STUDY TITLE

ION-ZC1: Anti-tumor efficacy study using syngeneic mouse melanoma xenograft model

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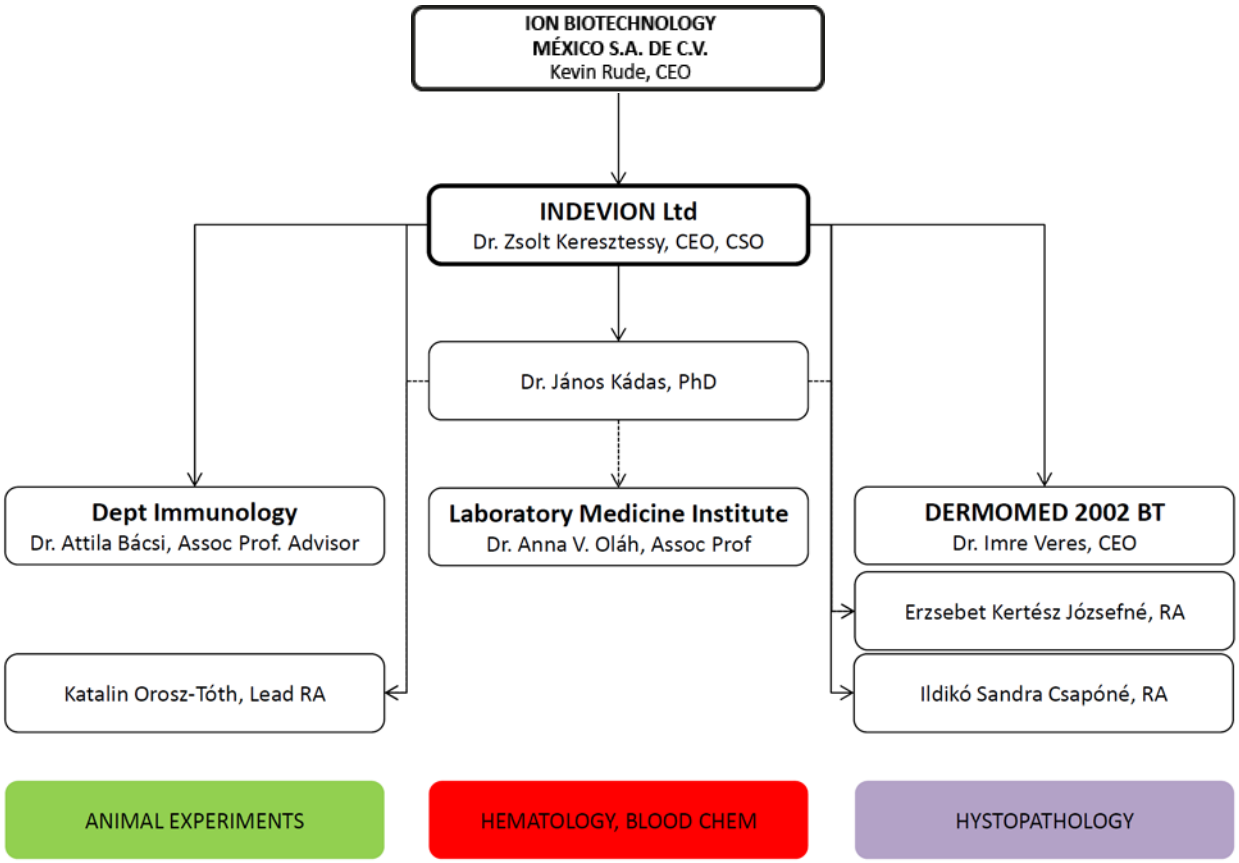
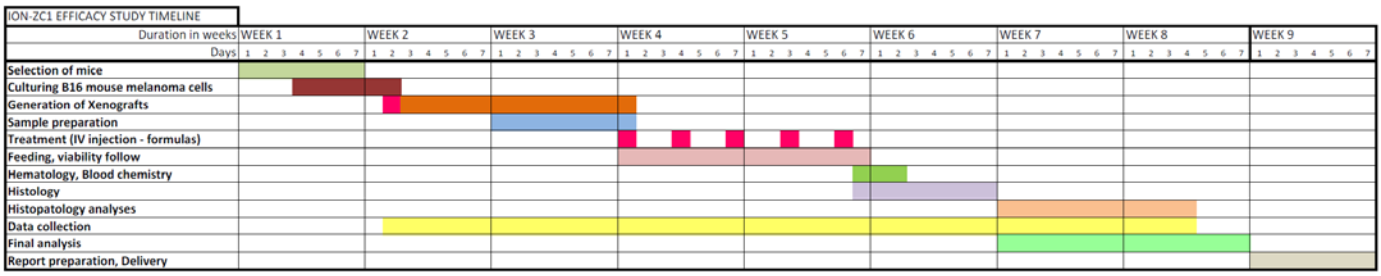
SUMMARIES AND OTHER INFORMATION

(1) ANTI-TUMOR EFFICACY STUDY TEAM - CONTRACTUAL FRAMEWORK



(2) STUDY TIMELINE



(3) GENERAL STUDY OUTCOME PLAN

Endpoint	Comment
In vivo	
>Tumor onset	Time to palpable tumor mass of predetermined size
>Tumor growth rate	Assessment of tumor volume throughout time
>Number of tumor-bearing animals	Frequency of cure
>Tumor burden *in vivo* at set time	Weight of tumor
>Tumor growth delay	Volume estimated (mm3) two-dimensional measurement
	Delay of time for tumor to reach specific volume
>Survival—life span	Increase in median survival time
>Survival— number alive	Percent cure at predefined time
Ex vivo	
>Gross pathology	Ulceration/central necrosis,
	invasion or tissue distribution and gross lesions
	Incidence of metastasis
	Gross count (lungs)
>Histopathology	H&E staining
	Organ histopathology
	Organs with metastases
	Tumor histopathology
	Tumor angiogenesis
	(Inflammatory cell infiltration)
>Hematology/Blood chemistry	Complete blood count, platelets, spleen, marrow
	Blood/spleen/marrow/thymus differential

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ION-ZC1: Anti-tumor efficacy study using syngeneic mouse xenograft model, B16 mouse melanoma model in C57BL/6J mice with intact immune system

PROTOCOL NO.:	ION-ZC1-EFF-IV-112015/1-3
AGENCY:	**III/10-KÁFH/2016,** DE MAB
STUDY NUMBER:	3-4-5/2015-2016
SPONSOR:	Ion Biotechnology México S.A. de C.V., Plaza Marina Local D24, Puerto Vallarta, Jalisco, México, C.P. 48335
TEST SUBSTANCE IDENTIFICATION:	ION-ZC1 (Also known as ION-Zn-Cu) LOT #:
DATE RECEIVED:	10TH JUNE 2015
PSL REFERENCE NO.:	
STUDY INITIATION DATE:	1 NOVEMBER 2015
DATES OF TESTS:	11 NOV 2015- 30 MAR 2016
NOTEBOOK NO.:	

1. PURPOSE

To provide information on the anti-tumor efficacy of ION-ZC1 by the intravenous injection (parenteral) route, using a syngeneic mouse xenograft model, more specifically a subcutaneous B16 mouse melanoma model in C57BL/6J mice (Charles River) with intact immune system.

2. SUMMARY

Anti-tumor efficacy study of ION-ZC1 was carried out in various doses of single and multiple intravenous injections (IV) on a syngeneic mouse xenograft model, namely subcutaneous B16 mouse melanoma in C57BL/6J mice (Charles River) with intact, more specifically, strong immune system. The subcutaneous model is widely used for the evaluation of therapy in many tumor models. To generate the tumor model, B16 mouse melanoma cell line (B16-F10, ATCC® CRL-6475™), was cultured and injected according to a standard protocol (*Willem W. Overwijk and Nicholas P. Restifo, Curr Protoc Immunol. 2001 May; CHAPTER: Unit–20.1. doi:10.1002/0471142735.im2001s39*.). Altogether 30 mice (both males and females) were selected for the study with an age range within 8-12 weeks. Upon subcutaneous injection of 1-10 x 10^5 cells on the back of the mice, B16 formed a palpable tumor in 5 to 10 days. Intravenously injectable formulations of ION-ZC1, containing the drug candidate at various concentrations, were prepared as dilution series in 5 ml final volumes, and filter sterilized. When preparing the solutions, the diluent was either (i) physiological saline with no pH adjustment (pH 1.5-2.4), or (ii) the pH of the first dilutions was adjusted to above 5 (pH 5.3) or (iii) pH of the dilutions was adjusted to above 5.4 (pH 5.45), using 0.1 mM bicarbonate buffer pH 9.2. The injection preparation series covered a range of ION-ZC1 concentrations between 0.39-6.25 % (Vol/Vol) or doses between 40-600 mg/kg body weight. Injections of “control tumor mice” (physiological salt or the same with added bicarbonate buffer) or “treated tumor mice” (various ION-ZC1 concentrations) were carried out with a frequency between once- every 2-3 days via the tail vein in a final volume of 0.06-0.2 ml/mouse/injection, and using either (i) a baby branule system, or a single use steal needle. During a period of 14 days following the first intravenous injections of tumor bearing control or treated mice, which were preferably applied at 10x10x10 mm tumor dimensions (where possible), were monitored, and *in vivo* outcomes, including tumor dimensions (using a caliper) to assess growth rate via tumor volume calculations (0.52 x Width x Length^2), survival time, body weight (every 2-3 days), and general health state and behaviour were recorded.

To reach *ex vivo* outcomes (as gross pathology: ulceration/central necrosis, invasion or tissue distribution, gross lesions, as histopathology: inflammatory cell infiltration, angiogenesis, metastasis, as hematology and blood chemistry), control and treated mice were sacrificed (after 14th day from the first IV injection or less) and whole blood (1.5 ml) was collected into standard vacuum blood collection tubes and submitted to routine human hematology and blood chemistry analysis. For histopathology investigations, 6 main organs (liver, lung, kidneys, spleen, heart, brain) and tumors or deformed organs of representative mice were isolated, collected and stored in formaline, followed by paraffin embedding (within a few days). Slides were prepared from paraffin embedded specimen and staining was performed (Hematoxilin/Eosine). Histopathology analysis was carried out by a specialist.

As one of the main outcomes of this efficacy study, intravenous injection of ION-ZC1 in various formulations on tumor growth rate (estimated via tumor size measurement and volume calculations against observation time) was found effective as an anti-tumor agent in the dose range of 40-350 mg/kg body weight (0.39 – 3.13 % ION-ZC1, 0.2 ml IV). Most effective formulations and treatment protocol were found to be when ION-ZC1 was diluted in physiological salt solution and the pH was adjusted to above 5 or 5.4, and the injections were applied 1-3 times over the 14-day observation period. For example, injection of ION-ZC1 at a dose of 60-70 mg/kg body weight (0.52 % ION-ZC1, 0.2 ml IV), tumor shrinkage of more than 50% was observed even after 3 days of application, followed by much lower tumor growth rate than in the control or none. Single injection at the dose of 90-100 mg/kg body weight (0.78 % ION-ZC1, 0.2 ml IV) also resulted in plateaued tumor growth or tumor some shrinkage, and a slower tumor growth compared to controls. When applying single ION-ZC1 intravenous injections in a formulation with no pH adjustment, effectiveness was observed to be lower or none than when pH adjusted formulations (pH above 5 or above 5.4) were used for IV treatment. Low pH intravenous injection of the compound resulted in higher mortalities compared to the pH adjusted IV solutions. For example, out of 16 mice 6 died within the timeframe of the study due to either tumor burden or injection intolerance/coagulation/ embolia (but not due to intoxication). ION-ZC1 IV did not have significant effect behaviour, except when high doses (above 350 mg/kg body weight) were injected, which resulted in e.g. numbness and slow moving). Body weight increased (except some non-significant decrease in case of high doses).

Abnormalities compared to control tumor mice were not noted for any of the animals when necropsied at the conclusion of the 14-day observation period. As most important results from histopathological analysis, we can state that (i) tumors isolated from mice treated with various concentrations of ION-ZC1 IV injections show massive necrosis, which is not pronounced as much in tumors isolated from control tumor mice; and (ii) blood vessels are much less frequent, less developed in the ION-ZC1 treated mice compared to control tumor mice. Furthermore (iii) ION-ZC1 injection results in spleen enlargement, which is remarkably (2-3 times) bigger in extent than that of the spleen of control (untreated) tumor mice, and consistent with ION-ZC1 inducing a strong anti-tumor immune response. *…(to be continued, supplied with new findings)*

3. MATERIALS

A) Test Substance (TDS Ion Biotechnology-ZCM1 25/ October/2015**) ION-ZC1 (Also known as ION-Zn-Cu)**

Property	**Unit**	**Min**	**Max**	**Method**
Redox Potential	mv		350 ASTM	D-1498
Concentration of Zinc	% w/w	4.5%	5.5%	AOAC/ 2006.03
Concentration of Copper	% w/w	1.8%	2.2%	AOAC/ 2006.03
pH	%/°C	0	1.0	ASTM E70-07

Property	**Unit**	**Value**	**Method**
Appearance		Blue liquid	ASTM D4176
Odor		Mild odor	
Boiling Point	°F/°C	220°F (104.4 °C)	
Specific Gravity	H2O=1	1.181	
Vapor Pressure	Mm Hg	0.1 mm at 68°F (20°C)	
Vapor Density	Air=1	1.00	
Solubility in Water		Very soluble, completely miscible	

B) Animals

3.B.1 Number of animals (Phase 1-2-3): 18 + 9 + 3 = **30**

3.B.2 Sex: males/females, nulliparous and non-pregnant

3.B.3 Species/Strain: Mouse/C57BL/6J

3.B.4. Age/Body weight: Young adult (8-12 weeks)/females 21.8 – 23.5 grams at experimental start

3.B.5. Source: SPF Animal Facility, University of Debrecen, Inbred from C57BL/6 - Charles River.

4. METHODS

A. Husbandry

4.A.1 Housing: The animals were singly housed in plastic caging, which confirms with the size recommendations given by the most recent Hungary regulations (40/2013. (II. 14.) Korm. rendelet - az állatkísérletekről).

4.A.2-6 At the Laboratory Animal Core Facility, specialized animal housing spaces include a quarantine suite, infectious biohazard containment suites.

All animal rooms and the operation rooms are ventilated with HEPA filtered air.

The technologies meet the requirements of FELASA recommendations and DIN EN ISO 9001 standards.

4.A.7 Water supply: Filtered tap water *ad libitum*.

4.A.8 Contamination: no known contaminants were foreseen to be present in the food or water at levels which would interfere with the results of this study.

Disease monitoring: Animals are housed separately by species and, when possible, by source and or microbiological status. All rodent colonies are housed and maintained under minimal specific pathogen free (SPF) conditions. In our SPF definition we are negative for the following animal pathogens:

http://bmbi.med.unideb.hu/teszt2/index.php?option=com_content&view=article&id=151:kiserleti-allathaz&catid=28:kozponti-laborok&Itemid=243&lang=en

B. Identification

4.B.1 Cage: Cage card is used to identify cages specifying study number, sex of the animal, and animal identification number within the study.

4.B.2 Animal: Cage identification

5. PROCEDURE

A. Preparation and Selection of Animals

Altogether 30 healthy mice (C57BL/6J) aged 8-12 weeks were selected after investigation of health state, weighing.

B.1. Preparation of Test Substances

To prepare the test substances, ION-ZC1 solution (100%) was mixed well before use.

(Version 1, 11/25/2015)

No pH controlled solutions

Serial dilutions were prepared in physiological salt, infusion grade (TEVA Pharmaceuticals), in 5 ml final volume, and at **12.5, 6.25, 3.13, 1.56 and 0.78 % (Vol/Vol)** and were also designated as 1x, 2x, 4x, 8x, 16x dilutions of the starting 12.5% stock solution. More specifically, the first dilution (12.5%) was prepared by adding 1250 µL ION-ZC1 into 8750 µL of physiological salt solution, then mixed well and filter sterilised using a 0.2 µ syringe filter (BD Biosciences). The rest of the dilutions were prepared by adding 5 mL of the 12.5 % solution into 5 mL physiological salt solution (sterile) to reach 6.25 %, and then repeated the process consecutively to obtain each test substance. Dilution preparations were carried out under a biological safety cabinet (Class II sterile cell culture hood). The pH of all dilutions was measured with a standard pH meter (Metrohm) and recorded.

ION-ZC1 dilution series in physiological salt:

Injection Code	1x	2x	4x	8x	16x
% (Vol/Vol)	12.5	6.25	3.13	1.56	0.78
pH	1.45	1.5	1.99	2.01	2.18

Adjustment of pH of the test substances to physiological values was not attempted to make sure the active complex in ION-ZC1 is not broken up (stable at low pH only).

(Version 2, . 11/30/2016)

pH controlled solutions pH >5.0 (0.1 M bicarbonate buffer, pH 9.2)

Serial dilutions were prepared using infusion grade physiological salt (TEVA Pharmaceuticals), and the pH was adjusted to above 5 using 0.1 M bicarbonate buffer pH 9.2.

In 5 ml final volume, and at **12.5, 6.25, 3.13, 1.56 and 0.78 % (Vol/Vol)** and were also designated as 1xpH, 2xpH, 4xpH, 8xpH, 16xpH dilutions of the starting 12.5% stock solution. More specifically, the first dilution (6.25%) was prepared by adding 625 µL 100% ION-ZC1 solution into 4375 µL of physiological salt solution, then the pH was slowly adjusted to above 5 adding 0.1 M bicarbonate buffer pH 9.2 dropwise (7 ml) to obtain 3.9%. Out of the former solution (2xpH) 8 ml was added to 2 ml of physiological salt solution to obtain 3.13% (4xpH), then 5 ml of the latter was added consecutively to obtain the rest of the dilutions (down to 0.78%). Solutions were filter sterilised using a 0.2 µ syringe filter (BD Biosciences). Dilution preparations were carried out under a biological safety cabinet (Class II sterile cell culture hood). The pH of all dilutions was measured with a standard pH meter (Metrohm) and recorded.

ION-ZC1 dilution series in physiological salt and adjusting pH with bicarbonate buffer

Injection Code	**2xpH**	**4xpH**	**8xpH**	**16xpH**
% (Vol/Vol)	6.25	3.13	1.56	0.78
pH	5.36	5.5	5.6	5.7
Effective % (Vol/Vol)	3.9	3.13	1.56	0.78

(Version 3 15/01/2016)

pH controlled solutions pH >5.4 (0.1 M bicarbonate buffer, pH9.2)

Serial dilutions were prepared using infusion grade physiological salt (TEVA Pharmaceuticals), and the pH was adjusted to above 5 using 0.1 M bicarbonate buffer pH 9.2. In 5 ml final volume, and at **6.25*, 3.13, 1.56 and 0.78 % (Vol/Vol)** and were also designated as 1xpH, 2xpH, 4xpH, 8xpH, 16xpH dilutions of the starting 12.5% stock solution. More specifically, the first dilution (6.25%) was prepared by adding 625 µL 100% ION-ZC1 solution into 4375 µL of physiological salt solution, then the pH was slowly adjusted to above 5 adding 0.1 M bicarbonate buffer pH 9.2 dropwise (7 ml) to obtain 3.9%. Out of the former solution (2xpH) 8 ml was added to 2 ml of physiological salt solution to obtain 3.13% (4xpH), then 5 ml of the latter was added consecutively to obtain the rest of the dilutions (down to 0.78%). Solutions were filter sterilised using a 0.2 µ syringe filter (BD Biosciences). Dilution preparations were carried out under a biological safety cabinet (Class II sterile cell culture hood). The pH of all dilutions was measured with a standard pH meter (Metrohm) and recorded.

ION-ZC1 dilution series in physiological salt and adjusting pH with bicarbonate buffer

Injection Code	**1xpH**	**2xpH**	**4xpH**	**8xpH**	**16xpH**
% (Vol/Vol)		6.25	3.13	1.56	0.78
pH		5.4	5.53	5.67	5.78
Effective % (Vol/Vol)		3.67*	1.84	0.92	0.46

Control injection solution was prepared by adding 1.25 ml 0.1 M bicarbonate buffer pH9.2 to 8.75 ml physiological salt solution with final pH 7.4

B.2 Generation of B16 mouse melanoma mouse tumor xenograft model in C57BL/6J animals

B16 is a syngeneic mouse model for human malignant melanoma, see original reference: (**Willem W. Overwijk** and **Nicholas P. Restifo**, *Curr Protoc Immunol*. 2001 May; CHAPTER: Unit–20.1. doi:10.1002/0471142735.im2001s39.)

"The subcutaneous model is widely used for the evaluation of therapy in many tumor models, including B16 melanoma. Upon subcutaneous injection, B16 will form a palpable tumor in 5 to 10 days and grow to a 1 × 1 × 1–cm tumor in 14 to 21 days. When allowed to grow larger, the tumors often become necrotic in the center and begin to ulcerate or bleed; it is advisable to sacrifice the mice before this point. The typical dose used is 1 × 105 cells/mouse, which is 1.5 to 2 times the minimal tumorigenic dose in normal C57BL/6 mice. It is important to note that, for subcutaneous tumor growth experiments, a consistent injection technique is extremely important. Each mouse should show a clearly visible, defined "bleb" upon injection; if not, a new mouse should be used. Mice without a clear "bleb" will show delayed tumor growth or no growth at all."

B.2.1. Materials

B16 culture, ≤50% confluent (see Support Protocol 1)
Trypsin/EDTA (Life Technologies)
Complete medium (CM; see recipe), 4°C
Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), ice cold
6- to 12-week-old female C57BL/6 mice
70% ethanol
50-ml conical centrifuge tubes
Centrifuge and Sorvall H-2000B rotor, 4°C
Disposable cell strainer (Falcon)
1-ml disposable syringes and 27½-G needles
Calipers
Additional reagents and equipment for trypsinizing cells, counting cells in a hemacytometer, and determining viability by trypan blue exclusion (*APPENDIX 3B*), restraint of mice (*UNIT 1.3*), ear tagging (*UNIT 1.5*), and subcutaneous injection of mice *(UNIT 1.6)*

B.2.2. B16 mouse melanoma cell culturing

1. Ensure that B16 cells are in the logarithmic growth phase when harvesting for injection, i.e., flasks should be ≤50% confluent.

Nondividing tumor cells from confluent flasks may take less well.

2. Aspirate medium, rinse flask briefly with 3 ml trypsin/EDTA, and aspirate again.

Rinsing helps remove fetal bovine serum (FBS), which otherwise dilutes the trypsin and inhibits proteolysis. APPENDIX 3B provides additional detail on trypsinization of cells.

3. Add 5 ml trypsin/EDTA and tilt flask to ensure that all cells are covered. Periodically, firmly tap side of flask until cells detach and slide down the culturing surface.

Do not leave cells in trypsin any longer than necessary, to ensure high viability.

4. Add 5 ml cold complete medium and pipet vigorously to obtain single-cell suspension.

5. Transfer to 50-ml conical centrifuge tube and add 40 ml cold CM to neutralize trypsin. Pellet cells for 10 min at 663 × *g* (in a Sorvall H-2000B rotor at 1500 rpm), 4°C.

6. Decant supernatant and resuspend cells in ice-cold HBSS, aiming for 1–5 × 106 cells/ml.

7. Pass suspension through disposable cell strainer to remove any clumps. Count live cells using trypan blue (*APPENDIX 3B*).

Viability should be >90%.

8. Adjust cell concentration to 1 × 106 cells/ml in ice-cold HBSS.

B.2.3. Inoculateting mice with B16 mouse melanoma cell culture

9. Relocate work area to facility where 6 to 12 week old female C57BL/6 mice are kept, maintaining cells on ice.

Inject cells as quickly as possible after preparation; viability slowly decreases over time, even on ice.

10. Resuspend cells by inverting tube several times. Fill 1-ml syringe with attached 27½-G needle.

11. Wet abdominal fur with 1 or 2 drops 70% ethanol, rub fur in a downward manner with one finger, then part fur in the middle with needle.

“Swipe” the hairs sideways by moving the needle perpendicular to the abdomen,

parting the hairs so that the skin becomes clearly visible.

12. Insert needle very superficially, so that it is visible through the semi-transparent skin. This requires some practice; the key is remaining very superficial without reemerging through the skin. UNIT 1.6 provides additional information on subcutaneous injections.

13. Slide needle 5 to 10 mm subcutaneously and inject 100 µl cell suspension; watch for appearance of a “bleb.”

Failure to insert needle far enough will result in leakage of tumor suspension when mice massage the area after injection. If no clear “bleb” results, sacrifice mouse and use a new one.

14. Gently withdraw needle and place mouse in cage.

Discard the last 0.5 ml in the syringe; typically tumor cells collect against the plunger, resulting in a disproportionately large amount of tumor cells in the last injection.

B.2.4. Randomizing mice and follow up

15. Ear tag mice (*UNIT 1.5*) to blind the experiment and randomize among cages.

16. Observe mice for tumor growth. Use calipers to measure perpendicular tumor diameters.

Tumors should become palpable in 5 to 10 days. Wetting fur with 70% ethanol facilitates early detection.

C. Dose Calculations

Dose range of the test substance to be applied (injected intravenously) in the tests was designed as follows. Intravenous injection volumes were maximised at 200 µL of the test substances (dilution series), based on pre-experimental injection trials. Higher volumes were not tolerated by the animals. Individual doses were calculated based on initial body weights of the animals, based on and according to the following:

ION-ZC1 specific Gravity: 1.181 (TDS Ion Biotechnology-ZCM1 25/October/2015)

Intravenous injection dose volume: 0.2 ml/mouse

mg/kg body weight calculation: (1.181 x 0.2 x1000 x ION-ZC1 % /100)/(mouse body weight (g) x 1000). Individual doses are listed in TABLE 1.

D. Application of Test Substance

Mice were injected with 60-200 µL of the ION-ZC1 dilutions intravenously (tail vein) using either a baby branule plastic needle system (see appendices), or a single use steal needle, the control animal received physiological salt solution or the same solution with added 12.5 % 0.1 M bicarbonate buffer, pH 9.2 (0 % ION-ZC1).

E. Body Weights

Individual body weights of the animals were recorded every two-three days prior and to and after IV injection of the test substance.

F. Cage-Side Observations

The animals were observed for mortality, signs of gross toxicity, and behavioural changes during the first several hours after application, and once daily or in two days thereafter over the study period. Particular attention was paid to the potential appearance of tremors, convulsions, salivation, diarrhea, coma. Tumor size (width and length) was measured using a caliper and recorded as W, L (mm). Large tumor size relative to body weight was notified to prevent unattended animal death and suffering, and allow in time sacrifice of animals. Tumor central necrosis was also observed and reported.

G. Necropsy, organ isolation

All mice were euthanised via cervical dislocation at the end of the observation period. Gross necropsies were performed on all animals. Six main organs (brain, lung, liver, heart, kidneys and spleen) as well as representative tumors or pathological parts were isolated and stored in formaline. Organ weights were determined upon proper isolation and organ weights to body weights (at necropsy) ratios were calculated.

H. Blood Sample collection and Hematology and Blood Chemistry Analyses

Final bleed was collected before euthanasia from all the animals, using standard vacuum blood collection tubes with coagulation control. Hematology analysis and Blood Chemistry parameters were determined at the Routine Diagnostics Laboratories of the Laboratory Medicine Institute, Clinical Center, University of Debrecen.

I. Histopathology analysis of organs and tumors

Formalin stored organs were paraffin embedded, slides were prepared by standard procedures. Single staining was carried out using H/E stain (around a 100 microscopic slides were prepared, see Appendices).

Organ sections were analysed for toxic markers by a trained pathologist (MD, Spec. Pathologist). Such markers included but not limited to (i) trabecularisation around blood vessels in the liver, (ii) lymphocytes surrounding dying hepatocytes in the liver, (iii) lesions in the brain, (iv) edema in the lungs, (v) lesions in the spleen, (vi) loss of integrity of glomeruli in the kidneys, etc.

In case of tumors, representative specimens were isolated, formalin stored and paraffin embedded, where it was applicable (one specimen in case of control tumor mice and one specimen in case of treated groups, 2x, 4x, 8x, 16xpH). Gross pathology and analysis prior to dissection included gross count of metastasis on the surface of the lungs, tumor invasion or tissue distribution, gross lesions, ulceration.

Histopathological analysis of the H/E stained slides included identification of metastasis primarily in the lungs and liver, and secondarily in other organs (bluish colored melanoma cells). Also, to observe necrosis, discolored cells (pinkish) with deformed nuclei were looked for. Angiogenesis was investigated via counting blood vessels in the tumor sections of the control and treated mice groups. Inflammatory cell infiltration was investigated visually. (To identify and differentiate

between white blood cell types, e.g. macrophages, B, and T cells, specific immuno-staining can be used in later studies, see in Appendix X. Cellular apoptosis can also be followed in the tumors via e.g. TUNNEL assay, see in Appendix Y).

Deformed extra organs were also submitted for histopathology slide generation and H/E staining. In this case a thickened small bowel section was isolated, formalin stored and paraffin embedded as a representative specimen.

6. STATISTICAL ANALYSIS

Statistical analysis was carried out on the outcomes of triplicate or duplicate animal experiments.

7. STUDY CONDUCT

See before, front page.

8. QUALITY ASSURANCE

ISO9001 University of Debrecen

9. AMENDMENTS TO THE PROTOCOL

ION-ZC1 formulation changes were applied over the phases of the study (See Section 5. PROCEDURES - B1. Preparation of the test substance).

10. DEVIATIONS FROM THE PROTOCOL

None

11. FINAL REPORT AND RECORDS TO BE MADE

All information regarding maintenance of equipment, calibration, storage, usage, and descriptions of the test substances, and all records which demonstrate adherence to the protocol will be maintained. Facility records will be archived.

The original, signed final report will be transferred to the Sponsor. A copy of the signed report together with the protocol and the raw data is maintained. Sponsor is offered to take possession of the records.

12. RESULTS

To generate the syngeneic tumor model animals (C57BL/6J – with intact immune system), B16 mouse melanoma cell line for xenografting was cultured and passaged according to standard protocol. Technical summary of the three phases of the study is presented below and in **Table 1-3**.

In **Phase 1** of the study, 18 mice were injected subcutaneously on the back with 10^6 B16 mouse melanoma cells per mouse. During the course of tumor model development, 16 mice developed palpable tumors (within 1-2 weeks), while 2 mice did not develop palpable tumors. Out of the 16 mice, 6 died within the timeframe of the study due to either tumor burden or injection intolerance (not due to intoxication), and 10 mice survived throughout (full length of) the study. The 3 control mice were terminated 10 days after control injection because of reaching high tumor burden/size/ulceration/bleeding. The 13 ION-ZC1 treated mice did not show ulceration or condition which would make termination necessary. 10 mice were injected with ION-ZC1 dilutions in physiological salt, while 3 mice were injected with 0,78% ION-ZC1 dilution in physiological salt, with pH adjusted to above 5 using 0.1 M bicarbonate buffer pH 9.2. ION-ZC1 IV injections were only applied once over the course of the testing in Phase 1 of the study (2 weeks) for practical reasons.

In **Phase 2** of the study, 9 mice were injected subcutaneously on the back with 5 x 10^5 B16 mouse melanoma cells per mouse. All 9 mice developed palpable tumors (1 weeks, 8-10 mm). Out of the 9 tumor mice, 7 mice were injected in the tail vein by single use steel needles applying 3 different ION-ZC1 double dilution series (6.25 % - 0.39%) in physiological salt, the pH of which was adjusted to 5.4 using 0.1 M bicarbonate buffer pH 9.2, (osmolarity of which is ~300 mOsm, therefore all dilutions of ION-ZC1 retained isoosmolarity). 2 Control (tumor model) mice were injected with physiological salt, pH adjusted to pH 7.4 using bicarbonate buffer pH 9.2, 0.1 M, 12.5 % (also isoosmotic). Efficacy test was timelined as 1-2 weeks for tumor model growth, 2 weeks testing, and ION-ZC1 or physiological salt injections were planned for every 2-3 days. Out of the 9 mice drawn into Phase 2 of the study, 3 mice died within the timeframe of the study due to injection intolerance (not due to intoxication) at ION-ZC1 concentration range 1.5-3 %. 3 mice died after the 3rd IV injection of ION-ZC1 dilutions probably due to overdosing the formulation, but not due to tumor burden, since tumor growth was inhibited by the formulation. 2 control mice died due to tumor burden at Day 30 of the study (Day 16 relative to first control injection). 1 mouse survived

throughout (full length of) the study (mouse was sacrifised at Day 13 relative to first ION-ZC1 injection). The 7 ION-ZC1 treated mice did not show ulceration or condition which would make termination necessary. ION-ZC1 IV injections were applied 3 times over the course of the testing phase of the study (~2 weeks) for practical reasons.

In **Phase 3** of the study, 3 mice (10 weeks old) were injected subcutaneously on the back with 5 x 10^5 B16 mouse melanoma cells per mouse. 3 mice developed palpable tumors (>1 weeks, at day 15, with 11-12 mm tumor diameter) and all 3 mice were injected in the tail vein with 3 different ION-ZC1 dilutions (0.78%, 0.486%, 0.36%) in physiological salt, pH adjusted to 5.4 using 0.1 M bicarbonate buffer pH 9.2, (osmolarity was ~300 – so all 3 dilutions retained isoosmolarity) with single use steel needles. ION-ZC1 IV injections were applied 2 times over the course of the testing with a 5-day gap between injections, which was less frequent compared to the frequency of injections applied in Phase 2. The 3ION-ZC1 treated mice did not show ulceration or condition which would make termination necessary. The 3 mice survived 13-14 days after the first injection, 1 died, 2 were sacrifised.
Outcomes were recorded as they were necessary, practical and possible to support statistically significant conclusions.

12.1 Behavior, symptoms and mortality

There were some mortalities in this study after the injections (see TABLE 1-3). Low pH intravenous injection of the compound resulted in higher mortalities compared to the pH adjusted IV solutions. For example, out of 16 mice 6 died in within the timeframe of the Phase 1 study due to either tumor burden or injection intolerance/coagulation/embolia (but not due to intoxication). ION-ZC1 IV did not have significant effect on behaviour, except when high doses (above 350 mg/kg body weight) were injected, which resulted in e.g. numbness and slow moving). Body weight increased (except some non-significant decrease in case of high doses) normally or extremely due to tumor size increase (which was pronounced in case of control tumor mice). After the treatment, some differences in eating and drinking patterns and physical activity were observed both in controls and treated mice. Tremors, convulsions, salivation, diarrhea, coma etc. or any signs of general toxicity

was not observed except when low pH (1.5-2.5) intravenous injection of the compound, and/or when high doses (above 350 mg/kg body weight) were injected.

12.2 Efficacy of ION-ZC1 as measured by tumor growth rate

Altogether 30 mice (both males and females) were selected for the study with an age range within 8-12 weeks. Upon subcutaneous injection of 1-10 x 10^5 cells on the back of the mice, B16 formed a palpable tumor in 5 to 10 days. Intravenously injectable formulations of ION-ZC1, containing the drug candidate at various concentrations, were prepared as dilution series in 5 ml final volumes, and filter sterilized (for dose conversions, please, see Table 6). When preparing the solutions, the diluent was either (i) physiological saline with no pH adjustment (pH 1.5-2.4), or (ii) the pH of the first dilutions was adjusted to above 5 (pH 5.3) or (iii) pH of the dilutions was adjusted to above 5.4 (pH 5.45), using 0.1 mM bicarbonate buffer pH 9.2. The injection preparation series covered a range of ION-ZC1 concentrations between 0.39-6.25 % (Vol/Vol) or doses between 40-600 mg/kg body weight. Injections of "control tumor mice" (physiological salt or the same with added bicarbonate buffer) or "treated tumor mice" (various ION-ZC1 concentrations) were carried out with a frequency between once- every 2-3 days via the tail vein in a final volume of 0.06-0.2 ml/mouse/injection, and using either (i) a baby branule system, or (ii) single use steal needles. During a period of 14 days following the first intravenous injections of tumor bearing control or treated mice, which were preferably applied at 10x10x10 mm tumor dimensions (where possible), were monitored, and *in vivo* outcomes were recorded, including tumor dimensions (using a caliper) to assess growth rate via tumor volume calculations (0.52 x Width x $Length^2$), survival time, body weight (every 2-3 days), and general health state and behaviour.

For dose conversions and all results, see relevant tables and figures: **Table 4, 6, 12, Figures 2-5**, **11-14, 16**. The following findings were obvious from Phase 1-3 of the study. The correlation between ION-ZC1 dose and the effect on tumor growth as lowering the rate or even dereasing tumor size (shrinkage) was dependent on some conditions. In **Phase 1**, where low pH (1.4-2.4) formulations of ION-ZC1 IV injections (single) were used (dilutions of a 12.5% stock to 2x, 4x, 8x times), the lower the dose was, the higher the effect could be observed on tumor growth (inhibition). There was, however, no positive correlation between ION-ZC1 dose and anti-tumor effect (**Figure 5**) but, instead, an optimal dose of the compound was identified (8x dilution, 1.56% ION-ZC1). When the pH of the formulation was adjusted to >5 and of a 16x dilution (0.78% ION-ZC1), a more

prononounced inhibitory effect on tumor growth was observed (compared to the above dilutions), with even tumor shrinkage happening within a few days after IV injections (**Figure 2-4**). In **Phase 2** of the study, where we used higher pH formulations (pH adjustment to ~<5.4) for injections a more frequent injection, an ION-ZC1 concentration/dose dependent effect on tumor growth was observed (**Figure 11-14**). Namely, when 24x and 32x dilutions of the 12.5% ION-ZC1 (corresponding to 0.39-0.52% ION-ZC1 concentration) was injected every 2-3 days, and altogether 3 times within the 14 day observation period, there was a positive correlation between injection dose and tumor growth inhibition. Furthermore, the 0.52% formulation (~50 mg/kg body weight dose), had a remarkable tumor shrinking effect even after the first ION-ZC1 IV injection, that is, the size of the tumor decreased >50% within a few days after the injection (Figure 11-13.) Correlation analysis of the results from this phase (2) is shown in Figure 14. In **Phase 3** of this study, where we used even higher pH formulations (>5.45) and less frequent IV injection (every 5 days, and 2 injections in total. Here (see **Figure 16**) we could not see such a strong correlation between dose and anti-tumor effect as in Phase 2. This data support and optimal tumor treatment schedule of ION-ZC1 IV as (i) injections every 3 days, (ii) using a ~50 mg/kg body weight dose, and (iii) in a formulation with pH betwee 5-5.4.

12.3 Body weights, organ weight/BW coefficients

Table 4-5, **11, 13** list individual body weights and doses of ION-ZC1 injected intravenously into the animals. **Figure 1 , 10 and 15** show body weight changes over the study period, including tumor model generation (31 days) study period. Weight gain was different between the control, where tumor size contributed to weight and the ION-ZC1-treated animals, where slower body weight gain or, in some sporadic cases, weight loss was observed. Organ weights were determined following euthanasia, necropsy and proper isolation of all six organs (TABLE 7), as well as tumors and deformed other organs, e.g. intestine (see Appendices for illustration). Organ weight/body weight coefficients were calculated for all six organs (TABLE 8). After weighing the organs, the organ weight/BW coefficients of heart, lung, liver, spleen, kidneys and brain were calculated as organ weight (wet weight, mg)/BW (g) x 100% (Figure 6-7 and TABLE 8). Trends of organ weight/BW coefficients are demonstrated on FIGURE 6-7 as a function of ION-ZC1 doses. The liver, and more slightly the brain and the kidnes are increasing in weight relative to body weight in case of ION-ZC1 dose 80-100 mg/kg body weight. Abnormalities compared to control tumor mice were not noted

for any of the animals when necropsied at the conclusion of the 14-day observation period. General observation was that (i) in the tumor model mice (both controls and treated animals) spleen was remarkably enlarged compared to non-tumor mice (See ION-ZC1 TOX Report), (ii) there was deformation of the colon in all tumor mice (see histopathology analysis in Part 2 of this study).

12.3 Hematological analysis

Hematological parameters in the blood as detected by the autoanalyzer are listed in TABLE 10. Intravenous injections of ION-ZC1 did not induce significant or acute hematological toxicity, when comparing to the results for the control tumor animals (Figure 9).

12.4 Blood biochemical analysis

Biochemical parameters in the serum as detected by the autoanalyzer are listed in Table 9. No significant differences were found in the serum levels of compounds determined (TABLE 9, Figure 8). One remarkable deviation in values of treated tumor mice from control tumor mice was in Alanine aminotransferase (GPT) (ALT), which was elevated in treated mice and may reflect some liver overload/damage (marked in Table 9). However, this was not observed in the ION-ZC1 toxicology studies. Hemolysis affected on parameter, namely CK, is marked and excluded from the analysis.

12.5. Histopathology detection

Altogether 73 paraffin embedded specimen was processed into H/E stained slides, namely six main organs plus tumors, and, in addition, a deformed intestine (from phase 1: K1-3,2x, 4x1-3, 8x,16x1-2, from phase 2: 16x3, See Table H1 and Table H2 for numbers of specimen and identification). Digital light microscopy was used to analyse and photo document the analysis (50-200x magnification). Results of the analysis are discussed below, but summarized here as follows.

Metastasis to the lungs or kidneys was not observed in either control or treated tumor mice. Tumor invasion was seen in blood vessels. Tumor metastasis was observed in small bowel walls. Tissue distribution and gross lesions were identified in the tumor sections. Ulceration was observed in vivo, but not represented on tumor sections. Massive necrosis was observed in ION-ZC1 treated

tumor sections. Differences in angiogenesis was discovered, that is less blood vessel density was detected in tumors isolated from the ION-ZC1 treated animals.

Inflammatory cell infiltration of tumors was not yet observed, need specific histochemistry analysis.

As preliminary results from histopathological detection, we can state that (i) tumors isolated from mice treated with various concentrations of ION-ZC1 IV injections show massive necrosis, which is not pronounced as much in tumors isolated from control tumor mice; and (ii) blood vessels are much less frequent, less developed in the ION-ZC1 treated mice compared to control tumor mice.

12.5.1. Organ histopathology

The six types of organ slides were stained with H/E and are presented in **Figure H1** as a composite. Besides control tumor and treated tumor mice, sections from non-tumor control mice were also included in the composite figure for comparative purpose. Individual and comparative histopathological analysis was carried out and discussed below by organs (see ref. *Peter Greaves (2012) Histopathology of Preclinical Toxicity Studies: Interpretation and Relevance – 4th Edition, Academic Press, NY - LINK*). In addition, organ sections were analysed for the presence of B16 melanoma metastasis primarily in the lungs and liver and secondarily in other organs (bluish colored melanoma cells).

>Lungs

The lungs of neither the control tumor mice nor the ION-ZC1 treated tumor mice showed B16 melanoma metastasis by visual observation. (Alpha-Vimentin immunohistochemical staining would be required to detect the presence of melanoma metastasis, see e.g. *Ebos JML et al. 2009, Accelerated Metastasis after Short-Term Treatment with a Potent Inhibitor of Tumor Angiogenesis. Cancer Cell 15, 232–239*). Also see **Figure H1** and **H2**. According to the literature, if it becomes metastatic, B16 melanoma cell mediate alterations of elastic fibers and collagen distribution (potential markers for clinical diagnosis of melanoma) in the lungs of C57/B6 mice. These result in the enlargement of pulmonar alveoli. In tumor mice, less EF is found around in the blood vessels and bronchioli, and enlarged alveoli occur in the interstitial of the inter-alveolar compared with normal mice (*Chien-Hsun Huang, et al. (2015) Hinokitiol Exerts Anticancer Activity through Downregulation of MMPs 9/2 and Enhancement of Catalase and SOD Enzymes: In Vivo Augmentation of Lung Histoarchitecture Molecules 2015, 20, 17720-17734;*

doi:10.3390/molecules201017720). In the specimen investigated, no obvious sign of alveolar expansion was observed compared to the lungs of non-tumor control mice. Secondarily, no sign of oedema or other pathological signs were found in the lungs of any group of tumor mice, which would mark toxicity caused by the intravenous injection of the compound (ION-ZC1). Upon disection, no sign of metastasis was observed in the lungs of either control or treated tumor mice (see ref. for visual signs of melanoma metastasis: *Shigeru Kakuta et al. (2002) Inhibition of B16 melanoma experimental metastasis by interferon-γ through direct inhibition of cell proliferation and activation of antitumour host mechanisms. Immunology 105(1), 92–100.*)

>Liver

Liver sections were also investigated primarily for the presence of B16 melanoma metastases. No obvious signs of metastasis were observed in the case of either control tumor or treated tumor mice. Secondarily, some moderate signs of hepatic damage in the form of (i) expanding inter-hepatocyte channels, (ii) trabecularisation around blood vessels, (iii) lymphocyte infiltration for clearance of dying hepatocytes, were observed in case of both control tumor mice and treated tumor mice (**Figure H2**). This may be attributed to the effect of tumor burden.
(see ref. *Inthisham NASSAR et al. (2010) Histopathological study of the hepatic and renal toxicity associated with the co-administration of Imatinib and Acetaminophen in a preclinical mouse model. Malaysian J Pathol 32(1), 1 – 11*). Upon disection, no sign of metastasis was observed on the surface of the liver of either control or treated tumor mice (see ref. for visual signs of melanoma metastasis: *Shigeru Kakuta et al. (2002) Inhibition of B16 melanoma experimental metastasis by interferon-γ through direct inhibition of cell proliferation and activation of antitumour host mechanisms. Immunology 105(1), 92–100.*)

>Brain

Brain sections were analysed for the presence of metastasis and also for signs of toxicity of ION-ZC1. No obvious mark of melanoma metastasis was discovered in the brain of either control tumor mice or ION-ZC1 treated tumor mice. No toxic effect (brain lesion) was seen in ION-ZC1 treated tumor mice (**Figure H2.** and see ref. *Moran Amit et al. (2013) Characterization of the melanoma brain metastatic niche in mice and humans. Cancer Med. 2013 Apr; 2(2): 155–163*)

>Spleen

The spleen contains vascular and lymphoid elements and is a site of hematopoiesis, and in some species, removal of effete, degenerate and aged red blood cells as well as particulate materials and circulating bacteria from the blood supply. The spleen is the site of direct and indirect toxicity and a target for some carcinogens and also a site for metastasis of malignant neoplasms arising in other sites *(ANDREW W. SUTTIE (2006) Histopathology of the Spleen. Toxicologic Pathology, 34:466–503).* Histopathological analysis of the spleens of neither control tumor mice nor ION-ZC1 treated tumor mice showed any obvious signs of either toxicity or metastasis (**Figure H1**). On the other hand, spleens isolated from both control and treated tumor mice are larger, as measured by organ weight/body weight indices, than in case of non-tumor normal or ION-ZC1 treated mice, as it was evident from previous toxicology studies of ION-ZC1 (**Table 8**, **Figure H2**). This is consistent with the presence of the B16 melanoma xenograft tumor, which elicits immune response (on splenomegaly in B16 melanoma as the sign of immune response: http://www.marietta.edu/~biol/capstone/2004_files/jurkovic.ppt). One of the key findings of this anti-tumor efficacy study on ION-ZC1 via IV administration is that the treated tumor mice had even larger spleens compared to control tumor mice (**Figure H2**). According to the literature, splenomegaly (as well as liver enlargement) is a good measure of immune response stimulating effects of drugs (see ref: *Feng-ying Huang et al. (2012) The antitumour activities induced by pegylated liposomal cytochalasin D in murine models, European Journal of Cancer (2012) 48, 2260– 2269*). Although the correlation of spleen size with ION-ZC1 dose is negative, this might reflect an optimal dose which induces the strongest immune response. Actually, low pH formulations of ION-ZC1 caused tumor growth arrest in a non-correlating way (dose versus tumor growth decrease). Lower doses had greater tumor suppressive effects, but once again, it is true only for the low pH set. The spleen data in **Figure H2** is from Phase 1 of the study, where low pH formulations (pH ~2-3) were used, but also a higher pH formulation of ION-ZC1 (16x pH 5, 94 mg/kg body weight) was applied in the same set of experiments (Phase 1). The highest effect on spleen size was observed in the case of the low pH formulations at lower doses (132,5-337,5 mg/kg body weight ION-ZC1) and in case of the high pH (5) formulation (dose of 94 mg/kg body weight). These findings can be consistent with the hypothesis that ION-ZC1 can induce an immune response to the tumor probably via necrotizing cancer cells and exposing them to the immune system.

>Kidneys

Histopathology analysis did not find any signs of kidney toxicity or metastasis of B16 melanoma cells in case of either control tumor mice or ION-ZC1 treated tumor mice. Glomeruli were intact and no sign of tubular degradation was discovered (see **Figure H1.** and ref. *Samar Omar Rabah (2010) Acute Taxol nephrotoxicity: Histological and ultrastructural studies of mice kidney parenchyma. Saudi J Biol Sci. 2010 Apr; 17(2): 105–114.)*

>Heart

No signs of heart damage or metastasis from B16 melanoma were discovered on the heart sections in the case of either control tumor mice or ION-ZC1 treated tumor mice (**Figure H1**).

>Intestine

In case of both control tumor mice and ION-ZC1 treated tumor mice, significant small bowel wall thickening was observed at necropsy. Therefore, one intestine section was submitted to histopathology analysis as a representative specimen. The microscopic picture is shown in **Figure H3** with H/E staining, which confirms metastasis of melanoma cells into the intestine.
As supported by human patient data, malignant melanoma is one of the most common malignancies to metastasize to the gastrointestinal (GI) tract. Metastases to the GI tract can present at the time of primary diagnosis or decades later as the first sign of recurrence. Symptoms may include abdominal pain, dysphagia, small bowel obstruction, hematemesis, and melena. (*Kelly V Liang et al. (2006) Metastatic malignant melanoma of the gastrointestinal tract. Mayo Clinic Proceedings 81(4):511-516*). (Further ref: *Laura Lamps et al. (2016) Neoplastic Gastrointestinal Pathology: An Illustrated Guide. Demos Medical Publishing, LLC., Metastasis to the small bowel. Page 244-245).* At visual observation of the thickening of the small intestine, the phenomenon was found to be more pronounced in control tumor animals than in ION-ZC1 treated animals (See **Figure H3 B**, and **Appendix H3**). This may be consistent with an anti-metastatic effect of ION-ZC1, but has to be confirmed statistically in further studies.

12.5.2 Tumor histopathology

Histopathological analysis of the H/E stained tumor slides (K1, 2x, 4x1, 8x, 16x1) was focused on the detection of necrosis as discolored cells (pinkish) with deformed nuclei, and to investigate angiogenesis within the tumors via counting blood vessels in the tumor sections of the control and treated mice. An attempt was also made to discover tumor infiltrating lymphocytes (inflammatory cell infiltration) although specific immunohistochemical staining will be required to investigate this phenomenon.

>Identification of elements in tumor sections

Tumor sections for 6 specimens (including control tumor and ION-ZC1 treated tumors) were analysed and live B16 melanoma cells, necrotic melanoma cells, and blood vessels were identified (see **Figure H4** and **Figure H5**). In addition, extraorgan metastatic melanoma cells were detected in the blood vessels of the tumors

>Angiogenesis in the tumors

Blood vessel count in tumor sections was compared between un-treated and ION-ZC1 treated tumor mice (**Figure H6**). Blood vessels were identified by the presence of RBC (red blood cells), and the presence of blood vessel wall. In Figure 5, blood vessels are marked with red circles, and blood vessel counts are graphed and tabled versus ION-ZC1 dose and control (0). The results show some negative correlation of blood vessel density (angiogenesis) with ION-ZC1 dose, which may reflect to the inhibitory effect of the compound on tumor angiogenesis. These results will have to be confirmed statistically later.

>Necrosis in the tumors

It seemed obvious by visual (light-microscopic) inspection at various magnifications that the necrotic regions (showing up as pinkish cells compared to deep bluish live melanoma cells) in the tumors were more extended in the control tumor than in the ION-ZC1 treated tumors (**Figure H4**). To estimate necrosis rate in the tumors, the Icy image analysis software was used. Spot detection by the software could differentiate between necrotic and live cells due to the difference in the cell bodies' color intensities (pale color of necrotic cells allowed detection of their nuclei). The image analysis is demonstrated and the results semi-quantitative necrosis rate estimation is shown in tumor slides with circled necrotic nuclei, and a graph and table presenting the number of the

necrotic nuclei versus ION-ZC1 doses compared to that of the un-treated control tumor **Figure H7**. From this preliminary investigation, we can conclude that ION-ZC1 has significant necrotic effect on tumor cells when administered intravenously (this finding will have to be confirmed statistically).

13. CONCLUSIONS

Anti-tumor efficacy study of ION-ZC1 was carried out in various doses of single and multiple intravenous injections (IV) on a syngeneic mouse xenograft model, namely subcutaneous B16 mouse melanoma in C57BL/6J mice (Charles River) with intact, more specifically, strong immune system. Injection of ION-ZC1 at a dose of 60-70 mg/kg body weight (0.52 % ION-ZC1, 0.2 ml IV), tumor shrinkage of more than 50% was observed even after 3 days of application, followed by much lower tumor growth rate than in the control or none. Single injection at the dose of 90-100 mg/kg body weight (0.78 % ION-ZC1, 0.2 ml IV) also resulted in plateaued tumor growth or tumor some shrinkage, and a slower tumor growth compared to controls.

As one of the main outcomes of this efficacy study, intravenous injection of ION-ZC1 in various formulations on tumor growth rate (estimated via tumor size measurement and volume calculations against observation time) was found effective as an anti-tumor agent in the dose range of 40-350 mg/kg body weight (0.39 – 3.13 % ION-ZC1, 0.2 ml IV). Most effective formulations and treatment protocol were found to be when ION-ZC1 was diluted in physiological salt solution and the pH was adjusted to above 5 or 5.4, and the injections were applied 1-3 times over the 14-day treatment period.

In this study, histopathological analysis of tumor sections, we can state that tumors isolated from mice treated with various concentrations of ION-ZC1 IV injections (i) show massive necrosis, which is not pronounced as much in tumors isolated from control tumor mice; and (ii) blood vessels are much less frequent, less developed in the ION-ZC1 treated mice compared to control tumor mice. Furthermore (iii) ION-ZC1 injection results in spleen enlargement, which is remarkably (2-3 times) bigger in extent than that of the spleen of control (untreated) tumor mice, and consistent with ION-ZC1 inducing a strong anti-tumor immune response.

REMARK

During the course of Phase 1 and 2, we managed

A) to optimise the tumor model assay

- less cell are injected, 5 x 10^5 cells per mice
- earlier we start the injections, 8-10 mm tumor size
- used multiple injections, every 3 days

B) to optimise the IV injectable

- pH adjusted to 5.4
- osmolarity is ISOOSMOTIC - calculated (9g/L sodium chloride+0.1M bicarbonate buffer: 300 mOsmol/L all)
- volume is now 50-100 microlitre
- ION-ZC1 cc should be less than 0.78% to be safe

>down to ION-ZC1 cc 0.39% all are very effective

IN VIVO FIGURES AND TABLES

Table 1. Phase 1 efficacy study technical summary

Phase 1 Eff tech summary				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	DAYS
Mouse No.	Cage code	ION-ZC1 IV injection code	ION-ZC1 IV injection description																																
1	I. ketrec	K	Control (phys salt)	B16													IV																		Sacrifised
2	II.ketrec	K	Control (phys salt)	B16													IV																		Sacrifised
3	III.ketrec	K	Control (phys salt)	B16													IV																		Sacrifised
4	II/2	2x	6.25% pH 2	B16													IV		died - instantly - coagulation																
5	IV/1	2x	6.25% pH 2	B16													IV		died - instantly - coagulation																
6	IV/2	2x	6.25% pH 2	B16													IV		died - instantly - coagulation																
7	II/1	4x	3.125% pH 2	B16														IV																		Sacrifised
8	III/1	4x	3.125% pH 2	B16														IV																		Sacrifised
9	IV.ketrec	4x	3.125% pH 2	B16														IV																		Sacrifised
10	II/3	8x	1.5625% pH 2	B16														IV																		Sacrifised
11	IV/3	8x	1.5625% pH 2	B16														IV	died - instantly - coagulation																
12	V.ketrec	8x	1.5625% pH 2	B16														IV	died - instantly - coagulation																
13	IV/4	2x	6.25%	B16																				IV	died - instantly - coagulation										
14	V/1	16xpH	0.78125% pH 5	B16																				IV												Sacrifised
15	V/2	16xpH	0.78125% pH 5	B16																				IV												Sacrifised
16	V/3	16xpH	0.78125% pH 5	B16																				IV												Sacrifised
17	V/4	0	none, no tumor	B16																																Not used
18	V/5	0	none, no tumor	B16																																Not used

Codes:

B16 melanoma cell injection subcutan	Xenograft model growth	IV injection of control (phys salt)	IV injection of ION-ZC1 (var cc.)	Monitoring of mice

Table 2. Phase 2 efficacy study technical summary

Phase 2 Eff tech summary

Mouse No.	Cage code	ION-ZC1 IV injection code	ION-ZC1 IV injection description (norm to 0.2 ml)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	DAYS
1	I. ketrec	K	Control (phys salt)	B16												IV				IV			IV		IV		IV		IV		Died				
2	I. ketrec	2x pH	6.25% pH 5.4	B16												IV		died - same day																	
3	I. ketrec	4x pH	3.125% pH 5.4	B16												IV		died - same day																	
4	II. ketrec	K	Control (phys salt)	B16												IV				IV			IV		IV		IV		IV		Died				
5	II. ketrec	4x pH	3.125% pH 5.4	B16												IV		died - same day																	
6	III. ketrec	16xpH	0.39% pH 5.4 dil	B16									IV			IV		IV									Sacrifised								
7	III. ketrec	16xpH	0.39% pH 5.4 dil	B16									IV			IV		IV						Died											
8	III. ketrec	8xpH	0.52% pH 5.4 dil	B16									IV		IV			IV						Died											
9	IV. ketrec	8xpH	0.52% pH 5.4 dil	B16															IV		IV		IV			Died									

Codes:

B16 melanoma cell injection subcutan	Xenograft model growth	IV injection of control (phys salt)	IV injection of ION-ZC1 (var cc.)	Monitoring of mice

Table 3. Phase 3 efficacy study technical summary

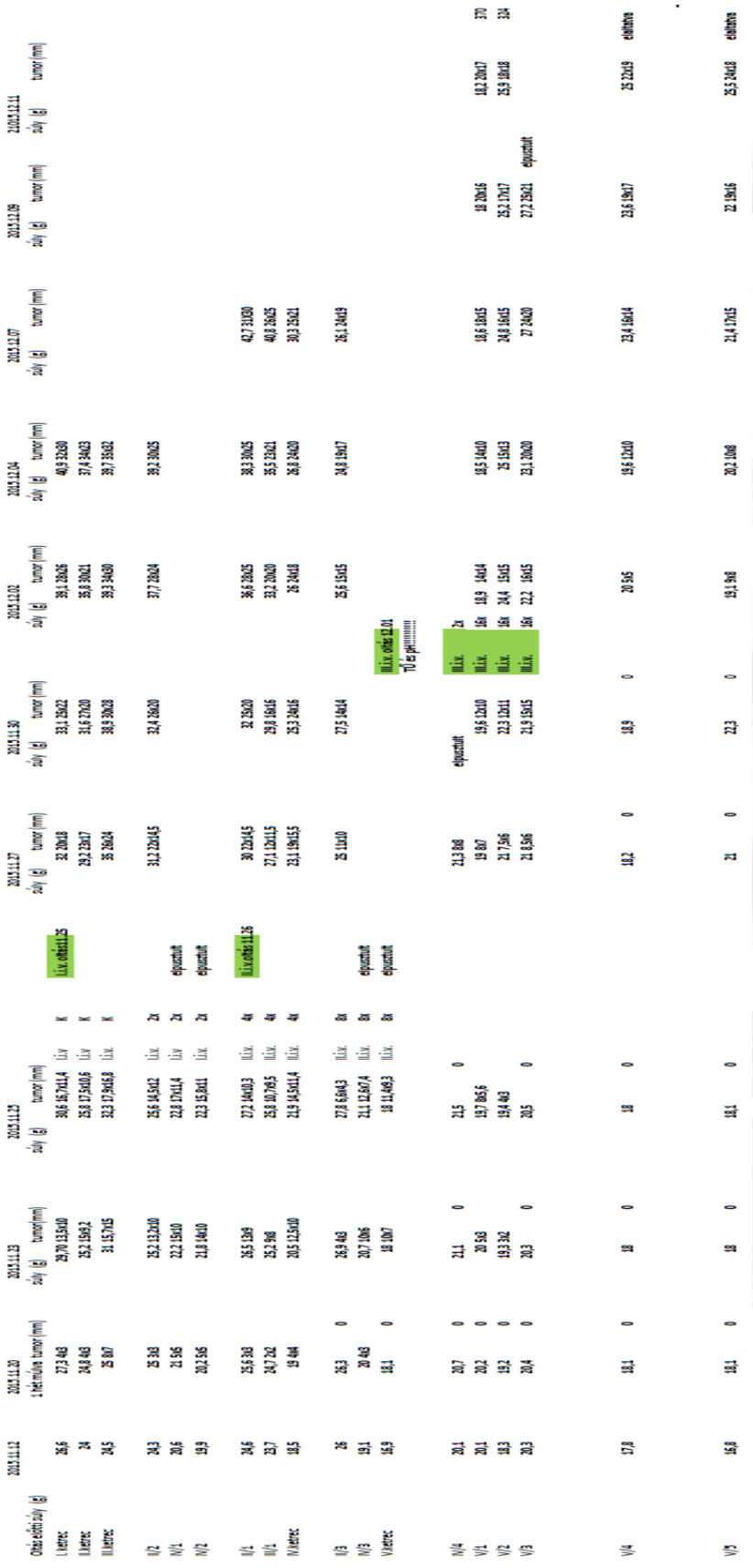
Phase 3 Eff tech summary

Mouse No.	Cage code	ION-ZC1 IV injection code	ION-ZC1 IV injection description (norm to 0.2 ml)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	DAYS
1	III. ketrec	8xpH5.4 100 µl	0.78% pH 5.4 dil	B16														IV				IV			IV					died					
2	III. ketrec	8xpH5.4 60 µl	0.52% pH 5.4 dil	B16														IV				IV			IV					sacrifised					
3	IV. ketrec	16xpH5.4 100 µl	0.39% pH 5.4 dil	B16														IV				IV			IV					sacrifised					

Codes:

B16 melanoma cell injection subcutan	Xenograft model growth	IV injection of control (phys salt)	IV injection of ION-ZC1 (var cc.)	Monitoring of mice

PHASE 1 DATA **Table 4.**



	DATE		B16 melanoma 2015.11.12 injection			2015.11.20				2015.11.23				BAM IV injection 2015.11.25				2015.11.26 estimate				2015.11.27			
	DAY																								
CODE	BAM IV		BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume
I. ketrec	K		26,6				27,3	4	3	18,72	29,70	13,5	10	702	30,6	16,7	11,4	1128,57264					32	20	18	3369,6
II.ketrec	K		24				24,8	4	3	18,72	25,2	15	9,2	660,192	25,8	17,5	10,6	1022,476					29,2	23	17	3456,44
III.ketrec	K		24,5				25	8	7	203,84	31	15,7	15	1836,9	32,3	17,9	16,8	2627,08992					35	26	24	7787,52
II/2	2x		24,3				25	3	3	14,04	25,2	13,2	10	686,4	25,6	14,5	12	1085,76					31,2	22	14,5	2405,26
IV/1	2x		20,6				21	5	5	65	22,2	15	10	780	22,8	17	11,4	1148,8464	died							
IV/2	2x		19,9				20,2	5	5	65	21,8	14	10	728	22,3	15,8	11	994,136	died							
II/1	4x		24,6				25,6	3	3	14,04	26,5	13	9	547,56	27,2	14	10,3	772,3352		15	11	943,8	30	22	14,5	2405,26
III/1	4x		23,7				24,7	2	2	4,16	25,2	9	8	299,52	25,8	10,7	9,5	502,151		12	10,5	687,96	27,1	12	11,5	825,24
IV.ketrec	4x		18,5				19	4	4	33,28	20,5	12,5	10	650	21,9	14,5	11,4	979,8984		15,5	12	1160,64	23,1	19	15,5	2373,67
II/3	8x		26				26,3	0	0	0	26,9	4	3	18,72	27,8	6,6	4,3	63,45768		7,9	5,4	119,78928	25	11	10	572
IV/3	8x		19,1				20	4	3	18,72	20,7	10	6	187,2	21,1	12,6	7,4	358,78752	died							
V.ketrec	8x		16,9				18,1	0	0	0	18	10	7	254,8	18	11,4	9,3	512,71272	died							
V/4	2x		20,1				20,7	0	0	0	21,1	0	0	0	21,5	0	0	0					21,3	8	8	266,24
V/1	16x		20,1				20,2	0	0	0	20	5	3	23,4	19,7	8	5,6	130,4576					19	8	7	203,84
V/2	16x		18,3				19,2	0	0	0	19,3	3	2	6,24	19,4	4	3	18,72					21	7,5	6	140,4
V/3	16x		20,3				20,4	0	0	0	20,3	0	0	0	20,5	0	0	0					21	8,5	6	159,12
V/4		0					18,1	0	0	0	18	0	0	0	18	0	0	0					18,2	0	0	0
V/5		0	17,8				18,1	0	0	0	18	0	0	0	18,1	0	0	0					21	0	0	0
		0	16,8																							

		BAM IV pH injection	2015.12.01				2015.12.02				2015.12.04				2015.12.07				2015.12.09				2015.12.11			
	2015.11.30																									
	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume	BW	TS Length	TS Width	T volume
	33,1	25	22	6292					39,1	28	26	9842,56	40,9	32	30	14976												
	31,6	27	20	5616					35,8	30	21	6879,6	37,4	34	23	9352,72												
	38,9	30	28	12230,4					39,3	34	30	15912	39,7	35	32	18636,8												
	32,4	26	20	5408					37,7	28	24	8386,56	39,2	30	25	9750												
	32	25	20	5200					36,6	28	25	9100	38,3	30	25	9750	42,7	31	30	14508								
	29,8	16	16	2139,92					33,2	20	20	4160	35,5	23	21	5274,36	40,8	26	25	8450								
	25,3	24	16	3194,88					26	24	18	4043,52	26,8	24	20	4992	30,3	25	21	5733								
	27,5	14	14	1426,88					25,6	15	15	1755	24,8	19	17	2855,32	26,1	24	19	4505,28								
died					2x IL IV																							
	19,6	12	10	624	16x pH				18,9	14	14	1426,88	18,5	14	10	728	18,6	18	15	2106	18	20	16	2662,4	18,2	20	17	3005,6
	22,3	12	11	755,04	16x pH				24,4	15	15	1755	25	15	13	1318,2	24,8	16	15	1872	25,2	17	17	2554,76	25,9	18	18	3032,64
	21,9	15	15	1755	16x pH				22,2	16	15	1872	23,1	20	20	4160	27	24	20	4992	27,2	25	21	5733				
	18,9	0	0	0					20	5	5	65	19,6	12	10	624	23,4	16	14	1630,72	23,6	19	17	2855,32	25	22	19	4129,84
	22,3	0	0	0					19,1	9	8	299,52	20,2	10	8	332,8	21,4	17	15	1989	22	19	16	2529,28	25,5	24	18	4043,52

Tv	B16													BAM IV																	
DATES	2015.11.12								2015.11.20			2015.12.23		2015.11.25	2015.11.26	2015.11.27			2015.11.30	2015.12.01	2015.12.02		2015.12.04			2015.12.07		2015.12.09		2015.12.11
Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Day relat	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
K	0								18,72			702		1128,573	3369,6				6292		9842,56		14976							
K	0								18,72			660,192		1022,476	3456,44				5616		6879,6		9352,72							
K	0								203,84			1836,9		2627,09	7787,52				12230,4		15912		18636,8							
K relative									0,01658732			0,6220246		1	2,985718				5,5751839		8,7212463		13,2699							
K relative									0,0183085			0,6456797		1	3,380461				5,4925495		6,7283731		9,14713							
K relative									0,07759156			0,6992147		1	2,964314				4,6554935		6,056892		7,09409							
K Rel Ave									0,03749579			0,6556397		1	3,110164				5,2410756		7,1688372		9,83702							
SD									0,03473461			0,0395472		0	0,234328				0,5088093		1,3857137		3,14516							

BAM IV estimate

DATE						2015.12.23		2015.11.25	2015.11.26	2015.11.27			2015.11.30	2015.12.01	2015.12.02			2015.12.04			2015.12.07		2015.12.09		2015.12.11					
Day relat	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16x pH						23,4		130,4576		203,84			624	800	1426,88		728			2106		2662,4		3005,6							
16x pH						6,24		18,72		140,4			755,04	950	1755		1318,2			1872		2554,76		3032,64							
16x pH						0		0		159,12			1755	2000	1872		4160			4992		5733								
16x pH rel						0,0375		0,20906667		0,326667			1		2,286667		1,166667			3,375		4,266667		4,816667							
16x pH rel						0,008264		0,02479339		0,18595			1		2,32438		1,745868			2,479339		3,383609		4,016529							
16x pH rel						0		0		0,090667			1		1,066667		2,37037			2,844444		3,266667									
16x pH rel ave						0,015255		0,07795335		0,201095			1		1,892571		1,760968			2,899594		3,638981		4,416598							
SD						0,019703		0,11422217		0,118727			0,59217615		0,715503		0,601994			0,45037		0,546728		0,565783							

DATE	2015.11.12								2015.11.20			2015.11.23		2015.11.25		2015.11.27			2015.11.30		2015.12.02		2015.12.04			2015.12.07				
Day relat	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
2x									14,04			686,4		1085,76		2405,26			5408		8386,56		9750							
2x relative																														
2x rel ave																														
SD																														

DATE	2015.11.12								2015.11.20			2015.11.23		2015.11.25		2015.11.27			2015.11.30		2015.12.02		2015.12.04			2015.12.07				
Day relat	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
4x									14,04			547,56		772,34		2405,26			5200		9100		9750			14508				
4x									4,16			299,52		502,15		825,24			2129,92		4160		5274,36			8450				
4x									33,28			650		979,9		2373,67			3194,88		4043,52		4992			5733				
4x rel																														
4x rel																														
4x rel																														
4x rel ave																														
SD																														

DATE	2015.11.12								2015.11.20			2015.11.23		2015.11.25		2015.11.27			2015.11.30		2015.12.02		2015.12.04			2015.12.07				
Day relat	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
8x												18,72		63,46		572			1426,88		1755		2885,32			4505				
8x rel																														
8x rel ave																														
SD																														

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Figure 1. Mouse body weight over the study period. Black arrows mark the days of first IV injection of mice (either with physiological salt/bicarbonate or with ION-ZC1 solutions).

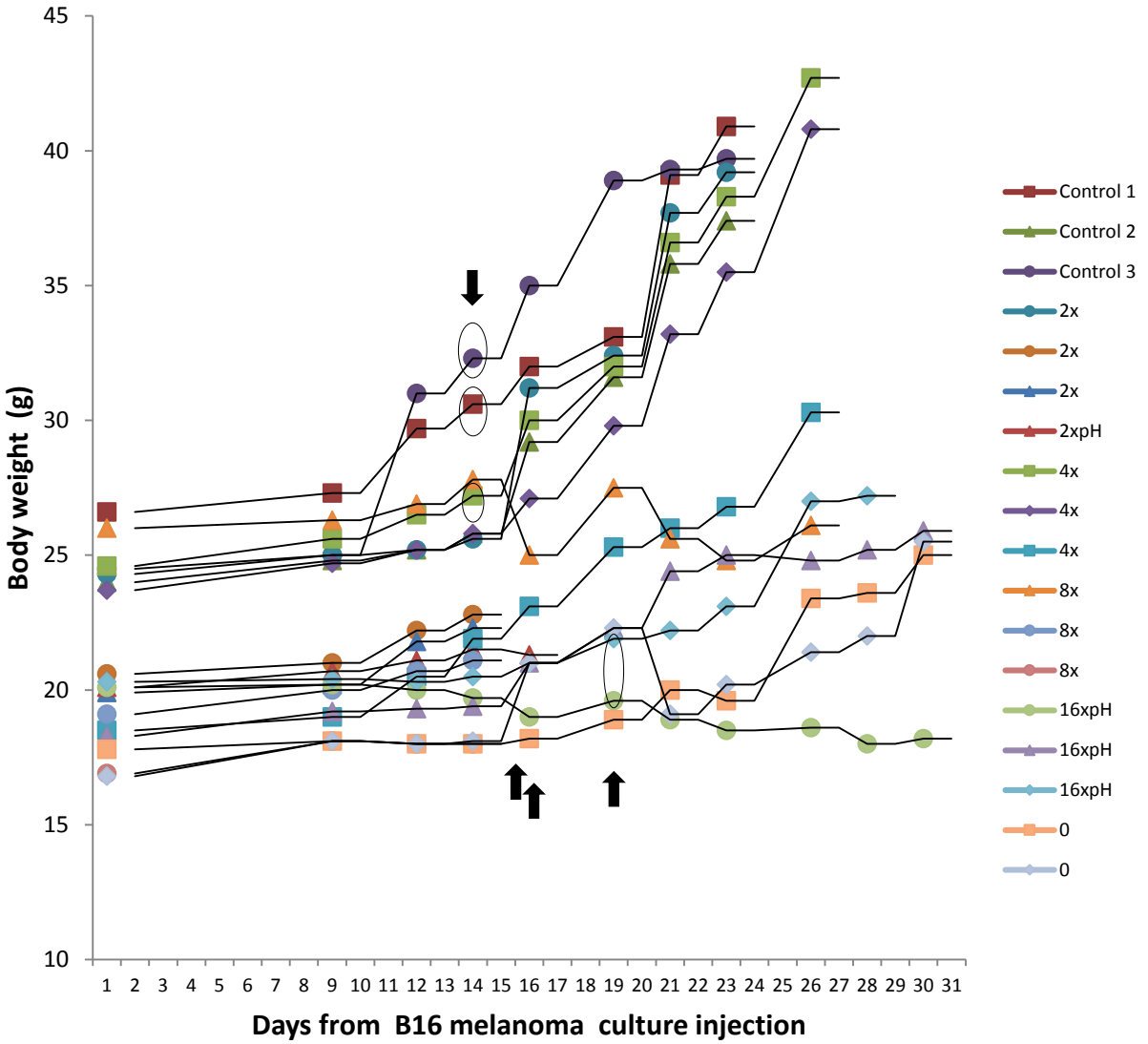

Table 5. Mouse body weights over the study period. Blue and red backrounds mark the days of IV injections of mice either with physiological salt/bicarbonate or with ION-ZC1 dilutions, respectively. Lack of data marks mice death.

DATE	2015.11.12							2015.11.20			2015.11.23		2015.11.25		2015.11.27			2015.11.30		2015.12.02		2015.12.04		2015.12.07		2015.12.09		2015.12.11	
Animals	Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
I. ketrec	Control 1	26,6								27,3			29,70		30,6		32			33,1		39,1		40,9							
II.ketrec	Control 2	24								24,8			25,2		25,8		29,2			31,6		35,8		37,4							
III. ketrec	Control 3	24,5								25			31		32,3		35			38,9		39,3		39,7							
II/2	2x	24,3								25			25,2		25,6		31,2			32,4		37,7		39,2							
IV/1	2x	20,6								21			22,2		22,8																
IV/2	2x	19,9								20,2			21,8		22,3																
IV/4	2xpH	20,1								20,7			21,1		21,5		21,3														
II/1	4x	24,6								25,6			26,5		27,2		30			32		36,6		38,3			42,7				
III/1	4x	23,7								24,7			25,2		25,8		27,1			29,8		33,2		35,5			40,8				
IV. ketrec	4x	18,5								19			20,5		21,9		23,1			25,3		26		26,8			30,3				
II/3	8x	26								26,3			26,9		27,8		25			27,5		25,6		24,8			26,1				
IV/3	8x	19,1								20			20,7		21,1																
V. ketrec	8x	16,9								18,1			18		18																
V/1	16xpH	20,1								20,2			20		19,7		19			19,6		18,9		18,5			18,6		18		18,2	
V/2	16xpH	18,3								19,2			19,3		19,4		21			22,3		24,4		25			24,8		25,2		25,9	
V/3	16xpH	20,3								20,4			20,3		20,5		21			21,9		22,2		23,1			27		27,2		
V/4	0	17,8								18,1			18		18		18,2			18,9		20		19,6			23,4		23,6		25
V/5	0	16,8								18,1			18		18,1		21			22,3		19,1		20,2			21,4		22		25,5

Figure 2. ION-ZC1 IV antitumor efficacy, 0.78 %, single injection, tumor size W x L (mm^2)
Average tumor sizes relative to the day of the first injection are graphed from the values measured in case of 3 mice.

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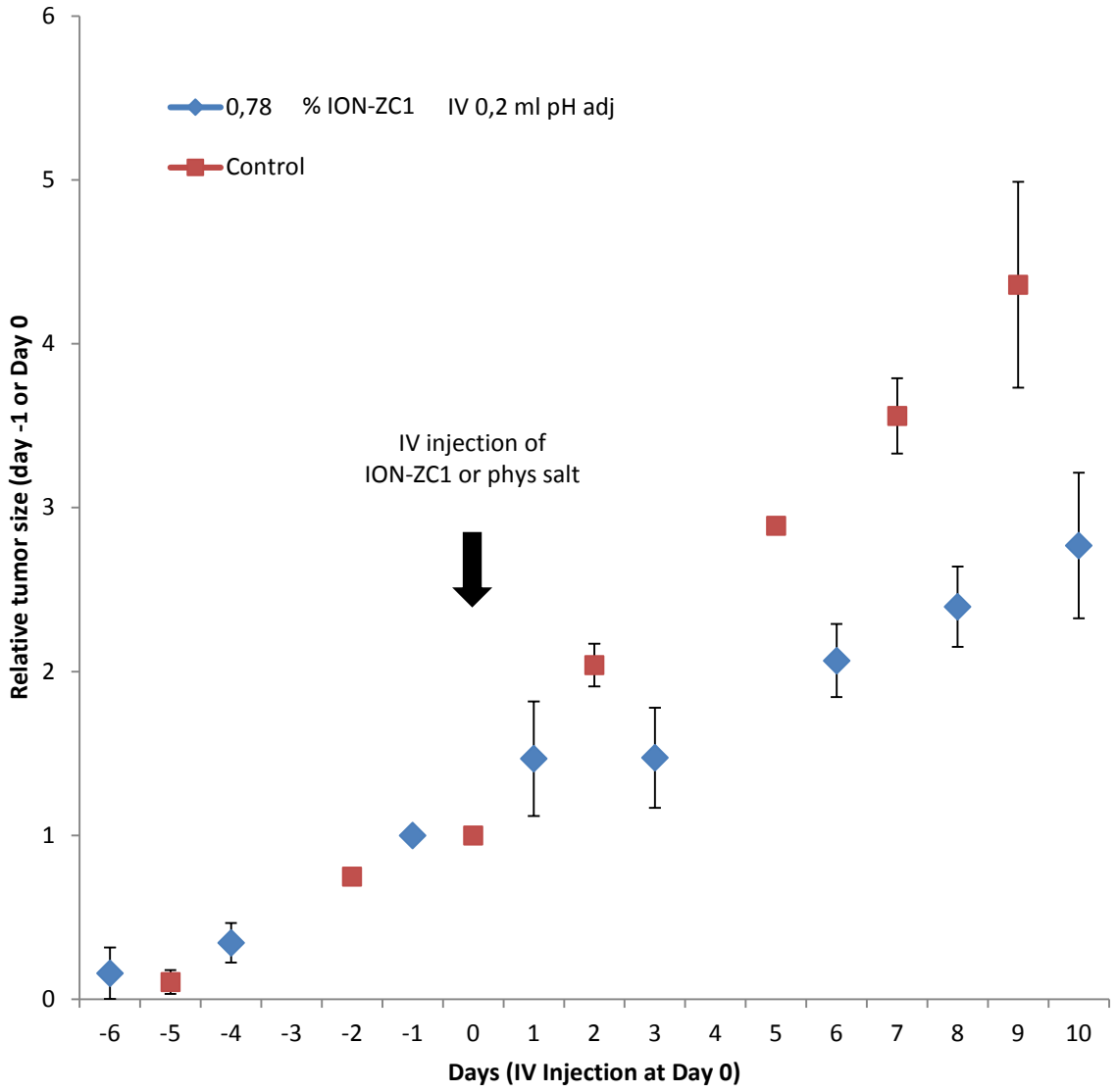


Figure 3.

ION-ZC1 IV antitumor efficacy, 0.78 %, single injection, tumor volume 0.52 x (W x L^2) (mm^3). Average tumor volumes relative to the day of the first injection are graphed from the values measured in case of 3 mice.

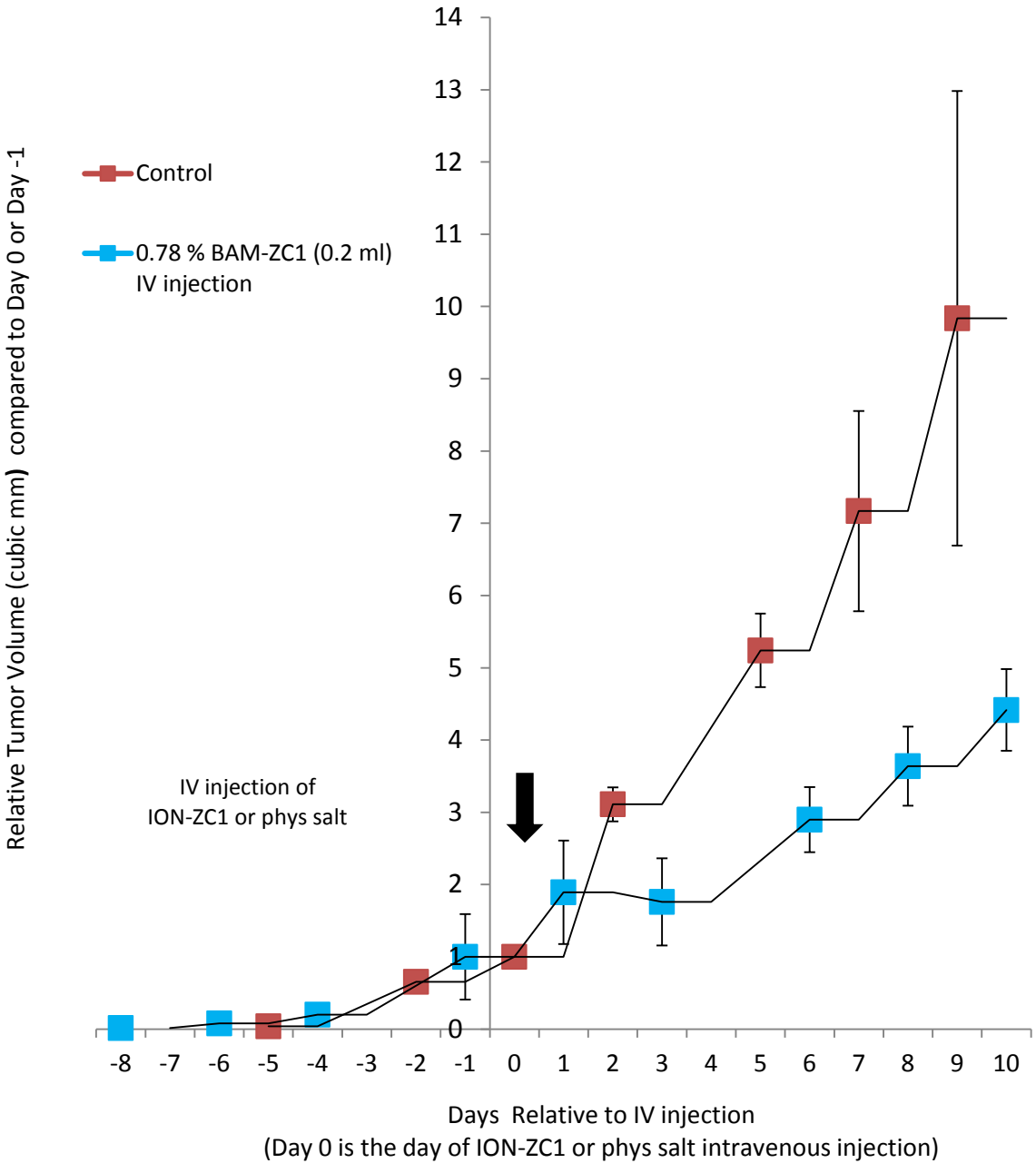


Figure 4.

ION-ZC1 IV antitumor efficacy, 0.78 %, single injection, tumor volume 0.52 x (W x L^2) (mm^3)

Absolute tumor volume over time (days relative to IV injections). Controls and 0.78% ION-ZC1 (0.2 ml) IV treated mice. Individual mouse data is graphed.

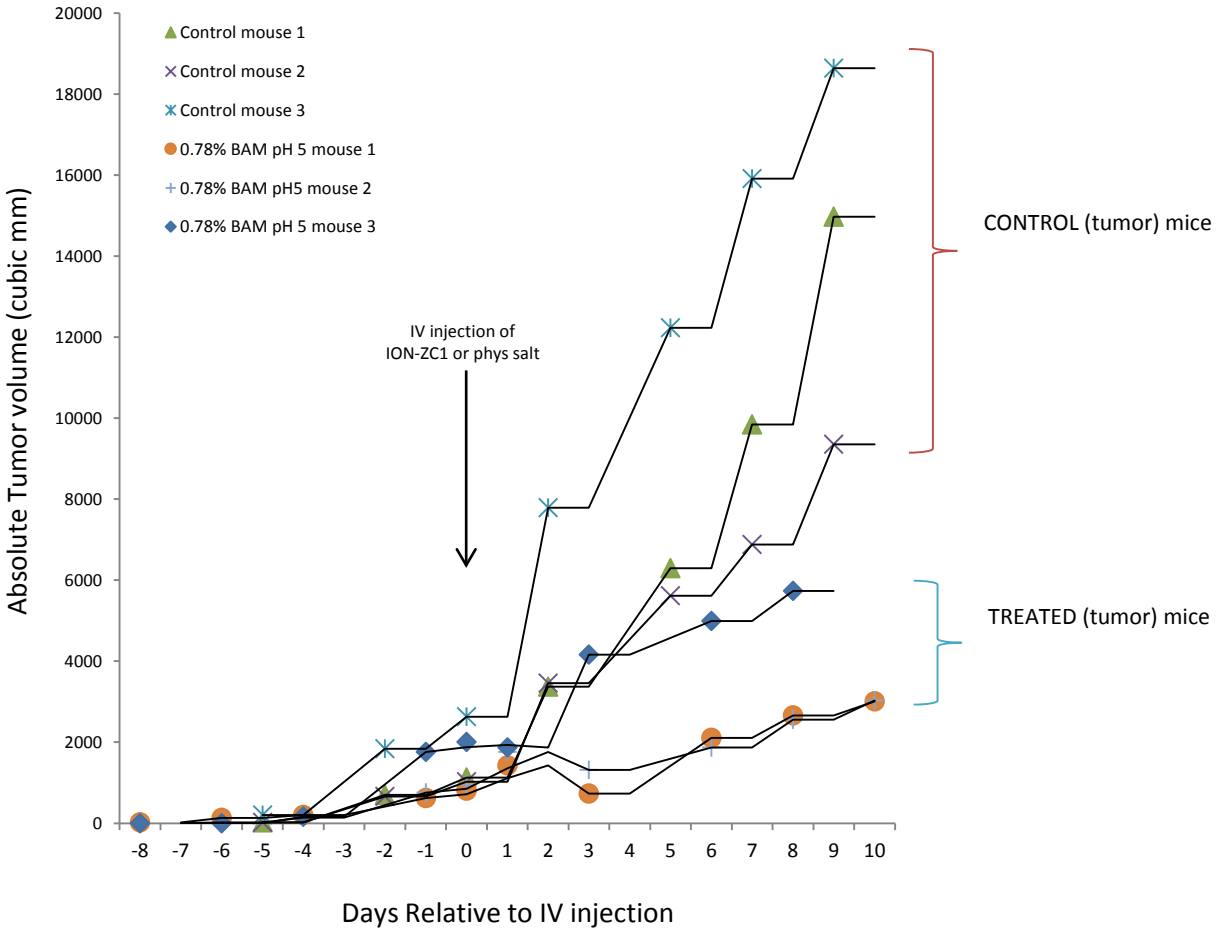


Figure 5.

ION-ZC1 IV antitumor efficacy, 1.5 %, single injection, tumor size W x L (mm^2)

Average tumor volumes relative to the day of the first injection are graphed.

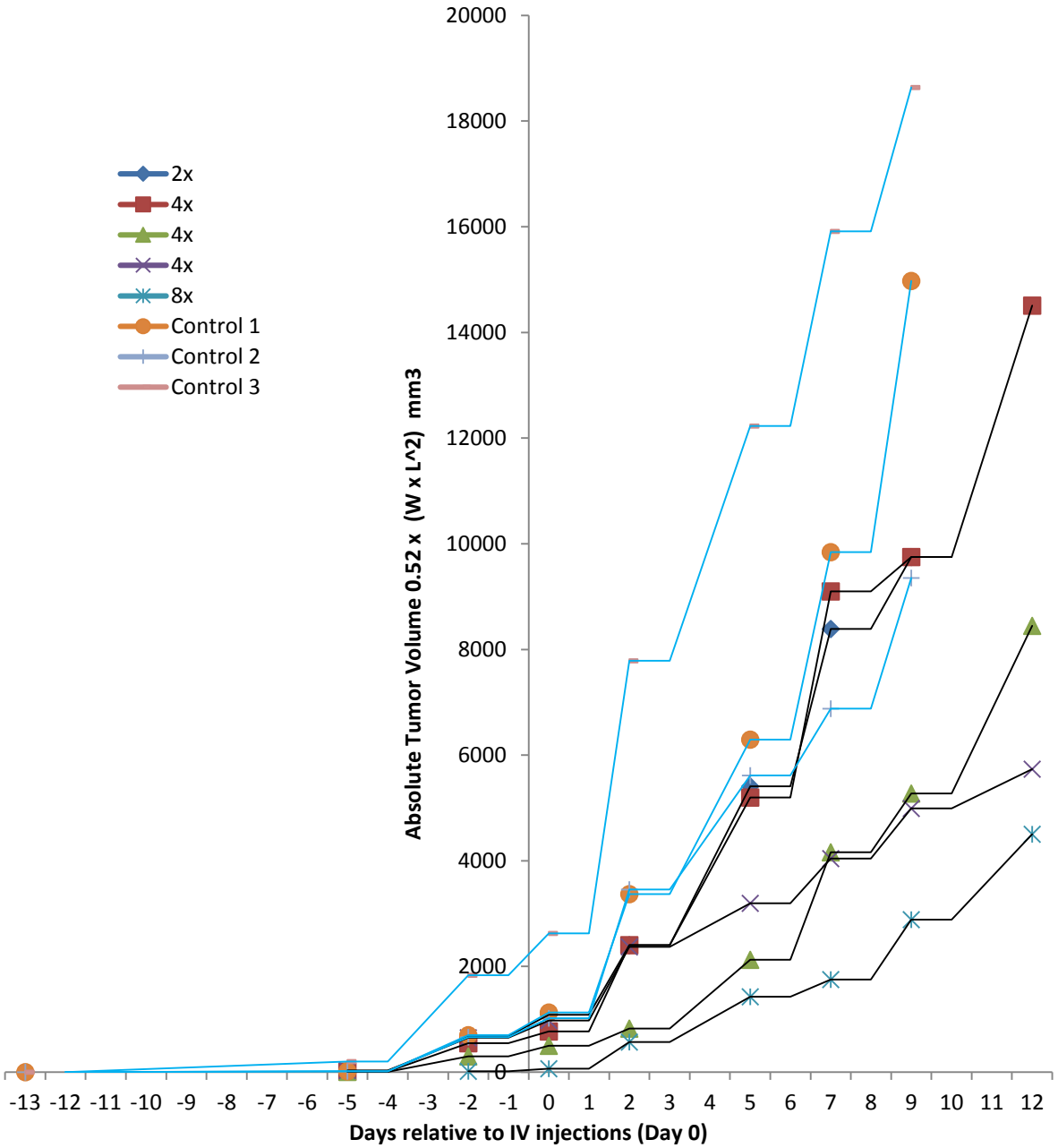


TABLE 6. Dose conversion from % to mg/kg body weight (Phase 1-3)

Mouse ID injection	ION-ZC1 (%) (IV: 0.2 ml/mice)	Body Weight before treatment (g)	Treatment dose mg/kg body weight	Gender of the mice
Phase 1				
Control 1	0	30.6	0.0	male
Control 2	0	25.8	0.0	male
Control 3	0	32.3	0.0	male
2x	6.25	25.6	576.7	male
2x	6.25	22.8	647.5	male
2x	6.25	22.3	662.0	male
2xpH	6.25	21.3	693.1	male
4x	3.13	27.2	271.8	male
4x	3.13	25.8	286.6	-
4x	3.13	21.9	337.6	female
8x	1.56	27.8	132.5	female
8x	1.56	21.1	174.6	-
8x	1.56	18	204.7	-
16xpH	0.78	19.6	94.0	female
16xpH	0.78	22.3	82.6	female
16xpH	0.78	21.9	84.1	-
0	0	18.9	0.0	-
0	0	22.3	0.0	-
Phase 2				
Control 1	0	30.6	0.0	male
Control 2	0	25.8	0.0	male
16xpH5.4	0.78	32.3	57.0	male
16xpH5.4	0.78	25.6	72.0	male
24xpH5.4	0.47	22.8	48.7	male
24xpH5.4	0.47	22.3	49.8	male
32xpH5.4	0.36	21.3	39.9	male
32xpH5.4	0.36	27.2	31.25	male
Phase 2				
16xpH5.4	0.78	30.0	61.4	male
24xpH5.4	0.47	24.0	46.2	male
32xpH5.4	0.36	27.5	30.9	male

ION-ZC1: Specific Gravity: 1.181
Intravenous injection dose volume: 0.06-0.2 ml/mouse mg/kg
Body weight calculation: (1.181 x 0.2 x1000 x ION-ZC1 % /100) / (mouse body weight in g) x 1000

EX VIVO DATA

TABLE 7. Organ weights after euthanasia and necropsy

Mouse ID injection	ION-ZC1 (%) (IV: 0.2 ml)	Treatment dose (mg/kg bw final)	HEART (g)	LUNG (g)	SPLEEN (g)	LIVER (g)	KIDNEY (g)	BRAIN (g)
Control 1	0	**0,0**	0,14	0,17	0,25	1,63	0,36	0,3
Control 2	0	**0,0**	0,16	0,19	0,27	1,79	0,4	0,32
Control 3	0	**0,0**	0,21	0,16	0,24	1,99	0,36	0,38
2x	6.25	**576,7**	0,19	0,18	0,27	1,7	0,29	0,4
2x	6.25	**647,5**	-	-	-	-	-	-
2x	6.25	**662,0**	-	-	-	-	-	-
2xpH	6.25	**693,1**	-	-	-	-	-	-
4x	3.13	**271,8**	0,16	0,16	0,39	2,19	0,36	0,5
4x	3.13	**286,6**	0,17	0,22	0,42	2,3	0,45	0,5
4x	3.13	**337,6**	0,18	0,14	0,4	1,35	0,32	0,38
8x	1.56	**132,5**	0,18	0,18	0,47	1,41	0,52	0,45
8x	1.56	**174,6**	-	-	-	-	-	-
8x	1.56	**204,7**	-	-	-	-	-	-
16xpH	0.78	**94,0**	0,15	0,29	0,29	1,68	0,29	0,5
16xpH	0.78	**82,6**	0,14	0,24	0,16	2	0,3	0,51
16xpH	0.78	**84,1**	-	-	-	-	-	-
0	0	**0,0**	-	-	-	-	-	-
0	0	**0,0**	-	-	-	-	-	-

...

EX VIVO DATA

TABLE 8. Organ weight/body weight coefficients

Mouse No.	ION-ZC1 (%) (IV: 0.2 ml)	Treatment dose (mg/kg bw final)	HEART	LUNG	SPLEEN	LIVER	KIDNEY	BRAIN	Body Weight before euthan. (g)
Control 1	0	**0,0**	0,003423	0,004156	0,006112	0,039853	0,008802	0,007335	40,9
Control 2	0	**0,0**	0,004278	0,00508	0,007219	0,047861	0,010695	0,008556	37,4
Control 3	0	**0,0**	0,00529	0,00403	0,006045	0,050126	0,009068	0,009572	39,7
2x	6.25	**576,7**	0,004847	0,004592	0,006888	0,043367	0,007398	0,010204	39,2
2x	6.25	**647,5**							22,8
2x	6.25	**662,0**							22,3
2xpH	6.25	**693,1**							21,3
4x	3.13	**271,8**	0,003747	0,003747	0,009133	0,051288	0,008431	0,01171	42,7
4x	3.13	**286,6**	0,004167	0,005392	0,010294	0,056373	0,011029	0,012255	40,8
4x	3.13	**337,6**	0,005941	0,00462	0,013201	0,044554	0,010561	0,012541	30,3
8x	1.56	**132,5**	0,006897	0,006897	0,018008	0,054023	0,019923	0,017241	26,1
8x	1.56	**174,6**							21,1
8x	1.56	**204,7**							18
16xpH	0.78	**94,0**	0,008242	0,015934	0,015934	0,092308	0,015934	0,027473	18,2
16xpH	0.78	**82,6**	0,005405	0,009266	0,006178	0,07722	0,011583	0,019691	25,9
16xpH	0.78	**84,1**							27,2
0	0	**0,0**							25
0	0	**0,0**							25,5

FIGURE 6.
Organ weights (wet weight, g) of all animals versus body weight (OW/BW, %) after euthanasia and necropsy.

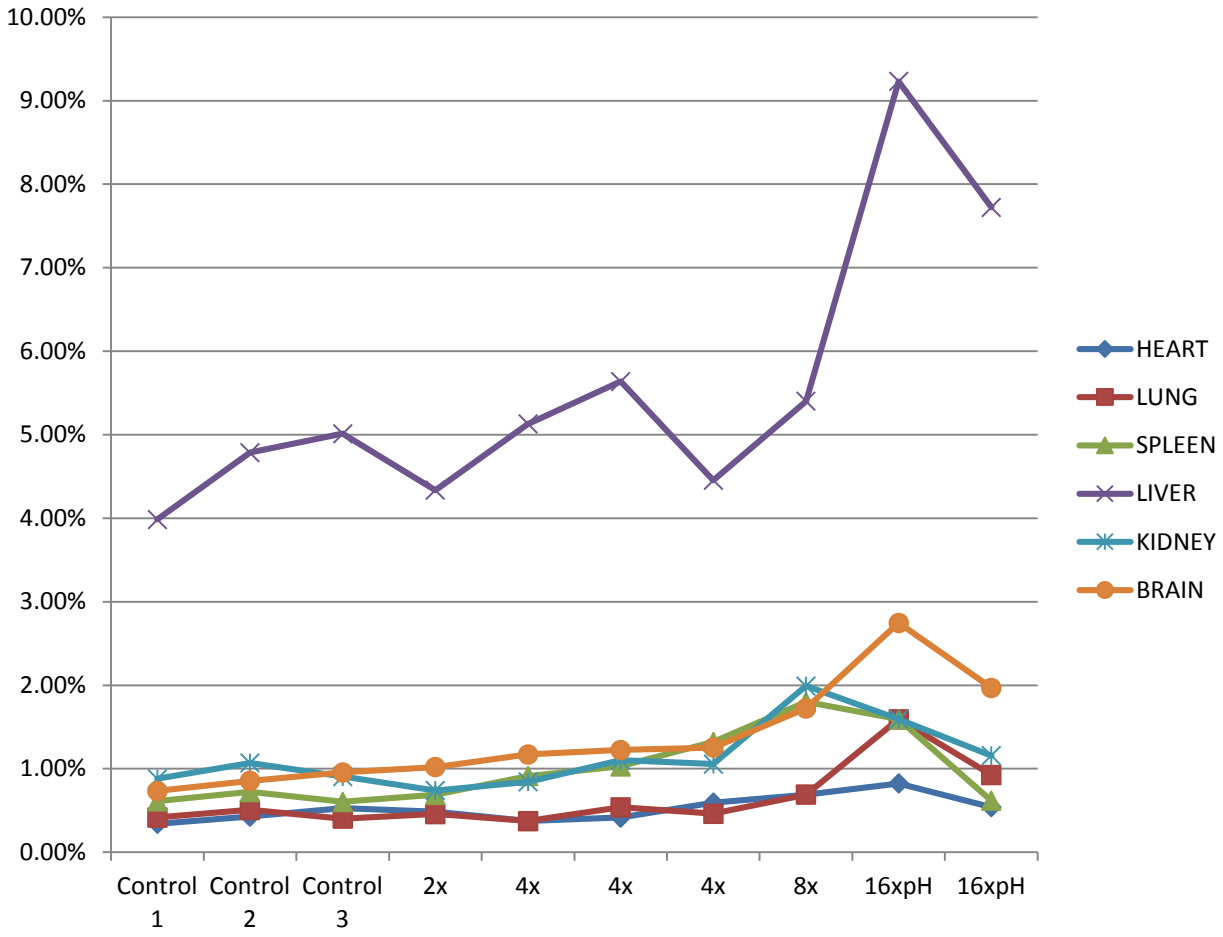


FIGURE 7.
Organ weights (wet weight, g) of all animals versus body weight (OW/BW, %) after euthanasia and necropsy by organ type

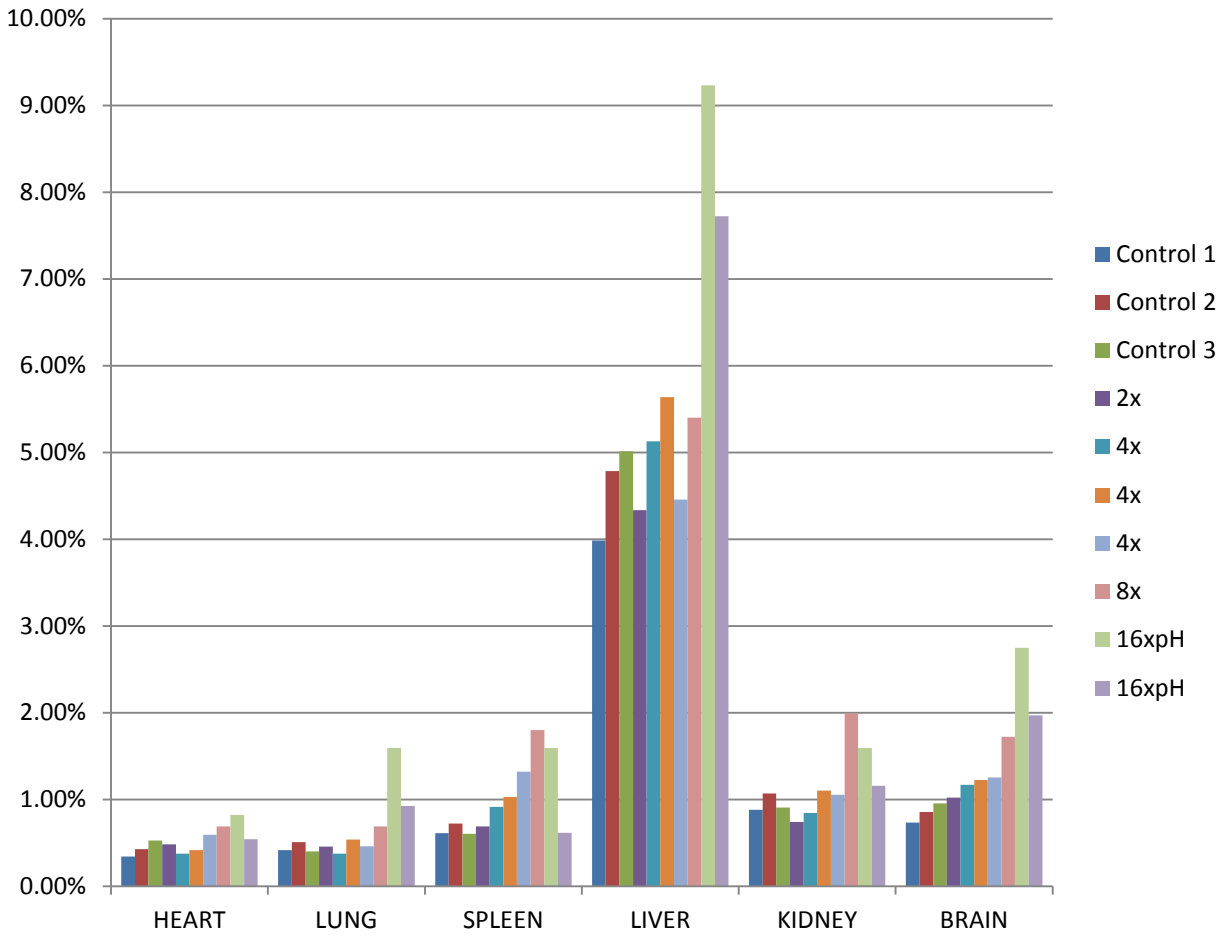


TABLE 9. Blood Chemical Parameters

Chemistry Parameters	Parameter CODE	DATA CODE	Dimension	Reference values Charles River C57BL/6 10 weeks Female 95% interval	Reference dimension (USA Conv. Units)	Conversion factor (x)(Merck manuals)	Reference values after conversion	Reference dimension after conversion	Control			6.25 % ION-ZC1	3.125 % ION-ZC1			1.5625 % ION-ZC1	0.78125 % ION-ZC1		Affected by hemolysis (yes/no)?
									1	2	3	1	1	2	3	1	1	2	
Total bilirubin (TBIL)	TBIL	SBIL3	umol/L	0,2-0,6	mg/dL	17,1 to umol/L	3,4 - 10,3	umol/L	1,7	3,6	4,6	2,5	3,6	1,5	0,7	1,7	1,9	1,5	No
Direct bilirubin (DBIL)	DBIL	BILD2	umol/L	0 - 1,71	umol/L	-	-	-	1	2,5	2	0,8	1	0,3	0,6	0,7	0,8	0,5	No
Indirect bilirubin (IBIL)	IBIL	No direct determination (TBIL -	umol/L	-	-	-	-	-	0,7	1,1	2,6	1,7	2,6	1,2	0,1	1	1,1	1	No
Alkaline phosphatase (ALP)	ALP	ALP2L	U/L	7,3-13,5	mg/dL				19	25	25	30	28	26	63	26	36	35	Small impact
Aspartate aminotransferase (GOT) (AST)	AST	Not determined	-	43-397	U/L	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alanine aminotransferase (GPT) (ALT)	ALT	ALTL	U/L	27-195	U/L	-	-	-	124	182	188	359	632	670	1489	141	548	550	Small impact
Blood Urea Nitrogene (BUN)	BUN	SUREA	mmol/L	5-26	mg/dL	0,357 to mmol/	1,8 - 9,3	mmol/L	16,2	9,2	10	8,4	11,2	9,2	20,1	12,5	11,9	20,7	Small impact
Creatinine (CREA)	CREA	SCRE2	umol/L	0,2-0,5	mg/dL	88,4 to umol/L	17,7 - 44,2	umol/L	0	15	16	5	14	9	12	6	9	8	No
Uric acid (URCA)	URCA	UA2	umol/L	0,08 - 0,1	mmol/L	1000 to umol/L	80 - 100	umol/L	156	93	265	173	157	203	158	161	236	141	No
Creatine kinase (CK)	CK	CKL	umol/L	182 - 998	U/L				1532	3665	15341	22000	30000	40000	20000	16457	35828	30000	High impact
Hemolysis index (H)	H	H	-	-	-	-	-	-	33	21	94	86	235	227	54	49	75	28	-
Lipemic index (L)	L	L	-	-	-	-	-	-	4	17	18	20	39	119	32	42	46	12	-
Icterus index (I)	I	I	-	-	-	-	-	-	2	1	2	2	2	0	0	5	2	1	-

Age-Related Reference Intervals of the Main Biochemical and Hematological Parameters in C57BL/6J, 129SV/EV and C3H/HeJ Mouse Strains
Cristina Mazzaccara et al.
PLoS ONE. 2008; 3(11): e3772.

ION-ZC1	stock 12,5%	%
DILUTIONS	1X	12,5
	2X	6,25
	4X	3,125
	8X	1,5625
	16X	0,78125
	Control	0

Fig 8. Blood Chemical Parameters versus ION-ZC1 injections (zooms)

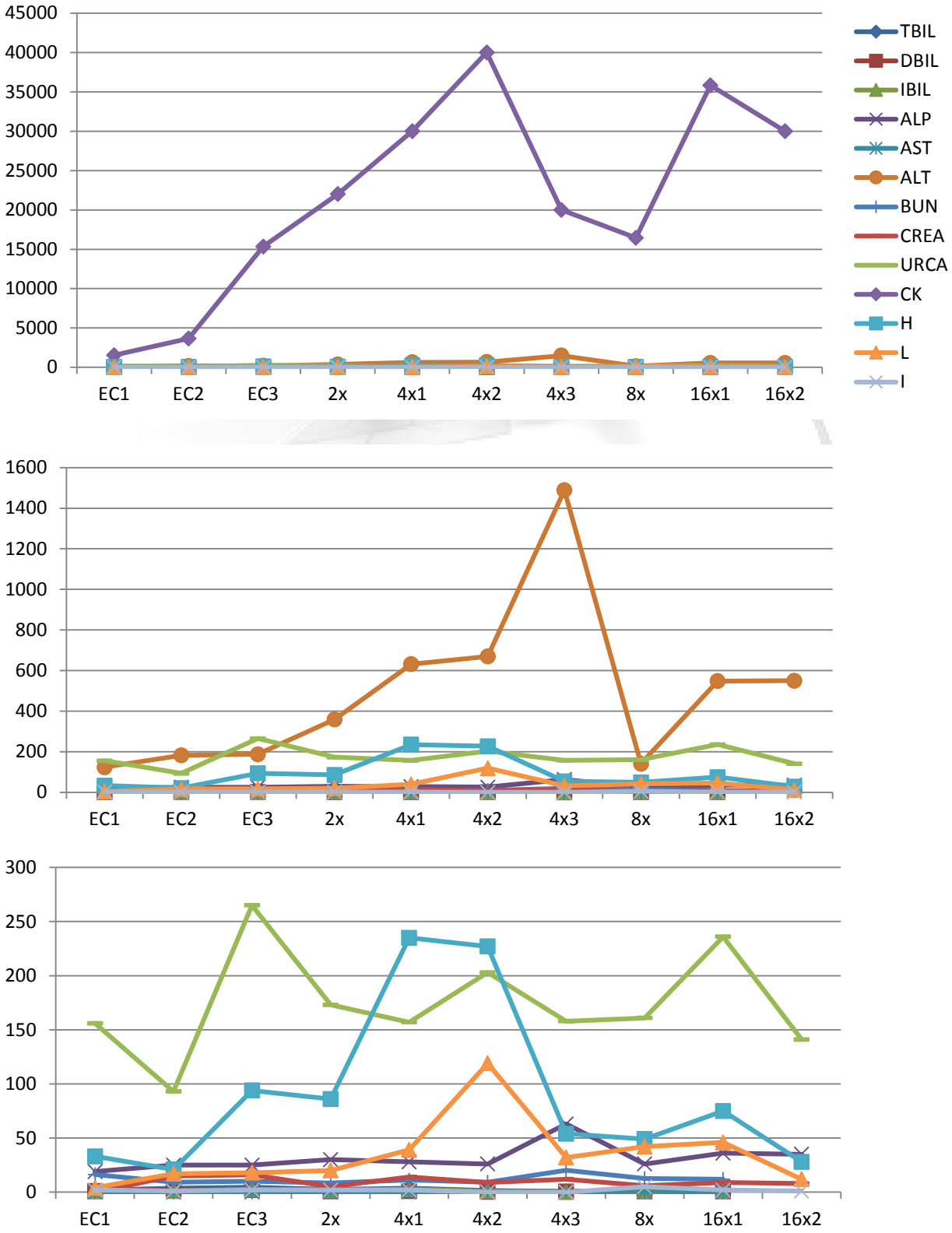


TABLE 10. Hematology Results

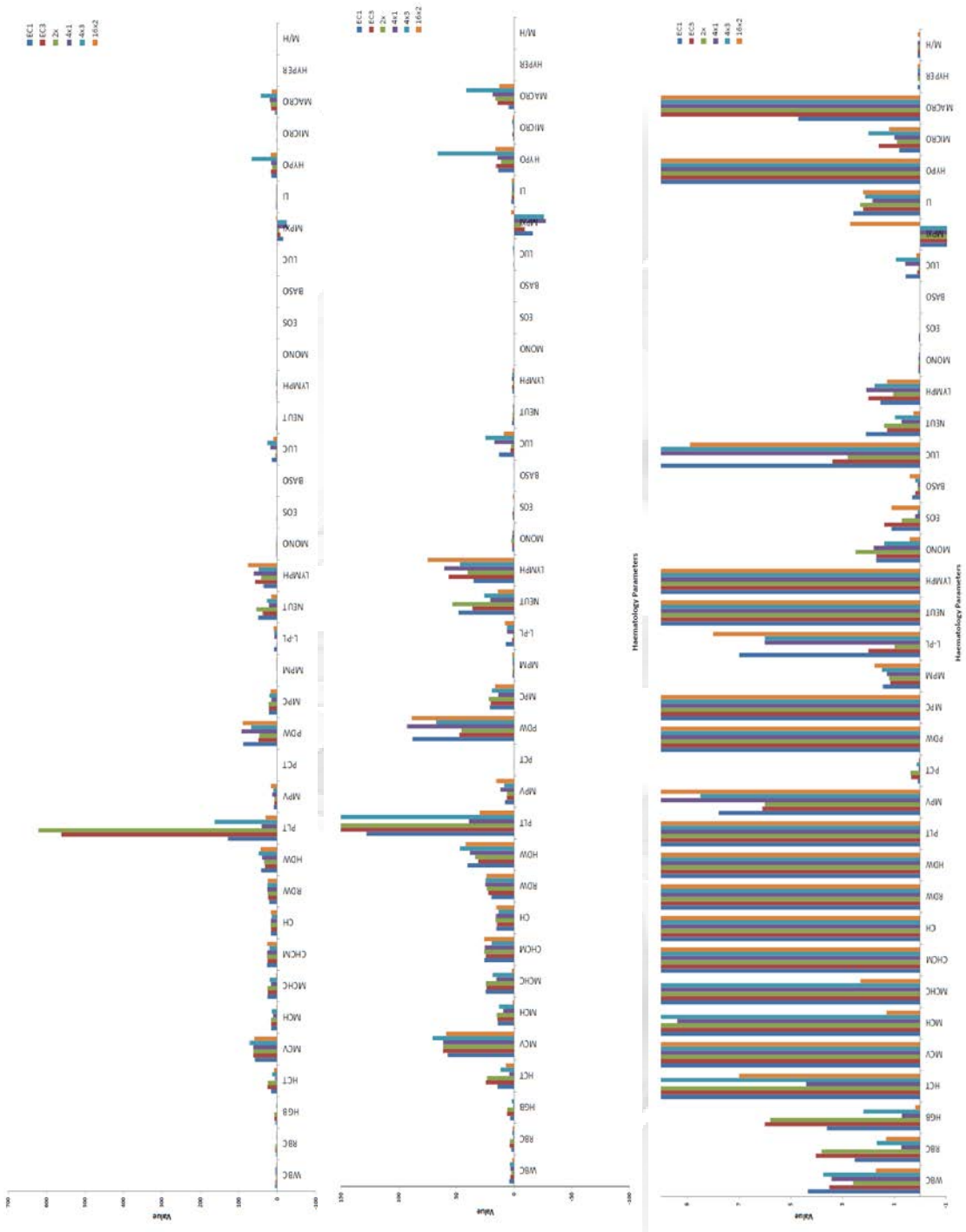
2015.10.26

Code	EC1	EC2	EC3	2x	4x1	4x2	4x3	8x	16x1	16x2
	Control			6.25 % ION-ZC1	3.125 % ION-ZC1			1.5625 % ION-ZC1	0.78125 % ION-ZC1	
	1	2	3	1	1	2	3	1	1	2
Number of white blood cells (WBC)	4,33		3,51	2,6	3,43		3,75			1,71
Number of red blood cell (RBC)	2,53		4,03	3,82	0,72		1,68			1,32
Hemoglobin (HGB)	3,6		6	5,8	0,7		2,2			0,2
Hematocrit / Red blood cell specific volume (HCT)	14,6		24,6	23,5	4,4		11,9			7
Average volume of red blood cells / Mean corpuscular volume (MCV)	57,9		61,7	61,4	61,4		71			59,3
Average mass of hemoglobin per red blood cell / Mean corpuscular hemoglobin (MCH)	14,4		14,8	15,2	9,4		13,2			1,3
Concentration of hemoglobin in a given volume of packed red blood cells / Mean corpuscular hemoglobin concentration (MCHC)	24,8		24,1	24,7	15,3		18,6			2,3
Cell hemoglobin concentration mean	26		24,6	26,2	25,8		19,3			26,2
Cell hemoglobin	15,4		15,1	15,9	15,6		13,7			15,5
Range of variation of red blood cells / Red blood cell distribution width (RDW, RDW-CV)	19,6		22,6	23,8	25,2		24,8			24,1
Cell hemoglobin concentration mean	40,3		31	33,9	38,1		47,3			42
Platelet count (PLT)	128		561	621	39		163			30
Average size of platelets / Mean platelet volume (MPV)	7,8		6,1	6	12		8,5			15,5
Mass of platelets / Plateletcrit (PCT)	0,1		0,34	0,37	0,05		0,14			0,05
Range of variation of platelets / Platelet distribution width (PDW, PDW-CV)	88,3		47,7	45,6	92,8		67,8			89
Mean platelet component	21,1		20,4	22	13,8		19,2			16,3
Mean platelet mass	1,43		1,15	1,19	1,29		1,47			1,77
Large Platelets	7		2	1	6		6			8
% **Neutrophyls**	48,4		36,3	53,7	20,6		26,2			14,3
% **Lymphocytes**	35,3		56,9	40,2	60,5		47,2			75
% **Monocytes**	1,7		1,7	2,5	1,8		1,4			0,4
% **Eosinophyls**	1,1		1,4	0,7	0,2		0,1			1,1
% **Basophyls**	0,3		0,2	0,1	0,1		0,2			0,4
% **Large unstained cells**	13,2		3,4	2,8	16,8		25			8,9
# **Neutrophyls**	2,1		1,28	1,4	0,71		0,98			0,24
# **Lymphocytes**	1,53		2	1,04	2,08		1,77			1,28
# **Monocytes**	0,07		0,06	0,06	0,06		0,05			0,01
# **Eosinophyls**	0,05		0,05	0,02	0,01		0			0,02
# **Basophyls**	0,01		0,01	0	0		0,01			0,01
# **Large unstained cells**	0,57		0,12	0,07	0,58		0,94			0,15
Mean peroxidase index	-16,4		-9	-5,8	-27,8		-26			2,7
Labeling index for labeled cells	2,59		2,21	2,31	1,85		2,14			2,21
Hypochromic red blood cells	13,9		15,6	11,3	14,8		66,4			16
Micro red blood cells	0,8		1,6	0,9	1		2			1,2
Macro red blood cells	4,7		14,6	16,1	18,5		41,4			12,9
Hyperchromic red blood cells	0,1		0	0,1	0,1		0,1			0,1
Micro / hypo ratio	0,1		0,1	0,1	0,1		0			0,1
Platelet clump	plus		-	-	-		-			-

Hematology	Parameters	CODE	Reference values Charles River C57BL/6 10 weeks Female 95% interval	Dimension
	Number of white blood cells (WBC)	WBC	3,9-13,94	x giga/L
	Number of red blood cell (RBC)	RBC	7,37-11,50	x tera/L
	Hemoglobin (HGB)	HGB	10,9-18,1	g/dL
	Hematocrit / Red blood cell specific volume (HCT)	HCT	37,2-58,0	(%)
	Average volume of red blood cells / Mean corpuscular volume (MCV)	MCV	42,6-55,6	fL
	Average mass of hemoglobin per red blood cell / Mean corpuscular hemoglobin (MCH)	MCH	13,0-16,8	pg
	Concentration of hemoglobin in a given volume of packed red blood cells / Mean corpuscular hemoglobin concentration (MCHC)	MCHC	26,0-35,9	g/dL
	Cell hemoglobin concentration mean	CHCM		g/dL
	Cell hemoglobin	CH		pg
	Range of variation of red blood cells / Red blood cell distribution width (RDW, RDW-CV)	RDW	16,1-21,1	(%)
	Cell hemoglobin concentration mean	HDW		g/L
	Platelet count (PLT)	PLT	565-1849	x giga/L
	Average size of platelets / Mean platelet volume (MPV)	MPV	4,3-5,6	fL
	Mass of platelets / Plateletcrit (PCT)	PCT		%
	Range of variation of platelets / Platelet distribution width (PDW, PDW-CV)	PDW		%
	Mean platelet component	MPC		g/L
	Mean platelet mass	MPM		pg
	Large Platelets	L-PL		x giga/L
%	**Neutrophyls**	NEUT	7,44-22,67	%
%	**Lymphocytes**	LYMPH	70,19-87,82	%
%	**Monocytes**	MONO	2,19-7,06	%
%	**Eosinophyls**	EOS	0,2-4,51	%
%	**Basophyls**	BASO	0,02-1,26	%
%	**Large unstained cells**	LUC		%
#	**Neutrophyls**	NEUT	0,42-2,55	x giga/L
#	**Lymphocytes**	LYMPH	2,88-10,92	x giga/L
#	**Monocytes**	MONO	0,17-0,69	x giga/L
#	**Eosinophyls**	EOS	0,01-0,50	x giga/L
#	**Basophyls**	BASO	0,00-0,14	x giga/L
#	**Large unstained cells**	LUC		x giga/L
	Mean peroxidase index	MPXI		
	Labeling index for labeled cells	LI		
	Hypochromic red blood cells	HYPO		%
	Micro red blood cells	MICRO		%
	Macro red blood cells	MACRO		%
	Hyperchromic red blood cells	HYPER		%
	Micro / hypo ratio	M/H		%
	Platelet clump	PLTPLMC		

ION-ZC1	stock 12,5%	%
DILUTIONS	1X	12,5
	2X	6,25
	4X	3,125
	8X	1,5625
	16X	0,78125
	Control	0

FIG 9. Hematology Results



Phase 2 DATA

Figure 10. Mouse body weight over the study period. Black arrows mark the days of first IV injection of mice (either with physiological salt/bicarbonate or with ION-ZC1 solutions).

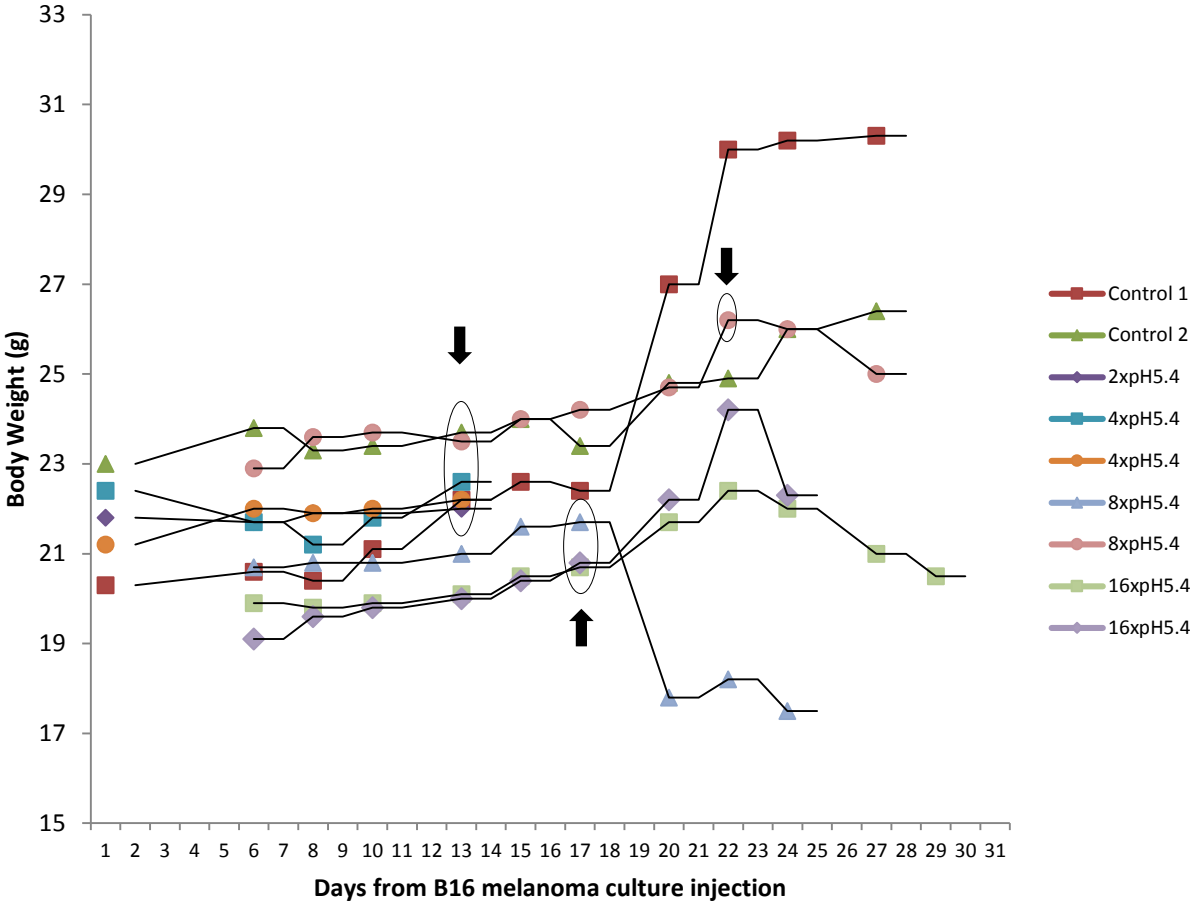
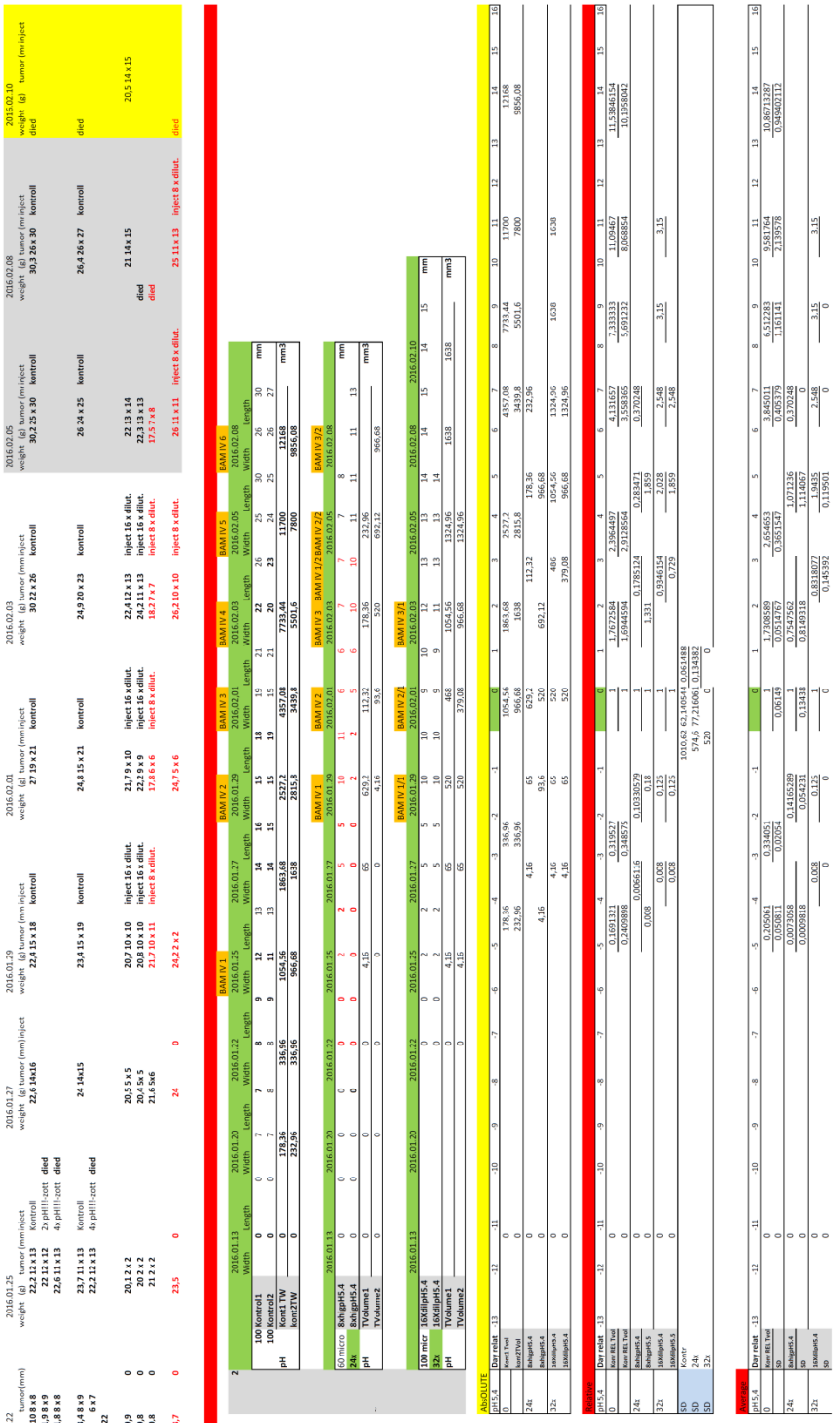


Table 11. Mouse body weights over the study period. Blue and red backrounds mark the days of IV injections of mice either with physiological salt/bicarbonate or with ION-ZC1 dilutions, respectively. Lack of data marks mice death.

DATE	2016.01.13					2016.01.18		2016.01.20		2016.01.22			2016.01.25		2016.01.27		2016.01.29			2016.02.01		2016.02.03		2016.02.05			2016.02.08		2016.02.10		
Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Control 1	20,3					20,6		20,40		21,10			22,2		22,6		22,4			27		30		30,2			30,3				
Control 2	23					23,8		23,3		23,4			23,7		24		23,4			24,8		24,9		26			26,4				
2xpH5.4	21,8					21,7		21,9		21,9			22																		
4xpH5.4	22,4					21,7		21,2		21,8			22,6																		
4xpH5.4	21,2					22		21,9		22			22,2																		
8xpH5.4						20,7		20,8		20,8			21		21,6		21,7			17,8		18,2		17,5							
8xpH5.4						22,9		23,6		23,7			23,5		24		24,2			24,7		26,2		26			25				
16xpH5.4						19,9		19,8		19,9			20,1		20,5		20,7			21,7		22,4		22			21		20,5		
16xpH5.4						19,1		19,6		19,8			20		20,4		20,8			22,2		24,2		22,3							

Table 12. Phase 2 data



Sub-tables:

1. original data recording

(tumor dimensions, injection dates and body weight measurements)

2. Tumor dimensions and calculated tumor volumes sorted by date

- Control mice – phys. salt IV
- 0.32% ION-ZC1 pH 5.4 IV
- 0.59% ION-ZC1 pH 5.4 IV

3. Absolute calculated tumor volumes sorted by days

- Control mice – phys. salt IV
- 0.32% ION-ZC1 pH 5.4 IV
- 0.59% ION-ZC1 pH 5.4 IV

4. Relative tumor volumes, compared to the size on the day of first IV injection, and sorted by days

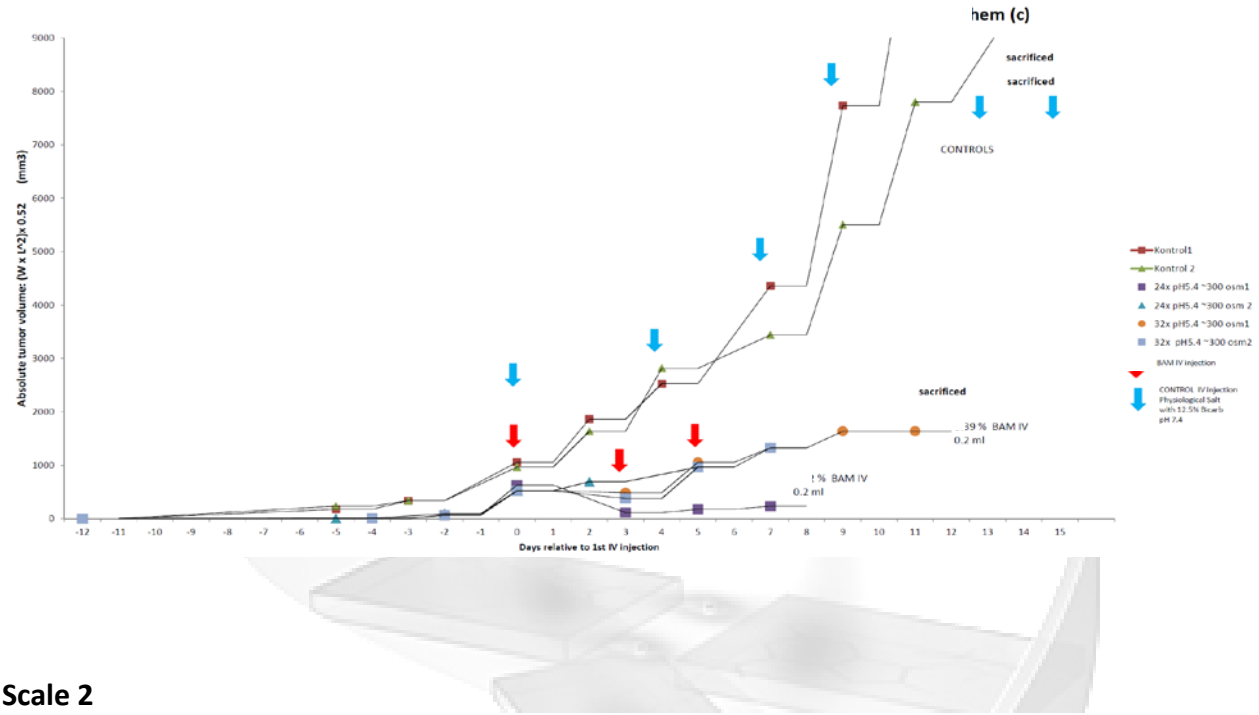
- Control mice – phys. salt IV
- 0.32% ION-ZC1 pH 5.4 IV
- 0.59% ION-ZC1 pH 5.4 IV

5. Averaged relative tumor volumes between mice and SD values, sorted by days

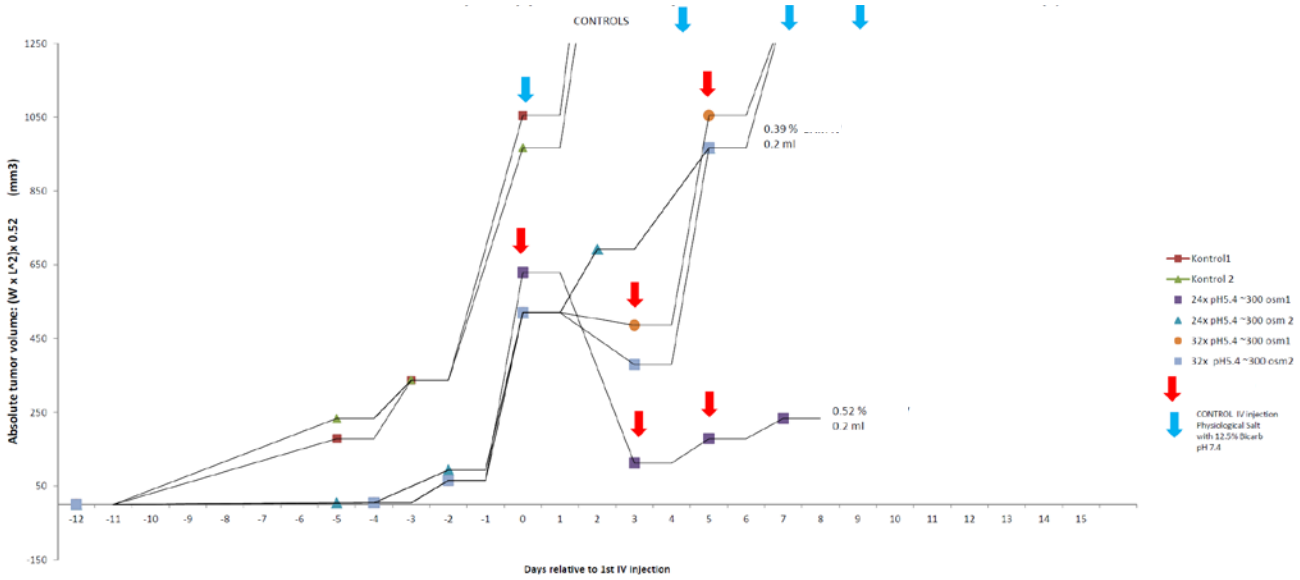
- Control phys. salt IV
- 0.32% ION-ZC1 pH 5.4 IV
- 0.59% ION-ZC1 pH 5.4 IV

Figure 11-12. Effect of ION-ZC1 injections on tumor growth. Absolute tumor volumes (compared to day 0, first injections) in case of individual mice (control tumor mice and treated tumor mice). Tumor volumes are given in mm3.

Scale 1

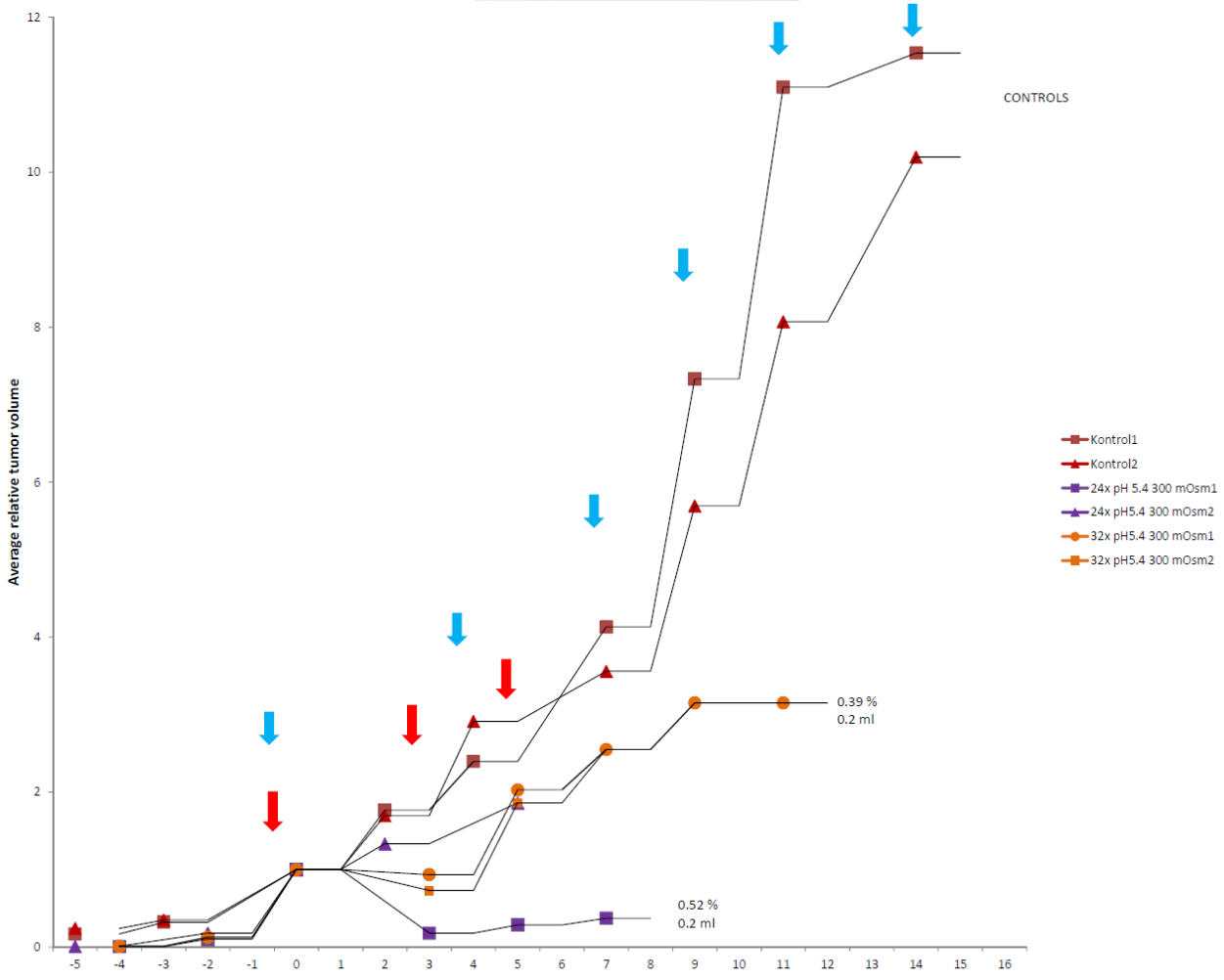


Scale 2



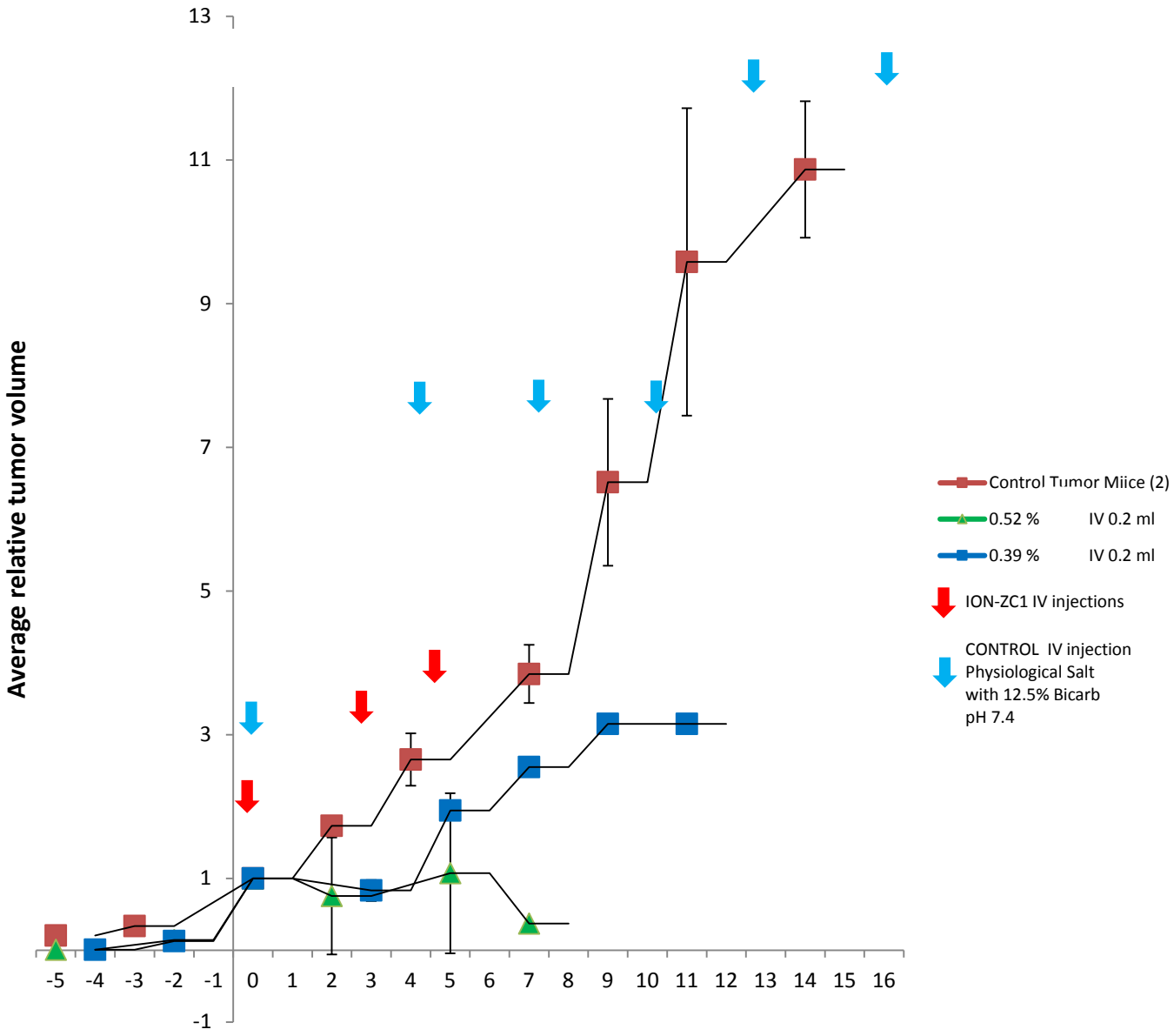
Red arrow: ION-ZC1 INJECTIONS (32x, 0.39%, 40 mg/kg body weight) (24x, 0.52%, 67 mg/kg body weight)

Figure 11-12 (continued). Effect of ION-ZC1 injections on tumor growth. Relative tumor volumes (compared to day 0, first injections) in case of individual mice (control tumor mice and treated tumor mice). Tumor volumes are given in mm3.



Red arrow: ION-ZC1 INJECTIONS (32x, 0.39%, 40 mg/kg body weight) (24x, 0.52%, 67 mg/kg body weight)

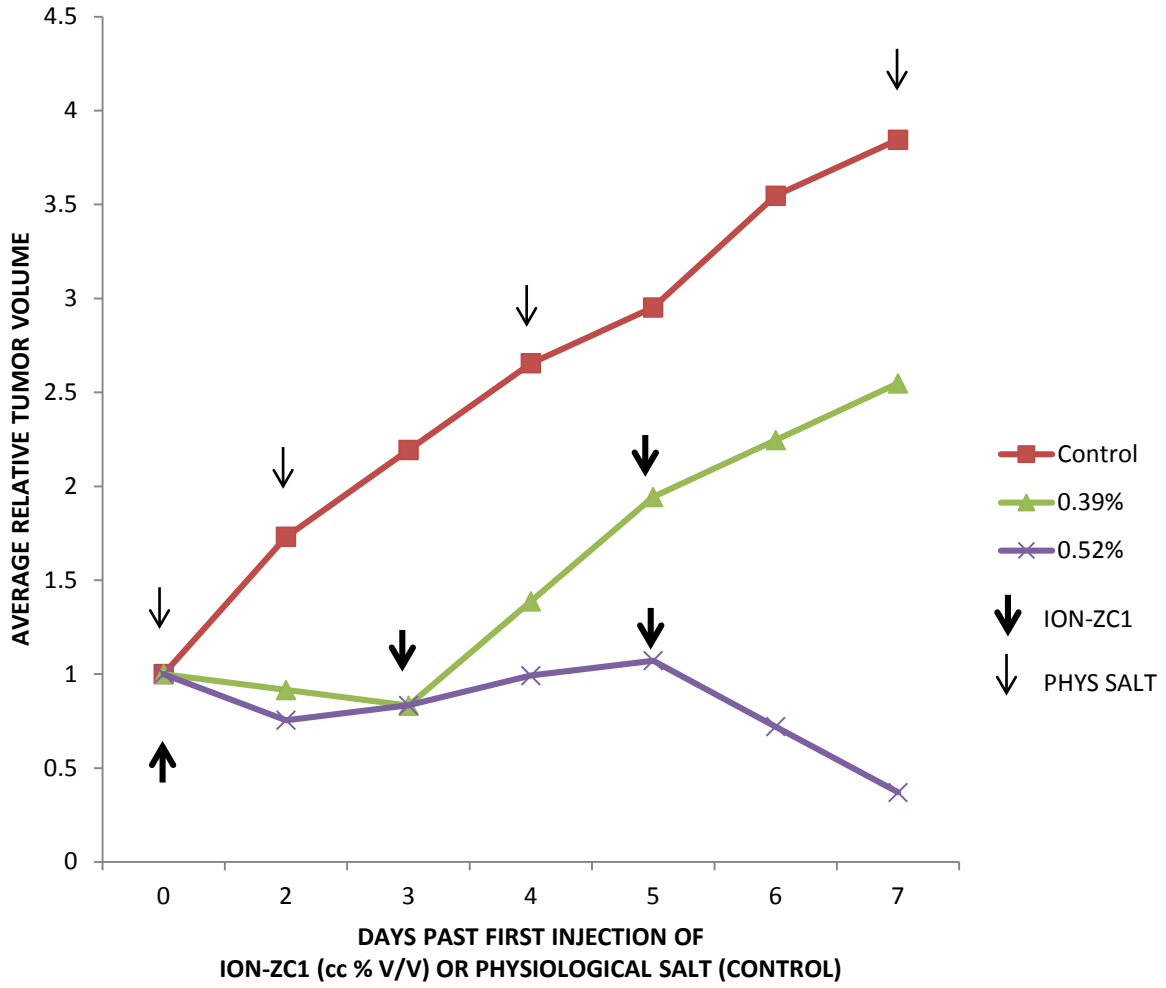
Figure 13. Effect of ION-ZC1 injections on tumor growth. Average relative tumor volumes (compared to day 0, first injections)**.**



Days Relative to 1st IV injection of ION-ZC1 or physiological salt (Day 0)

Red arrow: ION-ZC1 INJECTIONS (32x, 0.39%, 40 mg/kg body weight) (24x, 0.52%, 67 mg/kg body weight)

FIGURE 14. CORRELATION ANALYSIS. Effect of ION-ZC1 injections on tumor growth. Average relative tumor volumes (compared to day 0, first injections) are given in the table below the graph. Data from duplicate mice (3 x 2) were used and averaged. Data points from TV measurements are shown in black. Missing data points were estimated via linear regression, and marked as red. Correlation coefficients are shown in the table at the bottom. Injections are marked as arrows.



DAYS	Control	0.39%	0.52%	
0	1	1	1	
2	1,730859	0,915904	0,754756	
3	2,192756	0,831808	0,833876	0,912995868
4	2,654653	1,387654	0,992116	
5	2,952242	1,9435	1,071236	3,249831879
6	3,547421	2,24575	0,720742	
7	3,845011	2,548	0,370248	

CORRELATION

	Control	0.39%	0.52%
Control	1		
0.39%	0,899917	1	
0.52%	-0,55168	-0,51858	1

PHASE 3 DATA

Figure 15. Mouse body weights over the study period. Black arrows mark the days of IV injections of ION-ZC1 solutions. Individual mouse data is presented.

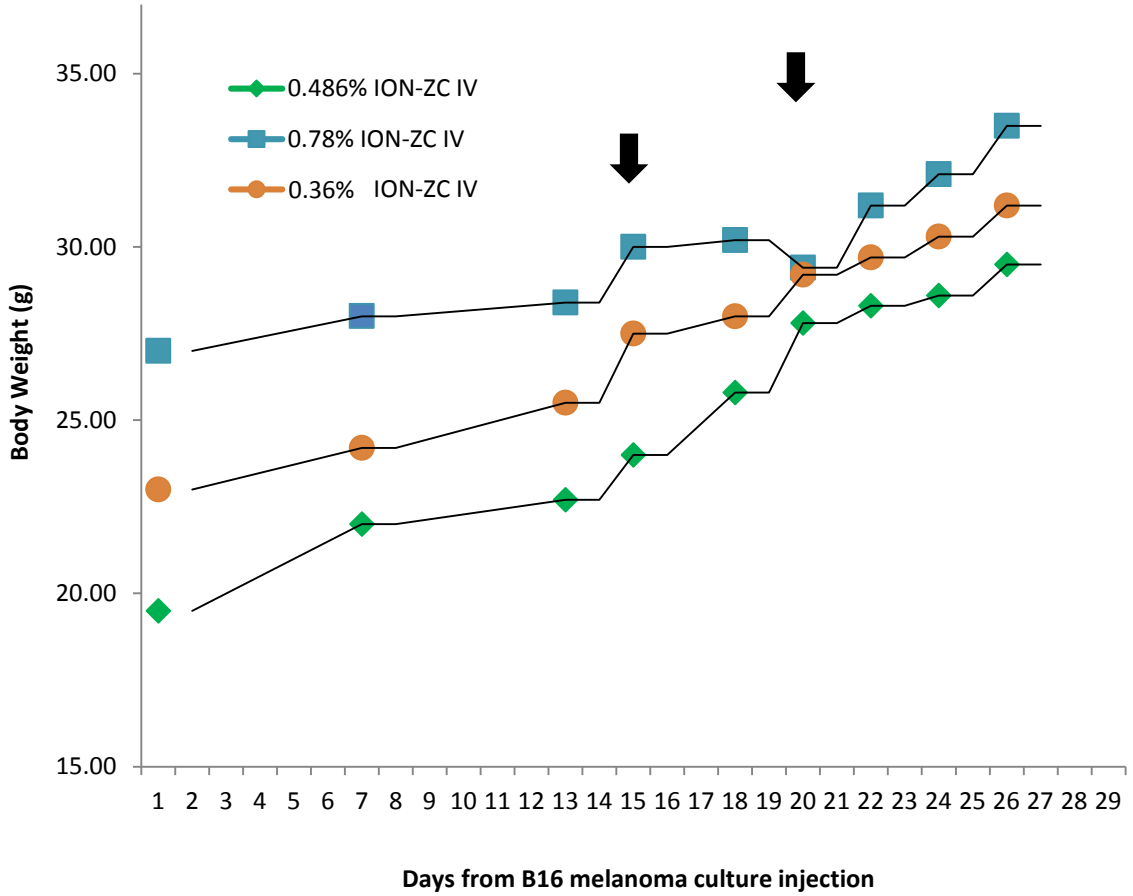
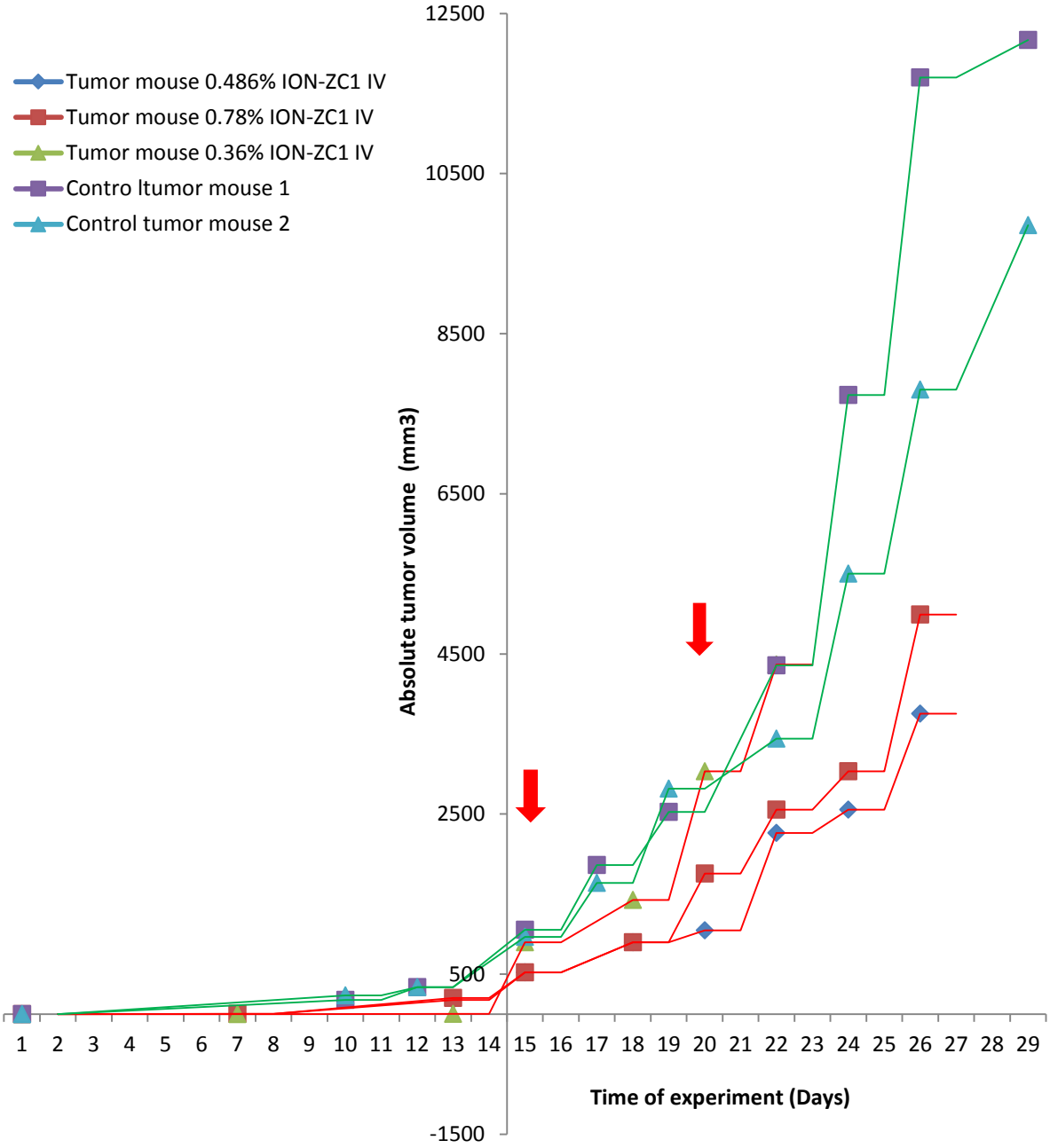


Table X. Mouse body weights over the study period. Blue and red backrounds mark the days of IV injections of mice either with ION-ZC1 dilutions, respectively.

		Mouse BW (g)																												
Mouse data		Date	2016.03.04					2016,03,10							2016,03,16		2016,03,18		2016,03,21			2016,03,23		2013,03,25		2013.03.27		2013,03,29			
Concentration	Dose (mg/kg body weight)	Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
0.486 % ION-ZC1	58.9		19,50						22						22,7		24			25,8		27,8		28,3		28,6		29,5		
0.78 % ION-ZC1	68.2		27						28						28,4		30			30,2		29,4		31,2		32,1		33,5		
0.36 % ION-ZC1	37.0		23						24,2						25,5		27,5			28		29,2		29,7		30,3		31,2		

Figure 16. Effect of ION-ZC1 injections on tumor growth. Absolute tumor volumes in case of individual mice (control tumor mice and treated tumor mice) starting from B16 melanoma culture injection (Day 1). Tumor volumes are given in mm3. Blue lines mark control tumor mice, red lines mark treated tumor mice. Red arrows represent ION-ZC1 intravenous injections (Day 15 and Day 20) for treated tumor mice. Data from control tumor mice are marked by green line, while data from treated tumor mice are marked by red lines in the graph.



HISTOPATHOLOGY FIGURES AND TABLES

Table H1.A

2015.12.17	HISTOPATHOLOGY	Total specimen
	specimen k1-3,2x, 4x1-3, 8x,16x1-2	66
Breakdown:		
	liver	10
Organs		
	kidneys	10
	spleen	10
	heart	10
	lungs	10
	brain	10
Tumor	control	1
	2x	1
	4x	1
	8x	1
	16x	1
Extra	intestine	1

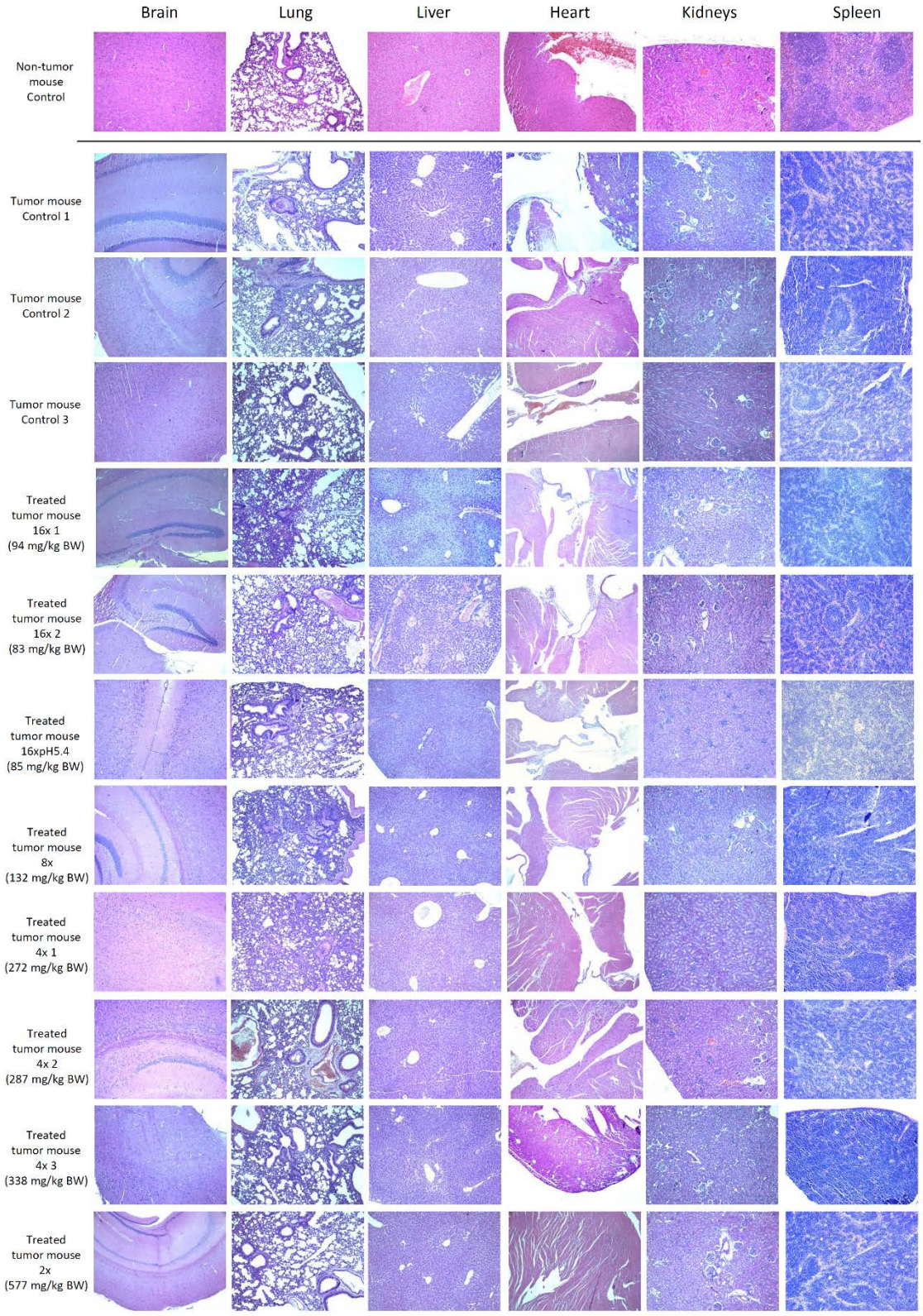
Table H1.B

2016.02.10	HISTOPATHOLOGY	Total specimen
	specimen 16x3	7
Breakdown:		
	liver	1
Organs		
	kidneys	1
	spleen	1
	heart	1
	lungs	1
	brain	1
Tumor	control	0
	2xpHnew	0
	4xpHnew	0
	8xpHnew	0
	16xpHnew	1
Extra	intestine	0

Table 2. Identification of specimen

K1-3	Control 1
	Control 2
	Control 3
2x	6.25%, pH 2
4x1-3	3.13 %, pH 2
	3.13 %, pH 2
	3.13 %, pH 2
8x	1.56% pH 2
16x1-2	0.78% pH 5
16x3	0.39% pH 5.4

Figure H1. Histopathology of the 6 main organs (brain, lungs, liver, heart, kidneys, spleen).

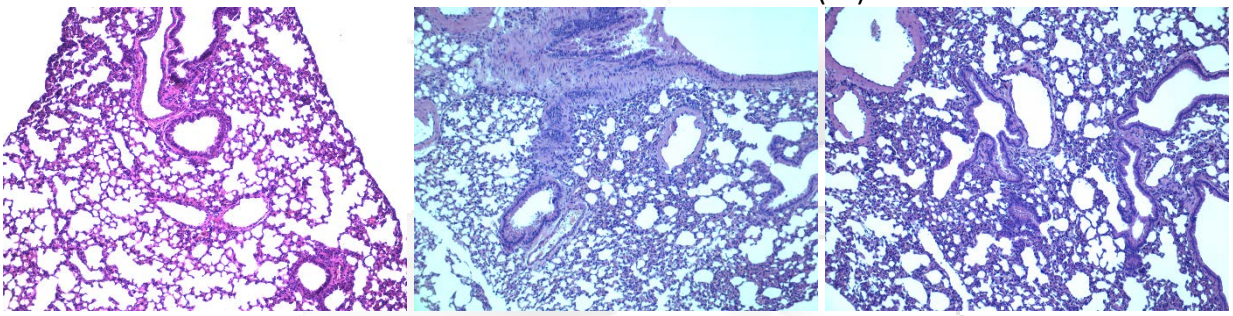


ION-ZC1 doses are specified as mg/kg body weight.

Figure H2. Organ histopathology detection examples

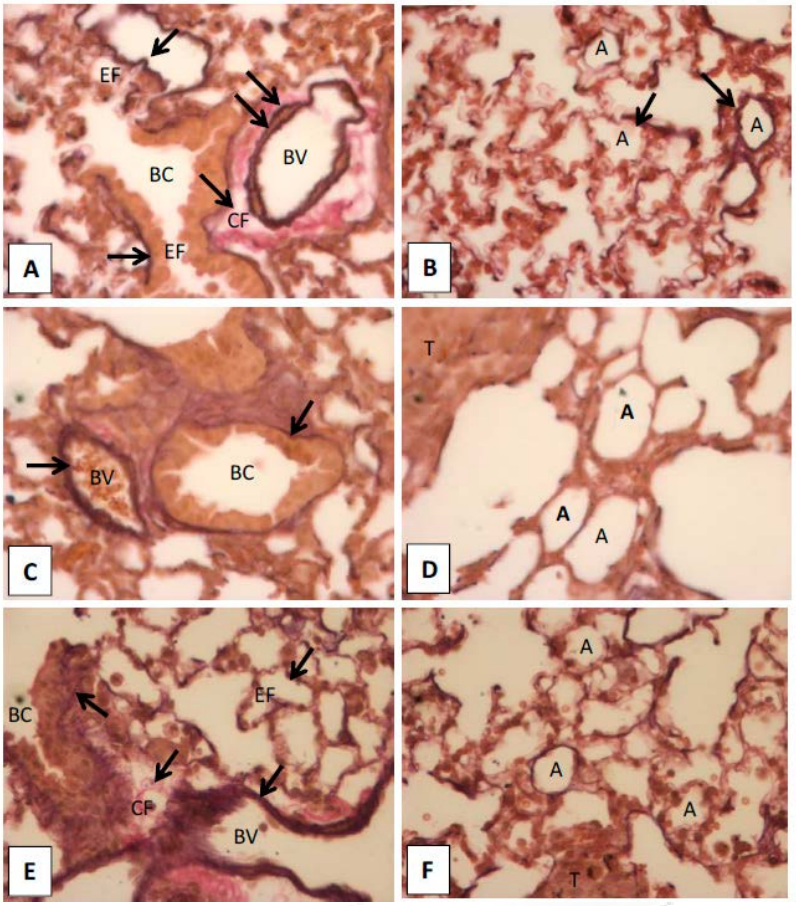
Lungs

Non-tumor control mouse | Control tumor mouse | (8x) ION-ZC1 treated tumor mouse



Literature

B16 melanoma metastasis enlarged alveoli occurred in the interstitial of the inter-alveolar occured

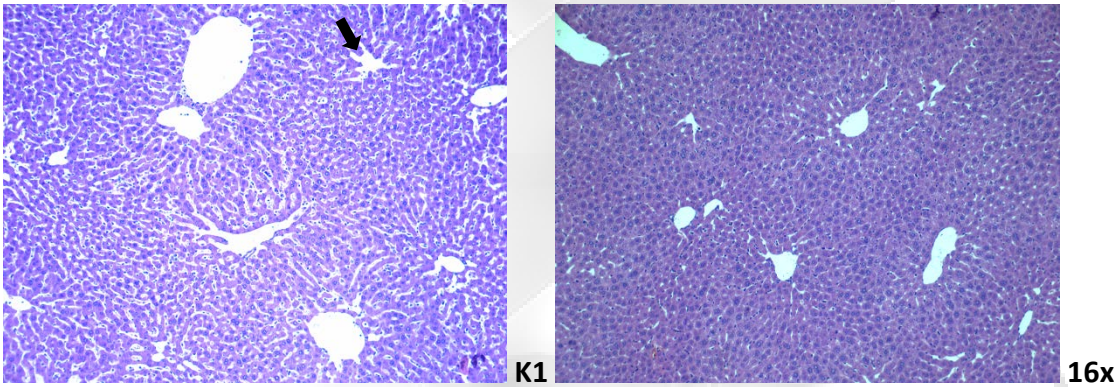


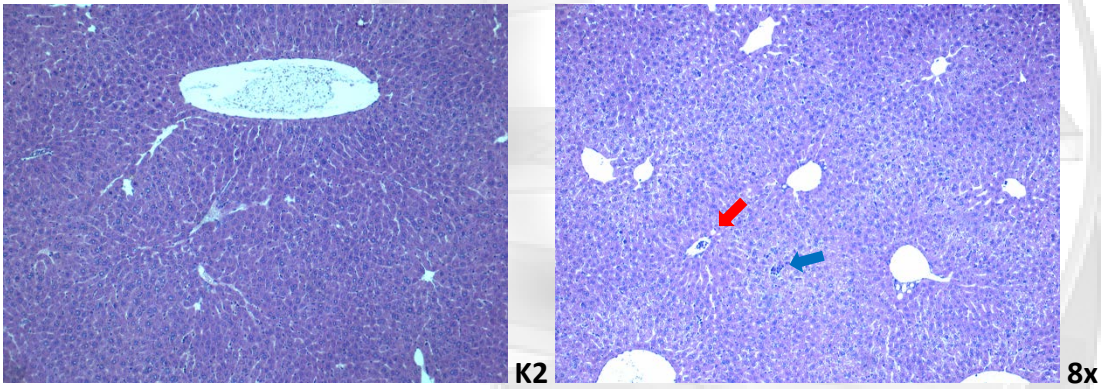
(**A**,**B**) are images of normal lung; (**C**,**D**) are images of lung with melanoma; (**E**,**F**) are images of cancer lung with hinokitiol-treatment. Bronchiole (BC), elastic fibers (EF), collagen fibers (CF), blood vessel (BV), alveoli (A), tumor (T). doi:10.3390/molecules201017720

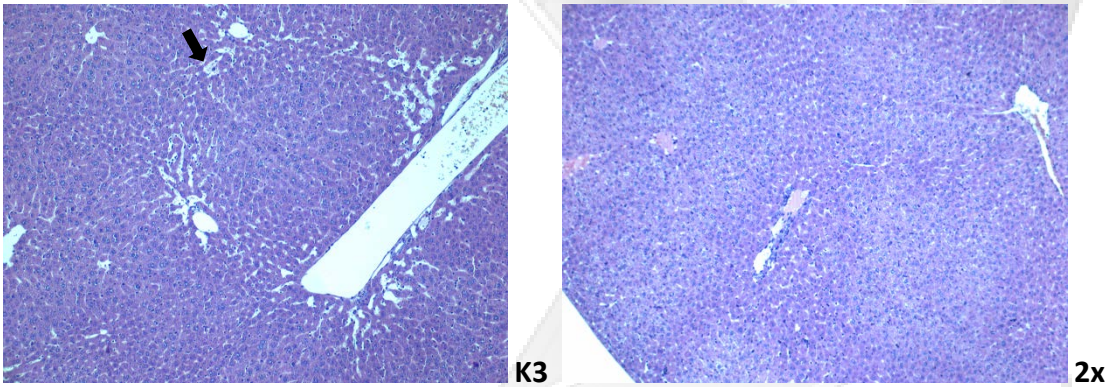
Figure H2. Organ histopathology detection examples (continued)

Liver

Control tumor mice (K1-3) **ION-ZC1 treated tumor mice (2x, 8x, 16x)**





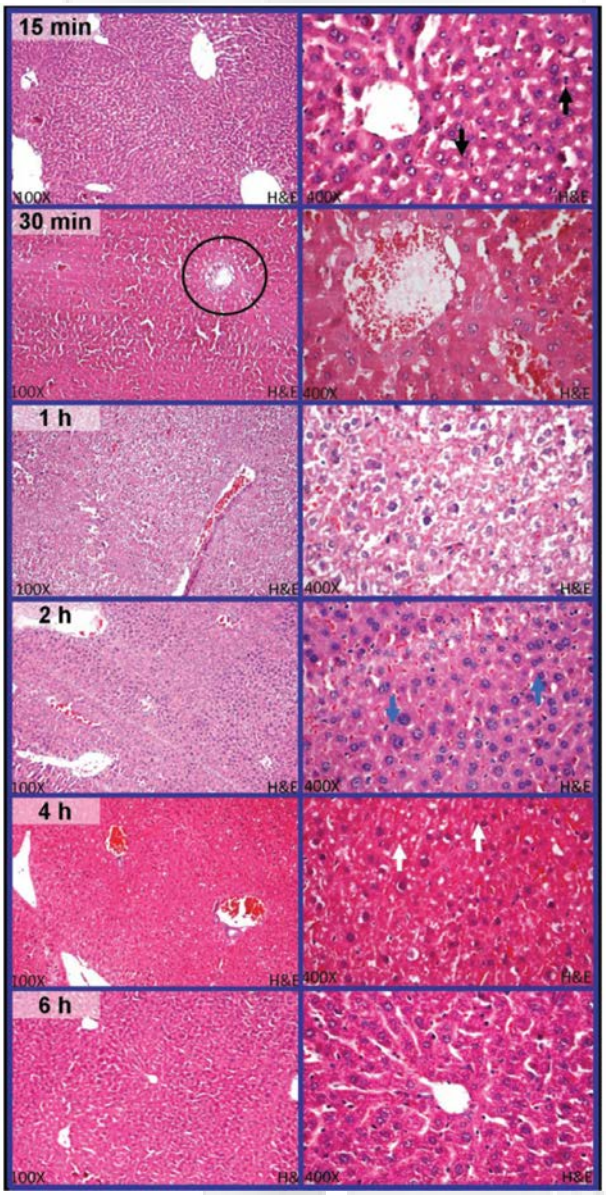


Dilated intersticial channels (black arrows), trabecularisation around blood vessels (red arrow) and lymphocyte infiltration (blue arrow).

Figure H2. Organ histopathology detection examples (continued)

Liver (continued)

Literature

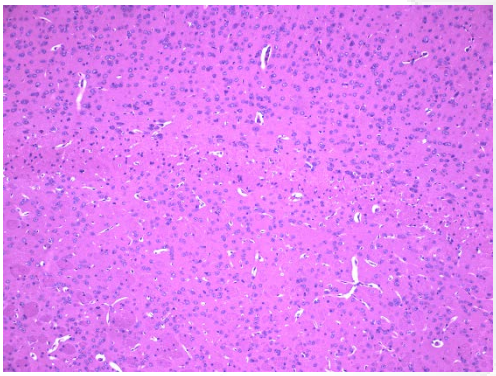


Photomicrographs of the liver after administration of 700 mg/kg IP acetaminophen. Mild sinusoidal congestion (black arrows), focal damage around the central vein (circled), feathery degeneration, loss of lobular architecture with damaged cellular outlines and congestion are seen at 1 h. By 2 h, nuclei have become condensed and pyknotic (blue arrow) which intensifi ed at 4 h with microvesicular fatty change (white arrows). By 6 h the damage has been reverted. *Malaysian J Pathol 32(1), 1 – 11*.

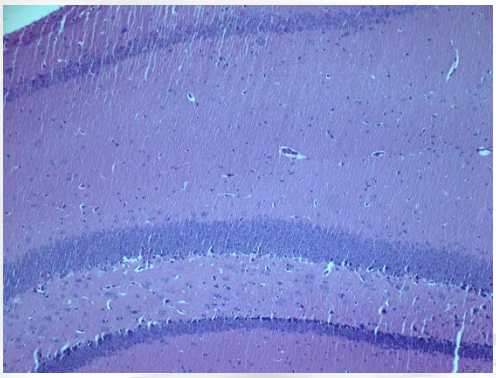
Figure H2. Organ histopathology detection examples (continued)

Brain

Non-tumor control

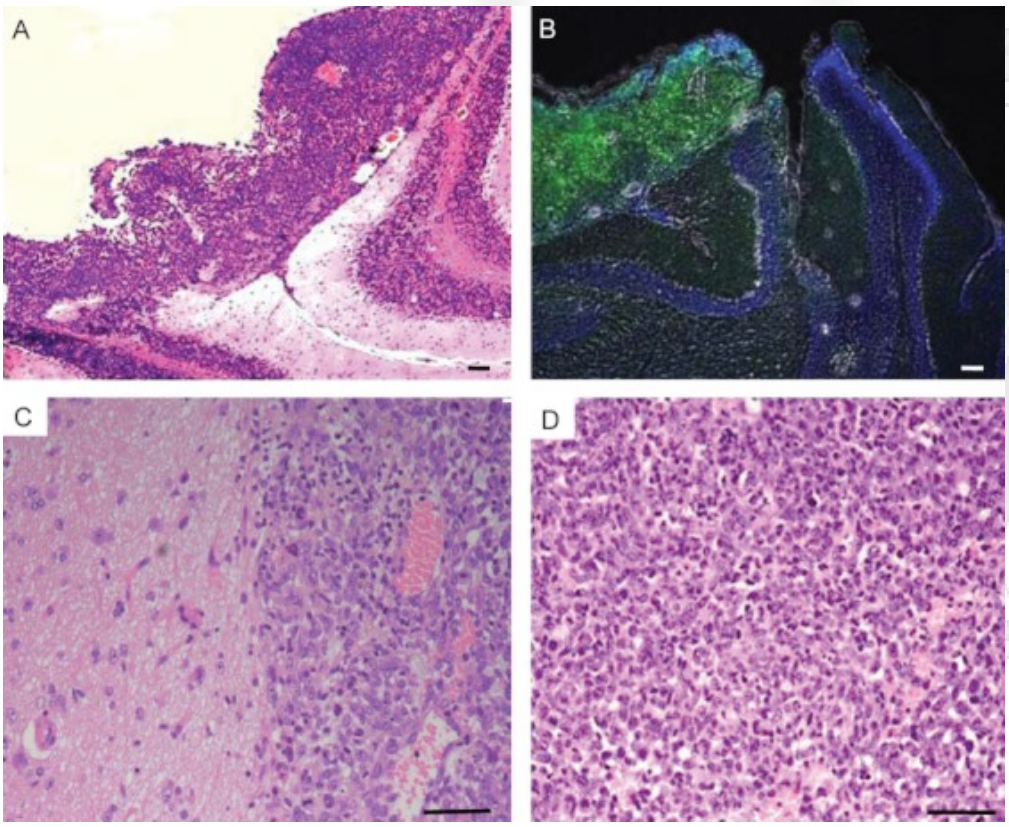


K1 tumor control



Literature:

B16-GFP melanoma cell metastasis in the brain



Histological characterization of brain metastatic melanoma. (A) Meningeal metastasis of mouse B16-GFP melanoma (hematoxylin and eosin 4×). (B) Immunofluorescent staining with anti-GFP Ab (green) and Dapi-Fluoromount-G (blue). (C) High-power field (20×) of tumor front. The tumor front was defined morphologically as the intersection of normal brain parenchyma with the tumor. (D) High-power field (20×) of the tumor core. Cores are considered as being the center of tumor where no normal brain tissue is visible within a single high-power field. All scale bars are 100 µm.

Cancer Med. 2013 Apr; 2(2): 155–163.

Figure H2. Organ histopathology detection examples (continued)

Spleen

OW/BW indices for the spleen in case of non-tumor mice and tumor mice (%)

Graph showing the relationship between relative spleen size (OW/BW indices) and ION-ZC1 dose

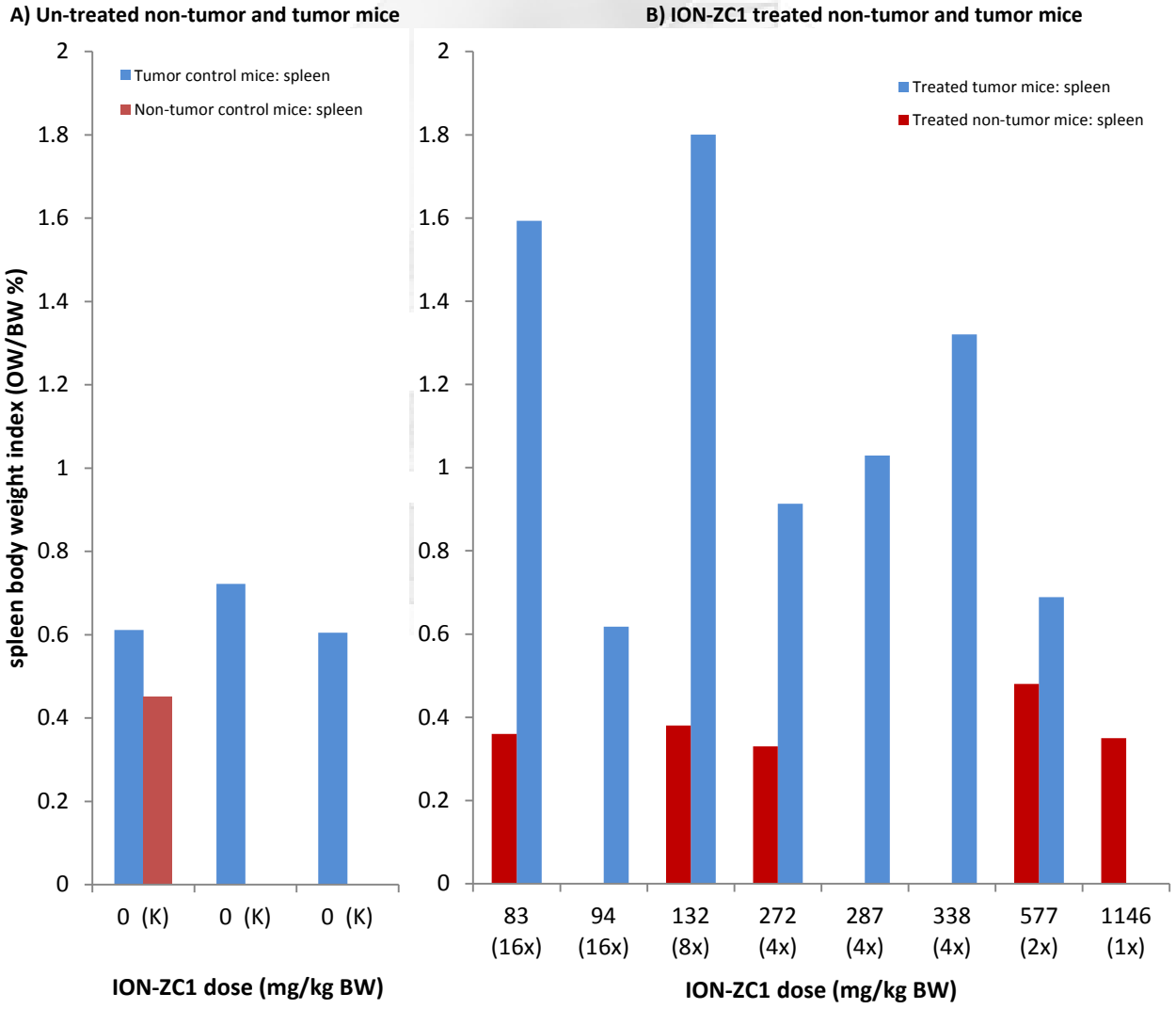


Figure H2. Organ histopathology detection examples (continued)

Spleen (continued)

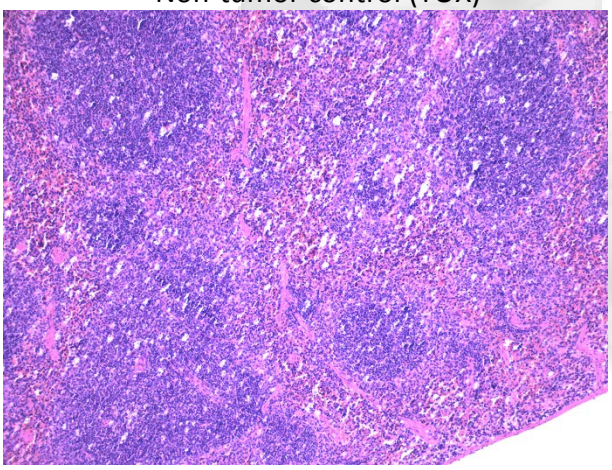
Tables summarizing spleen absolute weights (g) and relative weights (OW/BW %)

Mouse	TOX spleen	OW/BW %
1x	0,082	0,35
2x	0,11	0,48
4x	0,077	0,33
8x	0,088	0,38
16x	0,085	0,36
K	0,107	0,45

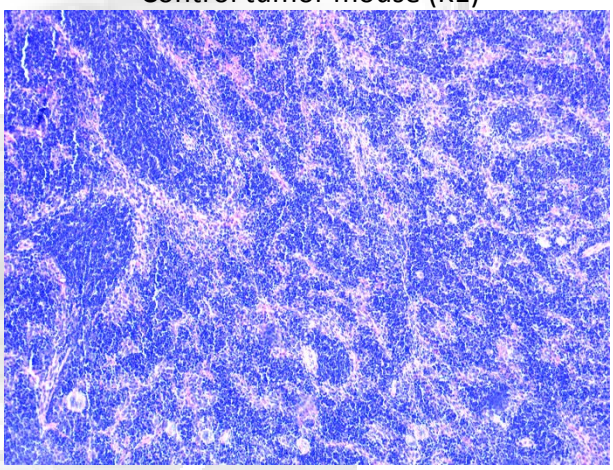
Mouse	Tumor spleen	OW/BW %
K	0,25	0,6112
K	0,27	0,7219
K	0,24	0,6045
2x	0,27	0,6888
4x	0,39	0,9133
4x	0,42	1,0294
4x	0,4	1,3201
8x	0,47	1,8008
16x	0,29	1,5934
16x	0,16	0,6178

Spleen histopathology slide examples

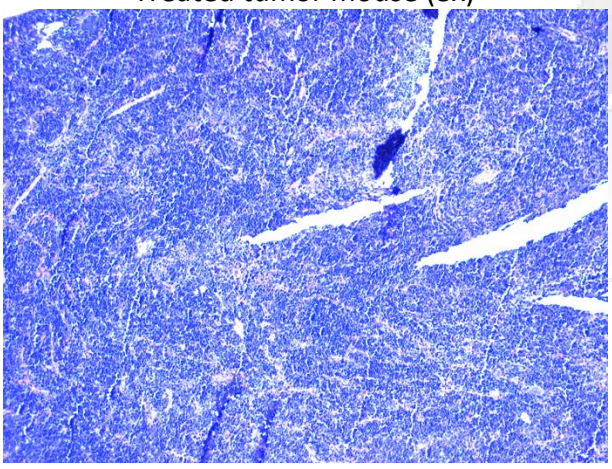
Non-tumor control (TOX)



Control tumor mouse (K1)



Treated tumor mouse (8x)



Treated tumor mouse (4x 2)

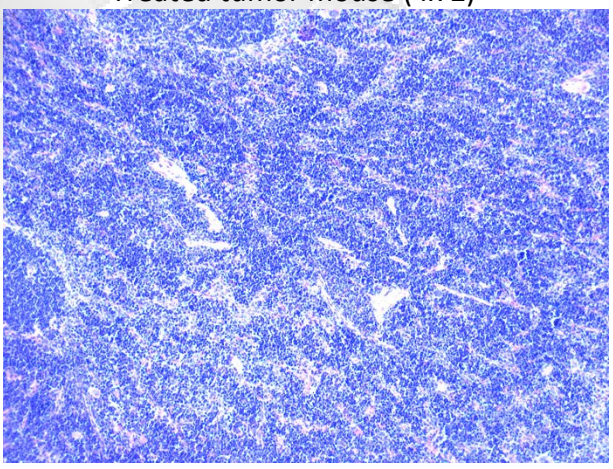
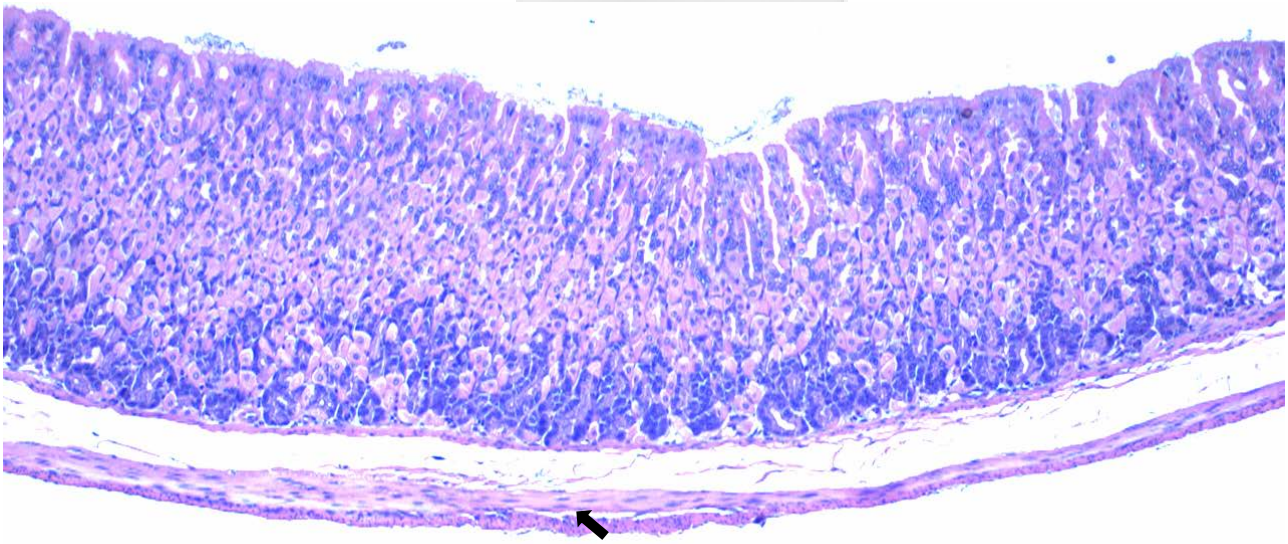


Figure H3. Intestine specimen with thickened small bowel wall due to melanoma metastasis. Black arrows mark B16 melanoma metastases in the walls of the small bowel. A) no significant thickening due to less developed metastasis and B) significant thickening due to more developed metastasis causing small bowel wall thickening.

A)



B)

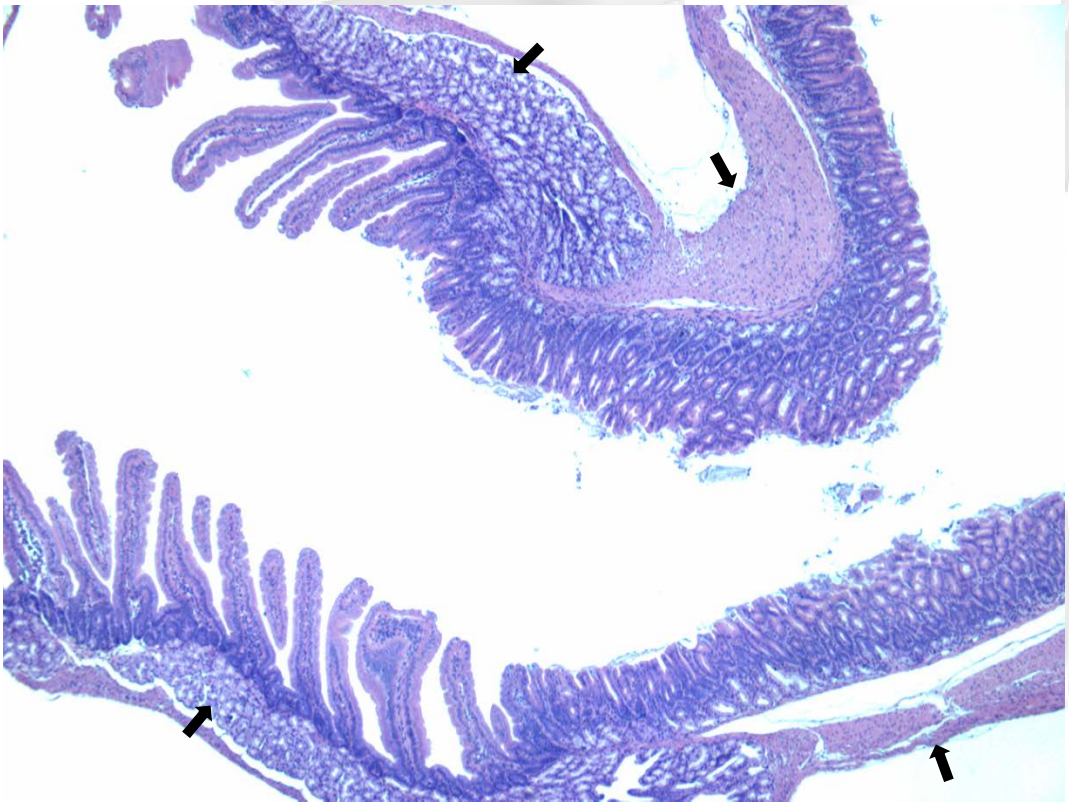


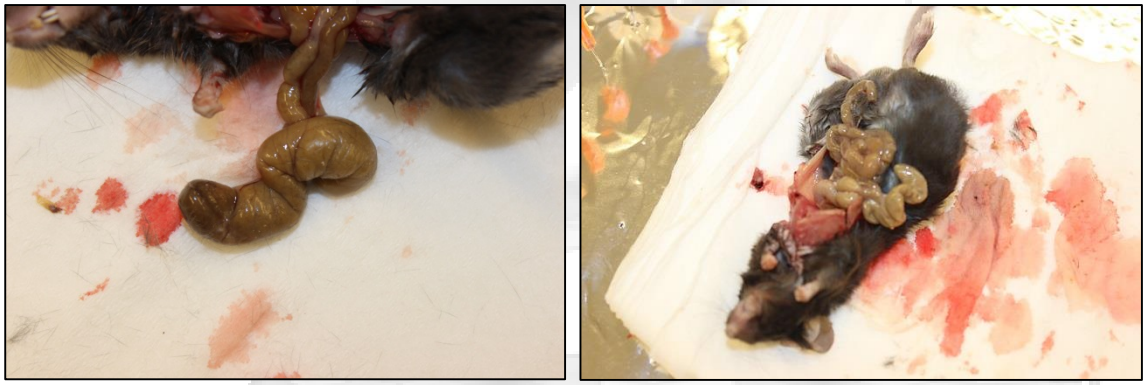
Figure H3. Intestine specimen with thickened small bowel wall due to melanoma metastasis (continued).

C) Thickened small bowel sections in control tumor and ION-ZC1 treated tumor mice.

D) Literature example of human condition with metastatic melanoma, causing small bowel deformation (Kelly V Liang et al. (2006))

C)

CONTROL **ION-ZC1 TREATED (2X)**



D)

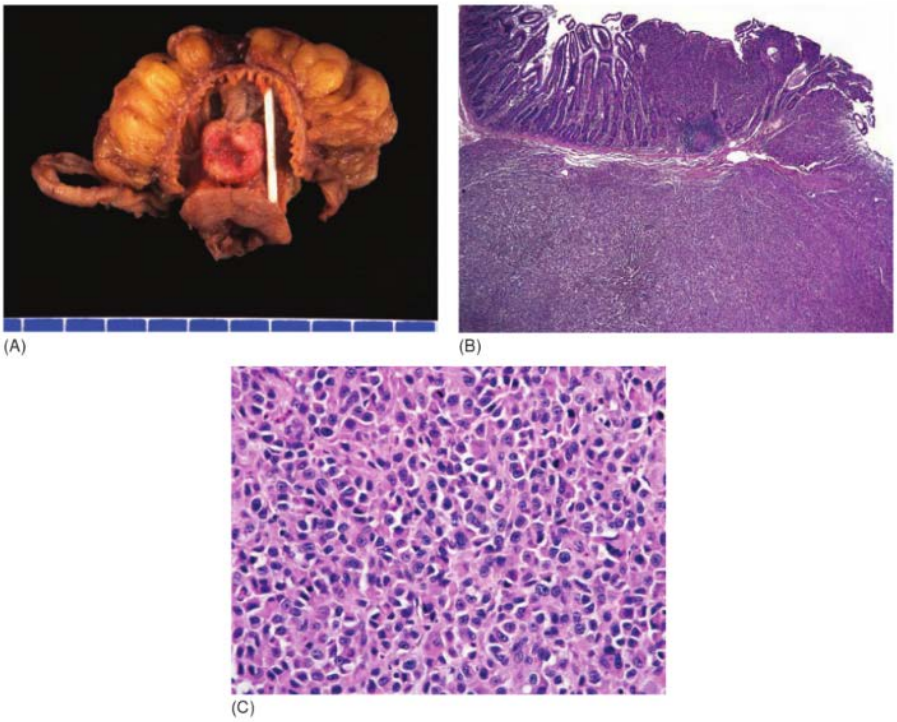
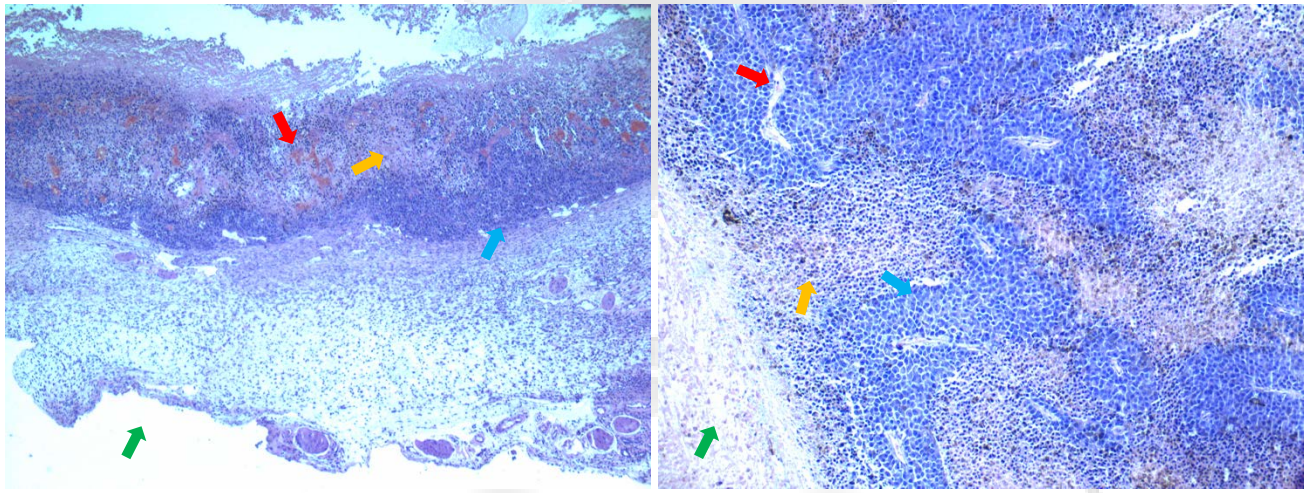


FIGURE 9.26 This example of metastatic melanoma to the small bowel presented as a mucosal polyp, mimicking a primary tumor (A). Another example of metastatic melanoma to the small bowel demonstrates a growth pattern centered in the submucosa, which extends into the surface epithelium (B). The tumor cells are dyscohesive with a plasmacytoid appearance and prominent nucleoli (C), typical of melanoma.

Figure H4. Tumor sections and histopathology detection of outcomes.

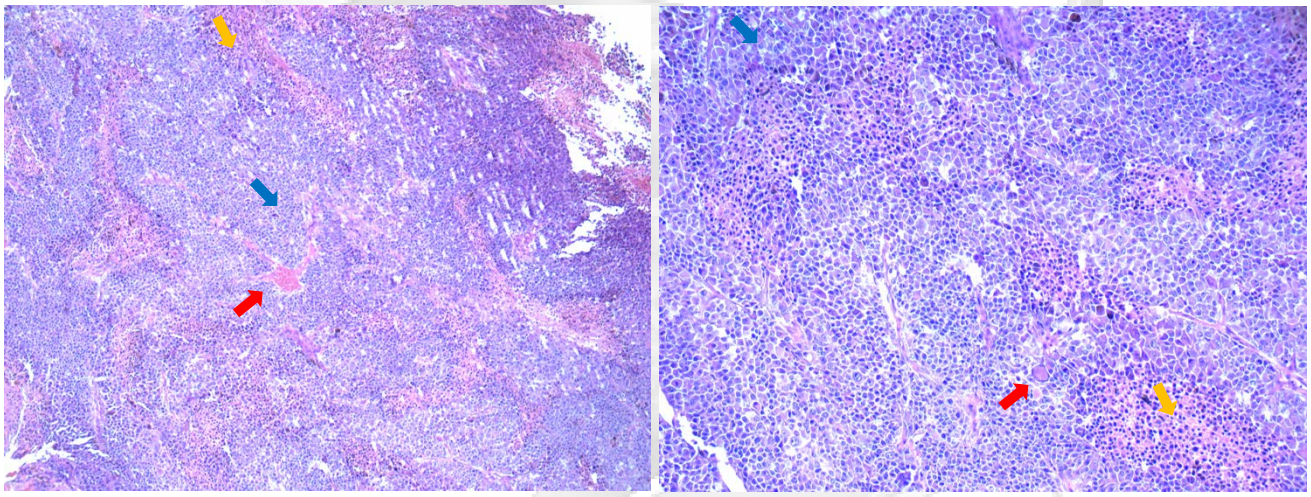
Control

50x **100x**



16x Treated (pH >5, 0.78 % ION-ZC1, 95 mg/kg body weight)

50x **100x**

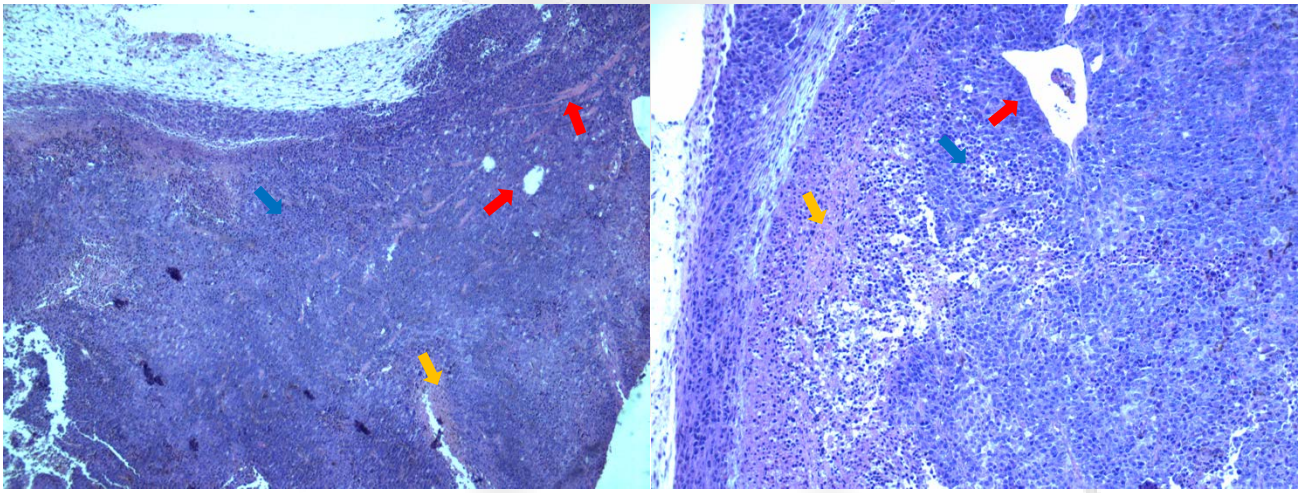


Blood vessels **Melanoma cells** **Necrotic melanoma cells** **Normal skin tissue**

Figure H4. Tumor sections and histopathology detection of outcomes (continued)

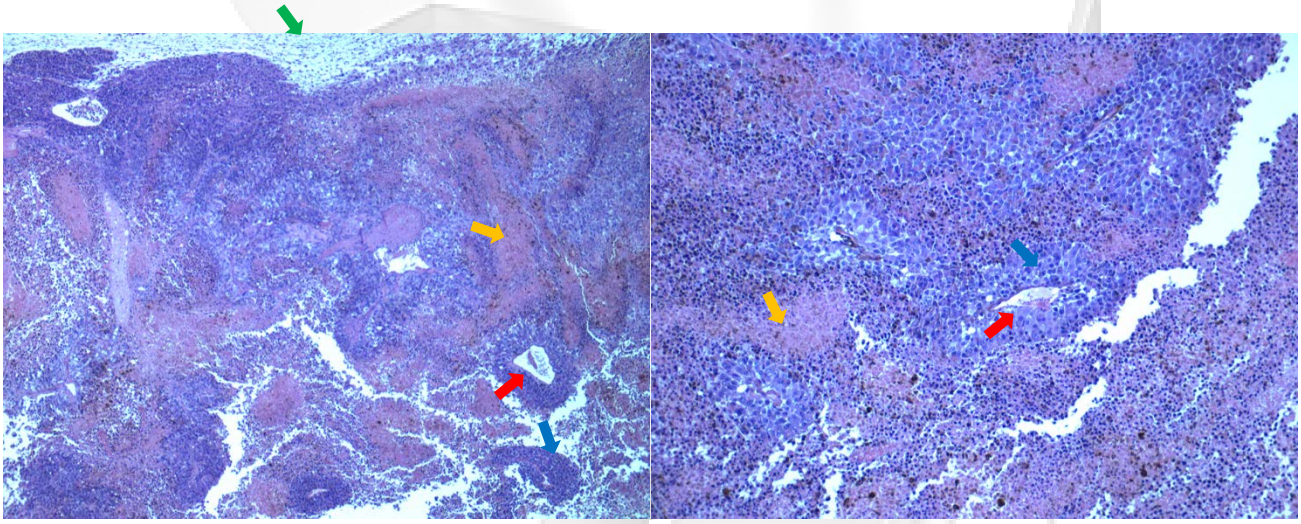
8x Treated (pH 2, 1.56 % ION-ZC1, 190 mg/kg body weight)

50x **100x**



4x3 Treated (pH 2, 3.13 % ION-ZC1, 380 mg/kg body weight)

50x **100x**



Blood vessels **Melanoma cells** **Necrotic melanoma cells** **Normal skin tissue**

Figure H4. Tumor sections and histopathology detection of outcomes (continued)

2x Treated (pH 2, 6.25 % ION-ZC1, 760 mg/kg body weight)

50x **100x**

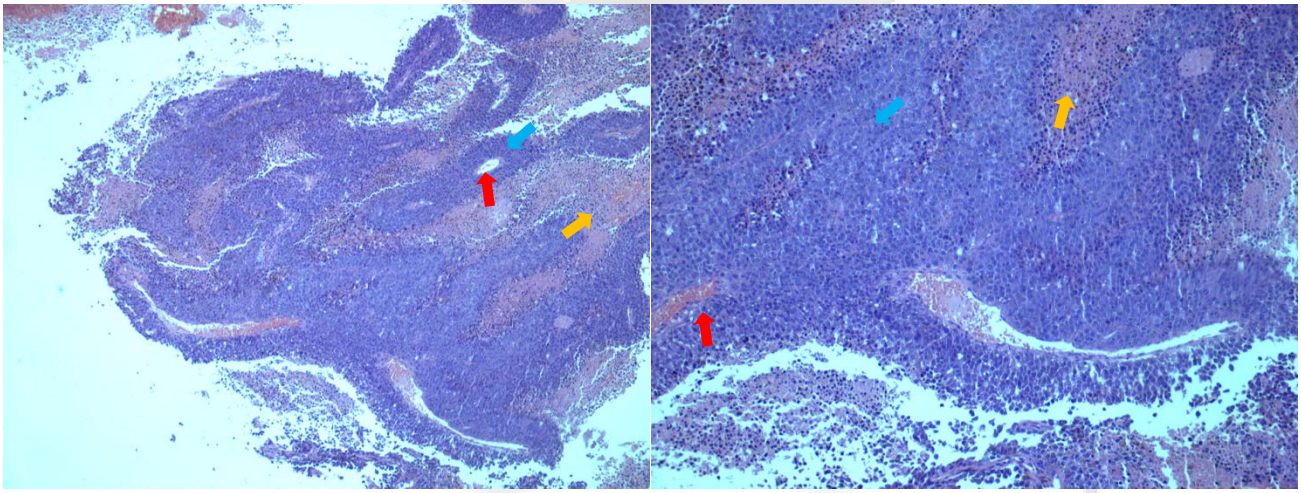
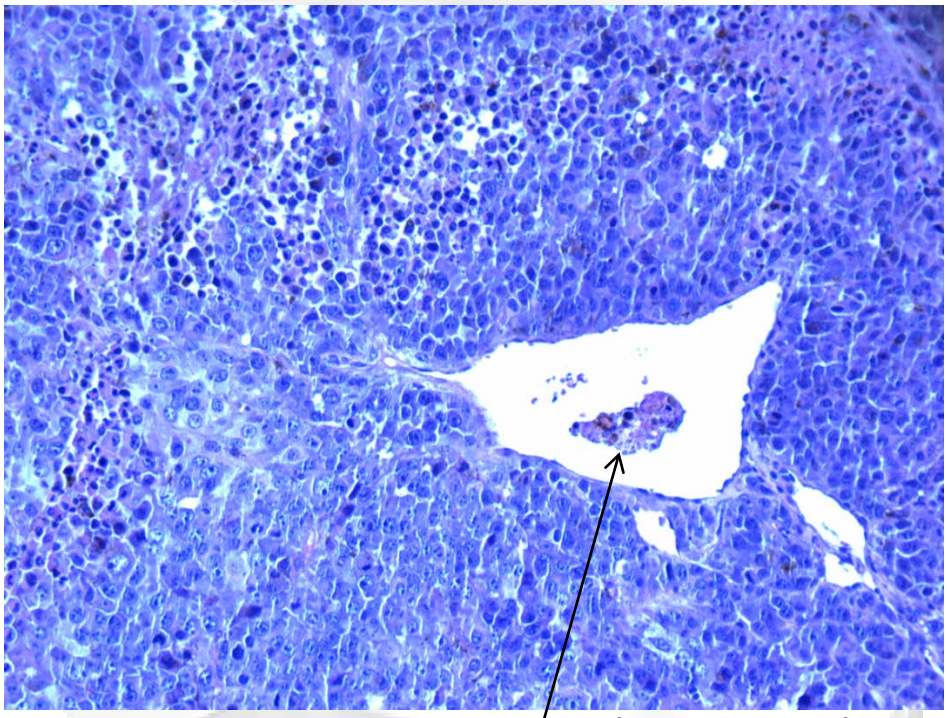


Figure H5. Tumor examples – blood metastasis, blood vessel cross-section

A) Tumor section with large blood vessel with melanoma cells
8x Treated (pH 2, 1.56 % ION-ZC1, 190 mg/kg body weight)
200x magnification



metastatic melanoma cells transorgan (in a blood vessel)

B) Blood vessel example
16x Treated (pH >5, 0.78 % ION-ZC1, 95 mg/kg body weight)
200x magnification

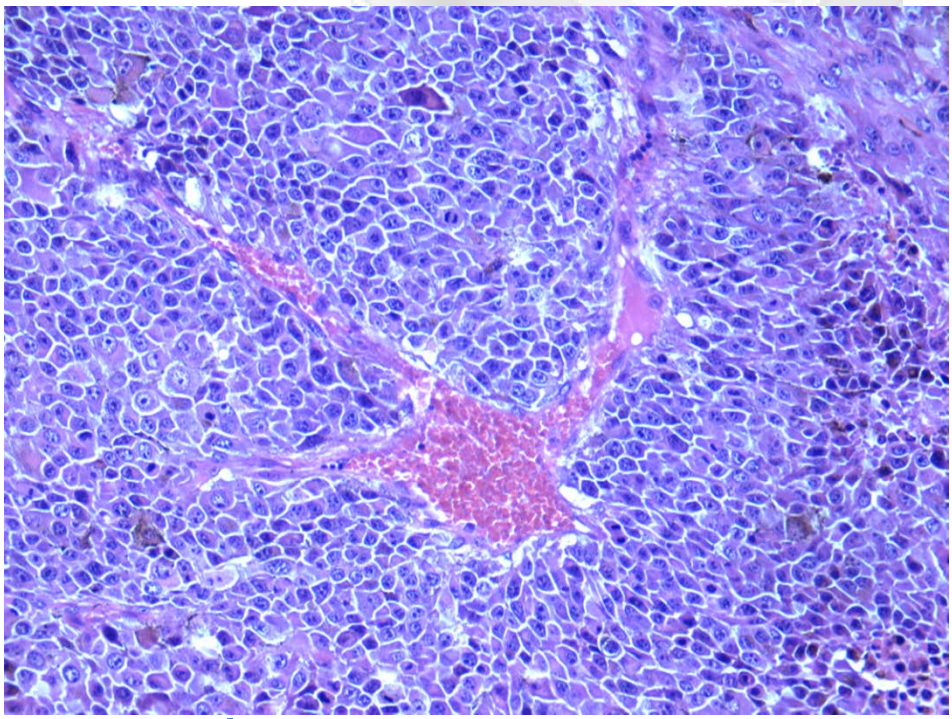
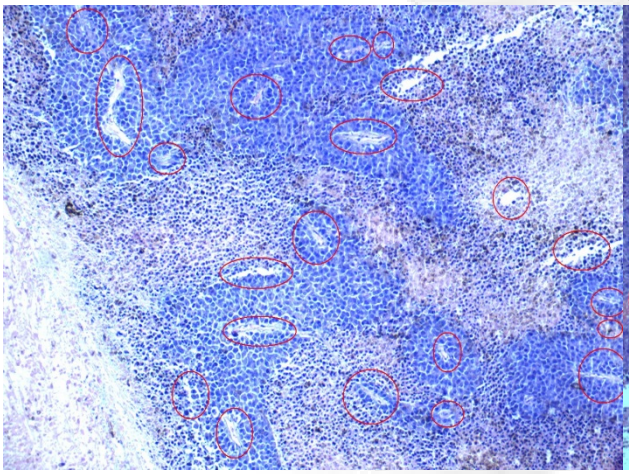


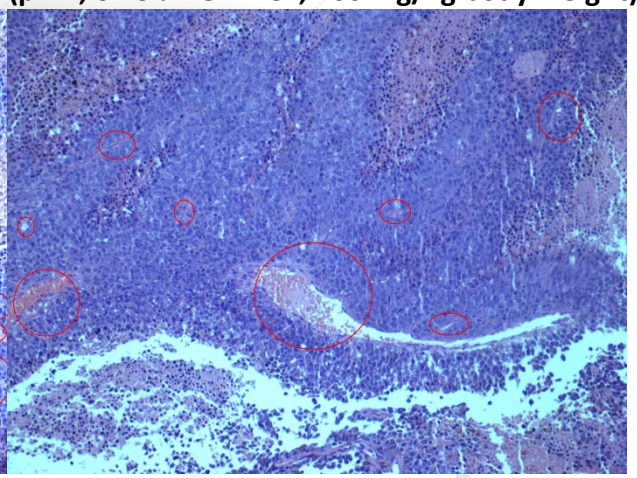
Figure H6. Blood vessel counting in tumor sections (magnification 100x).

A) Slides with blood vessels marked by red circles.

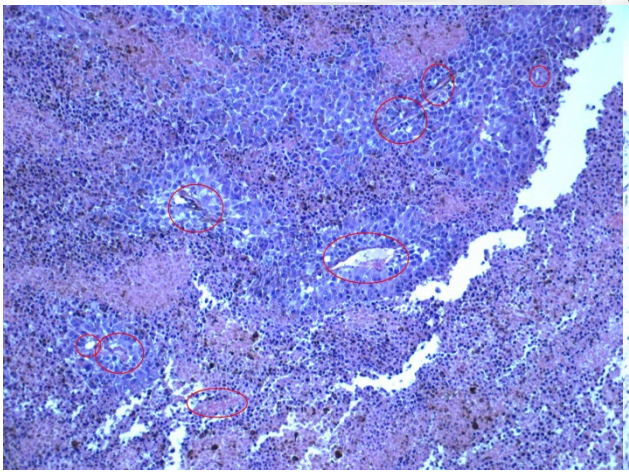
Control tumor mouse



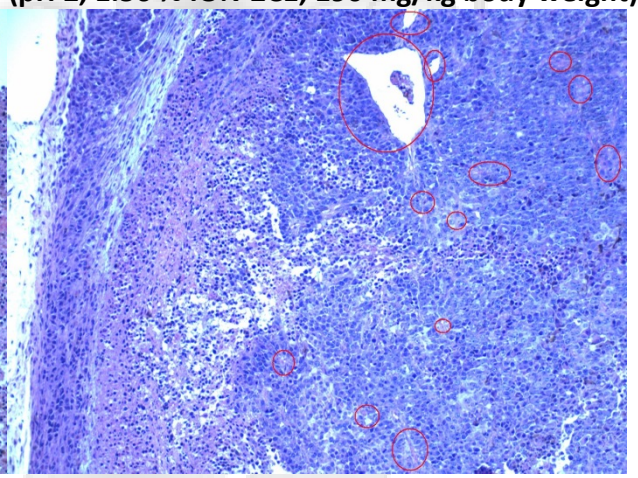
2x Treated (pH 2, 6.25 % ION-ZC1, 760 mg/kg body weight)



4x Treated (pH 2, 3.13 % ION-ZC1, 380 mg/kg body weight)



8x Treated (pH 2, 1.56 % ION-ZC1, 190 mg/kg body weight)



16x Treated (pH >5, 0.78 % ION-ZC1, 95 mg/kg body weight)

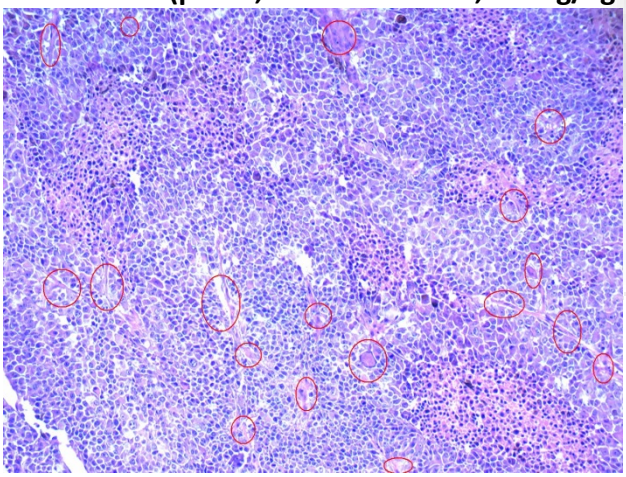


Figure H6. Blood vessel counting in tumor sections (continued)

B) Number of blood vessels versus ION-ZC1 dose.

Mouse tumor section ID	ION-ZC1 dose mg/kg body weight	Blood Vessel count
2x	760	**8**
4x	380	**8**
8x	190	**13**
16x	95	**18**
Control	0	**21**

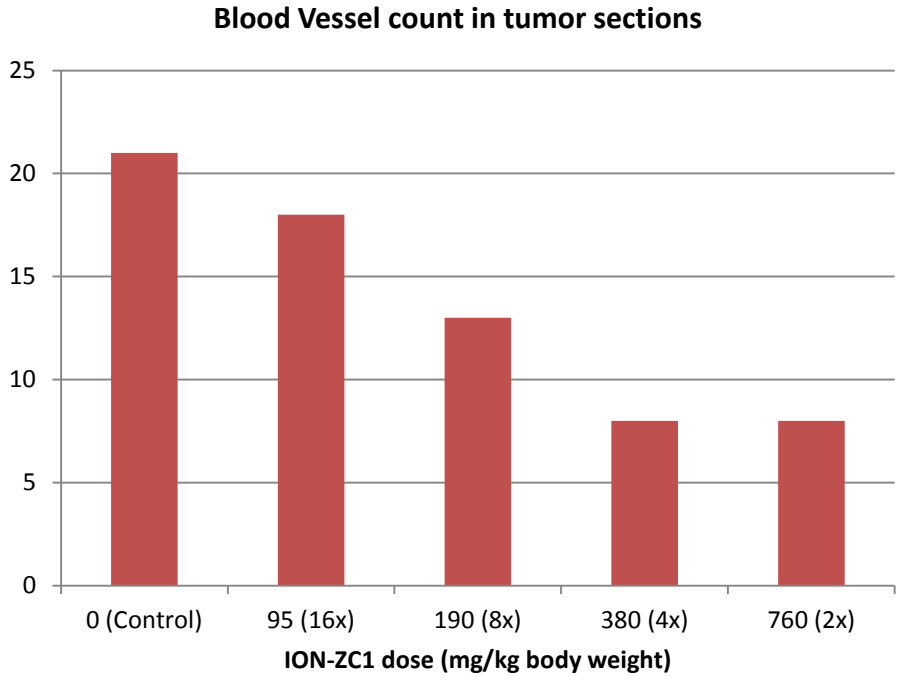


FIGURE H7. NECROSIS – IMAGE ANALYSIS
Estimation of necrosis rate **(ICY – SPOT DETECTION) –TYPE II**

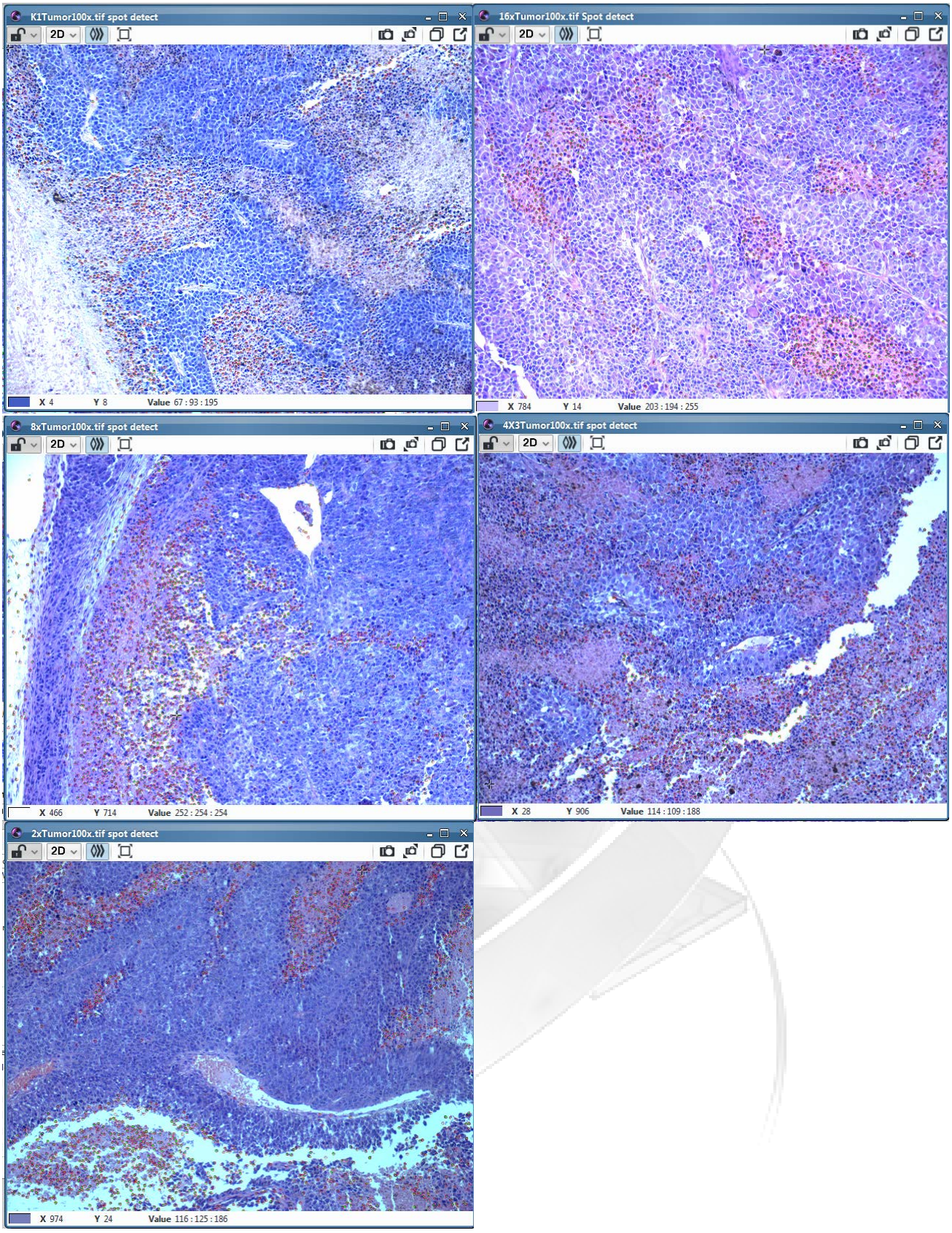
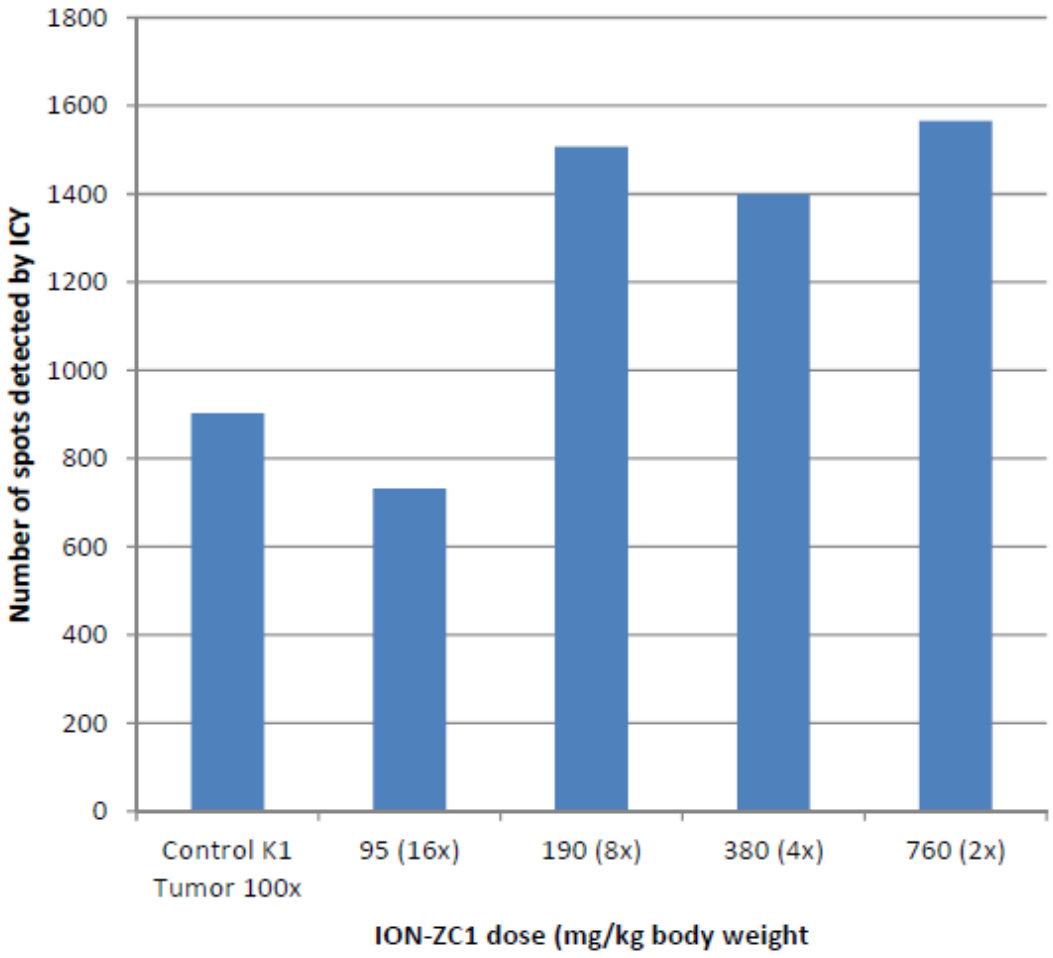


FIGURE H7. NECROSIS – IMAGE ANALYSIS (CONTINUED).
Number of spots detected by Icy to represent necrotic melanoma cell nuclei versus ION-ZC1 dose.



ION-ZC1 dose (designation)	No. Of spots (necrotic cell nuclei)
Control	902
95 mg/kg BW (16x)	732
190 mg/kg BW (8x)	1507
380 mg/kg BW (4x)	1399
760 mg/kg BW (2x)	1566

SIGNATURE

ION-ZC1

I THE UNDERSIGNED DECLARE THAT THE METHODS, RESULTS AND DATA CONTAINED IN THIS REPORT FAITHFULLY REFLECT THE PROCEDURES USED AND RAW DATA COLLECTED DURING THE STUDY.

DR ZSOLT KERESZTESSY

STUDY DIRECTOR

INDEVION KFT.
4032 Debrecen, Balassi Bálint u. 6. 1/10.
Cégjegyzékszám: 09-09-017601
Adószám: 14911680-1-09
Bankszlsz: 60600084-11053851

DATE: DEBRECEN 6TH JUNE 2016

APPENDICES

APPENDIX 1. GENERAL OUTCOMES USED IN ANTI-TUMOR EFFICACY STUDIES

Endpoint	Comment
In vivo	
Tumor onset	Time to palpable tumor mass of predetermined size
>Tumor growth rate	Assessment of tumor volume throughout time
>Number of tumor-bearing animals	Frequency of cure
>Tumor burden *in vivo* at set time	Weight of tumor
	Organ with metastases
>Tumor growth delay	Volume estimated (mm3) two-dimensional measurement
	Delay of time for tumor to reach specific volume
Tumor cell kill	Log10 total tumor cell kill
	Net log10 tumor cell kill
>Incidence of metastasis	Gross count (lungs)
	Cell count, resistance, florescence, 125IUdR uptake
>Survival—life span	Increase in median survival time
>Survival— number alive	Percent cure at predefined time
Ex vivo	
>Gross pathology	Ulceration/central necrosis,
	invasion or tissue distribution and gross lesions
	Metastasis.
	Angiogenesis
>Histopathology	H&E staining
	Morphometrics
	Inflammatory cell infiltration
	Mitotic index, cellular apoptosis ?
>Immunohistochemistry	T cell, macrophage, and DC infiltration
	Angiogenesis and lymphoangiogenesis ?
	Tumor cell apoptosis
	Enzyme and cytokine levels
Molecular pathology	Cytokines/chemokines or enzymes in serum or qRT-PCR of tumor, blood, spleen
>Hematology	Complete blood count, platelets, spleen, marrow
	Blood/spleen/marrow/thymus differential
>Immunology	Phenotype spleen, blood, tumor-infiltrating nonparenchymal cells and their function including qRT-PCR

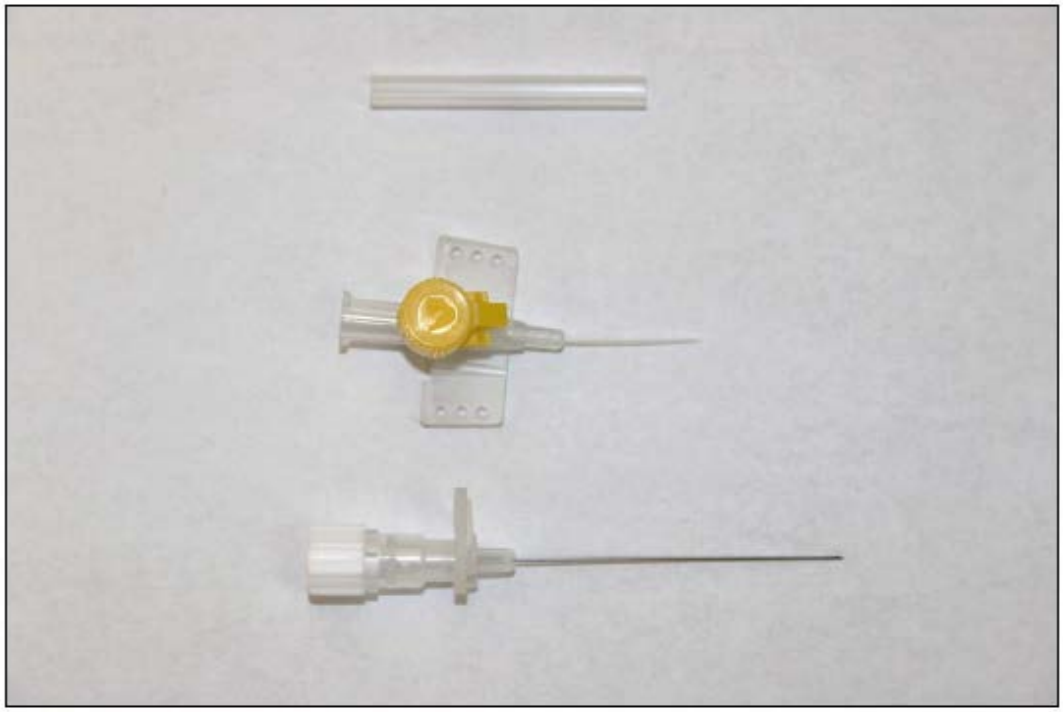
APPENDIX 2. C57BL/6 mouse

http://www.criver.com/products-services/basic-research/find-a-model/c57bl-6n-mouse

http://www.criver.com/files/pdfs/rms/c57bl6/rm_rm_d_c57bl6n_mouse.aspx

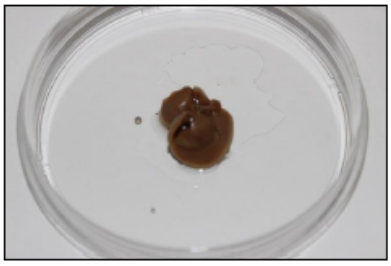
Baby branule

Injection (plastic) needle for the treatment



ORGANS

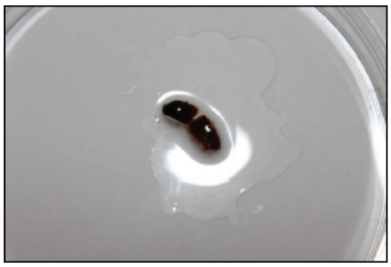
Organ photos from Control C57BL/6J mouse after dissection



Liver

Heart

Kidneys



Lien

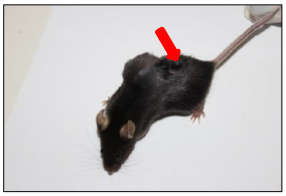
Lungs

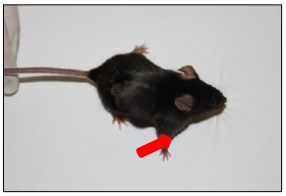
Brain

TUMOR MODEL GENERATION

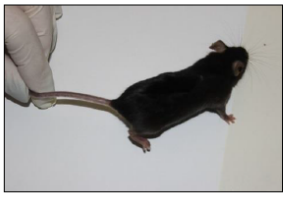
Photos of C57BL/6J mice three weeks after tumor generation

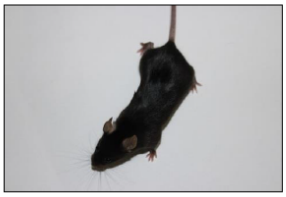
Mice 1. – 1x10^6 LLC (Tumor formation)



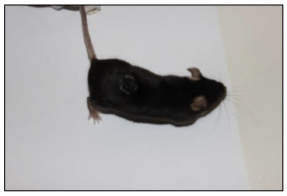


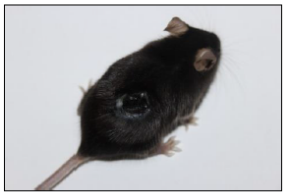
Mice 2. – 3x10^6 LLC (Tumor formation)





Mice 3. – 1x10^5 B16F10 (No tumor formation)





Mice 4. – 5x10^5 B16F10 (No tumor formation)

Appendix H1. Tumors and necropsy demonstration

Photos of Control C57BL/6J mice before sacrifice and after autopsy

Tumor formation from outside (1)

Tumor formation from outside (2)

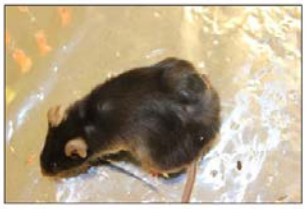
Thickened colon session (1)

Thickened colon session (2)

Tumor section

04.12.2015 1

Photos of treated (2x) C57BL/6J mice before sacrifice and after autopsy



Tumor formation from outside (1)

Tumor formation from outside (2)

Thickened colon session

Tumor section

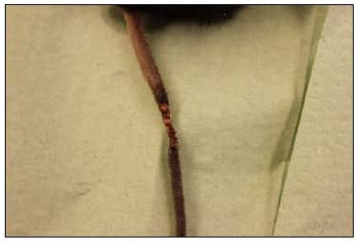
04.12.2015 2

Appendix H1. Tumors and necropsy demostration (continued)

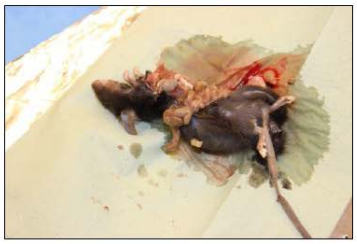
Photos of treated (4x) C57BL/6J mice before sacrifice and after autopsy

Tumor formation from outside (1)

Tumor formation from outside (2)



Injection site at the tail: caustic effect of the acidic pH



Thickened colon session

08.12.2015

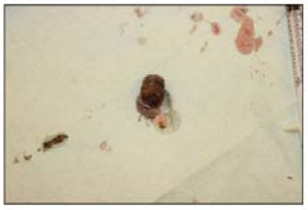
3

Photos of treated (8x) C57BL/6J mice before sacrifice and after autopsy

Tumor formation from outside

Injection site at the tail: healthy tissue, no effect

Thickened colon session



Thickened colon section

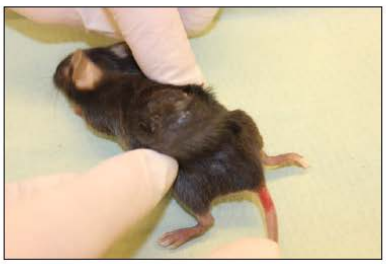
08.12.2015

4

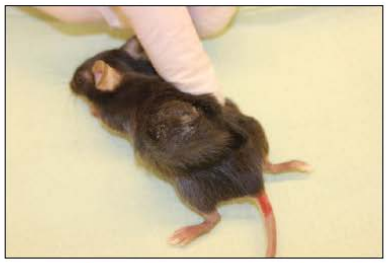
Appendix H1. Tumors and necropsy demonstration (continued)

Photos of treated (16x) C57BL/6J mice before sacrifice and after autopsy

Tumor formation from outside (1)



Tumor formation from outside (2)



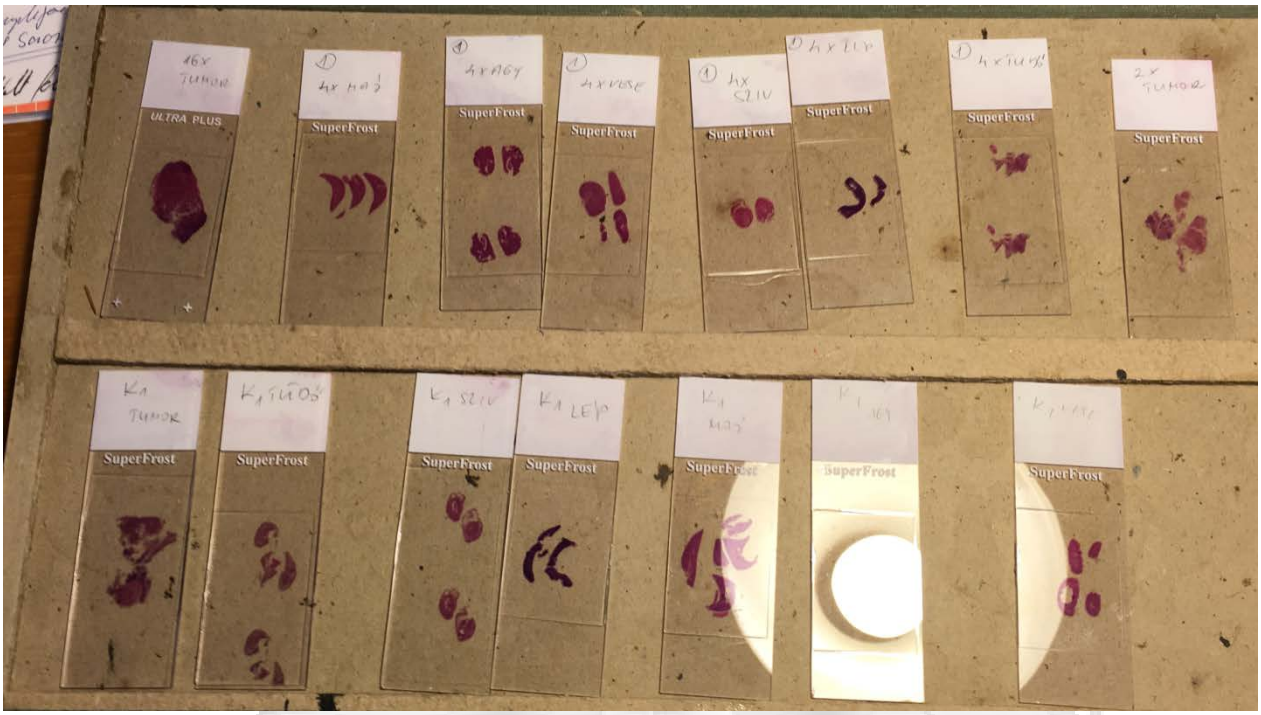
Tumor formation from outside (3)

Thickened colon session

11.12.2015

5

Appendix H2. HISTOPATHOLOGY SLIDES – examples for organs and tumors



Appendix H3. T cell MPh DC immunohistochemistry procedures

Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival.

Journal of Translational Medicine 2007, 5:62 doi:10.1186/1479-5876-5-62.

http://www.translational-medicine.com/content/pdf/1479-5876-5-62.pdf

Patient selection, immunohistochemistry and T cell assays

After institutional review board approval and informed consent, peripheral blood mononuclear cells from CRC patients were collected and frozen for T cell analysis. All analyses were performed in compliance with the Helsinki Declaration. HLA-A2-positive patients were tested for the presence of T-cell responses against the HLA-A*0201-presented T cell epitopes Ep-CAM p263-271, her-2/neu p654- 662, and CEA p571-579 by ELISPOT assay. HLA analysis and ELISPOT were performed as previously described [7,8]. Positive T-cell responses were also defined as previously described [7-9]. Immunohistochemistry for FOXP3, CD3, and CD8 had been performed on the same patient samples earlier [23]. Sufficient tumor sections for immunohistochemistry and sufficient clinical cancer were required for inclusion.

Immunohistochemistry and microscopic analysis

For immunostaining, 4 µm thick sections were cut, deparaffinized, and subjected to heat-induced epitope retrieval before incubation with antibodies. For this purpose, sections were immersed in sodium citrate buffer at pH 6.0 and heated in a high-pressure cooker. After cooking, the slides were rinsed in running water, washed with Tris-buffered saline, pH 7.4 and incubated with the primary antibodies. All primary antibodies employed are listed in Table 1. For double labelling S100/CD163, slides were incubated with the polyclonal rabbit antibody against S100 protein, blocked using a commercial peroxidase-blocking reagent and developed using the streptavidin peroxidase kit (Dako, Glostrup, Denmark). Sections were then incubated with the mouse monoclonal antibody against CD163, and the streptavidin AP kit (Dako) was used for detection. Alkaline phosphatase was developed using Fast Red as the chromogen, while peroxidase was visualized with diaminobenzidine chromogen as the substrate. Tonsillar tissue with follicular hyperplasia served as positive controls, and negative controls were performed by omitting the primary antibodies. Ten randomly chosen high power fields (1 HPF = 0,237 mm2) were analyzed for macrophage or dendritic cell infiltration in the tumor tissue and the tumor stroma and averaged in each case

Appendix H3. T cell MPh DC immunohistochemistry procedure (continued)

Dendritic Cells, T-Cell Infiltration, and Grp94 Expression in Cholangiocellular Carcinoma

HUMAN PATHOLOGY Volume 35, No. 7 (July 2004)

http://ac.els-cdn.com/S0046817704002242/1-s2.0-S0046817704002242-main.pdf?_tid=ab0d2554-bb8f-11e5-a9d7-00000aab0f6b&acdnat=1452866323_afdf96f4d980dc5113409213287585ce

Immunohistochemistry of CD83, CD1a, grp94, CD4, and CD8 Formalin-fixed, paraffin-embedded tissues were cut into 4-m-thick sections and mounted on glass slides coated with poly-L-lysine. The endogenous peroxidase activity was blocked for 30 minutes with 3% (v/v) hydrogen peroxidase. We used the tyramide signal amplification system (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. The sections were preincubated with TNB blocking buffer containing 0.1 mol Tris-HCl (pH 7.5), 0.15 mol NaCl, and 0.5% (w/v) blocking reagent (supplied in a kit). Subsequently, primary antibodies were applied and left overnight at 4°C. The primary antibodies used were anti-CD83 (Serotec, Oxford, UK), anti-CD1a (Serotec), anti-grp94 (NeoMarkers, Fremont, CA), anti-CD4 (Novocastra, Newcastle Upon Tyne, UK), and anti-CD8 (Dako, Glostrup, Denmark). The sections were washed in TNT wash buffer containing 0.1 mol Tris-HCl (pH 7.5), 0.15 mol NaCl, and 0.05% (v/v) Tween 20. Antibody binding was visualized using 3,3'-diaminobenzidine. After rinsing in water, cell nuclei were counterstained with hematoxylin, and the sections were dehydrated and coverslipped. For double immunostaining, sections were first incubated in 0.01 mol (pH 6.0) or 1 mmol (pH 8.0) ciytric acid buffer for 10 minutes after staining with alkaline phosphatase– conjugated streptavidin (Dako). The second step for the immunohistochemistry was done as described earlier using diaminobenzidene as the stain. As a negative control, normal mouse IgG (mouse primary antibody control; Zymed, San Francisco, CA) was used as the primary antibody. In 5 randomly chosen high-power fields (400) from 3 areas (CR, cancerous region; IM, invasive margin; and NC, noncancerous region), the numbers of cells positive for CD83, CD1a, CD8, and CD4 were counted using an Olympus BX50 microscope (Olympus, Lake Success, NY) and the same microscope objective. The immunohistochemical staining for grp94 was evaluated by 2 independent observers who were blinded to the source of the specimens, and the entire area of each section was observed. Immunoreactivity of cancer and non-cancer cells for grp94 was classified as negative () if 5% of the total number of cancer or non-cancer cells were positive, and as positive () if

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5% of the total number of cancer or non-cancer cells were positive.

Appendix H4. TUNEL Assay APOPTOSIS DETECTION in situ Roche

https://lifescience.roche.com/wcsstore/RASCatalogAssetStore/Articles/05242134001_05.08.pdf

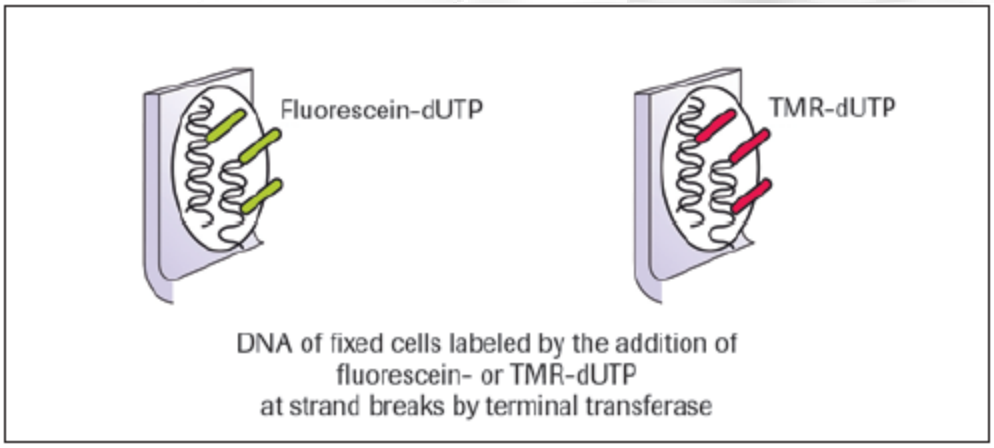
In Situ Cell Death Detection Kit, TMR red Cat. No. 12 156 792 910, 50 tests

Type Direct TUNEL labeling assay

Useful for Detection of DNA strand breaks in apoptotic cells by flow cytometry or fluorescence microscopy

Sample material Cells in suspension, adherent cells, cell smears, frozen or paraffin-embedded tissue sections

Test principle The assays use an optimized terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP or TMR-dUTP.



Method End-labeling of DNA with fluorescein-dUTP or tetramethylrhodamine-dUTP (TMRdUTP), followed by direct analysis of fluorescent cells

Significance of the kit This two In Situ Cell Death Detection Kits, measure and quantitate cell death (apoptosis) by labeling and detection of DNA strand breaks in individual cells by flow cytometry or fluorescence microscopy. The kits offer a direct TUNEL detection method, for maximum sensitivity and minimal background.

Sensitivity The enzymatic labeling allows the detection of an apoptotic event that occurs, prior to changes in morphology and even before DNA fragments become detectable in the cytoplasm. It detects early stage of DNA fragmentation in apoptotic cells. This is especially important if apoptosis is studied in vivo, e.g., in tissue sections, since apoptotic cells are rapidly and efficiently removed in vivo.

Specificity The amount of DNA strand breaks in apoptotic cells is so large that the degree of cell labeling in these assays is an adequate discriminator between apoptotic and necrotic cells.

Time 1–2 h (+ sample preparation, permeabilization, etc.)

Benefits Easily obtain results as no secondary detection system is required.

Identify apoptosis at a molecular level (DNA-strand breaks) and cells at the very early stages of apoptosis.

Achieve maximum sensitivity with minimal background.

Appendix H4. TUNEL Assay APOPTOSIS DETECTION in situ Roche (continued)

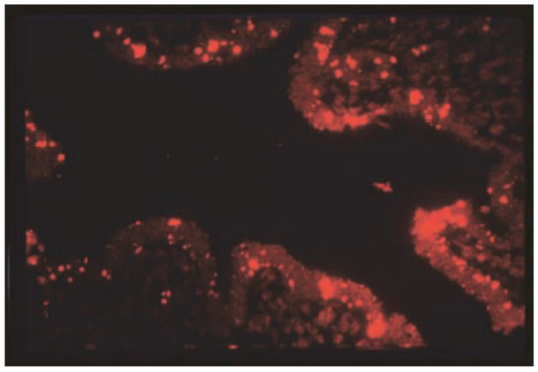
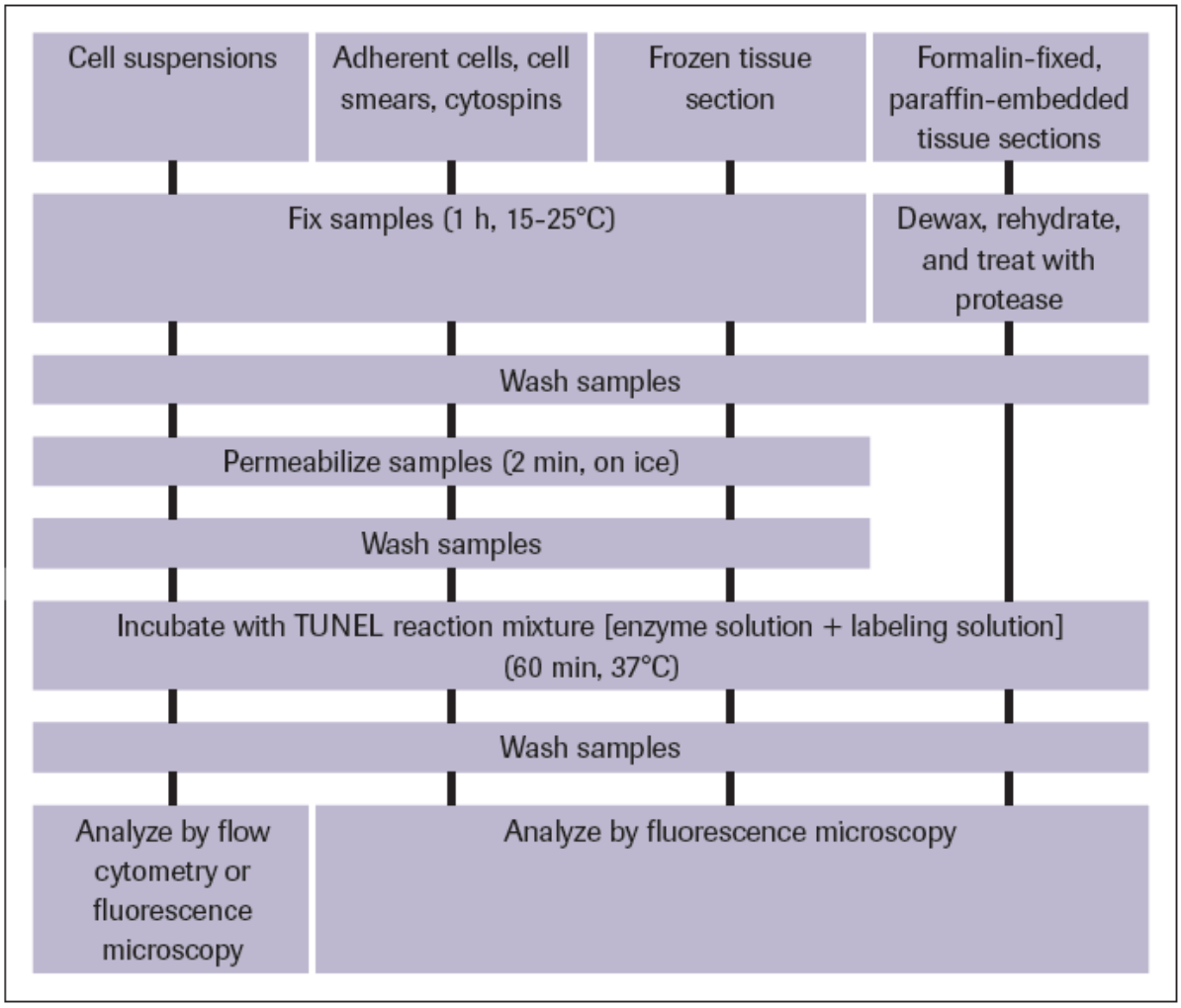
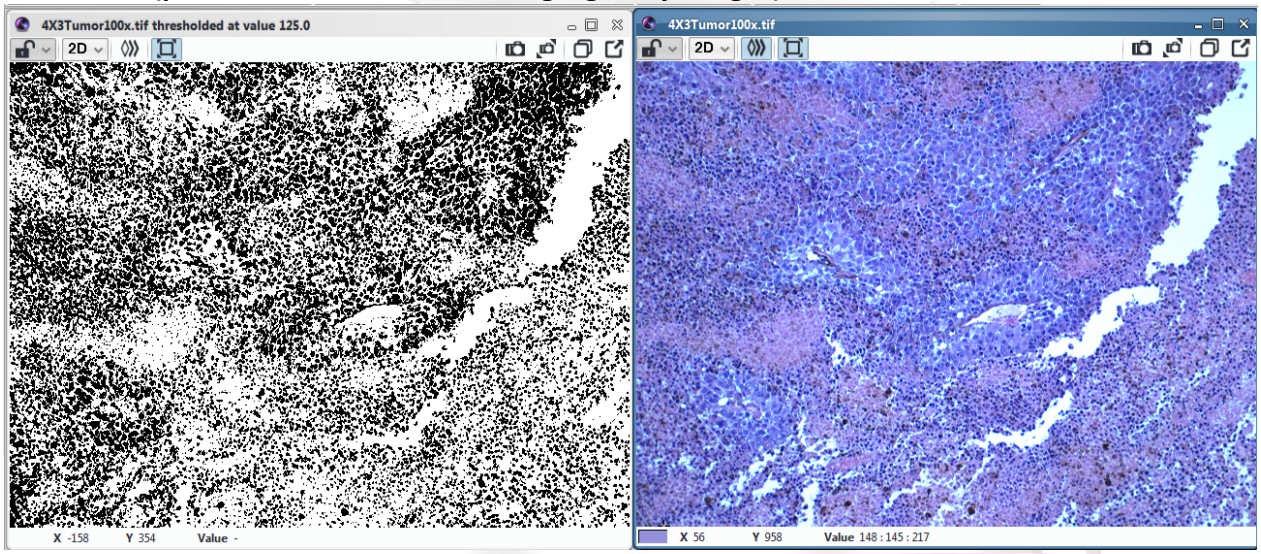


Figure 31: Use of the *In Situ* Cell Death Detection Kit, TMR red to detect apoptotic cells (red) by immunohistochemical staining. Tissue from rabbit endometrium was assayed with the kit and viewed under a fluorescence microscope. Apoptotic nuclei stain bright red; limited fluorescence is visible in background tissue.



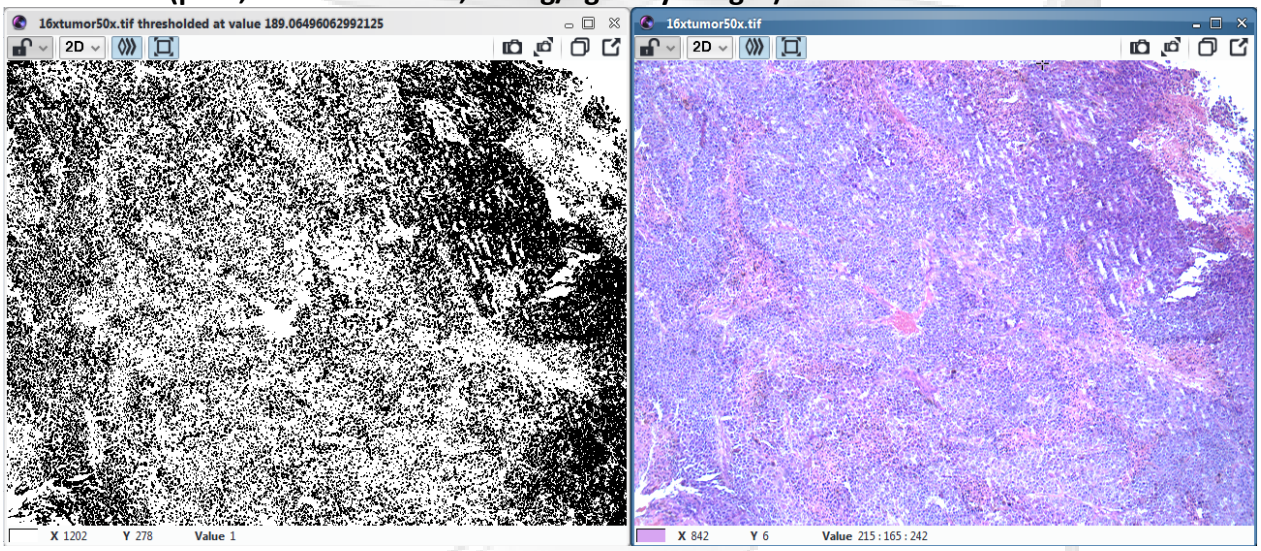
APPENDIX H5.
NECROSIS – IMAGE ANALYSIS - ALTERNATIVE
Estimation of necrosis rate **(ICY - THRESHOLD) –TYPE I**

4x Treated (pH 2, 3.13 % ION-ZC1, 380 mg/kg body weight)



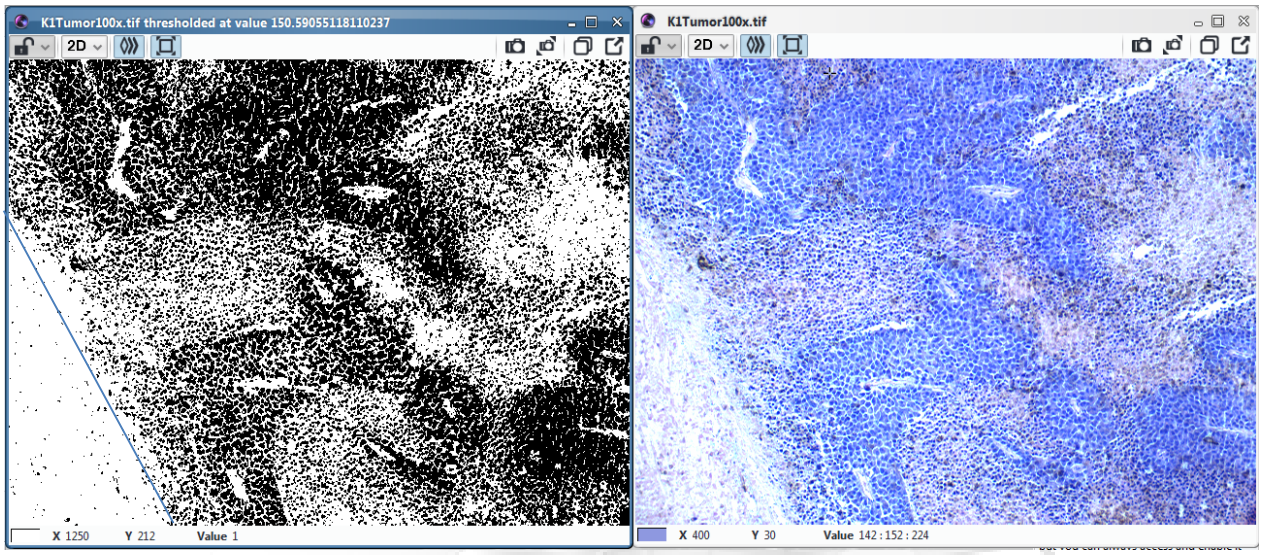
Necrotic (white): 728890 pixels
Alive (black): 499910 pixels

16x Treated (pH 5, 0.78 % ION-ZC1, 95 mg/kg body weight)



Necrotic (white): pixels 713670
Alive (black): 515130

K1 tumor control



Necrotic (white): 500125 pixels
Alive (black): 581179 pixels