The MEK inhibitor PD184352/CI-1040 selectively induces apoptosis in malignant schwannoma cell lines

Raymond R. Mattingly, Janice M. Kraniak, Joshua T. Dilworth, Patricia Mathieu, Beverly Bealmear, James E. Nowak, Joyce A. Benjamins, Michael A. Tainsky, and John J. Reiners, Jr

Wayne State University School of Medicine, Departments of Pharmacology (R.R.M., J.T.D., J.J.R.) and Neurology (B.B., J.A.B.), and Center for Molecular Medicine and Genetics (J.A.B., M.A.T.), 540 East Canfield Ave, Detroit MI 48201.

Barbara Ann Karmanos Cancer Institute, Programs in Molecular Biology and Human Genetics (R.R.M., J.A.K., J.E.N., M.A.T.) and in Proteases (J.J.R.), 110 East Warren Ave, Detroit MI 48201.

Wayne State University, Institute of Environmental Health Sciences (P.M., J.J.R.) and Environmental Health Sciences Center for Molecular & Cellular Toxicology with Human Applications (R.R.M., J.A.B., M.A.T., J.J.R.), 2727 Second Ave, Detroit MI 48201.
Running title: MEK inhibitor-induced schwannoma apoptosis

Corresponding author: Raymond R. Mattingly, Wayne State University Department of Pharmacology, 540 East Canfield Ave, Detroit MI 48201
Telephone: 313.577-6022. Fax: 313.577-6739
Email: r.mattingly@wayne.edu

Number of text pages: 40
Number of Tables: 1
Number of Figures: 9
Number of References: 39
Number of words in Abstract: 233; in Introduction: 671; in Discussion: 1671

Non-standard abbreviations:
AMC, amino-4-methylcoumarin; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; FTI, farnesyl transferase inhibitor; GAP, GTPase-activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase or ERK kinase; NF1, Type 1 neurofibromatosis; p90RSK, 90kDa ribosomal S6 kinase; Q-RT-PCR, quantitative real-time PCR

Recommended section assignment: Cellular and molecular
Abstract

Type 1 Neurofibromatosis (NF1) is a common autosomal dominant disorder that results in neuroectodermal tumors. The NF1 tumor-suppressor gene encodes neurofibromin, which includes a GTPase-activating domain for Ras inactivation. Affinity purification showed N-Ras to be the predominant activated isoform of Ras in 2 independent neurofibrosarcoma cell lines from NF1 patients (lines ST88-14 and NF90-8). These NF1 cells also demonstrated increased constitutive activity of the ERK1,2 MAP kinases as compared to a sporadic malignant schwannoma cell line that maintains neurofibromin expression (STS-26T). Thus MEK inhibitors may be a rational approach to NF1 therapy. The MEK inhibitors PD98059 [2’-amino-3’-methoxyflavone], PD184352 (CI-1040) [2-(2-chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3,4-difluoro-benzamide] and U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] all produced concentration-dependent suppression of the proliferation of the three cell lines. Individual MEK inhibitors had similar effects in all three cell lines. However, only the anti-proliferative effects of PD184352 correlated closely with the elimination of ERK1 and 2 MAP kinase activities. PD98059 was primarily cytostatic, whereas U0126 and PD184352 were cytotoxic. Only PD184352 induced apoptosis in all three lines, as indicated by morphology, activation of DEVDase, pro-caspase-3 cleavage, and the appearance of populations having sub G0/G1 DNA contents. The differential effects of the MEK inhibitors on cell survival were not dependent on p53 status or effects on the ERK5 pathway. PD184352 was also pro-apoptotic to primary rat Schwann cells. Hence, although PD184352 effectively killed neurofibrosarcoma cells, its effects on normal Schwann cells may limit its usefulness in the clinic.
Introduction

Type 1 neurofibromatosis (NF1) is an autosomal dominant disorder that, with a birth incidence of approximately 1 in 3000, represents the most commonly inherited cancer predisposition syndrome. In addition to a variety of other cutaneous, neurological, endocrine, cardiovascular, and orthopaedic manifestations, patients typically have one or more dermal neurofibromas. These benign tumors are comprised principally of Schwann cells, and are estimated to undergo malignant progression in about 10% of patients. Standard therapy of these tumors is primarily surgical and there is a need for effective pharmacological approaches (Packer et al., 2002).

The NF1 tumor-suppressor gene product, neurofibromin, is a large protein that includes a domain with GTPase-activating protein (GAP) activity towards Ras proto-oncogene products (DeClue et al., 1991). Loss of neurofibromin expression would be expected, therefore, to increase Ras activation, and this has been demonstrated in cells derived from NF1 patients (Basu et al., 1992; DeClue et al., 1992) and from transgenic mice that are deficient in neurofibromin (Kim et al., 1995). Farnesyltransferase inhibitors (FTIs) block the post-translational modification of Ras that is required for its function. They were developed with the initial expectation that they would provide a therapeutic approach to the approximately one quarter of human tumors that have mutationally active, oncogenic Ras (Adjei, 2001). They have also been proposed as potential therapeutics for tumors such as those from NF1 patients, which may be driven by an over-active proto-oncogenic Ras (Yan et al., 1995; Parada, 2000; Costa et al., 2002).
There are 4 human Ras proteins that are expressed from 3 genes (H-, K-, and N-Ras; K-Ras is alternatively spliced). There may be important functional differences between the Ras isoforms. N-Ras, for example, has been ascribed a particular role in cell survival in fibroblasts (Wolfman and Wolfman, 2000). The farnesyl transferase inhibitors were developed from biochemical experiments on H-Ras proteins, and it is likely that K-Ras (and perhaps also N-Ras) proteins can be alternatively modified by geranylgeranylation when farnesyl transferase activity is blocked (Adjei, 2001).

Another approach to therapeutically target an over-active Ras pathway is to block critical signal transduction cascades that are initiated by activated Ras. Ras stimulation of the Raf-MAP kinase kinase (MEK)-MAP kinase (ERK) pathway is often required for cell proliferation. Inhibitors of MEK have been used to block the growth of some tumors (Sebolt-Leopold et al., 1999). In NF1, this pathway has been shown to be important for proliferation of neurofibromin-deficient myeloid (Donovan et al., 2002) and astrocytoma (Lau et al., 2000) cells. To date, three agents have been used for the pharmacological inhibition of MEK. PD98059 [2’-amino-3’-methoxyflavone] and U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] both potently inhibit MEK1 in a variety of cell types when used in the low micromolar concentration range (Dudley et al., 1995; Favata et al., 1998; Reiners et al., 1998). A third inhibitor termed PD184352 (also called CI-1040) [2-(2-chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3,4-difluoro-benzamide] potently inhibits MEK1,2 activities in the high nanomolar range (Sebolt-Leopold et al., 1999). PD184352 has sufficient bioavailability for in vivo studies (Sebolt-Leopold et al., 1999), and has entered clinical trials (Allen et al., 2003).
In this study we tested the efficacy of the three MEK inhibitors, PD98059, U0126, and PD184352 in three cell lines derived from human malignant schwannomas: ST88-14 and NF90-8 from NF1 patients (DeClue et al., 1992) and STS-26T from a sporadic tumor in a non-NF1 patient (Dahlberg et al., 1993). The goal was to determine whether MEK inhibition may be a viable approach to suppress the proliferation of schwannomas, and whether there may be any increased dependence on the Ras/Raf/MEK/ERK cascade in NF1-derived tumors. The results confirm that Ras and ERK1,2 are constitutively activated in the neurofibromin-deficient cells, and further show that it is the N-Ras and K-Ras isoforms that are affected. To variable degrees, all three MEK inhibitors suppressed cell proliferation and induced G1 arrest. Only PD184352 potently induced apoptosis and blocked anchorage-independent growth. This cytotoxic effect occurred in both the NF1 and non-NF1 schwannoma cell lines and also in primary rat Schwann cells. This latter effect on normal cells may limit the usefulness of PD184352 in the treatment of NF1.
Methods

Cell Culture and Transfection. Human malignant schwannoma cell lines ST88-14 and NF90-8 (generous gift from T. Glover, University of Michigan) and STS-26T (generous gift from D. Scoles, Cedars-Sinai Medical Center) were maintained in RPMI with 5% fetal bovine serum (Hyclone, Ogden, UT) and 50 units/ml penicillin and 50 µg/ml streptomycin. Variations in culture conditions for particular experiments are stated below. COS-7 cells were cultured and transfected by calcium phosphate co-precipitation as previously described (Mattingly et al., 1994). The pRK5myc vector with inserts encoding H-Ras, K-Ras and N-Ras were generous gifts from J. Jackson (Scripps Research Institute, La Jolla, CA). Primary Schwann cells were isolated from sciatic nerves of neonatal Sprague-Dawley rats and cultured on poly-D-lysine coated glass coverslips as described previously (Skoff et al., 1998). Cultures were maintained in Eagle’s medium (Gibco BRL, Grand Island, NY) for 48 h before experimental manipulations to allow the cells to adhere to the coverslips.

Anchorage-independent Growth. ST88-14 and STS-26T cells were plated at 1 x 10^4 cells/ml in media with 15% fetal bovine serum and 0.35% agarose and grown for 14 days. Colonies were measured with a microscope fitted with a calibrated eye-piece and colonies ≥ 60 µm were counted as positive. Values for percent growth were calculated as # of positive colonies/# of cells plated x 100.

Flow Cytometry. Schwannoma cell lines were harvested and processed for FACS analyses of DNA content as described (Reiners et al., 1999). DNA analyses were made with a BD
Biosciences FACScalibur instrument (BD Biosciences, San Jose, CA). Percentages of cells in the G0/G1, S and G2/M stages of the cell cycle were determined with a DNA histogram-fitting program (MODFIT; Verity Software, Topsham, ME). When possible, a minimum of \(10^4\) events/sample were collected for subsequent analyses.

**DEVDase Assay.** The procedures used for the preparation of lysates and the assay of DEVDase using Ac-DEVD-AMC as the substrate have been described in detail (Caruso et al., 2004). Changes in fluorescence over time were converted into pmol of product by comparison with a standard curve made with AMC. DEVDase specific activities are reported as nmol product per minute per mg of protein. The bicinchoninic acid assay, using bovine serum albumin as a standard, was used to estimate protein concentrations.

**Western Blot Analyses.** The procedures used for the preparation of lysates and the western blot analyses of caspase-3 have been described in detail (Caruso et al., 2004). Whole cell lysates were prepared for western blotting of diphosphorylated ERK1,2 with a 1:2000 dilution of mouse monoclonal anti-phosphoERK1,2 (Sigma, St Louis, MO), and then stripped and re-probed for total ERK1,2 content with a 1:1000 dilution of a rabbit anti-ERK1,2 antibody (UBI, Charlottesville, VA). The procedures for assay of phosphorylated and then total ERK5, and for phosphorylated 90kDa ribosomal S6 kinase (p90RSK) and total p90RSK, were similar except that the antibodies used were a 1:325 dilution of rabbit anti-ERK5 (phosphothreonine-218/phosphotyrosine-220), 1:500 dilution of rabbit anti-ERK5, 1:1000 rabbit anti-p90RSK (phosphothreonine-359/phosphoserine-363), and 1:1000 dilution of rabbit anti-p90RSK (all from Cell Signalling Technology, Beverly, MA). Cell lysates were probed for Ras-
GAPp120 as described (Mattingly et al., 1994), for neurofibromin with a 1:1000 dilution of a rabbit polyclonal antibody against the C-terminal region of human neurofibromin (Santa Cruz Biotechnology, Santa Cruz, CA) and for β-tubulin with a 1:1000 dilution of the E7 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). Cell lysates were prepared for measurement of total and active/GTP-bound Ras, using an affinity pull-down reaction with beads pre-coated with a glutathione-S-transferase fusion of the Ras-binding domain of Raf (Taylor and Shalloway, 1996). Total Ras was detected with 1:250 anti-pan Ras (Transduction Laboratories, Lexington, KY). Whole cell lysates of COS cells were prepared for western blotting 48 h post-transfection. Transfected, epitope-tagged Ras proteins were detected with 1:5000 of the 9e10 anti-Myc monoclonal (Developmental Studies Hybridoma Bank). Isoform specific blotting of Ras proteins was performed with monoclonal antibodies. H-Ras was detected with a 1:100 dilution of sc-29 (Santa Cruz Biotechnology). K-Ras was detected with a combination of 1:100 sc-30 (Santa Cruz) plus 1:200 K-Ras Ab1 (Oncogene Research, San Diego, CA). N-Ras was detected with 1:200 sc-31 (Santa Cruz) plus 1:100 N-Ras Ab1 (Oncogene Research). Immune complexes were visualized by appropriate secondary antibodies and enhanced chemiluminescent detection and recorded on X-ray film.

**Quantification of gene expression by Q-RT-PCR.** Total RNA (1µg) was reverse transcribed into cDNA using Superscript II (Life Technologies, Gaithersburg, MD, USA). Q-RT-PCR was performed using the SYBR Green PCR detection kit (PE Biosystems, Warrington, United Kingdom) and run on the ABI 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). All methods for reactions and quantification were performed according to recommendations from the manufacturer, which can be found in the ABI User Bulletin #2.
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for quantification. The primer pair for N-Ras was: forward 5’-AGGGTTGTATGGGATTGCCA-3’, reverse 5’-TCAGGACCAGGGTGTCAGTG-3’. The primer pair for H-Ras was: forward 5’-TGTGCACAGACTGTCTTTGAACATC-3’, reverse 5’-TGATCCCATCTGTGCCCG. The primer pair for K-Ras was: forward 5’-AAGAGACCAAGGTGCAAGGC-3’, reverse 5’-TGACAACACTGGATGACCGTG. The target sequence for all Ras isoforms was located either wholly or partially in the non-coding sequence at the carboxy end of the mRNA transcript. The primer pair for GAPDH was: forward 5’-ATCAAGAAGGTGGTGAAGG-3’, reverse 5’-TGTCGCTGTGAAGTCAGAGG-3’.

Drugs and Other Reagents. PD98059, PD184352 and U0126 were purchased from Calbiochem (San Diego). PD184352/CI-1040 was also generously supplied by Pfizer, Inc. Control experiments demonstrated that the potency and efficacy of PD184352 from the two sources were identical. The MEK inhibitors were prepared as stock solutions in dimethyl sulfoxide and stored as frozen single-use aliquots at –80°C. Epidermal growth factor (EGF) was purchased from Invitrogen (Bethesda, MD).
Results

**Constitutive activation of wild-type Ras and ERK MAP kinase in NF1 cell lines.** Previous studies of cell lines derived from NF1 tumors indicated that expression of neurofibromin protein was either at a very low (line NF90-8) or undetectable (line ST88-14) level, while Ras-GAP p120 levels were maintained, and that Ras-GTP levels were increased (DeClue et al., 1992). In order to establish a system in which MEK inhibitors could be tested for potential therapeutic efficacy in NF1, we confirmed the phenotype of these NF1-derived lines in comparison to a cell line, STS-26T, that was derived from a sporadic malignant schwannoma in a non-NF patient. The data presented in Fig. 1A confirm that neurofibromin expression was not detectable in NF90-8 or ST88-14 cells, whereas expression of Ras-GAP p120 and the loading control β-tubulin were similar in all 3 cell lines. To investigate the activation status of Ras, we performed affinity pulldown reactions with the Ras-binding domain of Raf. This assay has the advantage over an alternative protocol that involves radiolabeling of nucleotide pools with inorganic phosphate (Mattingly et al., 1994) that Ras activation can be investigated under varied growth conditions (as opposed to the arrested state that occurs in the presence of mCi levels of $^{32}$P) (Taylor and Shalloway, 1996). The data in Fig. 1B confirm that total expression of Ras protein is similar in all 3 lines, and that Ras is activated in the NF1 lines under the 3 growth conditions tested: serum-starved for 24 h ($U$), serum-starved and then acutely stimulated for 5 min with serum ($S$), and cultured in normal growth medium ($C$). Ras activation was only detected in the STS-26T cells under the condition of acute serum stimulation. The degree of constitutive Ras activation in the NF1 lines exceeded the amount of Ras activation that could be induced in STS-26T cells by acute stimulation, and was also responsive to further increase by acute stimulation.
Since there are 3 Ras genes, we next addressed which Ras isoforms may contribute to the constitutive Ras activation that occurs in the NF1 cell lines. We first established conditions under which we could selectively and semi-quantitatively detect the individual Ras isoforms by western blotting. We expressed Myc-tagged versions of H-Ras, K-Ras and N-Ras in COS7 cells and then used the cell lysates to standardize the antibodies (Fig. 2A, upper panels). Blotting for the epitope tag verified the loading of the transfected Ras proteins and that the isoform-directed antibodies only detected the specific isoform intended. We then repeated the total and active Ras assays in the schwannoma cell lines with isoform-specific detection (Fig. 2A, lower panels). The pattern of expression of the isoforms was identical in all 3 lines, suggesting that this may be determined by cell lineage. N-Ras was easily detectable with short exposures, indicating that it is likely to be the predominant isoform in these cells. K-Ras was also detected, but typically required longer exposure times. We could not detect endogenous H-Ras protein under these conditions, or even when up to 150 µg of cell lysate protein per well was analyzed (data not shown). Both activated N-Ras and activated K-Ras were present in the NF1 lines. No activated Ras isoforms could be detected in the STS-26T cells under these conditions. To further investigate the expression of the individual Ras isoforms in the neurofibrosarcoma cell lines, quantitative, real-time PCR (Q-RT-PCR) was performed (Fig. 2B). The results showed that the mRNA for N-Ras was the most abundant, followed by that for H-Ras and then that for K-Ras. PCR amplification of genomic DNA followed by DNA sequence analysis demonstrated that there were no mutations in any of the exons of the Ras isoforms in any of these cell lines.
To determine whether the constitutively activated Ras that was present in the NF1 lines induced an activation of downstream signaling pathways, we assayed for the activated, dually phosphorylated ERK1,2 MAP kinases (Fig. 2C). Under normal growth conditions, we found both ST88-14 and NF90-8 cells had markedly more activated ERK1,2 than the STS-26T line. Even following serum starvation for 24 h, a significant amount of phosphorylated ERK1,2 was present in the NF1 lines. The ratio of relative expression of ERK1,2 was different between the neurofibrosarcoma cell lines. NF90-8 and STS-26T cells exhibited similar or greater expression levels of ERK2 (p42 MAP kinases) relative to ERK1 (p44 MAP kinase), while ST88-14 cells reproducibly showed higher expression of ERK1 (p44 MAP kinase) relative to ERK2 (p42 MAP kinase).

Inhibition of ERK MAP kinase activation by MEK inhibitors

In order to test the hypothesis that the increased amounts of phosphorylated ERK1,2 in the NF1 lines was due to Ras stimulation of the Raf/MEK/ERK cascade, we examined the abilities of 3 MEK inhibitors to block the activation of ERK1,2 in the malignant schwannoma cell lines. To ensure that the data on MEK inhibitor effects on ERK1,2 phosphorylation were collected under the same experimental conditions as used for cell cycle analyses, subsequent experiments on ERK activation were performed on cells grown for at least 24 h in the presence of 1% serum. The MEK inhibitors were tested for their effects on basal ERK1,2 phosphorylation and for their ability to block EGF-stimulated ERK1,2 activation (Fig. 3). All of the MEK inhibitors reduced basal ERK1,2 phosphorylation, with U0126 and PD184352 being fully effective at 1 µM. PD98059 was less potent. In dose-response analysis against EGF-stimulated ERK1,2 phosphorylation, PD184352 was fully inhibitory at 1 µM, while U0126 required 10 µM for full
inhibition, and PD98059 was only partially effective even at 20 µM. Equivalent data were also obtained for the effects of the inhibitors against ERK1,2 phosphorylation in cells stimulated by phorbol 12-myristate 13-acetate (data not shown).

**Inhibition of p90RSK phosphorylation by MEK inhibitors**

To establish whether the effects of the MEK inhibitors to block ERK1/2 activation produced inhibition of downstream signaling, we assayed the phosphorylation state of p90RSK at residues threonine-359-serine-363 (Frodin and Gammeltoft, 1999). One hour of treatment with PD184352 at 1 µM or U0126 at 10 µM could fully inhibit both the basal level of p90RSK phosphorylation and the increase that was induced by EGF stimulation (Fig. 4). After 48 hours of treatment with PD184352 at 1 µM or U0126 at 10 µM, the ability of EGF to stimulate an increase in p90RSK phosphorylation was also greatly reduced, indicating that inhibition of signal transduction through the MEK/ERK1/2 axis could be maintained.

**Cytostatic and cytotoxic effects of MEK inhibitors.** A concentration (1 µM) of U0126 sufficient to strongly suppress ERK1 and 2 activities (Fig. 3A) did not alter the proliferation of any schwannoma cell line (Figs. 5A,G,M). A concentration (10 µM) sufficient to totally eliminate ERK1 and 2 activities only weakly suppressed cell proliferation. This suppression correlated with the appearance of cytotoxicity (Figs. 5D,J,P). Higher concentrations (20 and 40 µM) of U0126 were increasingly cytotoxic (Figs. 5D,J,P).

PD98059 at 1 µM affected neither ERK activation status (Fig. 3A) nor cell proliferation (Fig. 5B,H,N) in any of the schwannoma cell lines. Thereafter, cell proliferation was progressively
suppressed by concentrations (10 – 40 µM) of PD98059 capable of reducing ERK1 and 2 activities (Figs. 3A, and 5B,H,N). PD98059-mediated suppressions of ST88-14 and STS-26T proliferation occurred in the absence of toxicity (Figs. 5E,Q). In contrast, the anti-proliferative effects of PD98059 on NF90-8 cultures were accompanied by some cytotoxicity (Fig. 5K).

PD184352 at 0.1 µM partially suppressed ERK phosphorylation (Fig. 3A) and the proliferations of all three schwannoma cell lines (Figs. 5C,I,O). Increasing the concentration to 1 µM, which totally suppressed ERK phosphorylation (Fig. 3A), almost totally suppressed the proliferations of all three cell lines (Figs. 5C,I,O). These effects were paralleled by marginal toxicity in the STS-26T cell line (Fig. 5R), and strong toxicities in the ST88-14 and NF90-8 cell lines. A higher concentration of PD184352 (10 µM) was very cytotoxic to all three lines.

Flow cytometric analyses of the DNA contents of ST88-14 cells (Fig. 6A) and STS-26T cells (Fig. 6C) indicated that concentrations of PD184352 sufficient to suppress proliferation perturbed cell cycle progression. Specifically, concentrations ≥ 1 µM induced the accumulation of G1 phase cells and the loss of S phase cells (Figs. 6A,C). In addition, in both cell types, a population of cells with sub G0/G1 DNA contents appeared within 24 h of treatment with 10 µM PD184352 (Figs. 6A,C), and accumulated upon further exposure. The anti-proliferative effects of 10 µM U0126 and 20 µM PD98059 on ST88-14 (Fig. 6B) and STS-26T (Fig. 6D) cultures also reflected the loss of S phase cells and an accumulation of G0/G1 phase cells. Neither of these MEK inhibitors induced the appearance/accumulation of populations having sub G0/G1 DNA contents in either cell line (data not presented). Flow cytometric analyses of DNA contents were also performed on the NF90-8 cell line following exposure to PD184352. In agreement
with the trypan blue exclusion data presented in Fig. 5L, exposure to ≥1 µM induced a rapid and extensive accumulation of cells having sub-G₀/G₁ DNA contents (data not presented).

**Pro-apoptotic effects of MEK inhibitors.** Exposure to 10 µM PD184352 caused similar morphological changes in all three cell lines. Fig. 7A depicts the effects of the MEK inhibitor on ST88-14 cultures. Within 48 h of treatment a majority of the cells had shrunken, rounded, and developed small blebs. These morphological traits, in conjunction with the accumulation of cells having sub-G₀/G₁ DNA contents during the same time frame (Fig. 6A), are characteristic of cells undergoing apoptosis. To confirm the development of apoptosis we also examined the cleavage of Ac-DEVD-AMC, a substrate of caspases-3 and –7. Exposure to 10 µM PD184352 resulted in a time-dependent activation of DEVDase (Fig. 7B). The kinetics of PD184352-induced DEVDase appearance correlated with the cleavage of pro-caspase-3 and appearance of one of its active subunits (Fig. 7C). Increases in DEVDase activity and pro-caspase 3 cleavage were also observed in response to 1 µM PD184352 (data not shown). In contrast to the effects of PD184352, DEVDase activities in ST88-14 cultures were unaffected by exposure of up to 40 µM U0126, and only slightly affected by 40 µM PD98059 (Fig. 7B). Although 40 µM U0126 was highly cytotoxic to ST88-14 cultures, dying cells did not exhibit the morphological characteristics of apoptosis.

**Effects of MEK inhibitors on anchorage-independent growth.** Formation of colonies during growth in soft agar culture is a condition that is thought to model malignant cell proliferation. ST88-14 and STS-26T cells formed robust colonies under anchorage-independent conditions and so could be evaluated for the effects of the MEK inhibitors on this activity (Table 1). Line
NF90-8 did not form colonies in soft agar in our test protocol. The results showed that PD184352 effectively suppressed anchorage-independent growth of both malignant schwannoma cell lines when used in the high nanomolar concentration range. The other two MEK inhibitors, PD98059 and U0126, were markedly less potent in this assay.

**Lack of effect of MEK inhibitors on ERK5 phosphorylation.** Since PD184352 potently induced apoptosis and blocked anchorage-independent growth, while PD98059 and U0126 did not, we addressed whether this difference could reflect differential abilities to inhibit ERK5 activation (Fig. 8). U0126 and PD98059 have been reported to block ERK5 phosphorylation in COS cells transfected with an epitope-tagged ERK5 construct (Kamakura et al., 1999), while PD184352 does not (Squires et al., 2002). Our studies showed that basal levels of ERK5 phosphorylation were undetectable in the two NF1 cell lines, and very low in STS-26T cells. Note that our analyses were performed under the same culture conditions where PD184352 produced a strong growth inhibitory and pro-apoptotic response (Figs. 5 - 7). In other cell types, ERK5 phosphorylation has been shown to be strongly induced by treatment with EGF (Kato et al., 1998; Mody et al., 2001). EGF was found to strongly stimulate ERK5 phosphorylation in all of the malignant schwannoma cell lines (Fig. 8). None of the 3 MEK inhibitors, when used at the concentrations at which they were effective inhibitors of ERK1,2 phosphorylation, significantly inhibited EGF-stimulated ERK5 activation.

**p53 status of malignant schwannomas.** Loss of p53 has been associated with some NF1 malignancies (Menon et al., 1990), and synergizes with neurofibromin deficiency to produce tumors in knock-out mouse models (Cichowski et al., 1999; Vogel et al., 1999). There are many
functional connections between the p53 and ERK MAP kinase pathways (Wu, 2004). It was therefore important to verify the p53 status of the malignant schwannoma cell lines. Lines ST88-14 and NF90-8 were previously reported to have wild-type p53 sequence as data not shown (DeClue et al., 1992). Sequencing of PCR products for p53 cDNA confirmed that ST88-14 cells express wild-type p53, but revealed a 22 base pair deletion in the third exon of p53 in NF90-8 cells. STS-26T cells were negative for expression of the p53 mRNA by northern blotting, negative for PCR products for p53 cDNA, and negative for p53 and p21 protein expression by western blotting. Southern analysis was consistent with the p53 gene being grossly intact despite the lack of expression (data not shown). Our results therefore show that PD184352 can induce apoptosis in 3 malignant schwannoma cell lines, one of which has wild-type p53, one of which has mutated p53, and the other of which is p53 null. These results suggest that p53 status does not determine the apoptotic outcome following PD184352 treatment of transformed Schwann cells.

Pro-apoptotic effect of PD184352 in primary rat Schwann cells. PD184352 potently induced apoptosis in human malignant schwannoma cell lines. We therefore tested whether PD184352 was cytotoxic to primary Schwann cells to determine whether the pro-apoptotic effect was specific to the transformed phenotype. Primary rat Schwann cells were isolated from the sciatic nerve and treated with the MEK inhibitors. Morphological analysis (Fig. 9) demonstrated that neither PD98059 nor U0126 at concentrations up to 20 µM had much effect on primary Schwann cells. Assays of cell viability confirmed that 99% of the Schwann cells were viable under control incubations and that at least 96% of the cells remained viable after 72 h treatment with PD98059 or U0126. Treatment with 10 µM or 20 µM PD184352, however, induced a profound
morphological change in the Schwann cell cultures (Fig. 8) that indicated cytotoxicity. In agreement with the morphological assessment, PD184352 at 20 µM reduced cell viability to 38% within 48 h of treatment.
Discussion

NF1 is one of the most common autosomal dominant human genetic diseases, with about half of all cases representing new mutations that are not present in either parent (Klose et al., 1998). The disease is highly penetrant but has variable clinical presentation, with manifestations in cutaneous, neurological, endocrine, cardiovascular, gastrointestinal, haematological, and orthopaedic systems. Although this collage of symptoms could be due to varied loss of several putative functions of the neurofibromin protein in patients with different mutations, there is substantial evidence that the most prominent NF1 symptoms, including the increased risk of malignancies, derive from increased Ras-GTP levels and proliferation in neural crest-derived cells (Klose et al., 1998). The most powerful evidence for this contention may be analysis of the Arg to Pro mutation at residue 1276 of neurofibromin. This point mutation does not grossly alter the secondary or tertiary structure of neurofibromin; nor does it reduce its expression level. This mutation does, however, completely block the Ras-GAP activity of neurofibromin. The family that carries this mutation has multi-symptom NF1 disease, including malignant schwannoma (Klose et al., 1998).

A rational therapy for NF1 may therefore be to inhibit Ras signaling. A direct approach to inhibit Ras would be to block its required post-translational processing, e.g. by inhibition of farnesyl transferase. In pre-clinical studies, for example, FTIs have been shown to block proliferation of a human neurofibrosarcoma cell line (Yan et al., 1995), and of Schwann cells derived from a neurofibromin-deficient mouse (Kim et al., 1997). FTI administration also reversed learning deficits in the heterozygous NF1⁺⁻ mouse (Costa et al., 2002). There is a
currently accruing phase 2 clinical trial of an FTI for progressing plexiform neurofibromas in NF1 (protocol 01-C-0222). It has previously been noted that FTI treatment is not very effective at inhibiting proliferation of haematopoietic cells from neurofibromin-deficient mice, which the authors attributed to lack of ability of the FTI to inhibit the processing of K- and N-Ras (Mahgoub et al., 1999). Functional connections between neurofibromin deficiency and N-Ras activation have also previously been made in astrocytes (Bajenaru et al., 2001). Our data show that 3 human malignant schwannoma cell lines all predominantly express N-Ras and K-Ras, and that those isoforms are activated in the neurofibromin-deficient lines. The absence of detectable H-Ras activation in these neurofibrosarcoma cell lines could suggest that FTIs may not be particularly effective against these tumors, although it is possible that the actual therapeutic target of FTIs may be another farnesylated protein, such as RhoB (Mattingly et al., 2002). It is also significant that Q-RT-PCR demonstrates that, while the mRNA for N-Ras is the most abundant as we would expect from the western blotting results, the mRNA for H-Ras is present at similar or greater levels than that for K-Ras. Thus our inability to detect H-Ras by western blotting could have several explanations. On the one hand, the level of H-Ras protein may indeed by very low, which would suggest that there is strong control of H-Ras expression at the translational or degradation levels. On the other hand, and despite our attempts to standardize the detection of the Ras isoforms by western blotting using recombinant standards, it may be that the H-Ras isoform-selective antibody is insufficiently sensitive.

Ras controls cell proliferation and survival through its activation of downstream signaling cascades. Ras-GTP is a potent activator of the Raf/MEK1,2/ERK1,2 MAP kinase cascade. Our data confirm that Ras is constitutively activated in neurofibromin-deficient schwannoma cells
and that this Ras activation is accompanied by a constitutive increase in ERK1,2 phosphorylation. It is therefore reasonable to investigate whether small molecules that can prevent ERK1,2 activation may be useful in NF1.

The most promising pharmacological target within the Raf/MEK/ERK cascade has been MEK (Allen et al., 2003). Three compounds have been described as selective inhibitors of MEK. The first of these was PD98059, which inhibits MEK1 at low μM concentrations (Dudley et al., 1995). This compound has been extensively used in in vitro studies, including the demonstration that 50 μM PD98059 can block the increased proliferation of astrocytes that are haploinsufficient for neurofibromin (Gutmann et al., 1999). A second effective MEK inhibitor is U0126, which inhibits MEK1,2 in the high nM range in tissue culture studies (Favata et al., 1998). A third MEK inhibitor is PD184352/CI-1040, which is both more potent and has sufficient bioavailability that it can be used in mice (Sebolt-Leopold et al., 1999) and the clinic (Allen et al., 2003). To differing degrees the MEK inhibitors suppressed the proliferations of the three schwannoma cell lines, and reduced their cellular phosphorylated ERK1/2 contents. The rank order for MEK potency and efficacy for these two activities was PD184352 > U0126 > PD98059. The anti-proliferative activities of the three MEK inhibitors were only seen at concentrations that reduced phosphorylated ERK1/2 contents. However, in the cases of PD98059 and U0126, ERK1/2 inhibition did not equate with anti-proliferative effects. For example, whereas exposure to 1 μM U0126 or 10 μM PD98059 eliminated ERK1/2 activities in all three cell lines grown in 1% serum (Fig. 3), those concentrations of U0126 had no effect on proliferation, and PD98059 was only weakly cytostatic (Fig. 5). Higher concentrations of U0126 were cytotoxic. In contrast, the anti-proliferative and ERK1/2 inhibitory activities of PD184352
correlated with one another. In addition, PD184352 differed from the other MEK inhibitors in its ability to induce apoptosis. Although U0126 was cytotoxic to all three cell lines, the death that occurred did not have the characteristics of apoptosis. Either PD184352 induces a pro-apoptotic effect that is unique to itself, or PD184352 lacks an activity that is induced by PD98059 and U0126 that suppress the initiation/development of apoptosis. Interestingly, PD184352 was without any notable pro-apoptotic effect on SKNSH human neuroblastoma cells or MCF10A human breast epithelial cells (data not shown). Thus the cytotoxic effect of PD184352 is probably dependent on one or more aspects of the Schwann cell lineage, although a less likely possibility is that it could depend on a property of primary cells that is retained by the schwannoma cells but not the other two cell lines. One relevant property of Schwann cells may be that they are sensitive to cytokine-induced apoptosis (Skoff et al., 1998). It will be interesting, therefore, to explore whether the cytotoxic effect of PD184352 may be related to the cytotoxic program normally induced by cytokines. If so, such a finding could imply that schwannomas may also retain some susceptibility to cytokine-induced apoptosis.

One potential distinction in the actions of the MEK inhibitors could be in their abilities to inhibit the MEK5 that is responsible for ERK5 phosphorylation. Both PD98059 and U0126 have been reported to be effective inhibitors of MEK5 (Kamakura et al., 1999; Karihaloo et al., 2001), whereas PD184352 is not (Squires et al., 2002). Our data, however, suggest that this distinction is unlikely to explain the differential induction of apoptosis in malignant schwannomas following treatment with MEK inhibitors. We could not detect ERK5 phosphorylation in the NF1 lines under the growth conditions in which the cytostatic/pro-apoptotic effects were defined. Since ERK5 does not seem to be significantly activated in the relevant growth environment, inhibition
of this pathway is unlikely to explain the effects of the drugs. Nevertheless, it is possible that there is a particular fraction of ERK5 that is activated and critical for a pro-survival outcome, but that it is not adequately detected during assays of total cellular ERK5 phosphorylation. It is significant, therefore, that pretreatment with the MEK inhibitors at concentrations sufficient to inhibit EGF stimulation of ERK1,2 phosphorylation had no effect on EGF-stimulated ERK5 phosphorylation. Our data agree somewhat with the study by Mody et al. (Mody et al., 2001), who compared the effects of the 3 MEK inhibitors against both ERK1,2 and ERK5 phosphorylation in HeLa cells. Their data show that higher concentrations of PD98059, U0126, and PD184352 are required to inhibit ERK5 phosphorylation than to inhibit ERK1,2 phosphorylation. In that study, which was performed by adding drugs to cells that had been starved for serum overnight prior to treatment, PD98059 inhibited ERK5 phosphorylation only partially with a 100 µM pretreatment; while U0126 and PD184352 partially inhibited ERK5 phosphorylation at 10 µM. Our experiments were performed in cells in the presence of 1% fetal calf serum to ensure that data were collected under the same experimental conditions used to investigate effects on cell proliferation and viability. Under these conditions, we found that although PD184352 was less potent as a MEK inhibitor than it is under serum-free conditions (data not shown), it was still highly effective. Both PD184352 at 1 µM and U0126 at 10 µM were able to suppress signaling to p90RSK activation through the MEK/ERK1/2 pathway for at least 48 hours’ despite their lack of effect on ERK5 phosphorylation. Thus it is unlikely that effects on ERK5 activation explain the differential effects of the MEK inhibitors on malignant schwannomas.
A rational therapy for NF1 will presumably target the underlying molecular defect that drives the aberrant proliferation of neuroectodermal cells. It is noteworthy, therefore, that the effects of all the MEK inhibitors were largely indistinguishable between the NF1 cell lines and the control malignant schwannoma from a non-NF patient. The control cell line maintains neurofibromin expression and low levels of Ras and ERK1,2 activation, and yet was equally sensitive to the anti-proliferative effects of the MEK inhibitors. The MEK inhibitors were expected to be most effective against tumors, such as those with oncogenic Ras, that had constitutively high levels of ERK MAP kinase activation (Sebolt-Leopold et al., 1999). This does not seem to be a decisive determinant of response, however, since some tumors with high ERK phosphorylation are resistant to the effects of MEK inhibitors, while other malignancies with low constitutive ERK phosphorylation may be sensitive (Allen et al., 2003). Our data with the malignant schwannoma cell lines suggest that PD184352 may provide an effective therapy for this tumor type whether it is NF1-derived or sporadic, although the pro-apoptotic effect of PD184352 in primary rat Schwann cells indicates that its effects on the peripheral nervous system should be carefully assessed.
Acknowledgements

We thank Dr T. Glover for providing the malignant NF1 cell lines, Dr. D. Scoles for providing the STS-26T cell line, and Dr J. Jackson for providing the expression vectors for Myc-tagged Ras proteins. Dr R. Lisak provided critical expertise and support from the Mary Parker Neuroscience Fund for the studies with rat Schwann cells. We also acknowledge the generous gift of PD184352/CI-1040 from Pfizer, Inc., and technical support for this project from A. Akpabio and I. Laer.
References


Footnote:

This work was supported by grants DAMD-17-00-1-0544 and DAMD-17-03-1-0182 from the Department of the Army. The content of the information does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred. This project was assisted by the services of the Imaging and Cytometry and Genomics Facility Cores, which are supported by National Institutes of Environmental Health Sciences and National Cancer Institute grants P30ES06639 and P30CA22453.
Figure Legends

Figure One. Constitutive Ras activation in neurofibromin-deficient schwannoma cells.

A. Extracts of cell lines (approximately 20 µg protein per lane) derived from NF1 tumors [lines ST88-14 (88) and NF90-8 (90)] or from a sporadic, non-NF1 tumor [line STS-26T (26)] were subjected to western blotting analyses of neurofibromin, Ras-GAPp120, and β-tubulin. A representative result from 3 independent experiments is shown. No neurofibromin protein was detected in either of the NF1 lines, even with prolonged exposures to film. B, Lysates were prepared from cells that had been cultured for 24 h in the absence of serum (U), or followed by acute serum stimulation for 5 min (S), or in the continuous presence of serum (C). Two thirds of the lysate (typically 100 µg protein) was subjected to affinity purification for active Ras with the Ras binding domain of Raf (RBD) coupled to GST; one third of the lysate (typically 50 µg protein) was precipitated with tricholoroacetic acid (TCA) to analyze the total Ras content. Samples were assayed for Ras by SDS-PAGE and western blotting, using a pan-reactive Ras monoclonal antibody. Results are representative of 3 independent experiments.

Figure Two. Constitutive N-Ras and ERK MAP kinase activation in neurofibromin-deficient schwannoma cells.

A. Ras proteins with Myc epitope tags were expressed in COS-7 cells and the lysates run in parallel to characterize monoclonal antibodies against H–, K–, and N–Ras. Relative loading of the Ras standards was confirmed by western blotting with the 9e10 anti-Myc monoclonal antibody (upper panels). Ras activation experiments were performed as for Fig. 1B, except that...
the blots were probed with the isoform-selective Ras antibodies; the data for H– and K–Ras are from prolonged exposures, the data for N–Ras is from a brief exposure. The results are representative of data from 6 independent experiments. B, The mRNAs for H–, K–, and N–Ras were amplified by Q-RT-PCR relative to GAPDH as an internal standard. The results shown are mean ± S.E.M. from 3 independent RNA preparations that were analyzed separately for each cell line. The data for relative expression levels are expressed as ΔCt values, and reflect the cycle at which specific product is detected relative to GAPDH. Thus a lower value represents a higher level of that mRNA, and is on a log2 scale. C, Monolayers of malignant schwannoma cell lines were cultured as shown for 24 h prior to lysis and assay of active and total ERK1,2 content by western blotting. The results shown are representative of 5-8 independent experiments. Densitometry of films from these experiments demonstrated that, when cultured in 5% fetal calf serum, ST88-14 cells showed a 3-fold increase and NF90-8 cells showed an 8-fold increase in the amount of active ERK1,2 relative to that present in STS-26T cells. When cultured in serum-free medium, ST88-14 cells showed a 1.6-fold increase and NF90-8 cells showed a 1.4-fold increase in the amount of active ERK1,2 relative to that in STS-26T cells.

**Figure Three. Inhibition of ERK1,2 phosphorylation in malignant schwannoma cell lines.**

Monolayers of ST88-14, NF90-8 and STS-26T malignant schwannoma cells were shifted to media with 1% fetal calf serum for ~24 h. Cultures were then pretreated with MEK inhibitors or an equivalent volume of the DMSO vehicle for 1 h prior to addition of 10 ng/ml EGF or its vehicle (0.1 mg/ml bovine serum albumin) for 5 min. Data shown are western blots for
active/dually-phosphorylated ERK1,2 that were then stripped and reprobed for total ERK1,2 and are representative of results from 3 independent experiments.

**Figure Four. Inhibition of p90RSK phosphorylation in STS-26T malignant schwannoma cells.** Monolayers of STS-26T cells were shifted to media with 1% fetal calf serum for 48 h of culture. Cultures were treated with MEK inhibitors ($U$: 10 µM U0126; $P$: 1 µM PD184352; $D$: DMSO vehicle) either continuously from the time of media change or those inhibitors were added for the last 1 h of culture. The cells were then stimulated by addition of 10 ng/ml EGF or its vehicle (0.1 mg/ml bovine serum albumin) for 10 min and cell lysates prepared. Data shown are western blots for active/dually-phosphorylated p90RSK that were then stripped and reprobed for total p90RSK.

**Figure Five. Cytostatic and cytotoxic effects of MEK inhibitors on malignant schwannoma cell lines.** Approximately 22 h after plating in media with 1% fetal calf serum, cultures of ST88-14 (panels A-F), NF90-8 (panels G-L), and STS-26T (panels M-R) were harvested for analyses of cell numbers and trypan blue permeability (an index of viability). Other cultures were treated with DMSO (closed circle), or 0.1 µM (open diamond), 1 µM (open circle), 10 µM (open triangle), 20 µM (open square), or 40 µM (open upside-down triangle) MEK inhibitor. These cultures were harvested at various times after treatment as shown. Data represent means ± SD of three culture dishes. Similar data were obtained in a second independent experiment.
Figure Six. Cell cycle analyses of malignant schwannoma cell lines treated with MEK inhibitors. Approximately 22 h after plating in media with 1% fetal calf serum, cultures of ST88-14 (A,B) and STS-26T (C,D) cells were treated with DMSO, or 1, 10 or 20 µM MEK inhibitor. Specific conditions are noted in the figure. Treated cultures were harvested 24 and 48 h after treatment for analyses of DNA contents by flow cytometry. The row of three numbers in each of the figures in panels A and C indicate the cell cycle phase distribution of the non-apoptotic population, as determined by MODFIT. Similar data were obtained in an additional independent experiment.

Figure Seven. Pro-apoptotic activities of MEK inhibitors in ST88-14 cell cultures.

Subconfluent ST88-14 cultures were re-fed with media containing 1% fetal calf serum ~20 h prior to exposure to DMSO or varied concentrations of MEK inhibitors. A, ST88-14 cultures were photographed ~48 h after being treated with DMSO or 10 µM PD184352. B, ST88-14 cultures were harvested at various times after treatment with DMSO (cross), 10 µM PD184352 (open triangle), 40 µM U0126 (open square) or 40 µM PD98059 (open circle). Data represent means ± SD of triplicate analyses. Similar data were obtained in a second independent experiment. C, ST88-14 cultures were treated with either DMSO or 10 µM PD184352 for the indicated time prior to being harvested for analyses of pro-caspase-3 activation by western blot. Arrow indicates the 17 kDa cleavage product of pro-caspase-3. Similar data were obtained in a second independent experiment.
Figure Eight. **ERK5 phosphorylation in malignant schwannoma cell lines.** Monolayers of ST88-14, NF90-8 and STS-26T malignant schwannoma cells were shifted to media with 1% fetal calf serum for ~24 h. Cultures were then pretreated with MEK inhibitors or an equivalent volume of the DMSO vehicle for 1 h prior to addition of 10 ng/ml EGF or its vehicle (0.1 mg/ml bovine serum albumin) for 5 min. Data shown are western blots for active/phosphorylated ERK5 that were then stripped and reprobed for total ERK5 and are representative of results from 3 independent experiments.

Figure Nine. **Cytotoxicity of PD184352 in primary rat Schwann cell cultures.** Primary rat Schwann cell cultures were photographed after 72 h treatment with MEK inhibitors as indicated. Similar data were obtained in a second independent experiment.
Table 1. Inhibition of Anchorage-independent Growth by MEK Inhibitors

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<sup>a</sup>indicates the concentration that demonstrated a 50% inhibition of growth in soft agar as compared to vehicle treated controls.
Figure One

A

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Figure Three

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**Active p90RSK**

**Total p90RSK**
Figure Five
Figure Six

A. 

B. 

C. 

D.
Figure Seven

A

Control
PD184352 (10 M)

B

![Graph showing time vs. nmol product/min/mg protein](chart.png)

C

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Pro-caspase-3
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Figure Nine