUVEC cells

Two hASC ampoules ($0.5x10^6$ cells/ml/ampoule) were thawed and cell cultures were prepared according to SOP/M/0072-6.0 Chapter 4.2. hASC cells were cultured in 10 ml of hASC cell culture medium in 75 cm^2 cell culture flasks in a cell culture incubator at +37 ˚C, 5 % CO_2, humidified air for 4 days prior to plating into 48-well plates.

HUVECs (1 ampoule containing $0.5x10^6$ cells/ml) were thawed and cell cultures were prepared according to SOP/M/0072-6.0 Chapter 4.3. HUVEC cells were cultured (separately from hASC cells) in 10 ml of HUVEC cell culture medium in 75 cm^2 cell culture flasks in a cell culture incubator at +37 ˚C, 5 % CO_2, humidified air for 3 days prior to plating into 48-well plates with hASC cells.

On **Day -1**, co-culture of hASC and HUVEC cells were prepared as instructed in SOP/M/0072-6.0 Chapter 4.4. First, hASCs were seeded at a density of 20 000 cells/cm^2, 22 000 cells/ 48-multiwell plate well in HUVEC cell culture medium, then after 1-4 hours, HUVECs were seeded on top of hASCs. HUVEC cell density was 4 000 cells/cm^2, 4400 cells/ 48-multiwell plate well. The cells were let to attach overnight in an incubator at +37 ˚C, 5 % CO_2, humidified air before the exposure started.

Four 48-well plates were prepared (one for tubule formation assay, one for cytotoxicity assay for both Suramin and D-Mannitol)

7.2. Preparation of stocks and dilutions from test items

The stocks and dilution series were always prepared freshly on the day of use, i.e. for Exposure 1 on **Day 0**, and for Exposure 2 on **Day 3**. Weighing was performed beforehand.

A 2 mM Suramin stock (2.858 mg/ml) was prepared in VAM stimulation solution, and then dilution series in VAM stimulation solution using 2.15 as dilution factor (see Table III and IV). Each Suramin concentration was tested in three replicates.

Table III. Preparation 2 mM Suramin stocks in VAM stimulation solution.

	Test 1 SUR	**Test 2 SUR**	**Test 3 SUR**	**Test 4 SUR**	**Test 5 SUR**
Suramin stock (2.858 mg/ml)	Weighed/Dissolution volume	Weighed/Dissolution volume	Weighed/Dissolution volume	Weighed/Dissolution volume	Weighed/Dissolution volume
1.exposure	29.41 mg/ 10.29 ml	28.78 mg/ 10.07 ml	29.18 mg/ 10.210 ml	29.61 mg/ 10.360 ml	28.71 mg/ 10.045 ml
2.exposure	28.70 mg/ 10.04 ml	28.94 mg/ 10.13 ml	29.05 mg/ 10.164 ml	28.97 mg/ 10.136 ml	28.82 mg/ 10.084 ml

Table IV. Preparation of Suramin dilution series in VAM stimulation solution.

Tube	Suramin µl	VAM-stimulation solution µl	Final concentration in exposure mM
1	stock	-	2
2	4000↓	4600	0.93
3	4000↓	4600	0.43
4	4000↓	4600	0.20
5	4000↓	4600	0.094
6	4000↓	4600	0.044
7	4000↓	4600	0.020
8	4000	4600	0.0094

A 10 mM D-Mannitol stock (1.82 mg/ml) was prepared in VAM stimulation solution, and then dilution series in VAM stimulation solution using 3.16 as dilution factor (see Table V and VI). Each D-Mannitol concentration was tested in three parallels.

Table V. Preparation 10 mM D-Mannitol stocks in VAM stimulation solution

	Test 1 SUR	Test 2 SUR	Test 3 SUR	Test 4 SUR	Test 5 SUR
D-Mannitol (1.82 mg/ml)	Weighed/Dissolution volume	Weighed/Dissolution volume	Weighed/Dissolution volume	Weighed/Dissolution volume	Weighed/Dissolution volume
1.exposure	19.01 mg/ 10.45 ml	19.46 mg/ 10.69 ml	18.76 mg/ 10.308 ml	18.71 mg/ 10.280 ml	18.82 mg/ 10.341 ml
2.exposure	18.94 mg/ 10.41 ml	19.09 mg/ 10.49 ml	18.84 mg/ 10.352 ml	18.74 mg/ 10.297 ml	18.57 mg/ 10.203 ml

Table VI. Preparation of D-Mannitol dilution series in VAM stimulation solution.

Tube	D-Mannitol µl	VAM-stimulation solution µl	Final concentration in exposure mM
1	stock	-	10.00
2	2000↓	4320	3.16
3	2000↓	4320	1.00
4	2000↓	4320	0.317
5	2000↓	4320	0.100
6	2000↓	4320	0.0317
7	2000↓	4320	0.0100
8	2000	4320	0.00318

7.3. Exposures

The exposures were performed according to SOP/M/0072-6.0 Chapter 4.6.
The plate layout shown in Figure 1 was used.

	1	2	3	4	5	6	7	8
A		VC	C2	C4	C6	C8	LTC	
B		VC	C2	C4	C6	C8	LTC	
C		VC	C2	C4	C6	C8	LTC	
D		PC	C1	C3	C5	C7	VC	
E		PC	C1	C3	C5	C7	VC	
F		PC	C1	C3	C5	C7	VC	

Figure 1. Plate layout for exposure.
C1-C8 test item concentrations: C1 highest, C8 lowest concentration
VC: vehicle control = VAM stimulation solution
LTC: low tubule control = VAM solution
PC 0.2 mM Suramin

On **Day 0**, the cells were exposed to test items, low tubule controls and vehicle/tubule controls and positive control using three replicate wells, 500 µl /well. (Exposure 1).

The HUVEC cell culture medium was removed from the wells with cocultures using Pasteur pipettes connected to a suction pump, 3 wells at a time. The order of pipetting was: LTC controls, VC controls, then test items starting from the lowest concentration to the highest concentration. Positive controls were pipetted at the end. The total pipetting time (during which cells were exposed to room air) was maximally 12 minutes.

On **Day 3** the exposure was repeated (Exposure 2) using freshly prepared test item solutions and controls.

The cell exposure lasted in a cell culture incubator at +37 ˚C, 5 % CO_2, humidified air till **Day 6** after which NRU cytotoxicity assay (7.4) and immunocytochemical staining (7.5) were performed.

7.4. Cytotoxicity test (NRU assay)

On **Day 6,** the cytotoxicity assay (NRU assay) was performed according to SOP/M/0072-6.0, Chapter 4.7. to one 48-well plate with Suramin and one 48-well plate with D-Mannitol.

Before starting the NRU assay, each plate was examined with a phase contrast microscope to identify systemic cell seeding errors. After microscoping, the exposure medium was removed from the wells by dumping and blotting the plate carefully on tissue. The wells were washed with DPBS (500 µl/well) and D-PBS was removed. NR working solution (50 µg/ml neutral red in VAM solution) was added to all wells. The well plates were incubated at +37° C, 5% CO_2 for ~2.0 hrs. After incubation, the NR working solution was removed and the washing was repeated with DPBS (500 µl/well). 250 µl of NR desorption solution (49 parts water, 50 parts ethanol, 1 part acetic acid, freshly prepared) was added to all wells. The plate was shaken for about 30 minutes on a microtiter plate shaker (protected from light). The plate was kept still for about 5 minutes after removing from the shaker. The absorption was measured at 540 nm (ABS_{540}). The absorbance data (i.e., original data) was printed out, and also saved electronically for subsequent analysis.

7.5. Immunocytochemical staining

On **Day 6,** the immunocytochemical staining was performed to the second 48-well plates with Suramin and D-Mannitol according to SOP/M/0072-6.0, Chapter 4.8. Tubules were stained against specific endothelial cell marker von Willebrand Factor (vWF), collagen IV was not used.

First, the cell cultures were washed twice with of DPBS (500 µl/well) and then fixed with ice-cold 70% ethanol for 20 min. After two washings, the second DPBS was left in the wells and staining was continued next day. The next phases were permeabilization with 0.5% Triton X-100 (120 µl/well) for 15 min followed by two washings and blocking with Vector animal-free blocking agent (120 µl/well) for 30 min. Blocking agent was removed and without washing, primary antibody solution against von Willebrand factor (1:100 dilution, 120 µl/well) was pipetted to the wells and incubated overnight. Next day, after two washings, secondary antibody (anti-rabbit Alexa Fluor 568, 1:400 dilution, 120 µl/well) was incubated for 45 min. Again, two washings were performed, and DPBS was left in the wells. The vascular (tubule) structures were photographed directly from the 48 multiwell plates using inverted microscope (see 7.6).

7.6. Imaging of tubules

The imaging of immunostained tubules was performed according to SOP/M/0072-6.0, Chapter 4.9. Images of the multiwell plates were taken using Nikon Ti-S inverted fluorescence microscope.

Appropriate excitation and emission wavelength for Alexa 568 nm was used. From each well, 5x5 image grids were taken using 10x objective and 200 ms exposure time. The quality of the images was inspected after imaging.

The tiff images were the raw data. In the AngioTool analysis of tubule formation, merged images 2500x2500 px 8-bit were used after converting them to jpeg format.

7.7. Validation of Excel calculation templates

Microsoft Excel® (Excel) calculation templates, i.e. SOP/M/0072-6.0, Appendix 9 (for calculating NRU cytotoxicity results) and Appendix 12 (for calculating tubule formation results) with respective model data (Appendix 10 and Appendix 13, and model result (Appendix 11 and 14) were validated before they were used in the calculation of the results. The template validation was performed according to SOP/S/0001-8.0.

The sample size $\sqrt{n+1}$ for the template validation was used. Hence, all operations and calculations of $\sqrt{36+1}=7$ wells[2] from the 48-well plate were verified. The 7 wells were randomly chosen eg. using Excel´s random generator 7* [=RANDBETWEEN(1;36)]. The correct model data, either cytotoxicity or tubule inhibition data, was pasted to the respective template. Then data transfers and calculation formulas of the chosen wells were verified.

Correspondence of the results calculated by the templates with the model result of Appendix 11 (NRU cytotoxicity assay) or Appendix 14 (tubule formation) was checked.

[2]Each value on the template represent one well in the 48-well plate. Only 36 wells are in use in the 48-well plate.

7.8. Validation of the analysis software AngioTool

The software validated for the analysis of tubule structures is AngioTool, is developed by US National Cancer Institute. The software detects vascular structures from fluorescence images.

In the validation, the correct performance of AngioTool analysis was verified. The images from VM0019 Test1 SUR and VM0019 Test1 MAN were used in the verification. The analysis software was used as instructed in the SOP/M/0072-6.0. Chapter 6.3.

First, the correct recognition of tubules by AngioTool was ensured by eye (so that the software drew the lines on the tubule structures correctly). Thereafter, the analysis of tubule length by the software was compared to manual classification of the same images. Manual classification was performed according to Appendix 2 of the validation study plan.

7.9. Analysis of results

The analysis of the NRU cytotoxicity results was performed using the validated excel calculation template according to SOP/M/0072-6.0 Chapter 6.2. The results from multimode reader were pasted to the designated areas in the calculation template and study specific info was filled in. The template automatically calculated the results giving the mean, stdev and N of each test item dilutions and controls. The results were normalized so that the vehicle control was set as 100 and all other treatments were calculated against it.

Tubule images were analyzed using AngioTool according to SOP/M/0072-6.0 Chapter 6.3. The von Willebrand factor - stained images were opened one at a time, vessel diameter and intensity was adjusted when necessary. Von Willebrand factor stains endothelial cells even though they have not formed tubules. Usually, a large number of single cells or unspecific structures (clusters of cells) were detected in the background especially in highest concentrations of Suramin and in low tubule controls. These were removed by visually choosing appropriate values for vessel diameter and intensity.

The results of the tubule analysis were saved in an excel file by the AngioTool software. The analysed images were saved in folder VM0019, and its treatment-specific sub-folders: VM0019-Test1-SUR, VM0019-Test1-MAN, VM0019-Test2-SUR, VM0019-Test2-MAN, VM0019-Test3-SUR, VM0019-TEST3-MAN, VM0019-Test4-SUR, VM0019-Test4-MAN, VM0019-Test5-SUR, VM0019-Test5-MAN. The excel file created by AngioTool was opened and contents from Image Name and Total Vessels Length columns were pasted to the Calculation template for tubule formation assay (Appendix 12 in SOP/M/0072-6.0). Unusable wells were determined and excluded from the data analysis (e.g. when tubule structures were fully or > 1/3 detached). Again, the template automatically calculated the results giving the mean, stdev and N of each test item dilutions and controls.
The results were normalized so that the vehicle control was set as 100 and all other treatments were calculated against it.

The normalized data from both NRU and tubule formation analyses was transported from Excel to GraphPad Prism 9 for drawing of dose response curves

and calculating IC_{50} values. Sigmoidal, 4PL, X is log(concentration) equation was used in IC_{50} calculations. IC_{50} values could not be calculated from the D-Mannitol results because there was no such inhibition that could be used in the calculations. The 20% cytotoxicity and 30% tubule inhibition values were interpolated from dose response curves by GraphPad Prism. These results were used for classifications of test items and evaluation the specificity of the result (see Interpretation of results)

For repeatability evaluation, a comparison of logIC50s of dose response curves of individual repeats was performed with ANOVA Using GraphPad Prism software. The analysis used was ordinary One-way ANOVA assuming equal SDs where Mean was $logIC_{50}$, SEM was Sy.X and N was Degrees of Freedom +1 obtained from IC_{50} analysis results.

The analysis could not be performed for D-Mannitol because D-Mannitol did not show inhibitory effect on tubule formation with logIC50s.

Interpretation of results

Test item was classified a specific inhibitor of vascular formation if it inhibited tubule formation at least 30% at lower than 20% cytotoxicity concentrations. If > 30% inhibition of tubule formation appeared with > 20% cytotoxicity, it was considered cytotoxicity-related, not specific.

If there was no inhibition of tubule formation the test item was classified as non-inhibitor of vascular formation.

8. DATA INTEGRITY

It was verified that the data from the plate reader was transferred correctly to the Excel files (by visually comparing the data sets). It was also verified that the data from Excel files was transferred to GraphPad Prism correctly (by visually comparing the data sets).

9. RESULTS

The tubule formation results calculated with excel templates are shown in Tables VII and VIII and NRU cytotoxicity results in Tables IX and X.

In tubule formation tests, Suramin showed an inhibitory effect. The results for D-Mannitol did not show any inhibition of tubule formation. Either test item was highly cytotoxic.

Table VII Tubule formation analysed after 6-day Suramin exposure from immunostained tubules

mM	SUR-Test1			SUR-Test2			SUR-Test3			SUR-Test4			SUR-Test5			SUR-Manual		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0,000	100	8,05	4	100,00	11,00	5	100	7,45	3	100	13,28	6	100,00	8,61	6	100	15,31	6
0,0094	91,42	16,39	3	99,12	7,03	2			0	108,73	17,32	3	110,20	7,80	2	112,50	0,00	3
0,02	96,96	3,59	3	102,63	22,54	3	104,12	NA	1	105,65	18,71	3	107,39	8,53	2	112,50	0,00	3
0,044	90,69	18,79	3	105,07	1,90	3	111,82	5,96	3	107,06	4,56	3	127,87	4,49	3	112,50	0,00	3
0,094	97,3	7,60	3	111,80	9,32	3	109,54	16,98	3	94,8	4,49	3	107,74	7,91	3	106,25	10,83	3
0,2	62,42	14,85	3	75,45	3,35	3	79,71	13,04	3	65,52	18,42	3	67,14	14,93	3	75,00	18,75	3

0,43	20,38	3,90	3	19,26	1,17	3	18,9	7,90	3	14,44	6,56	3	14,54	6,99	3	31,25	10,83	3
0,93	25,26	1,68	3	35,53	13,35	3	20,07	7,58	3	11,13	0,57	3	29,00	10,30	3	25,00	10,83	3
2	9,03	1,85	3	12,41	3,48	3	6,21	4,27	3	6,90	2,78	3	9,10	2,52	3	18,75	0,00	3

Table VIII Tubule formation analysed after 6-day Mannitol exposure from immunostained tubules

mM	MAN-Test1			MAN-Test2			MAN-Test3			MAN-Test4			MAN-Test5			MAN-Manual		
	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
0,000	100	16,79	6	100	13,28	5	100	15,91	6	100	15,51	6	100	2,83	4	100	15,21	6
0,00318	100,74	2,75	2	108,15	5,79	2	111,46	NA	1	115,28	2,19	2	107,79	NA	1	109,09	0,00	2
0,01	112,72	25,08	2	102,54	10,77	2	81,60	NA	1	93,19	12,80	3	101,88	4,50	2	103,03	10,50	3
0,0317	105,74	24,26	2	102,12	NA	1	102,60	3,94	3	115,97	3,59	3	110,7	12,13	2	109,09	0,00	3
0,1	111,91	20,48	2	120,77	NA	1	100,93	25,2	2	100,9	26,30	2	96,26	NA	1	103,03	10,50	3
0,317	97,91	18,22	3	103,29	NA	1	110,95	2,66	2	125,86	11,02	3	91,75	NA	1	109,09	0,00	3
1	109,11	14,34	3	121,77	NA	1	90,69	9,79	2	107,05	20,83	3	88,87	NA	1	103,03	10,50	3
3,17	109,92	23,30	3	108,40	9,62	3	115,16	8,39	3	123,08	3,69	3	105,94	9,46	3	109,09	0,00	3
10	107,04	20,10	2	100,80	NA	1	103,11	13,06	3	96,43	34,59	2	99,14	12,23	2	96,97	20,99	3

Table IX Cytotoxicity of Suramin after 6-day exposure measured with NRU assay

mM	SUR-Test1			SUR-Test2			SUR-Test3			SUR-Test4			SUR-Test5		
	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
0,000	100	9,98	6	100	3,44	6	100	7,59	6	100	8,98	6	100	3,37	6
0,0094	110,52	10,94	3	109,67	4,19	3	108,81	0,64	3	109,9	3,18	3	108,21	6,67	3
0,02	115,60	8,43	3	117,53	6,69	3	118,23	4,10	3	111,01	9,50	3	118,52	11,35	3
0,044	133,88	8,60	3	130,07	6,70	3	130,92	3,19	3	126,62	8,09	3	133,99	6,42	3
0,094	145,7	6,44	3	148,68	3,48	3	155,92	11,28	3	139,72	9,86	3	146,64	9,96	3
0,2	175,44	20,14	3	167,94	6,00	3	179,47	6,40	3	162,80	23,49	3	161,14	5,98	3
0,43	154,35	0,58	3	175,59	8,39	3	171,77	0,50	3	163,90	4,69	3	188,37	6,90	3
0,93	79,73	7,38	3	97,57	16,08	3	92,22	16,77	3	92,71	5,32	3	94,88	6,94	3
2	64,30	3,92	3	65,70	6,27	3	44,84	39,4	3	91,12	13,39	3	97,85	8,46	3

Table X Cytotoxicity of D-Mannitol after 6-day exposure measured with NRU assay

mM	MAN-Test1			MAN-Test2			MAN-Test3			MAN-Test4			MAN-Test5		
	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N	MEAN	SD	N
0,000	100	10,41	6	100	4,49	6	100	4,05	6	100	5,96	6	100	6,86	6
0,00318	92,49	0,81	3	98,79	6,88	3	101,47	8,60	3	98,35	3,63	3	97,38	3,65	3
0,01	92,48	11,15	3	98,95	5	3	99,60	3,16	3	99,20	35,91	3	104,05	5,66	3
0,0317	94,44	2,69	3	96,85	4,12	3	97,90	4,23	3	99,95	8,67	3	97,83	3,29	3
0,1	91,17	6,87	3	96,24	4,8	3	94,02	2,23	3	98,92	5,46	2	96,99	3,34	3
0,317	94,22	6,47	3	95,84	1,53	3	100,06	6,22	3	99,64	5,2	3	103,44	1,81	3
1	93,18	8,55	3	97,85	2,57	3	97,90	3,72	3	115,95	20,31	3	101,02	5,86	3
3,17	92,69	2,36	3	101,81	1,35	3	103,37	3,79	3	113,01	6,16	3	101,54	2,65	3
10	81,25	17,21	3	132,77	26,2	3	113,31	31,25	3	112	15,52	3	103,33	8,20	3

Dose response curves drawn for tubule formation assay by GraphPad Prism are shown in Figures 2 and 3. Suramin (Fig.2) showed a clear sigmoidal dose response, while D-Mannitol (Fig.3) did not show any such effect.

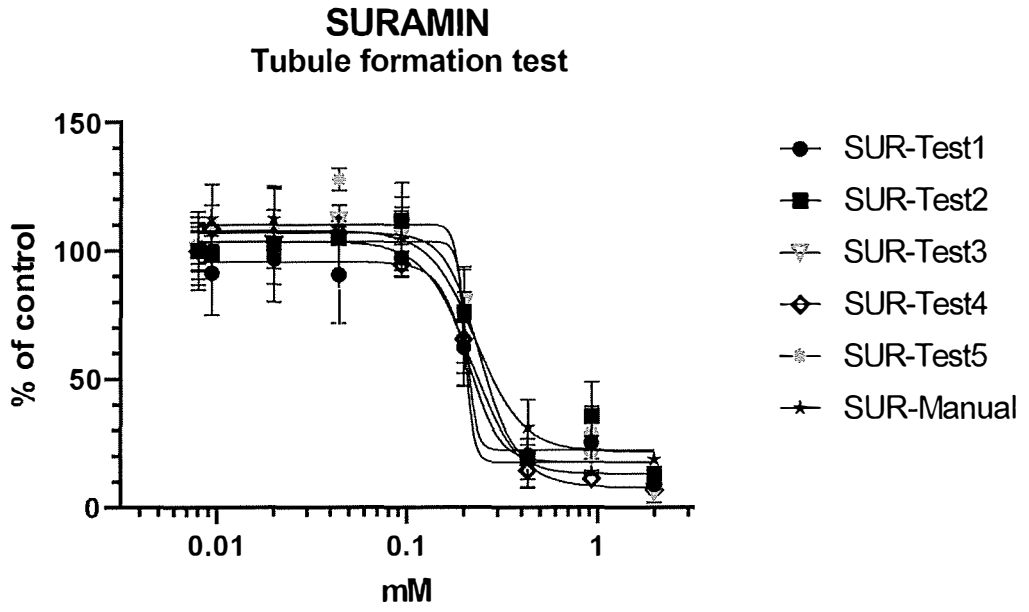

Figure 2. Dose response curves of tubule formation tests after 6-day exposure to Suramin. Manual means visual evaluation during AngioTool validation.

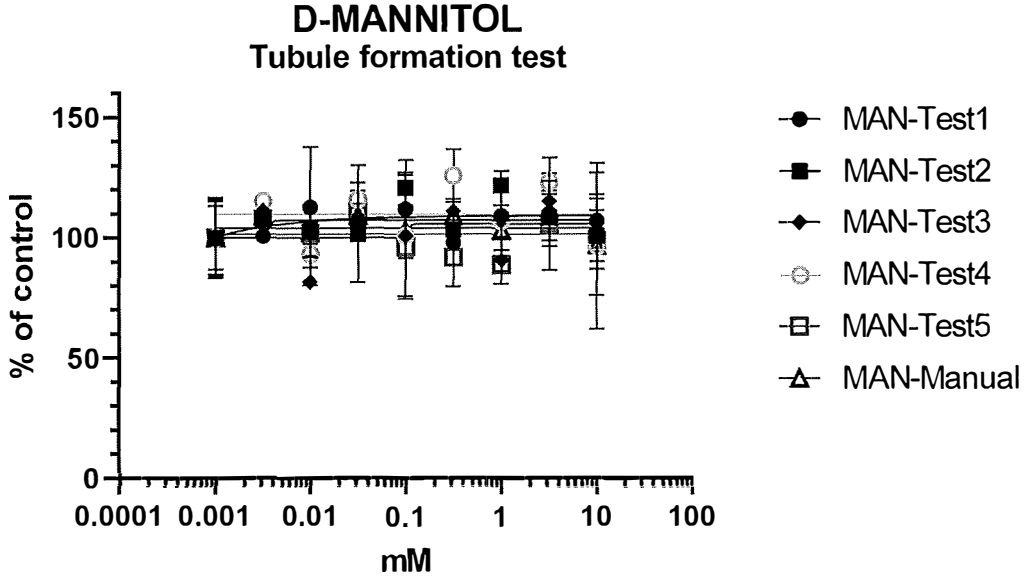

Figure 3. Dose response curves of tubule formation tests after 6-day exposure to D-Mannitol. Manual means visual evaluation during AngioTool validation.

GraphPad IC_{50} analysis of NRU cytotoxicity measurements with neutral red uptake assay with Suramin showed first a strong hormesis-like effect in lower than 0.43 mM concentrations, thereafter a rapid decline in viability (see Figure 4). Suramin is washed out before neutral red incubation starts, therefore the result is not due to direct interaction with Suramin.

D-Mannitol was not generally cytotoxic, but the highest 10 mM concentration was diverging (see Figure 5).

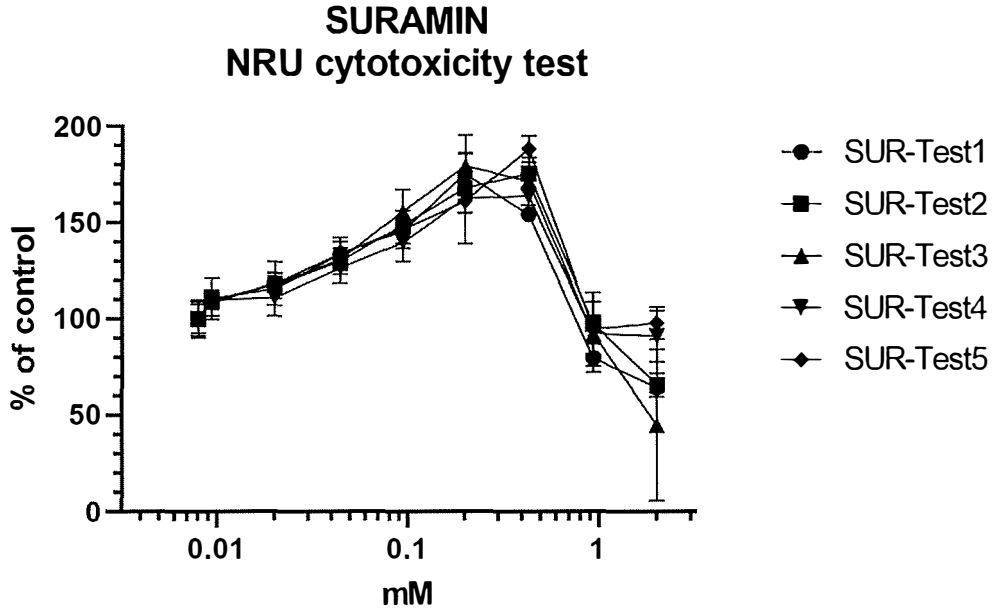

Figure 4. NRU cytotoxicity dose response curves after 6-day exposure to Suramin

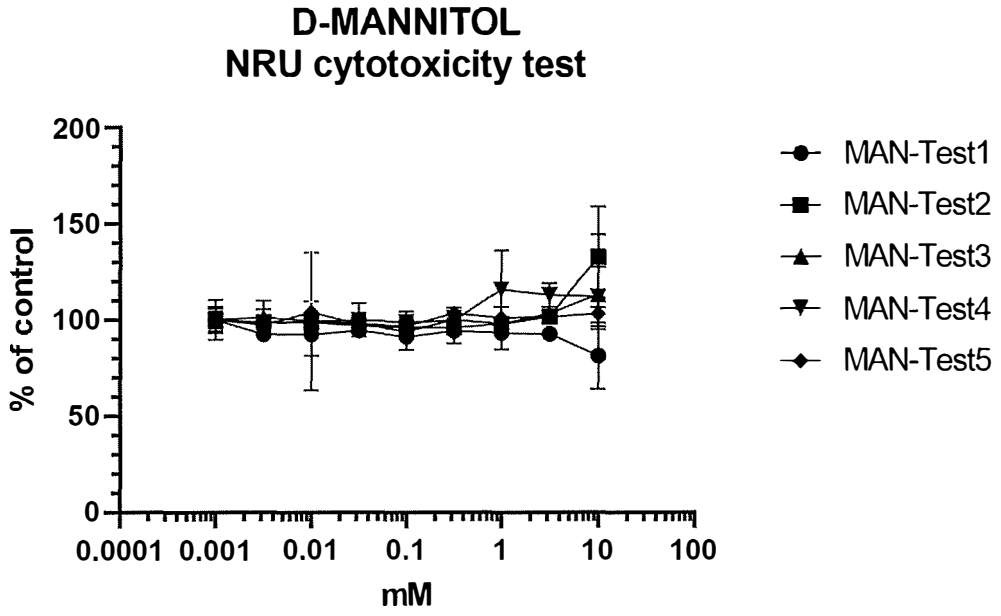

Figure 5. NRU cytotoxicity dose response curves after 6-day exposure to D-Mannitol

IC_{50} values of Suramin calculated with GraphPad Prism are shown in Table XI. For D-mannitol no IC_{50} could be calculated.

Table XI. IC_{50} calculation of Suramin using Sigmoidal, 4PL, X is log(concentration) equation (GraphPad Prism)

	SUR-Test1	SUR-Test2	SUR-Test3	SUR-Test4	SUR-Test5	SUR-Manual
LogIC50	-0,6744	-0,6832	-0,6179	-0,6547	-0,6959	-0,6349
IC50: mM	0,2116	0,2074	0,2411	0,2215	0,2014	0,2318
R squared (Goodness of fit)	0,9277	0,9200	0,9549	0,9383	0,9299	0,9271

Interpolated values for 30% tubule inhibition and 20% cytotoxicity in tests using Suramin are shown in Table XII. The test item Suramin was classified as specific inhibitor of tubule formation because > 30% tubule inhibition appeared at < 20% cytotoxicity concentrations (as defined in the criteria). In tests 4 and 5 cytotoxicity did not reach 20 %.
D-Mannitol was classified as non-inhibitor of vascular (tubule) formation because there was no tubule inhibition and no cytotoxicity.

Table XII. Interpolated values (by GraphPad Prism 9) for 30% tubule inhibition and 20% cytotoxicity and classification of test item Suramin.

mM (Interpolated)	SUR-Test1	SUR-Test2	SUR-Test3	SUR-Test4	SUR-Test5
30% tubule inhibition	0,186	0,202	0,221	0,185	0,199
20% cytotoxicity	0.930	0.931	0.944	-	-
Classification*	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Criterion	Passed	Passed	Passed	Passed	Passed

*Test item is classified a specific inhibitor

10. ASSESSMENT OF THE PERFORMANCE/VALIDITY OF THE TEST METHOD

10.1. Technical validity

The tests were technically accepted since following criteria were met:

- Vehicle/tubule controls: there was intense tubule network present in VC cultures in all tests
- Low tubule controls: there were less or no tubules present compared to VC controls in all tests
- The coefficiency of variation (%CV) of VC samples was always <18%. See results in Table XIV

Table XIV. Coefficient of variation (CV%) of VC samples in tests 1-5

Suramin	Test 1	Test 2	Test3	Test4	Test5
TF	8.05	11.00	7.45	13.28	8.61
NRU	9.98	3.44	7.59	8.98	3.37
Mannitol	Test 1	Test 2	Test3	Test4	Test5
TF	16.79	13.28	15.91	15.51	2.83
NRU	10.41	4.49	4.05	5.96	6.86

- In every multiwell plate there were three positive control wells (PC; 0.2 mM Suramin). Tubule formation compared to VC controls in positive controls ranged from 32.95 - 53.93% in a concentration area that was not cytotoxic. This is >30% inhibition which fulfills the acceptance criterium in the SOP.

10.2. Acceptance criteria for the test items

Criteria for reliability (relevance) and repeatability were met as follows (see also Table XII):

Relevance:

1. The classification of Suramin: Suramin was classified as an Inhibitor of tubule formation in all five tests (criterion was passed)
2. The classification of D-Mannitol: D-mannitol was classified as a non-Inhibitor of tubule formation in all five tests (criterion was passed)

Repeatability

1. The tests were repeatable. In the five tubule formation analyses using Suramin, repeats did not differ statistically ($p>0.05$) from each other, the p value was >0.999. In the analysis, the logIC50s of dose response curves were compared using one-way ANOVA (see Table XV). D-Mannitol had no logIC50s to enable analysis, but it showed repeatably no inhibition of tubule formation.

Table XV. One-way ANOVA analysis of logIC50s of Suramin of Tests 1-5 and manual analysis. The analysis used was ordinary One-way ANOVA assuming equal SDs. The mean was $logIC_{50}$, SEM was Sy.X and N was Degrees of Freedom +1 obtained from IC_{50} analysis results. GraphPad Prism 9 software was used.

Values used in the analysis:

Test1-SUR			Test2-SUR			Test3-SUR			Test4-SUR			Test5-SUR			SUR-MANUAL		
MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
-0,6744	10,59	25	-0,6832	11,72	25	-0,6179	10,33	19	-0,6547	11,35	27	-0,6959	12,31	25	-0,6349	11,12	27

Analysis results:

	LOgIC50 ANOVA
Table Analyzed	SUR
Data sets analyzed	A-F
ANOVA summary	
F	6,375e-006
P value	>0,9999
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R squared	2,245e-007
Brown-Forsythe test	
F (DFn, DFd)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	
Bartlett's test	
Bartlett's statistic (corrected)	2,321

P value	0,8032
P value summary	ns
Are SDs significantly different (P < 0.05)?	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0,1020	5	0,02040	F (5, 142) = 6,375e-006	P>0,9999
Residual (within columns)	454359	142	3200		
Total	454359	147			

Data summary	
Number of treatments (columns)	6
Number of values (total)	148

10.3. Acceptance of the Microsoft Excel templates

MicroSoft Excel templates were accepted:
1) Data transfers performed by the template were correct (checked visually)
2) Calculations performed by the template were correct (formulas were correct)
Note! In the calculation template for tubule formation the row titles (rows 78, 89) were in reverse order which was corrected.

10.4. Acceptance criteria for the AngioTool software

The acceptance criteria for AngioTool software were fulfilled:
1) The vascular structures were recognized correctly by the software as shown by the lines drawn by the software on the tubule structures (checked visually)
2)) Manual reading of tubule structure and AngioTool software analysis of tubule length correlated: The images that got the highest value in manual reading got the highest value also in AngioTool analysis and the images that got the lowest value in manual reading got the lowest value also in AngioTool analysis (see tables VII and VIII).
Also, the dose response comparison of logIC50s revealed that manual analysis did not differ statistically from automated analysis. Note! In manual reading also partly detached areas could be evaluated.
3) The classification of Suramin was *inhibitory effect*, and the classification of D-Mannitol was *non-inhibitory effect* in both AngioTool software analysis and manual reading.

11. AMENDMENTS

There were no amendments to the validation study plan.

12. DEVIATIONS

There was one deviation from the validation study plan. In the definition of the study, word “disruptors” was changed to “signalling”. “A joint EURL ECVAM - coordinated validation of in vitro methods for detecting of modulators of thyroid hormone disruptors 8c (Part 1) and FHAIVE’s in-house follow-up validation of the test method” reads now “A joint EURL ECVAM -coordinated validation of in vitro methods for detecting of modulators of thyroid hormone signalling 8c (Part 1) and FHAIVE’s in-house follow-up validation of the test method”. This change has no effect on the outcome of the validation.

13. DOCUMENTATION

This validation report provides a true and complete record of the data generated. The validation study was conducted according to the validation study plan. There was one deviation that did not affect the results.

14. IMPLEMENTATION OF THE TEST/CONCLUSIONS

The vasculogenesis/angiogenesis test method validation passed the technical, relevance and repeatability criteria. Positive test item Suramin was classified as an inhibitor of vascular formation and the negative test item D-Mannitol was classified as a non-inhibitor of vascular formation in all five tests performed. The test method can be implemented as soon as the relevant SOP/M/0072 is updated.

Suggested SOP update includes corrected calculation template 12 for Calculation of tubule formation results (the order of concentrations needs to be reversed) update of Appendix 5 (Appendix is now not practical for two exposures; microscopical inspection section can be modified afterwards, it should be locked after inspection), Appendix 3 should be corrected to chronological order.

Additionally, low tubule controls (LTC) are not used in the calculations and do not add value to the results. On the contrary, cultures tend to detach easily and are difficult for automatic analysis. LTC is proposed to be removed from the SOP.

Detachment of cell structures from the bottom of wells was a concern (at the worst case 36% of the wells had to be rejected). A criterion for acceptance will be added to SOP/M/0072.

15. DISTRIBUTION OF THE VALIDATION STUDY REPORT

Copies of Validation Study Report will be distributed to the QA and EURL ECVAM.

16. REFERENCES

OECD 2016, Position Paper Regarding the Relationship between the OECD Principles of GLP and ISO/IEC 17025, OECD Series on Principles of GLP and Compliance Monitoring, No. 18, OECD Publishing, Paris.

OECD (2018), Guidance Document on Good In Vitro Method Practices (GIVIMP), OECD Series on Testing and Assessment, No. 286, OECD Publishing, Paris, https://doi.org/10.1787/9789264304796-en.

Liu X, Zheng N, Shi YN, Yuan J, Li L. Thyroid hormone induced angiogenesis through the integrin αvβ3/protein kinase D/histone deacetylase 5 signaling pathway. J Mol Endocrinol. 2014 Apr 28;52(3):245-54. doi: 10.1530/JME-13-0252. PMID: 24532656.

Luidens MK, Mousa SA, Davis FB, Lin HY, Davis PJ. Thyroid hormone and angiogenesis. Vascul Pharmacol. 2010 Mar-Apr;52(3-4):142-5. doi: 10.1016/j.vph.2009.10.007. Epub 2009 Oct 30. PMID: 19879961.

Davis PJ, Sudha T, Lin HY, Mousa SA. Thyroid Hormone, Hormone Analogs, and Angiogenesis. Compr Physiol. 2015 Dec 15;6(1):353-62. doi: 10.1002/cphy.c150011. PMID: 26756636.

Rajabi S, Dehghan MH, Dastmalchi R, Jalali Mashayekhi F, Salami S, Hedayati M. The roles and role-players in thyroid cancer angiogenesis. Endocr J. 2019 Apr 25;66(4):277-293. doi: 10.1507/endocrj.EJ18-0537. Epub 2019 Mar 6. PMID: 30842365.

Balzan S, Del Carratore R , Nardulli C, Sabatino L , Lubrano V and Iervasi G:The Stimulative Effect of T3 and T4 on Human Myocardial Endothelial Cell Proliferation, Migration and Angiogenesis. J Clin Exp Cardiolog 2013, 4:12 DOI: 10.4172/2155-9880.1000280

Toimela T, Huttala O, Sabell E, Mannerström M, Sarkanen JR, Ylikomi T, Heinonen T: Intra-laboratory validated human cell-based in vitro vasculogenesis/angiogenesis test with serum-free medium. Reprod Toxicol. 2017 Jun;70:116-125. doi: 10.1016/j.reprotox.2016.11.015. Epub 2016 Nov 30.

Huttala O, Vuorenpää H, Toimela T, Uotila J, Kuokkanen H, Ylikomi T, Sarkanen JR, Heinonen T. Human vascular model with defined stimulation medium – A characterisation study. Altex 32(2):125-36, 2015.

Sarkanen JR, Vuorenpää H, Huttala O, Mannerström B, Kuokkanen H, Miettinen S, Heinonen T, Ylikomi T: Adipose stromal cell tubule network model provides a versatile tool for vascular research and tissue engineering. Cells Tissues Organs. 196(5):385-97, 2012.

Ai S, Cheng XW, Inoue A, Nakamura K, Okumura K, Iguchi A, Murohara T, Kuzuya M. Angiogenic activity of bFGF and VEGF suppressed by proteolytic cleavage by neutrophil elastase. Biochem Biophys Res Commun. 2007 Dec 14;364(2):395-401. Epub 2007 Oct 15.

Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NF, Wheatley DN. 1999. An in vitro model of angiogenesis: basic features. Angiogenesis 3(4):335-344.

Donovan D, Brown NJ, Bishop ET, Lewis CE. 2001. Comparison of three in vitro human 'angiogenesis' assays with capillaries formed in vivo. Angiogenesis 4(2):113-121.

Friis T, Kjaer Sorensen B, Engel AM, Rygaard J, Houen G. 2003. A quantitative ELISA-based co-culture angiogenesis and cell proliferation assay. Apmis 111(6):658-668.

Nakatsu MN, Hughes CC. An optimized three-dimensional in vitro model for the analysis of angiogenesis. Methods Enzymol. 2008;443:65-82

Certificate of Analysis

PromoCell

Human Mesenchymal Stem Cells from Adipose Tissue (hMSC-AT)

Product Description

Product Name	hMSC-AT-c	hMSC-AT-p
Order Number	C-12977	C-12978
Lot Number	453Z005.6	
Amount per Unit	≥ 500.000 cells in Cryo-SFM (Order No.: C-29910)	≥ 500.000 cells in MSC Growth Medium 2 (Order No.: C-28009)
Condition	Cell solution cryopreserved	Proliferating cell culture
Package Size	1.2 ml Cryo-Vial	T25 cell culture flask
Stage of Culture	thawing and seeding results in passage 2 (3rd culture)	shipped in passage 2 (3rd culture)
Date of Manufacture	09/2019	---
Expiry Date	n/a if stored under defined condition (Instruction Manual)	
QC Evaluation Medium	MSC Growth Medium 2 (Order No.: C-28009) MSC Adipogenic Differentiation Medium 2 (Order No.: C-28016) MSC Chondrogenic Differentiation Medium (Order No.: C-28012) MSC Osteogenic Differentiation Medium (Order No.: C-28013)	

Donor information

Donor Age / Sex / Race	26 / female / caucasian
Tissue / Localisation	subcutaneous adipose tissue / breast

APPROVED "TISSUE DONOR" PROGRAM

The tissue used by PromoCell for the isolation of human cell cultures is derived from donors who have signed an informed consent form, which outlines in detail the purpose of the donation and the procedure for processing the tissue (www.promocell.com/ethics).

Results of Analysis

Growth Characteristics	Test Method	Specification	Result
Cell Count	Automated fluorescent live / dead cell staining method (Viacount Assay / Muse Cell Analyzer, Millipore)	≥ 500.000 cells	700.000 cells
Viability		≥ 75 %	85 %
Population Doublings	Test performed by thawing cryopreserved cells and using PromoCell's standardized culture system and procedures. The stated values may vary under customer culture conditions.	≥ 10 PD	pass
Median Population Doubling Time incl. lag phase over 10 PDs		≤ 30 h	23 h
Identity & Purity			
	Analysed in passage 3 using a Guava Flow Cytometer (Millipore)		
CD73 / CD90 / CD105	Antibodies provided by Miltenyi Biotec MSC Phenotyping Kit human No. 130-095-198.	≥ 90 %	100 %
CD14 / CD34 / CD45		≤ 10 %	1 %
CD19 / HLA-DR	Antibodies provided by BioLegend No. 302208 and No. 307604.	≤ 10 %	5 %
Potency			
Adipogenesis	Sudan III staining performed with P3 or P4 cells on day 12-14	pass	pass
Chondrogenesis	Alcian Blue staining performed with P3 or P4 cells on day 21-23	pass	pass
Osteogenesis	Alizarin Red S staining performed with P3 or P4 cells on day 12-14	pass	pass
Sterility and Virus Testing			
Bacteria, Fungi	Growth Promotion Test	negative	negative
Mycoplasma Genus, Mycoplasma Pulmonis	PCR	negative	negative
HIV-1, HIV-2	PCR	negative	negative
HBV, HCV	PCR	negative	negative
HTLV-1, HTLV-2	PCR	negative	negative

See MSC Analysis Application Notes for reference protocols: http://www.promocell.com/application-notes

Michael Heinbuch, Head of Quality Control

Printed 270821 MW

Date: Nov 25, 2019

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Certificate of Analysis

PromoCell

Human Umbilical Vein Endothelial Cells (HUVEC), pooled

Description

Product Name	HUVEC-c pooled / HUVEC-p pooled
Order Number	C-12203 / C-12253
Lot Number	466Z022
Donor Age / Sex / Race	newborn / female / caucasian newborn / male / caucasian newborn / male / caucasian
Tissue / Localisation	umbilical cord / umbilical vein
Number of Viable Cells	500.000
Freezing Medium	Cryo-SFM (Order No.: C-29910)
QC Evaluation Medium	Endothelial Cell Growth Medium (Order No.: C-22010)
Stage of Culture	HUVEC-c: thawing and seeding results in passage 1 (2nd culture) HUVEC-p: shipped in passage 1 (2nd culture)

Viability & Growth Characteristics

Parameter	Test Method*	Result
Viability	Automated Viability Test	76 %
Population Doubling (PD) Time in Log Phase	Growth Promotion Test	12.1 h / PD
Population Doublings	Growth Promotion Test	>15 PD*

Phenotypic Characterization (tested within the first two passages)

Parameter	Test Method*	Result
CD31	Flow Cytometry	positive
vWF / Factor VIII-related antigen	Flow Cytometry	positive
DiI-Ac-LDL uptake	Flow Cytometry	positive
Smooth muscle α-actin	Flow Cytometry	negative

Test for microbiological contaminants and infectious viruses

Parameter	Test Method	Result
Bacteria, Fungi	Sterility Test	negative
Mycoplasma Genus, M. Pulmonis	PCR	negative
HIV-1, HIV-2	PCR	negative
HBV, HCV	PCR	negative
HTLV-1, HTLV-2	PCR	negative

* Using PromoCell's standardized culture system and procedures. The stated values may vary under customer culture conditions.

The tissue used by PromoCell for the isolation of human cell cultures is derived from donors who have signed an informed consent form, which outlines in detail the purpose of the donation and the procedure for processing the tissue (www.promocell.com/ethics).

Michael Heinbuch
Head of Quality Control

Date: Nov 26, 2020

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CERTIFICATE of ANALYSIS

Cayman CHEMICAL

Suramin (sodium salt)

8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid, hexasodium salt

Item No. 11126 • Batch No. 0609411

Purity Specification: ≥98%
Molecular Formula : C51H34N6O23S6 • 6Na
CAS Number: 129-46-4 Formula Weight: 1429.1 Expiry date: 17MAR2023

Overview

Tests	Results
Mass spec	M+Na: 1451.0
[illegible]	Purity: 100 %

Reviewed and approved by: Jennifer LaBrecque

Jen LaBrecque

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Printed 16US 22 Mv

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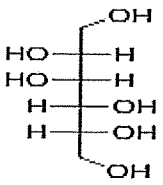
Certificate of Analysis

Product Name:
D-Mannitol - ≥98%

Product Number:	**M4125**
Batch Number:	**WXBD1141V**
Brand:	SIAL
CAS Number:	69-65-8
Formula:	C6H14O6
Formula Weight:	182,17 g/mol
Quality Release Date:	17 OCT 2019
Recommended Retest Date:	OCT 2025



Test	Specification	Result
Appearance (Color)	White	White
Appearance (Form)	Powder	Powder
Solubility (Colour)	Colorless	Colorless
Solubility (Turbidity) 100 mg/mL , H20	Clear	Clear
Specific Rotation C= 1 in acidified Molybdate,25 Deg C	137 - 145 deg	137 deg
Proton NMR spectrum	Conforms to Structure	Conforms
Purity (GC)	≥ 98 %	100 %

Steven Chen, Manager
Quality Control
Wuxi, China CN