Ketotifen Modulates Mast Cell Chemotaxis to Kit-Ligand, but Does Not Impact Mast Cell Numbers, Degranulation, or Tumor Behavior in Neurofibromas of Nf1-Deficient Mice

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Abstract

Neurofibromatosis Type 1 (NF1) is one of the most common genetic tumor predisposition syndromes in humans. Mutant NFI results in dysregulated RAS allowing neoplasms throughout the neuroaxis. Plexiform neurofibromas (pNF) afflict up to 50% of patients with NF1. They are complex tumors of the peripheral nerve that cause major morbidity via nerve dysregulation and mortality via conversion to malignant sarcoma. Genetically engineered mouse models (GEMM) of NF1 provide valuable insights for the identification of therapies that have utility in people with pNF. Preclinical studies in GEMMs implicate mast cells and the c-Kit/Kit ligand pathway in pNF tumorigenesis. Kit ligand is a potent chemokine secreted by tumorigenic, NFI-deficient Schwann cells. Ketotifen is an FDA-approved drug for the treatment of allergic conjunctivitis and asthma that promotes mast cell stabilization and has been used in prior case studies to treat or prevent pNFs. This study investigated the effect of ketotifen on mast cell infiltration and degranulation in the presence and absence of Kit ligand provocation and the effect of ketotifen on shrinking or preventing pNF formation in the Nf1fl/fl;NemoCre” GEMM. Ketotifen decreased mast cell infiltration in response to exogenous Kit ligand administration, but did not affect mast cell degranulation. Importantly, ketotifen did not reduce mast cell numbers or activity in pNF and did not prevent pNF formation or decrease the volume of established pNF despite administration of pharmacologically active doses. These findings suggest that ketotifen has limited use as monotherapy to prevent or reduce pNF burden in the setting of NF1 mutations.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant cancer predisposition syndrome with a prevalence of 1 in 2,600 to 3,000 across all populations of the world (1, 2). NF1 syndrome is caused by mutations of the NF1 tumor-suppressor gene encoding the protein neurofibromin, which functions as a GTPase-activating protein for p21 Ras. Loss of both alleles of Nf1 in the tumor-initiating cells results in constitutive activation of Ras and its downstream effectors, ultimately leading to tumor formation. NFI is the murine homologue of the human NF1. It is highly conserved both structurally and in biochemical and cellular function across species.

Plexiform neurofibromas (pNF) are a hallmark manifestation of NF1. These multicellular tumors arise from peripheral nerves and afflict approximately 50% of patients with NF1 (3, 4). pNFs frequently result in debilitating disfigurement, organ dysfunction (3–6), neurologic dysfunction (7, 8), chronic pain (9), and have a propensity for malignant conversion (1, 2, 10). Furthermore, pNFs are resistant to many targeted and standard chemotherapies and, due to their intercalation within nerves, cannot be completely removed without sacrificing functioning nerve (11–14). pNF consist of a mixture of Schwann cells, fibroblasts, endothelial cells, and inflammatory mast cells (15–19). NF1 (NFI)-deficient Schwann cells are the established tumorigenic cell of origin for pNF formation (16, 19). However, detailed serial dissection studies show that mast cells are the first microenvironment cell drawn to the tumorigenic nidus (15, 17, 18, 20). Furthermore, genetic and adoptive transfer studies in genetically engineered mice show that tumorigenic initiation is dependent on c-Kit/Kit ligand, the key chemokine and growth factor for all...
aspects of mast cell development and function (15, 17, 18). Nf1-deficient Schwann cells secrete pathologically elevated levels of Kit ligand (17, 20, 21). Thus, through Nf1-deficient Schwann cell-mediated Kit ligand secretion, mast cells are recruited to the site of the tumorigenic nidus where they release multiple cytokines and matrix metalloproteinases that promote neoangiogenesis, fibroblast recruitment, and collagen deposition (15, 17, 18, 20–23).

Our group has shown that targeting c-Kit/Kit ligand with imatinib mesylate leads to significant reduction in pNF size in preclinical models of pNF and that imatinib had similar activity in humans; particularly young children with Nf1-associated pNF (18, 22, 24).

Ketotifen is a mast cell stabilizer and noncompetitive H1-antihistamine approved for the treatment of asthma and allergic conjunctivitis that is well tolerated and inexpensive. Small, nonrandomized human trials dating to the 1980s have evaluated ketotifen in people with Nf1 based on the rationale of mast cell stabilization (25–27). These studies reported subjective activity of ketotifen with the largest study being a combination of a controlled study and an observation study of 52 patients given 2 to 4 mg ketotifen per day for up to 78 months and the endpoint being patient reports of benefit based on recall (26). No formal measurements of tumor growth or tumor reduction were obtained in these studies (25–27). Although the data reported are limited in nature, they suggest that pharmacologic mast cell stabilization with ketotifen may have therapeutic benefit for people with Nf1-associated neurofibromas. Given the safety profile of ketotifen in patients treated for asthma, validation of its efficacy in tumor reduction or prevention is of great value to the Nf1 patient community.

A limitation in the use of effective therapies for many pediatric orphan disease cancers is the availability of adequate patient populations to formally test drug efficacy. The development of genetically engineered mouse models (GEMM) that closely recapitulate the developmental pathogenesis of human tumors provides an opportunity to test therapies in preclinical models in a way that was not available previously (28). There are now multiple instances where preclinical studies in Nf1 GEMMs have led to the identification of drugs that are active in patients (24, 28, 29).

Importantly, studying GEMMs provides the opportunity to critically examine the mechanism of action of small molecules and ensure adequate pharmacokinetics (PK) are achieved before assessing efficacy endpoints.

The aim of this study was to investigate the efficacy and mechanism of action of ketotifen in the treatment of Nf1-associated pNF. The primary objective was to determine whether ketotifen monotherapy is sufficient to reduce or prevent pNF formation in a highly validated GEMM of pNF (18, 24). In addition, given ketotifen’s hypothesized role in ameliorating or preventing pNF formation via stabilization of the mast cell, we conducted a series of experiments to test whether ketotifen effectively prevents mast cell recruitment and degranulation in response to Kit ligand stimulation in this model (30, 31).

Methods and Materials

Study approval

The animal protocol #10932 was approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine and all studies were carried out accordingly.

Animals, kit ligand, and ketotifen administration

The Nf1fl/fl;PostnCre+ mice were developed and bred by the Clapp Laboratory utilizing tools reported previously (18). Tumor formation is driven by the early embryonic conditional deletion of Nf1 in Schwann cell progenitors via the Periostin (Postn) promoter and Cre recombinase enzyme (32, 33). Nf1fl/fl;PostnCre+ mice develop neurofibromas with complete penetrance that are measurable at roughly four months of age. Ketotifen fumarate was purchased from Sigma-Aldrich. The chemical structure of ketotifen fumarate is available in PubChem, PubChem CID: 5282408 (34). In the prevention and intervention therapeutic studies, ketotifen 1 mg/mL was prepared in ddH2O and administered via oral gavage at 10 mg/kg once daily seven days/week. Vehicle-treated Nf1fl/fl;PostnCre+ mice were administered 0.1 mg/d water via oral gavage. The control and treatment cohorts of mice were weighed daily to determine the safety and tolerability of ketotifen throughout the duration of the treatment period. Following 12 weeks of treatment, mice in the prevention study (water/vehicle-treated n = 6, ketotifen-treated n = 6) were sacrificed at four months of age (Fig. 1A) and mice in the tumor treatment study (water/vehicle-treated n = 15, ketotifen-treated n = 14) were sacrificed at seven months of age (Fig. 2A).

To determine whether ketotifen had an in vivo effect on mast cell infiltration and degranulation, 4-month-old Nf1fl/fl mice were administered subcutaneous Kit Ligand as previously described (35). Briefly, Nf1fl/fl mice were pre-treated with 10 mg/kg/d of ketotifen or 10 mg/kg/d of water administered via oral gavage from days 0 to 7. On day 7, a micro-osmotic pump was implanted and the mice received a continuous infusion of 20 μg/kg/d Kit ligand or control PBS, as previously described (35). Oral ketotifen treatment or water was continued throughout days 7–14. On day 14, skin biopsies near the site of the micro-osmotic pump were harvested and stained with toluidine blue for quantification of mast cell infiltration and degranulation (Fig. 3A).

Quantification of ketotifen

Ketotifen was quantified in plasma and nerve tissue samples from ketotifen-treated Nf1fl/fl;PostnCre+ mice (n = 3) by HPLC-MS/MS (Agilent 1200 HPLC and ABI 3200 MS/MS; ref. 36). The animals received a single dose of 10 mg/kg ketotifen. Plasma samples were obtained at 1, 2, 4, 8, and 24 hours post-ketotifen dose. Tissue samples from sciatic, brachial, and trigeminal nerves were obtained at four and 24 hours post-ketotifen dose. A method to quantify ketotifen in plasma was developed using temazepam as the internal standard, liquid–liquid extraction, and HPLC-MS/MS. Variability was minimized in the method by using methyl tert-butyl ether instead of ethyl acetate, dichloroethane, or hexane-ethyl acetate as the solvent and polypropylene tubes instead of glass tubes. The mobile phase uses formic acid instead of ammonium acetate. The lower limit of quantification is one ng/mL using 20 μL of plasma. A set of eight standards were run for each batch of plasma or tissue samples.

Nerve/tumor volume quantitation

Following microscopic dissection of the spinal proximal nerves, nerve volume was measured by calipers and calculated by the established approximation for the volume of a spheroid, 0.52 × (width)2 × length. Four proximal nerves per mouse were measured as previously described (18).
Histology and immunohistochemistry
To examine tumor morphology and the extent of mast cell infiltration, spinal proximal nerves, and peripheral nerves were dissected from 4 to 7 month old $N_f1^{fl/ox}$, $PostnCre^+$ mice, fixed in 10% formalin, processed through graded alcohols, xylenes, and molten paraffin, embedded in paraffin, and subsequently sectioned and stained with hematoxylin and eosin (H&E), toluidine blue (to identify mast cells) and Masson’s trichrome stain for collagen and tumor quantification.

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**Figure 1.** Ketotifen treatment fails to prevent neurofibroma genesis in NF1-deficient pups. 

**A.** Schematic of experimental design of tumor prevention study. 

**B.** Proximal nerve volume in vehicle and ketotifen-treated $N_f1^{fl/ox}$, $PostnCre^+$ mice ($P = 0.9012, \text{ns}$). 

**C.** Histological analysis of toluidine blue-stained nerve tissue from water/vehicle- and ketotifen-treated $N_f1^{fl/ox}$, $PostnCre^+$ mice. Images were obtained using a x40 objective lens. Black arrows point to representative infiltrating mast cells. Red arrows point to representative degranulating mast cells. 

**D.** Quantitation of mast cell infiltration in water/vehicle- and ketotifen-treated $N_f1^{fl/ox}$, $PostnCre^+$ mice ($P = 0.8295, \text{ns}$). 

**E.** Quantitation of the percentage of degranulating mast cells in water/vehicle- and ketotifen-treated $N_f1^{fl/ox}$, $PostnCre^+$ mice ($P = 0.5495, \text{ns}$).
Figure 2.
Ketotifen fails to inhibit established neurofibroma progression in Nf1-deficient mice. A, Schematic of experimental design of established tumor regression study. B, Proximal nerve root volume in water/vehicle and ketotifen-treated Nf1lox/lox;PostnCre+ mice (P > 0.05, ns) and in WT Nf1lox/lox;PostnCre- mice (water/vehicle-treated vs. WT ****, P < 0.0001; ketotifen-treated vs. WT ***, P < 0.0001). C, Quantitation of tumors in water/vehicle and ketotifen-treated Nf1lox/lox;PostnCre+ mice (P > 0.05, ns and in WT Nf1lox/lox;PostnCre- mice (water/vehicle-treated vs. WT ***, P = 0.0010; ketotifen-treated vs. WT ***, P = 0.0004). D, Histological analysis of toluidine blue-stained tumor tissue from water/vehicle- and ketotifen-treated Nf1lox/lox;PostnCre+ mice. Images were obtained using a ×40 objective lens. Black arrows point to representative infiltrating mast cells. Red arrows point to representative degranulating mast cells. E, Quantitation of mast cell infiltration in water/vehicle- and ketotifen-treated Nf1lox/lox;PostnCre+ mice (P = 0.0821, ns). F, Quantitation of the percentage of degranulating mast cells in water/vehicle and ketotifen-treated Nf1lox/lox;PostnCre+ mice (P = 0.5686, ns).
Figure 3.
Effect of Ketotifen on Kit-mediated mast cell infiltration and degranulation in vivo. A, Schematic of experimental design in ketotifen-pretreated/treated Nf1−/− mice with continuous Kit ligand or PBS subcutaneous infusion. B, Histological analysis demonstrating toluidine blue-stained intact versus degranulating mast cells in the skin following a 7 day Kit or PBS infusion. Image obtained using a ×100 objective lens with oil. C, Histological analysis of toluidine blue-stained skin biopsy of 20 μg/kg/d Kit ligand infusion-mediated and PBS infusion-mediated mast cell infiltration in ketotifen-treated and water/vehicle-treated 4-month-old Nf1−/− mice. Images were obtained using a ×40 objective lens. Black arrows point to representative infiltrating mast cells. Red arrows point to representative degranulating mast cells. D, Quantification of toluidine blue-stained skin biopsy of 20 μg/kg/d Kit ligand infusion-mediated and PBS infusion-mediated mast cell infiltration per high-powered field in ketotifen-treated and water vehicle 4-month-old Nf1−/− mice (for PBS pump vs. Kit ligand pump, ***P < 0.0001; for PBS pump vs. ketotifen-treated PBS pump, P > 0.05, ns; for Kit ligand pump vs. ketotifen-treated Kit ligand pump, ***, P < 0.0001). E, Quantitation of toluidine blue-stained skin biopsy of 20 μg/kg/d Kit ligand infusion-mediated and PBS infusion-mediated absolute number degranulating mast cells per high-powered field (for PBS pump vs. Kit ligand pump, P > 0.05, ns; for PBS pump vs. ketotifen-treated PBS pump, P > 0.05, ns; for Kit ligand pump vs. ketotifen-treated Kit ligand pump, P > 0.05, ns).
Data Analysis

Statistical methods

GraphPad Prism 5.0 and 6.0 was used to perform all statistical analyses. Comparison of the means for nerve root volume, tumor number, Ki-67 proliferation index, number of infiltrating mast cells, number of degranulating mast cells, and percentage of degranulating mast cells was performed using the two-tailed, unpaired Student t test and one-way analysis of variance (ANOVA) with Tukey’s test post-hoc analysis. P values of < 0.05 were considered statistically significant for all tests.

Pharmacokinetic data analysis

PK parameters for ketotifen, including AUC and t½, were estimated using noncompartmental methods with add-ins in Excel. The maximum plasma concentration (Cmax) was obtained from the data. The AUC from zero to infinity (AUC∞) was estimated from the AUC0-t (time zero to the last quantifiable concentration Clast) and the AUC from Clast to infinity, Clast/t½, where t½ is the rate constant of elimination.

Image Analysis

Toluidine blue is a histological stain specific for mast cells. To avoid user bias, toluidine blue-stained histological images were scanned in on a Leica ScanScope and HALO software v2.0. 0.1038 was used for image analysis in scoring total and degranulating mast cells. The Cytonuclear algorithm was used to quantify mast cells in the skin. Using a toluidine blue stain, the Cytonuclear algorithm uses a dark blue positive stain (0.992, 0.65558, 0.219 RGB OD) against a light blue (1.176, 1.260, 0.621 RGB OD) background. The intensity threshold was narrowed from the standard immunostain algorithm of 0.112, 0.287, 0.445 (weak, moderate, strong) to 0.300, 0.340, 0.355 (weak, moderate, strong). Two sets of analyses were run, only altering the minimum nuclear roundness parameter to 0.355 (weak, moderate, strong). Two sets of analyses were run, narrowing from the standard immunostain algorithm of 0.112, 0.287, 0.445 (weak, moderate, strong) to 0.300, 0.340, 0.355 (weak, moderate, strong) to 0.300, 0.340, 0.355 (weak, moderate, strong). Two sets of analyses were run, only altering the minimum nuclear roundness parameter to 0.355 (weak, moderate, strong). Two sets of analyses were run, narrowing from the standard immunostain algorithm of 0.112, 0.287, 0.445 (weak, moderate, strong) to 0.300, 0.340, 0.355 (weak, moderate, strong) to 0.300, 0.340, 0.355 (weak, moderate, strong).

Results

Ketotifen does not prevent neurofibroma genesis in Nf1fl/fl, PostnCre mice

A major goal for genetic tumor predisposition syndromes like NF1 is the prevention of tumor formation. Given the high therapeutic index of ketotifen and the mechanistic link between mast cells, c-Kit/Kit ligand, and pNF formation we hypothesized ketotifen may prevent or alter the latency of tumorigenesis. Nf1fl/fl, PostnCre mice begin to acquire Schwann cell hyperplasia at approximately 4 to 6 weeks of age before the formation of multiple pNFs. To test our hypothesis, we used the experimental design outlined in Fig. 1A. Nf1fl/fl, PostnCre mice (n = 6) were treated with 10 mg/kg of ketotifen from 5 to 6 weeks post-natal, before the genesis of pNFs, until 4 months of age when they were sacrificed. At necropsy, there was no difference in nerve hyperplasia between the treatment and water/vehicle-treated Nf1fl/fl, PostnCre mice (Fig. 1B, ns). Furthermore, microscopic evaluation revealed that ketotifen treatment did not reduce the number of mast cells infiltrating nerve tissue (Fig. 1C and D, ns) nor did it reduce the percentage of degranulating mast cells (Fig. 1C and E, ns).

Ketotifen does not reduce proximal nerve root volume or tumor number in Nf1fl/fl, PostnCre mice

Nf1fl/fl, PostnCre mice uniformly acquire multiple pNFs by 4 months of age. To model the treatment of human pNF-harboring NF1, we followed the experimental design outlined in Fig. 2A. Nf1fl/fl, PostnCre mice were treated from 4 to 7 months of age to assess the impact of ketotifen on established tumors. There was no statistical difference in proximal nerve root volume (Fig. 2B, ns) or tumor number (Fig. 2C, ns) between ketotifen (n = 14) and water/vehicle-treated (n = 14) Nf1fl/fl, PostnCre groups. Tumor number ranged from 0-22 in water/vehicle-treated Nf1fl/fl, PostnCre mice and 0-20 in the ketotifen-treated Nf1fl/fl, PostnCre mice at 7 months. Both ketotifen and water/vehicle-treated Nf1fl/fl, PostnCre mice demonstrated a significantly greater proximal nerve root volume compared with historic age, sex and strain-related Nf1fl/fl, PostnCre WT mice that did not develop tumors (Fig. 2B and C). Furthermore, microscopic evidence revealed no significant difference in the quantity of mast cells infiltrating peripheral nerve tumor tissue in ketotifen-treated mice compared with mice receiving water vehicle (Fig. 2D and E). Finally, ketotifen treatment failed to reduce the percentage of degranulating mast cells when compared with water/vehicle-treated mice (Fig. 2D and F).

Effect of Kit ligand and ketotifen, alone or in combination, on mast cell infiltration and degranulation in skin

Prior work in genetically engineered mice established that the c-Kit/Kit ligand pathway is central in pNF tumor initiation (18, 20–22). This work has been replicated using a variety of Cre drivers with similar results. Given that c-Kit impacts the development, migration, proliferation, and degranulation of mast cells in vitro and in vivo we assessed the impact of ketotifen on preventing mast cell infiltration and degranulation following a Kit ligand infusion using an established protocol (17, 20, 35–38). Consistent with the previous studies, Kit ligand infusion revealed a statistically significant increase in mast cell infiltration (Fig. 3C and D, P < 0.0001). Kit ligand infusion had no impact on the percentage of degranulating mast cells relative to PBS infusion (Fig. 3E, P > 0.05). We then evaluated the effect of ketotifen on mast cell infiltration and degranulation in the presence and absence of Kit ligand infusion. In mice receiving Kit ligand infusion, ketotifen treatment decreased the number of infiltrating mast cells when compared with water treatment (Fig. 3D, P < 0.0001). However, ketotifen treatment failed to induce a significant decrease in the percentage of degranulating mast cells (Fig. 3E, ns) in mice receiving the Kit ligand infusion. These results indicate that ketotifen is not effective in inhibiting all Kit ligand-mediated mast cell functions in Nf1fl/fl, PostnCre mice, including the inhibition of Kit-mediated mast cell degranulation, at least at pharmacologic concentrations used in these experiments.

Pharmacokinetics of ketotifen in Nf1-deficient mice

After a single dose of ketotifen at 10 mg/kg in Nf1fl/fl, PostnCre tumor-bearing mice, the maximum plasma concentration was 40 ng/mL, AUC∞, 119 ng/mL-h, and t½, 29.9 hours.
These data are slightly greater than the values estimated in humans (36). The concentration of ketotifen in sciatic, brachial, and trigeminal nerve tissue was also measured at 4 and 24 hours after a single dose of ketotifen 10 mg/kg (n = 6) to confirm that the drug reached the site of tumor initiation. At 4 hours, the mean concentration of ketotifen in all nerves measured was 14.56 ng/g of nerve tissue (Fig. 4B), whereas at 24 hours the concentration of ketotifen in all nerve tissue samples was below the level of quantification.

Safety and tolerability

The tolerability of ketotifen in Nf1fl/fl;PostnCre+ mice was measured with daily weights throughout the 12-week treatment period of established tumors. There was no significant difference between the vehicle-treated (n = 15) versus ketotifen-treated (n = 14) cohorts in weight.

Discussion

Neurofibromas are the hallmark tumor of the NF1 syndrome. pNFs start very early in life (many are thought to be congenital) and grow rapidly through early childhood resulting in nerve sheath tumors that cause pain, neurologic dysfunction, disfigurement and roughly 10% of the time convert to the highly aggressive sarcoma, malignant peripheral nerve sheath tumor (1–3, 5–10). Given their prevalence and the lack of effective therapies to prevent or reduce the impact of these tumors, major efforts from multiple laboratories are being pursued to reduce the size and morbidity associated with these tumors (28). The drugs being used currently in preclinical models and in clinical trials are overwhelmingly small-molecule targeted therapies (28). Although some of these therapies show significant promise, there are common, off-target adverse effects that limit their use. For example, though overall largely well-tolerated, both imatinib mesylate and selumetinib caused a variety of symptoms, including gastrointestinal reactions, edema, muscle cramps, fatigue and acneiform rash that limit their long-term tolerability (24, 29, 39, 40). Furthermore, the cost of these novel drugs could be substantial and long-term safety is unknown. Thus, the hypothesis that a drug like ketotifen that has a high therapeutic index, is low cost and shown to be safe for long-term use with a hint of clinical activity in prior studies provided the rationale for formal testing in a preclinical model of NF1 pNF.

Specifically, the clinical studies using ketotifen in patients with NF1 conducted in the 1980s and 1990s preceded the current
understanding of the cellular and molecular pathways underlying the genesis of pNFs and hence, mechanism was not examined. In addition, the prior clinical studies with ketotifen used efficacy evaluations that are outside of the recommended endpoints for pNF in the modern era (41–43). Hence, the community is left with reports of symptomatic improvement with non-validated measures and an interesting hypothesis about the biologic effect of ketotifen on pNF without evidence of mechanism. Given this conundrum, we conducted a formal preclinical study to evaluate the PK, mechanism of action and efficacy of ketotifen in a manner similar to preclinical studies pursued with imatinib mesylate and selumetinib before clinical use (17, 29).

We found that maximum drug concentrations in both plasma (40 ng/ml) and target tissue sites (14.56 ng/g), including sciatic, brachial, and trigeminal nerve with 10 mg/kg ketotifen were significantly greater than the levels achieved in published human plasma PK studies (2 mg/kg, Cmax 449 pg/mL) even after adjusting for the dose administered, and had t1/2 (29.9 hours) significantly longer than that previously reported in the plasma of human patients (4.4 hours; Fig. 4A and B; ref. 36). There is no published human tissue PK for ketotifen. Thus, we conclude that both systemic and tissue-specific drug concentrations were sufficient to achieve biologic effects.

Regrettably, we found ketotifen did not alter tumor burden when treatment was started after tumor formation in Nf1+/-;PostnCre<sup>+</sup> mice (Fig. 2B and C). This is consistent with case series reporting that ketotifen had no effect on established tumors in adults with NF1 (44). Nf1 is characterized by rapid growth of pNFs beginning in infancy and throughout childhood (8, 45, 46). Therefore, the identification of a drug that prevents early tumor initiation and progression would have great therapeutic value. On the basis of prior unpublished studies in our laboratory, we have demonstrated that Nf1+/-;PostnCre<sup>+</sup> mice acquire Schwann cell hyperplasia at approximately 4 to 6 weeks of age and acquire multiple pNFs by four months of age. In order to model a pNF prevention therapeutic strategy in mice, Nf1<sup>lox/lox</sup>;PostnCre<sup>+</sup> mice were treated from 5 or 6 weeks of age until four months of age with ketotifen (Fig. 1A). Again, at the conclusion of the 12-week treatment period, there was no difference in proximal nerve root volume or mast cell infiltration between vehicle and ketotifen-treated cohorts of Nf1<sup>lox/lox</sup>;PostnCre<sup>+</sup> mice (Fig. 1B–E).

The interaction between Schwann cells, the established tumorigenic cell in pNF, and the tumor microenvironment has been demonstrated in multiple GEMMs (17, 18, 20, 21). Kit ligand is one key mediator of tumor initiation and progression (17, 20–22, 24). The exact mechanism by which c-Kit<sup>+</sup> positive cells, such as mast cells, function to induce tumor development in response to Kit stimulation remains incompletely understood. However, prior work by our laboratory established that Kit ligand secreted at pathologically elevated levels by Nf1-deficient Schwann cells promotes multiple aspects of mast cell development and function, including migration, proliferation, survival, degranulation, and secretion of de novo synthesized cytokines independent of pre-stored granules (17, 20–22, 24). The pre-stored and de novo synthesized biologically active products released by mast cells (matrix metalloproteinases, angiogenin, bFGF, MCP-1, VEGF and IL-8) play essential roles in tumor biology by promoting angiogenesis, monocyte recruitment, and tissue remodeling (38, 47). The present studies demonstrate that ketotifen fails to exert a meaningful impact in vivo on mast cell infiltration, degranulation, or on tumor development in both established tumors and in a prevention model. Interestingly, a recent genetic study demonstrated that significant declines in Kit ligand and mast cell numbers in existing pNFs have minimal impact on tumor growth (48). It is possible that other infiltrating immune lineages, including macrophages, which are abundant constituents of the pNF microenvironment may play a critical role in tumor progression. This concept warrants further investigation. Though we cannot exclude the potential of species-specific differences in pharmacologic activity, there has been high fidelity of this preclinical model and subsequent clinical trials using other targeted therapeutics, including with the modulation of the c-Kit pathway (18, 24, 28). Collectively, our data suggest that ketotifen fails to modulate mast cell infiltration or degranulation in pNF and is insufficient as monotherapy for the treatment and prevention of these tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.A. Burks, S.D. Rhodes, W.K. Bessler, S. Chen, A. Smith, J.R. Gehlhausen, M. Jacobsen, G.E. Sandusky, D.R. Jones, J.O. Blakeley

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