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ORIGINAL ARTICLE

Selective Inhibition of HIF1α Expression by $ZnSO_4$ Has Antitumoral Effects in Human Melanoma

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Abstract

Zinc as an essential trace metal is a ubiquitous component of various molecules of the cell. Studies indicated that it may modulate functions of various cancer cell types, and can even inhibit metastasis formation in experimental models. In melanoma, zinc was shown to affect melanin production and to induce apoptosis. Using human melanoma cell lines, we have tested the effects of $ZnSO_4$ on cell proliferation, survival, migration as well as in vivo on experimental liver colony formation. We have found that $ZnSO_4$ has antiproliferative and proapoptotic effects in vitro. In SCID mice intraperitoneal administration of $ZnSO_4$ specifically inhibited liver colony formation without affecting primary tumor growth. To reveal the molecular mechanisms of action of zinc in human melanoma, we have tested mRNA expression of zinc finger transcription factors and found a strong inhibitory effect on HIF1α, as compared to WT1 whereas HIF2α and MTF1 expression was unaffected. Immunohistochemical detection of HIF1α protein in liver metastases confirmed its decreased nuclear expression after in vivo $ZnSO_4$ treatment. These data indicate that in human melanoma zinc administration may have an antimetastatic effect due to a selective downregulation of HIF1α.

Keywords $ZnSO_4$ · Human melanoma · HIF1α · Metastasis inhibition

Introduction

Zinc is an essential trace element for cells and plays a broad role in cellular functions. By the help of specific zinc transporters it provides an irreplaceable factor for several intracellular proteins which can be classified as zinc-dependent enzymes (hydrolases, ligases, transferases, oxidoreductases), transcription factors, signaling proteins and other zinc-dependent proteins.

The majority of human cancers is characterized by decreased intratumoral zinc levels [1, 2]. Furthermore, decreased plasma zinc levels were also detected in various types of cancer such as colorectal [3], gastric [4], head and neck [5] or breast cancers [6]. Nutritional supplements for cancer patients frequently contain zinc although zinc deficiency is not a universal phenomenon in this disease. Unlike in case of other metallic elements, zinc overdose does not have a toxic effect on normal cells, accordingly, no significant side effects were reported in this patient group except for metal allergy [7, 8]. In an experimental model we have provided the first evidence for the direct antitumoral and antimetastatic effect of $ZnSO_4$ in murine Lewis lung carcinoma [9]. Later on it was shown by others that zinc has antitumoral effect on human prostate [10] or breast cancer cells as well [11]. Recently, it was found that zinc has a newly recognized function, it can regulate gene expression in human renal cell carcinoma and colorectal cancer cell lines [12, 13].

Malignant melanoma is one of the most aggressive human tumor types resistant to chemo- and radiotherapy. Although a significant improvement was recently achieved in the treatment of this tumor type by using target therapies (BRAF, MEK inhibitors) as well as immunotherapy (anti-CTLA-4 or anti-PD-1/PD-L1 antibodies), the survival of patients with advanced melanoma is still incomparable to that of other cancer patients [14]. In most cancer types maximal benefit can be

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achieved by combination of the target therapies with classical cytotoxic ones but in melanoma this cannot be applied. Recently it was revealed that zinc may regulate melanin production and melanoma cell survival [15], accordingly, we have aimed at revisiting the possible antitumoral effect of zinc in human melanoma.

Materials and Methods

Cell Lines

The HT168-M1 cell line is a BRAF mutant human melanoma cell line derived from A2058 by in vivo selection for liver colony forming potential [16]. HT199 is a BRAF mutant human melanoma cell line established in our laboratory [17], and the BRAF mutant WM983B was kindly provided by M. Herlyn, Wistar Institute, Philadelphia, PA. As normal controls, primary cultures of human skin fibroblasts and microvascular endothelial cells have been used. Melanoma cells were cultured in RPMI1640 medium (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma), fibroblasts were cultured in Medium 199 supplemented with 10% FBS while endothelial cells were cultured in DMEM/F12HAM 1:1 supplemented with 20% FBS. Cells were kept at 37 °C at 5% CO_2 as monolayers. Cell suspensions were produced using either 0.02% EDTA (melanoma) or trypsin-EDTA (fibroblasts and endothelial cells).

Cell Proliferation Assay

Cells (3-5 × 10^3) were plated in 96-well plates in RPMI/FCS and after 24 h treated with $ZnSO_4$ for 96 h in the presence of 2.5% FBS. At the end of the experiment 0.5 mg/ml MTT solution was added to the wells for 4 h at 37 °C, the supernatant was discharged, the cell layer was covered by DMSO and absorbance was measured at 570 nm with a Bio-Rad microplate reader (Bio-Rad, Hercules, CA).

Viability Assay

Cells were cultured in 24-well plates using 2 × 10^4 cells/well as above. After treatment with $ZnSO_4$ for 24 h, the monolayer was detached with 0.02% EDTA, the suspension was stained with Trypan-Blue dye, and the ratio of living cells was determined under lightmicroscope.

Apoptosis Assay

Cells (5 × 10^5/well) were cultured in 6-well plates as above. After treatment with $ZnSO_4$ for 24 h, cells were detached with 0.02% EDTA, and fixed in 70% EtOH at -20 °C. After 1 h incubation with propidium-iodide and RNase (Cystain PI Absolute T, Partec, Görlitz, Germany), we determined the amount of DNA in cells by flow cytometer (CyFlow, Partec). The percentage of the apoptotic cells (sub-G1 fraction) was analyzed by the FlowMax software.

Matrix Adhesion

24-well plates have been preincubated with fibronectin (Sigma-Aldrich, St.Louis, MO), collagen-I-III (isolated from rat tail) or PBS at 37 °C. Tumor cells were incubated on matrix proteins for 45 min at 37 °C in 24-well plates using 2 × 10^4 cells/well in the presence of various concentrations of $ZnSO_4$ diluted in serum-free RPMI. Non-adherent cells were washed off by RPMI, adherent cells were fixed with 10% trichloroacetic acid, and stained with sulforhodamine B dye for 15 min. Plates were repeatedly washed with 1% acetic acid to remove excess dye. Protein-bound dye was dissolved in 10 mM Tris buffer and then optical density was measured at 570 nm using a microplate reader (Bio-Rad).

Migration Analysis

To investigate cell migration videomicroscopic analysis was carried out as described earlier. [18]. Cells were plated in 24-well plate with complete growth medium to overnight attachment. For the videomicroscopic examinations the medium was changed to CO_2-independent medium (Gibco-BRL Life Technologies, UK) with 10% FCS. The culture plate was kept in a 37 °C custom designed incubator built around an inverted phase-contrast microscope (World Precision Instruments, USA). Images of 3 neighboring microscopic fields were taken of each well every 5 min for 1 day before and 1 day after the treatment with $ZnSO_4$. Migration data was obtained by analyzing the images from the phase contrast microscope with a cell-tracking program enabling manual marking of individual cells and recording their position parameters into data files. The parameter migrated distance was calculated by averaging for each cell the displacement for each time interval up to 20 h in the after treatment. For each condition at least 20 cells were tracked from three fields of views and all experiments were repeated twice.

Liver Colony Formation Assay

HT168-M1 human melanoma cells were injected intrasplenically into female SCID mice (10^4 cells/animal). Animals were treated with $ZnSO_4$ intraperitoneally at concentrations of 100, 500, and 1000 mg/bwkg/day, started at day 4. Physiological saline served as control. Study groups contained 9–10 animals. At termination (when the weight loss of animals achieved a minimum of 20%) animals were treated with Nembutal, the weight of spleen and liver were measured and liver surface colonies were counted under stereomicroscope. Animal experiments have been approved by the local IRB.

Expression of Zinc Finger Proteins

HT168-M1 cells were placed in 6-well tissue culture plates (6×10^3/well) in RPMI 1640 medium containing 5% FBS. After overnight incubation at 37 °C, adherent cells were treated with $ZnSO_4$ at final concentration of 100 μM for 10 h. Total RNA was extracted from the adherent cells using TRIzol® Reagent (Life Technologies, Thermo Fischer Scientific, Waltham, MA) according to the manufacturer's protocol. One μg of total RNA was reverse transcribed using oligo(dT)/random primer (2.5 μM) and MMLV reverse transcriptase (200 unit/μl, Finnzyme®, Fischer Scientific). The reaction mixture was incubated at 37 °C for 50 min, heated at 85 °C for 10 min. The primers were designed by ArrayDesigner software. DNA amplifications were performed using AmpliTaq Gold® 360 PCR Master Mix (Fischer Scientific) and Mastercycler gradient thermal cycler supplied by Eppendorf (Hamburg, Germany) at 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 59 °C for 1 min, chain elongation at 72 °C for 2 min. After amplification 10 μl of PCR products were separated on 3% agarose gel and stained with ethidium bromide.

For quantitative measurement of the expressed genes (HIF1A, HIF2A, MTF1 and WT1) q-PCR reactions were used. Each 25 μl reaction mixture contained 12.5 μl of 2X iQ SYBR® GreenSupermix (Bio-Rad), 0.5 μl of each primer for final concentration of 200 nM and 11.5 μl of the diluted cDNA. The primers used are presented in Table 1. Cycling conditions comprised 3 min of iTaq™ DNA polymerase activation at 95 °C, 40 cycles at 95 °C for 30 s, at 55 °C for 30 s and at 72 °C for 1 min. Starting quantities were defined on the basis of standard five-fold dilution series (1X-625X) carried out with control cDNA of human K562 cell line. Relative expression of examined genes was determined by normalizing starting quantities to the housekeeping beta-actin to starting quantities from the same cDNA sample.

Immunohistochemistry of HIF1α Protein

Metastasis containing SCID mice liver samples have been fixed in 10% buffered formaldehyde overnight and embedded into paraffin. HIF1α protein was detected by immunohistochemistry on 5 μm sections using anti-human HIF1α polyclonal rabbit antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:100 and incubated for 60 min at room temperature. After buffer washings sections were incubated with secondary antibody using EnVision Flex mini kit (Dako, Glostrup, Denmark), and HRP was visualized with diaminobenzidine chromogen. Cell nuclei were counterstained with hematoxylin. Semiquantitative evaluation of nuclear HIF1α protein expression was performed with light microscopy counting 3 individual fields assessing a minimum of 50 tumor cells/field.

Statistical Analysis

For statistical evaluation of the results of in vitro experiments Student's t test was used, while those of the in vivo experiments were analyzed with Mann-Whitney U-test.

Results

Three day long in vitro $ZnSO_4$ exposure resulted in inhibition of cell proliferation of human melanoma cells in the concentration range of 100–300 μM, while human fibroblasts (and endothelial cells – data not shown) were unaffected (Fig. 1a). One day in vitro exposure of $ZnSO_4$ of human melanoma cells induced apoptosis in human melanoma cells (Fig. 1b). Meanwhile 45 min in vitro exposure to $ZnSO_4$ did not alter melanoma cell adhesion to FN or collagen III (Fig.2a) and did not affect 20 h haptotactic migration either (Fig.2b), suggesting a selective negative impact on cell proliferation/survival.

Parenteral administration (ip.) of $ZnSO_4$ into SCID mice after intrasplenic injection of HT168-M1 human melanoma cells did not affect the primary tumor weight in the spleen nor liver weight. (Fig.3a) However, the in vivo $ZnSO_4$ treatment of mice inhibited liver colony formation even at the lowest dose applied (100 mg/bwkg) without a clear dose dependence (Fig. 3b).

Based on previous reports, we have analyzed the expression of the zinc finger transcription factors HIF1α and HIF2α in HT168-M1 cells by RT-PCR following 10 h treatment with 100 μM $ZnSO_4$. In untreated melanoma cells HIF1α expression was found to be predominant over HIF2α. Data indicate

Table 1 Primer sets used throughout the studies

Gene name	Ref. seq. Number	Sense primer	Antisense primer
HIF1A	NM_001530.2	AACTTCTGGATGCTGGTGATT	TGTTCATAGTTCTTCCTCGGC
WT1	NM_001198552	GTTACAGCACGGTCACCTTC	GTGGCTCCTAAGTTCATCTGATT
HIF2	NM_001430	TCCTGAGTGAGATTGAGAAG	GCTTGGTGAATAGGAAGTTAC
MTF1	NM_005955	GTCCAGACAACAACATCATC	CAATAAGAACAGTGGTCCTATC

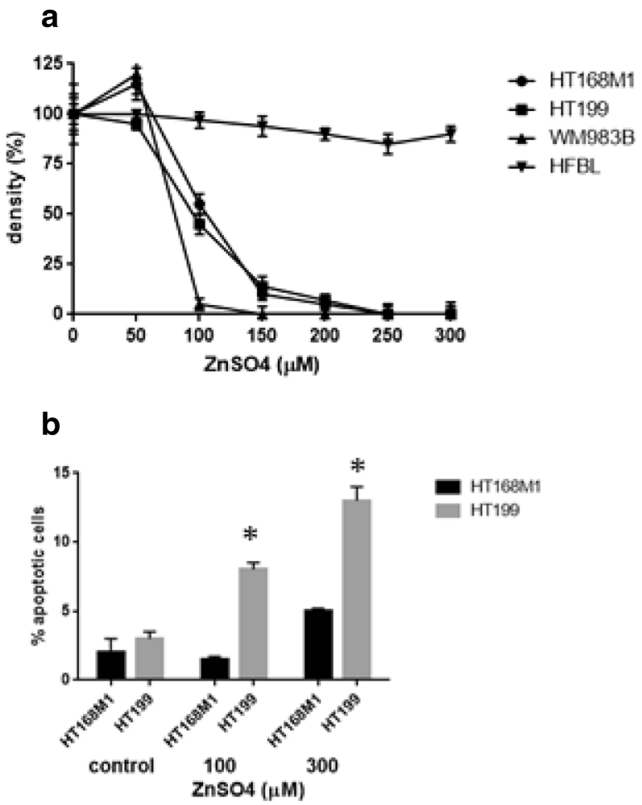

Fig. 1 In vitro effects of $ZnSO_4$ on the growth of human melanoma cells. A) Effect on proliferation of melanoma cells and fibroblasts (MTT test). HFBL = human fibroblast. Data presented in relative cell density expressed in % of control and represent mean ± SD. B) Apoptosis induction by $ZnSO_4$ exposure in melanoma cells. Data presented in % of apoptotic cells and shown as mean ± SD. *$p < 0.05$

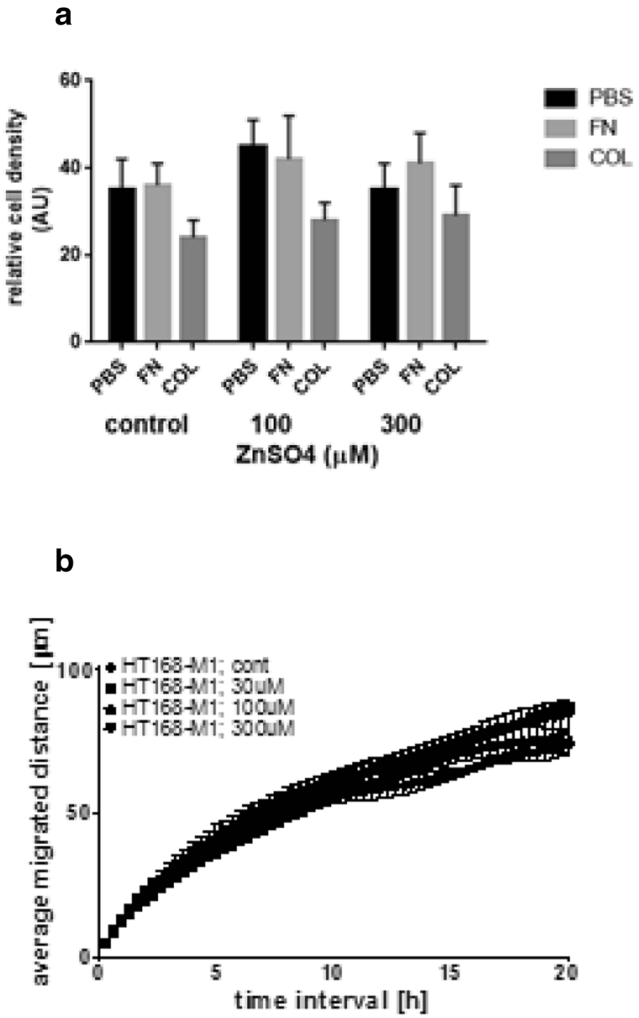

Fig. 2 In vitro effects of $ZnSO_4$ on matrix adhesion and migration of HT168-M1 human melanoma cells. **a** Effect on adhesion to matrix proteins in vitro. Data presented in cell density and represent mean ± SD. **b** Effect on haptotactic migration. Average migrated distance was evaluated by videomicroscopy for the first 20 h after treatment. Data are shown as average ± SEM from at least two independent measurements and three microscopic fields of view

that in melanoma cells the expression of HIF1α significantly decreased following $ZnSO_4$ exposure, while a moderate increase was detected in HIF2α. $ZnSO_4$ also inhibited the expression of WT1, while the expression of MTF1 was unaffected. (Fig.4) To test if HIF1α protein expression is affected by in vivo $ZnSO_4$ treatment (500 μg/bw), we have analyzed liver metastases of HT168-M1 cells in SCID mice using immunohistochemistry. In the control group metastatic foci contained frequent nuclear reaction for HIF1α beside the less intense cytoplasmic one (Fig. 5a) unlike in liver metastases of $ZnSO_4$-treated animals where nuclear labeling was rare (Fig. 5b). Quantification of nuclear HIF1α labeling of liver metastases indicated a significant decrease in HIF1α protein expression. (Fig.5d).

Discussion

We have provided evidence that $ZnSO_4$ exposure of human melanoma in vitro and in vivo has an antitumoral effect. In vitro $ZnSO_4$ inhibited proliferation and induced apoptosis while in vivo the treatment was antimetastatic without affecting primary tumor growth. It is of note that human fibroblasts as well as endothelial cells were insensitive to $ZnSO_4$ exposure. In theory, the in vivo antimetastatic effect could be explained by a pleiotropic effect of $ZnSO_4$ on the host immune system [19], but it is highly unlikely since the model system was SCID mice, therefore a direct antitumoral mechanism may be operational. We have shown that this effect does not include melanoma cell interactions with extracellular matrices and motility-independent.

Zinc was reported to regulate apoptosis of various cancers, however, its effect can be either proapoptotic (breast, prostate, ovarian cancer) or antiapoptotic (pancreatic cancer) [20]. The mechanism of action of zinc in apoptosis involves mitochondrial apoptogenesis, is mediated by BAX and is independent of p53 [20].

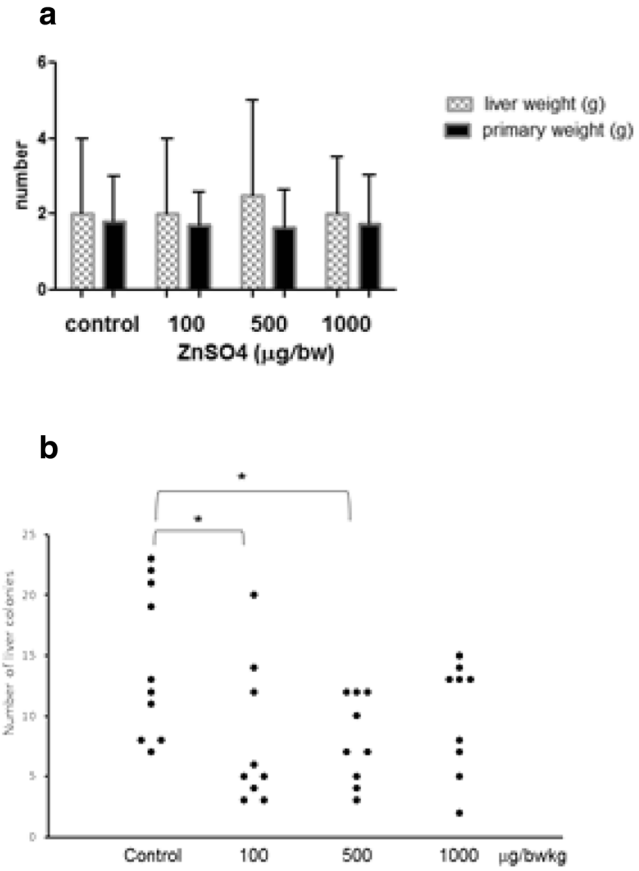

Fig. 3 Effect of $ZnSO_4$ on the spleen primary tumor and the liver colony formation by HT168-M1 melanoma cells injected intrasplenically (10^4 cells/animal) in SCID mice. **a** Effect on spleen and liver weight expressed in gr. Data are mean ± SD. **b** Effect on liver colony formation. Dots represent liver colony numbers of individual animals. $^*p < 0.05$

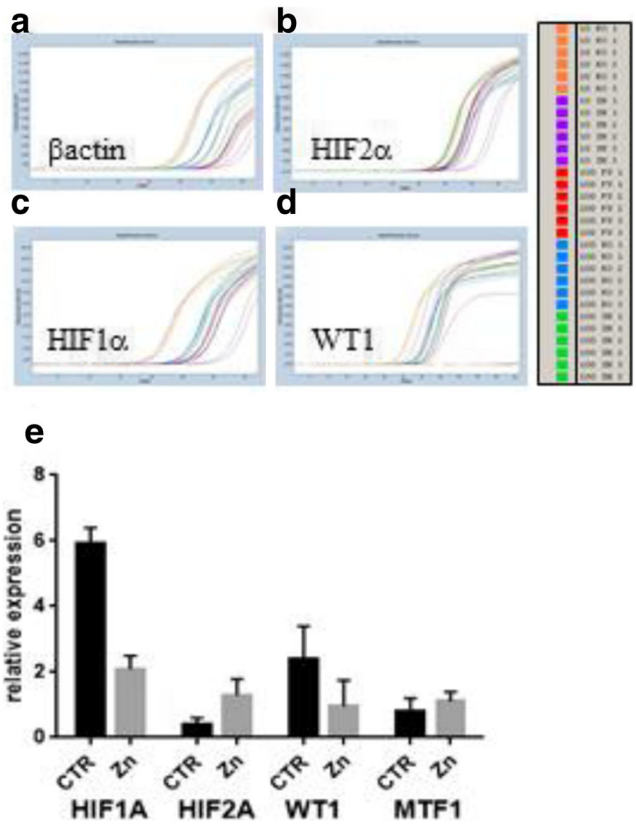

Fig. 4 Effect of $ZnSO_4$ treatment on the mRNA expression of zinc-transcription factors. Cultured HT168-M1 cells were treated with 10 or 100 μM $ZnSO_4$ for 10 h at 37 °C and mRNA was isolated from adherent cells and the expression of HIF1α, HIF2α, WT1 and MTF1 was determined by q-PCR using β-actin as control. A-D are original amplification curves of β-actin control, HIF2α, HIF1α and WT1, respectively. E) Relative mRNA expression of transcription factors as compared to β-actin = 1. Each data point is mean ± SD ($n = 6$). $^*p < 0.05$

Recently it was shown in some normal and tumor cells that zinc exposure fundamentally affects gene expression patterns which is mediated through MTF1 transcription factor. Such a zinc exposure affects the expression among others methallothioneins, CYP450, KL4 transcription factor or R-ras3 [12, 13]. More interestingly, recently it was also demonstrated that zinc exposure in vitro inhibits HIF expression in astrocytes [21] and renal cancer cells [22]. Previous studies revealed that cobalt, another rare metallic element, also regulates HIF expression but on the contrary to zinc, it stabilizes the protein, leading to „chemical hypoxia" condition [23]. We provided evidence that under our experimental conditions short exposure to relatively low concentration of $ZnSO_4$ selectively inhibits HIF1α expression unlike of the HIF2α, in BRAF mutant human melanoma cells. Zinc exposure affected the expression of another zinc finger transcription factor WT1 (which was a minor zinc-finger transcription factor in BRAF-mutant melanoma cell) while it was ineffective in case of MTF1. Two different possible mechanisms can be operational behind these zinc effects: induction of an inhibitory HIF1α variant (HIF1aZ) [24] or induction of a HIF1α negative regulator, HIPK2 [25].

HIF1α is constitutively expressed in human melanoma cells due to ROS and NFκB activity [26]. Furthermore, human melanoma carrying mutant BRAF is characterized by increased HIF1α expression [27]. Meanwhile, data suggest that the increased HIF1α expression in human skin primary melanoma has no prognostic effect [28]. In a genetically engineered mouse melanoma model, however, it was demonstrated that in BRAF mutant melanoma tumor progression is mediated by HIF1α through constitutive activity of SRC [29]. In another recent study using the same human melanoma model which was used in this work, it was found that experimental liver metastatization was dependent on HIF1α and RhoA expressions [30].

Cancer patients are taking various vitamins and trace element supplements including those containing zinc. Our data strongly suggest that a zinc-containing supplement can be beneficial for melanoma patients since human melanoma is

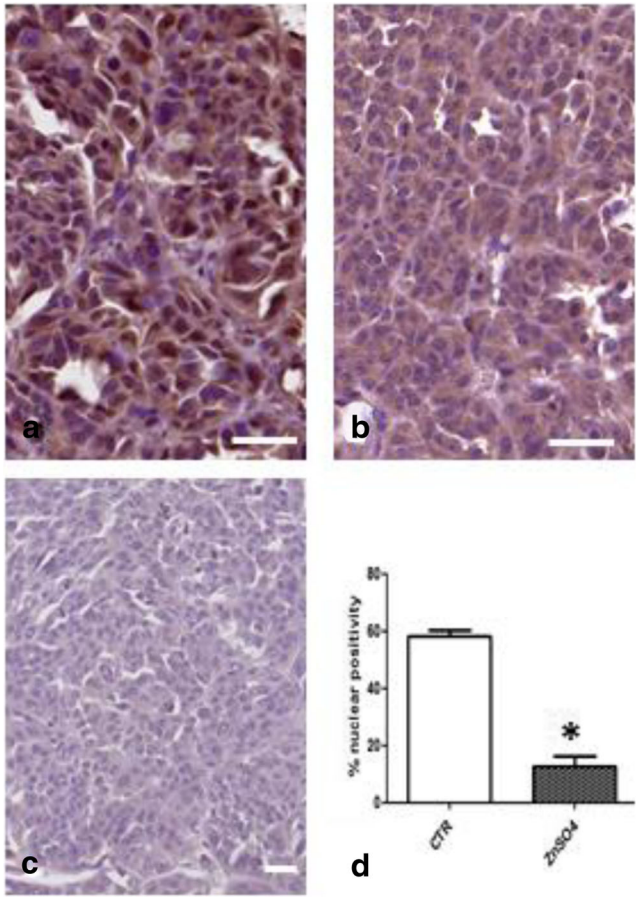

Fig. 5 Effect of in vivo $ZnSO_4$ treatment (500 μg/bw) on HIF1α protein expression in liver metastases of HT168-M1 melanoma in SCID mice as detected by immunohistochemistry (brown color). **a** Untreated control metastasis. Note the intense nuclear labeling (brown color) of the majority of tumor cells beside the cytoplasmic one. **b** $ZnSO_4$ treated liver metastasis. The cytoplasm of the majority of tumor cells is positive (brown color) but there is no nuclear labeling. **c** Negative control of immunohistochemistry (untreated melanoma metastasis). All sections have been counterstained with Hematoxylin (blue) to label nuclei. Bars represent 50 μm. D) Quantitative measurement of nuclear HIF1α protein positivity in melanoma liver metastases. Data are expressed in % of positivity and are means of three fields (± SD). * = $p < 0.05$

sensitive to zinc. However, evidence based medicine requires carefully designed clinical trials where zinc supplement must be combined with the most effective anti-melanoma therapies: mutant BRAF inhibitors or immunotherapy.

HIF overexpression, either induced by hypoxia or constitutive, is a hallmark of various cancer types and is the master regulator of the angiogenic phenotype, therefore, is a molecular cancer target. Unfortunately, few specific or selective HIF inhibitors have been developed and they were barely tested clinically [31, 32]. Previous reports and our recent data strongly suggest that zinc could well be a pharmacological HIF1α expression inhibitor which can be developed further due to low toxicity and relative selectivity.

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Compliance with Ethical Standards

Conflict of Interest The authors of this manuscript declare no conflict of interest concerning of this manuscript.

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