Inhibitory Effect of Pirfenidone on Glioblastoma Cell Lines: Implications for Treatment of Neurofibromatosis

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder characterized by diverse cutaneous, neurological, skeletal, and neoplastic manifestations with no standard drug treatment options available. Pirfenidone is an oral agent undergoing clinical investigation in NF1, where its antifibrotic properties are used to target the fibrotic component of plexiform neurofibromas. The effect of pirfenidone on other cells in these complex tumors is unknown. We studied the effects of pirfenidone on malignant glioma cell lines to take advantage of easily cultured and rapidly growing cells with similar biologic properties to NF1-derived tumors. On a standardized MTS assay, pirfenidone showed dose-dependent inhibition of cell proliferation. Using a tritiated thymidine assay, dose-dependent DNA synthesis inhibition was seen as early as 2 hours after treatment. Gelatin and casein zymography showed inhibition of matrix metallopeptidase 2 (MMP-2) activity at a time point when no inhibition of proliferation was noted. Western blotting with an antibody to epidermal growth factor receptor (EGFR) phosphotyrosine residues documented dose-dependent inhibition of EGFR

phosphorylation. An 8-hour measurement of apoptosis using propidium iodide flow cytometry revealed a large sub-G_0 population of cells upon treatment of cells with 10 mg/mL of pirfenidone. These results suggest that pirfenidone has antitumor effects on malignant gliomas, which is partly mediated through inhibition of EGFR phosphorylation.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disorder with an incidence of 1 in 3000. The NF1 gene is a tumor suppressor located on chromosome 17q11.2. It encodes neurofibromin, a negative regulator of the Ras oncogene, the inactivation of which leads to cell proliferation and tumor development.[1,2]

NF1 is characterized by diverse progressive cutaneous, neurological, skeletal, and neoplastic manifestations. Patients with NF1 have an increased risk of developing tumors of the central and peripheral nervous system including plexiform neurofibromas (27%), optic gliomas (15-20%), pheochromocytomas (1%), malignant peripheral nerve sheath tumors (5%), and neurofibrosarcomas (6%).[3,4] They are a major source of morbidity, causing severe pain, impairment of nerve function, and, in some cases, development of malignant peripheral nerve sheath tumors. Although less frequent than peripheral nervous system tumors central nervous system (CNS) tumors are important because they may lead to major morbidity and mortality, despite the fact that most of them are grade I pilocytic astrocytomas.[5-7]

Optic pathway[8] and brainstem[9] gliomas are the most prevalent CNS tumors in NF1. Even though most studies in children show that these tumors are less aggressive than their counterparts in non-NF1 patients,[10-13] their behavior is unpredictable, and they often have a progressive course, causing debilitating neurological consequences. Although they are considered typical for the pediatric age group, it has been shown that brainstem gliomas could lead to increased mortality in NF1 adults.[14,15] There is no currently approved effective drug therapy or radiotherapy for most neurofibromatosis-associated tumors, and surgical options are limited to select patients with resectable tumors.

Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone) is a new broad-spectrum antifibrotic drug that has been shown to inhibit fibroblast growth and collagen synthesis, reportedly through inhibition of transforming growth factor-β (TGF-β). Pirfenidone modulates the action of other cytokines, including platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), intercellular adhesion molecule-1 (ICAM-1),[16] and $TGF\alpha_1$-induced fibronectin synthesis.[17]

Antiproliferative properties have been noted in vitro in fibroblasts[18] and leiomyoma/myometrial cells[19] and in vivo in vascular smooth muscle.[20,21] Anti-invasive properties have been noted in vitro in fibroblast[18] and in vivo in vascular smooth muscle.[20] Antifibrotic effects are well-documented in vitro and in vivo, indicating the therapeutic potential in many fibrosing conditions.[22-27]

Pirfenidone showed anti-fibrotic effects and therapeutic potential in treatment of pulmonary fibrosis[28] as well as peritoneal sclerosis and desmoid tumors.[29] We recently showed that oral pirfenidone significantly inhibits survival of human neurofibroma xenografts in severe combined immunodeficiency disease (SCID) mice.[30] Pirfenidone is currently being used in clinical trials in patients with NF1 and plexiform neurofibromas, where its antifibrotic activity is aimed at the fibrotic component of these tumors. However, the main proliferative potential in neurofibromatosis-

related tumors is unknown, and the role of other cells in these complex tumors cannot be ignored. Explanted neurofibromas frequently have a neural component overgrown by the fibroblasts, such that they are very difficult to study. Therefore, we chose to evaluate the effect of pirfenidone on the neural component of neurofibromatosis-related tumors using glioma cell lines in vitro. In addition, we wanted to explore the potential for the use of pirfenidone in the treatment of progressive brainstem and optic glioma in patients with NF1.

MATERIALS AND METHODS

Reagents and Cell Culture

The glioma cell lines used include SKMG3 (overexpressing EGFR), U118 and U87 (not overexpressing EGFR), HS463 (mutant p53), and D37 (wild type p53). The non-glioma cell lines used included two cell lines known to overexpress EGFR: A431 (squamous cell carcinoma) and MDA468 (breast cancer). All cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco modified Eagle medium (DMEM; Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 nM glutamine, and 1% penicillin/streptomycin, and maintained at 37˚C in a humidified incubator with 5% CO_2. Cells were grown to subconfluent monolayers, washed with phosphate-buffered saline (PBS), incubated with serum-free DMEM for 24 hours, pulsed with drug and/or EGF, and then harvested using a cell lifter at various time points.

Stimulation with EGF and Pirfenidone

Pirfenidone was provided by Marnac, Inc. (Dallas, TX). The stock solution of pirfenidone was prepared in 100% acetone (100 mg/mL) and stored at -20˚C. The drug was diluted in distilled water immediately before treatment. Cells were stimulated with 10 ng/mL EGF (Upstate Biotechnology, Lake Placid, NY) 15 minutes after incubation with pirfenidone.

MTS Assay

A standardized MTS assay (CellTiter $96^{R}AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay, Promega Corporation, Madison WI) was employed to determine cell viability. Cells in exponential growth were harvested and plated in 96-well plates (2000 cells/well in 100 µL of standard growth medium). Each treatment condition was tested in six replicate wells. Cells were incubated overnight, serum starved for 24 hours, and then graded concentrations of pirfenidone were added to the wells in 20 µL of media. Cells were incubated at 37˚C for 72 hours and then processed for the MTS assay according to the manufacturer's instructions. After incubation of cells with the MTS reagent for 2 hours, absorbance at 490 nM was measured in a spectrophotometer.

[^{3}H]-THYMIDINE ASSAY

Western Blot Analysis

For tritiated thymidine incorporation assays, tumor cells were cultured in 96-well plates (1000 cells/plate) for 48 hours in 10% serum-enriched media. Cells were then serum starved for 48 hours. These quiescent cells were washed and then treated with various doses of pirfenidone (0.01, 0.1, 1.0 and 10 mg/mL). After 2, 6, and 12 hours, the cells received 0.2 µCi/well [^{3}H]-thymidine (New England Nuclear, Boston, MA), and the incubation was continued for a further 50 minutes. Subsequently, cells were harvested on glass fiber paper (FilterMAT, Skatron Instruments, Sterling, VA), using an automatic cell harvester (Semi-12 Cell Harvester, Skatron Instruments, Sterling, VA). The incorporated radioactivity was measured with a liquid scintillation counter (LS6500, Beckman Coulter, Fullerton,

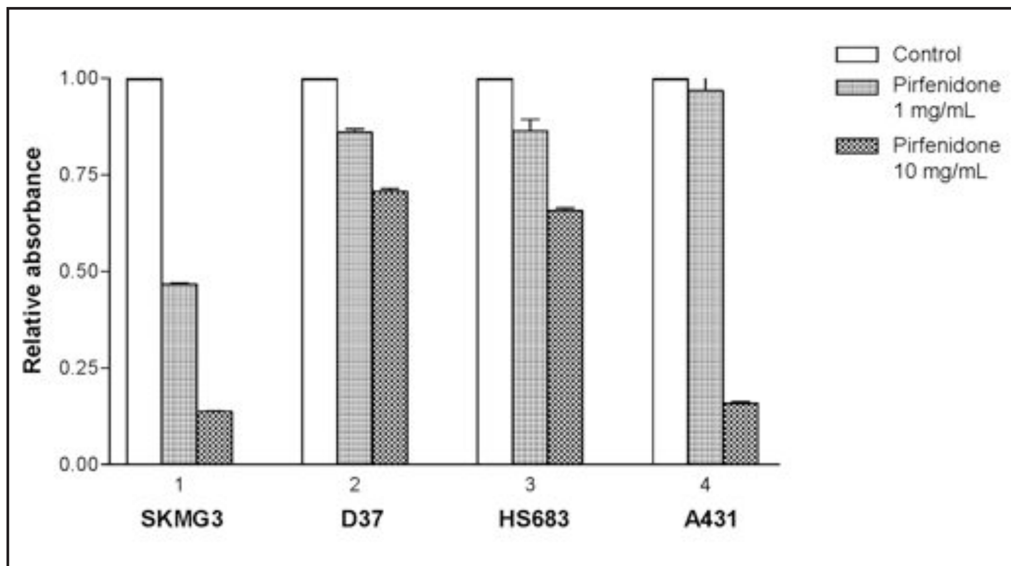

Figure 1. Dose-dependent effect of pirfenidone on cell proliferation in a panel of different glioblastoma cell lines. Serum-starved cells were treated with pirfenidone (1 mg/mL and 10 mg/mL). A standardized MTS assay was employed to determine cell proliferation in response to varying concentrations of pirfenidone: SKMG3 (cell line 1), D37 (cell line 2), and HS683 (cell line 3). The antiproliferative effect was independent of p53 mutation status of the cell lines. The non-glioblastoma cell line (A431; squamous cell carcinoma, cell line 4), overexpressing EGFR, also showed sensitivity to pirfenidone.

CA). All experiments were done in triplicate.

For the preparation of whole cell lysates, 2 minutes after EGF stimulation, cells were washed with PBS and harvested in protein extraction buffer [20% sodium dodecyl sulfate (SDS), pH 7.4 tris, 2-β-mercaptoethanol] using a cell lifter, centrifuged at 1200 x *g*, and the supernatant was used for Western blot analyses. Both adherent and detached cells were collected and were subjected to lysis in the lysis buffer (25 mM Tris–HCl pH 7.6, 50 mM NaCl, 2% Nonidet P-40, 0.5% deoxycholate, 0.2% SDS). After determination of protein concentrations, equal amounts of cell lysate were separated by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blotted first with a primary antibody against phospho EGFR (Upstate Biotechnology, Lake Placid, NY) and subsequently with an appropriate horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology, Rockford, IL). Blots were visualized by autoradiography (CL-X Posurefilm, Pierce Biotechnology, Rockford, IL).
Western blotting for phospho-EGFR, Bax (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3 (Cell Signaling, Beverly, MA), cleaved caspase-3 (Cell Signaling, Beverly, MA), gelsolin (BD Transduction Labs, Lexington, KY), and PKC-δ (Santa Cruz Biotechnology, Santa Cruz, CA) was conducted using appropriate primary and secondary antibodies (Pierce Biotechnology, Rockford, IL).

Zymography

For acquisition of conditioned media, one million cells were grown in culture dishes, washed with PBS, incubated with serum-free DMEM for 24 hours, and treated with pirfenidone. Twenty-four hours later, supernatant (conditioned media) was harvested and stored at -20°C. Four parts of medium containing

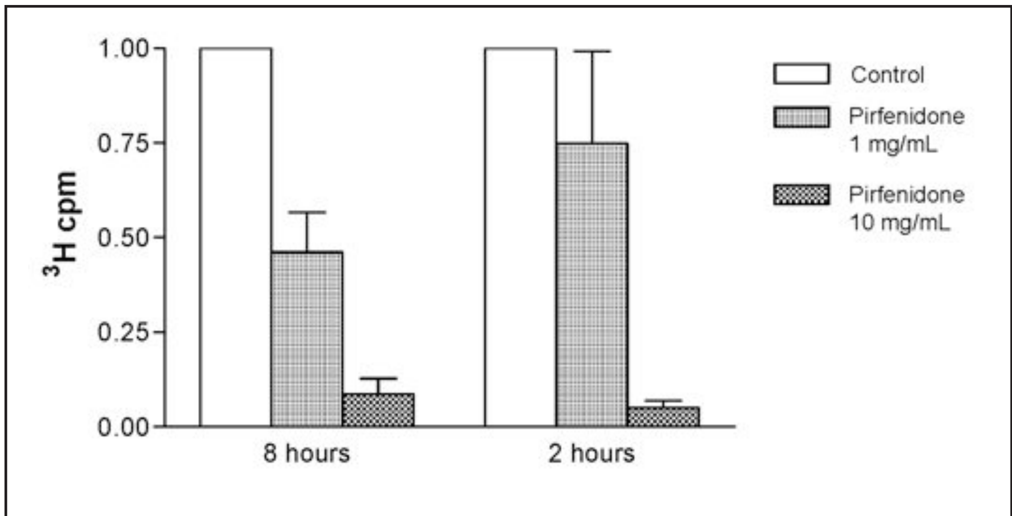

Figure 2. Dose-dependent inhibition of DNA synthesis in the SKMG3 cell line, based on (3H)-thymidine incorporation and expressed as counts per minute (cpm). Left, Effects on DNA synthesis 8 hours after treatment with pirfenidone. Right, 2 hours after treatment with pirfenidone.

equal amounts of protein (20 μg) were mixed with one part of Laemmle sample buffer minus the reductant before electrophoresis. MMP-2 and MMP-9 activity were analyzed on SDS-polyacrylamide gels impregnated with 0.1% (w/v) gelatin and 10% (w/v) polyacrylamide as described previously.[31] The gels were run at constant current and then washed twice for 30 minutes in 50 mM Tris-HCl, pH 7.4, plus 2.5% Triton X-100; the gels were then incubated overnight at 37˚C in 50 mM Tris-HCl, pH 7.6; 10 mM $CaCl_2$; 150 mM NaCl; and 0.05% NaN_3 to allow the gelatinase to digest the gelatin structure. Gels were stained with Coomassie Blue for 30 minutes, followed by 1 hour of destaining. The proteolytic activity appeared as clear bands (zones of gelatin degradation) on a blue background at M_r 92,000 for MMP-9 and M_r 72,000 for MMP-2. As all samples were normalized to the cell number from the derivative culture, comparisons between the relative levels of proteolytic activity could be made.

Flow Cytometry

Cells were fixed in 70% ethanol diluted in PBS, and the samples were stored at -20°C. The fixed cells were resuspended in PBS containing 20 μg/mL propidium iodide and 100 μg/mL boiled RNase A and incubated for 30 minutes at 37°C before flow cytometric analysis on a Becton Dickinson FACScan; 20,000 ungated events were collected. Cell cycle distribution was determined using the ModFit software package (Verity Software House, Topsham, ME) after excluding doublets and clumps by gating on the DNA pulse-width versus pulse-area displays.

RESULTS

Pirfenidone Inhibits Cell Proliferation

The effects of pirfenidone on proliferation were examined in a panel of six glioma cell lines using a MTS assay. This assay relies on the bioreduction of a tetrazolium compound by metabolically active cells into a soluble formazan salt, which then can be quantitated using a spectrophotometer. As seen in Figure 1, incubation with 10 mg/mL pirfenidone for 72 hours significantly inhibited the proliferation of SKMG-3, D37, and HS683 cells. SKMG3 is a glioma cell line

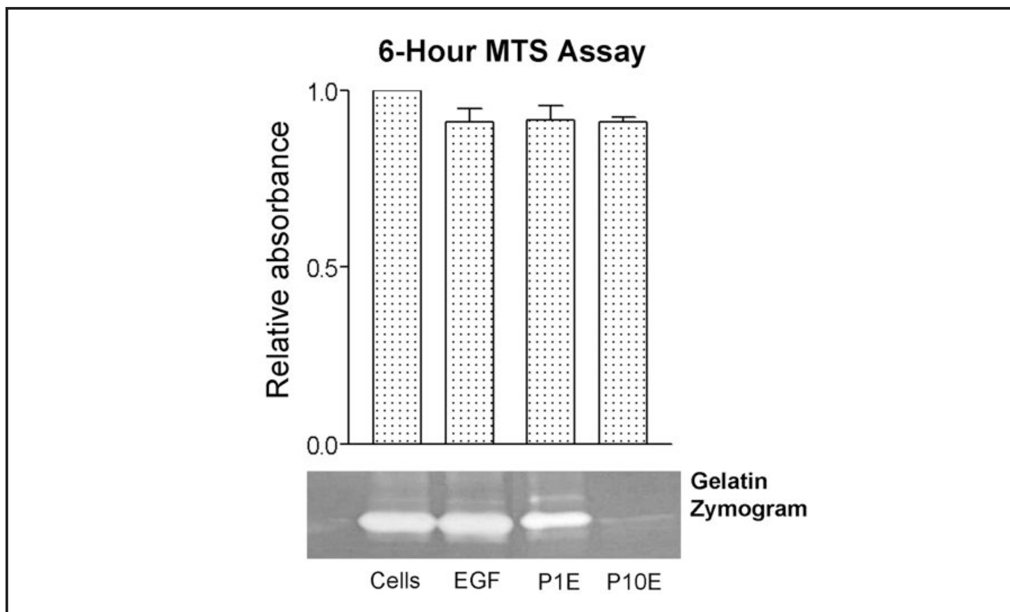

Figure 3. Lower panel, Gelatin zymography shows inhibition of matrix metalloproteinase-2 by 10 mg/mL (P10E) of pirfenidone. This effect was observed 6 hours after exposure to pirfenidone. Upper panel, A simultaneously performed proliferation assay did not demonstrate negative effects on cell proliferation at the same drug concentration. Left to right, The gelatin zymogram shows cells without stimulation or treatment (Cells); serum-starved cells stimulated by EGF in a concentration of 10 ng/mL, not treated with pirfenidone (ECG); serum-starved cells stimulated with EGF and treated with 1 mg/mL pirfenidone (P1E); and serum-starved cells stimulated with EGF and treated with 10 mg/mL pirfenidone (P10E).

that overexpresses EGFR and has mutations in PTEN and p53. Inhibition of proliferation was dose-dependent and had an IC_{50} of 0.9 mg/mL. D37 is a glioma cell line that does not overexpress EGFR and has no PTEN or p53 mutation. HS683 is a glioma cell line that does not overexpress EGFR and has no PTEN mutation but does have a p53 mutation. Two later glioma cell lines showed similar, but less potent, inhibition of proliferation (results not shown). We then looked at a non-glioma cell line that overexpresses EGFR and found that pirfenidone also strongly inhibits proliferation of A431 (a squamous cell carcinoma cell line).

Pirfenidone Inhibits DNA Synthesis

The effects of pirfenidone on DNA synthesis were examined using a tritiated thymidine assay. This assay relies on the ability of cells actively synthesizing DNA to incorporate tritiated thymidine into their newly synthesized DNA; the incorporated radioactivity can then be quantitated using a liquid scintillation counter. As seen in Figure 2, incubation with 10 mg/mL pirfenidone for 8 hours significantly inhibited DNA synthesis. DNA synthesis inhibition was dose dependent and had an IC_{50} of 0.3 mg/mL. The inhibition of DNA synthesis was seen as early as 2 hours after incubation with pirfenidone.

Pirfenidone Inhibits MMP Activity

The effects of pirfenidone on tumor cell motility and invasiveness were examined by determining MMP activity as a surrogate marker, using zymography. This assay relies on the ability of MMPs to degrade the substrate that has been incorporated into a Western blot gel; the gel can then be stained with Coomassie Blue allowing for visualization of bands

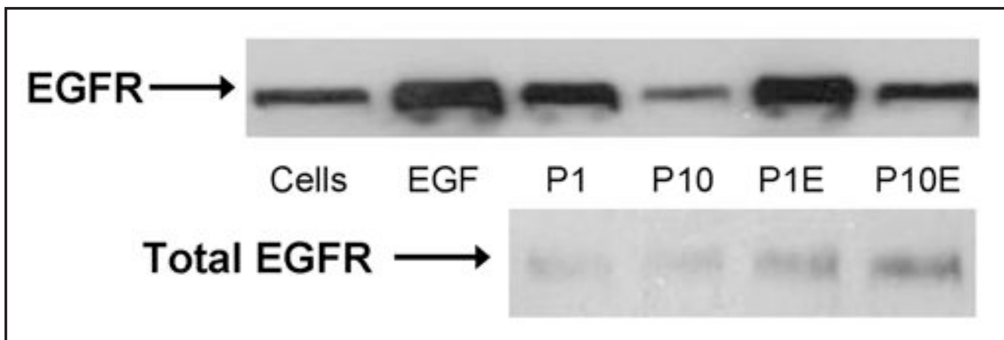

Figure 4. Dose-dependent inhibition of EGF-induced EGFR phosphorylation by pirfenidone in SKMG3 cell line. Western blot was performed with antibody to EGFR phosphotyrosine residues. Cells: without stimulation or treatment; EGF: serum-starved cells stimulated by EGF in concentration of 10 ng/mL, not treated with pirfenidone; P1: serum-starved cells treated with 1 mg/mL pirfenidone; P10: serum-starved cells treated with 1 mg/mL pirfenidone; P1E: serum-starved cells stimulated with EGF and treated with 1 mg/mL pirfenidone; P10E: serum-starved cells stimulated with EGF and treated with 10 mg/mL pirfenidone, after EGF.

of proteolytic activity corresponding to the molecular weight of the proteases. As seen on the zymogram in Figure 3, incubation with pirfenidone for 6 hours significantly inhibited MMP activity, both when stimulated by EGF (lower graph) and in the absence of EGF stimulation (not shown). The graph above the zymogram shows the lack of inhibition of proliferation (MTS assay) at this time point. The inhibition of MMP activity is likely a direct effect of pirfenidone and not a consequence of inhibition of proliferation.

Pirfenidone Inhibits EGFR Phosphorylation

The effect of pirfenidone on EGFR activity was examined using a Western blot for phosphorylation of EGFR. As seen in Figure 4, stimulation of cells with EGF leads to increased phosphorylation of EGFR. Pirfenidone inhibits phosphorylation of EGFR in a dose-dependent manner. This effect is noted both in EGF-stimulated and in non-EGF-stimulated cells.

Pirfenidone Induces Apoptosis

The nature of pirfenidone's inhibition of proliferation was investigated using an assay for apoptosis. Propidium iodide flow cytometric assessment of cell cycle distribution showed accumulation of cells in sub-G_0 phase upon treatment with 10 mg/mL pirfenidone (Figure 5). Confirmation of apoptosis was done by Western blot analysis of known pathways of apoptosis, showing activation of the pro-apoptotic protein Bax, leading to activation of pro-caspase-3 and also the degradation of PKC-δ (Figure 6). These results suggest a unique mechanism by which pirfenidone exerts its antitumor effects and the possible apoptotic pathway driving this response.

DISCUSSION

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder with impressive clinical variability. The hallmark of NF1 is the development of cutaneous pigmentary manifestations, peripheral neurofibromas (including plexiform neurofibromas), and increased predisposition to CNS tumors including optic glioma and astrocytoma. Treatment of peripheral neurofibromas is generally limited to complete resection of symptomatic lesions, but in many instances, complete resection is not feasible due to the location of the tumor. Therefore, it is desirable to have other modalities of treatment available to arrest the growth

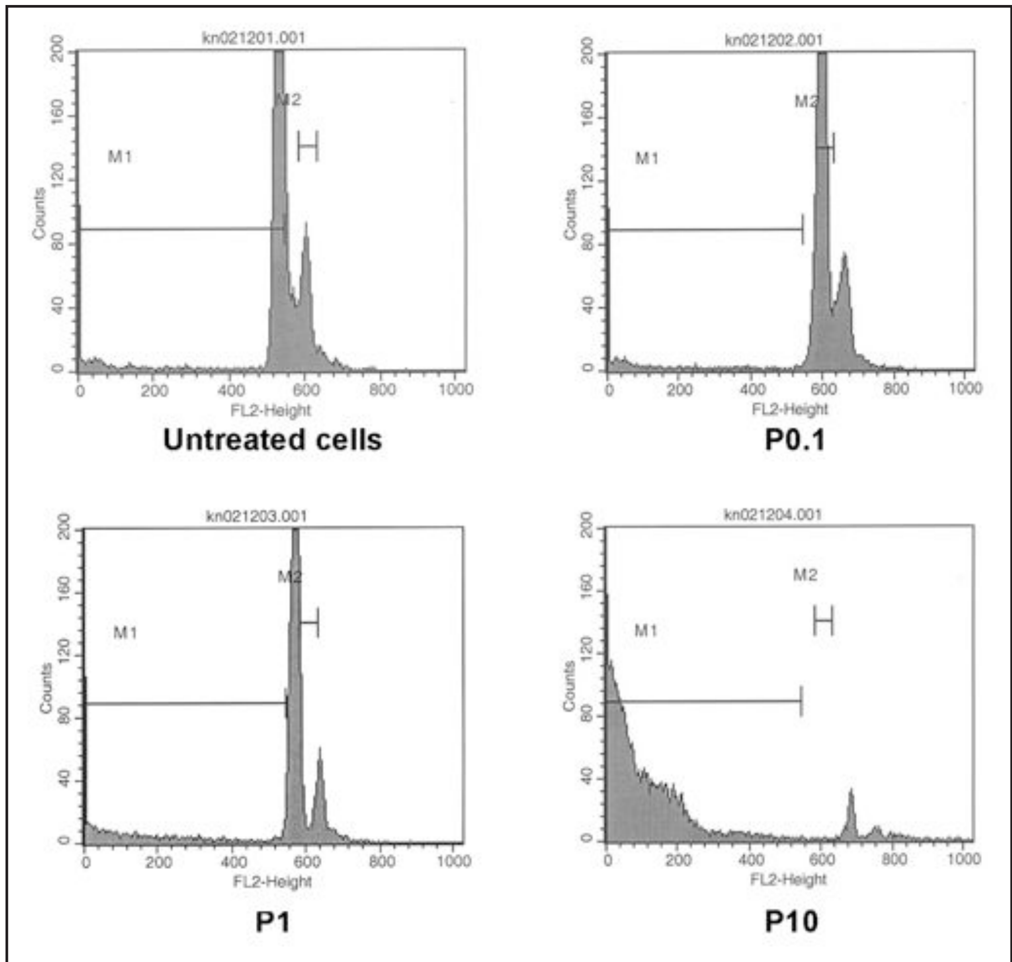

Figure 5. Pirfenidone-induced apoptosis on the SKMG3 cell line. *Panel 4, lower-right (P10),* Propidium iodide flow cytometry after exposure to various concentrations of pirfenidone. Upon treatment with 10 mg/mL, there was a large sub-G_0 population of cells (pirfenidone-induced apoptosis at higher concentrations). *Panels 1-3, top left, top right, and bottom left,* Effects of pirfenidone on untreated cells and in a concentration of 0.1 mg/mL (P0.1) and 1 mg/mL (P1).

of these tumors or to complement subtotal resection.

Astrocytomas are the most common CNS tumors in NF1, and pilocytic astrocytomas (WHO grade I) are the main histological subtype.[32,33] Pilocytic astrocytomas are usually characterized by a stable or a very slow progressive course, but in some patients tumor location and growth result in severe neurological damage and/or death. Treatment of progressive optic and brainstem gliomas is also suboptimal, and trials of radiotherapy and cytostatics commonly fail. Unlike the benign histology of indolent intracranial gliomas, a high proportion of progressive tumors are higher-grade astrocytomas (grade II, III, and IV). This is consistent with previous data from NF1 brainstem gliomas,[9,11] suggesting that symptomatic/progressive tumors may be associated with a more aggressive histological subtype.[15]

Presently, there is no established effective therapy for either progressive neurofibromas or for progressive gliomas and astrocytomas, which are major contributors to morbidity and

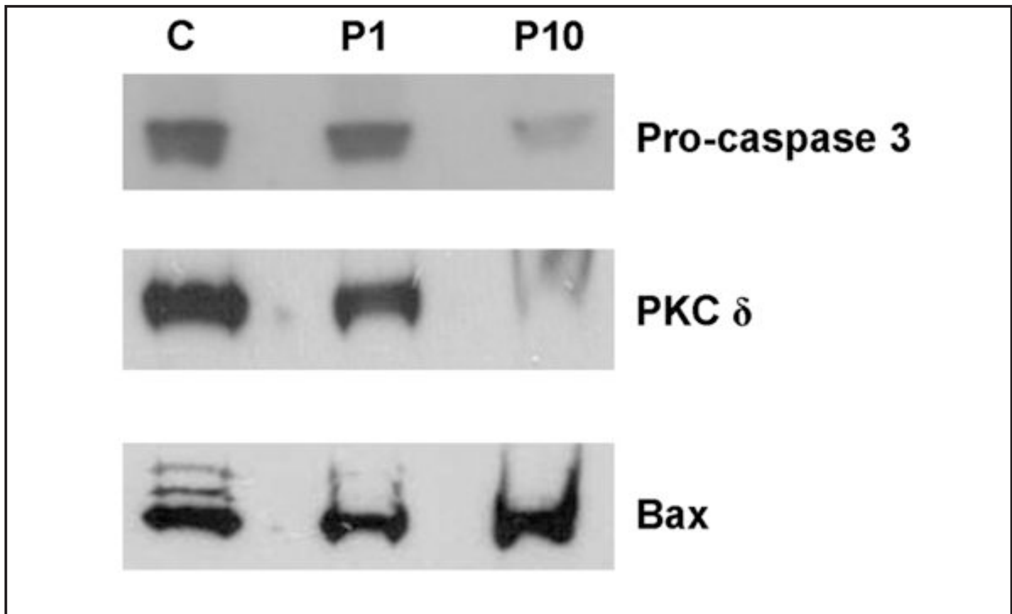

Figure 6. Western blot shows surrogate markers for apoptosis. Top panel, activation of pro-caspase 3 to caspase 3. Middle panel, degradation of PKC-δ. Bottom panel, increase in Bax in response to treatment with pirfenidone, 1 mg/mL (P1) and 10 mg/mL (P10) and without treatment lower panel, cells only).

mortality in patients with NF1. Attempts to study benign neurofibromas and low-grade gliomas in vitro are hampered by the difficulty in growing cultures of cells demonstrating the representative features of the index tumor. In the case of neurofibromas, the fibroblast component typically overgrows the neural component. Therefore, we chose to study the antitumor effects of pirfenidone on the neural component of neurofibromas, as well as its possible inhibitory effect on NF1-associated gliomas, by using malignant glioma cell lines.

Pirfenidone is a well-tolerated oral agent with good CNS penetrance.[34] Human studies show only mild gastrointestinal side effects and virtually no toxicity at doses of up to 2400 mg/day in adults. Following ingestion of a single dose of 400 mg, an average serum concentration of 3.97 µg/mL was achieved in 15 minutes. Our study of pirfenidone in vitro on a panel of GBM cell lines demonstrates dose-dependent inhibition of proliferation with an IC_{50} of 0.9 mg/mL. The antiproliferative effects were independent of the cell lines' p53 mutation status. Glioma cell lines overexpressing EGFR were more sensitive to this effect. Dose-dependent inhibition of DNA synthesis was seen as early as 2 hours after pirfenidone treatment (IC_{50} of 3 mg/mL).

Intriguingly, the antiproliferative effect was partially mediated by induction of apoptosis. Eight-hour measurement of apoptosis using propidium iodide flow cytometry revealed a large sub-G_0 population of cells upon treatment of cells with 10 mg/mL of pirfenidone. Programmed cell death was associated with increased levels of the pro-apoptotic protein Bax as well as increased caspase-3 activation. This is a novel and unusual mechanism of action for a cytostatic agent in malignant gliomas. Unfortunately, the high concentrations of drug required to produce these effects might not be clinically

achievable in rapidly growing malignant tumors. However, it may be more relevant in the treatment of indolent or slow-growing tumors, where lower concentrations of pirfenidone for prolonged periods of time might achieve significant antitumor effects. Our in vivo studies would suggest that this is probably the case. Ongoing clinical studies will further illuminate our understanding of the clinical uses of this agent.

Despite pirfenidone's known antifibrotic effect, which is putatively mediated through inhibition of TGF-β signaling, we also found a dose-dependent inhibition of EGFR phosphorylation. It has been previously shown that EGFR expression may play a role in NF1 tumorigenesis and Schwann cell transformation,[35] such that the documented anti-EGFR activity of pirfenidone may further reinforce its potential in the treatment of actively growing NF1-related tumors, including progressive intracranial gliomas. Furthermore, there might be some inhibition of matrix metalloproteinase-2 (MMP-2) activity at doses of pirfenidone that do not inhibit proliferation.

These results suggest that there may be a clinical role for pirfenidone in the treatment of slow-growing tumors associated with NF1. The effect of pirfenidone might be attributed to its activity against both the fibroblast component and the neural component of neurofibromas. Given that pirfenidone is a highly lipophilic drug that readily transverses the intact blood-brain barrier and has potent antitumor activity against gliomas in vitro, it is possible that it might have activity not only against progressive neurofibromas, but also low-grade gliomas seen frequently in patients with NF1.

CONCLUSIONS

Pirfenidone is a well-tolerated oral agent already being used in clinical trials for plexiform neurofibromas in patients with NF1. We report pirfenidone's potent antitumor effects on the neural component of these tumors, including inhibition of DNA synthesis, cell proliferation, and invasion as well as induction of programmed cell death. Our results suggest that these effects are partially mediated through inhibition of EGFR phosphorylation. We propose to further validate these results in vivo and correlate our findings with clinical outcomes seen in ongoing clinical trials. We also propose further studies to define role of pirfenidone in treatment of low-grade gliomas associated with NF1.

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