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In Vitro and *In Vivo* Studies of Efficacy of Nilotinib on Plexiform Neurofibromas

Dissertation

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1. SYNOPSIS

1.1. Introduction

1.1.1. Plexiform neurofibroma (PNF)

PNFs are benign tumors of subcutaneous or visceral peripheral nerves (Fig. 1) [1-2]. These tumors appear as tangled, gelatinous, thick tendrils involving the trunks of large nerves [3]. They grow along the length of a nerve and involve multiple nerve fascicles and branches, resulting in a diffuse mass of thickened nerve fibers covered with a proteinaceous matrix [1, 4-5]. In less common situations, they may be modular and multiple discrete tumors may develop on nerve trunks [6]. As the lesion develops, there is an increase in number of Schwann cells and/or perineurial cells [3, 6]. Superficial PNFs are characterized by extensive thickening and hyperpigmentation of the skin and some subcutaneous PNFs may be evident on palpation [7, 8].

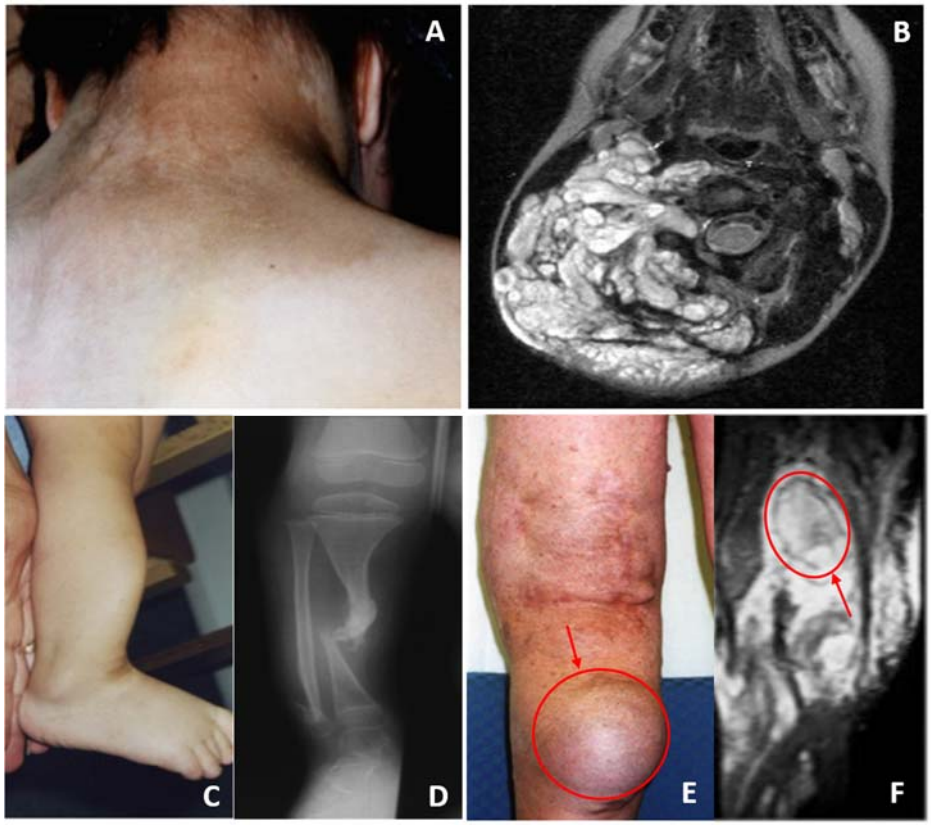

Fig. 1. External appearance (A, C, E) and magnetic resonance imaging (B, D, F) of plexiform neurofibromas. E: plexiform neurofibroma with malignant transformed part marked in the red circle.

Typically, PNFs can be limited to deep soft-tissue, or may affect the skin and superficial muscle [9]. The tumors in skin or underlying muscle may produce a change in appearance such as a noticeable increase in size or deformation of nearby tissue [10, 11]. PNFs can also displace or penetrate neighboring organs or invade into bone and induce significant clinical consequences, including dysfunction, pain, severe disfigurement, organ compression [12]. More seriously, they can progress to malignant peripheral nerve sheath tumors (MPNSTs), which is the leading cause of death [13]. Thus, clinical investigation alone does not determine whether a PNF occurs superficially or involves deep tissues. Generally, magnetic resonance imaging (MRI) is the best method for characterization of the growth pattern of these heterogeneous tumors [2].

Most PNFs are associated with neurofibromatosis type 1 (NF1), an autosomal dominant, multisystem disorder [14-16]. Previous genetic research proved that PNF is caused by heterozygous inactivation of the *NF1* gene, which is a tumor suppressor gene located on chromosome 17 at q11.2 [17, 18]. The *NF1* gene codes for a protein called neurofibromin, which is associated with increased activation of Ras and decreased adenylyl cyclase (AC) activity (Fig. 2) [19, 20]. The majority of *NF1* mutations result in the loss of function of the gene product, leading to uncontrolled cell proliferation and the formation of neurofibroma tumors [21].

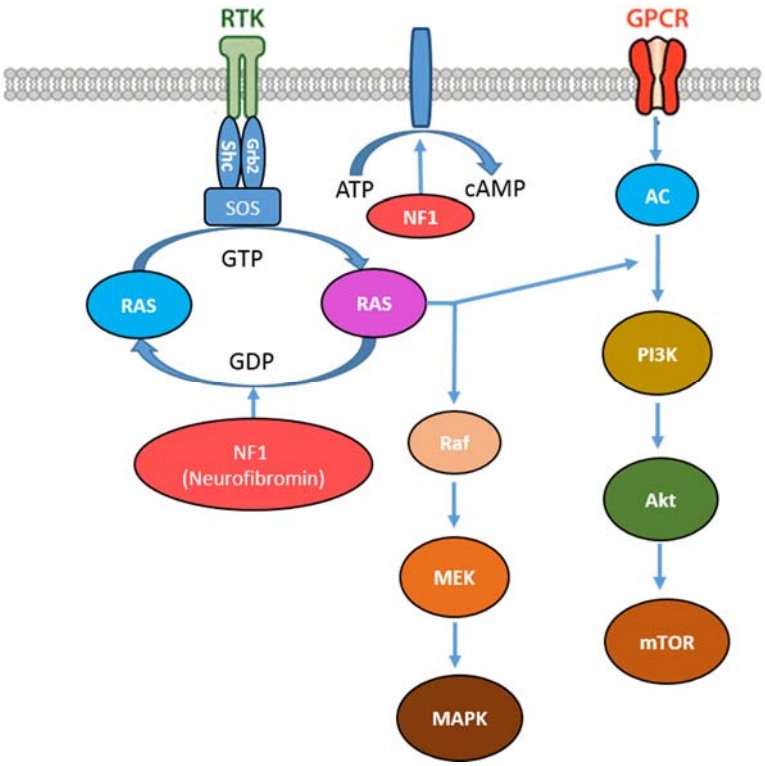

Fig. 2. The function of *NF1* gene and neurofibromin in the pathogenesis of plexiform

neurofibroma. The *NF1* gene codes for neurofibromin that functions as a negative regulator of Ras-MAPK signaling cascade. On the other hand, neurofibromin acts as an activator of adenylate cyclase (AC) and modulates phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR cascade (Boyd *et al*., 2009; Brems *et al*., 2009). RTK, receptor tyrosine kinase; Grb2, growth factor receptor-bound protein 2; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Raf, murine sarcoma viral oncogene homologue; MEK, mitogen-activated protein kinase or extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; GPCR, G protein-coupled receptor ERK; mTOR, mammalian target of rapamycin.

1.1.2. MPNST

MPNST refers to any malignancy arising from a peripheral nerve or demonstrating nerve sheath differentiation, excluding those tumors that originate from the epineurium or peripheral nerve vasculature [22]. Histologically, MPNSTs are classified as malignant soft-tissue sarcomas with an incidence of 1 per 100 000 population and involve large and medium nerves most commonly in the buttock, thigh, brachial plexus and paraspinal regions [23]. They are aggressive, rare tumors presenting as a rapidly growing and painful lump. These tumors infiltrate surrounding tissues, relapse commonly after surgery and may metastasise early to lung, liver, brain, soft tissue, bone, regional lymph nodes, skin and retroperitoneum [14, 24]. MPNSTs are difficult to detect and are associated with poor prognosis unless wide excision of the tumor is undertaken before local invasion or distant metastasis can occur. Generally, the 5-year survival rate is only 21%. In addition, the aberrant molecular pathways that underlie this malignant transformation are still largely unknown, and considerable effort is being directed towards the molecular defects involved [25, 26].

1.1.3. Treatment of PNF

To date, PNFs are amenable only to surgical removal. However, since these tumors often infiltrate adjacent tissues, complete resection is usually not possible without damaging nerves and healthy tissues [27, 28]. In addition, there is also no established medical therapy available for such patients. Thus, many trials have been undertaken or are ongoing to discover and test medical treatments for the various manifestations of NF1, primarily progressive or disabling PNFs and MPNSTs. For instance, sirolimus, targets mTOR and is the focus of a multicenter trial for PNFs [29]. A previous study reported the improved cognition in NF1 following treatment with lovastatin in mice [30]. However, a large, randomized, double-blind trial failed

to show any significant improvement after 3 months of treatment with simvastatin in the cognitive abilities of children with NF1 [31]. Another study with NF1 patients used the combination of intense pulsed-radio frequency and topical vitamin D_3 to treat freckling and café-au-lait spots. The results indicated that freckling and repigmentation was improved after treatment [32].

1.1.4. Imatinib mesylate

Imatinib mesylate (Glivec), a receptor tyrosine kinase inhibitor, targets platelet-derived growth factor receptor (PDGFR), c-KIT, BCR-ABL and Arg-kinase. This drug exhibited efficacy for some cancers and is the first-line treatment for chronic myeloid leukaemia (CML) today [33, 34]. Previous studies have found amplification and mutations of PDGFR-α and KIT in MPNSTs, and wide expression of ligand PDGFR-α in MPNSTs and neurofibromas [35-37]. A recent study in our laboratory revealed expression of PDGFR-α and PDGFR-β also in PNFs and in PNF-derived primary Schwann cells. Imatinib had high potency against the PDGFR-α and PDGFR-β, suppressed proliferation of PNF-derived Schwann cells *in vitro* and induced regression of PNFs xenografts *in vivo* [27]. Another *in vitro* study demonstrated that imatinib not only decreased the activation of c-KIT, PDGFR, but also inhibited ERK-1/2, AKT and FAK [38]. A recent phase 2 trial reported >20% shrinkage of PNFs in 26% of the NF1 patients treated with imatinib [28].

1.1.5. Nilotinib

Nilotinib (AMN107), is a new, orally active tyrosine kinase inhibitor with similar potency to that of imatinib against the c-KIT, PDGFR-α, PDGFR-β and discoidin domain receptors (DDRs), but has a number of advantages including a different toxicity profile and a lower incidence of fluid retention [39-42]. The chemical structure of nilotinib preserves the aminopyrimidine and amide pharmacophores of imatinib but incorporates substituents alternative to the highly basic N-methyl-piperazine of imatinib, thereby leading to greater lipophilicity [42]. A recent assessment reported statistically significant advantage of nilotinib compared with imatinib for cytogenetic response and major molecular response in CML. Haematological, non-haematological events (nausea, diarrhoea, vomiting and muscle spasm events) and oedema events were more frequent for imatinib. In contrast, headache, pruritus and alopecia events were more frequent for nilotinib [43]. The data from another study showed that nilotinib inhibited ABL-catalyzed peptide substrate phosphorylation with a 20-fold higher potency than imatinib [39].

1.1.6. Aims of this study

In this study, the efficacy of nilotinib on PNFs was examined *in vitro* and *in vivo*. *In vitro* studies were carried out using primary Schwann cells derived from PNFs and using cells of 3 established MPNST cell lines. Efficacy of nilotinib was examined by monitoring cell proliferation, vitality, viability, apoptosis and collagenase activity. From a subset of the PNFs, fibroblasts were also cultured as paired non-tumor cells controls and treated with nilotinib to assess the selectivity of nilotinib. *In vivo* studies were carried out by xenografting PNF fragments onto sciatic nerve of immunodeficient nude mice and treating these mice with nilotinib and imatinib. Efficacy of the drugs was followed by means of monitoring the size change of the grafts using ultrasound.

1.2. Material and Methods

1.2.1. Patients

PNF tumor tissues were obtained from eight patients (age range 4-60 years, mean 30.1± 20.0 years; five male and three female) who underwent surgery at the Department of Maxillofacial Surgery, University Hospital Hamburg-Eppendorf. Diagnosis of NF1 was conducted according to the modified National Institutes of Health (NIH) criteria [44]. All patients gave informed written consent, and the Institutional Review Board approved the study (OB-061/05). Tumor tissues from two patients, a 6-year-old male and a 12-year-old female, were used for animal experiments.

All specimens were kept in Hanks buffered saline (Gibco, Paisley, UK) immediately after surgical removal. One part of each specimen was used to confirm the pathological diagnosis of the tumor (Institute of Neuropathology, University Hospital Hamburg-Eppendorf). Another part was snap frozen in liquid nitrogen and stored at -80℃. The remaining tissue was used to establish primary Schwann cell and fibroblast cultures.

1.2.2. Cell culture

Three MPNST cell lines used in the study, S462, S1507 and S1844, were established in our laboratory [14, 24].

Primary Schwann cells were derived from PNFs. Upon surgery, part of the resected PNF was kept in sterile phosphate-buffered saline (PBS; Gibco) and transported into the laboratory where the skin, fat and connective tissues were removed. The remaining tumor tissues were incubated in Dulbecco's modified eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco), 500 U/ml penicillin or streptomycin (Gibco), 2 mM glutamine (Gibco) and 1 mM sodium pyruvate (Biochrom, Berlin, Germany), at 37℃ and 5% CO_2. After 1 day, tissues were cut into 2-3 mm^3 fascicles and digested in the same medium with 0.5 mg/ml collagenase and dispase (Sigma, St. Louis, MO) at 37℃ and 10% CO_2. After 24 h, digested tissue fascicles were mechanically dissociated by straining through a 100 µm steel mesh screen (Partec, Görlitz, Germany). The resulting single cell suspension was cultured under either standard culture conditions or conditions optimized for Schwann cells.

For selective culture of Schwann cell, DMEM was complemented with 0.5 mM 3-iso-butyl-L-methylxanthine (IBMX; Sigma), 2 nM human heregulin (rHRG$\beta1_{177–244}$; provided by Dr. Steven Carrol, University Alabama, USA) and 2.5 mM insulin (Sigma). The culture

surfaces of the flasks were coated with 4 μM of natural mouse laminin (Gibco), and CO_2 was kept at 10%. After the initial 24 h of culture, the medium was changed to Schwann cell medium [9].

For fibroblast culture, DMEM was supplemented with 10% FBS (Gibco), 2 mM glutamine (Gibco), 0.1 mM 2-mercaptoethanol, 500 U/ml penicillin and 500 g/ml streptomycin, and 1 mM sodium pyruvate (Biochrom).

To enrich Schwann cells and fibroblasts, differential detachment using controlled trypsinization was applied [45]. The cells were expanded selectively for four to six passages.

1.2.3. Cell immunofluorescence staining

Cells were seeded on glass cover slips and fixed when subconfluent in 4% paraformaldehyde (PFA; Sigma). For Schwann cell staining, cells were stained with 2 μg/ml rabbit anti-human S100 antibody (DAKO, Copenhagen, Denmark), 10 μg/ml secondary FITC-conjugated anti-rabbit antibody (DAKO), and 0.5 μg/ml propidium iodide (PI; Molecular Probes, Leiden, Netherlands), all diluted in DMEM with 10% FBS. The percentage of Schwann cells was determined as the number of S100-positive cells divided by the number of nuclei stained with 4'-6-diamidino-2-phenylindole (DAPI; Vysis Inc., Downers Grove, USA). In this study, the primary cultures which had more than 85% S100 positive cells were selected for the tests.

To stain fibroblasts, cells were incubated with 2 μg/ml anti-fibroblast antibody (CD90; DAKO, Copenhagen, Denmark), 2 μg/ml secondary FITC-conjugated goat anti-mouse IgG (DAKO), and 0.5 μg/ml PI, all diluted in DMEM with 10% FBS.

1.2.4. Drug treatment

Cells were cultured with nilotinib (Novartis, Switzerland) at various concentrations (0, 2, 4, 6, 8 and 10 μM) for 10 days for proliferation and vitality assays, 48 h for viability, and 24 h for apoptosis and collagenase assays. Primary Schwann cells from the PNF (T2463) were simultaneously treated with nilotinib and imatinib (Novartis, Switzerland) for proliferation and vitality assays, each at 0, 5, 10, 15 and 20 μM for 10 days. Since the stock solution of nilotinib was in dimethyl sulfoxide (DMSO), the medium with 0.2% of DMSO was used as control. Medium containing nilotinib or imatinib was refreshed daily, extrapolating from the 18 h half-life of nilotinib in human serum [46]. Functional assays were carried out according to the request of the following tests.

1.2.5. Proliferation

Five hundred cells were cultured in 96-well plates and incubated with nilotinib and imatinib as described above. The colorimetric 5-bromodeoxyuridine (BrdU) assay, a fast and simple method to quantitate cell proliferation by measuring DNA synthesis, was carried out according to the manufacturer's instructions (Roche, Mannheim, Germany). The absorbance was measured at 450 nm (reference wavelength: 690 nm) by the Model 680 Microplate Reader (Bio-Rad, Tokyo, Japan). Each concentration was tested in 12 replicates.

1.2.6. Vitality

Cell vitality was detected using a XTT kit (Roche), which is designed for the spectrophotometric quantification of cell growth and vitality. Five hundred cells were cultured in 96-well plates and treated by nilotinib or imatinib with different concentrations. Then, 50 μl XTT labeling mixture was used for each well and incubated in a humidified atmosphere for 5 h. The absorbance was measured using the Model 680 Microplate Reader at 450 nm with a reference wavelength at 650 nm. Each concentration was tested in 12 replicates.

1.2.7. Viability

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. In this study, 50 000 cells were cultured in 6-well plates and incubated with nilotinib at 37°C, 10% CO_2 for 48 h. Then, the cells were harvested using 0.05% trypsin-EDTA (Sigma) and resuspended in PBS with a concentration of 1×10^5 cells/ml. Twenty-microliter of cell suspension was mixed with the same volume of 0.4% trypan blue (Sigma) and the cells were counted in a hemacytometer. Proportion of unstained cells (viable) against the total number of cells within the grids was counted. Viability was defined as proportion of viable cells treated with nilotinib to that of untreated cells.

1.2.8. Apoptosis

Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenance of tissue homeostasis. In this study, cell apoptosis induced by the drug was examined using the kit of annexin-V-binding assay (BD Biosciences, Mannheim, Germany). Since the flow cytometry-based assay requires a large number of cells, only

MPNST cells could be studied. Briefly, MPNST cells of S462, S1507 and S1844 were treated with nilotinib (2, 6 and 10 μM) for 24 h and harvested by trypsinization. The cells were washed twice with cold PBS and resuspended in binding buffer at a concentration of 2×10^6 cells/ml, then stained with 5 μl FITC-labeled annexin V dye and PI for 15 min in the dark at room temperature (RT), and subsequently analyzed with a FACSCalibur (Becton-Dickinson, Heidelberg, Germany) using Cell Quest Software (Becton-Dickinson) calculating the percentage of apoptotic and/or necrotic cells.

1.2.9. Collagenase activity

Cancer progression has been shown to be correlated with the expression of extracellular proteinases such as matrix metalloproteinases (MMPs) [47]. Collagenase is one of the members of MMPs family and highly expressed in a large number of animal and human tumors than corresponding benign tissues. This is because tumor cells can produce collagenase or induce host cells to produce collagenase [48]. In this study, collagenase activity was determined in medium of cell cultures treated with nilotinib for 24 h, using a collagenase assay kit (Chondrex, WA, USA). Fluorescence of digested substrate at 490 nm excitation and 520 nm emission was measured by a Tecan SpectraFluor Plus well plate reader (Tecan, Mannedorf, Switzerland).

1.2.10. Animal and tumor implantation

Forty-two female athymic nude mice (nu/nu Balb/c, 6 weeks) were obtained from Charles River Laboratories (Sulzfeld, Germany). The care and use of animals were carried out in strict accordance with the local research council's guide (Approval number: Hamburg 112/11). Tumor tissues from freshly resected PNFs were placed in martrigel (BD Biosciences) and cut into 4-8 mm^3 pieces. The mice were anaesthetised with a mixture of xylasin and ketamin. A small incision was made into the skin to expose the right sciatic nerve and an incision was made into the sciatic nerve, under which one tumor piece was orthtopically implanted (Fig. 3A, B).

After confirming successful xenografting by ultrasound scanning one week later, the 42 mice were allocated into two groups: group 1 had 19 mice that were divided into two subgroups, 9 as control without any treatment and 10 for nilotinib treatment with a dose of 75 mg/kg/day; the remaining 23 mice in group 2 were divided into three subsets, 8 as control, 8 and 7 mice were administered nilotinib and imatinib with a dose of 100 mg/kg/day, respectively.

For the treatment, nilotinib (as the hydrochloride salt) was diluted in 10% N-methyl-pyrrolidinone (NMP; Sigma) and 90% polyethylene glycol 300 (PEG 300; Sigma). Imatinib (as the mesylate salt) was dissolved in sterile water. Oral administration was started on day 7 after implantation and was continued to day 35. Body weights of the mice were recorded daily and the drug dosage was adjusted accordingly. Food consumption and general condition of the mice were monitored daily. At the end of the treatment, animal blood was collected 3 h after the last oral administration of nilotinib for plasma preparation. Then, nilotinib concentration was measured using a Micromass Quattro Liquid Chromatography triple-quadrupole mass spectrometer (LC-MS; Beverly, MA, USA) [49].

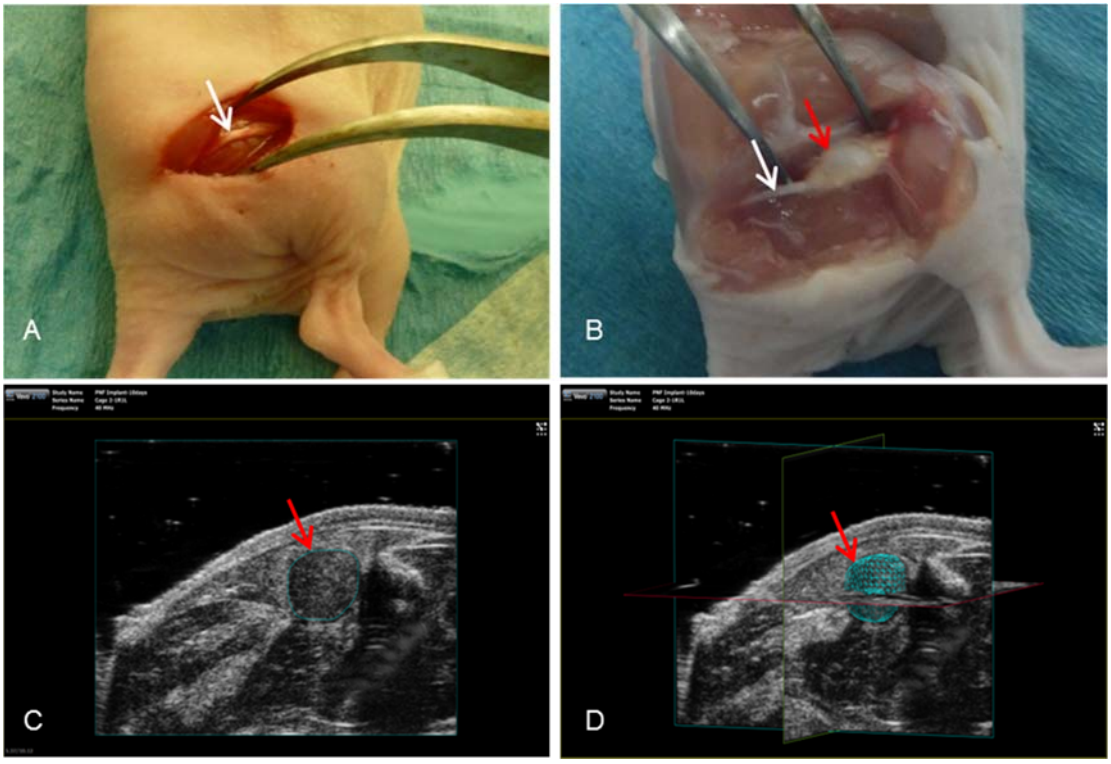

Fig. 3. Xenograft on sciatic nerve in mouse. (A) The exposed sciatic nerve (white arrow) for implantation, (B) a PNF xenograft (red arrow) integrated onto the sciatic nerve, (C) images and (D) three-dimensional reconstruction of a xenograft (red arrow) by a Vevo 2100 micro-imaging system.

1.2.11. Sonographic measurement of xenografts

High frequency 'micro-ultrasound' has steadily evolved in the post-genomic era as a rapid, comparatively inexpensive imaging tool for studying normal development and models of human disease in small animals [50]. Our previous results demonstrated that high frequency sonographic measuring is a superior non-invasive method for monitoring grafts of solid

tumors in mice and is especially valuable for tracing effect of drug treatment at multiple time-points without sacrificing the animals [51]. In the present study, ultrasound monitoring was performed using a Vevo 2100 micro-imaging system (VisualSonics, Amsterdam, Netherlands), which is a high-frequency, high-resolution digital imaging platform with linear technology and color Doppler mode. Xenografts were verified after 7 days post transplantation and measured weekly during the whole treatment period of 4 weeks. Three-dimensional images of the xenografts were generated and analyzed using the Vevo software version 5.0.0 to calculate their size in volume (Fig. 3C, D).

1.2.12. Activation of natural killer cells

Activation of natural killer cells in the mice was measured in cytotoxicity of non-adherent spleen cells. Briefly, after killing the mice, the spleen of each mouse was removed aseptically and placed in a tube containing Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) with FBS. Then, the spleen cells were dissociated using the Ficoll-Paque PLUS (GE Healthcare, Freiburg, Germany) as previously described [52]. Cytotoxic efficacy of the non-adherent spleen cells was assessed by adding them as effector cells to PNF-derived Schwann cells as target cells at a ratio of 1:10 for 4 h using the CytoTox 96® Non-Radioactive Cytotoxicity assay (Promega, Fitchburg, WI).

1.2.13. Statistical analysis

Statistical analysis and calculation of 50% inhibitory concentration (IC_{50}) were carried out using SPSS 19.0 software (Chicago, USA). Relative proliferation and vitality were calculated as ratio of absorbance of treated cells to controls. Data of treated cells at various concentrations of the drug were compared to those of untreated cells using Student's *t*-test. Sizes of each xenograft and body weight of the mice were normalized against the corresponding initial values. Time course of size change of the xenografts and body weight increase of the mice in the three groups were compared with each other using a linear mixed model. Pearson correlation coefficients were calculated for the relationships between reduced tumor volumes and cell cytotoxicity, and between reduced tumor volumes and nilotinib concentrations. Data were represented as mean±standard deviation (SD). $P<0.05$ was considered significant.

1.3. Results

1.3.1 Efficacy of nilotinib on PNF *in vitro*

1.3.1.1 Tumor cells

MPNST cells were cultured under standard condition and exhibited a neuron-like morphology such as large cell bodies, polygonal with more abundant cytoplasm and lacked long processes (Fig. 4A), and the nuclei were stained with DAPI and shown in blue (Fig. 4D, G).

Schwann cells from PNFs were cultured in the optimized medium and exhibited an elongated spindle-shaped morphology with a prominent nucleus (Fig. 4B). These cells were stained with S100 and shown in green (Fig. 4E, H).

Fibroblasts from PNFs displayed bipolar or multipolar elongated shapes (Fig. 4C) and shown in red after stained by CD90 (Fig. 4F, I).

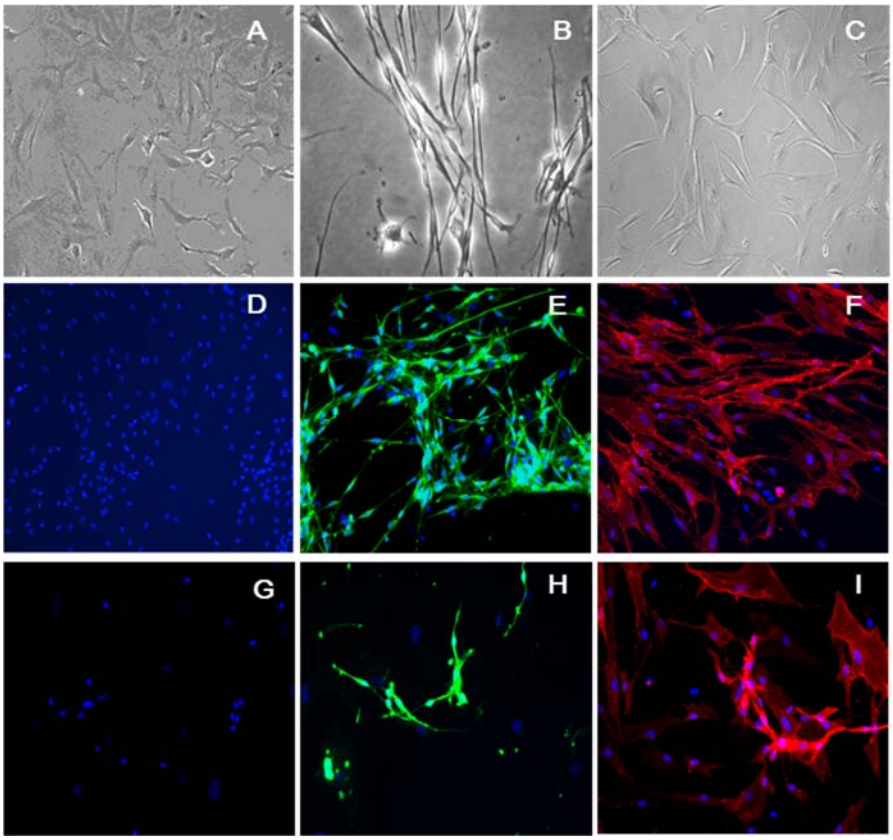

Fig. 4. Images of living cells (A-C) and cells stained with immunofluorescent antibodies (D-I). A-C: phase-contrast images of living MPNST cells, Schwann cells and fibroblasts, respectively; D, G: nuclei of MPNST cells stained with DAPI (blue); E: S100 positive cells cultured under conditions optimized for Schwann cells (green); F: CD90 positive cells

cultured under standard conditions for fibroblasts (red); G-I: 10-day nilotinib-treatment (10 μM) decreased the number of MPNST cells (G), Schwann cells (H) and fibroblasts (I).

1.3.1.2. Efficacy of nilotinib on MPNST cell

Nilotinib inhibited proliferation and vitality of all MPNST cell lines in a dose-dependent manner as revealed by both BrdU and XTT assays (Table 1). For MPNST cells S462, S1507 and S1844, the mean IC_{50} values were 3.1, 6.7 and 8.3 μM by BrdU assay (Fig. 5A), and 2.3, 5.8 and 10.8 μM by XTT assay (Fig. 5B), respectively. Efficacy of nilotinib on cell viability was also seen in trypan blue assay (Fig. 5C) with similar IC_{50}. Among the three MPNST cell lines, S462 cells were most sensitive to nilotinib and had the lowest IC_{50} in all three assays.

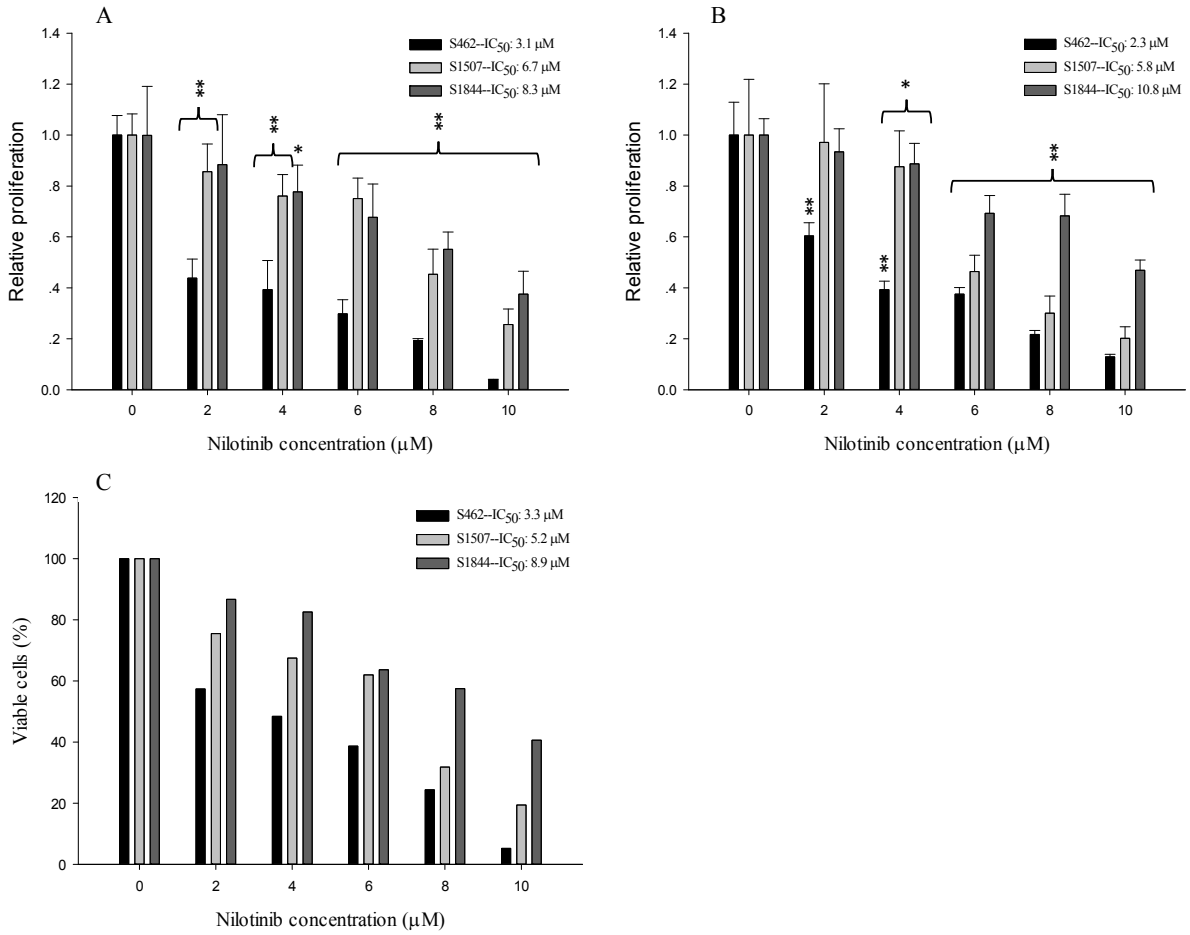

Fig. 5. Effect of nilotinib on cell proliferation, vitality and viability of MPNST cells using BrdU (A), XTT (B) and trypan blue (C) assays, respectively. Data are absorbance (A and B) or cell number (C) normalized against that of DMSO controls. Values differing significantly from untreated cells are marked with *(P<0.05) or ** (P<0.005).

1.3.1.3. Effect of nilotinib on Schwann cell and fibroblast

Nilotinib also constrained proliferation, vitality and viability of Schwann cells, the tumor cells from PNFs, in a dose-dependent manner with IC_{50} varying from 3.9 to 9.8 μM (Fig. 6, Table 1). From all the eight PNFs, we also cultured and tested fibroblasts which are non-tumor cells. The mean IC_{50} values of nilotinib for proliferation of fibroblasts ranged from 5.4

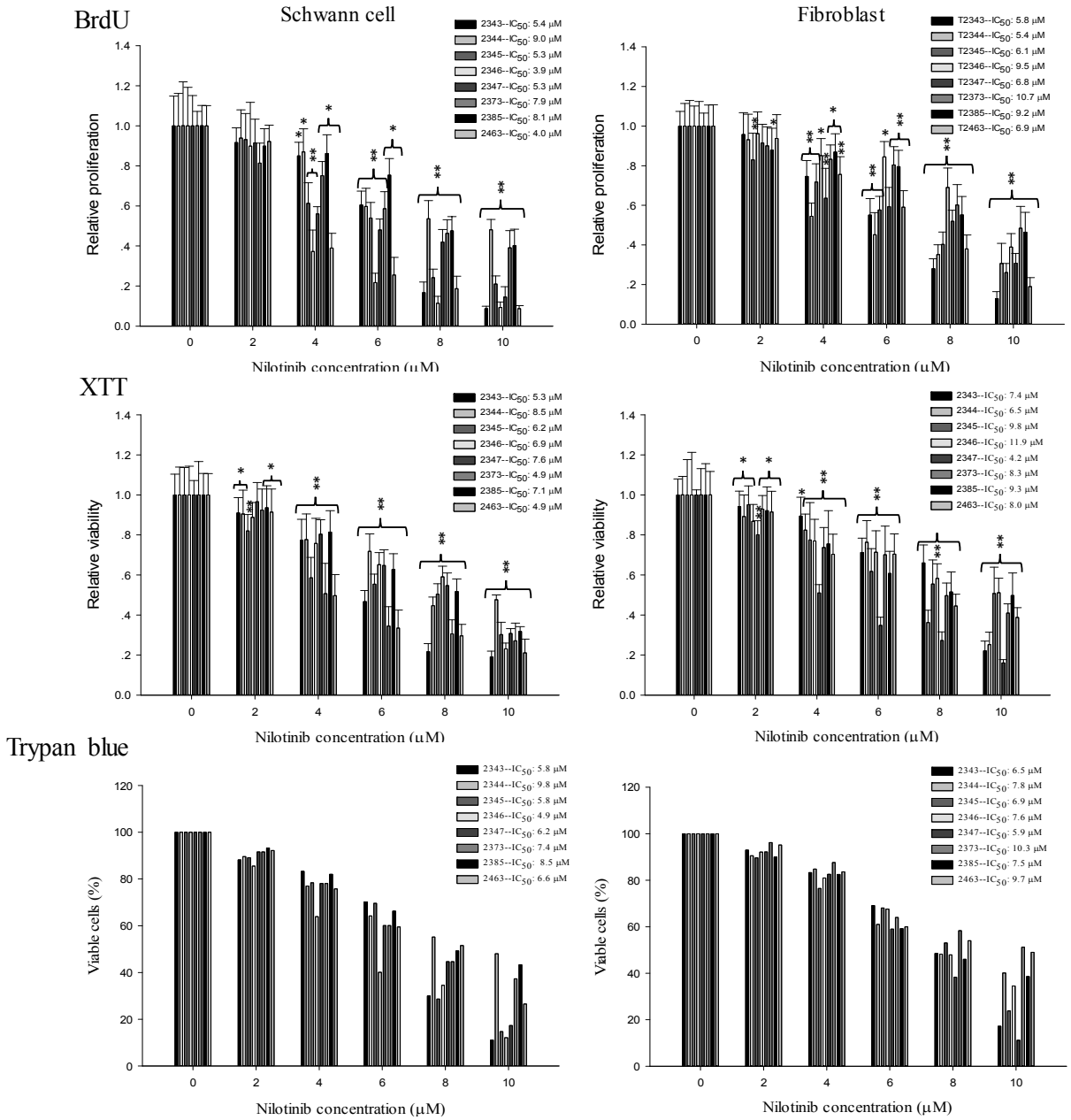

Fig. 6. Effect of nilotinib on Schwann cell and fibroblast using BrdU, XTT and trypan blue assays. Data are normalized against that of DMSO controls. Values differing significantly from untreated cells are marked with *(P<0.05) or ** (P<0.005).

Table 1. IC_{50} and 95% confidence (μM) of nilotinib on proliferation, vitality and numbers of cells

Cells	Proliferation		Vitality		Cell number	
	Sch	F	Sch	F	Sch	F
T 2346	**3.9** (3.0-3.8)	**9.5** (7.0-11.7)	**6.9** (5.1-9.3)	**11.9** (9.4-25.8)	**4.9** (4.2-6.3)	**7.6** (4.8-20.2)
T 2463	**4.0** (3.4-9.1)	**6.9** (8.9-11.9)	**4.9** (3.1-5.9)	**8.0** (3.7-15.0)	**6.6** (3.9-27.8)	**9.7** (3.9-23.1)
T 2345	**5.3** (4.2-6.7)	**6.1** (5.0-7.3)	**6.2** (5.2-7.3)	**9.8** (3.3-10.2)	**5.8** (3.8-7.0)	**6.9** (4.2-9.2)
T 2347	**5.3** (3.7-6.8)	**6.8** (6.1-7.8)	**7.6** (5.6-19.9)	**4.2** (2.8-19.6)	**6.2** (3.4-7.1)	**5.9** (5.1-7.4)
T 2343	**5.4** (4.0-6.5)	**5.8** (4.9-7.5)	**5.3** (4.4-7.1)	**7.4** (6.5-9.2)	**5.8** (4.7-7.4)	**6.5** (4.9-7.5)
T 2373	**7.9** (3.1-15.2)	**10.7** (4.0-16.1)	**4.9** (2.2-11.9)	**8.3** (3.2-14.1)	**7.4** (5.4-19.8)	**10.3** (4.2-22.5)
T 2385	**8.1** (2.9-16.7)	**9.2** (3.1-22.7)	**7.1** (5.1-12.3)	**9.3** (3.5-16.7)	**8.5** (5.3-12.7)	**7.5** (4.1-11.7)
T 2344	**9.0** (7.7-10.8)	**5.4** (3.8-6.7)	**8.5** (6.9-11.9)	**6.5** (4.0-9.1)	**9.8** (6.8-19.0)	**7.8** (6.2-10.1)
MPNST S462	**3.1** (2.0-3.8)		**2.3** (1.2-3.6)		**3.3** (2.7-4.0)	
MPNST S1507	**6.7** (5.0-7.8)		**5.8** (3.7-6.7)		**5.2** (3.8-6.6)	
MPNST S1844	**8.3** (7.9-9.9)		**10.8** (8.4-12.4)		**8.9** (7.8-11.0)	

IC_{50}: 50% inhibitory concentration; Sch: Schwann cells; F: fibroblasts

to 10.7 μM, generally higher than that for Schwann cells. For 5 (62 %) PNFs, T2343, T2345, T2346, T2373 and T2463, the mean IC_{50} values were lower for Schwann cells than for fibroblasts for all three parameters (proliferation, vitality and viability), indicating a good selectivity of nilotinib. In another PNF (T2385), IC_{50} for proliferation and vitality was lower for Schwann cells than for fibroblasts, but on the contrary in viability assay. In addition, IC_{50} for proliferation of PNF (T2347) was lower for Schwann cells while that for vitality and

viability not. For the last PNF (T2344), IC_{50} was higher for Schwann cells than for fibroblasts regarding all three parameters.

Furthermore, both nilotinib and imatinib dose-dependently inhibited proliferation and vitality of primary Schwann cells from the PNF (T2463) which was involved in the animal experiments. The mean IC_{50} values of nilotinib were 4.0 and 4.7 μM, respectively, much lower than the 12.4 and 14.6 μM of imatinib (Fig. 7).

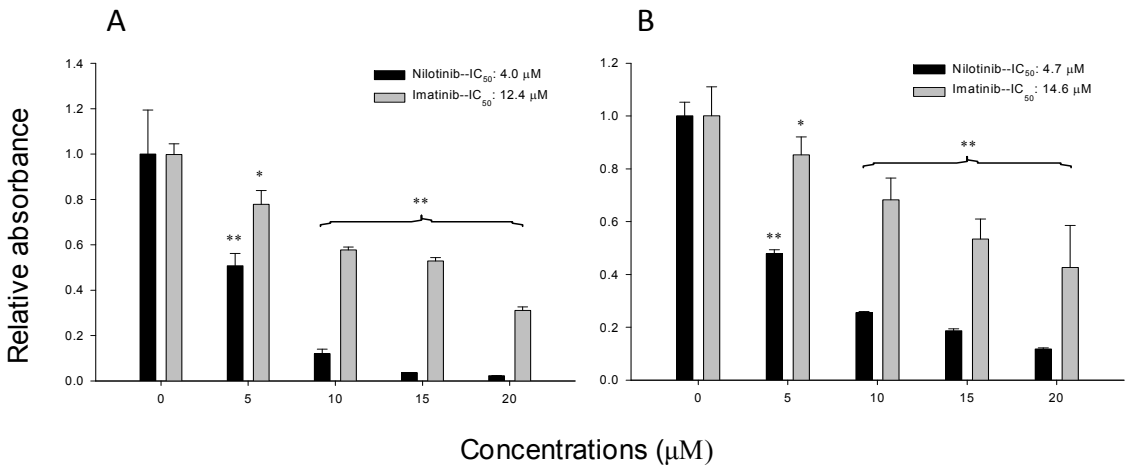

Fig. 7. Effects of imatinib and nilotinib on proliferation (A) and vitality (B) of PNF-derived Schwann cells. Data are absorbance normalized to that of untreated controls. Significant (P<0.05) and highly significant (P<0.005) differences were marked with * and **, respectively.

In the trypan blue assays, the cells looking faint or dark blue within the grid were counted as dead cells. Proportion of viable cells against the total number of cells was calculated. After nilotinib treatment, the number of viable cells decreased along with the increasing of drug concentrations. The morphology of Schwann cells after treated by various nilotinib concentrations showed in Fig. 8.

1.3.1.4. Cell apoptosis

Nilotinib treatment induced apoptosis in cells of all three MPNST cell lines in a dose-dependent manner up to the concentration of 6 μM, but dropped at 10 μM nilotinib, possibly due to cytotoxicity of the drug at higher concentrations (Fig. 9).

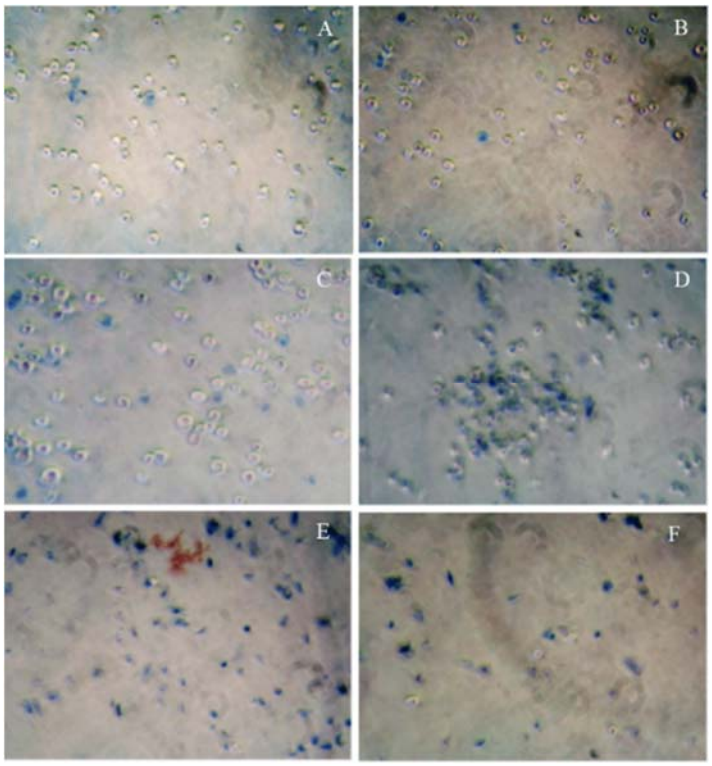

Fig. 8. Viable Schwann cells (unstained) decreased while dead Schwann cells (stained blue) increased along with increasing concentration of nilotinib. Figures (A-F) were cells treated by 0, 2, 4, 6, 8 and 10 μM nilotinib, respectively.

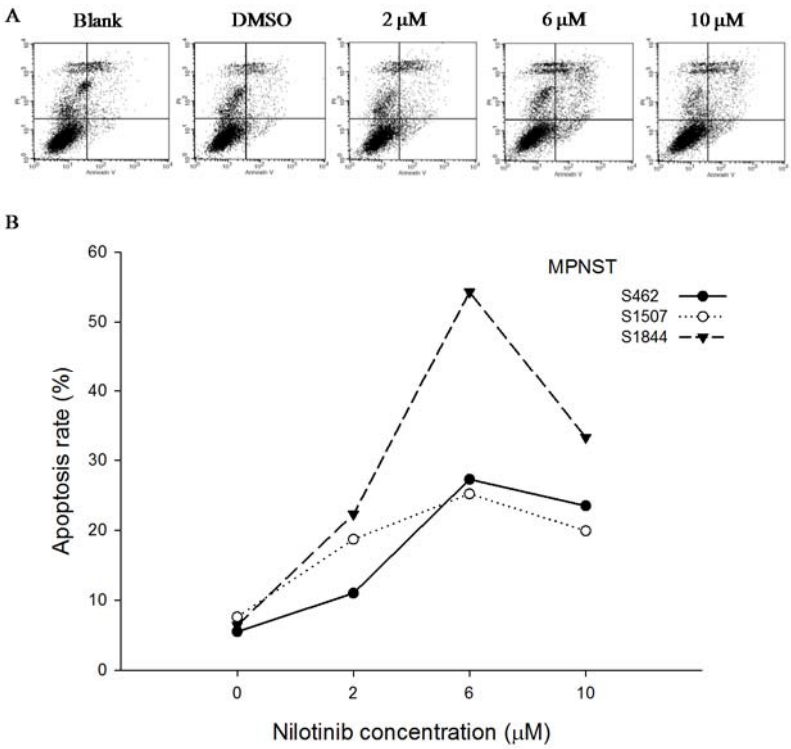

Fig. 9. Apoptosis of three MPNST cell lines after nilotinib-treatment. A: FACS of annexin V/PI-stained MPNST S462 cells cultured at various nilotinib concentrations. In each panel,

four distinct cell populations are visible: normal viable cells (lower left quadrant), apoptotic cells (lower right quadrant), late apoptotic cells (upper right quadrant) and necrotic cells (upper left quadrant). B: effect of nilotinib on apoptosis rates of three MPNST cell lines at various nilotinib concentrations.

1.3.1.5. Effect of nilotinib on collagenase activity

Collagenase activity varied largely among the cultures. Highest collagenase activity was detected in Schwann cells derived from a PNF (T2345). In contrast, no activity was detected in MPNST S1507 and Schwann cells from another PNF (T2346). In two PNF-Schwann cell cultures and two MPNST cell lines, collagenase activity was higher than 2 U/ml. In other cultures, it was below 1.1 U/ml. Nilotinib treatment for 24 h reduced collagenase activity in a dose-dependent manner with IC_{50} of 0.3-2.8 μM. At a concentration of 4 μM, nilotinib suppressed collagenase activity down to 1 U/ml in all cultures (Fig. 10).

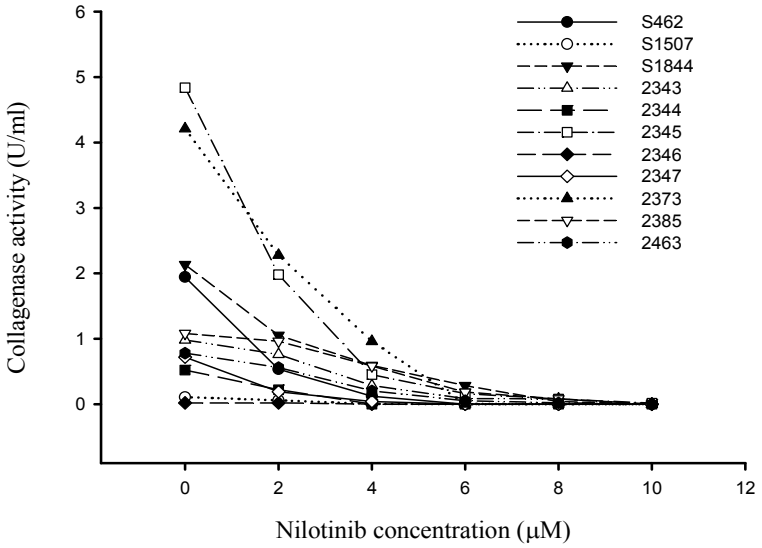

Fig. 10. Decrease of collagenase activity in MPNST cells and Schwann cells after nilotinib-treatment. Collagenase activity varied among MPNST cells and PNF derived Schwann cells, but all decreased to below 1 U/ml at 4 μM nilotinib.

1.3.2 Efficacy of nilotinib *in vivo*

1.3.2.1 Tolerance of mice to nilotinib

The food consumption and general behavior of all mice were monitored and no difference was observed between the groups during the experiment. All animals tolerated the treatments

well without observable signs of toxicity and no gross pathologic abnormalities were noted at necropsy.

After the nilotinib-treatment with a dose of 75 mg/kg/day, no difference of body weight was observed when compared with the control mice.

For the mice treated with 100 mg/kg/day nilotinib and imatinib, the body weights increased 10% ($23.9_{\pm 1.0}$ to $26.4_{\pm 1.6}$ g) in the control group and 7% ($23.4_{\pm 1.8}$ to $25.1_{\pm 1.8}$ g) in the nilotinib group but not in the imatinib group (2%, $23.3_{\pm 1.2}$ to $23.7_{\pm 1.7}$ g) over the 28 days of treatment period (Fig. 11). Only the difference between the imatinib group and the control group was significant ($P<0.05$).

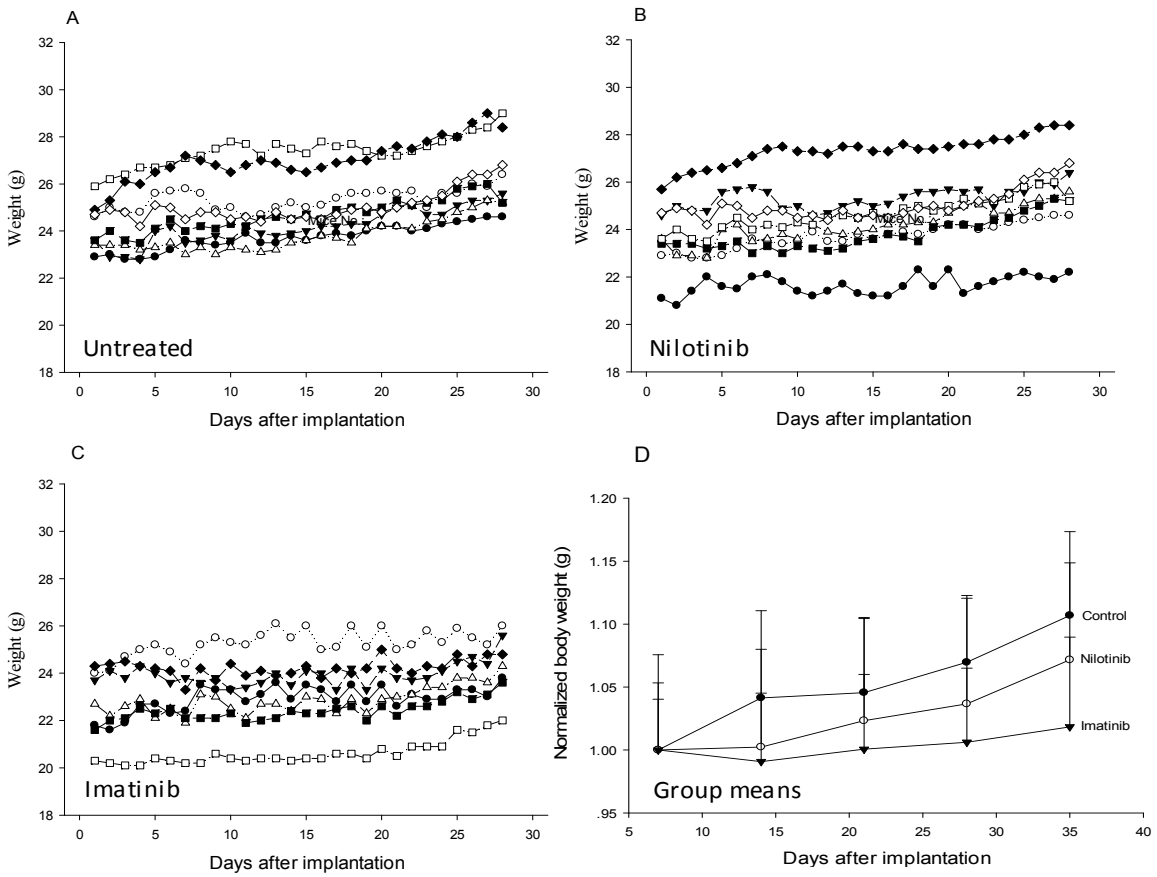

Fig. 11. Change of body weights of mice in the three groups over whole experiment period of 35 days in the untreated (A), nilotinib (B) and imatinib (C) groups. (D) Only the difference between the imatinib group and the control group was significant ($P<0.05$).

1.3.2.2 Plasma nilotinib concentration

Peak plasma nilotinib concentration was $6.6_{\pm 1.1}$ μM (Fig. 12). No correlation was observed between the extent of reduction of tumor volumes and the plasma nilotinib concentrations

(r=0.24, *P*>0.05).

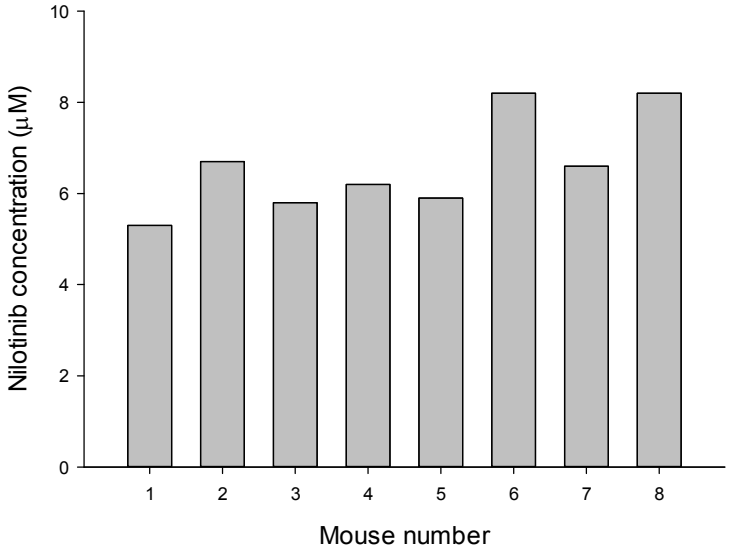

Fig. 12. The peak plasma nilotinib concentrations of all 8 mice in the nilotinib treated group.

1.3.2.3 Volume changes of tumor grafts

After treatment by nilotinib with a dose of 75 mg/kg/day, there was no difference in grafts volume between the treated and control groups (Fig. 13, *P*>0.05).

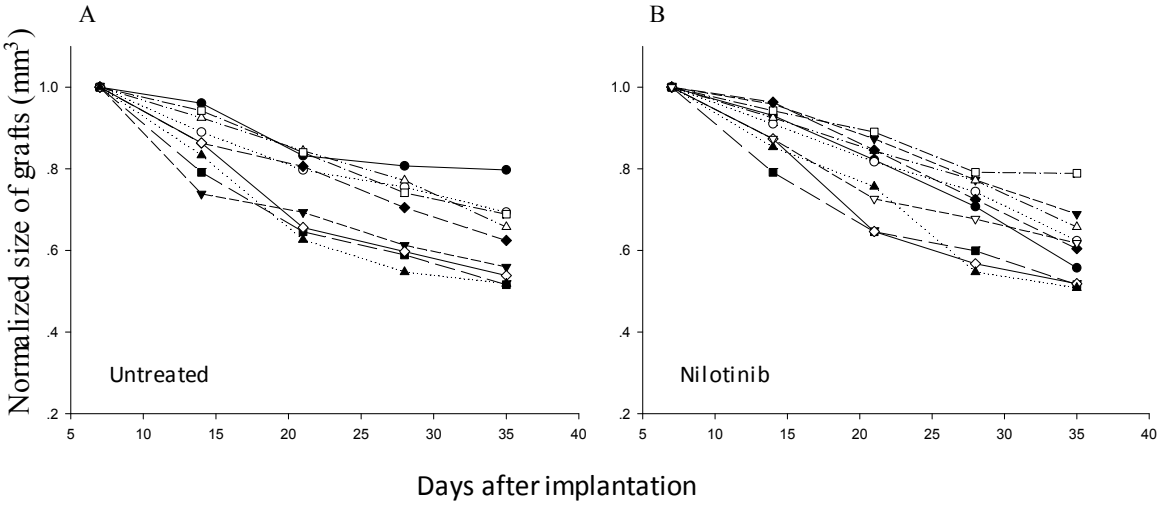

Fig. 13. Volume changes of PNF grafts in the mice without treatment (A) and treated by nilotinib (B) with a dose of 75 mg/kg/day. No difference was observed between the two groups (*P*>0.05).

The initial sizes of the xenografts in group 2 (100 mg/kg/day) were comparable among the

control, nilotinib and imatinib groups, which were $6.0_{\pm 3.7}$ mm^3, $5.9_{\pm 2.5}$ mm^3 and $5.2_{\pm 3.4}$ mm^3, respectively. Grafts decreased in size in all mice for the first two weeks and stabilized and slightly decreased in untreated mice (Fig. 14A). In contrast, size of the xenografts decreased in mice treated with nilotinib (Fig. 14B) or imatinib (Fig. 14C) continued. The decrease in xenograft size was significantly more profound in the nilotinib group than in the untreated group ($68_{\pm 7}$% vs. $33_{\pm 8}$%, $P<0.05$) and than in the imatinib group ($47_{\pm 15}$%, $P<0.05$, Fig. 14D).

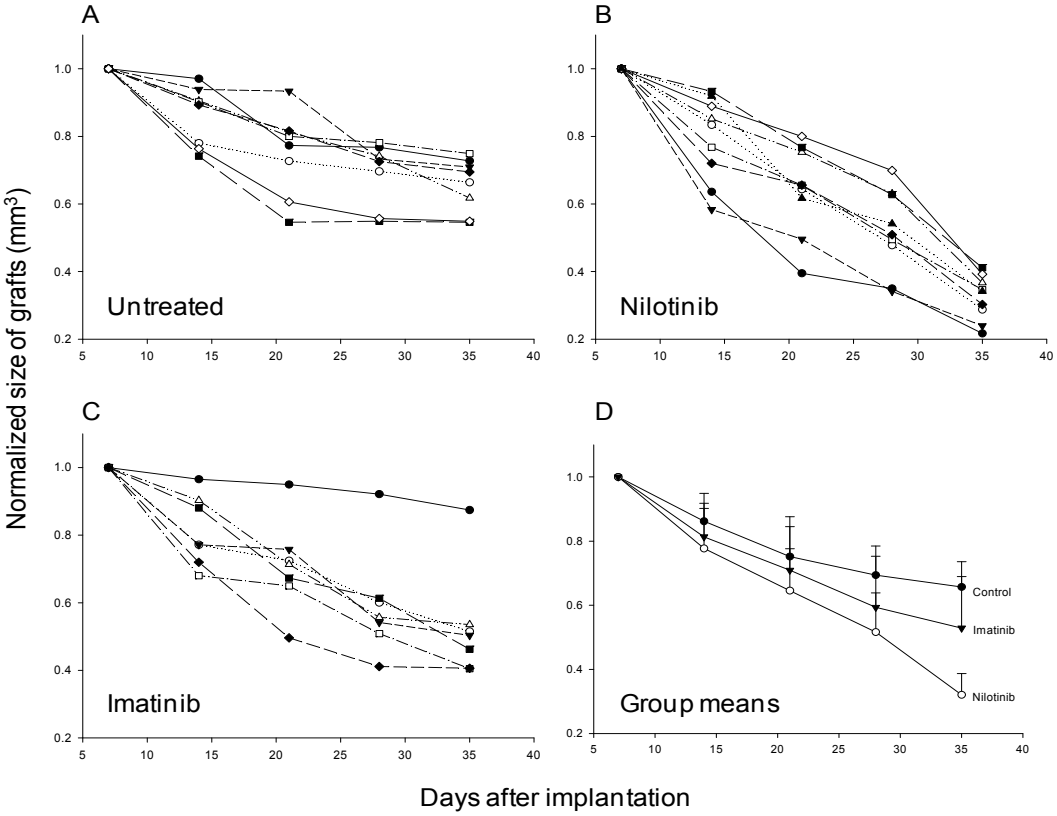

Fig. 14. Change of xenograft size in each mouse over the 4-weeks of treatment period in the untreated (A), nilotinib (B) and imatinib (C) groups. Group-means (D) differed significantly among the three groups ($P<0.05$).

1.3.2.4. Cytotoxicity of mouse spleen cells

Imatinib and nilotinib elevated cytotoxicity of mouse spleen cells on cultured PNF Schwann cells significantly, $21.1_{\pm 7.2}$% and $17.6_{\pm 6.2}$%, respectively vs. $12.5_{\pm 7.1}$% in spleen cells of untreated mice whereas imatinib mesylate was significantly more potent than nilotinib ($P<0.05$, Fig. 15). There was no correlation between the reduction of tumor volumes and the cytotoxicity of mouse spleen cells ($r=0.53$, $P>0.05$).

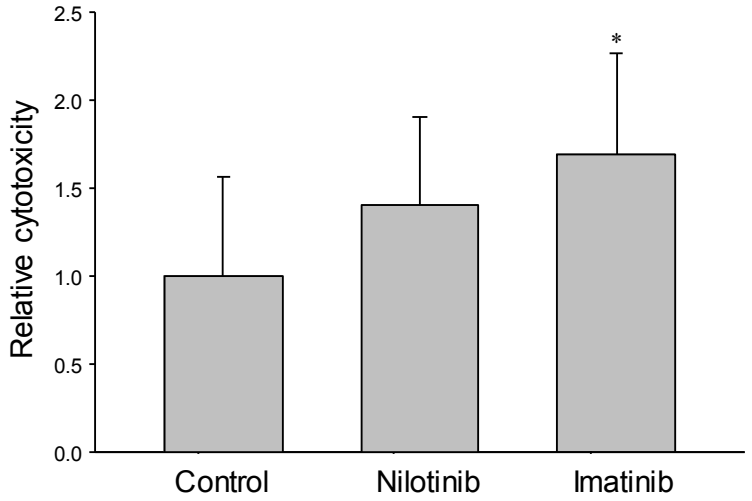

Fig. 15. Imatinib significantly elevated cytotoxicity of mouse spleen cells on cultured PNF Schwann cells (*P<0.05). The elevation by nilotinib was not significant.

1.4. Discussion

The results of the study showed that nilotinib dose-dependently inhibited proliferation, vitality and viability of all tested PNF-derived primary Schwann cells and MPNST cell lines with mean IC_{50} of 3.9-9.8 μM and 2.3-10.8 μM, respectively.

Nilotinib also had inhibitory effects on fibroblasts. However, the mean IC_{50} values ranged from 4.2 to 11.9 μM, which were higher than that for Schwann cells. Generally, lower IC_{50} values on tumor cells than on non-tumor cells may be considered as a clue to the good selectivity of a drug. In the case of nilotinib, the mean IC_{50} values for Schwann cells were lower than that for fibroblasts for five out of the eight PNFs studied, indicating good selectivity for the five PNFs. For the other three PNFs, IC_{50} values for some parameters were lower for fibroblasts than for the Schwann cells. All these differences are rather small and their clinical relevance remains to be clarified. However, the difference in the mean IC_{50} values for Schwann cells and fibroblasts may also or solely be due to cell type dependent sensitivity to the drug. Nevertheless, the concept of comparing efficacies on tumor cells and non-tumor cells in a primary culture is a step toward addressing drug-selectivity *in vitro*.

Tanaka et al. reported that the treatment of adult patients with chronic and accelerated phase CML with resistance or intolerance to prior therapy, nilotinib is dosed at 400 mg twice daily with a $1.95_{\pm 1.04}$ μM steady-state and a $4.27_{\pm 1.47}$ μM peak plasma concentration [43]. In the present study, 4 μM nilotinib, compatible to that of plasma concentration in patients of CML receiving nilotinib therapy, can significantly inhibit the proliferation and vitality of both Schwann cells and MPNST cells ($P<0.05$). Thus, these findings could be of clinical relevance.

The apoptotic analysis in all three MPNST cell lines showed that nilotinib induced the cell apoptosis in a dose-dependent manner up to the concentration of 6 μM, but dropped at 10 μM nilotinib, possibly due to cytotoxicity of the drug at higher concentrations. Ammoun et al. also found that 1 μM nilotinib inhibited the basal ERK and PDGFR-β activity, but lost the effect at a higher concentration (10 μM) due to the inhibition of a negative feedback loop [56].

Increased synthesis of certain extracellular matrix components, such as expression of MMPs, can contribute to tumor growth and progression. Collagenase, a member of the MMPs family, is generally expressed at low levels in normal tissues and up-regulated during tissue remodeling, wound healing and cancer progression [57]. In this study, higher collagenase activity was detected in some of the PNF-derived Schwann cells and some MPNST lines. Treatment with nilotinib for 24 h dramatically decreased collagenase activity in all cells to the base-line, indicating anti-oncogenic effect of the drug.

Owing to their infiltrating nature, unfavorite localization and other features, many PNFs are unresectable and many others can only be resected to limited extent in order to avoid damaging adjacent nerves, organs and tissues. Further, the multi-features and diverse lesions of NF1 may not all be the result of the same pathologic mechanism and may not respond to the same treatments. A recent phase 2 trial for imatinib reported subjective clinical improvement in airway patency, bladder control and extremity motor function in several cases [28]. Another pilot study addressing safety/efficacy of nilotinib for PNFs is ongoing currently [58]. In this study, fragments of human PNF tumors were semi-orthotopically implanted into injured sciatic nerve of nude mice. No growth was observed in any of the xenografts, in concordance with the result of the natural history study of PNF, which showed no growth in the majority of cases and very slow growth over years in the few cases where the tumors do grow. It is therefore unreasonable to expect any detectable growth of small xenografts in short experiment periods of less than 2 months. In fact, xenografts tend to shrink in the 2 to 3 weeks after the implantation, likely due to clearance of pre-grafting and post-grafting cell death. Drug effect can therefore be described as an increase in graft size reduction, which is more profound after 2 to 3 weeks. Indeed, we could detect significantly more grafts size reduction in the drug-treated mice than in the untreated control mice. Furthermore, the significantly more potent effect of nilotinib over imatinib could also be demonstrated in this *in vivo* model. This is of high relevance for patients with PNF, which suffer mostly from the secondary tumor size effects.

Recently, a growth deceleration of PNF was reported in children treated with imatinib [59]. In concordance, we also observed a deceleration of body weight increase in mice treated with imatinib. Judging from body weight, nilotinib was better tolerated than imatinib by the mice, indicating a better side effect profile of the former.

Since off-target effects of imatinib have been reported, among them the effect of activating natural killer cells [60]. Cytotoxicity of spleen cells from the treated mice was measured in this study. The results indicated an elevated cytotoxicity of spleen cells in imatinib-treated mice and to a lesser extent, in nilotinib-treated mice. This finding suggests that imatinib might have an immune-activating component in its anti-PNF effect, such that its pharmacological mechanism differs from that of nilotinib.

In summary, the results of this study indicate that nilotinib is effective against NF1-associated tumor cells and it is more potent than imatinib both *in vitro* and *in vivo*, suggesting the potential clinical application of nilotinib for PNFs.

2. LIST OF ABBREVIATIONS

%	Percent
℃	Celsius degree
AC	Adenylyl cyclase
BrdU	5-bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
CML	Chronic myelogenous leukemia
CO_2	Carbon Dioxide
DAPI	4'-6-diamidino-2-phenylindole
DDR	Discoidin domain receptor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
ERK	Extracellular signal-regulated kinase
ERK1/2	Extracellular signal-regulated kinase 1/2
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
h	Hour
HRGβ1	Heregulin β1
IBMX	3-iso-butyl-L-methylxanthine
IC_{50}	50% inhibitory concentration
IgG	Immunoglobulin G
kg	Kilogram
LC-MS	Liquid chromatography-tandem mass spectrometry
MAPK	Mitogen-activated protein kinase

MEK	Mitogen-activated protein kinase
mg	Milligram
min	Minute
ml	Milliliter
mm^3	Cubic millimeter
mM	Millimole
MMPs	Matrix metalloproteinases
MPNST	malignant peripheral nerve sheath tumor
mTOR	Mammalian target of rapamycin
NF1	Neurofibromatosis type 1
NIH	National Institutes of Health
nm	Nanometer
nM	Nanomole
NMP	N-methyl-pyrrolidinone
PBS	Phosphate-buffered saline
PDGFR-α	Platelet-derived growth factor receptor-α
PDGFR-β	Platelet-derived growth factor receptor-β
PEG300	Polyethylene glycol 300
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PNF	Plexiform neurofibroma
Raf	Murine sarcoma viral oncogene homologue
$rHRG\beta1_{177-244}$	Recombinant peptide of HRGβ1 residues 177-244
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RTK	Receptor tyrosine kinase
SD	Standard deviation
U	Unit
XTT	2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-(phenylamino) carbonyl]-2H-tetrazolium hydroxid
µg	Microgram
µl	Microliter
µm	Micrometer

μM	Micromole

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4. **PUBLICATION 1**

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LABORATORY INVESTIGATION

Efficacy and selectivity of nilotinib on NF1-associated tumors in vitro

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Abstract Neurofibromatosis type 1 is a tumor suppressor gene disorder which predisposes patients to cutaneous neurofibromas, plexiform neurofibromas (PNFs) and malignant peripheral nerve sheath tumors (MPNSTs) among other neoplasias and manifestation. In this study, we examined the efficiency of nilotinib on PNF-derived Schwann cells and on cells of established MPNST lines in vitro. Nilotinib treatment for 10 days led to decreased proliferation, viability and vitality of the cells with 50 % inhibitory concentration (IC_{50}) for proliferation varying from 3.1 to 9.0 μM. We further addressed selectivity of the drug for tumor cells by simultaneously examining its efficacy on tumor cells (Schwann cells) and non-tumor cells (fibroblasts) from the same tumor. For four out of the six PNFs studied, IC_{50} was lower in Schwann cells than in fibroblasts for all parameters measured (proliferation, vitality and viability), indicating good drug selectivity. In addition, nilotinib induced apoptosis and suppressed collagenase activity. Our results suggest that nilotinib may provide a treatment option for some PNFs and MPNSTs and our in vitro model of comparative treatment on tumor and non-tumor cells may provide a prototype of preclinical drug screening system toward personalized treatment.

Keywords Neurofibromatosis type 1 · Plexiform neurofibroma · Malignant peripheral nerve sheath tumors · Nilotinib · Drug selectivity

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Background

Neurofibromatosis type 1 (NF1) is an autosomal dominant, multisystem disorder with the hallmark of café-au-lait spots and neurofibromas [1, 2]. Plexiform neurofibromas (PNFs) are also frequent (30–50 %) in NF1 patients [3, 4]. These benign tumors can displace and/or penetrate neighboring organs, impair functions, cause pain and serious disfigurement, and have high risk of malignant transformation into malignant peripheral nerve sheath tumors (MPNSTs) which is the leading cause of NF1-related death [5]. To date, treatment of PNFs has been limited to surgical intervention. However, since the tumors often infiltrate adjacent tissues, complete resection is usually not possible without damaging nerves and healthy tissues. There is no established medical therapy available for PNFs and MPNSTs so far [6, 7].

Previously, we found amplification and mutations of PDGFR-α and KIT in MPNSTs, and wide expression of ligand PDGFR-α in MPNSTs and neurofibromas. Also focal c-Kit expression was detected in some MPNSTs [8]. Our more recent study further revealed expression of PDGFR-α and PDGFR-β in PNFs and in PNF-derived primary Schwann cells. We found that imatinib mesylate, a receptor tyrosine kinase inhibitor with high potency against

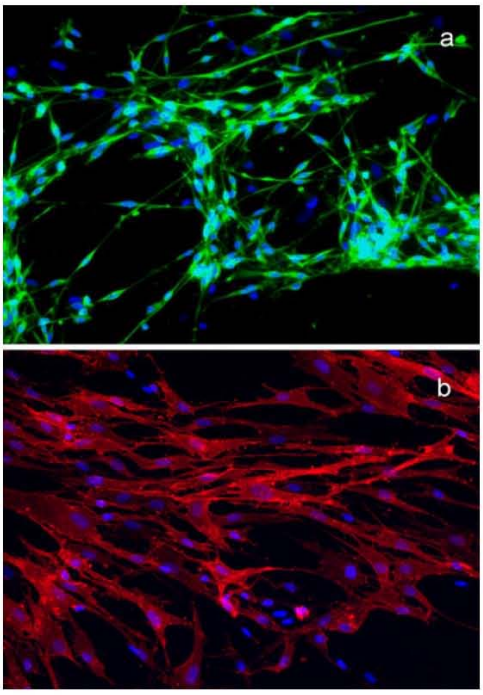

Fig. 1 Schwann cells (**a**) and fibroblasts (**b**) derived from a PNF, stained with S100 and CD90, respectively

the platelet-derived growth factor receptors, suppresses proliferation of PNF-derived Schwann cells in vitro and induced regression of PNFs xenografts in vivo [1]. A recent phase 2 trial reported >20 % shrinkage of PNFs in 26 % of the NF1 patients treated with imatinib mesylate [6].

Nilotinib is a new drug with similar potency to that of imatinib against the c-KIT, PDGFR-α, PDGFR-β and discoidin domain receptors, but has a number of advantages including a different toxicity profile and a lower incidence of fluid retention [9–12]. A recent assessment reported statistically significant advantage of nilotinib compared with imatinib for cytogenetic response and major molecular response in chronic myeloid leukaemia. Haematological, non-haematological events (nausea, diarrhoea, vomiting and muscle spasm events) and oedema events were more frequent for imatinib. In contrast, headache, pruritus and alopecia events were more frequent for nilotinib [10].

In this study, we examined the efficacy of nilotinib on PNF-derived primary Schwann cells and established MPNST cell lines in vitro, by means of monitoring cell proliferation, vitality, viability, apoptosis and collagenase activity. Furthermore we addressed selectivity of nilotinib for tumor cells by comparing its efficacy on tumor cells (Schwann cells) and non-tumor cells (fibroblasts) from the same PNF.

Materials and methods

Cell cultures

PNFs tumor tissues were obtained from six unrelated NF1 patients who underwent surgery at the Department of Maxillofacial Surgery, University Hospital Hamburg Eppendorf. Diagnosis of NF1 was conducted according to the modified National Institutes of Health criteria [13]. All patients gave informed written consent and the Institutional Review Board approved the study. All the specimens were kept in Hanks buffered saline immediately after surgical removal. A portion of each tumor was stored at −80 °C, and the remaining tissue was used to establish primary Schwann cell and fibroblast cultures under conditions optimized for Schwann cell and standard condition, respectively as previously described [1]. Only Schwann cell cultures with more than 85 % S100 positive cells were included in this study (Fig. 1a). Fibroblasts (Fig. 1b) were identified by staining with 2 μg/mL anti-fibroblast antibody CD90 (DAKO, Copenhagen, Denmark).

Three MPNST cell lines used in the study, S462, S1507 and S1844, were established and genetically verified in our laboratory [4, 7].

Nilotinib treatment

Cells were cultured with nilotinib (Novartis, Switzerland) at various concentrations (0, 2, 4, 6, 8 and 10 μM) for 10 days for proliferation and vitality assays, 48 h for viability, and 24 h for apoptosis and collagenase assays. Since stock solution of nilotinib was in DMSO, a medium with 0.2 % of DMSO was used as control. Medium containing nilotinib was refreshed daily, considering the 18 h half-life of nilotinib in human serum [9].

Proliferation, apoptosis and collagenase assays

Proliferation and vitality were monitored using a colorimetric bromodeoxyuridine assay and a XTT assay (Roche, Mannheim, Germany), respectively. Relative proliferation and vitality were calculated as ratio of absorbance at 450 nm of treated cells to controls. Each drug concentration was tested in 12 replicates. The proportion of viable cells after drug treatment was determined by direct counting with 0.4 % trypan blue solution (Sigma, MO, USA). Viability was defined as proportion of viable cells treated with nilotinib to that of untreated cells.

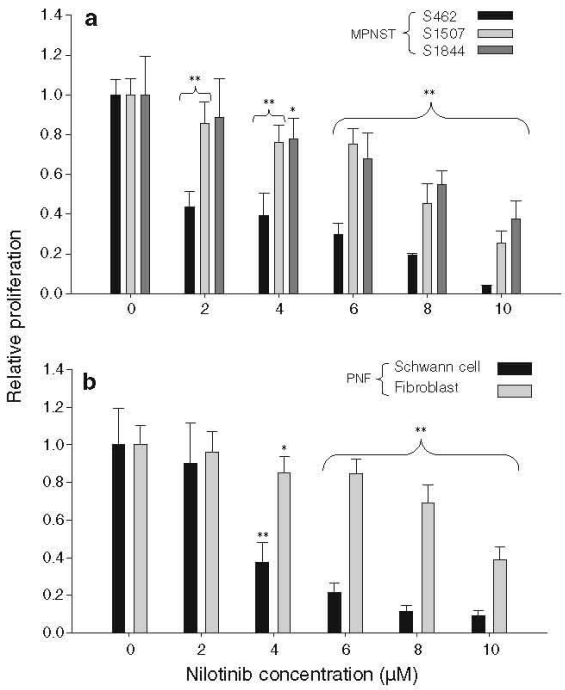

Fig. 2 Efficacy of nilotinib on proliferation of cells of three MPNST lines (**a**), Schwann cells and fibroblasts derived from a PNF (**b**). Proliferation was normalized to that of DMSO controls by dividing the absorbance at 450 nm of the former with that of the latter. Values differing significantly from untreated cells are marked with * ($P < 0.05$) or ** ($P < 0.005$)

Apoptosis was examined using Annexin-V-binding assay (BD Biosciences, Mannheim, Germany). Since the flow cytometry-based assay requires a large number of cells, only MPNST cells could be studied. Briefly, MPNST cells of S462, S1507 and S1844 were treated with nilotinib medium (0, 2, 6 and 10 μM) for 24 h and collected by trypsinization. The cells were then stained with 5 μL of FITC-labeled Annexin V dye and propidium iodide for 15 min in the dark at room temperature at a concentration of 2×10^6 cells/mL, and subsequently analyzed with a flow cytometer using Cell Quest Software (BD Biosciences), calculating the percentage of apoptotic and/or necrotic cells.

Collagenase activity was determined in medium of cell cultures treated with nilotinib for 24 h, using a collagenase assay kit (Chondrex, WA, USA). Fluorescence of digested substrate at 490 nm excitation and 520 nm emission was measured using fluorescence reader.

Statistical analysis

Proliferation and vitality of treated cells at various concentration of nilotinib were compared to those of untreated cells using *t* test.

Results

Nilotinib reduced cell proliferation, vitality and viability

Nilotinib dose-dependently inhibited proliferation (Fig. 2a, b), vitality and viability of all tested PNF-derived primary Schwann cells and MPNST lines with mean 50 % inhibitory concentration (IC_{50}) of 3.1–9.0 μM for proliferation (Table 1). MPNST S462 cells were most sensitive to nilotinib and had the lowest IC_{50} of 3.1 μM.

From all the six PNFs, we also cultured and tested fibroblasts which are non-tumor cells. The IC_{50} values of nilotinib for proliferation of fibroblasts ranged from 5.4 to 10.7 μM, generally higher than that for Schwann cells. For 4 (67 %) PNFs, IC_{50} was lower for Schwann cells than for fibroblasts for all three parameters (proliferation, vitality and viability), indicating a good selectivity of nilotinib. In another PNF, IC_{50} for proliferation was lower for Schwann cells while that for vitality and viability not (Table 1). For the last PNF (#2344), IC_{50} was higher for Schwann cells than for fibroblasts regarding all three parameters.

Apoptosis

Nilotinib treatment induced apoptosis in cells of all three MPNST lines in a dose-dependent manner up to the concentration of 6 μM (Fig. 3). Apoptosis dropped at 10 μM nilotinib, possibly due to cytotoxicity of the drug at higher concentrations.

Effect of nilotinib on collagenase activity

Collagenase activity varied largely among the cultures. In two PNF-Schwann cell cultures and two MPNST cell lines, it was higher than 2 U/mL. In other cultures, it was below 1 U/mL (Fig. 4). Nilotinib treatment for 24 h reduced collagenase activity in a dose-dependent manner with IC_{50} of 0.3–2.8 μM. At a concentration of 4 μM, nilotinib suppressed collagenase activity down to <1 U/mL in all cultures (Fig. 4).

Discussion

We found an inhibitory effect of nilotinib on proliferation, vitality and viability of PNF and MPNST cells with mean IC_{50} values in the range of 3.1 to 9.0 μM, lower than those of imatinib in our previous study which were higher than 10 μM [1]. Further, it has to be considered that nilotinib-treatment period in the present study was 10 days, only approximately one-third of that of imatinib-treatment in our previous study which was 28 days. Therefore, nilotinib

Table 1 IC_{50} and 95 % confidence (μM) of nilotinib on proliferation, viability and numbers of cells

Cells	Proliferation		Viability		Cell number	
	Sch	F	Sch	F	Sch	F
PNF 2346	3.9 (3.0–3.8)	9.5 (7.0–11.7)	6.9 (5.1–9.3)	11.9 (9.4–25.8)	4.9 (4.2–6.3)	7.6 (4.8–20.2)
PNF 2345	5.3 (4.2–6.7)	6.1 (5.0–7.3)	6.2 (5.2–7.3)	9.8 (3.3–10.2)	5.8 (3.8–7.0)	6.9 (4.2–9.2)
PNF 2347	5.3 (3.7–6.8)	6.8 (6.1–7.8)	7.6 (5.6–19.9)	4.2 (2.8–19.6)	6.2 (3.4–7.1)	5.9 (5.1–7.4)
PNF 2343	5.4 (4.0–6.5)	5.8 (4.9–7.5)	5.3 (4.4–7.1)	7.4 (6.5–9.2)	5.8 (4.7–7.4)	6.5 (4.9–7.5)
PNF 2373	7.9 (6.4–9.1)	10.7 (8.9–11.9)	4.9 (3.1–5.9)	8.3 (3.7–15.0)	7.4 (3.9–27.8)	10.3 (3.9–23.1)
PNF 2344	9.0 (7.7–10.8)	5.4 (3.8–6.7)	8.5 (6.9–11.9)	6.5 (4.0–9.1)	9.8 (6.8–19.0)	7.8 (6.2–10.1)
MPNST S462	3.1 (2.0–3.8)		2.3 (1.2–3.6)		3.3 (2.7–4.0)	
MPNST S1507	6.7 (5.0–7.8)		5.8 (3.7–6.7)		5.2 (3.8–6.6)	
MPNST S1844	8.3 (7.9–9.9)		10.8 (8.4–12.4)		8.9 (7.8–11.0)	

Sch Schwann cells, *F* fibroblasts

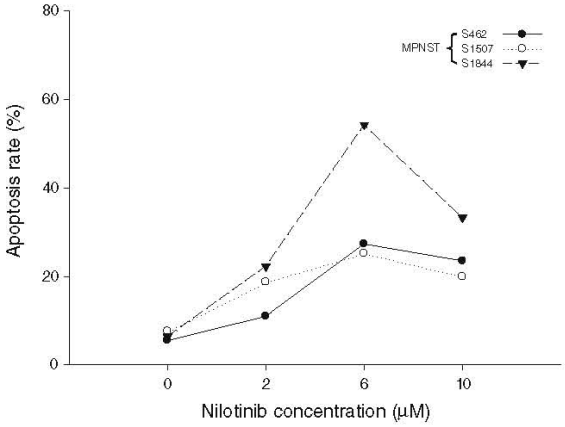

Fig. 3 Effect of nilotinib on apoptosis rates of three MPNST cell lines

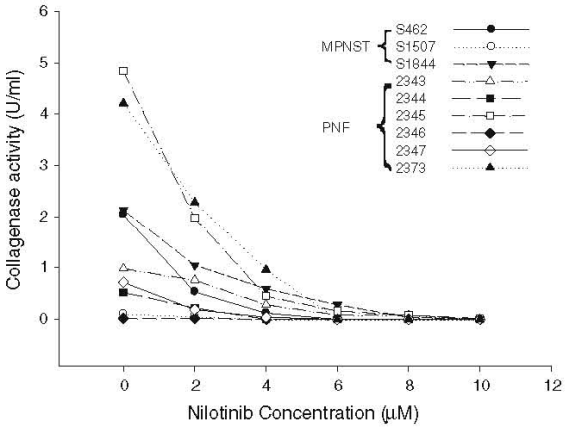

Fig. 4 Collagenase activity varied among MPNST cells and PNF-derived Schwann cells, but all decreased to below 1 U/mL at 4 μM nilotinib

seems to be more potent than imatinib for NF1-associated tumor cells in vitro. The shorter period of 10 days of drug treatment was set for the current experiments because we included fibroblasts as controls which grow much faster and die after 10 days due to overgrowth. Further studies should compare efficacy of imatinib and nilotinib directly in the same experimental setting.

For the treatment of adult patients with chronic and accelerated phase chronic myeloid leukemia with resistance or intolerance to prior therapy, nilotinib is dosed at 400 mg twice daily with a 1.95 ± 1.04 μM steady-state and a 4.27 ± 1.47 μM peak plasma concentration [10]. IC_{50} values in our study are slightly higher, but within the same order and thus our findings could be of clinical relevance.

A special feature of the present study was the assessment of drug selectivity. Cell cultures derived from tumors enable testing drugs in vitro for their efficacy to some extent but rarely their selectivity which is however of decisive importance for clinical application. We approached this issue by comparing efficacy of nilotinib on tumor cells and non-tumor cells derived from the same tumor. We and others have previously shown that loss of heterozygosity of the *NF1* gene are present only in Schwann cells but not in fibroblasts derived from same cutaneous neurofibromas [14, 15], and we confirmed this finding also in PNFs (unpublished data), providing genetic evidence that Schwann cells are the tumor cells in PNFs while fibroblasts are non-tumor cells. Generally, lower IC_{50} values on tumor cells than on non-tumor cells may be considered as an indication of good selectivity of a drug. In the case of nilotinib, the IC_{50} value for Schwann cells was lower than that for fibroblasts for four out of the six PNFs studied, indicating good selectivity for these PNFs. For the other two PNFs, IC_{50} values were lower for fibroblasts than for the Schwann cells. All these differences are rather small and their clinical relevance remains to be clarified.

Furthermore, the difference in the IC_{50} values for Schwann cells and fibroblasts may also or solely be due to cell type-dependent sensitivity to the drug. Nevertheless, this way of thinking of comparing efficacies on tumor and non-tumor cells is a step toward addressing drug-selectivity in vitro.

Collagenase, an extra cellular matrix component, is generally expressed at low levels in normal tissues and up-regulated in tissue remodeling, wound healing and oncogenesis [16]. Higher collagenase activity was detected in some of the PNF-derived Schwann cells and some MPNST lines. Nilotinib-treatment reduced collagenase activity in all cells to the base-line, indicating anti-oncogenic effect of the drug.

Due to their infiltrating nature, unfavorite localization and other features, many PNFs are unresectable and many others can only be resected to limited extent in order to avoid damaging adjacent nerves, organs and tissues. A recent phase 2 trial for imatinib mesylate reported subjective clinical improvement in airway patency, bladder control and extremity motor function in several cases [6]. Currently, we are testing efficacy of nilonitib in vivo in a series of experiments by xenografting PNF fragments onto sciatic nerve of immunodeficient mice. Also pilot studies addressing safety/efficacy of nilotinib for PNFs is ongoing [17]. Should nilotinib be more potent and have less side effect than imatinib mesylate, it will provide an alternative treatment option especially for symptomatic PNFs which are unresectable due to the tumour itself or other circumstances such as the patient's general condition.

We further demonstrated that comparing efficacy of a drug on tumour cells and non-tumour cells derived from the same tumor enables testing drug selectivity in vitro and thus may open a new perspective for preclinical drug-screening and for individualized drug-selection towards personalized medicine, for PNFs, MPNSTs and also other tumors.

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Conflict of interest The authors declare that they have no further conflict of interest.

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5. PUBLICATION 2

Nilotinib is more potent than imatinib for treating plexiform neurofibroma *in vitro* and *in vivo*. PLoS ONE, 2014, 9 (10): e107760.

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Nilotinib Is More Potent than Imatinib for Treating Plexiform Neurofibroma *In Vitro* and *In Vivo*

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Abstract

Plexiform neurofibromas (PNFs) are benign nerve sheath tumors mostly associated with neurofibromatosis type 1. They often extend through multiple layers of tissue and therefore cannot be treated satisfactorily by surgery. Nilotinib is a tyrosine kinase inhibitor used to treat leukemia, with advantages over the prototype imatinib in terms of potency and selectivity towards BCR-ABL, and the DDR, PDGFR, and KIT receptor kinases. In this study, we compared efficacies of the two drugs on cultured cells of PNF *in vitro* and on xenografted tumor fragments on sciatic nerve of athymic nude mice. Xenografts were monitored weekly using a high resolution ultrasound measurement. Treatment with nilotinib at a daily dose of 100 mg/kg for four weeks led to a reduction of the graft $sizes_{std}$ by $68_{\pm 7}$% in the 8 treated mice, significantly more than the $33_{\pm 8}$% reduction in the 8 untreated mice ($P<0.05$) and the $47_{\pm 15}$% in the 7 mice treated with imatinib ($P<0.05$). The peak plasma nilotinib concentration $6.6_{\pm 1.1}$ μM is within the pharmacological range of clinical application. Imatinib, but not nilotinib significantly hindered body weight increase of the mice and elevated cytotoxicity of mouse spleen cells ($P<0.05$). Our results suggest that nilotinib may be more potent than imatinib for treating PNFs and may also be better tolerated. Imatinib seems to have some off-target effect in elevating immunity.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

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Competing Interests: The co-author Paul W. Manley is employed by Novartis. Novartis has financed the study, has developed the drug nilotinib (as well as Glivec) and holds the following patents on nilotinib: Pyrimidylaminobenzamide derivatives for treatment of neurofibromatosis US 8604045 B2. The authors confirm that this does not alter their adherence to PLOS ONE policies on sharing data and materials.

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Introduction

Plexiform neurofibromas (PNFs) are benign tumors originating from peripheral nerve sheath and mostly associated with neurofibromatosis type 1 (NF1), a tumor suppressor gene syndrome [1]. Depending on their location, size and growth type, PNFs can cause pain and disfigurement, functional impairment of vision, mobility, bladder and bowel [2]. PNFs have a high risk of malignant transformation into malignant peripheral nerve sheath tumors (MPNST) which is the leading cause of NF1-related death [3 5]. The lifetime risk of MPNST for NF1-patients has been estimated to be about 8 to 13% and thus is more than 1000 times higher for these patients than for the general population. The lifetime risk to develop an MPNST increases to 30 50% in patients with NF1 and PNF [6 8]. Since PNFs often extend through multiple layers of tissue, total resection is usually not possible without damaging functions and organs [9 10].

Nilotinib (Tasigna; Novartis Pharmaceuticals) is an orally active tyrosine kinase inhibitor which targets ABL (and the oncogenic BCR-ABL), together with several receptor tyrosine kinases including those for stem cell factor (c-KIT), collagen (DDR-1/-2) and platelet-derived growth factor (PDGFR-α/-β) [11 13]. Nilotinib has a number of advantages over imatinib (Glivec; Novartis Pharmaceuticals), including a different toxicity profile and a lower incidence of fluid retention. A recent clinical trial of newly diagnosed chronic myelogenous leukemia indicated that nilotinib was superior to imatinib in terms of potency and selectivity of BCR-ABL inhibition; reduction of progression events; absence of a response plateau [14,15].

Our more recent study revealed an inhibitory effect of nilotinib on proliferation, viability and vitality of PNF-derived Schwann cells and nerve sheath tumor cells *in vitro* with 50% inhibitory concentration (IC_{50}) values lower than that of imatinib in our previous study [16,17]. However, the experimental settings were different in the two *in vitro* studies and an *in vivo* study was not performed. In the present study, we comparatively studied efficacies of the two drugs on PNF *in vitro* on cultured tumor cells and *in vivo* on xenografted tumor fragments in mice.

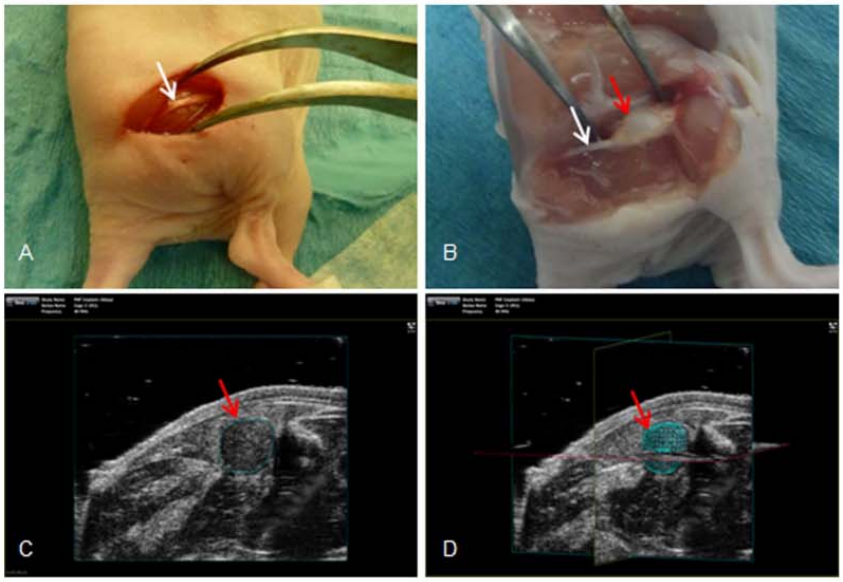

Figure 1. Xenograft on sciatic nerve in mouse. (A) The exposed sciatic nerve (white arrow) for implantation, (B) a PNF xenograft (red arrow) integrated onto the sciatic nerve, (C) images and (D) three-dimensional reconstruction of a xenograft by (red arrow) a Vevo 2100 micro-imaging system.
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Materials and Methods

Ethical approvals

For the donation of human biological samples approval OB-061/05 by the Institutional Review Board of the University Hospital Hamburg-Eppendorf; for animal studies approval Hamburg 112/11 by the Animal Care and Use Committee of Hamburg.

Specimen and *in vitro* study

The donor of the study specimen was a 12-year-old female NF1 patient, diagnosed according to the modified National Institutes of Health criteria [18]. A parent of the patient gave informed written consent in addition to assent from the patient. The Institutional Review Board approved the study (OB-061/05). Her PNF was operated at the Department of Maxillofacial Surgery, University Hospital Hamburg-Eppendorf. A part of the tumor was kept in Hanks buffered saline and delivered into the laboratory for cell culture and for xenografting. Schwann cells from the PNF were cultured and identified as previously described [16].

After ensuring purity of 85%, PNF-derived Schwann cells were treated with nilotinib and imatinib (Novartis Pharma AG, Switzerland), each at 0, 5, 10, 15 and 20 μM for 10 days. Cell proliferation and viability assays were performed as previously described [16].

RNA expression comparison of PNF-segments

To assess variability between PNF grafts, we performed RNA-sequencing from 4 pieces of the original tumor sample and from one unrelated PNF. RNA was isolated from snap-frozen tumors using Trizol. and mRNA was reverse transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsabd, CA). Sequencing libraries were prepared using TruSeq Stranded Total RNA Sample Preparation kit (Illumina, San Diego, CA) Libraries were sequenced on a Genome Analyzer IIx (Illumina, San Diego, CA). Alignment and analysis was performed using a Galaxy server and open source Chipster software (http://chipster.csc.fi/) as well as R bioconductor (http://www.bioconductor.org/) tools for calculating similarity clusters on a Galaxy server.

Xenografting and treatment

The care and use of laboratory animals were carried out in strict accordance with the local animal care and use committee's research council's guide (Approval No.: Hamburg 112/11). Athymic nude mice (female, nu/nu Balb/c, 6 weeks, 20 g) were obtained from Charles River Laboratories (Sulzfeld, Germany).

The xenografting of fragments of PNF was carried out as described previously [17,19]. Briefly, a small incision was made into the skin to expose the right sciatic nerve and an incision was made into the sciatic nerve, under which one tumor piece was orthtopically implanted (Fig. 1A, B). After confirming successful xenografting by ultrasound scanning one week later, the 23 mice were randomly allocated into three groups: 8 as controls, 8 for nilotinib treatment and 7 for imatinib treatment.

Nilotinib (as the hydrochloride salt) was diluted in 10% N-methyl-pyrrolidinone and 90% polyethylene glycol 300. Imatinib (as the mesylate salt) was dissolved in sterile water. Oral administration at a daily dose of 100 mg/kg of the drugs was started on day 7 after implantation and was continued to day 35. Body weights of the mice were recorded daily and the drug dosage was adjusted accordingly. Food consumption and general condition of the mice were monitored weekly.

At the end of the treatment, animal blood was collected 3 hours after the last oral administration of nilotinib for plasma preparation. Nilotinib concentration was measured using liquid chromatography-tandem mass spectrometry [20].

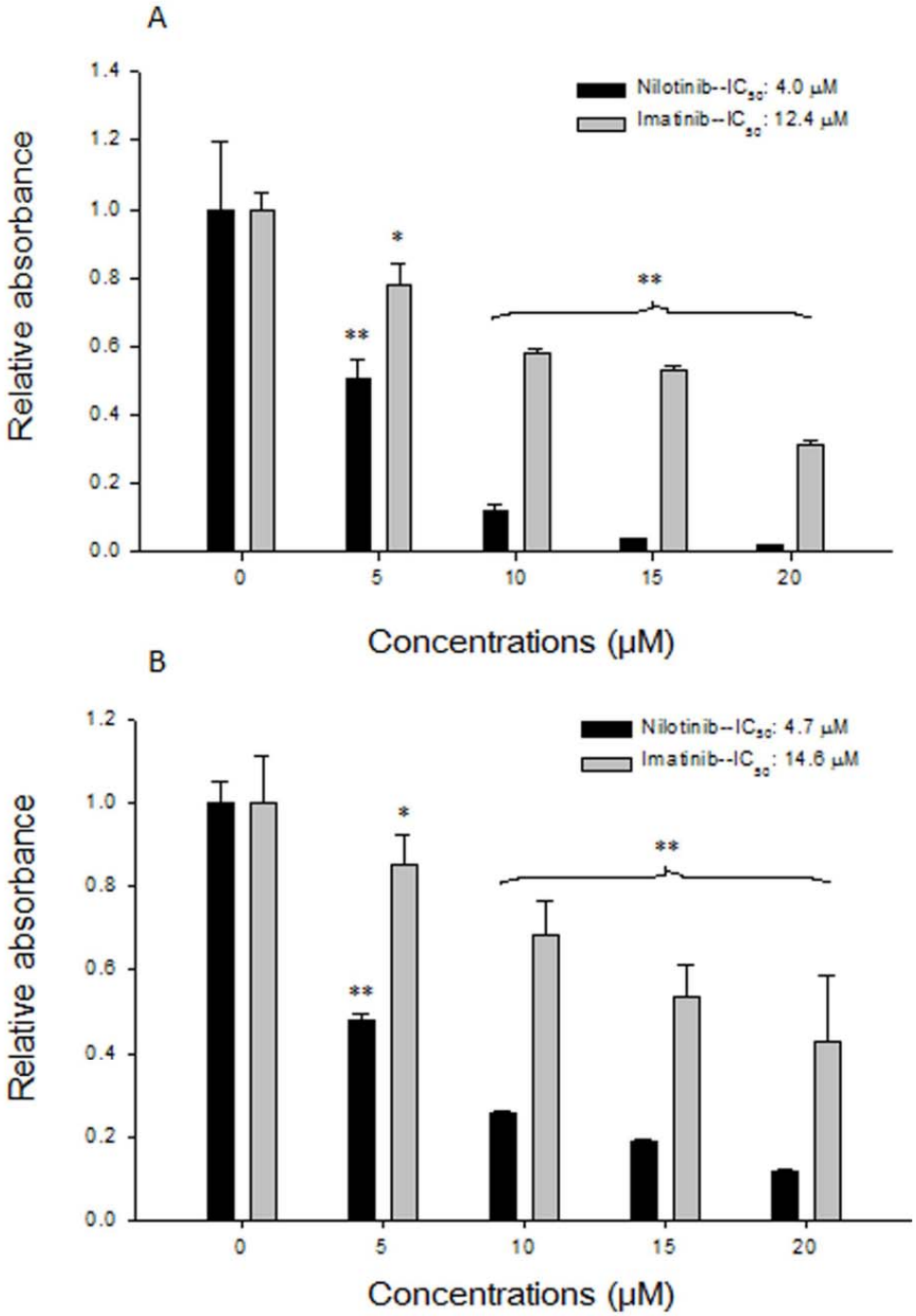

Figure 2. Effects of imatinib and nilotinib on proliferation (A) and viability (B) of PNF-derived Schwann cells. Data are absorbance normalized to that of untreated controls. Significant ($P<0.05$) and highly significant ($P<0.001$) differences were marked with * and **, respectively. doi:10.1371/journal.pone.0107760.g002

Ultrasound monitoring of xenografts

Sonographic measurement was performed using a Vevo 2100 micro-imaging system (VisualSonics, Amsterdam, Netherlands), which is a high-frequency, high-resolution digital imaging platform with linear technology and color Doppler mode. Xenografts were verified after 7 days post transplantation and measured weekly during the whole treatment period of 4 weeks. Three-dimensional

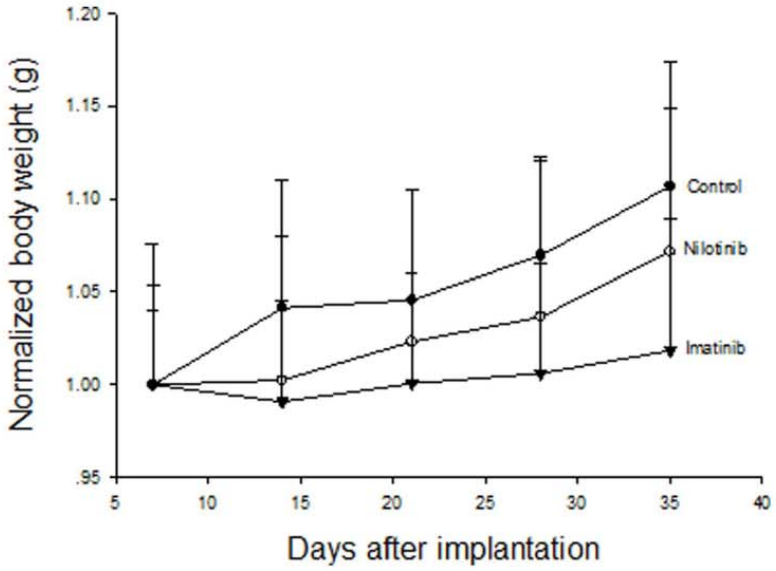

Figure 3. Change of body weights of mice in the three groups over whole experiment period of 35 days. Standard deviations are shown in single direction.
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images of the xenografts were generated and analyzed using the Vevo software version 5.0.0 to calculate their size in volume (Fig. 1B–D).

Cytotoxicity of mouse spleen cells

Non-adherent spleen cells were harvested from the mice after erythrocyte- depriving and 3 hours adherence at 37°C. Cytotoxic efficacy of the non-adherent spleen cells was assessed by adding them as effector cells to PNF-derived Schwann cells as target cells at a ratio of 1:10 for 4 h using the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega, Fitchburg, WI).

Statistical analysis

The effect of the drugs *in vitro* was evaluated using Student's *t*-test and the IC_{50} was calculated. Sizes of each xenograft and body weight of the mice were normalized against the corresponding initial values. Time course of size change of the xenografts and body weight increase of the mice in the three groups were compared with each other using a linear mixed model. Pearson correlation coefficients were calculated for the relationships between the reduction of tumor volumes and the cell cytotoxicity, and between the reduction of tumor volumes and the nilotinib concentrations. $P<0.05$ was considered significant. All averaged data were represented as the $\text{mean}_{\pm\text{standard deviation}}$.

Results

Nilotinib inhibits PNF cells more potently than imatinib

Both nilotinib and imatinib dose-dependently inhibited proliferation and viability of the PNF-derived Schwann cells (Fig. 2). However, the IC_{50} values of nilotinib were 4.0 and 4.7 μM, much lower than the 12.4 and 14.6 μM of imatinib.

Nilotinib suppressed PNF-xenografts more potently than imatinib

All animals tolerated xenografting and treatments well without visible signs of toxicity and gross abnormalities. General conditions of mice were also compatible among the untreated and the two treated groups. The body weights of mice increased 10% ($23.9_{\pm1.0}$ to $26.4_{\pm1.6}$g) in the control group and 7% ($23.4_{\pm1.8}$ to $25.1_{\pm1.8}$g) in the nilotinib group but not in the imatinib group (2% = $23.3_{\pm1.2}$ to $23.7_{\pm1.7}$g) over the 28-days of treatment period (Fig. 3). Only the difference between the imatinib group and the control group was significant ($P<0.05$).

The initial sizes of the xenografts were comparable among the control, nilotinib and imatinib groups, which were $6.0_{\pm3.7}$ mm^3, $5.9_{\pm2.5}$ mm^3 and $5.2_{\pm3.4}$ mm^3, respectively. Grafts decreased in size in all mice for the first two weeks and stabilized and slightly decreased in untreated mice (Fig. 4A). In contrast, size of the xenografts decreased in mice treated with nilotinib (Fig. 4B) or imatinib (Fig. 4C) continued. The decrease in xenograft size was significantly more profound in the nilotinib group than in the untreated group ($68_{\pm7}$% vs. $33_{\pm8}$%, $P<0.05$) and than in the imatinib group ($47_{\pm15}$%, $P<0.05$, Fig. 4D).

Peak plasma nilotinib concentration was $6.6_{\pm1.1}$ μM. No correlation was observed between the extent of reduction of tumor volumes and the plasma nilotinib concentrations ($r = 0.24$, $P>0.05$). RNA sequencing of 4 different pieces from the original PNF that was used for grafting showed similar but not identical expression patterns, indicating biological variability of the different pieces (data not shown).

Cytotoxicity of mouse spleen cells

Imatinib and nilotinib elevated cytotoxicity of mouse spleen cells on cultured PNF Schwann cells significantly, $21.1_{\pm7.2}$% and $17.6_{\pm6.2}$%, respectively vs. $12.5_{\pm7.1}$% in spleen cells of untreated mice whereas imatinib was significantly more potent than nilotinib ($P<0.05$, Fig. 5). There was no correlation between the reduction

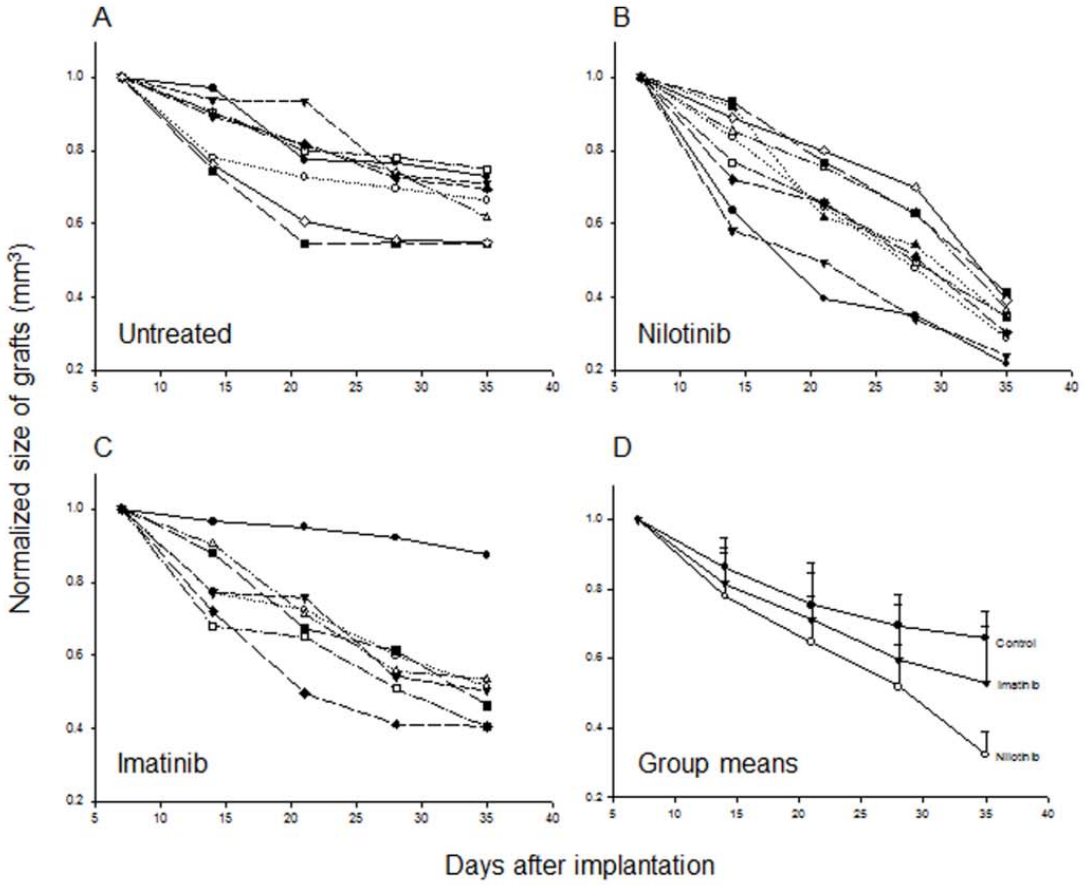

Figure 4. Change of xenograft size in each mouse over the 4-weeks of treatment period in the untreated (A), nilotinib (B) and imatinib (C) groups. Group-means (D) differed significantly among the three groups (P<0.05).
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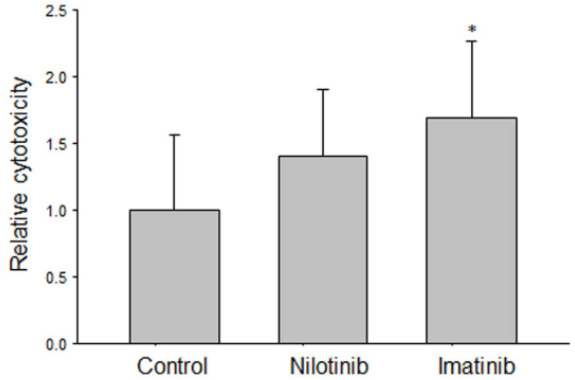

Figure 5. Imatinib significantly (*P<0.05) elevated cytotoxicity of mouse spleen cells on cultured PNF Schwann cells. The elevation by nilotinib was not significant.
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of tumor volumes and the cytotoxcity of mouse spleen cells (r = 0.53, P>0.05).

Discussion

We showed that nilotinib inhibited proliferation of PNF-derived Schwann cells *in vitro* substantially more potently than imatinib. The applied IC_{50} values of 4.0 μM and 12.4 μM were compatible to the plasma concentrations of the two drugs in patients [12,21], legitimating their potential clinical application for PNF.

No growth was observed in any of the xenografts, in concordance with the result of the natural history study of PNF, which showed no growth in the majority of cases and very slow growth over years in the few cases where the tumors do grow. It is therefore unreasonable to expect any detectable growth of small xenografts in short experiment periods of less than 2 months. In fact, xenografts tend to shrink in the 2 to 3 weeks after the implantation, likely due to clearance of pre-grafting and post-grafting cell death. Drug effect can therefore be described as an increase in graft size reduction, which is more profound after 2 to 3 weeks. Indeed, we could detect significantly more grafts size reduction in the drug-treated mice than in the untreated control mice. Furthermore, the significantly more potent effect of nilotinib over imatinib could also be demonstrated in this *in vivo* model.

This is of high relevance for patients with PNF, which suffer mostly from the secondary tumor size effects.

Recently, a growth deceleration of PNF was reported in children treated with imatinib [22]. In concordance, we also observed a deceleration of body weight increase in mice treated with imatinib. Judging from body weight, nilotinib was better tolerated than imatinib by the mice, indicating a better side effect profile of the former.

Since off-target effects of imatinib have been reported, among them the effect of activating natural killer cells [23], we measured cytotoxicity of spleen cells of the treated mice. We found elevated cytotoxicity of spleen cells in imatinib -treated mice and to a lesser extent, in nilotinib-treated mice. This finding suggests that imatinib might have an immune-activating component in its anti-PNF effect, such that its pharmacological mechanism differs from that of nilotinib.

In summary, our data reveal a more potent antitumor effect of nilotinib on PNF than imatinib *in vitro* and *in vivo*, suggesting the potential clinical application of nilotinib for PNFs.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: AK LK PWM VFM. Performed the experiments: JW MF YS WAN REF. Analyzed the data: JW MF LK AK WAN. Contributed reagents/materials/analysis tools: VFM REF WAN YS PWM. Wrote the paper: JW LK MF AK PWM.

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6. PUBLICATION 3

Preclinical assessment of drug-response for plexiform neurofibroma using primary cultures. J Clin Neurol, 2014 (accepted)

Preclinical assessment of drug-response for plexiform neurofibroma using primary cultures

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Running title: Assessment of drug efficacy and specificity

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Conflict of Interest

Pending patent: "Verfahren zur Bestimmung der Wirksamkeit und/oder Spezifität Eines Wirkstoffs"

Inventor: Lan Kluwe

Holder: University Medical Center Hamburg-Eppendorf

Status: Examination was positive and will be filed in Oct. 2014

The authors confirm that this article content has no further conflict of interest.

Abstract

Background and Purpose: Individualized drug-test for each tumor, in analogue to antibiotic-tests for infectious diseases, would be highly desirable in personalized cancer care.

Methods: We conceived a strategy to use primary cultures containing tumor and non-tumor stromal cells for testing drug-response regarding both efficacy and specificity. In this pilot study, we implemented this concept to four primary cultures derived from plexiform neurofibromas. Responses to drugs were measured by following dose-dependent changes of proportions of tumor cells and non-tumor cells which were determined by means of staining them with specific antibodies. Viability and cytotoxicity of total cells in cultures were also measured using proliferation and cytotoxicity assays

Results: Numbers of total cells decreased after drug-treatment, in concordance to the decreased proliferation and increased cytotoxicity. Proportions of Schwann cells and fibroblasts changed dose-dependently while the patterns varied from tumor to tumor and from drug to drug. The highly variable *in vitro* drug-responses likely reflect highly variable responses of tumors to therapies in patients.

Conclusions: Our preliminary results suggest that the concept of assessing *in vitro* drug-response using primary cultures is feasible yet demands extensive further development toward application in preclinical drug-selection and in drug-discovery.

Keywords

Personalized medicine, drug selection, preclinical test, specificity, primary culture, *in vitro* testing

Introduction

Responses of cancer patients to chemotherapies vary largely.[1] An individualized laboratory drug-test for each tumor, in analogue to antibiotic-tests for infectious diseases, would facilitate drug-choice in personalized cancer treatment.[2, 3] Cell lines and animal models are not suitable for such purpose.[4, 5] By contrast, primary cultures provide a promising laboratory model since they can be obtained from most resected tumors in a short time, contain multiple cell-populations and therefore represent the heterogeneous reality in tumors better than cell lines.[6] However, the heterogeneity is also a technical obstacle since conventional assays measure parameters of all cells in a culture but cannot assign the obtained values separately to tumor and non-tumor stromal cells.

Toward solving this problem, we conceived a strategy which assesses relative drug effect on tumor cells and non-tumor cells in a primary culture by following the change of their proportions. Furthermore, effect of a drug on non-tumor cells provides an *in vitro* indication for its specificity. In this pilot study, we implemented this concept using plexiform neurofibroma (PNF) as a model.

PNFs are benign tumors of the peripheral nerves and are mostly associated with neurofibromatosis type 1 (NF1), an autosomal dominant disorder caused by heterozygotic inactivation of the *NF1* tumor suppressor gene.[7, 8] Approximately half of the patients develop PNFs.[9,10] Depending on their location, size and growth type, PNFs can cause pain, serious disfigurement and functional impairment.[9, 10] PNFs have a high risk of malignant transformation into malignant peripheral nerve sheath tumors (MPNST) which is the leading cause of NF1-related death.[11] To date, surgical intervention is the established treatment of this kind of tumors. However, since the tumors often infiltrate adjacent tissues, complete resection is usually not possible without damaging nerves and healthy tissues.[12] Non-surgical therapies are being developed. For example, a phase 2 trial for imatinib mesylate reported subjective clinical improvement in airway patency, bladder control and extremity motor function in several cases.[13] Our own *in vitro* and *in vivo* studies showed that nilotinib is more potent than imatinib for PNFs.[14, 15] A pilot studies addressing safety/efficacy of nilotinib for PNFs is ongoing. Generally, efficacy and side-effect of the drugs for PNFs vary largely among cell lines, primary cultures, tumors and patients.[13, 14] Severe side-effect is frequently the cause for patient drop-out in clinical trials. An individualized preclinical test for drug-efficacy and specificity therefore would greatly facilitate the therapy decision, drug-choice and dose-range for each patient.

PNFs consist mainly of Schwann cells and fibroblasts at various ratios. Schwann cells are known to be the tumor cells since they bear the causative somatic alterations while the fibroblasts not.[16, 17] Schwann cells and fibroblasts are different types of cells and therefore can be specifically stained with antibodies. In this way, we determined proportions of tumor cells and non-tumor cells in cultures treated with two different drugs at various concentrations.

Methods

Tumor tissues were obtained from four unrelated patients who underwent surgery. All patients gave informed written consent and the Institutional Review Board approved the study (OB-061/05). All specimens were anonymised and cultured under conditions enhancing growth of Schwann cell.[16]

After 3-5 passages of expanding, cells of each culture were seeded in 8-compartment chamber-slides at a density of 10,000/well and treated with nilotinib and imatinib at various concentrations over 5 days. After the treatment, the slides were double stained with antibodies against S100 and CD90, and counterstained with DAPI for the nuclei.[14] For each drug-concentration, S100-positive cells and CD90-positive cells were counted manually on a photo taken under fluorescence microscope. More than 200 of total cells were counted for each drug-concentration using ImageJ 1.48 software. The percent proportions of Schwann cells and fibroblasts were calculated. Cells negative for both S100 and CD100 were not included in the calculation.

Viability and cytotoxicity of total cells in cultures treated as above were measured using XTT and LDH assays (Roche, Germany), respectively. Cells of the same culture were seeded in wells of a 96-plate at 500/well and parameters at each drug concentration were measured in 6 replicates. IC_{50} and CC_{50}, defined as the concentrations of a drug at 50% of maximum viability and cytotoxicity in a culture, respectively, were calculated using a Probit-analysis.

Results

Schwann cells and fibroblasts were specifically stained with S100 (Fig. 1A, C, G) and CD90 (Fig. 1B, D, G) antibodies, respectively. After drug-treatment, numbers of total cells decreased (Fig. 1C, D), in concordance to the decreased viability and increased cytotoxicity (IC_{50} and CC_{50} in Fig. 2). Immunofluorescence images were disposed (Fig. 1E) and the stained cells were counted by ImageJ software (Fig. 1F). A considerable portion of cells were negative for both (Fig. 1G), they were not included in the subsequent calculation of the proportion of tumor and non-tumor cells.

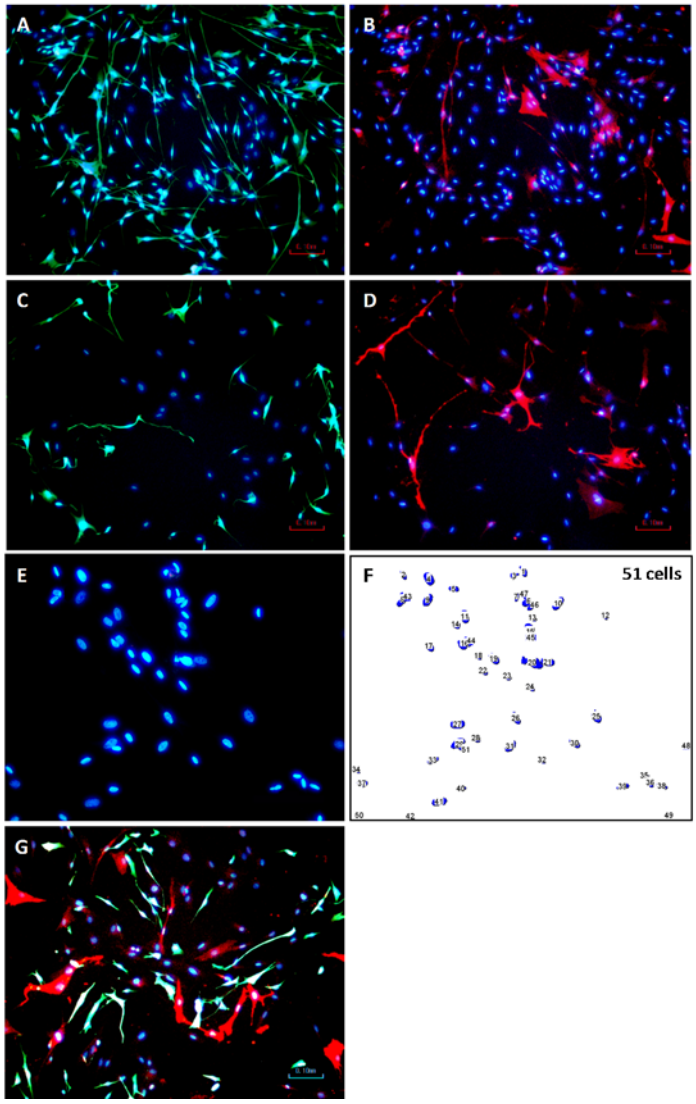

Fig. 1. Immunostaining with antibodies against S100 (green in **A**, **C** and **G**) for Schwann cells and CD90 (red in **B**, **D** and **G**) for fibroblasts. Nuclei were counterstained with DAPI (blue). **A, B**: the same cells without treatment stained with S100 and CD90, respectively; **C, D**: cells treated with 20 μM nilotinib for 5 days and stained by S100 and CD90, respectively; **E**, **F**: immunofluorescence pictures pre- and post-calculating disposed by ImageJ software; **G**:

superposed S100, CD90 and DAPI staining showing cells neither positive for S100 nor for CD90.

Proportions of Schwann cells and fibroblasts at each drug-concentration were calculated from the number of S100 positive and the CD90 positive cells in a defined area. These proportions changed dose-dependently but the patterns varied from tumor to tumor and from drug to drug (Fig. 2). Good drug-response, defined as continuous and substantial decrease of the proportion of the tumor cells, was observed in the culture derived from tumor No.1 for both nilotinib and imatinib (Fig. 2A, B). By contrast, culture of tumor No.2 responded well to nilotinib (Fig. 2C) but poorly to imatinib (Fig. 2D). Culture of tumor No. 3 responded well to imatinib (Fig. 2F) and less well to nilotinib (Fig. 2E) while culture of tumor No. 4 responded poorly to both (Fig. 2G, H).

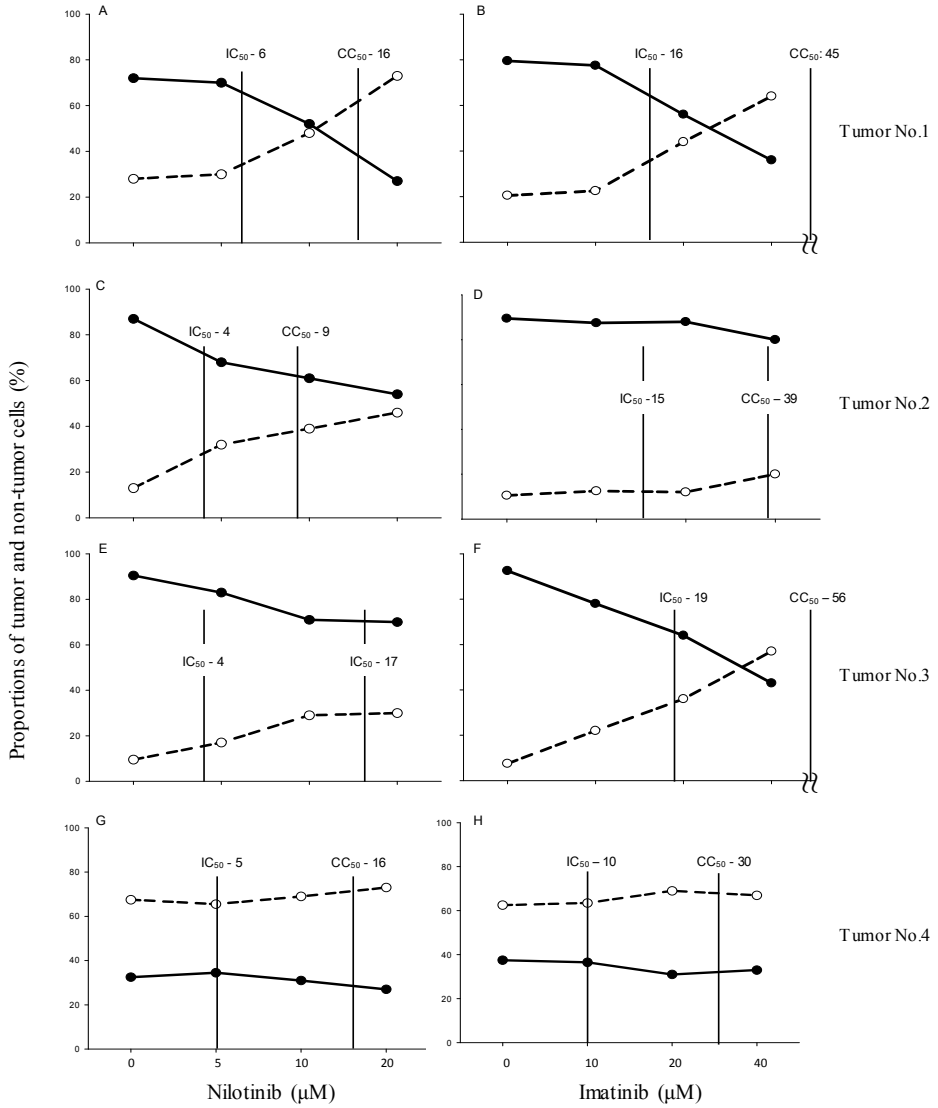

Fig. 2. Proportions of tumor cells (solid line) and non-tumor cells (broken line) in cultures derived from 4 unrelated tumors at various concentrations of two drugs. IC_{50} (concentration at

50% of maximum viability) and CC_{50} (concentration at 50% maximum cytotoxicity) were determined for total cells in separate cultures derived from the corresponding tumors.

Discussion

We illustrated the principle of an *in vitro* test for response of cultured tumor cells and non-tumor cells to anticancer drugs and demonstrated its basic feasibility. The concept of measuring proportions of tumor cells and non-tumor cells in a mixed culture enables (1) use of primary cultures and (2) assessment of drug-specificity.

Specificity of a drug is difficult to assess in the laboratory, largely due to the lack of suitable testing tools. Our initial strategy tackling this issue was to compare drug-efficacies on paired cultures of tumor cells and non-tumor cells derived from the same tumor but enriched separately under different conditions.[14] In the present study, we improved our approach by means of assessing drug-efficacies on tumor cells and non-tumor cells simultaneously in the same culture under the same condition. With this approach, the presence of non-tumor cells in primary cultures can be turned from a technical obstacle into a methodical advantage in sense of enabling assessment of drug-specificity.

As expected, efficacy and specificity of the drugs on cultures varied largely from tumor to tumor, likely reflecting the highly variable response of tumors to the treatment and side-effects in the patients, as reported by studies.[14]

In the present study, discrimination of tumor cells and non-tumor cells was based on staining them with respective specific antibodies. However, this immunostaining-based relative quantification of tumor cells can only be applied in special cases where tumor cells and non-tumor cells in the culture are different cell types which can be distinguished from each other with antibodies. Generally, phenotypes are not ideal parameters for quantifying tumor cells in a mixed culture since (1) distributions of a phenotype in tumor cells and non-tumor cells usually overlap, (2) phenotypes vary depending on cultural conditions and (3) phenotypes are difficult to quantify and their relation to quantity of tumor cells is not straightforward.

By contrast, a genetic alteration (for example a *p53* mutation) is a clear-cut parameter which is present exclusively in the tumor cells. A genotype is stable and can be quantified by modern technologies such as digital PCR and fluorescence in situ hybridization. Today, frequently altered genes and regions are known for most tumor-entities (Cancer Genome Atlas; http://cancergenome.nih.gov) and therefore for each tumor, one or more alterations can be identified within days. Once identified, a tumor-specific alteration can be used to quantify tumor cells in the derived cultures treated with various drugs at various concentrations. Subsequently, dose-dependent change of amounts of tumor cells and non-tumor cells can be

obtained (Fig. 3). Furthermore, by quantify a defined genetic alteration (for example, kras mutation), dose-dependent change of the corresponding subpolulation of the tumor cells can be followed, providing a tool to study target and work-mechanism of drugs.

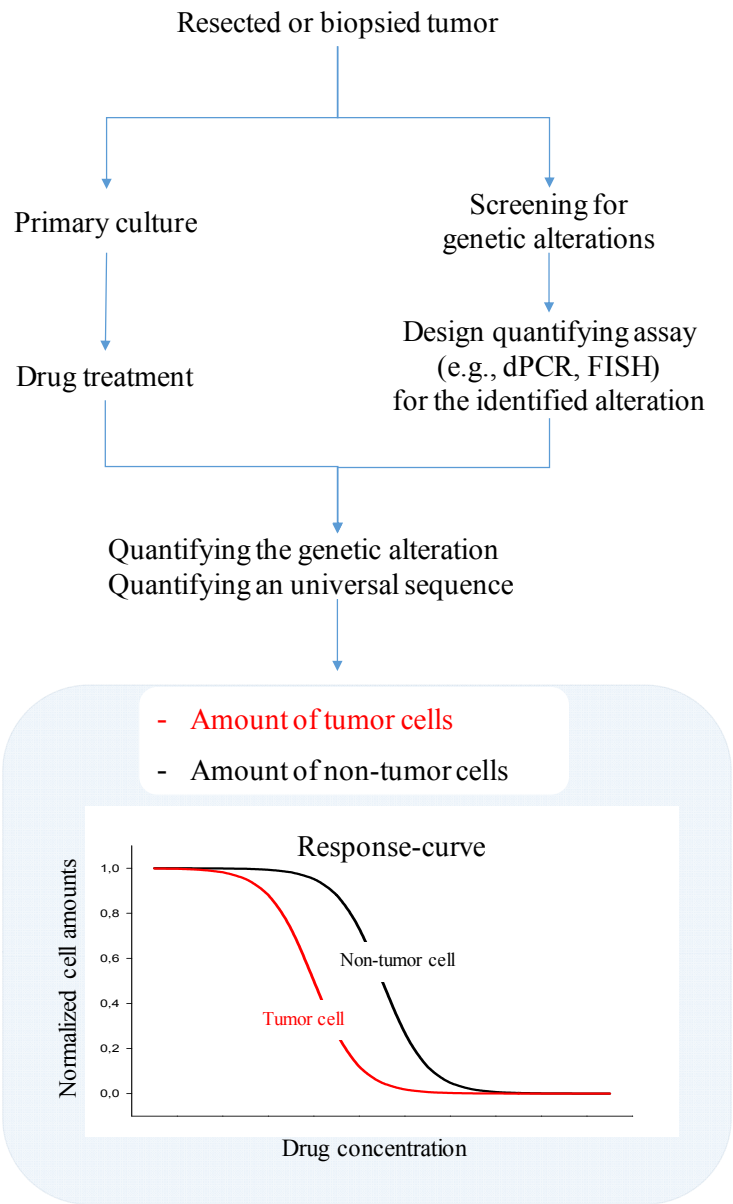

Fig. 3. Illustration of the concept of a genetic preclinical drug-testing. A resected tumor will be subjected to (1) cell culture and (2) genetic screening for alterations. The cultured cells will be treated with various drugs and living tumor cells will be quantified by measuring tumor-specific genetic alterations. Amount of total cells can be quantified by measuring a universal DNA sequence, from which amount of non-tumor cells can be derived. dPCR, digital PCR; FISH, fluorescence in situ hybridization.

Studies are in progress where non-tumor cells are spiked into the cultures of established cell lines of MPNST. These cultures are being treated with various drugs and the tumor cells and non-tumor cells will be quantified using genetic alterations of the former. Furthermore, studies using primary cultures of other malignant tumors are in planning.

Though primary cultures represent real tumors better than cell lines, they are still a simplified model. Therefore it is unlikely that the *in vitro* drug-response and the clinical response will correlate well right away for all drugs. However, there are various strategies, for example, 3-dimensional culture, hanging-drop culture and organoid-culture, for adjusting and optimizing the *in vitro* settings. Establishing adequate correlations for some of the drugs will also be a good start for translational application of the genetic-based *in vitro* drug-testing.

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7. ABSTRACT

Plexiform neurofibromas (PNFs) are benign tumors from peripheral nerves and mostly associated with neurofibromatosis type 1. They often extend through multiple layers of tissue and therefore cannot be treated satisfactorily by surgery. Nilotinib is a tyrosine kinase inhibitor used to treat leukemia, with advantages over the prototype imatinib in terms of potency and selectivity towards BCR-ABL, DDR, PDGFR, and KIT receptor kinases.

The efficacies of nilotinib and imatinib on the primary cultures containing Schwann cells and fibroblasts derived from PNFs were tested *in vitro*, by means of monitoring cell proliferation, vitality, viability, apoptosis and collagenase activity. For *in vivo* experiment, fragments of PNFs were xenografted onto sciatic nerve of athymic nude mice and these mice were treated with nilotinib and imatinib, respectively. Xenografts were monitored weekly using a high resolution ultrasound measurement and size-change was followed by calculating their volumes. Peak plasma nilotinib concentration was measured 3 h after the last oral administration using liquid chromatography-tandem mass spectrometry. Activation of natural killer cells were examined by measured cytotoxicity of non-adherent mouse spleen cells of the mice as effector cells to PNF-derived Schwann cells as target cells at a ratio of 1:10.

Nilotinib treatment led to decreased proliferation, vitality and viability of all cells with 50% inhibitory concentration (IC_{50}) varying from 2.3 to 11.9 μM. For five out of the eight PNFs studied, IC_{50} was lower in Schwann cells than in fibroblasts for all parameters measured, suggesting good drug-selectivity for Schwann cells which are the tumor cells in PNFs. In addition, nilotinib induced apoptosis and suppressed collagenase activity. *In vivo*, nilotinib-treatment at a daily dose of 100 mg/kg for four weeks led to a reduction of the graft $volume_{std}$ by $68_{\pm 7}$%, significantly more than the $33_{\pm 8}$% reduction in the untreated mice ($P < 0.05$) and the $47_{\pm 15}$% in the mice treated with imatinib ($P<0.05$). The peak plasma nilotinib concentration $6.6_{\pm 1.1}$ μM is within the pharmacological range of clinical application. Imatinib significantly elevated cytotoxicity of mouse spleen cells on PNF-derived Schwann cells when compared with the control group (21.1±7.2% vs. 12.5±7.1%).

The results suggest that nilotinib is more potent than imatinib for treating PNFs and therefore should be further explored as a potential drug for some PNFs.

ZUSAMMENFASSUNG

Plexiforme Neurofibrome (PNF) sind benigne Tumore peripherer Nerven und meist mit Neurofibromatose Typ 1 assoziiert. Sie erstrecken sich häufig über mehrere Gewebeschichten, so dass eine vollständige chirurgische Resektion erschwert wird. Nilotinib ist ein Tyrosinkinaseinhibitor, welcher Anwendung in der Leukämie-Therapie findet und gegenüber dem Prototypen Imatinib potenter, aber auch selektiver gegen BCR-ABL, DDR, PDGFR und KIT- Rezeptorkinasen gerichtet ist.

Die Wirksamkeit von Nilotinib und Imatinib wurde *in vitro* an PNF-Primär kulturen, bestehend aus Schwann-Zellen und Fibroblasten getestet, wobei die Zellproliferation, Vitalität, Viabilität, Apoptose und Kollagenaseaktivität gemessen wurde. Für *in vivo* Experimente wurden PNF-Tumorstücke am Nervus ischiadicus athymischer Nacktmäuse implantiert und anschließend mit Nilotinib bzw. Imatinib behandelt. Die xenotransplantierten Tumore wurden wöchentlich mittels eines Kleintierultraschallgeräts dargestellt und Größenveränderungen des Tumorvolumens berechnet sowie der maximale Plasmaspiegel von Nilotinib drei Stunden nach der letzten oralen Applikation mittels Flüssigkeitschromatographie und Massenspektrometrie bestimmt. Die Aktivität natürlicher Killerzellen konnte durch Zytotoxizitätsmessungen nicht-adhärenter muriner Milzzellen als Effektorzellen gegen Schwann-Zellen aus PNFs, als Target, in einem Verhältnis von 1:10 gemessen werden.

Die Nilotinib-Behandlung führte *in vitro* zu einer verringerten Zellproliferation, Vitalität und Viabilität mit einer 50%igen inhibitorischen Konzentration (IC_{50}) zwischen 2,3 und 11,9 µM. Für fünf der acht untersuchten PNFs war der IC_{50}, im Vergleich zu Fibroblastenzellen in Schwann-Zell-Primärkulturen geringer, was auf eine Selektivität gegen Schwann-Zellen, als beschriebene Tumorzellen in PNFs, hindeutet. Nilotinib induzierte zudem Apoptose und supprimierte die Kollagenaseaktivität. *In vivo* führte die tägliche Gabe von 100 mg/kg Nilotinib über vier Wochen zu einer Tumorreduktion von $68_{\pm 7}$% und war somit signifikant stärker als in der unbehandelten Gruppe mit $33_{\pm 8}$% ($P<0.05$) sowie der Imatinib-Gruppe mit $47_{\pm 15}$% ($P<0.05$). Der maximale Plasmaspiegel von Nilotinib lag bei $6.6_{\pm 1.1}$ µM und somit im pharmakologischen Bereich klinischer Applikation. Imatinib erhöhte zudem signifikant die Zytotoxizität muriner Milzzellen auf Schwann-Zellen aus PNFs im Vergleich zur Kontrollgruppe ($21.1_{\pm 7.2}$% vs. $12.5_{\pm 7.1}$%).

Die Ergebnisse deuten darauf hin, das Nilotinib potenter ist als Imatinib und somit für die Behandlung von plexiformen Neurofibromen als potentielles Medikament weiter untersucht werden sollte.

8. CONTRIBUTIONS FOR PUBLICATION

Publication 1:

I have carried out the *in vitro* experiments, collected and evaluated the data, prepared the figures and contributed in drafting and critical reading the manuscript.

Publication 2:

I have taken on the *in vitro* and *in vivo* experiments, harvested and analyzed all data, designed the figures and prepared the first draft of the manuscript, and revised the manuscript according to the reviewers' comments.

Publication 3:

I have carried out the experiments, collected and analyzed the data, prepared some figures and contributed in drafting and critical reading the manuscript, and controlled publication decision.

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10. RESUME

Due to protection of personal information, the curriculum vitae of Jiang Wei can not be published.

11. EIDESSTATTLICHE ERKLÄRUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: Jiang Wei .....