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Enhancing effect of calcipotriol on efficacy of imatinib and nilotinib for cells derived from plexiform neurofibroma *in vitro*

Dissertation

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SYNOPSIS

1. Introduction

1.1. Plexiform neurofibroma

Neurofibromatosis type 1 (*NF1*), also known as peripheral neurofibromatosis[1] or von Recklinghausen neurofibromatosis, is a genetic disorder caused by a single gene defect which affects multiple organs, with a prevalence of approximately 1:3000 [2]. The mutated gene of this inherited genetic disorder is in the *NF1* tumor-suppressor gene, composed of 60 exons spanning 350kb of genomic data and located on chromosome 17q11.2[3]. *NF1* gene encodes a 220kDa cytoplasmic protein called neurofibromin which works as a negative regulator of the RAS mitogen activated protein kinase pathway[4]. The inactivation of *NF1* gene results in the complete loss of functional neurofibromin and initiates the pathogenic process that leads to the formation of neurofibroma[5]. Furthermore, this gene defect is associated with a wide range of symptoms from physical disfiguration and pain to cognitive disability. The common symptoms include multiple skin alteration called café-au-lait spots, axillary freckling in inguinal regions, brownish-red spots in the iris called Lish nodules, distinctive osseous lesion, vision disorders and learning disabilities (Fig.1).

Plexiform neurofibromas (PNF) is a common type of tumor in individuals

with *NF1*, about half of the patients with *NF1* will develop PNF. PNFs are benign tumors usually occur in subcutaneous tissues or visceral peripheral nerves[6,7]. Previous studies of our own group and others showed that Schwann cells are tumor cells and fibroblast cells are non-tumor cells in PNF[8,9]. They grow along the nerve sheath and may involve multiple fascicles and branches of nerve, which can develop a proteinaceous matrix and cover the thickened nerve fibers[10]. PNFs arise in various parts of the body, including head, neck, pelvis, and extremities. They can cause dysfunction such as pain, cosmetic abnormalities, organ compression and functional deficits[11]. PNF can be diagnosed readily in childhood and grow most rapidly during this period[12]. Studies have found that PNFs are at a significant risk (about 10%) of transforming into a malignant peripheral nerve sheath tumor (MPNST)[13].

Surgical resection is the standard treatment for PNF to date, but the indications and timing of resection remain controversal[14]. As PNFs often exhibit features of local invasion into adjacent tissues, the nerves and healthy tissues are often damaged during the resection[15]. Currently, there is no accepted medical therapy or cure, although several agents that target different signaling pathways implied in the pathogenesis of PNF are being developed and some of them are in various stages of clinical trials[16–18].

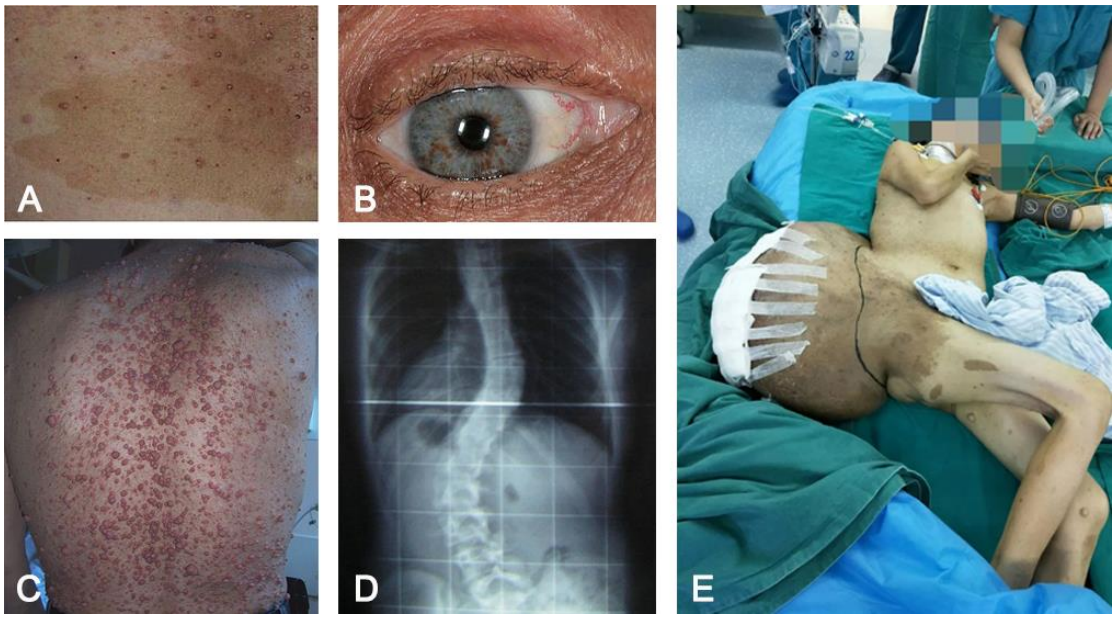


Figure 1. Symptoms of *NF1*： Café au lait spots[19] (A); Lish nodules[20] (B); Neurofibroma of the skin[21] (C); Scoliosis[22] (D); Huge superficial tumor (E, our unpublished data, Henan cancer hospital, 2017).

1.2. Vitamin D

$1,25(OH)_2D_3$ is the hormonally active metabolite of vitamin D, which is the natural ligand for the vitamin D receptor (VDR) and is one of the key regulators of bone metabolism and calcium homeostasis. VDR has also been found to participate in the development of the nervous system and was detected in neurons of several adult brain areas as well as primary astrocytes[23,24]. Studies have shown that VDR is expressed by relatively high levels in Schwann cells in the presence of $1,25(OH)_2D_3$[25]. Numerous investigations have shown that vitamin D and its analogues reveal anticancer activity both *in vitro*[26] and *in vivo*[27–29]. Vitamin D is engaged in regulating the cellular proliferation, apoptosis,

oxidative stress, inflammation, matrix homeostasis and angiogenesis, through interaction with the nuclear VDR and affecting target gene transcription, making vitamin D an attractive candidate therapeutic for cancer and cardiovascular diseases[30–32]. In chemotherapy, studies indicate that vitamin D triggers anticancer activity synergistically in combination with a diverse array of chemotherapeutics, steroids and tyrosine kinase inhibitors[33].

Serum 25-hydroxyvitamin D (25OH-vitamin D) concentration was identified as the appropriate functional indicator of human vitamin D status[34]. There has been a poor consensus in defining normal levels of serum 25-hydroxyvitamin D level. The 2011 Endocrine Society guideline[35] set the desirable 25OH-vitamin D level at 75 nM while the Institute of Medicine reported that 50 nM of the 25OH-vitamin D level meets the requirements of at least 97.5% of the population[36].

Several recent pieces of evidence indicate that the low serum vitamin D concentrations are associated with an increased risk of tumors. Our previous study reported significantly lower serum vitamin D concentrations in *NF1* patients (Figure. 2A, B). In addition, this serum vitamin D level is inversely correlated with burden of dermal neurofibromas (Fig. 2C)[37]. Another study reported that application of vitamin D3 is effective in improving the pigmentation of café au lait spots and suppressing the development of neurofibromas in patients with von Recklinghausen's disease[38].

A previous in vitro study addressed the possible effect of an active vitamin

D analogue calcipotriol on human glioblastoma cells T98G[26]. The cells were treated with calcipotriol at a concentration range of 0.001 – 10 µM for six days. Significant inhibition of vitality was obtained for all concentrations (Fig. 3). Another study reported that when tamoxifen was used in combination with calcitriol or its analogs calcipotriol against human breast cancer cell line MCF-7 cells[39], statistically significant proliferation inhibition was observed compared to tamoxifen used alone (Table.1).

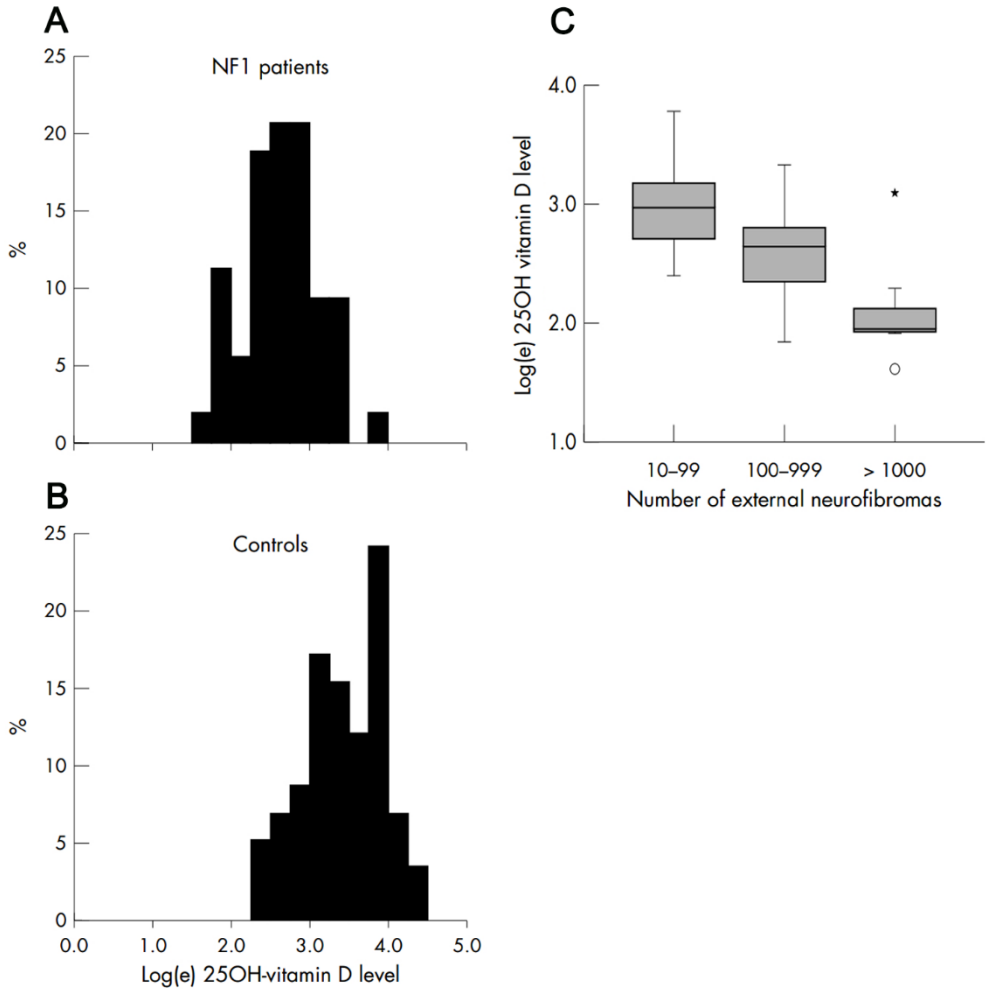


Figure 2. Distribution of serum 25-hydroxyvitamin D concentrations in 55 *NF1*

patients (A) and 58 healthy control subjects (B). Association between estimated number of dermal neurofibromas and serum 25-hydroxyvitamin D concentration in 47 patients with *NF1* (C).

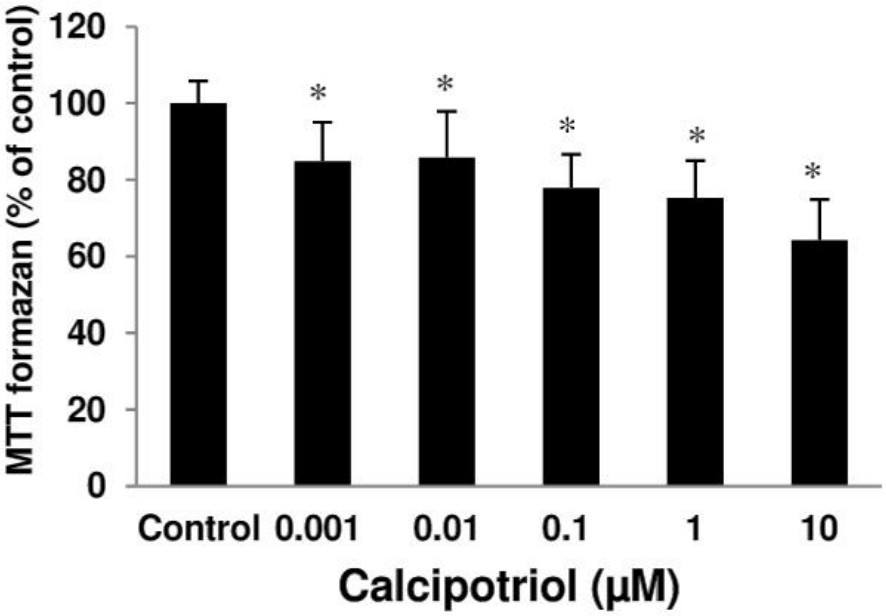


Figure 3. T98G human glioblastoma cells were treated with calcipotriol for six days. Vitality is presented as percentage of untreated control cells with standard deviation. Significant difference ($p < 0.05$) to untreated cells is marked with *.

Table 1. The proliferation inhibition of MCF-7 cells exposed to calcitriol or calcipotriol and tamoxifen.

Compounds	Vitamin D analogs [nM]	Concentration of tamoxifen (TX) [µg/mL]			
		1	0.1	0.01	0
TX alone	0	21.6 ± 2.7	5.30 ± 3.3	−2.5 ± 3.3	-
TX + Calcitriol	1	19.0 ± 4.1	6.7 ± 3.5	-1.7 ± 3.8	−3.3 ± 5.1
	10	32.9 ± 1.0	14.9 ± 3.1	7.2 ± 4.4	6.7 ± 5.4
	100	62.4 ± 7.4	50.0 ± 10.3 *	47.0 ± 13.3	48.2 ±11.1
TX + Calcipotriol	1	26.7 ± 4.8	6.7 ± 3.5	3.6 ± 3.5	1.6 ± 3.5
	10	34.4 ± 2.7	14.9 ± 3.1	10.7 ± 2.9	8.5 ± 5.0
	100	59.3 ± 4.7 *	50.1 ± 10.3 *	44.6 ± 8.6	44.8 ± 10.9

* $p \leqslant 0.05$ as compared to tamoxifen (ANOVA Kruskal-Wallis test). Mean ± SD is resented.

1.3. Imatinib and Nilotinib

Imatinib mesylate (Gleevec/Glivec) is a commercially available receptor tyrosine kinase inhibitor (TKI), blocking the platelet-derived growth factor receptor (PDGFR), ATPase activity of the kinase c-Abl, Abl-related gene, c-KIT, and discoid domain receptor-1[1]. This pharmaceutical was primarily used in the in patients with chronic myeloid leukemia and gastrointestinal stromal tumors, transforming previously lethal diseases into chronic illnesses. Previous studies have found wide expression of ligand PDGFR-α in neurofibromas and MPNSTs[40,41]. Amplification and mutations of PDGFR-α and KIT were also found in MPNSTs[42]. Our laboratory previously discovered that PDGFR-α and PDGFR-β is expressed in PNFs as well as PNF-derived primary Schwann cells. Imatinib had high potency to suppress proliferation of PNF-derived Schwann cells *in vitro* and induce regression of PNFs xenografts *in vivo*[15]. A recent phase 2 trial found 26% of the *NF1* patients had a 20% or more

decrease in tumor volume (relatively small tumors) with treatment of imatinib.[16]

Nilotinib, in the form of the hydrochloride monohydrate salt, is the second-generation tyrosine kinase inhibitor. It was initially approved for the treatment of imatinib-resistant patients and had been the primary treatment option since 2007[43]. The structure of nilotinib preserves the amide pharmacophores and aminopyrimidine of imatinib but incorporates substituents alternative to the highly basic N-methyl-piperazine of imatinib[44]. It selectively targets BCR-ABL and also has potent activity against discoidin domain receptor, stem cell factor receptor, PDGFR tyrosine kinases and colony stimulating factor receptor-1 tyrosine kinases[45–47]. Nilotinib is 30-fold more potent than imatinib with high affinity and selectivity on BCR/ABL, and also in inhibiting proliferation of Bcr-Abl-expressing cells[48,49]. Recent assessments reported that the administration of oral nilotinib demonstrated significant and durable response rates in treatment for the patients with Ph+ CML-CP or CML-AP who were resistant to imatinib therapy[50,51]. The data from another study showed that nilotinib was rarely associated with the adverse effects seen with imatinib such as fluid retention, edema, cramps, and weight gain, or with pleural effusions[52]. Our previous study revealed that nilotinib is more potent than imatinib for treating plexiform neurofibroma *in vitro* and *in vivo*[53].

1.4. Aims of this study

Like pharmaceuticals in general, drugs for PNF including imatinib and nilotinib also have various toxic effects. Therefore it is desirable to keep dose as low as possible while not sacrificing therapeutic efficacy. This study is aimed to examine the possible enhancing effect of calcipotriol, an active analogue of vitamin D, on the efficacy of imatinib and nilotinib for PNF-derived tumor and non-tumor cells *in vitro*. Positive results may lead to consideration of a new strategy of including vitamin D in the treatment concept for PNF aiming to reducing dose of medication and consequently side effects.

From a total of 10 anonymous PNF specimens, tumor cells (Schwann cells) and non-tumor cells (Fibroblasts) were selectively enriched in separate primary cultures. These cultures were then treated with imatinib and nilotinib in the presence of calcipotriol, an analogue of the physiologically active metabolite of vitamin D3, at 0, 10, 100 and 1000 nM. Proliferation and vitality were measured. Response curves, IC_{50} and combination index in the presence of vitamin D were evaluated.

2. Material and Methods

2.1. Culturing tumor cells

- Standard DMEM: Dulbecco's Modified Eagle Medium (DMEM; Thermofisher) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermofisher), 2mM glutamine (Thermofisher), 500U/ml penicillin and 500µg/ml streptomycin (Thermofisher)

- Schwann cell medium: Standard DMEM supplemented with 2nM human heregulin (rHRG-$\beta1_{177-244}$; provided by Dr. Steven Carrol, University Alabama, USA), 0.5nM 3-iso-butyl-L-methylxanthine (IBMX; Sigma), 2.5nM insulin (Sigma).

- Surface coating for Schwann cells: 4µM natural mouse laminin (Thermofisher) overnight. Wash twice with PBS. Store at 4°C for up to 2 weeks.

PNFs were obtained from surgically resected tissue, which were kept in Dulbecco's Phosphate Buffered Saline (Thermofisher) and transported to the laboratory. All specimens were anonymized. According to the local regulation, anonymized application of rest surgical specimens does not require approval of an ethical review board. However, the protocol has to be reported to the local authority for privacy protection, which was carried out on our part. At a surgery, part of a PNF was taken and sent in for pathological diagnosis. However, since PNFs are often large and therefore only a part of a tumor can be pathologically examined. Rest specimens are usually discarded as

biological waste. With consent from the patients, this waste was saved for the study.

After removing the excess skins, fats and muscles, the tumor tissues were kept in standard DMEM at 37℃ and 5% CO_2 in a humidified incubator overnight. The next day, tumor tissues were minced with sterile scissors and digested in the same medium plus 10mg/ml collagenase (Worthington, New Jersey, USA) and dispase (Thermofisher) at 37℃. After 24h, digested tissues were filtered by a 100μm mesh (PARTEC; Gorlitz, Germany). Cells were washed and resuspended in selective culture medium respectively.

To enrich Schwann cells, tumor cells dissociated from the PNF tissues were cultured in Schwann cells medium in flask coated with laminin. Flasks were coated with laminin before seeding cells.

Fibroblasts were cultured in standard DMEM.

All cultures were incubated in a humidified incubator with 5% CO_2 at 37℃. Cells were expanded for 3-5 passages[54].

2.2. Immunofluorescence staining

To identify Schwann cells and fibroblasts, immunofluorescence staining was carried out with S100 for the former and the CD90 for the latter. Cells were grown on coverslips and fixed in 4% paraformaldehyde (Santa Cruz Biotechnology; USA), permealized with 100% methanol, washed with PBS and

labeled with primary antibodies overnight at 4℃: rabbit anti-human S100 antibody (DAKO, Copenhagen, Denmark) at 1:200 dilution or mouse anti CD90 antibody (Dianova, Hamburg, Germany) at 1:200 dilution. The cells were then incubated with secondary anti-rabbit antibody conjugated with Alexa Fluor 488 or anti-mouse antibody conjugated with Alexa Fluor 568 (Thermofisher) at 1:500 dilutions for 1h at room temperature, followed by nuclei staining for 1min with DAPI (SIGMA). After washings, coverslips were mounted on glass slides with permanent mounting medium (VECTOR; California; USA), stained cells were examined under a Nikon ECLIPSE E200 fluorescence microscope.

2.3. Drug treatment

10^3 cells were seeded in each well of 96-well plates with 100µl medium. For each measuring point, cells were seeded in 4 replicates. After one day incubation to let the cells attach to the surface, drugs were added to final concentrations of 0, 5, 10, 15, 20, 25 µM for imatinib (Novartis, Switzerland), and 0, 2, 4, 6, 8, 10 µM for nilotinib (Novartis, Switzerland). Control and treatment wells were treated with DMSO to equalize total volume across all wells. In addition to each of the above drugs and concentrations, calcipotriol (LEO Pharma A/S, Dänemark) was added to 0, 10, 100 and 1000 nM. Calcipotriol is an analogue of the physiologically active metabolite of vitamin

D3. Cells were treated for 10 days. Medium containing the drugs was refreshed every day. After the treatment, proliferation and vitality of the cells were measured as following.

2.4. Assays

Proliferation

Was measured using a BrdU cell proliferation Elisa kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. BrdU assay is a colorimetric immunoassay. Cells in proliferation can integrate the thymidine analogue 5-bromo-2'-deoxyuridine during the S phase of the cell cycle. Following denaturation, BrdU can be detected using an enzyme-linked immunosorbent assay (ELISA) with anti-BrdU antibodies. The absorbance indicates the extent of newly synthesized DNA as a parameter of proliferation. The absorbance was measured in a plate-spectrophotometer using 450nm wavelength with 690nm as a reference.

Vitality

Was measured using a XTT assay kit (Roche, Mannheim, Germany) following the provided instruction. The kit includes a 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide which can be reduced by mitochondrial dehydrogenases of viable cells and metabolized to a colored formazan dye. The amount of produced formazan

dye is indicative of number of vital cells. After treatment, the culture medium was removed. Cells were washed with phosphate buffer solution (PBS) and refilled with 100µl normal culture medium. 50µl XTT labeling mixture (50:1, XTT/electron coupling reagent solution) was added to each well. Following gentle mixing of the samples and an incubation period of 4h at 37℃, the absorbance of each well was measured in a spectrophotometer for microplates at 450nm with a reference wavelength at 650nm.

2.5. Data Evaluation

Statistical analysis was performed using a SPSS Version 17.0 (Chicago, IL, USA). Relative proliferation and vitality were calculated as ratio of inhibition of treated cells to controls with no drugs. Vitamin values (50% inhibitory concentration) were calculated for each experiment separately and the mean values ± standard deviation were presented. The comparison of IC_{50} between Schwann cells and fibroblast cells carried out by Student's *t*-test. The combination effects for calcipotriol and imatinib/nilotinib were analyzed using the Chou-Talalay method[55]. The interaction between two agents was calculated by means of combination index (CI) as follows:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

In the denominators, $(Dx)_1$ is the doses of Drug 1 alone giving the inhibits

effect x%; $(Dx)_2$ is the doses of Drug 2 alone giving the inhibits effect x%. In the numerators; $(D)_1$ is the portion of Drug 1 in combination $(D)_1 + (D)_2$ inhibits effect x%; $(D)_2$ is the portion of Drug 2 in combination $(D)_1 + (D)_2$ inhibits effect x%.

The values for the combination index indicated the following quantitative definition:

$0.9 < CI \leqslant 1.1$: additive effect

$CI \leqslant 0.9$: synergism

$CI > 1.1$: antagonism

The CI values were calculated using CompuSyn software (www.combosyn.com)[56]. The statistical significance among several groups was analyzed by Kruskal-Wallis ANOVA. Data were presented as mean ± S.E.M. The statistical significance was indicated with asterisks (*).* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

3. Results

3.1. Tumor cells and non-tumor cells in culture

Schwann cells from 10 PNFs were successfully enriched under the special condition optimized for this type of cells. The cells exhibited typical bipolar morphologies with elongated processes containing a prominent nucleus (Fig. 4B). They were stained with S100 (green in Fig. 4E) which is a well known marker for Schwann cells.

Fibroblast cells grew under standard cultural condition and displayed a branched cytoplasm surrounding a speckled, elliptical nucleus. Those cells had the elongated flattened form, oblong or triangular (Fig. 4C) and were stained with CD90 in red (Fig. 4F).

The purity of each cell type was assessed by staining, and both of them were confirmed to have a purity grade of more than 85%.

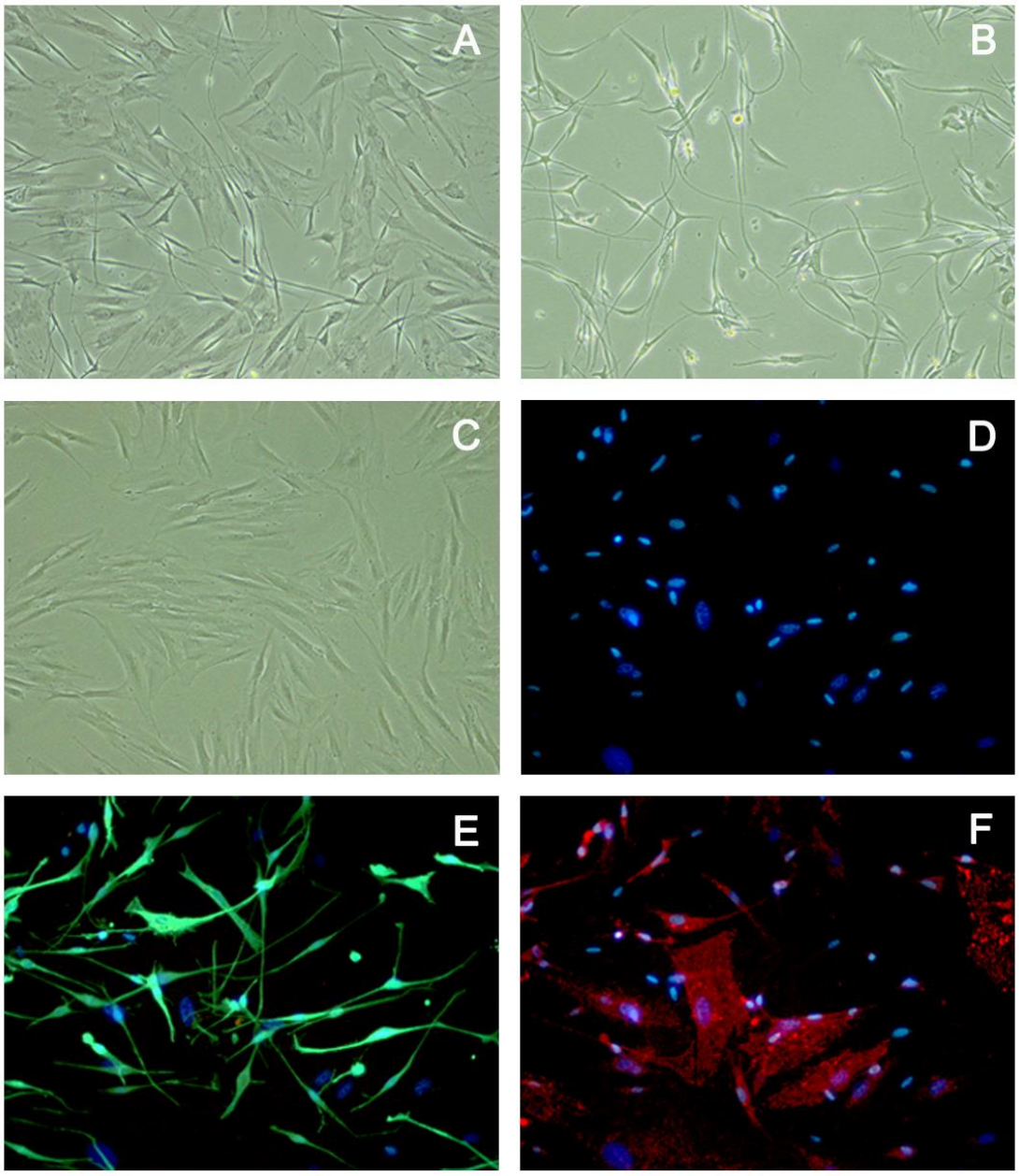
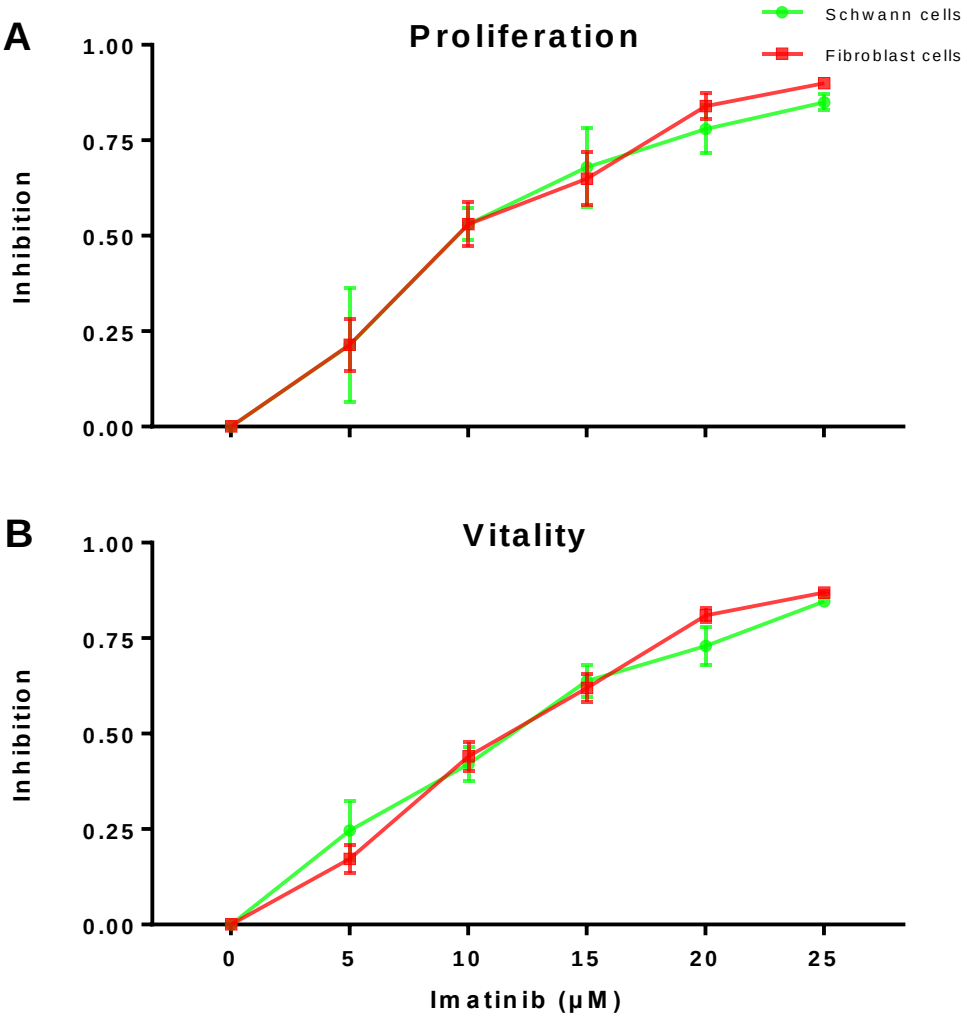


Figure 4. Images of living cells (A-C) and cells after immunofluorescent staining (D-F). A: The first passage of primary cell culture from PNF tissue; B: Schwann cells cultured in the selective medium in the third generation; C: The third generation of fibroblast cells. D: nuclei of the cells stained with DAPI (blue); E: S100 positive Schwann cells (green); F: CD90 positive fibroblast cells (red).

3.2. Effect of imatinib and nilotinib on PNF-derived tumor and non-tumor cells

Schwann cells and fibroblast cells derived from a total of 10 PNFs were all affected by imatinib and nilotinib. Both proliferation and vitality were inhibited by these two drugs in a dose-dependent manner. An example of the inhibition of cells derived from a PNF T1972 is shown in Fig.5. Similar response was also observed for cells derived from other PNFs.



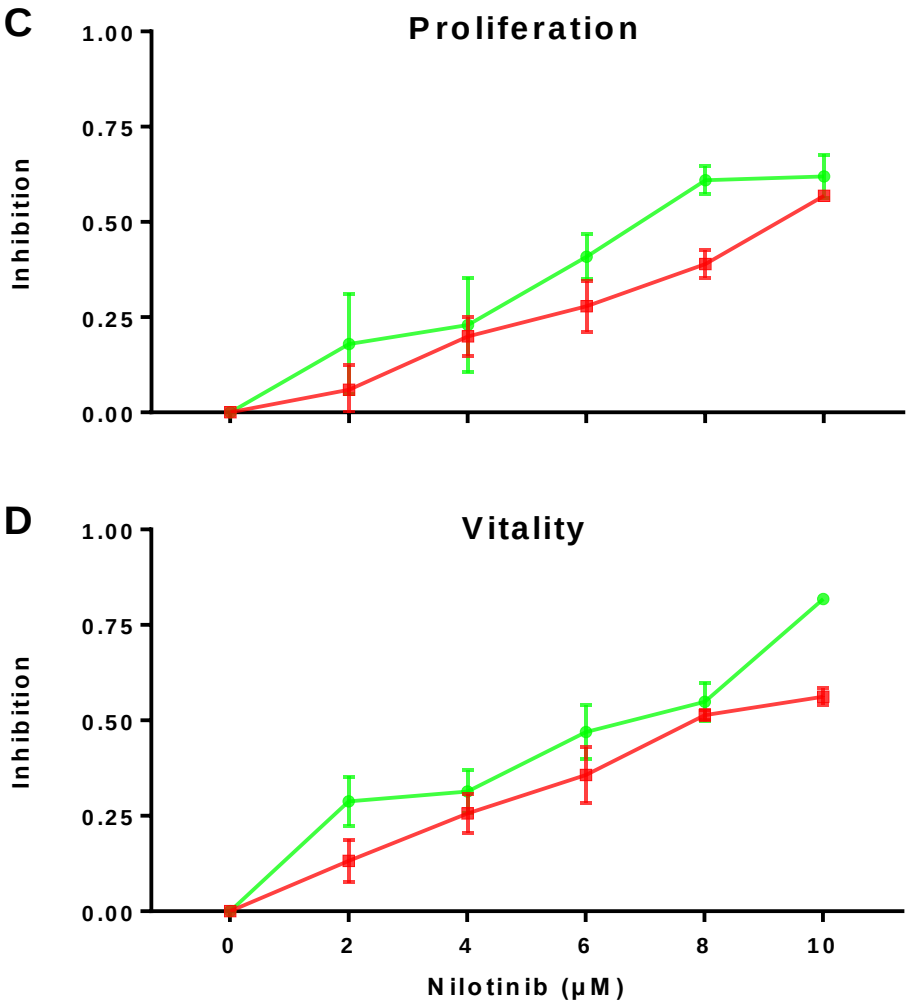


Figure 5. Cells derived from a PNF T1972. Imatinib (A, B) and nilotinib (C, D) inhibits proliferation and vitality of Schwann cells and fibroblast cells.

The mean IC_{50} values of imatinib and nilotinib for cells derived from the 10 PNFs were summarized in table 2. Briefly, the value range of imatinib for Schwann cells and fibroblasts were compatible: 9.5 - 13.0 vs. 9.0 - 11.2 µM. By contrast, the value range nilotinib for Schwann cells was lower than that for fibroblasts: 3.7 - 7.9 vs. 5.5 – 9.5 µM.

IC_{50} values were further compared between Schwann cells and fibroblasts

derived from same PNF. For imanitib, IC_{50} measured in proliferation were compatible for all 10 pairs of Schwann cells and fibroblasts and no significant difference was found in any of them (Fig. 6A). When measured in vitality, the relation varied largely from pair to pair (Fig. 6B). In 4 cases, IC_{50} was significantly lower in fibroblasts while in 2 cases the vice versa.

In the case of nilotinib, IC_{50} values were lower for fibroblasts than for Schwann cells in more cases than vice versa (Fig. 6C, D). However, there are cases where the relation differs when measured in different assays. For example, for the cells derived from the PNF T3212, IC_{50} was lower in fibroblasts regarding proliferation but high in fibroblasts regarding vitality (Fig. 6C, D).

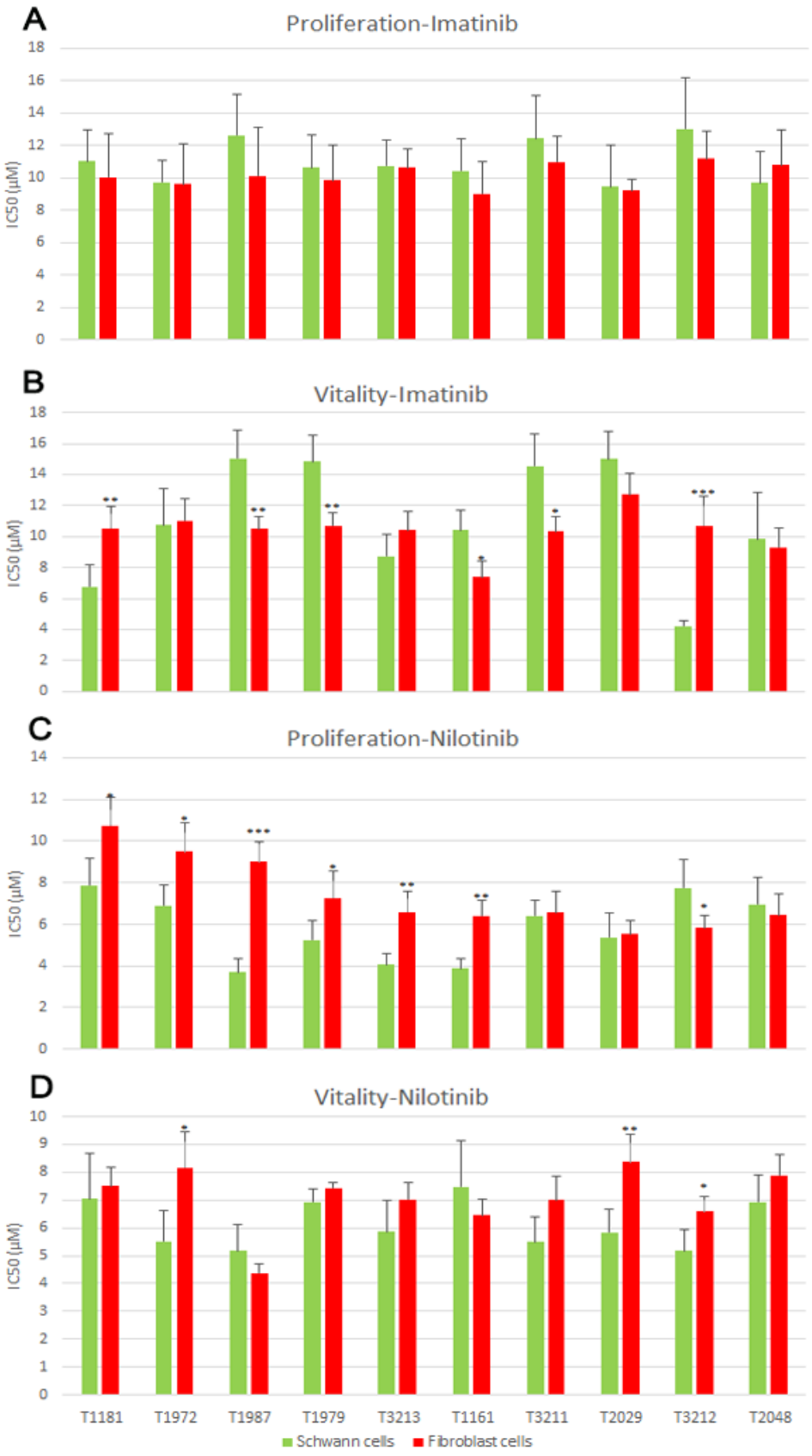


Figure 6. IC_{50} of imatinib and nilotinib for proliferation and vitality of pairs of Schwann cells and fibroblast cells from PNFs. * P<0.05, ** P<0.01, ***

P<0.001

3.3. Effect of calcipotriol on PNF-derived cells

Calcipotriol alone exhibited weak dosed-dependent inhibitory effect on PNF-derived Schwann cells and fibroblasts. An example is given for the inhibition on cells derived from a PNF T1161 (Fig. 7). In this case, the maximal inhibition was around 40%. For all cultures from the 10 PNF, the maximal inhibition range from 11.1% to 50.4%

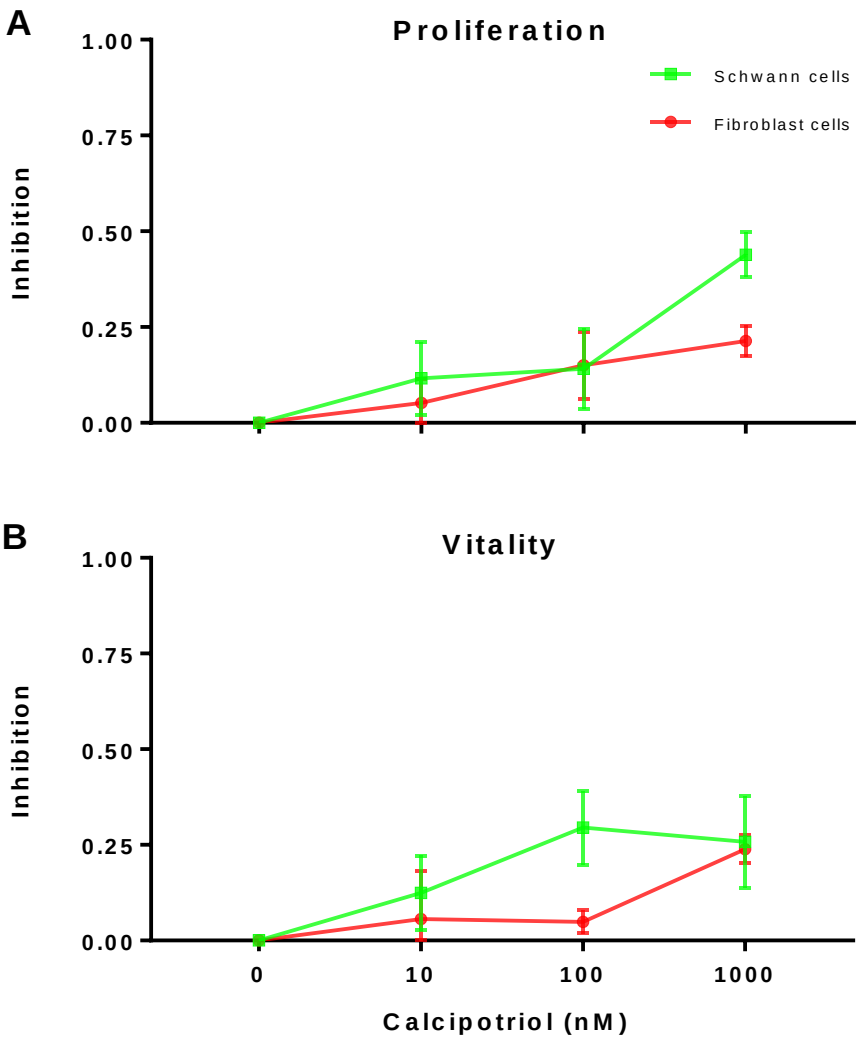


Figure 7. Cells derived from a PNF T1161. Calcipotriol alone inhibits

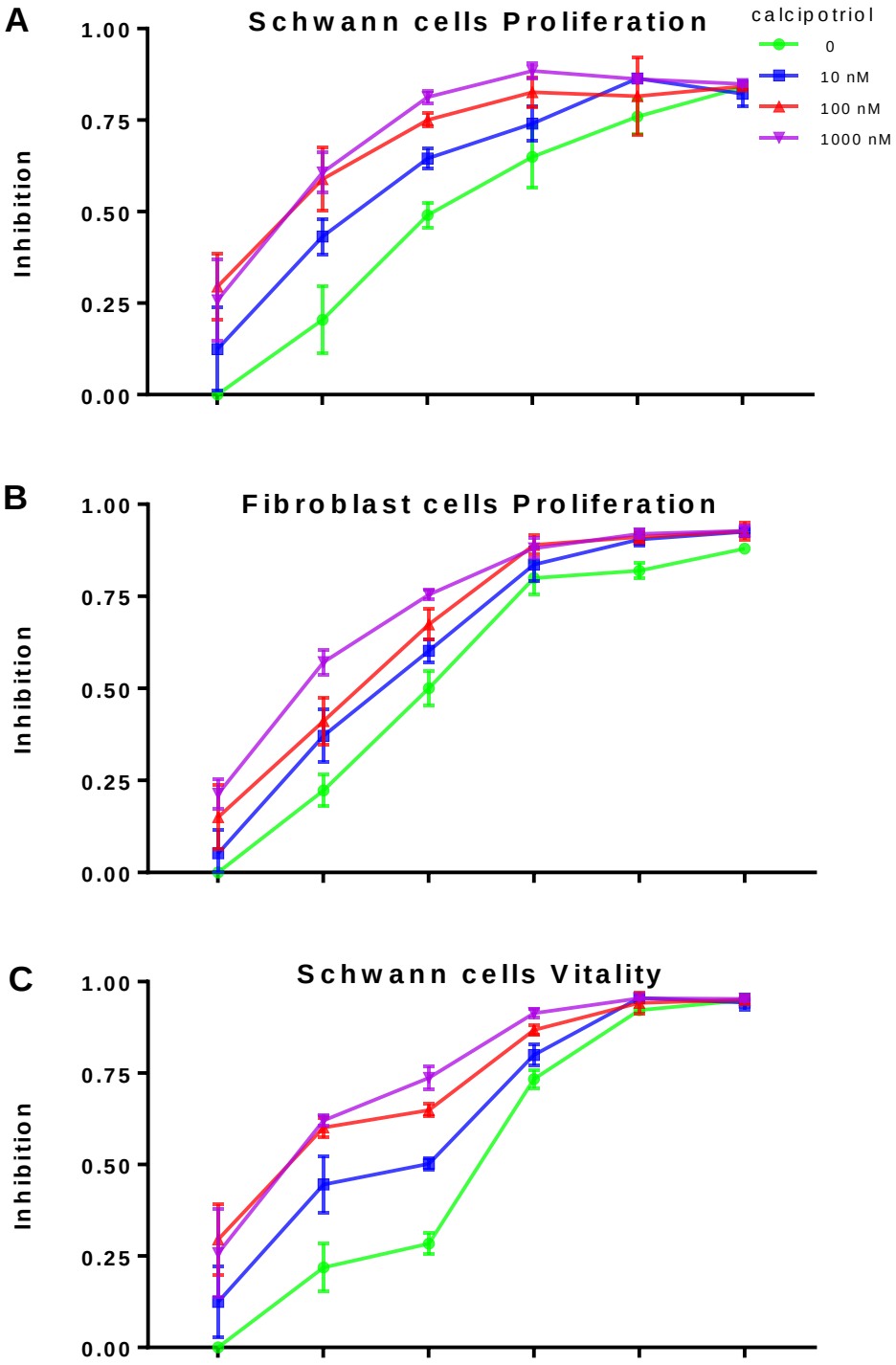
proliferation (A, B) and vitality (C, D) of Schwann cells and fibroblast cells.

3.4. Effect of vitamin D on efficacy of imatinib and nilotinib for PNF-derived cells

When calcipotriol was added to the treatment, a dose-dependent increase in inhibition was observed. This effect was detectable for proliferation and vitality, with both imatinib and nilotinib, and also for both Schwann cells and Fibroblast cells (Fig.8). The enhancing effect of calcipotriol is most prominent at zero concentration of imatinib and nilotinib and decreased along with increasing concentration of the two drugs. At the highest dose of imatinib and nilotinib, the effect of calcipotriol nearly vanished.

Dose-effect data analysis revealed an inversed correlation of IC_{50} of imatinib and nilotinib with calsipotriol dose. In the presence of 1000 nM calcipotriol, IC_{50} of imatinib and nilotinib were reduced for approximately 1.5 to 5 folds (Table 2 and Table 3). These results mean that for a certain therapeutic efficacy, less imatinib and nilotinib are needed when calcipotriol is added.

However, this enhancing effect of calcipotriol on the efficacy of imatinib and nilotinib was similar for Schwann cells and fibroblasts, indicating that it is rather non-specific.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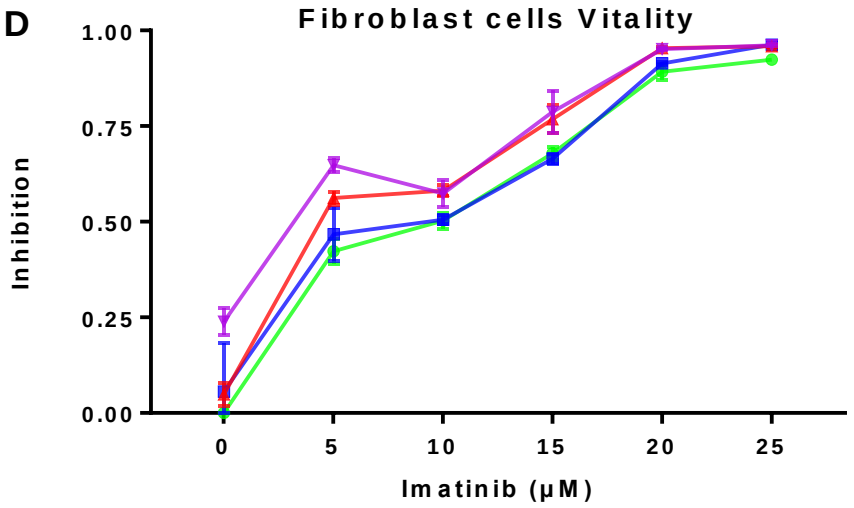
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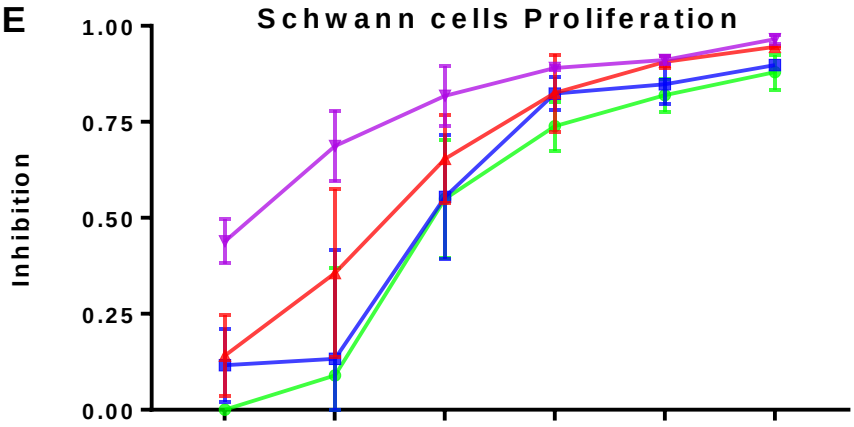
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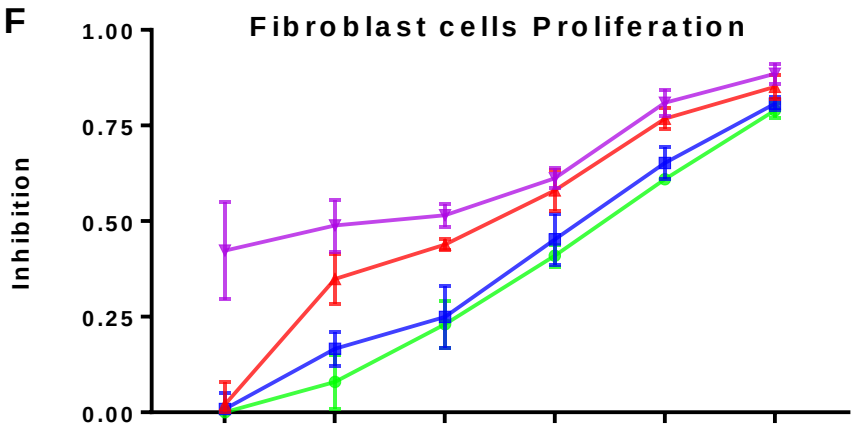
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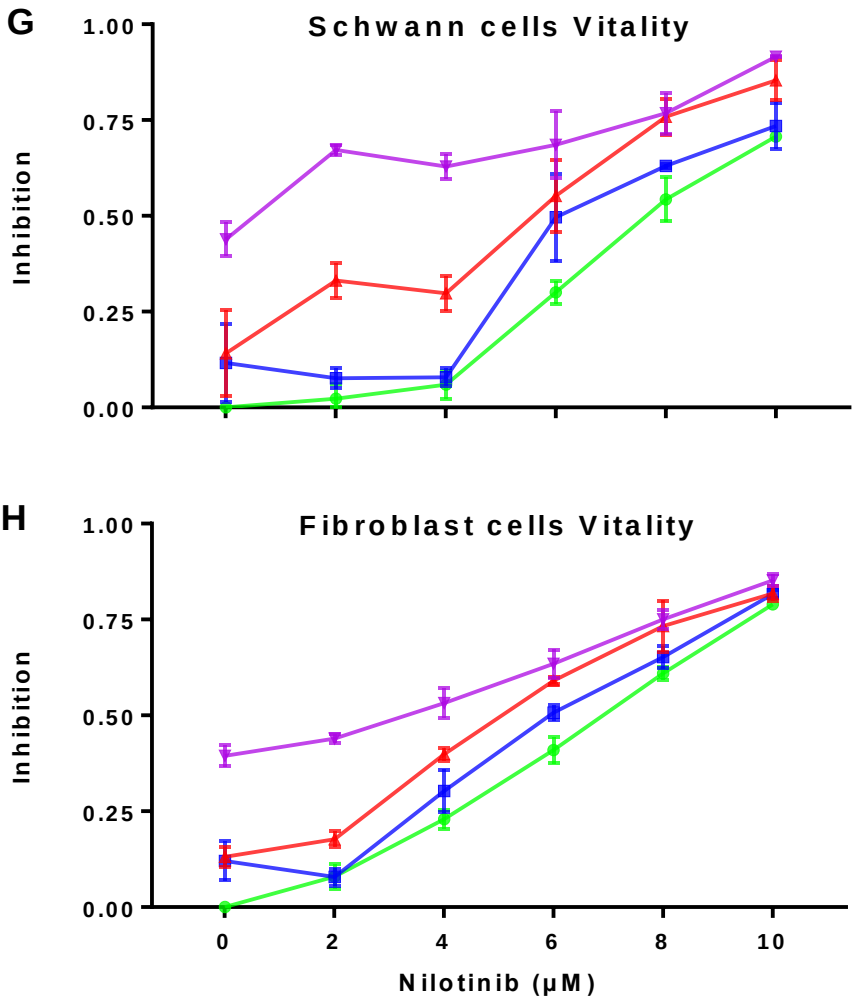

Figure 8. Inhibitory effect of imatinib or nilotinib on proliferation and vitality of Schwann cells and fibroblasts in presence of calcipotriol at 0, 10, 100 and 1000 nM. Drugs, cell type and measured feature are given directly in the graphic.

Table 2. IC_{50} of imatinib and nilotinib in presence of calcipotriol for cell proliferation

Schwann cells Proliferation (Cal=calcipotriol)

	Imatinib				Nilotinib			
Cal(nM)	0	10	100	1000	0	10	100	1000

T1181	11.0±2.0	8.2±2.4	6.6±0.3	5.8±0.4	7.9±1.3	6.1±2.4	3.7±0.4	2.3±0.2
T1972	9.8±1.4	7.3±0.3	2.8±1.2	3.2±1.1	6.9±1.0	4.6±1.7	2.7±0.	2.8±0.1
T1987	12.6±2.6	11.0±2.4	7.9±1.4	7.2±1.1	3.7±0.7	3.7±0.3	3.5±0.1	2.3±0.
T1979	10.6±2.1	7.7±2.5	7.0±0.8	5.3±0.4	5.3±0.9	4.3±1.6	3.1±0.5	2.0±0.3
T3213	10.8±1.6	9.0±1.4	6.2±0.2	5.3±0.3	4.1±0.5	3.3±0.7	2.6±0.2	1.9±0.5
T1161	10.4±2.0	6.2±0.5	3.0±1.0	2.7±0.6	3.9±0.5	3.6±1.1	2.5±0.3	1.1±0.4
T3211	12.5±2.7	11.2±2.1	7.7±1.4	6.2±1.3	6.4±0.8	5.1±0.7	3.4±0.2	3.3±0.1
T2029	9.5±2.5	7.9±2.0	5.8±1.1	5.1±0.4	5.4±1.2	5.1±0.5	3.4±0.9	2.9±1.0
T3212	13.0±3.1	9.2±1.6	8.4±1.9	7.2±0.2	7.7±1.4	8.4±3.1	4.2±1.6	2.9±0.8
T2048	9.7±2.0	8.4±1.2	6.0±0.5	3.5±1.7	7.0±1.3	5.6±0.9	4.6±0.4	3.6±0.9

Fibroblast cells Proliferation

	Imatinib				Nilotinib			
Cal (nM)	0	10	100	1000	0	10	100	1000
T1181	10.1±2.6	9.3±0.7	6.1±0.2	5.6±0.5	10.7±1.3	7.3±2.3	4.2±0.6	1.7±1.4
T1972	9.6±2.5	9.4±0.8	5.8±0.1	5.4±0.6	9.5±1.4	8.8±2.6	5.5±1.2	2.0±1.5
T1987	10.1±3.0	9.9±0.6	7.3±1.1	7.2±1.5	9.0±1.0	2.0±1.6	1.2±1.3	0.5±0.4
T1979	9.9±2.2	9.6±0.8	7.3±0.7	6.3±1.1	7.3±1.3	5.8±1.1	5.2±0.5	3.0±1.2
T3213	10.7±1.1	9.2±0.2	5.2±0.4	4.9±0.3	6.6±1.0	6.1±0.7	3.9±0.6	2.7±0.9
T1161	9.0±1.9	6.3±0.8	5.2±0.2	4.3±0.2	6.4±0.7	5.9±0.3	3.9±0.6	2.7±0.3
T3211	11.0±1.6	9.5±0.9	6.5±0.2	4.1±1.6	6.6±1.0	5.9±1.2	4.8±0.4	3.7±1.0
T2029	9.2±0.7	6.4±0.4	2.8±1.0	3.1±1.0	5.5±0.7	3.7±1.5	2.3±0.6	2.3±0.4

T3212	11.2±1.7	8.3±1.6	6.1±0.8	5.2±0.8	5.8±0.6	4.1±1.2	3.0±0.6	2.9±0.3
T2048	10.8±2.2	9.1±1.9	6.5±0.8	6.3±0.8	6.4±1.0	5.9±0.9	5.2±0.3	2.7±1.0

Table 3. IC_{50} of imatinib and nilotinib in combined with calcipotriol in vitality assay.

Schwann cells Vitality

	Imatinib				Nilotinib			
Cal (nM)	0	10	100	1000	0	10	100	1000
T1181	6.8±1.4	5.5±1.0	4.2±0.2	4.7±0.1	7.1±1.6	5.3±1.4	3.0±0.5	1.7±0.2
T1972	10.8±2.3	9.2±0.5	7.2±0.1	6.6±0.8	5.5±1.1	5.2±1.0	2.4±0.6	0.9±0.7
T1987	15.0±1.8	12.6±1.0	9.1±1.8	7.8±1.2	5.2±1.0	4.9±0.1	4.6±0.6	2.6±0.6
T1979	14.9±1.7	13.6±1.7	10.0±1.2	8.5±1.2	6.9±0.5	4.9±1.5	3.2±0.6	3.3±0.4
T3213	8.7±1.5	7.2±1.3	5.6±0.5	4.2±0.9	5.9±1.1	5.0±0.6	4.1±0.2	3.2±0.8
T1161	10.4±1.3	5.9±0.6	4.6±0.4	4.1±0.1	7.5±1.7	6.5±1.9	4.8±1.4	1.3±0.6
T3211	14.5±2.1	13.1±0.8	10.4±1.1	7.5±0.1	5.5±0.9	4.8±0.9	3.7±0.2	2.1±0.7
T2029	15.0±1.8	13.3±1.4	9.8±1.1	9.0±0.8	5.8±0.9	6.1±0.5	5.7±0.5	2.7±0.5
T3212	4.2±0.3	4.3±0.6	3.5±0.6	3.5±0.5	5.2±0.7	4.6±0.3	3.0±0.2	2.4±0.6
T2048	9.8±3.0	7.8±0.8	6.5±0.8	5.9±0.8	6.9±1.0	6.86±1.1	4.0±1.	3.5±1.2

Fibroblast cells Vitality

	Imatinib				Nilotinib			
Cal (nM)	0	10	100	1000	0	10	100	1000

T1181	10.5±1.5	10.9±0.4	9.6±0.4	6.0±1.3	7.5±0.7	7.7±0.4	5.4±1.0	2.8±0.6
T1972	11.0±1.4	11.8±0.3	10.6±0.2	7±1.3	8.2±1.3	7.8±0.8	5.6±1.5	2.7±1
T1987	10.5±0.8	7.2±0.6	5.9±0.1	4.4±0.1	4.4±0.4	3.9±0.1	2.7±0.3	2.0±0.3
T1979	10.7±0.9	10.6±0.7	4.4±0.3	1.8±0.4	7.4±0.2	5.2±1.4	2.9±1.7	1.2±0.7
T3213	10.4±1.2	9.6±2.5	5.0±2.2	3.4±1.1	7.0±0.6	4.7±1.8	3.2±0.6	2.8±0.7
T1161	7.4±1.1	6.9±1.1	5.4±0.8	4.5±0.7	6.5±0.6	5.8±0.5	4.8±0.3	3.0±0.6
T3211	10.3±1.0	9.4±1.2	5.0±0.8	3.7±0.2	7±0.9	6.2±0.6	3.1±0.6	1.8±0.5
T2029	12.7±1.4	11.6±0.6	9.0±0.5	6.8±0.6	8.4±1.0	7.5±2.0	4.6±0.6	1.9±1.3
T3212	10.7±1.9	9.6±0.8	6.5±0.4	5.3±0.5	6.6±0.6	5.7±0.9	4.5±0.4	3.3±0.7
T2048	9.3±1.2	9.1±0.1	6.1±0.3	5.2±0.4	7.9±0.7	7.1±0.9	4.7±0.7	1.9±0.5

IC_{50} of imatinib and nilotinib in each concentration of calcipotriol. Compared to the IC_{50} when calcipotriol = 0, * P<0.05 (blue), ** P<0.01 (yellow), *** P<0.001 (red) (Kruskal-Wallis ANOVA test)

Combination index analysis revealed synergistic (CI $\leqslant$ 0.9) effects of calcipotriol with imatinib or nilotinib in majority of cases, especially for calcipotriol at a concentration of $\geqslant$100nM (table 4 and table 5, green cells). These results suggest that the combination of calcipotriol with imatinib and nilotinib may have beneficial effects for the treatment of PNF derived cells *in vitro*.

Table 4. Combination index (CI) of imatinib and nilotinib in combination with

vitamin D in proliferation assay.

Schwann cells Proliferation

		Imatinib					Nilotinib				
		5 μM	10 μM	15 μM	20 μM	25 μM	2 μM	4 μM	6 μM	8 μM	10 μM
T1181	10 nM	0.6	0.9	1.1	0.9	1.0	0.5	1.1	1.1	0.5	0.7
	100 nM	0.6	0.6	1.0	0.7	0.9	0.3	0.6	0.7	0.5	0.5
	1000 nM	0.7	0.8	0.8	0.6	1.0	0.3	0.5	0.5	0.4	0.4
T1972	10 nM	0.7	0.8	0.9	0.8	0.9	0.5	0.9	0.7	0.6	0.9
	100 nM	0.4	0.6	0.5	0.9	0.9	0.4	0.5	0.5	0.6	0.8
	1000 nM	0.4	0.5	0.5	0.9	1.0	0.9	1.1	0.8	0.6	0.7
T1987	10 nM	0.7	0.8	1.0	1.1	1.0	1.1	0.8	0.9	0.9	1.1
	100 nM	0.6	0.7	0.6	1.0	1.0	1.1	0.8	0.7	1.0	1.0
	1000 nM	0.5	0.7	0.7	1.0	1.0	0.6	0.6	0.6	0.7	0.9
T1979	10 nM	0.6	0.8	1.0	0.9	1.0	0.7	0.9	0.9	0.9	0.7
	100 nM	0.6	0.8	0.9	0.9	0.9	0.5	0.8	0.8	0.8	0.6
	1000 nM	0.7	0.7	0.8	0.6	0.9	0.4	0.6	0.6	0.8	0.5
T3213	10 nM	0.8	0.9	1.0	0.7	0.7	0.7	1.0	0.8	1.0	1.0
	100 nM	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.8	0.8	0.9
	1000 nM	0.6	0.7	0.7	0.7	0.7	0.6	0.6	0.7	0.7	0.9
T1161	10 nM	0.6	0.7	0.8	0.7	1.0	1.3	0.9	0.8	1.0	1.0
	100 nM	0.4	0.5	0.6	0.9	1.0	0.7	0.7	0.8	0.8	0.8
	1000 nM	0.4	0.4	0.5	0.7	0.9	0.4	0.5	0.6	0.8	0.7
T3211	10 nM	0.6	1.1	1.0	1.1	0.9	0.6	0.9	0.8	0.8	0.9
	100 nM	0.5	0.9	0.6	0.9	0.9	0.5	0.6	0.8	0.8	0.9
	1000 nM	0.5	0.7	0.6	1.0	0.9	1.4	1.6	0.9	0.7	0.7
T2029	10 nM	0.8	0.9	1.0	1.2	1.0	0.8	1.4	1.2	0.8	1.0
	100 nM	0.6	0.8	0.6	1.0	1.0	0.6	1.0	1.0	0.7	0.9
	1000 nM	0.6	0.7	0.7	1.1	1.0	2.6	1.8	1.1	0.8	0.8
T3212	10 nM	0.7	0.9	1.0	0.9	0.9	0.9	1.1	1.0	0.9	1.0
	100 nM	0.6	0.9	0.9	0.9	0.9	0.6	0.9	0.7	0.7	0.7
	1000 nM	1.4	0.8	1.0	0.8	1.0	3.0	1.0	0.8	0.9	0.7
T2048	10 nM	0.9	0.8	1.1	0.9	0.8	1.7	0.8	0.8	0.8	0.8
	100 nM	0.6	0.7	0.9	0.9	0.8	0.9	0.7	0.8	0.6	0.5
	1000 nM	0.4	0.6	0.7	0.7	0.9	0.8	0.7	0.7	0.6	0.5

Fibroblast cells Proliferation

		Imatinib					Nilotinib				
		5 μM	10 μM	15 μM	20 μM	25 μM	2 μM	4 μM	6 μM	8 μM	10 μM
T1181	10 nM	1.0	1.1	0.9	0.8	0.7	0.4	0.7	0.8	0.8	0.6
	100 nM	0.8	0.8	0.7	0.8	0.7	0.4	0.5	0.8	0.5	0.5
	1000 nM	0.6	0.8	0.7	0.7	0.7	0.3	0.4	0.7	0.4	0.5
T1972	10 nM	0.9	1.1	1.2	0.8	0.7	0.7	0.8	0.9	1.0	0.8
	100 nM	0.7	0.8	0.9	0.7	0.7	0.4	0.6	0.8	0.5	0.7
	1000 nM	0.6	0.7	0.9	0.7	0.7	0.4	0.4	0.6	0.6	0.7
T1987	10 nM	0.7	0.9	0.9	1.0	1.3	0.2	0.4	0.5	0.4	0.5
	100 nM	0.5	0.7	0.9	1.0	1.2	0.2	0.4	0.3	0.4	0.4
	1000 nM	0.5	0.7	0.9	0.9	1.2	0.1	0.3	0.2	0.3	0.4
T1979	10 nM	0.9	1.0	1.0	1.0	1.0	0.7	0.6	0.9	1.1	0.7
	100 nM	0.7	0.8	0.9	0.9	1.0	0.7	0.7	0.7	1.0	0.6
	1000 nM	0.6	0.9	0.9	0.9	1.0	0.6	0.7	0.8	1.0	0.6
T3213	10 nM	0.9	0.7	1.0	1.1	1.0	0.7	1.2	1.1	0.9	0.8
	100 nM	0.6	0.8	0.9	1.1	1.0	0.6	1.0	0.9	0.8	0.8
	1000 nM	1.3	1.3	1.6	1.6	1.3	2.0	1.8	1.0	0.9	0.6
T1161	10 nM	0.7	0.9	0.7	0.7	0.8	0.7	1.0	1.0	1.0	0.8
	100 nM	0.7	0.8	0.6	0.7	0.8	0.5	0.8	0.9	0.8	0.7
	1000 nM	0.5	0.6	0.6	0.7	0.8	0.8	1.0	1.1	0.8	0.7
T3211	10 nM	0.8	0.8	1.1	0.9	0.8	1.7	0.8	0.9	0.9	0.8
	100 nM	0.6	0.7	0.9	0.8	0.7	1.0	0.7	0.9	0.8	0.7
	1000 nM	0.4	0.7	0.7	0.8	0.7	0.9	0.7	0.8	0.9	0.7
T2029	10 nM	0.7	0.9	0.9	0.8	0.7	0.7	0.7	0.7	0.8	0.8
	100 nM	0.4	0.6	0.5	0.8	0.8	0.5	0.5	0.5	0.8	0.7
	1000 nM	0.4	0.5	0.4	0.9	0.9	1.0	0.8	0.8	0.8	0.6
T3212	10 nM	0.7	0.8	0.8	1.0	0.8	0.6	0.8	0.8	0.8	0.9
	100 nM	0.6	0.7	0.5	0.9	0.8	0.5	0.6	0.7	0.8	0.8
	1000 nM	0.5	0.6	0.6	0.9	0.8	0.8	0.8	0.7	0.8	0.7
T2048	10 nM	0.7	0.8	1.1	1.3	0.8	1.0	0.7	1.2	0.8	0.9
	100 nM	0.6	0.7	0.8	1.1	0.9	1.6	0.7	0.8	0.9	0.8
	1000 nM	0.6	0.7	0.8	1.2	0.8	0.4	0.4	0.5	0.6	0.8

CI=Combination index. Red: CI > 1.1, antagonism; Yellow: 0.9 < CI $\leqslant$ 1.1, additive effect; Green: CI $\leqslant$ 0.9, synergism.

Table 5. Combination index (CI) of imatinib and nilotinib in combined with vitamin D in vitality assay.

Schwann cells Vitality

		Imatinib					Nilotinib				
		5 μM	10 μM	15 μM	20 μM	25 μM	2 μM	4 μM	6 μM	8 μM	10 μM
T1181	10 nM	0.7	1.2	1.0	0.9	1.0	0.5	0.9	1.1	0.5	0.7
	100 nM	0.7	0.9	0.8	0.9	0.9	0.3	0.6	0.7	0.4	0.6
	1000 nM	0.6	1.0	1.0	0.9	1.0	0.2	0.6	0.5	0.4	0.4
T1972	10 nM	1.0	0.8	0.8	1.0	0.8	0.6	1.1	1.3	1.3	0.5
	100 nM	0.6	1.0	0.7	1.0	0.9	0.4	0.7	0.9	0.9	0.3
	1000 nM	1.1	1.0	0.8	0.9	0.9	0.3	0.3	0.5	0.7	0.2
T1987	10 nM	0.6	0.9	1.2	0.9	0.9	0.8	1.1	1.0	1.0	0.9
	100 nM	0.5	0.8	0.9	0.8	0.9	0.8	0.9	0.9	0.9	0.8
	1000 nM	0.7	0.7	0.8	0.8	0.9	0.4	0.6	0.7	0.9	0.8
T1979	10 nM	0.6	1.1	1.3	0.9	0.9	0.5	0.8	0.7	0.7	1.0
	100 nM	0.5	0.9	0.9	0.8	0.9	0.4	0.6	0.6	0.7	0.8
	1000 nM	0.5	0.7	0.9	0.8	0.9	0.7	1.0	0.7	0.7	0.7
T3213	10 nM	0.3	0.6	0.8	0.9	1.0	0.1	0.4	0.5	0.8	0.8
	100 nM	0.5	0.7	0.8	0.9	1.0	0.3	0.5	0.6	0.8	0.9
	1000 nM	0.6	0.7	0.8	0.9	1.0	0.4	0.6	0.6	0.8	0.9
T1161	10 nM	0.6	1.0	1.0	0.7	1.0	2.5	2.9	0.8	0.9	0.9
	100 nM	0.5	0.8	0.8	0.8	0.9	0.5	0.9	0.7	0.7	0.7
	1000 nM	0.4	0.7	0.7	0.7	0.9	0.3	0.5	0.6	0.7	0.6
T3211	10 nM	0.9	1.2	1.0	0.8	0.8	0.2	0.3	0.5	0.8	0.9
	100 nM	0.6	0.9	0.9	0.6	0.8	0.3	0.5	0.6	0.8	0.9
	1000 nM	0.5	0.7	0.9	0.6	0.8	0.6	0.5	0.7	0.9	0.9
T2029	10 nM	0.6	1.0	1.3	0.9	0.9	0.8	0.9	1.1	1.0	1.0
	100 nM	0.5	0.9	0.9	0.8	0.9	5.9	0.8	1.3	0.9	1.0
	1000 nM	0.5	0.9	0.9	0.8	0.9	0.5	0.5	0.9	0.8	1.0
T3212	10 nM	0.5	0.8	0.9	1.0	1.0	0.8	1.1	0.9	1.0	0.9
	100 nM	0.7	0.8	0.9	1.0	1.0	0.7	0.9	0.8	0.9	0.9
	1000 nM	0.7	0.7	0.9	1.0	1.0	2.0	1.4	0.7	0.9	0.7
T2048	10 nM	0.8	0.8	0.9	1.0	0.8	0.8	1.2	1.4	1.0	0.7
	100 nM	0.6	1.0	0.7	0.9	0.9	0.6	1.0	1.0	0.8	0.5
	1000 nM	1.0	0.9	0.8	0.8	0.9	2.8	2.8	1.1	1.0	0.5

Fibroblast cells Vitality

		Imatinib					Nilotinib				
		5 μM	10 μM	15 μM	20 μM	25 μM	2 μM	4 μM	6 μM	8 μM	10 μM
T1181	10 nM	1.0	1.0	1.1	1.1	1.0	1.1	0.9	1.4	0.9	1.0
	100 nM	0.8	0.9	1.0	1.1	0.9	0.6	0.7	1.3	0.8	0.5
	1000 nM	0.6	0.7	0.8	0.9	0.8	0.4	0.5	0.8	0.5	0.6
T1972	10 nM	1.1	1.1	1.2	1.0	1.0	0.1	0.2	0.4	0.5	0.6
	100 nM	0.9	1.0	1.1	1.0	0.9	0.3	0.3	0.4	0.6	0.8
	1000 nM	0.7	0.8	0.9	0.9	0.8	0.5	0.5	0.6	0.7	0.8
T1987	10 nM	0.7	0.8	0.9	0.7	0.8	0.8	0.9	1.0	0.9	1.0
	100 nM	0.6	0.7	0.7	0.7	0.7	0.6	0.8	0.9	0.7	0.8
	1000 nM	0.5	0.6	0.8	0.6	0.7	1.0	1.0	1.0	0.7	0.8
T1979	10 nM	0.8	0.8	1.1	1.2	1.1	1.0	0.7	0.9	0.8	0.8
	100 nM	0.4	0.7	0.8	0.9	1.0	0.3	0.5	0.8	0.6	0.7
	1000 nM	0.3	0.5	0.6	0.8	1.0	0.2	0.4	0.6	0.6	0.6
T3213	10 nM	0.8	1.0	1.1	1.0	1.1	1.1	0.8	1.0	0.8	0.9
	100 nM	0.5	0.7	0.7	1.0	1.0	0.8	0.7	1.0	0.7	0.8
	1000 nM	0.5	0.5	0.6	1.0	1.0	0.7	0.7	0.8	0.8	0.7
T1161	10 nM	0.7	1.3	1.4	0.7	0.6	3.0	0.9	0.9	1.0	0.8
	100 nM	0.6	1.1	1.0	0.5	0.6	2.0	0.8	0.8	0.8	0.8
	1000 nM	0.5	1.1	1.0	0.5	0.6	0.7	0.7	0.8	0.8	0.7
T3211	10 nM	0.8	1.0	1.1	1.0	1.0	0.7	1.1	1.2	0.9	0.7
	100 nM	0.5	0.7	1.1	1.0	1.0	0.5	0.6	0.9	0.7	0.6
	1000 nM	0.6	0.7	1.0	1.0	0.8	1.0	0.7	0.9	0.8	0.5
T2029	10 nM	1.1	1.0	1.0	0.7	0.8	0.5	0.8	0.8	1.0	0.8
	100 nM	0.9	0.7	0.7	0.7	0.8	0.4	0.6	0.7	0.5	0.6
	1000 nM	0.6	0.7	0.7	0.7	0.8	0.3	0.4	0.6	0.4	0.7
T3212	10 nM	0.9	1.0	0.9	0.8	1.0	0.7	1.1	1.3	0.8	0.7
	100 nM	0.7	0.7	0.8	0.6	0.9	0.5	0.9	1.1	0.7	0.6
	1000 nM	0.5	0.6	0.7	0.6	0.9	1.1	1.0	1.0	0.6	0.6
T2048	10 nM	1.0	1.1	1.0	0.8	0.7	0.7	0.9	0.8	0.9	1.0
	100 nM	0.7	0.8	0.7	0.7	0.7	0.5	0.7	0.7	0.6	0.8
	1000 nM	0.5	0.7	0.7	0.7	0.7	0.3	0.4	0.5	0.6	0.8

CI=Combination index. Red: CI > 1.1, antagonism; Yellow: 0.9 < CI $\leqslant$ 1.1, additive effect; Green: CI $\leqslant$ 0.9, synergism.

4. Discussion

In this study, imatinib and nilotinib were confirmed to inhibit the proliferation and vitality of Schwann cells and fibroblasts derived from PNFs in a dose-dependent manner[53].

PNFs comprise mostly of Schwann cells and fibroblasts. Previous studies of our own group[55] and others revealed that Schwann cells are the tumor cells while fibroblasts are not. In this study, we successfully cultivated those two types of cells separately and treated them with imatinib and nilotinib. Both reagents decreased cellular proliferation and vitality. For nilotinib, the mean IC_{50} values were generally higher for fibroblasts cells than for Schwann cells. By contrast, imatinib showed no significant difference for those two types of cells. These results may suggest that nilotinib has a better selectivity than imatinib, which is in accord with our previous observation that nilotinib inhibited proliferation of PNF-derived Schwann cells *in vitro* substantially more potently than imatinib[53].

We further showed that imatinib and nilotinib exhibited higher inhibitory efficacy on PNF-derived Schwann cells and fibroblast cells when combined with calcipotriol. Since the enhancing effect on tumor cells (Schwann cells) and non-tumor cells (fibroblasts) was compatible, it may be non-specific. Combination analysis suggested synergic effect of calcipotriol with imatinib and nilotinib. However, the variation of efficacy is large and therefore we would rather interpret the effects as additive ones.

The enhancing effect of calcipotriol on efficacy of imatinib and nilotinib is substantial for calcipotriol of ⩾ 100nM. Physiological range of serum 25OH-vitamin D is 50-100nM[57]. Vitamin D toxicity related hypercalcemia occurs usually only when serum 25OH-vitamin D levels are above 300 to 375 nM[58,59]. Individuals may have high levels of serum 25OH-vitamin D from 250 nM up to 375 nM without associated hypercalcemia[60]. Therefore, the clinical increase of serum 25OH-vitamin D levels to 100 – 200nM by means of vitamin D substitution for enhancing efficacy of imatinib and nilotinib is feasible. Keeping vitamin D at higher level yet the within physiological range may allow dose reduction of the drugs and thus alleviate the toxicities. These findings might be important as the continuous usage of tyrosine kinase inhibitors in chemotherapy may result in adverse effects, such as weight loss, fatigue, neutropenia, neurotoxicity, hepatotoxicity, cardiotoxicity, and so on[61].

A previous study showed that vitamin D inhibited maturation and promoted apoptosis of mast cells, which relate to the vitamin D dose and VDR expression[62]. VDR is expressed in both Schwann cells[25] and fibroblast cells[63]. The underlying mechanisms by which vitamin D alone suppresses proliferation and reduces vitality of PNF-derived Schwann cells and fibroblasts is unclear. Several studies have reported that vitamin D induces a significant G0/G1 arrest which resulted in increasing expression of p21 and p27, cyclin-dependent kinase inhibitors[64–67]. It also had been shown that the combined use of AG1296 (a PDGFR inhibitor) and vitamin D enhances

inducible oxidative metabolism and other various features of vitamin D-related monocytic differentiation. By contrast, the enhancing effect of vitamin D would be limited when used in combination with cytotoxic drugs which require cells to be actively dividing to have an inhibition effect. As vitamin D causes cell cycle arrest and apoptosis, the enhancing activity worked in an ideal way when used with imatinib and nilotinib, because those tyrosine kinase inhibitors are targeted therapies and do not require cells to be actively dividing to take effect[68]. In addition, there is also data suggesting that vitamin D enhances the inhibitive effects of anti-cancer drugs through the production of reactive oxygen species and loss of mitochondrial membrane potential[69].

In summary, our *in vitro* data demonstrate that nilotinib is more potent and has better selectivity than imatinib on PNF-derived tumor cells *in vitro*, and calcipotriol enhanced the inhibitiory activity of both imatinib and nilotinib. These findings suggest the potential clinical application value of sustaining vitamin D at high level (100nM) for patients receiving tyrosine kinase inhibitors for the treatment of their PNF. This strategy may enable a dose-reduction of imatinib and nilotinib, and consequently reduce side effects.

List of Abbreviations

%	Percent
℃	Celsius degree
BrdU	5-bromodeoxyuridine
CML	Chronic myelogenous leukemia
CI	Combination index
CO2	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
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
h	Hour
HRGβ1	Heregulin β1
IBMX	3-iso-butyl-L-methylxanthine
IC_{50}	50% inhibitory concentration

IgG	Immunoglobulin G
kg	Kilogram
MAPK	Mitogen-activated protein kinase
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimole
µM	Micromole
mM	Manomole
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
PAK1	p21-activated kinase 1
PBS	Phosphate-buffered saline
PDGFR-α	Platelet-derived growth factor receptor-α
PDGFR-β	Platelet-derived growth factor receptor-β
PI	Propidium iodide

PNF	Plexiform neurofibroma
RAC1	Ras-related C3 botulinum toxin substrate 1
Raf	Murine sarcoma viral oncogene homologue
RT	Room temperature
RTK	Receptor tyrosine kinase
TKI	Tyrosine kinase inhibitor
U	Unit
VDR	vitamin D receptor
XTT	2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-(phenyl amino) carbonyl]-2H-tetrazolium hydroxid

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SUMMARY

Numerous studies have demonstrated that vitamin D or its analogues possess the antitumor effect in a variety of tumor models, especially in combination with multiple chemotherapy agents. Plexiform neurofibromas (PNFs) are benign tumors arising from the peripheral nerve sheath and most associated with Neurofibromatosis type 1 (*NF1*). Schwann cells and fibroblast cells are the predominant cell types of tumor cells and non-tumor cells in PNF respectively. In the previous study, we presented data on the anticancer efficacies of two tyrosine kinase inhibitors (imatinib and nilotinib) on cultured cells of PNF *in vitro* and *in vivo*. This study aims to examine the possible effect of calcipotriol, which is an analogue of the physiologically active metabolite of vitamin D, on efficacy of imatinib and nilotinib on cells derived plexiform neurofibromas *in vitro*.

Schwann cells and fibroblast cells derived from 10 different PNFs were treated with imatinib (0, 5, 10, 15, 20, 25 μM) and nilotinib (0, 2, 4, 6, 8, 10 μM) in absence or presence of vitamin D (0, 10, 100, 1000 nM). Cell proliferation and vitality were determined by BrdU and XTT assay kits. Possible synergistic effect was evaluated by combination index.

Imatinib and nilotinib provided dose-dependent suppression of Schwann cells and fibroblast cells. Nilotinib showed a higher efficacy on Schwann cells than on fibroblast, indicating a good selectivity. By contrast, imatinib has similar efficacy on the two types of PNF-derived cells. When used in combination with calcipotriol, the IC_{50} of imatinib and nilotinib were generally 1.5 to 5 folds lower than when used alone. Combination Index revealed possible synergistic effect of calcipotriol at higher concentration ($\geqslant$100 nM) with imatinib and nilotinib for anti-proliferation and anti-vitality activity on PNF-derived cells.

Our results suggest that nilotinib may be more potent and have better selectivity than imatinib for targeting PNF-tumor cells. Vitamin D analogue calcipotriol may potentiate the efficacy of imatinib and nilotinib on PNFs. Sustaining vitamin D at high levels (100nM) may provide a strategy for reducing dose of tyrosine kinase inhibitor on PNFs and thus mitigate the side effect. Since high vitamin D levels are not harmful but generally beneficial, the above strategy may also be applicable for chemotherapies for other tumors and cancers.

Zusammenfassung

Zahlreiche Studien konnten bereits zeigen, dass Vitamin D und seine Analoga, insbesondere in Kombination mit zahlreichen Chemotherapeutika, in einer Vielzahl von Tumormodellen eine tumorhemmende Wirkung besitzen. Plexiforme Neurofibrome (PNFs) sind gutartige Tumore, die aus peripheren Nervenscheiden entstehen und am häufigsten mit der Neurofibromatose vom Typ 1 (NF1) assoziiert sind. Schwann-Zellen und Fibroblastenzellen sind die vorherrschenden Zelltypen von Tumorzellen bzw. Nicht-Tumorzellen in PNFs. In einer vorhergehenden Studie haben wir Daten zur tumorhemmenden Wirkung von zwei Tyrosinkinase-Inhibitoren (Imatinib und Nilotinib) auf kultivierte PNF-Zellen in vitro und in vivo präsentiert. Die aktuelle Untersuchung zielte darauf ab, einen möglichen Einfluss von Calcipotriol, welches ein Analogon des physiologisch aktiven Metaboliten von Vitamin D ist, auf die Wirksamkeit von Imatinib und Nilotinib auf aus plexiformen Neurofibromen gewonnenen Zellen in vitro zu untersuchen.

Aus 10 verschiedenen PNF´s isolierte Schwann- und Fibroblastenzellen, wurden mit Imatinib (0, 5, 10, 15, 20, 25 µM) und Nilotinib (0, 2, 4, 6, 8, 10 µM) mit oder ohne Zusatz von Vitamin-D (0, 10, 100, 1000 nM) behandelt. Zellproliferation und Vitalität wurden mit Hilfe von BrdU- und XTT-Tests bestimmt. Ein möglicher synergistischer Effekt wurde durch Bestimmung eines Kombinationsindexes bewertet.

Imatinib und Nilotinib zeigten eine dosisabhängige Hemmung der Vitalität und der Proliferation von Schwann- und Fibroblasten-Zellen. Nilotinib zeigte dabei eine stärkere Wirkung auf Schwann-Zellen als auf Fibroblasten, was auf eine gute Selektivität hinweist. Im Gegensatz dazu zeigte Imatinib bei beiden aus den PNF´s gewonnenen Zellarten eine ähnlich starke Wirkung. Bei Kombination der beiden Tyrosinkinsaeinhibitoren (TKI) mit Calcipotriol waren die mittleren inhibitorischen Konzentrationen (IC_{50}) von Imatinib und Nilotinib durchgängig 1,5 bis 5 mal niedriger als bei alleiniger Anwendung des jeweiligen TKI. Der Kombinationsindex zeigte für die Ansätze mit Calcipotriol in höherer Konzentration ($\geqslant$ 100 nM) in Kombination mit Imatinib bzw. Nilotinib einen möglichen synergistischen Effekt bezüglich der proliferations- und vitalitätssupprimierenden Wirkung der TKI´s auf die aus den PNF´s gewonnen Zellarten.

Unsere Ergebnisse legen nahe, dass Nilotinib möglicherweise potenter ist und eine bessere Selektivität als Imatinib für das Targeting von PNF-Tumorzellen aufweist. Das Vitamin-D-Analogon Calcipotriol kann dabei die Wirksamkeit von Imatinib und Nilotinib bei PNFs potenzieren. Die Etablierung von hohen Vitamin-D-Konzentrationen (100 nM) kann eine Strategie zur Verringerung Tyrosinkinase-Inhibitor-Dosis bei der Behandlung von PNFs sein und somit die Nebenwirkungen der TKI´s mildern. Da ein hoher Vitamin-D-Spiegel im Allgemeinen nicht schädlich, sondern eher von Vorteil ist, kann die genannte Strategie auch bei Chemotherapien anderer Tumorentitäten anwendbar sein.

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Sep, 2010 - Jun, 2012: Master of Medicine, Zhengzhou University, Zhengzhou, PR of China

Sep, 2005 - Jun, 2010: Bachelor of Medicine, Zhengzhou University, Zhengzhou, PR of China

Work Experience

Jul, 2012 - Oct, 2015: Department of Orthopedics, Henan Cancer Hospital, Professional Direction: Treatment of bone and soft tissue Tumors

Awards

Medical and Technological Progress Award 2017, First Prize

New Medical Technology Introduction Award 2015, First Prize

Medical and Technological Progress Award 2014, Second Prize

Medical and Technological Progress Award 2013, First Prize

Outstanding Graduates Awards, 2012

Graduate Student Scholarship, 2011, First Prize

Outstanding Graduates Awards, 2010

National Scholarship for Encouragement, 2009

National Scholarship, 2008

The HSBC Bank Scholarship, 2008

Zhengzhou University Scholarship 2006, 2008, 2010, First Prize

Zhengzhou University Scholarship 2007, 2009, Second Prize

Publications

Zhao Yao, Liu Yilin, Wang Limin (2012). Preliminary results of Activ C artificial disc replacement for cervical spondylosis. Chinese Journal of Spine and Spinal Cord, 2012, 22(10)/ 10.3969/j.issn.1004-406X.2012.10.03

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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.
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