


RESEARCH NOTE

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# Testing ATRA and MEK inhibitor PD0325901 effectiveness in a nude mouse model for human MPNST xenografts

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## Abstract

**Objective:** Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas characterized by high recurrence rates and early metastases. These tumors arise more frequently within neurofibromatosis type 1 (NF1) and present with resistance during standard chemotherapy leading to increased mortality and morbidity in those patients. *In vitro* all-*trans* retinoic acid (ATRA) and MEK inhibitors (MEKi) were shown to inhibit tumor proliferation, especially when applied in combination. Therefore, we established a nude mouse model to investigate if treatment of xenografts derived from NF1 associated S462 and T265 MPNST cells respond to ATRA and the MEKi PD0325901.

**Results:** We demonstrated that human NF1 associated MPNST derived from S462 but not T265 cells form solid subcutaneous tumors in Foxn1 nude mice but not in Balb/c, SHO or Shorn mice. We verified a characteristic staining pattern of human MPNST xenografts by immunohistochemistry. Therapeutic effects of ATRA and/or MEKi PD0325901 on growth of S462 MPNST xenografts in Foxn1 nude mice were not demonstrated *in vitro*, as we did not observe significant suppression of MPNST growth compared with placebo treatment.

**Keywords:** Malignant peripheral nerve sheath tumors (MPNST), Neurofibromatosis type 1 (NF1), Nude mouse model, All-*trans* retinoic acid (ATRA), MEK inhibitor (MEKi), S462, T265, PD0325901, Xenograft model

## Introduction

Neurofibromatosis type 1 (NF1) is a risk factor for the development of malignant peripheral nerve sheath tumors (MPNST), which are associated with poor prognosis due to high recurrence and early metastases [1, 2]. In NF1, MPNST tend to arise from plexiform neurofibromas as a consequence of *NF1* mutations and LOH in Schwann cells, leading to activation of Ras signaling [3]. Treatment involves surgical removal and chemotherapy. However, MPNST display significant resistance to standard chemotherapy [4, 5], therefore adapted therapies are necessary.

Aberrant MAPK cascade activation (Raf/Mek/Erk) resulting from neurofibromin inactivation is involved in MPNST formation. A recent study detected several MEK inhibitors (MEKi) to be active in NF1-associated MPNST [6] and the small molecule MEKi and multi kinase inhibitor sorafenib has shown anti-tumor properties in MPNST *in vitro* [6–8]. The effect of MEKi on MPNST can be increased by co-treatment with agents such as ATRA, BMP2, mTOR kinase inhibitors (AZD8055, RAD001), PAK1/2/3 inhibitors and photothermal therapy *in vitro*, as well as with PAK inhibitors and photothermal therapy in xenograft models [9–14]. Pre-clinical transgenic mouse models demonstrated efficacy of MEKi when admitted alone, however increased MEKi properties were seen when combined with RAD001 [8, 14, 15]. Unfortunately, studies testing sorafenib showed only minimal response in MPNST patients [16].

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We recently identified a crucial role of retinoic acid in MPNST, suggesting a potential therapeutic option as shown in other cancers [9, 17, 18]. Combination therapy of ATRA is used not only to overcome resistance but also to enhance therapeutic effects. Thus, a combination of retinoic acid with interferon alpha 2a in progressive metastatic renal cell carcinoma, or with histone deacetylase-inhibitor valproic acid in refractory and high-risk acute myeloid leukemia, demonstrated beneficial effects [19, 20]. In neuroblastoma cells, inhibition of MAPK cascade downstream Ras has been shown to restore ATRA responsiveness [21, 22].

In the current study we have attempted to verify whether ATRA and MEKi, both alone and in combination, exhibit efficacy in a xenograft nude mouse model for human MPNST, as we have recently demonstrated in vitro [9, 17, 18]. A combination of ATRA and MEKi may provide a novel promising therapeutic approach for MPNST.

## Main text

### Materials and methods

#### Cell culture and colony formation assay

Human MPNST cell lines S462 and T265 were described previously [23–26]. Cells were cultured in DMEM (4.5 g/L glucose, 2 mM L-glutamine, 10% (v/v) FBS, 100 U/mL penicillin/streptomycin and 1 mM sodium pyruvate). Clonogenic assays were performed as described elsewhere with specifications: 300 cells per well were seeded in a 6-well plate and incubated for 14 days. [27] Following incubation, cells were washed with PBS and incubated with staining/fixation solution (6% glutaraldehyde/0.5% crystal violet/PBS) for 30 min. Colonies were defined as accumulation of >50 cells. Images were taken per well (Olympus SZX12 microscope) using Adobe Photoshop CS5 (Adobe Systems Software Ireland Limited 2010), and colonies were counted using the cell counter tool of ImageJ (NIH United States 2014).

#### Xenotransplantation and ATRA quantification

Experiments were approved by the federal state authority of nature, environment and consumer protection of Nordrhein-Westfalen ("Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen", LANUV) (27.08.2013, reference 84-02.04.2013.A275). Balb/c Nude (Balb/cAnNRj-Foxn1nu/Foxn1nu, Janvier), Foxn1 (Nu/Nu) Nude (Crl:NU-Foxn1nu, Charles River), SHO® (SCID Hairless Outbred, Crl:SHO-PrkdcscidHrhr, Charles River) and Shorn (ShrN NOD SCID, NOD.Cg-PrkdcscidHrhr/NCrHsd, Harlan) mice were scheduled for xenotransplantations. Female mice were housed in single cages and all strains lacked hair and T cells. SHO® and Shorn mice also lacked B cells, and Shorn mice

additional partially lacked NK cells. We followed published criteria for generating xenografts.

At study initiation nude mice were 5–7 weeks old. A total of  $5 \times 10^6$  cells (T265, S462) within 30% Matrigel™ (Corning, GFR)/DMEM were implanted by subcutaneous injection (150 µL) into the right and/or left flank.

Blood was collected on day 21 of treatment, 2–3 h after oral application. Plasma was extracted immediately from blood by centrifugation ( $1000 \times g$ , 5 min). ATRA plasma levels were analyzed as described elsewhere [28].

#### Pharmacological treatment, tumor volume quantification and preparation

Daily oral treatment started 42 days after subcutaneous implantation over a period of 28 days. Ten mice were treated with ATRA (15 mg/kg) or PD0325901 (10 mg/kg). Intake was scored (1 = full intake, 0.5 = incomplete intake, 0 = no intake) and mean values were calculated. Tumor volume was measured three times a week using a digital caliper and calculated according to the formula:  $v = L \times W^2 (\pi/6)$  (L-longest diameter, W-width) [29].

Intra-cardiac perfusion was used for euthanization of mice. Under deep anaesthesia (1.8% (v/v) ketamine and 0.3% xylazine in PBS), peritoneal cavity, thorax, left heart ventricle and the right heart atrium were opened, and 10 mL PBS were injected into the ventricle. For fixation of mice tissues injection of 10 mL 4% paraformaldehyde followed PBS intra-cardiac perfusion. Tumors were dissected and stored in 4% PFA for 24 h followed by paraffin embedding. Tumors were dehydrated in increasing alcohol concentrations in a Tissue-Tek VIP system (Sakura) and embedded in paraffin. Immunohistochemistry of MPNST xenografts was performed using antibodies against S100 (monoclonal, mouse, 10 µg/mL, Ventana S100-4C4.9) to demonstrate human Schwann cell origin, anti-Ki67 (monoclonal, mouse, 0.4 µg/mL, Ventana Ki-67-30.9) to evaluate proliferation fraction, anti-CD34 (monoclonal, mouse, 0.8 µg/mL, Ventana QBEnd/10) to demonstrate vessel and stem cell formation, anti-p53 (monoclonal, mouse, Ventana Bp-53-11), anti-p16 (monoclonal, mouse, 1 µg/mL, Ventana 725-4713), anti-vimentin (vimentin, monoclonal, mouse, 2.5 µg/mL, Ventana V9 and anti-actin (monoclonal, mouse, 0.02 µg/mL, Cell Margue 1A4). Staining was visualized using the ultra-View™ Universal Alkaline Phosphatase Red Detection Kit (Ventana, A-PAP method). Staining was performed in the automated staining system Ventana BenchMark XT according to the standard protocols.

#### Statistical analysis

ANOVA followed by post hoc *t* test was used to compare differences between groups. Kruskal–Wallis test was applied to compare groups of 4 mice for weight gain. A *p*

value  $<0.05$  was defined to be significant. The SPSS program (IBM SPSS Statistics Standard) was applied.

## Results

We investigated four strains and cell lines T265 and S462. To determine tumor formation capability colony formation assays were performed and yielded  $42 \pm 6$  colonies of T265 cells and  $21 \pm 5$  colonies of S462 cells from 300 cells plated.

Once clonogenic potential had been demonstrated, we tested strains for tumor formation capability.  $2 \times 10^6$ ,  $5 \times 10^6$ , and  $10 \times 10^6$  MPNST cells were subcutaneously transplanted into the right flank of Balb/c mice (6 groups of 4–5 mice). After 8 weeks tumor volumes did not reach  $70 \text{ mm}^3$ . Differences with respect to cell number were not seen. To test other strains for xenotransplantation,  $5 \times 10^6$  MPNST cells were transplanted into the right and left flank (2 mice/strain, 2 transplants/mouse) of Foxn1, SHO and Shorn mice, and growth was monitored for 8 weeks. Transplants of T265 cells did not generate tumors in Foxn1 mice whereas SHO and Shorn mice developed one flank tumor each (2 tumors/4 transplants) with only 1 tumor gaining a volume  $>70 \text{ mm}^3$ . Transplantation of S462 cells resulted in complete tumor development in all Foxn1 mice (4/4), and all tumors reached volumes  $>70 \text{ mm}^3$  48 days after transplantation. SHO mice developed 3/4 tumors, but volumes did not reach  $70 \text{ mm}^3$ . Only one tumor in Shorn mice (1/4) below  $70 \text{ mm}^3$  was seen. MPNST pattern of xenografts was confirmed histologically.

Beforehand, we had checked in Balb/c mice if application of ATRA and MEKi was tolerated and if oral application was sufficiently fast and complete. Oral administration of ATRA was prescribed by the veterinarian. Oral intake of MEKi is published in literature [15]. In our analysis intake of 0.2 g chocolate with ATRA was identical to chocolate with DMSO (placebo group) (mean intake: 1). Due to its strong flavor, the MEKi had to be served with 0.2 g peanut butter, still 1 mouse showed low intake ( $<0.5$ ). ATRA and MEKi treatment as well as combination was also pre-checked to determine potential side effects in groups of 4 mice per agent as well as placebo (DMSO). We did not observe major side effects, in fact temporary unilateral eye irritation was the only impairment seen independently of treatment in all groups (sparing SHO mice) which did not meet the criteria for exclusion. None of the mice presented with significant weight loss ( $>20\%$ ): by Kruskal–Wallis test we did not detect significant differences due to treatment regimens (placebo/chocolate, ATRA/chocolate, MEKi/peanut butter, control/normal diet). Macroscopy and histological examination of brain, spleen, kidney, stomach,

liver and heart of all animals was without any pathology at the end of experiments in all animals.

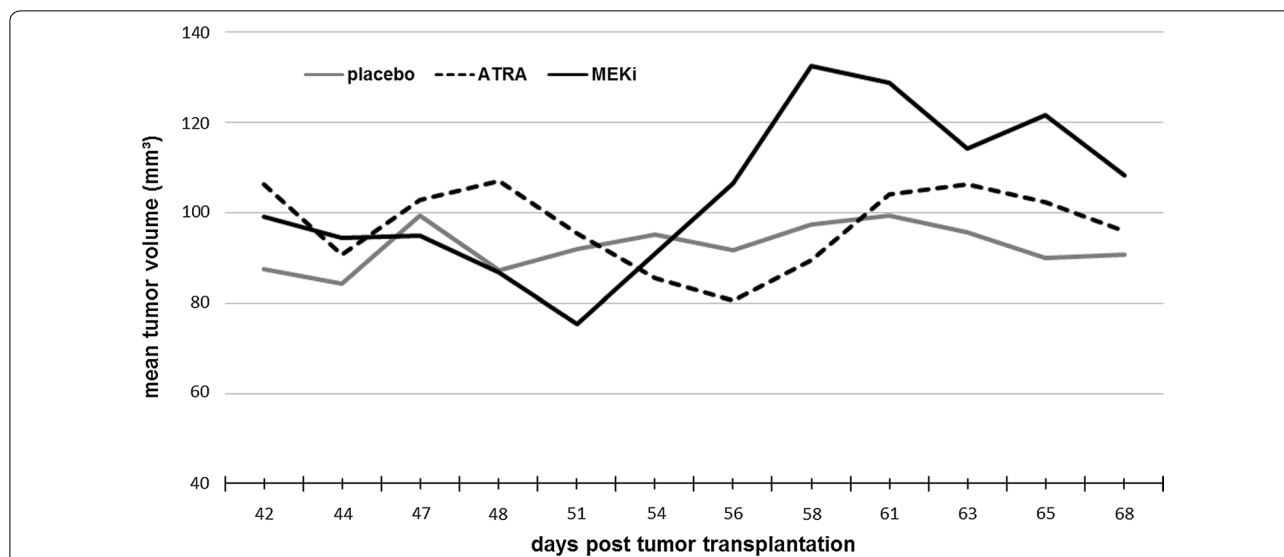
From the above experiments, we selected S462 cells for transplantation into the right flank of Foxn1 mice. At day 42 after transplantation placebo, ATRA and MEKi, both alone and in combination, were administered until day 68. At day 42 mean tumor volume of the placebo group ( $n=10$ ) was  $87.6 (\pm 31.4 \text{ SD}) \text{ mm}^3$ , of the ATRA group ( $n=9$ ) was  $106.2 (\pm 11.9 \text{ SD}) \text{ mm}^3$ , and the MEKi group ( $n=10$ ) was  $99.2 (\pm 51.3 \text{ SD})$ .

ATRA plasma levels following ATRA administration were determined on day 21. Levels observed in the ATRA group were  $2.76 \mu\text{M}$  (mean  $\pm 2.07$ ; range 0.41–5.84), compared to levels below the detection limit of  $0.07 \mu\text{M}$  in the placebo group. These exposures in treated animals were consistent with levels of 1–5  $\mu\text{M}$  observed in human acute promyelocytic leukemia patients.

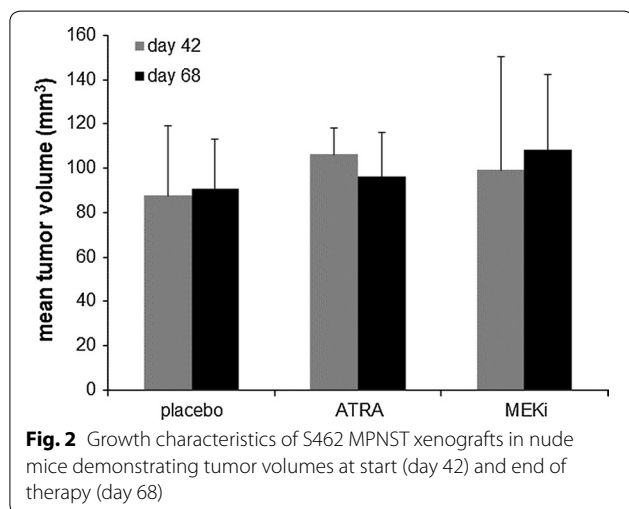
Unexpectedly, Foxn1 mice of ATRA-MEKi combination group did not show sufficient food intake as we had trialed in Balb/c: Compared to placebo group (intake: 1 during the whole treatment period) ATRA/MEKi group showed low intake rates. We therefore exclude this group from experiments.

Mean tumor volume at the end of experiments (day 68) were as follows:  $90.7 (\pm 22.2 \text{ SD}) \text{ mm}^3$  in the placebo group ( $n=10$ ),  $96.0 (\pm 19.9 \text{ SD}) \text{ mm}^3$  in the ATRA group ( $n=9$ ), and  $108.3 (\pm 34.1 \text{ SD})$  in the MEKi group ( $n=10$ ). All data are shown in Additional file 1. Comparing mean tumor volumes at the end of experiments revealed no statistically significant differences as compared to the untreated placebo group (Figs. 1, 2). Mean tumor volumes were higher in the ATRA group at the start of the experiments and a decrease in tumor volumes was seen following treatment, nevertheless differences between first and last day of treatment were statistically not significant. The animals treated with MEKi also did not show significant changes of tumor volumes. Principally, we defined tumor regression (that may have been observed independently from the poor tumor growth characteristics of the placebo) as  $\geq 50\%$  reduction of volume compared to volume at the start of treatment. However, no treatment group or single animals met this criterion.

Finally, S462 xenografts were subjected to HE stain and immunohistochemistry. Typical MPNST morphology was confirmed by neuropathological examination (Fig. 3). Marked inflammation was not seen. Areas of low cellular density and myxoid change or adipose tissue within tumors that were otherwise highly cellular indicating focal differentiation within the MPNST (Triton tumor-like) was identified in some cases. Expression of S100 and a strong nuclear expression of p53 were detected indicating additional *TP53* mutations in MPNST and no genotype change of the transplants. Nuclear expression



**Fig. 1** Growth characteristics (mean tumor volumes) of S462 MPNST xenografts are shown for ATRA and MEKi treatment and placebo



**Fig. 2** Growth characteristics of S462 MPNST xenografts in nude mice demonstrating tumor volumes at start (day 42) and end of therapy (day 68)

of Ki-67 reached 40–45%. As MPNST typically acquire *CDKN2A* mutations transplanted S462 cells demonstrated loss of p16 as well. To exclude minor histopathological changes all tumors of animals were investigated using H&E staining and immunohistochemistry in this study, tumors of the main experiments were also cutted in 3 μm series for evaluation.

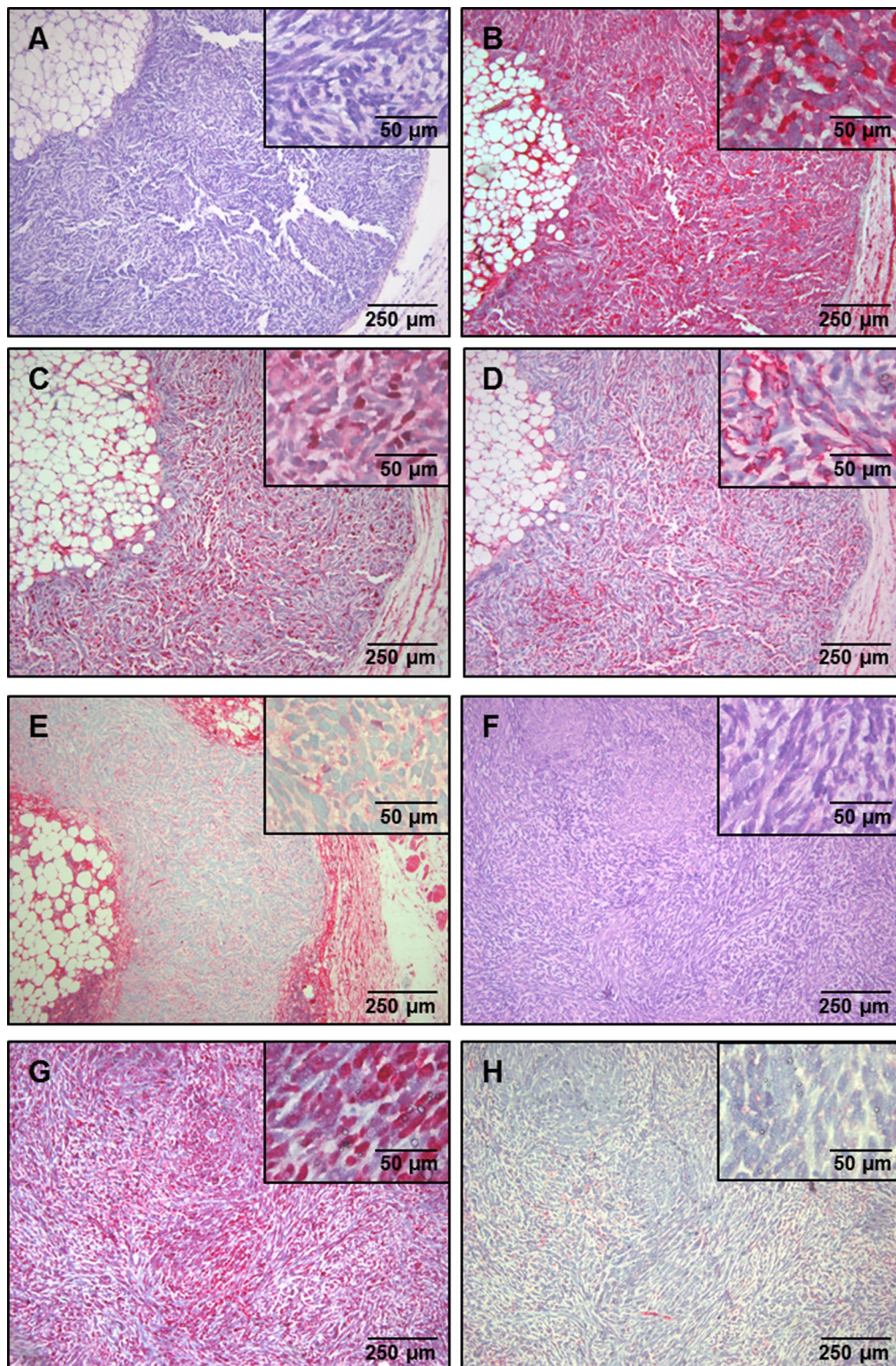
**Conclusions**

In contrast to other in vivo studies, we observed that neither ATRA nor MEKi therapy significantly reduced growth of S462 derived xenografts in Fox1 nude mice. Due to several limitations we expect that tumor

formation capability, agent administration, mouse strain and treatment period are critical parameters, that need to be exhaustedly examined before excluding MEKi and/or ATRA treatment as sufficient agents against MPNST.

**Limitations**

- Reduced acceptance of oral administration led to exclusion of an important subgroup (ATRA/MEKi) from our study. Thus, intraperitoneal application that allows exact dose control may be more suitable in future experiments.
- Although tumor formation capability of S462 cells was reliably pre-tested in Foxn1 mice, tumor volumes did not reach values described in literature (750–2500 mm<sup>3</sup>) [15, 30, 31] in almost three studies with S462 cells or with other MPNST cell lines [29, 30, 32, 33]. Thus, selecting more suitable mouse strains and longer treatment periods may improve validity.
- Tumor growth was not seen in Balb/c mice at all, although those have been successfully used in several ATRA treatment studies for human tumor xenografts including medulloblastoma, melanoma and others [34, 35]. T265 and S462 MPNST cells seem not to bear sufficient tumor formation capacity in this otherwise well investigated strain. Thus, selection of other MPNST cell lines seems necessary to test the hypothesis (at least in Balb/c mice).
- Measuring small tumors with a caliper hampers significance. MRI or ultrasound may be more suitable to control tumor size.



**Fig. 3** Histomorphological features S462 MPNST xenografts. **A** HE staining of a highly cellular MPNST xenograft. **B** Tumor cells stain positive S100 (nuclear and cytoplasmic). **C** A high Ki-67 labelling index of 45% was determined by MIB-1 staining. **D** Staining for CD34 was found to be positive for single cells of the xenograft. **E** Staining for actin was present only in single cells. **F** HE staining of a hyper-cellular MPNST xenograft. **G** Strong nuclear p53 expression and **H** absence of p16 expression

- Insufficient growth characteristics may be due to Triton tumor-like differentiation within MPNST xenografts, however we cannot provide detailed investigations.

## Additional file

**Additional file 1.** Mean tumor volumes. Description of data: Mean volumes of transplanted MPNST (mm<sup>3</sup>) of two treatment groups (mice treated with ATRA and with MEKi) and of placebo group. Tumors were measured from day 7 until day 68 post transplantation. Treatment started at day 42. Average volumes and standard deviation over the whole group per day as well as difference of volumes between day 42 and 68 are given.

## Abbreviations

ATRA: all-*trans* retinoic acid; BMP2: bone morphogenetic Protein 2; DMEM: Dulbecco's modified eagle's medium; ERK: extracellular-signal regulated kinase; LOH: loss of heterozygosity; MAPK: mitogen-activated protein kinase; MEK: mitogen-activated protein kinase; MEKi: MEK inhibitor; mTOR: mechanistic target of rapamycin; MPNST: malignant peripheral nerve sheath tumor; NF1: neurofibromatosis type 1; PAK: p21-Activated Kinase; RAF: rapidly accelerated fibrosarcoma; RAS: rat sarcoma.

## Authors' contributions

The study was planned and coordinated by AH, SFH and VoS. Animal experiments were carried out by SFH, LC, VS, and VoS. Quantification of ATRA plasma level was performed by GV, PB and MZ. GV organized and analyzed quantification of ATRA plasma level. Histological evaluation was done by AH, SFH and LC. Data collection, analysis, interpretation and manuscript writing was performed by AH, SFH and LC. VFM supplied MPNST cells and revised the manuscript critically for intellectual content. All authors read and approved the manuscript.

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## Competing interests

The authors declare that they have no competing interests.

## Availability of data and material

All data generated or analyzed during this study are included in this published article and its Additional file 1.

## Consent for publication

Not applicable.

## Ethics approval and consent to participate

Material from patients was not included into the study. Animal experiments were approved by the federal state authority of nature, environment and consumer protection of Nordrhein-Westfalen ("Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen", LANUV) (27.08.2013, reference 84-02.04.2013.A275).

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