Title: Therapeutic Potential of HSP90 Inhibition for Neurofibromatosis type 2

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Running title: HSP90 Inhibition for NF2

Keywords: NF2, HSP90 inhibitors, Transcriptome

Financial support:
This work was supported by a Drug Discovery Initiative Award, Children’s Tumor Foundation, to M.G., and by the House Research Institute.

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Disclosure of Potential Conflicts of Interest:

R. Chen is, Z.M. Jaffer and A.E. Rubenstein were, employees of Nexgenix Pharmaceuticals Inc., a privately held, for-profit entity holding the rights to NXD30001 and its analog compounds. The other authors disclosed no potential conflicts of interest.

The abbreviations used:

NF2; neurofibromatosis type 2, VS; vestibular schwannoma, HSP90; heat shock protein 90, NGS; next generation sequencing, 17-AAG; 17-allylamino-17-demethxy-geldanamycin, 17-DMAG; 17-dimethylaminoethylamino-17-demethoxy-geldanamycin

Word count:

Abstract, 204; Translational relevance, 134; Text, 4968

Total number of figures and tables:

4 figures and 2 tables, 1 supplementary materials, 2 each of supplementary figures and tables.
Abstract

**Purpose:** The growth and survival of NF2-deficient cells are enhanced by the activation of multiple signaling pathways including ErbBs/IGF-1R/Met, PI3K/Akt, and Ras/Raf/Mek/Erk1/2. The chaperone protein HSP90 is essential for the stabilization of these signaling molecules. The aim of the study was to characterize the effect of HSP90 inhibition in various NF2-deficient models.

**Experimental Design:** We tested efficacy of the small molecule NXD30001, which has been shown to be a potent HSP90 inhibitor. The anti-proliferative activity of NXD30001 was tested in NF2-deficient cell lines and in human primary schwannoma and meningioma cultures *in vitro*. The anti-tumor efficacy of HSP90 inhibition *in vivo* was verified in two allograft models and in one NF2 transgenic model. The underlying molecular alteration was further characterized by a global transcriptome approach.

**Results:** NXD30001 induced degradation of client proteins in and suppressed proliferation of NF2-deficient cells. Differential expression analysis identified subsets of genes implicated in cell proliferation, cell survival, vascularization, and Schwann cell differentiation, whose expression was altered by NXD30001 treatment. The results demonstrated that NXD30001 in NF2-deficient schwannoma suppressed multiple pathways necessary for tumorigenesis.

**Conclusions:** HSP90 inhibition demonstrating significant antitumor activity against NF2-related tumor cells *in vitro* and *in vivo*, and represents a promising option for novel NF2 therapies.
Translational Relevance

For sporadic NF2-deficient vestibular schwannoma, surgical removal is both an effective and sufficient intervention in most cases. However, NF2 patients have high risks of developing cranial, spinal and peripheral nerve schwannoma and meningioma at a younger age, the majority of whom present with hearing loss and deafness. Moreover, a significant proportion of meningiomas are inoperable. Therefore, development of effective chemotherapy would be most beneficial for overall NF2 tumor prevention. HSP90 inhibitors have acquired significant importance in the field of cancer chemotherapy in recent years. In particular, the response of ERBB2 (HER2)-positive breast cancer has offered promise for the use of HSP90 inhibitors as a novel therapeutic alternative, a strategy that is currently in clinical trials. Given their partial dependence on ErbB signaling, NF2-related tumors are readily amenable with this strategy.
Introduction

Vestibular schwannomas (VS) account for approximately 5-10 percent of all tumors inside the skull (1); about 1 out of every 100,000 individuals per year develops a VS (2). Conventional treatment of this benign tumor includes surgical removal and radiotherapy but to date no validated chemotherapy is available due to poor response to tested interventions (3). Both sporadic and familial forms of VS lack expression of a functional NF2 protein, merlin/schwannomin (4, 5). While sporadic VS occur in later stages of life, the hallmark of familial NF2 is the development of early-onset, bilateral VS, often associated with other cranial and spinal nerves schwannomas, meningiomas, and ependymomas that frequently requires repeated invasive surgeries. Hence, less invasive chemotherapy will be highly beneficial to the NF2 patients for tumor control and prevention.

Merlin is a unique member of the ezrin-radixin-moesin (ERM) gene family with tumor suppressing activities (6). Its over-expression was shown to decrease cell growth concomitantly with cell cycle arrest and apoptosis (7, 8), whereas its depletion by antisense oligonucleotides resulted in increased cell proliferation (9). Consistently, mice heterozygous for NF2 inactivation (10), homozygous for tissue-specific NF2 inactivation (11), and transgenic for expression of a human dominant-negative NF2 mutant (12) developed a range of tumors. Merlin is involved in cellular function by providing the link between the actin cytoskeleton and multiple membrane-associated proteins, which are essential for processing extracellular signals, cell adhesion, and cytoskeletal architecture (reviewed in (13, 14)). Various biological pathways were suggested for merlin’s involvement in cell proliferation control, including negatively regulation of Rac pathway necessary for Ras transformation (15), and contact inhibition of growth through interaction with CD44 (16). Recently, merlin’s translocation into the nucleus was demonstrated to suppress tumorigenesis by inhibiting the nuclear E3 ubiquitin ligase CRL4 (DCAF1) implicated in DNA replication and cell cycle progression (17).

Multiple links between merlin and its interacting proteins suggested the activation of various signaling pathways in NF2-related tumors, which present a challenge for developing targeted therapeutics
for NF2. Heat shock protein 90 (HSP90) is a ubiquitous molecular chaperone that is responsible for maintaining a subset of proteins involved in cell proliferation and transformation (18). HSP90 inhibition induces proteasomal degradation of its client proteins, providing an attractive therapeutic strategy that can simultaneously suppress multiple signaling pathways. HSP90 is often found over-expressed in malignant tumors, and its elevated level was shown to correlate with poor survival among patients (19). A study demonstrated that the HSP90 complexes in tumor cells possess greater affinity to an HSP90 inhibitor 17-AAG, thereby promising selectivity for targeting tumor cells over normal cells (20). There is single report suggesting the efficacy of HSP90 inhibition in NF2 (21). Many known client proteins of HSP90 were found to be co-activated in human NF2-related tumors, such as ERBBs, AKT, and MET (22) (23) and our unpublished observations. Studies also showed the role of PDGFR and Integrin-FAK pathways in the growth of schwannoma (24) (25). Accordingly, we hypothesize that targeting HSP90 will be efficacious for NF2 therapeutics.

In this study, we aimed to evaluate the efficacy of HSP90 inhibition in NF2-deficient cells in cell culture systems (hereafter referred to as ‘in vitro’) and in mouse allograft and in a genetically modified NF2 mouse model (‘in vivo’). The novel small molecule inhibitor series Pochonin and Pochoximes, developed on the radicicol scaffold, were shown to be highly potent and specific inhibitors of HSP90 with anti-proliferative activity (26, 27). One of the lead compounds NXD30001 (pochoximeA) penetrated the blood-brain and blood-spinal cord barriers and accumulated in nervous tissues (28), a pre-requisite for treating tumors in the central/peripheral nervous system including some NF2-related tumors, such as ependymomas. In order to further characterize the underlying molecular mechanisms of action, we utilized the next-generation sequencing (NGS) approach for assessing the global abundance of transcripts. Treatment of NF2-deficient cells with NXD30001 in vitro resulted in the depletion of multiple signaling molecules implicated in NF2. Concomitantly, in vivo administration of NXD30001 reduced growth of NF2-deficient tumors, and further gene expression analysis identified multiple biological pathways that may contribute the efficacy of HSP90 inhibition against NF2-related tumorigenesis.
Materials and Methods

Reagents

The structure of NXD30001 and its formulation has been described elsewhere (27). Details are in Supplementary Materials.

Cell lines and primary cultures

Mouse Nf2−/− embryonic Schwann cells, ESF-FC1801, and mouse schwannoma cells expressing the human Sch-Δ(39–121) mutant, 08031-9, were derived in our laboratory. An immortalized human schwannoma cells HEI193 was a gift from Dr. Lim (House Research Institute, Los Angeles, CA). The human schwannoma and meningioma, and the mouse Schwann cell primary cultures, were freshly prepared and used before exceeding five passages. Details are in Supplementary Materials.

Cell viability and proliferation assay

MTS-based monolayer cell proliferation assay (Promega) and soft agar clonogenic assay were used to test cell viability and proliferation at 72 hours and at 3 weeks of drug administration in vitro, respectively. The drug concentration that inhibits cell growth by 50% (GI50) was determined using Prism 5.0 (GraphPad Software). Details are in Supplementary Materials.

Allograft models and antitumor efficacy assay

All animal experiments were performed in strict adherence to the NIH guidance in the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. The ESC-FC1801 cells, and the transplantable schwannoma homogenate, 08031-9, were grafted subcutaneously in nude and in FVB/N syngenic mice, respectively. Mice were subjected to intraperitoneal injection of NXD30001 at 100mg/kg/day, three days a week for four weeks. Details are in Supplementary Materials.
Pharmacokinetic analysis
The concentration of NXD30001 in the plasma and grafted tumors was measured following single dose and repeated dose administration. The assay was performed at Cerep Inc. (Redmond, WA). Details are in Supplementary Materials.

Morphometric analysis of Schwann cell tumorlets in NF2 transgenic mice
F1 mice of NF2 transgenic mice TgP0-Sch-Δ(39–121)-27, 5 weeks of age, were subjected to oral administration of 17-DMAG (Alvespimycin) at 10mg/kg/day or 20mg/kg/day, three days a week for eight weeks. The development of Schwann cell tumorlets in the spinal nerve roots was histopathologically scored at the endpoint, and was compared between drug-treated and vehicle-treated mice. Details are in Supplementary Materials.

Quantitative RT-PCR analysis
Total RNA was isolated, reverse transcribed, then subjected for TaqMan® gene expression assay using the 7500 Real-Time PCR system (Applied Biosystems). The relative quantification (RQ) was determined against freshly dissected mouse sciatic nerve (= 1.0). Details are in Supplementary Materials.

Western blotting
Cultured cells (5 x 10^5) and pulverized frozen tumor samples were lysed in RIPA buffer and the total proteins were extracted, separated, and transferred using standard procedures. Antibodies used for Western blotting are in Supplementary Materials.

Immunocytochemistry, immunohistochemistry, TUNEL and BrdU staining
For the detection of S100β, cells were grown on cover glass, fixed and stained using anti-S100β antibody and Alexa594-conjugated secondary antibody. The apoptotic cells were fixed and labeled using
DeadEnd™ Fluorometric TUNEL System (Promega). The cells undergoing DNA replication were detected using BrdU staining Kit (Invitrogen). For detection of vascularization, paraffin sections of grafted tumors were subjected for PECAM immunohistochemistry followed by Hematoxylin-Eosin (H&E) staining. All images were acquired using the AxioImager.M1 microscope (Carl Zeiss). Details are in Supplementary Materials.

**Cell cycle analysis**

The distribution of cells in different phases of cell cycle was measured by flow cytometry. The ESC-FC1801 cells (1x10^6/10-mm dish) were treated with NXD30001 for 0, 24, 48, 72 hours, then fixed in 70% ethanol and incubated in PBS-T containing 10 \( \mu \)g/mL propidium iodide (PI) and 100 \( \mu \)g/mL RNase for 1 hour. The cells were analyzed on the FACSArria II platform (BD Bioscience).

**Mutation analysis of NF2-related human schwannomas and meningiomas**

NF2 mutations were screened on the genomic DNA isolated from patient-derived schwannoma and meningioma by direct sequencing of 17 exons and the adjacent splice sites (Supplementary Table S1). Loss of heterozygosity (LOH) was determined by SALSA® MLPA® kit for NF2 (#P044-B1, MRC-Holland). The automated sequencing and the MLPA analysis were performed at Laragene Inc. (Los Angeles, CA).

**Transcriptome analysis by high-throughput mRNA sequencing**

Total RNA from matched ESC-FC1801 cultured cells and allograft tumors (NXD30001 treated and untreated, n=1) was used for global sequencing. The expression of 35,604 gene loci in the mouse genome was reported by Cufflinks in the unit of ‘fragments per kilobase of exon per million fragments mapped’ (FPKM). The data has been submitted to GEO with the accession number GSE40187. Differentially expressed genes were subjected to pathway analysis using MetaCore (Thomson Reuters). Details are in
Supplementary Materials.

**Statistical Analysis.**

Quantitative data are presented as means ± SD when applicable. Statistical analysis was carried out using two-tailed unpaired *t* test on GraphPad Prism 5.0 Software. *P* < 0.05 was considered statistically significant. For the transcriptome analysis, genes were considered differentially expressed by the Cufflinks software when the false discovery rate (FDR) corrected *p*-value after Benjamini-Hochberg correction for multiple testing was less than 0.05.
Results

Elevated HSP90 expression in NF2-related human schwannomas.

We previously conducted an RNA expression study using microarrays comparing human schwannomas from NF2 patients with human saphenous nerves from normal individuals (Manent, manuscript in preparation). We found the expression of HSP90AA and HSP90AB (encoding HSP90) was elevated in schwannoma when compared with normal nerves (Fig. 1A): HSP90AA probes 1 and 2 were up-regulated 2.7- and 2.1-folds, respectively. The HSP90AA probe 4 and the HSP90AB probe 4 were also increased in the schwannoma samples. These results indicated an increased requirement of HSP90 in NF2-related schwannomas.

Growth inhibition and client protein degradation by NXD30001 in NF2-deficient cells in vitro.

Two NF2-deficient cell models were used in this study: mouse embryonic Schwann cells ESC-FC1801 lacking Nf2 exon 2, and mouse schwannoma cells 08031-9 expressing a human Sch-Δ(39–121) dominant-negative mutant of NF2. The expression of the Schwann cell marker S100β was positive in >99% of the 08031-9 cells, comparable to the mouse Schwann cell (mSC) primary culture, but was found to be low in the ESC-FC1801 cells (Fig. 1B). Quantitative PCR confirmed the lower S100β expression in ESC-FC1801, whereas p75Ngfr was higher in ESC-FC1801 than that in 08031-9 (Fig. 1C). In human vestibular schwannoma (hVS), expression of ERBB2 and ERBB3 was upregulated compared to that in normal nerves, whereas EGFR was predominant in the immortalized schwannoma cells HEI193, which lacked ERBB3 (Fig. 1D, top). Consistent with their neuregulin-dependency, ESC-FC1801 and 08031-9 expressed high levels of ErbB2 and ErbB3 but lacked Egfr (Fig. 1D, bottom), showing similar expression pattern to hVS.

HSP90 inhibition by NXD30001 effectively inhibited the growth of the NF2-deficient cells at low nanomolar concentration after 72hrs exposure in vitro (Fig. 2A). ESC-FC1801 was ten-fold more sensitive to the drug compared to the normal mSC, signifying the selectivity of the drug for the Schwann cells.
lacking NF2. NXD30001 at 100nM and 1000nM induced nuclear DNA fragmentation, a hallmark of apoptosis, in a small percentage of the cells (Fig. 2B and Supplementary Fig. S1): NF2<sup>−/−</sup> Schwann cells ESC-FC1801 were more sensitive to apoptosis than NF2-deficient schwannoma cells HEI193 and 08031-9, but less sensitive than normal mSC. No apoptotic cell was observed in the patient-derived schwannoma primary cultures (data not shown). The results indicated that NF2-deficient cells acquired resistance to apoptosis, and the primary effect of HSP90 inhibition in the schwannoma cells was cytostatic rather than cytotoxic.

Long-term effect of NXD30001 in the Nf2-deficient cells in vitro was evaluated by soft agar clonogenic assay. Because of their benign nature, primary cultures of mouse Schwann cells or human schwannoma cells do not form colonies in this assay, and were therefore excluded from the analysis. NXD30001 inhibited colony formation (both in number and in size) of ESC-FC1801, 08031-9, and HEI193 in a dose dependent manner (Fig. 2C). In contrast to the 72-hr cell viability assay, the anti-proliferative efficacy in the long-term culture was similar in the three cell lines tested, with \( \text{GI}_{50} = 11.8 \text{nM}, 10.8 \text{nM}, \text{and } 10.0 \text{nM}, \) respectively. The result indicated that the pathway leading to growth inhibition was independent of the NF2 status, since NF2<sup>−/−</sup> ESC-FC1801, HEI193 with isoform 3, and 08031-9 carrying mutant NF2 responded similarly to the drug.

Western blot analysis revealed higher baseline expression of HSP90 and its client proteins ErbB2, c-Met, Axl, c-Raf, and Cdk4, in the mouse NF2-deficient cells than in the NF2<sup>+/+</sup> mSC (Fig. 2D). Upon HSP90 inhibition, HSP90 and HSP70 increased concomitantly, whereas client proteins showed dose-dependent degradation at 24 hours. Of the concentrations tested, the minimum dose sufficient to deplete client proteins was 100nM for ESC-FC1801, 1000nM for 08031-9, and between 100nM and 1000nM for HEI193, which corresponded with their \( \text{GI}_{50} \) in the cell viability assay. In mSC, on the other hand, some client proteins, such as Axl, c-Raf, and Akt, increased at lower doses, and the degradation only occurred at 10000nM, demonstrating resistance to protein degradation by HSP90 inhibition in normal Schwann cells.
Given that drug efficacy can differ between different species, primary cultures of patient-derived schwannoma and meningioma were also tested in the cell viability assay. The GI50 for ten human schwannoma and meningioma ranged from 20nM to 1μM (Table 1). The NF2 mutation analysis on the original tumor samples revealed no obvious correlation between drug sensitivity and the NF2 status. However, the mitotically active cells of the sporadic vestibular schwannoma-5, which carried bi-allelic nonsense/frameshift NF2 mutations, demonstrated high sensitivity to the drug. Likewise, highly proliferating spinal schwannoma-1 and meningioma-2, which were derived from a single NF2 patient with history of multiple lesions, responded significantly to the drug.

**Antitumor efficacy of NXD30001 in NF2-deficient allograft models in vivo**

NXD30001 was administered for 4 weeks in two subcutaneous allograft models; ESC-FC1801 cells inoculated in nude mice, and 08031-9 mouse schwannoma homogenate inoculated in syngenic FVB/N mice. In nude mice, NXD30001 exhibited severe toxicity when administered at 100mg/kg/day, 3d/week, which resulted in progressive weight loss (Fig. 3A, upper-left panels), compelling the change of schedule to 2d/week after the first two weeks. By contrast, FVB/N mice tolerated the same regime without major weight loss (Fig. 3A, upper-right panel) or other clinical toxicities. In both models, the tumor growth was significantly inhibited in the drug-treated group (Fig. 3A, lower panels, p<0.001 and p<0.0001, respectively).

The concentration of NXD30001 in the ESC-FC1801 tumors after repeated administration reached above 1μg/g, a dose equivalent to ~2μM in vitro (Fig. 3B, left). Furthermore, a single administration (100mg/kg) to the 08031-9 tumor bearing FVB/N mice demonstrated selective drug accumulation in the tumors (Fig. 3B, right), resulting in the tumor/plasma ratio of AUC(0-t) as 8.9. HSP90 client and related proteins (Axl, p-Akt, p-Erk, and Cdk4) were reduced after 4-week drug administration in the ESC-FC1801 tumors (Fig. 3C). Consistently, cellular proliferation in the drug-treated tumors, evaluated by the BrdU incorporation, was suppressed in the drug-treated tumors (Fig. 3D).
Evaluation of HSP90 inhibition against orthotopic tumor development in NF2 transgenic mice

The NF2 transgenic mouse model expressing a human Sch-Δ(39–121) dominant-negative mutant protein was previously created and characterized (12). 100% of the heterozygous transgenic mice in the (FVB/NCrIxC3H/HeNCrl)F1 background were found to develop multiple benign Schwann cell tumorlets in the spinal nerve roots by 3 months of age (Fig. 3E). In order to evaluate HSP90 inhibition as a preventive strategy in NF2, we carried out drug efficacy test against schwannoma development using a well-characterized HSP90 inhibitor 17-DMAG on our NF2 transgenic mice. No weight loss was observed during the 8-week treatment with 17-DMAG at 10mg/kg/day and 20mg/kg/day, 3d/week (Fig. 3F). About a hundred microscopic fields per each group, covering equivalent nerve root areas, from the lumber and sacral spines were subjected to morphometric analysis (Fig. 3G). Both the number of tumorlets per root area and the ratio of tumorlet/root area were dose-dependently reduced after HSP90 inhibition (Fig. 3H, I).

Global transcriptional alterations induced by NXD30001 in the NF2-deficient cells

Next generation sequencing (NGS) was utilized to characterize the transcriptional consequences of HSP90 inhibition in the NF2-deficient cells. The expression profiles of cultured ESC-FC1801 cells (control vs. treated with 1μM NXD30001 for 24 hours) were further compared with those of ESC-FC1801 tumors (control vs. treated with 100mg/kg/day, 3d/week for two weeks) to provide the added context and contribution of the microenvironment for growing tumors (Supplementary Fig. S2A). About 27 million paired end reads of RNA sequences per sample (100bp in size, Illumina HiSeq 2000) from the in vitro experimental groups, and about 10 million single end reads (76bp in size, Illumina GAIIx) from in vivo experimental groups, were obtained from TopHat. The absolute values of gene expression for 35,604 gene loci were determined by Cufflinks and Cufflinks' differential expression analysis (Cuffdiff) (Supplementary Fig. S2B). The differential expression in fold change was highly reproducible by qPCR analysis performed on selected genes (Supplementary Table S2).

Alterations of HSP90 and its interacting proteins (www.picard.ch/downloads/HSP90interactors)
upon HSP90 inhibition involved both up- and down-regulation (Supplementary Fig. S2C, individual data in the GEO database). In vitro, significant increase of HSP90aa1, HSP90ab1, Hspa8 (HSC70), Hspa5 (GRP-78), and Hsf1 demonstrated the well-known response to HSP90 inhibition and heat shock. Subsets of up-regulated transcripts also indicated various cellular responses, including induction of apoptosis (Dedd2, Dapk3), autophagy (Map1lc3a, Map1lc3b, Gabarapll2), oxidative stress response (Pink1), inflammatory response (F2r), and anti-apoptotic response (Sgkl). On the other hand, survivin (Birk5), an apoptosis inhibitor and essential regulator of mitosis, was among the most significantly down-regulated transcripts whose expression was reduced to less than 5% of control. Similarly, transcripts of centrioles-associated tubulins (Tubdl and Tube1), majority of mitotic kinases (Cdk1, Cdk6, Cdk11, Aurkb, Plk1, Plk4), DNA polymerases (Pola1, Pola2, Polh), DNA helicase (Blm), DNA damage checkpoint and repair proteins (Chek1&2, Rad50, Mre11a), cell cycle proteins (Ccnb1&2), and some histone proteins were concomitantly silenced after the HSP90 inhibition in vitro. By contrast, transcriptional alteration in vivo, included different gene sets: Most of the chaperone proteins, mitotic components, and DNA modulators remained unchanged. Instead, modification was observed in many cytoskeletal (actin, tubulin, myosin) and histone components. However, the increase of apoptosis-related genes (Dedd, Ask1) was in accordance with the apoptotic response in vitro. Transcriptional changes in the HSP90 clients implicated in NF2 included up-regulation of Pdgfrb, Erbb3, and Lats2, down-regulation in Egfr, Vegfr2, Esr1, and Akt3. The results demonstrated that the overall consequences are complex and involve multiple responses, such as possible feedback required for the growth and survival of ESC-FC1801 tumor graft.

Pathway analysis of HSP90 inhibition in NF2-deficient cells

Next, we aimed to systematically identify enriched pathways and regulatory networks that were affected by NXD30001. Cuffdiff revealed changes in approximately 3% of the total genes; 1383 and 965 genes in vitro and in vivo, respectively, with 128 genes in common (Supplementary Fig. S2B). Remarkably, there was almost no overlap in the affected biological processes between in vitro and in vivo conditions (Table
In vitro, there were significant changes exclusively in a gene set associated with cell cycle and DNA damage (Table 2, top). NXD30001 increased the expression of genes known to negatively regulate cell proliferation (Cgref1, Chfr, Dusp1), while it depleted the expression of genes essential for cell cycle progression, DNA replication, and DNA repair. These include phase specific cyclins (Cena2, Ccnb1&2, Ccnd1), cell division cycle proteins (Cdc14a, Cdc25c), initiator of DNA replication (Prim1&2), DNA polymerases (Polal&2, Polh), DNA methyltransferase (Dnmt1), mitotic kinases (Aurka,b, Bub1, Bub1b), and anaphase promoting complex (APC) (Anapc1,7). The major DNA repair checkpoint proteins Ataxia telangiectasia mutated (Atm) and ATM and RAD3-related (Atr), as well as DNA repair proteins (Brca1&2) were also significantly down-regulated. These changes potentially affect all phases of the cell cycle, thereby inhibiting cell proliferation.

In vivo, on the other hand, there were changes in genes implicated in diverse biological pathways (Table 2, bottom): Cell adhesion promoting genes Adam11&23, Cadm4, Mcam, Chl1, Cdh13, Dlg1, integrins, laminins, and collagens, some of which were shown to be absent in certain cancers, were found to be up-regulated. Conversely, merlin-interacting hyaluronate receptor CD44 whose expression is higher in Schwannoma (29) was down-regulated. Many genes involved in blood vessel development were also deregulated; the reduced expression of vascular endothelial specific cadherin (Cdh5) and endothelial marker Pecam1, together with complete lack of hemoglobin expression, indicated the overall suppression of vascularization. Remarkably, genes associated with myelination and peripheral nervous system development, such as Ngfr, Erbb3, Egr2, Sox10, Pmp22, and Plp1, were concomitantly up-regulated in the NXD30001-treated tumor.

Experimental validation of gene expression profiles
In order to validate the transcriptome analysis, we examined the selected biological processes altered by NXD30001. We identified severe down-regulation of genes essential for DNA synthesis, cell cycle
progression, and cell division in vitro. Consistently, cell cycle analysis revealed depletion of cells in S phase and accumulation in G2/M phase after NXD30001 treatment (Fig. 4A). The increasing population of PI-unstained cells over time corresponded with the induction of apoptosis. Immunocytochemical staining confirmed that BrdU-incorporation was significantly suppressed after NXD30001 treatment (Fig. 4B). The results concluded that NXD30001 induced cell cycle arrest at multiple phases followed by apoptosis in ESC-FC1801 cells in vitro.

In our in vivo model, genes involved in angiogenesis were found to be deregulated in the drug-treated tumor. Accordingly, we performed immunohistochemistry of endothelial marker PECAM-1, which revealed that the ESC-FC1801 tumors contained fewer blood vessels than the 08031-9 tumors (Fig. 4C). Upon HSP90 inhibition, the number of blood vessels significantly decreased in the drug-treated tumors.

Many genes that encode major components of myelin sheath, Pmp22, Mbp, Mpz, and Prx, were concomitantly up-regulated in the drug-treated tumor, indicating that HSP90 inhibition in the NF2-deficient cells induced re-differentiation towards Schwann cell lineage. To test this hypothesis, we examined the expression of Pmp22, Mpz, and Mbp mRNA without or with NXD30001 in the ESC-FC1801 and 08031-9 cells (1μM for 24 hours), and their grafted tumors (100mg/kg/day, 2-3d/week for 4 weeks). Both tumors and cells demonstrated up-regulation of the myelin-specific genes, particularly Pmp22 (Fig. 4D). The effect was broader in the ESC-FC1801 cells, as no significant induction of Mpz was detected in 08031-9 cells. Remarkably, the proteins of these genes were not detectable in the drug-treated cells despite the induction of their mRNA in vitro (Fig. 4E).
Discussion

In this study, we validated the efficacy of a novel HSP90 inhibitor NXD30001 against NF2-related tumors in vitro and in vivo. Exposure to NXD30001 resulted in degradation of major client proteins activated in NF2 concomitantly with growth suppression in the NF2-deficient cells. Degradation occurred at lower doses in all NF2-deficient cells tested than in the normal Schwann cells in vitro. On the other hand, cells derived from schwannoma (08031-9, HEI-193, and human tumor primary cultures) manifested partial resistance to apoptosis. Hence, HSP90 inhibition in NF2-deficient cells involves both cytostatic and cytotoxic events, which together determined the overall anti-proliferative efficacy. NXD30001 accumulated selectively in the tumors well above the GI_{50} in vitro, hence, we conclude that the observed anti-tumor activity in vivo is primarily attributable to the HSP90 inhibition.

The NF2-deficient cells used in this study displayed differential baseline gene expression, reflecting their difference of origin and adaptation to the in vitro culture. For example, absence of S100β and presence of Ngfrp75 in the ESC-FC1801 cells indicated their immature nature as compared to that of 08031-9 (30). Remarkably, the predominant expression of Erbb2 and Erbb3 in ESC-FC1801 resembled that of human schwannomas, which may have contributed to its selective sensitivity to the drug, as exemplified by the study on melanoma cells where response to HSP90 inhibition partly correlated with the expression of ERBB2 (31). Nonetheless, the long-term exposure to the NXD30001 in the clonogenic assay and in the allograft models demonstrated comparable anti-proliferative effect in all cell lines tested, implying that HSP90 inhibition by repeated dose may be cumulative, and the effect is independent of the NF2 status.

Our allograft models of two different NF2-deficient cells ESC-FC1801 and 08031-9, and the NF2 transgenic mouse model TgP0-Sch-Delta(39–121)-27 provide valuable tools essential for preclinical study for NF2. In view of immune system as body’s major defense mechanism against cancer development, the use of immunocompetent FVB/N mice is beneficial for the evaluation of anti-tumor activity of a drug. Furthermore, the preclinical use of a genetically modified mouse model of NF2 schwannomagenesis
allows us to address the drug delivery to the peripheral nerves for the efficacy studies. Because of their extra-axial nature, schwannomas are not protected by the blood-brain barrier (32). By using different HSP90 inhibitors in multiple NF2 models in vivo, we ascertained the efficacy of HSP90 inhibition in the NF2 tumorigenesis.

The drug sensitivity to NXD30001 in the patient-derived schwannoma and meningioma primary cells in vitro was variable. This implied that the clinical response of human tumors to HSP90 inhibition may also differ significantly between patients. We identified a few schwannomas/meningioma cells with significant response to the HSP90 inhibition, which carry truncating mutations and/or are of spinal origin from patients with severe clinical phenotypes (i.e., younger age of onset, existence of multiple lesions, and larger tumor size). These primary cells sustained advanced proliferation over time despite that most human schwannoma/meningioma cells undergo progressive senescence in a short period in vitro. Nonsense or frameshift NF2 mutations have been associated to more severe disease phenotypes in NF2 (33). Also, spinal tumors are associated with severe NF2 phenotypes (34). Further analysis with larger number of cases will be necessary to evaluate whether the NF2 patients with malignant phenotypes have therapeutic advantage in HSP90 inhibition.

The molecular consequences caused by HSP90 inhibition in NF2-related tumors are of great interest, also in the context of understanding NF2 tumorigenesis and therapeutic targets. Our transcriptome analysis recapitulated many biological responses reported for HSP90 inhibition, including cell cycle arrest, induction of apoptosis, modification of cell adhesion and cytoskeletons, inhibition of angiogenesis, and induction of cell differentiation. Furthermore, our data demonstrated striking differences in gene expression between the short-term HSP90 inhibition in cell culture environment and the long-term administration in the microenvironment of solid tumor. While many of the cell cycle associated genes and DNA damage response genes are the known HSP90 clients, the primary effect of NXD30001 in vitro was exclusively enriched in suppression of cell cycle transit, DNA replication and repair, spindle assembly and chromosome separation. HSP90 protein was shown to be concentrated on the centrosomes, whose
inhibition caused abnormal centrosome segregation and maturation, aberrant cell division spindles, and impaired chromosome segregation (35). Recently, Lyman et al. systematically demonstrated three types of perturbation in the cell cycle, at M-phase, G2-phase, and G1-phase, by different HSP90 inhibitors in various cancer cells, signifying the multiple involvement of HSP90 in cell proliferation (36). NXD30001 also down-regulated the expression of anti-apoptotic survivin (37), whose expression is positively regulated by ErbB2 signaling (38). Our experimental validation demonstrating cell cycle arrest and apoptosis was in good agreement with these results, which further indicated the potential of HSP90 inhibition for sensitizing NF2-deficient cells to radiotherapy through suppression of DNA damage repair mechanisms and cell survival.

The effect of NXD30001 in vivo involved complex biological changes including cell adhesion, extra-cellular matrix remodeling, vascularization, and differentiation, where much of merlin’s function is also implicated. Although NXD30001 did not necessarily reverse the individual gene expression deregulated in human schwannoma (39, 40), the transcriptional alteration induced by NXD30001 and by the restoration of wild-type merlin in the NF2−/− ESC-FC1801 cells overlapped strikingly (17), including down-regulation of survivin, Aurkb, Brca2, Ccna2, Ccnd1, and Dedd2. Moreover, a number of molecular markers for tumorigenesis were altered toward expression profile preferable for therapeutic outcome. These results confirmed the beneficial effect of targeting HSP90 in NF2.

There is no evidence that merlin directly regulates the expression of HSP90. However, a recent study reported the co-immunoprecipitation of merlin with HSP70, which forms a multichaperone complex with HSP90 (21), implying that merlin may be regulated by or involved in the function of this complex. Identification of potential specificity of HSP90 inhibition in the NF2-deficient tumors will be the next step of the study. CD44 is one of the few transcripts that responded to HSP90 inhibition with significant decrease both in vitro and in vivo. Merlin functions in contact inhibition of growth by inhibiting CD44 binding of its ligand hyaluronate (HA) (16, 41). On the other hand, CD44-HA interaction was shown to be essential for the activation of ErbB2 in cancerous cells, inhibition of which caused disassembly of a
constitutive, lipidraft-associated, signaling complex, containing phosphorylated-ErbB2, CD44, ezrin, PI3K, Hsp90 and cdc37(42). Hence, it is conceivable that merlin restoration and Hsp90 inhibition share a common molecular effect to suppress ErbB2 signaling complex in NF2−/− cells through disruption of CD44-HA interaction and complex assembly.

The present study also demonstrated that the HSP90 inhibition in NF2-deficient cells simultaneously increased the transcription of myelin sheath specific genes as well as the core transcription factors for Schwann cell differentiation, which are down-regulated in the human VS (43). Recently, the mTOR inhibitor rapamycin was shown to improve myelination of Schwann cells of neuropathic mice by increasing the expression of myelin specific genes (44). Loss of NF2 constitutively activates mTORC1 (45), whereas geldanamycin suppresses the mTOR-raptor signaling pathway (46), hence, the up-regulation of myelin specific genes by NXD30001 may be accomplished through inhibition of the mTOR signaling pathway. Property of HSP90 inhibitors to induce differentiation has been reported in different tumor cells: Ansamycin induced functional differentiation in ERBB2-positive breast cancer cells (47). Likewise, radicicol increased expression of erythroid differentiation marker in myelogenous leukemia cells (48). Our transcriptome data may facilitate the identification of common mechanisms shared in the HSP90 inhibition and differentiation of tumor cells of different origins.

Taken together, HSP90 inhibition demonstrated significant anti-tumor efficacy in NF2-deficient cells in vitro and in vivo. Our findings provide sufficient preclinical evidence for the benefit of the HSP90 inhibition against NF2-related tumors in human patients.
Acknowledgements

We thank Dr. Mark Schwartz for providing surgical human NF2 tumor samples. We are grateful to Drs. Rick Friedman and Jeffrey Ohmen for coordination in transcriptome analysis, and Ms. Benedicte Chareyre and Ms. Tyan Ly for technical support.

Grant support

This work was supported by a Drug Discovery Initiative award, Children’s Tumor Foundation, to Marco Giovannini, and by the House Research Institute.

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Figure legends

**Fig. 1.** A, Heatmap of HSP90 gene expression in the human schwannoma. Expression of HSP90AA and HSP90AB (each with four different probe sets) in NF2-related schwannoma (n=9) were compared to that in human Schwann cells of saphenous nerves (n=3). Of the 8 probe sets, three HSP90AA and one HSP90AB probes were statistically different from normal (shown in boxed, FDR = 0.005, 9479 probe sets total). B, NF2-deficient cell lines HEI193, ESC-FC1801, and 08031-9, and the mouse Schwann cell (mSC) primary culture were shown with cytosolic Schwann cell marker S100β and nuclear marker Hoechst 33258. C, The differential expression of Schwann cell markers (S100β, p75 Ngfr) in ESC-FC1801, and 08031-9. The relative mRNA level was expressed against normal sciatic nerve. D, Expression of receptor tyrosine kinases ErbBs and Pdgfrs in the human vestibular schwannoma (hVS) and in the NF2-deficient cell models. The relative mRNA level was expressed against normal nerve. Columns, mean; bars, SD.

**Fig. 2.** Anti-proliferative effect of NXD30001 in NF2-deficient Schwann/schwannoma cell lines. A, Dose response to NXD30001 was determined for NF2-deficient cells along with the mouse Schwann cell (mSC) primary culture. Cells were treated with NXD30001 for 72hrs and the number of viable cells was quantified. B, NF2-deficient cells were treated with 100nM and 1000nM NXD30001 in vitro and apoptotic cells were detected by fluorometric TUNEL assay. Percentage of apoptotic cells was quantified from 500–1000 cells. C, NF2-deficient cells were cultured as single cell suspension for 2–3 weeks in the soft agar media with or without NXD30001. Numbers of colonies (n=3 per group) were quantified relative to the vehicle-treated control. D, NF2-deficient cells were treated with various concentrations of NXD30001 for 24hrs and the dose dependent client protein degradation was displayed by Western blot analysis. Columns, mean; bars, SD.

**Fig. 3.** Antitumor efficacy and tolerability of NXD30001 in vivo NF2 models. A, Tumor-bearing mice were injected with NXD30001 at the days indicated with arrows, and the body weight and the tumor volume were monitored. ESC-FC1801 allograft tumors in female nude mice (n=10 per group), and 08031-9 allograft tumors in FVB/N syngenic mice (n=15 per group) were treated with 100mg/kg/day, 3d/week. B, Concentration of NXD30001 in the plasma (upper) and tumor (lower) was determined by LC/MS-MS. Left, measurement in the ESC-FC1801 tumors treated with 100mg/kg/day, 2-3d/week for 4 weeks and collected 1hr after the last injection. Right, measurement in the 08031-9 tumors treated with
a single dose of 100mg/kg and collected at indicated time (n=3 per group).  

C, Pharmacodynamic analysis of ESC-FC1801 tumors treated with 100mg/kg/day of NXD30001, 2-3d/week for 2 and 4 weeks, showing down-regulation of multiple HSP90 client proteins.  

D, The mitotic index of the grafted 08031-9 tumors was compared by the percentage of BrdU positive cells (arrows) scored from three independent sections per tumor.  

E, Morphometric analysis of Schwann cell tumorlets (red arrows) in NF2 transgenic mice.  

F, 5-week old transgenic mice were treated with 10mg/kg/day or 20mg/kg/day of 17-DMAG (n=4 per group) at the days indicated with arrows for 8 weeks, and their body weight was monitored.  

G, Number of microscopic fields and the total root area analyzed per group. Number of tumorlets per nerve root area (H), and the ratio of tumor area to root area (I), demonstrating reduced development of tumorlets in 17-DMAG treated mice. Points and columns, mean; bars, SD.

**Fig. 4.** Experimental validation of transcriptome analysis.  

A, Effects of NXD30001 on cell cycle in ESC-FC1801 cells treated with DMSO or 1μM NXD30001. DNA content was measured by flow-cytometry. An arrow indicates depletion of cells in S phase, and unstained cells (asterisk) correspond with apoptotic cells.  

B, DNA synthesis in ESC-FC1801 cells was shown by immunocytochemistry using anti-BrdU antibody.  

C, Vascular regression caused by NXD30001, 100mg/kg/day, 3 times/week for 4 weeks, in ESC-FC1801 and 08031-9 tumors. Immunohistochemical images and the mean number of PECAM positive blood vessels in the representative sections of the tumors sampled on day 14 (ESC-FC1801) and day 28 (08031-9) were shown.  

D, Up-regulation of myelin specific markers by NXD30001 in NF2-deficient cells. Quantitative PCR was performed on the total RNA isolated from the vehicle or drug-treated tumors (n=3~8) and cultured cells (n=3) of FC-1801 and 08031-9. The relative values were expressed against normal sciatic nerve.  

E, Western blot of myelin specific proteins in the ESF-FC1801 cells treated with NXD30001. Columns, mean; bars, SD.
Figure 2

A

Cell viability (% of control)

NXD30001 (nM)

- HEI193 (DMEM) GI_{50} = 14.4nM
- ESC-FC1801 (N2) GI_{50} = 1.8nM
- mSC primary (N2) GI_{50} = 19.1nM
- 08031-9 (PM) GI_{50} = 29.9nM
- mSC primary (PM) GI_{50} = 40.8nM

B

% of apoptotic cells

24hr 48hr 72hr

- HEI193
- mSC
- ESC-FC1801
- 08031-9

C

Control 3nM 10nM 30nM

ESC-FC1801 08031-9

HEI193

2mm

D

Number of colonies (% of control)

NXD30001 (nM)

- ESC-FC1801
- 08031-9
- HEI193

ErbB2 ErbB3 c-Met Axl p-PI3K PI3K c-Raf p-Akt Akt p-S6 S6 p-Erk1/2 Erk1/2 Cyclin D1 Cdk4 HSP90 HSP70 β-tubulin

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Figure 3

Author Manuscript Published OnlineFirst on May 28, 2013; DOI: 10.1158/1078-0432.CCR-12-3167
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

(A) ESC FC1801 allograft in nude mice
08031-9 allograft in FVB/N mice

(B) ESC-FC1801 tumors
1 hr after last dose
08031-9 tumors
single dose

(C) Vehicle 100mg/kg 100mg/kg
2 weeks 4 weeks
ErbB2

(c-Met)
Axl
p-Akt/Akt
Akt
p-Erk1/2
Erk1/2
Cdk4
Cyclin D1
Tubulin

(D) BrdU positive cells (%)

**p<0.005

(E) 1 field = 710 μm x 530 μm

(F) Vehicle
10mg/kg/day
20mg/kg/day

(G) Number of fields
Total root area (μm²)

(H) 17-DMAG 10mg/kg 17-DMAG 20mg/kg

(I) Tumor / root area ratio

* p<0.05
** p<0.01

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Figure 4

A

Control

G1

S

G2/M

24 hr

48 hr

72 hr

B

Control

NXD30001 (24hr)

C

Control

NXD30001

ESC-FC1801

08031-9

x200

D

ESC-FC1801

08031-9

Pmp22

**P<0.001

*P<0.05

**P<0.01

Mpz

***P<0.001

Mbp

*P<0.05

E

ESC-FC1801

PECAM-1 positives / field

Control

1nM

10nM

100nM

1μM

10 μM

Sciatic nerve

Control

NXD30001

08031-9

PECAM-1 positives / field

Control

NXD30001

*P<0.05
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<th>Primary Cells</th>
<th>GI50 (nM)</th>
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<th>Age</th>
<th>NF2 Gene mutation</th>
<th>Predicted effect</th>
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<td>HEI193 (Immortalized)</td>
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<td>NF2</td>
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<td>r.1575_1737del</td>
<td>Hemizygous loss of Ch 22</td>
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<td></td>
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<td></td>
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<td>p.Val525_Leu595del</td>
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<td>Vestibular schwannoma-1</td>
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<td>c.784C&gt;G</td>
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<td>Vestibular schwannoma-13</td>
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<td>Sporadic</td>
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<td>p.Arg160fs</td>
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<td>c.1445delCinsTGG</td>
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<td>Spinal meningioma-2</td>
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<td>NF2#</td>
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<td>c.1445delCinsTGG</td>
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<td>1050.0</td>
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<td>53</td>
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<td>39</td>
<td>c.1021C&gt;T</td>
<td>p.Arg341*</td>
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NOTE: Classification of sporadic or NF2-associated status was based on the diagnoses from referring physicians. Mutations are listed with the numbering of bases showing alterations relative to the cDNA sequence with the initiator ATG beginning at base=1. GI50 is determined for 72hr drug treatment. *, the tumors were two spinal cord tumors from the same patient. c., cDNA; r., mRNA; p., protein sequences; LOH, loss of heterozygosity; N.A., not analyzed.
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<th>Genes (fold change down)</th>
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<td>Motif family, Hoxb2, Hoxb3</td>
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Therapeutic Potential of HSP90 Inhibition for Neurofibromatosis type 2

Karo Tanaka, Ascia Eskin, Fabrice Chareyre, et al.

Clin Cancer Res Published OnlineFirst May 28, 2013.

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