

Primary research

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Sulindac derivatives inhibit cell growth and induce apoptosis in primary cells from malignant peripheral nerve sheath tumors of NF1-patients

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Abstract

Background: Malignant peripheral nerve sheath tumors (MPNSTs) are neoplasms leading to death in most cases. Patients with Neurofibromatosis type I have an increased risk of developing this malignancy. The metabolites of the inactive prodrug Sulindac, Sulindac Sulfide and Sulindac Sulfone (Exisulind) are new chemopreventive agents that show promising results in the treatment of different cancer types. In this study we examined the antineoplastic effect of these compounds on primary cells derived from two MPNSTs of Neurofibromatosis type I patients.

Results: Exisulind and Sulindac Sulfide showed a dramatic time- and dose-dependent growth inhibitory effect with IC50-values of 120 μ M and 63 μ M, respectively. The decrease in viability of the tested cells correlated with induction of apoptosis. Treatment with 500 μ M Exisulind and 125 μ M Sulindac Sulfide for a period of 2 days increased the rate of apoptosis 21-27-fold compared to untreated cells. Reduced expression of RAS-GTP and phosphorylated ERK1/2 was detected in treated MPNST cells. Moreover, elevated levels of phosphorylated SAPK/JNK were found after drug treatment, and low activation of cleaved caspase-3 was seen.

Conclusions: Our results suggest that this class of compounds may be of therapeutic benefit for Neurofibromatosis type I patients with MPNST.

Background

The malignant peripheral nerve sheath tumor (MPNST) is one of the most aggressive neoplasias of soft tissue, characterized by neurological deficits, pain and a rapid increase in size. Surgical removals or amputations do not prevent from recurrences with increased morbidity and fatality. More than 50% of individuals with MPNSTs also have neurofibromatosis type 1 (NF1), and approximately

10% of NF1 patients develop MPNSTs, of whom only 21% survive for five years after diagnosis [1,2]. NF1 is a common genetic disease with an incidence of 1:3500 [3,4], caused by mutations of the *NF1* tumor suppressor gene located on chromosome 17q11.2 [5]. One proposed function of the *NF1* gene product neurofibromin is the downregulation of activated RAS, based on the conversion of RAS-GTP to RAS-GDP via its GTPase enzymatic activity

[6]. However, the loss of *NF1* gene function is not the unique molecular lesion in these tumors, inactivation of *p53* and *p16* gene regions seem to play a crucial role in the malignant transformation of MPNSTs [7,8]. To this date, there is no effective treatment of *NF1* patients with this malignancy [9]. Our interest focused on the therapeutic use of oral, non-toxic agents which may be able to control the progression of MPNSTs.

Sulindac, a nonsteroidal anti-inflammatory drug (NSAID), is known to prevent recurrence and reduce colorectal polyps in number and size in patients with familial adenomatous polyposis [10]. Sulindac is a pro-drug that is metabolized to a Sulfide and Sulfone derivative *in vivo*. The biological mechanism of the antineoplastic effect of both Sulindac metabolites is the selective induction of apoptosis, found in human breast-, lung-, prostate- and colon-cancer cell lines [11-14]. Furthermore, the tumor-preventing effect of the Sulfone metabolite has been shown in animal models of colon [15], breast [16] and lung cancer [17].

The anti-inflammatory properties of the Sulfide metabolite are supposed to be mediated by cyclooxygenase (COX) inhibition that results in a reduction of prostaglandin synthesis [18]. In contrast, the Sulfone metabolite (Exisulind) is a pro-apoptotic drug that inhibits cancer growth without inhibiting COX and independent of prostaglandin synthesis, *p53* induction and cell cycle arrest [11,13,18,19]. In a colon cancer cell line Exisulind showed induction of apoptosis as a result of its ability to inhibit cGMP-dependent phosphodiesterase (PDE), followed by elevated cGMP-levels and increased protein kinase G (PKG) activity [20]. Furthermore, PKG activates the JNK pathway via phosphorylation and activation of MEKK1 [21,22]. Activation of JNK plays a critical role in gene transcription for mediation of apoptosis, usually involving cleavage of caspases and PARP [23]. Recent studies implicate that both Sulindac metabolites inhibit the RAS signaling pathway, reducing the levels of activated (phosphorylated) ERK1/2 [24,25], that indirectly regulates gene transcription responsible for enhanced cell proliferation and differentiation. However, the exact mechanisms of apoptosis induced by Exisulind and Sulindac Sulfide remain unknown.

In clinical studies Exisulind showed antineoplastic effects and has been well tolerated by most colorectal and prostate cancer patients [19,26]. Lung cancer patients treated with twice-daily oral doses exhibited peak concentrations of Exisulind that were equivalent to those required for *in vitro* effects [27]. Exisulind applied in combination with the semisynthetic drug Docetaxel indicates additive or synergistic chemopreventive properties in *in vitro* and *in vivo* experiments [28].

In this study, we examined the effect of both Sulindac metabolites on two primary MPNST cell lines from *NF1* patients *in vitro*. Additionally, we tested the activity of both drugs on the RAS- and JNK-signaling pathway.

Results

Cell growth inhibition

Exisulind and Sulindac Sulfide were examined for their ability to inhibit growth of two MPNST cell lines. Exisulind was added to the media at a final concentration of 100, 250 and 500 μM and Sulindac Sulfide of 50, 100 and 125 μM . Both Sulindac derivatives exhibited a dramatic dose-dependent growth inhibition of the two MPNST derived cell lines (Fig. 1). This effect was more apparent after 4 days of treatment than after 2 days (Fig. 1). The corresponding mean IC_{50} values of both cell lines after 96 h of treatment were 120 μM for Exisulind, and 63 μM for Sulindac Sulfide. The proliferation rate of cells did not change when the medium was supplemented with DMSO at concentrations used in drug treatment (0.2%).

Viability and apoptosis

To determine the relation between growth inhibition and apoptosis, we measured the number of viable cells and the amount of DNA-fragmentation simultaneously. The viability of the cells decreased drastically after 48 h of drug treatment (Fig. 2a,2b). At the highest applied concentrations of Exisulind and Sulindac Sulfide, only 1–33% of the cells were actively metabolizing compared to cells treated with the vehicle alone. As shown in figures 2c,2d, the reduction of viable cell number is paralleled by the increase of apoptosis rates up to 25–45% for 500 μM Exisulind and up to 43–60% for 125 μM Sulindac Sulfide, determined by photometrical quantification of DNA-fragmentation. Supplementation of DMSO to the culture medium at concentrations used for drug treatment had no effect on cell death.

Apoptosis induction by Sulindac metabolites was further examined by TUNEL assay on both cell lines. Figure 3 displays a time dependent induction of apoptosis in MPNST cell line S462 treated with 500 μM Exisulind and 125 μM Sulindac Sulfide. In both cell lines, the apoptosis rate increased 3–5 fold after 24 h treatment with Exisulind in comparison to untreated cells, and 7–21 fold after 48 h treatment. Similarly, 24 h treatment with Sulindac Sulfide caused a 3–4 fold increase in the proportion of apoptotic cells, and a 10–27 fold increase after 48 h treatment (Table 1).

Apparent alterations in cell morphology and detachment from the culture surface were observed after 48 h of treatment with 125 μM Sulindac Sulfide and 500 μM Exisulind in both cell lines, when cells became sparse and rounded (Fig. 4b). Cell shrinkage, nuclear condensation and

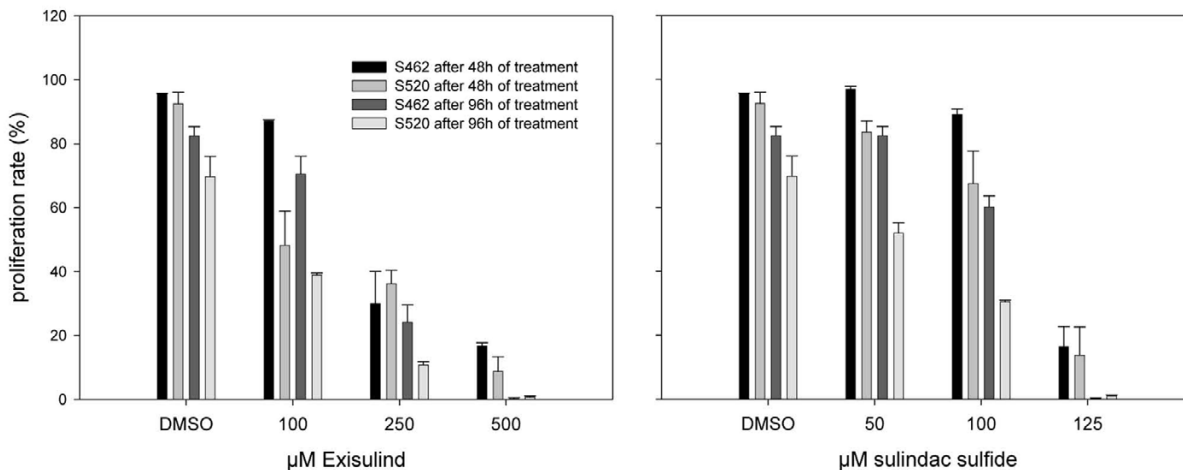


Figure 1

Growth inhibition of MPNST cell lines S462 and S520 Cells were plated at a density of 15000/well on 12 mm coverslips and treated with indicated concentrations of Exisulind and Sulindac Sulfide for 48 h and 96 h. Cell growth was measured by BrdU-incorporation. The final DMSO concentration used here did not exceed 0.2% and had no effect on cell growth. The values represent the means and standard deviations of triplicates.

formation of apoptotic bodies were visible on propidium iodide labeled cell nuclei, which are classical characteristics of apoptosis and not necrosis (Fig. 4c).

RAS signaling

To ascertain the effect of the Sulindac metabolites on the RAS signaling pathway, we determined the levels of RAS-GTP by affinity precipitation and western blotting with a monoclonal RAS antibody, as well as activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). After 24 h treatment with the Sulindac metabolites, reduction of RAS-GTP levels were observed in both cell lines, slightly more pronounced in Exisulind treated cells (Fig. 5a). In concordance, phosphorylation of ERK1 (p44) and ERK2 (p42) was also reduced after the treatment. No changes in basal ERK1/2 levels were observed (Fig. 5a). Under serum-free conditions a significant reduction of phospho-ERK1/2 activation was detected already after 8 h of treatment (data not shown).

The effects of Sulindac metabolites were further examined after stimulation with epidermal growth factor (EGF). The RAS-GTP level did not change after EGF stimulation, neither after additional treatment with Sulindac derivatives. Phosphorylation of ERK1/2 was upregulated by EGF stimulation at similar extents in treated and untreated cells (Fig. 5c). Phosphorylation of AKT, an inhibitor of several apoptotic pathways and important player in survival sign-

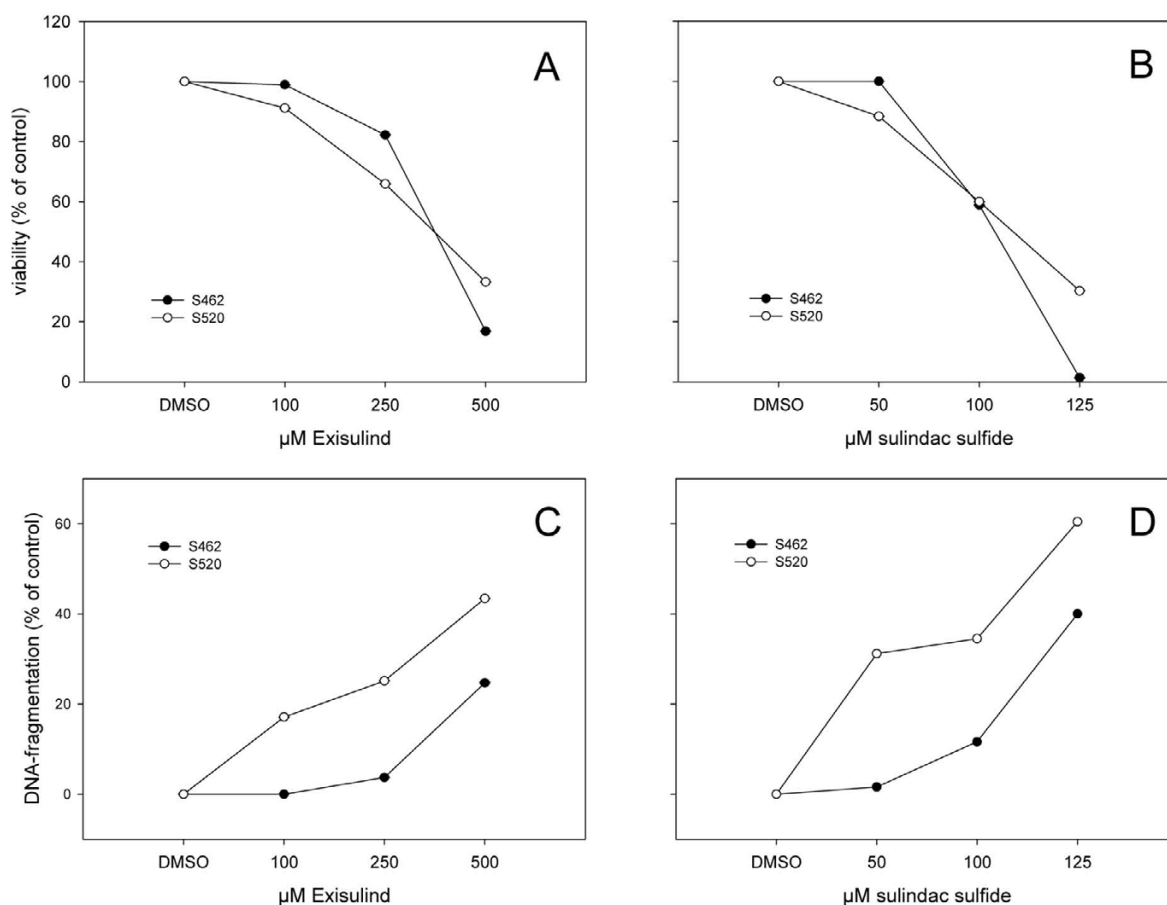
aling, also increased upon EGF stimulation. Additional treatment with Sulindac metabolites did not alter the EGF-elevated phospho-AKT level.

SAPK/JNK and caspase-3 activation

The stress-activated protein kinase/ Jun-terminal kinase SAPK/JNK, a mediator of apoptosis, was activated (phosphorylated) 16 h after treatment with either of the Sulindac metabolites (not shown), and increased further after 24 h and 48 h (Fig. 6). Treatment with DHA, a drug known to induce apoptosis in mouse MPNST cells (personal communication of Andreas Kurtz) had no effect on phosphorylation of this protein.

In addition, cleavage of caspase-3, a common downstream regulator of SAPK/JNK [23,34], was assayed. No cleaved caspase-3 was detected by western blotting after 24 h and 48 h treatment with Sulindac metabolites or DHA (Fig. 6). Furthermore, no consistent differences in caspase-3 activation were found after 8 h–16 h of treatment (data not shown).

In order to verify the obtained results, an additional colorimetric caspase-3 assay was performed. After 24 h of treatment, Exisulind and Sulindac Sulfide treated cells showed only low levels of caspase-3 enzymatic activity, 0.7 and 3.5 Units respectively. By comparison, 12.7 Units of protease activity were measured after addition of

**Figure 2**

Correlation of growth inhibition (reduction of viable cell number) and apoptosis The increase of apoptotic cells is paralleled by reduction of viable cell number at the corresponding concentrations. A-B: Viability of cell lines S462 and S520 were measured by their ability to reduce XTT metabolically to a purple formazan product after 48 h of treatment with Exisulind and Sulindac Sulfide at different concentrations. The percentage of viable cells was determined setting the absorbance of cells treated with the vehicle as 100%. C-D: Percentages of apoptotic cells were determined by photometric quantification of DNA- and histone-fragmentation after 48 h of drug treatment. The absorbance mean values of treated samples (triplicates) were normalized to the corresponding control of untreated cells and the maximal absorbance from the test compound included in the assay was used as positive control (100% apoptosis).

Staurosporin (positive control) to the culture medium for 24 h in the same cells. Thus, the Sulindac derivatives were shown to induce apoptosis in the presence of low caspase-3 activity.

Discussion

In this study, we demonstrated that the Sulfide and Sulfone Sulindac metabolites inhibit the proliferation of tumor cells derived from human MPNSTs. We observed induction of apoptosis in association with reduction of viable cell number, and showed that apoptosis primarily

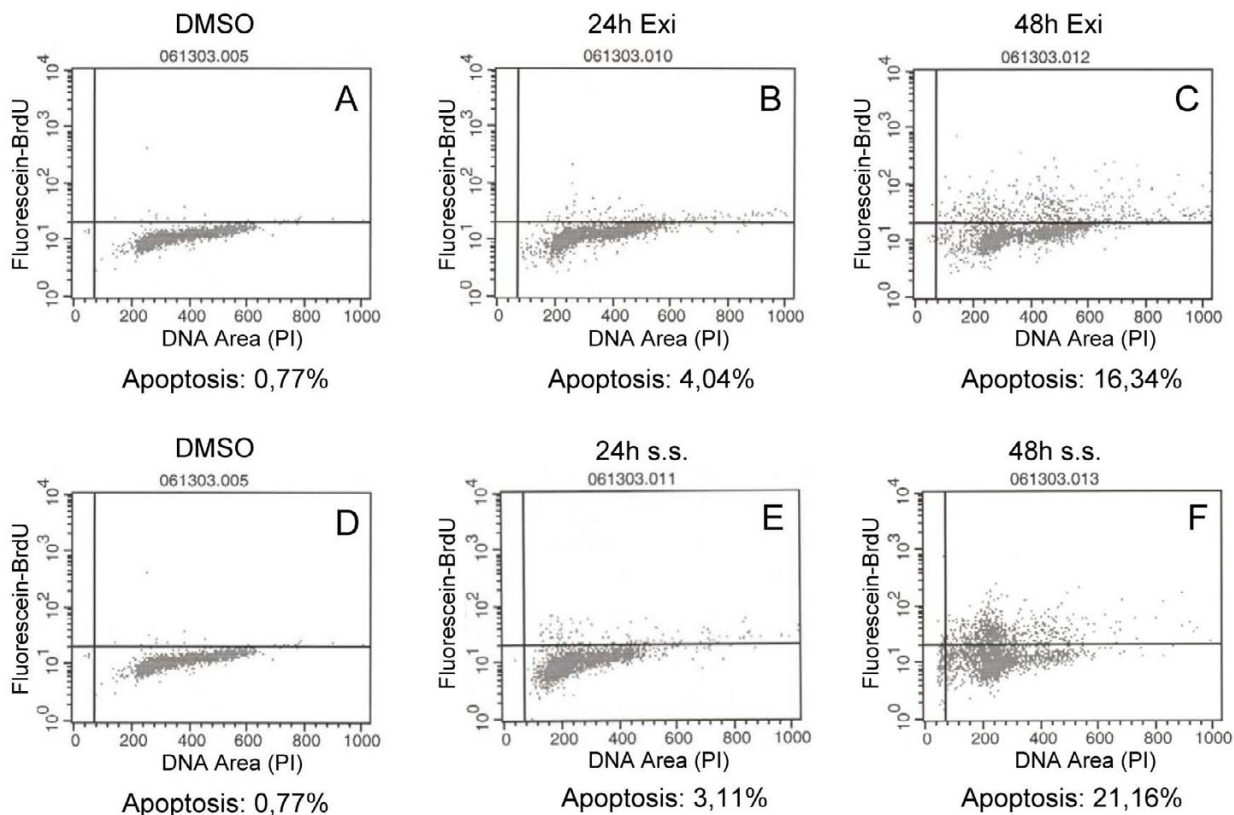


Figure 3

TUNEL assay of cell line S462 showing time dependent induction of apoptosis Cells were either treated with 0.2% DMSO for 48 h (A, D), 500 μ M Exisulind (Exi) for 24 h (B) and 48 h (C) or 125 μ M Sulindac Sulfide (s. s.) for 24 h (E) and 48 h (F). The percentage of gated cells (upper box) represents the ratio of apoptotic cells (FITC-BrdU) to the total number of cells (PI), values are presented beneath each display.

Table 1: Percentage of apoptotic cells after 24 h and 48 h of drug-treatment

cell line	DMSO	24 h treatment		48 h treatment	
		Exisulind	S. Sulfide	Exisulind	S. Sulfide
S462	0,77	4,04	3,11	16,34	21,16
S520	4,66	16,04	16,18	33,56	48,03

contributes to growth inhibition of the MPNST cells. Exisulind and Sulindac Sulfide completely inhibited MPNST cell growth due to induction of apoptosis at doses of 500 μ M and 125 μ M, respectively, comparable to those used in previous studies on other tumor types [11,13,14]. The drugs were effective at these doses in 10% serum,

while in serum free conditions induction of apoptosis was more rapid and growth inhibition more complete (data not shown). A previous study showed that Exisulind induced apoptosis only in prostate cancer cells, but not in normal epithelial prostate cells [13]. We used normal (*NF1*+/-) fibroblasts from the MPNST-donor as control

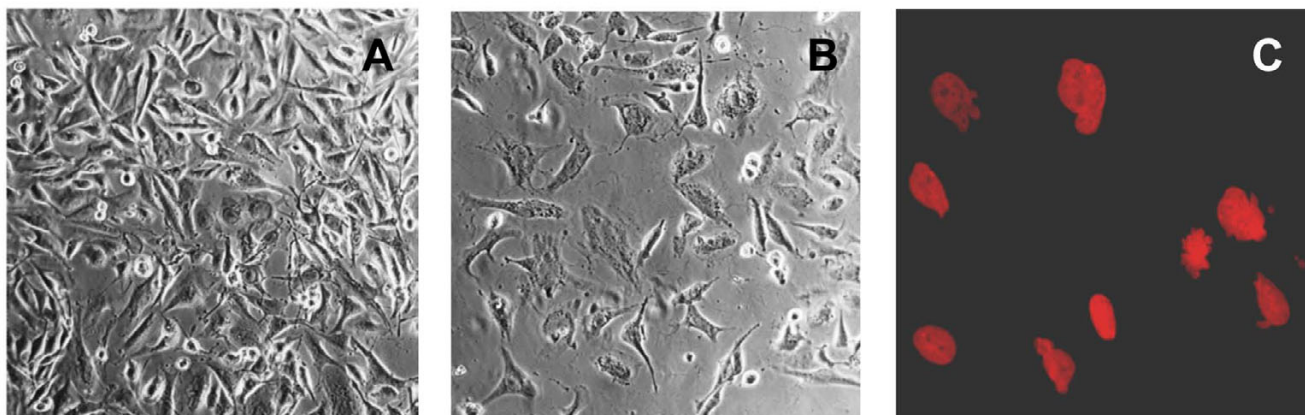


Figure 4

Changes in cell morphology characteristic for apoptosis Morphologic changes became evident after 48 h of treatment with 500 μ M Exisulind or 125 μ M Sulindac Sulfide. A-C: Phase contrast photomicrographs of S462 cells before (A) and after Exisulind (B) treatment. Cell shrinkage, nuclear condensation and formation of apoptotic bodies shown on immunocytochemically labeled cell nuclei with PI after Sulindac Sulfide treatment (C) are classical characteristics of apoptosis and not necrosis.

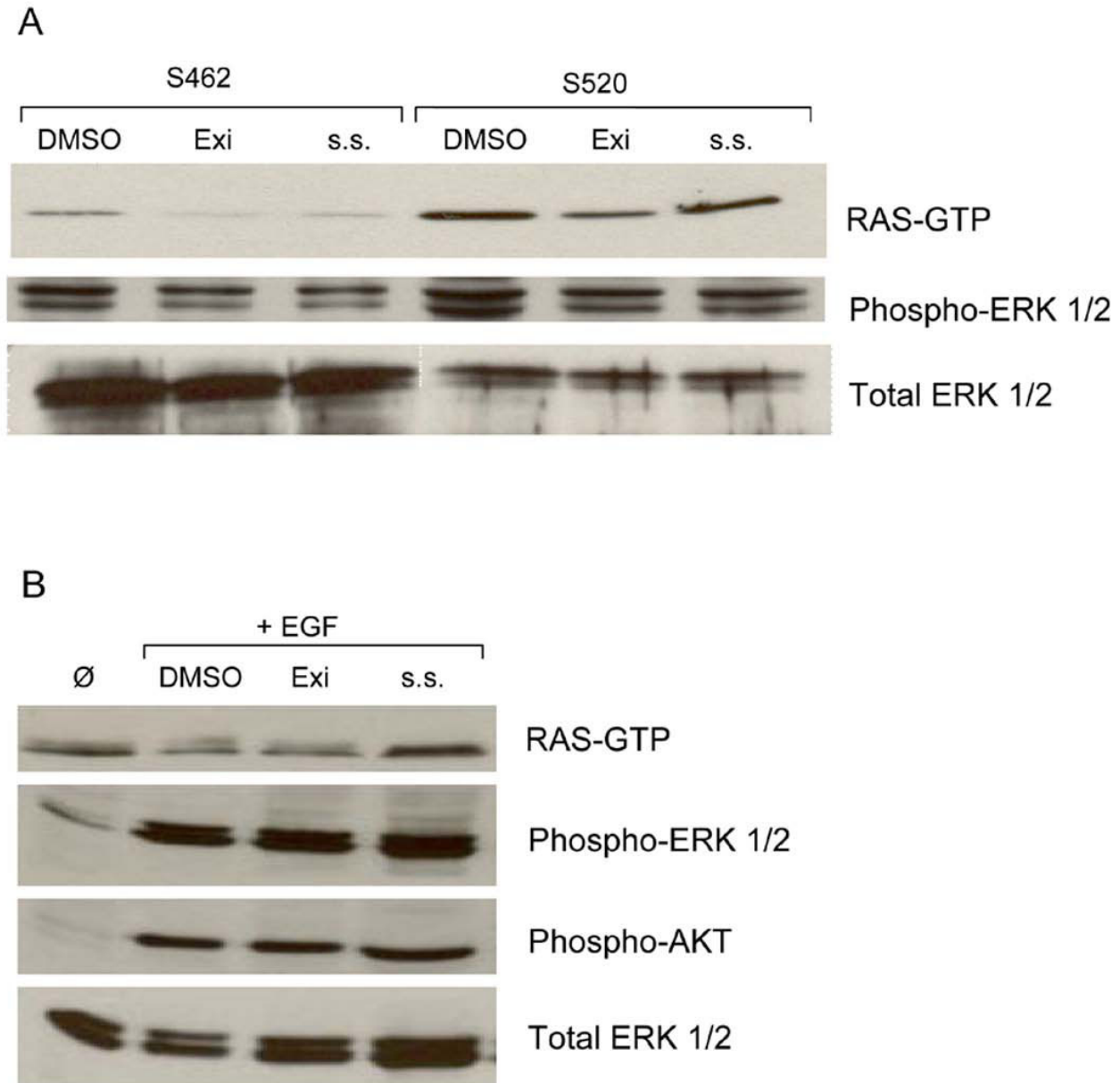
cells to test the effects of Exisulind and found that growth of these cells was only slightly inhibited after drug treatment (data not shown). Since Exisulind causes phosphodiesterase (PDE) inhibition in tumor cells [20,21], this differential effect on normal versus tumor cells may in part be explained by the overexpression of cGMP-PDE in most neoplasias compared to normal cells [19].

Increased levels of RAS-GTP have previously been shown in MPNSTs from NF1 patients [29-31], caused perhaps by loss of the RAS-GAP-function of the *NF1* gene product, neurofibromin. In the present study both, Exisulind and Sulindac Sulfide significantly reduced the levels of RAS-GTP and phospho-ERK1/2 – a downstream effector in the RAS pathway. Basal ERK1/2 levels were not affected by the treatment, thus excluding cytotoxic effects of the drugs and demonstrating specific effects on the RAS-ERK activation cascade during MPNST-cell growth inhibition. In mouse MPNST-derived cells, epidermal growth factor (EGF) was shown to stimulate proliferation in association with activation of phospho-ERK1/2 [32]. Interestingly, exposure of the human MPNST-derived cells to EGF did not increase RAS-GTP levels, yet strongly induced phosphorylation of ERK1/2. One explanation for this effect may be that MPNST cells express maximal levels of RAS-GTP, thus no additional effect of EGF could be detected. Apparently, activation of ERK1/2 by EGF stimulation was independent of increased RAS-GTP levels. In addition, no effects on RAS-GTP and phospho-ERK1/2 levels by Sulindac derivatives were observed in the presence of EGF. Since EGF-stimulated cells still undergo drug-induced

apoptosis, the observed reduction in cell number is independent of the state of RAS-pathway activation.

While apoptosis induction seems to be the predominant effect of Sulindac derivatives on MPNST-cells, AKT, an active inhibitor of apoptosis, is most likely not involved in mediating this effect, since its phosphorylated form is not present in these cells under any conditions, besides the EGF-stimulated condition. On the other hand, it was previously shown that the JNK-pathway is associated with the biological mechanism of action of the Sulindac metabolites in human colon cells [21]. In MPNST-cells, the activation of phospho-SAPK/JNK preceded apoptosis and reached its peak at 24 h treatment, while the observed apoptosis rate still increased after 48 h of treatment, indicating a causative association. Furthermore, Sulindac metabolites showed low activation of caspase-3, a common downstream regulator of SAPK/JNK [23,33]. These caspase-3 levels were probably too low for the antibody-based detection.

Sulindac Sulfide and Sulfone are dissimilar compounds according to their pharmacological classification: the first is a NSAID and decreases prostaglandin synthesis by inhibition of COX-1 and -2, whereas the Sulfone metabolite does not act as a NSAID. However, the effects of both drugs on growth inhibition, induction of apoptosis, RAS pathway inhibition and JNK-activation seem to be comparable, suggesting that their apoptotic activity is independent of COX inhibition.

**Figure 5****Inhibition of RAS-GTP and phosphorylated ERK1/2 in human MPNST cells exposed to Sulindac derivatives A:**

Cell lines S462 and S520 were grown in DMEM with 10% serum and treated at 80–90% confluency with either 0.2% DMSO, 500 μ M Exisulind (Exi) or 125 μ M Sulindac Sulfide (s. s.). Cells were lysed after 24 h and RAS was immunoprecipitated and detected by western blotting with an anti-RAS antibody, the same lysates were blotted for phosphorylated (phospho-) and basal (total-) ERK1/2. B: Upregulation of phosphorylated ERK1/2 and AKT for treated and untreated cells after addition of EGF, whereas the RAS-GTP level remains unchanged at all conditions. Cell line S462 was starved over night and then Sulindac metabolites were supplemented at concentrations mentioned above in DMEM containing 0.1% serum. EGF was added after 24 h of treatment, cells were lysed 15 min later and after western blotting incubated with phosphorylated and unphosphorylated ERK1/2, phospho-AKT and RAS-antibodies.

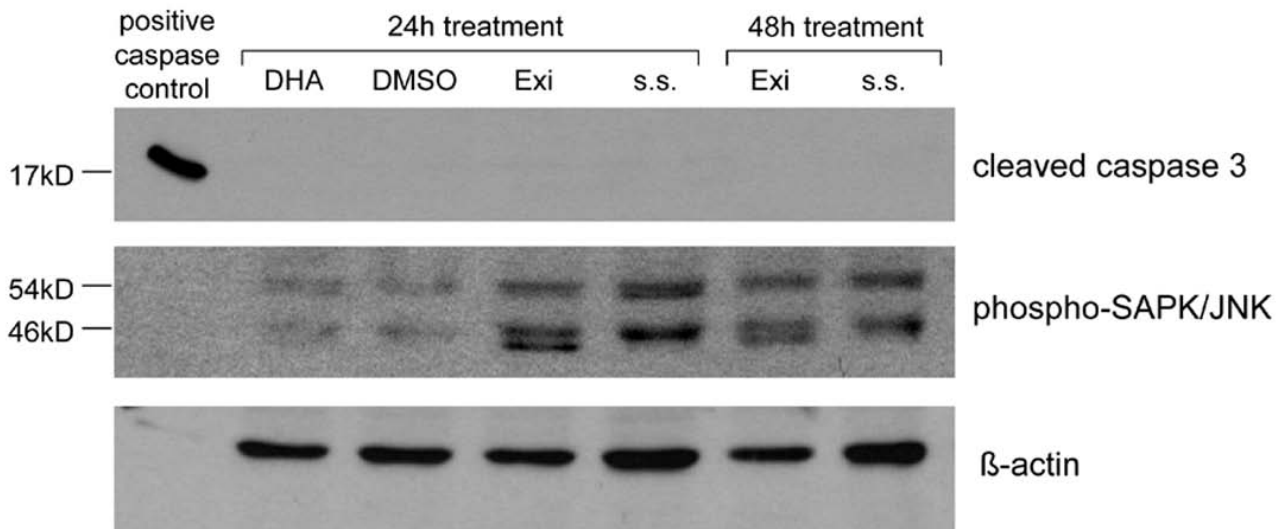


Figure 6

Activation of SAPK/JNK but not caspase-3 after treatment with Sulindac metabolites The activation of phospho-SAPK/JNK (dimer: 46 kD + 54 kD) reached the highest level after 24 h of treatment with Exisulind (Exi) and Sulindac Sulfide (s. s.), but no effect was seen on cleaved caspase-3 activation. DHA did not have any effect on neither JNK nor caspase-3 activation. Cell line S462 was grown in DMEM with 10% serum and treated at 80–90% confluency with 0.2% DMSO, 500 μ M Exisulind (Exi) or 125 μ M Sulindac Sulfide (s. s.) and blots were incubated with either phosphorylated SAPK/JNK or cleaved caspase-3 antibody. The positive control for caspase activation is a mouse MPNST cell line treated 7 h with 30 μ M DHA.

The antineoplastic effect of the Sulindac derivatives was more pronounced for the Sulfide metabolite than for Exisulind, as shown in this and in previous studies [11,13,14]. Anyhow, long-term application of Sulindac Sulfide may cause irritations in the upper gastrointestinal tract due to COX inhibition [34]. In contrast, Exisulind has been well tolerated by most patients in phase III familial adenomatous polyposis (FAP) trials and is currently being evaluated in patients with lung, prostate, colon and breast cancer [19]. Furthermore, positive results could be observed with Exisulind in *in vivo* animal models of several cancer types [15-17].

Conclusions

In summary, our results provide evidence that both Sulindac metabolites effectively suppress the growth of MPNST cells *in vitro* at non toxic doses. This promising finding opens for the first time a new perspective in drug treatment for patients with this kind of malignancy.

Methods

Cell lines and tissue culture

Cells were isolated from 2 MPNSTs from NF1 patients diagnosed according to the NIH criteria, and primary cell cultures were established (S462 and S520) and verified genetically [35]. Cells were grown in DMEM (Gibco, Paisley, UK) supplemented with 10% FBS (Gibco), 500 U/ml penicillin/streptomycin (Gibco), 2 mM glutamine (Biochrom, Berlin, Germany), and 1 mM sodium pyruvate (Biochrom), in a humidified atmosphere at 37°C and 10% CO₂.

Cell growth inhibition

Cells were plated in 24-well plates with coverslips (\varnothing 12 mm) at a density of 1.5×10^4 cells/well and were allowed to settle down over night. After 24 h, Sulindac Sulfide and Exisulind (Calbiochem, San Diego, CA) dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) were added at various concentrations to the media. Cell growth inhibition was measured by 5-Bromo-2'deoxyuridine (BrdU) incorporation as described previously [35] after 48 h and 96 h of treatment. The proliferation rate is determined as the ratio of proliferating cells to the total

number of cells, counted in triplicates of 500 cells. The IC_{50} values for growth inhibition were calculated using Microsoft Excel 2000 software. The final DMSO concentration used here did not exceed 0.2% and had no effect on cell growth.

Viability

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h, Sulindac Sulfide and Exisulind were added at various concentrations in triplicates. After a time period of 48 h, the tetrazolium salt XTT was supplemented to the medium, according to the manufacturers instructions (Cell proliferation kit II XTT; Roche, Basel, Swiss). Only viable cells can reduce XTT metabolically to a purple formazan product, which can be quantified spectrophotometrically. The viability was determined as percent of control cells (cells treated with the vehicle alone were defined as 100% viable).

Apoptosis assays

Photometric Quantification

Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h, Sulindac Sulfide and Exisulind were added at various concentrations to the media in triplicates. The cell death detection elisa^{plus} assay (Roche) was performed according to the manufacturers instructions with cells treated for 48 h. The absorbance values of drug treated samples – equivalent to the amount of DNA-fragmentation – were normalized with the corresponding negative control of cells treated with DMSO. The maximal absorbance from the test compound included in the assay was defined as positive control (100% apoptosis).

Nuclei morphology

Changes in cell-nuclei morphology characteristic for apoptosis were examined on cells grown on coverslips using a fluorescence microscope. Therefore, cells were fixed in 4% paraformaldehyde after 48 h of drug treatment, permeabilized in ice-cold methanol and incubated for 15 min at room temperature with 0.5 μ g/ml propidium iodide (PI; Molecular Probes, Leiden, Netherlands).

TUNEL assay

TUNEL (terminal deoxynucleotidyltransferase nick end labeling) assay (APO-BRDU KIT; BD Biosciences, San Jose, CA) was performed according to the manufacturers instructions. Briefly, 7.5×10^5 cells were seeded on 6 cm dishes and treated 24 h later with 500 μ M Exisulind, 125 μ M Sulindac Sulfide or 0.2% DMSO. After 24 h and 48 h of treatment, cells were collected by trypsinization and fixed in 1% paraformaldehyde. After incorporation of BrdUTPs, cells were visualized using FITC-labeled anti-BrdU antibody. The amount of total cellular DNA is obtained by staining cells with propidium iodide/RNase buffer. Flow cytometric analysis displays the amount of total

DNA in red on the X-axis and the FITC-BrdU incorporated nuclei in green on the Y-axis.

Western blot analysis

Cells were scraped from plates 24 h after drug treatment and resuspended in lysis buffer (25 mM Hepes pH7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA and 2% Glycerol). RAS affinity precipitation with Raf-1 RBD agarose beads was performed according to the manufacturers instructions (RAS activation assay kit; Upstate, San Diego, CA). Samples were prepared for SDS-PAGE, followed by electroblotting onto nitrocellulose membrane. Before overnight-incubation at 4°C with monoclonal Anti-RAS clone RAS10 antibody (1 μ g/ml), the blot was blocked 30 min in Tris-buffered normal saline (TBS) with 3% non-fat dry milk and 0.05% Tween-20 (BioRad, Hercules, CA) at room temperature. Separate blots were blocked overnight at 4°C in TBS with 5% BSA (Sigma) and 0.05% Tween-20 and incubated with Anti-phospho-Erk1/2 (1 μ g/ml), Anti-Erk1/2 (1 μ g/ml) or Anti-phospho-AKT (1 μ g/ml) (Upstate) at room temperature for 2 h. Immunoreactive protein was detected by incubating blots with horseradish peroxidase-conjugated secondary antibody for 1 h followed by chemiluminescent substrate for 1 min, and visualized by luminography.

In a separate experiment cells were starved over night without serum before drug treatment. 500 μ M Exisulind or 75 μ M Sulindac Sulfide was then supplemented at 0.1% FBS concentration, and after 24 h incubation time 20 ng/ml epidermal growth factor (EGF; Invitrogen, Carlsbad, CA) was added for 15 min to the media. Lysates were treated as described above.

For the detection of caspase-3 and phosphorylated JNK, cells were lysed in CHAPS-lysis buffer (50 mM Pipes/HCl pH6.5, 2 mM EDTA, 0.1% Chaps, 20 μ g/ml Leupeptin, 10 μ g/ml Pepstatin A, 10 μ g/ml Aprotinin, 5 mM DTT, 1 mM PMSF) after 24 h and 48 h of drug treatment with either one of the Sulindac derivatives or docosahexaenoic acid (DHA) and loaded onto SDS-PAGE. One positive caspase-control was loaded, a mouse MPNST cell line treated for 7 h with 30 μ M DHA. After SDS-gel electrophoresis and transfer, nitrocellulose membranes were blocked in TBS/Tween with 5% non-fat dry milk for 1 h and incubated with Anti-cleaved-caspase-3 or Anti-phospho-SAPK/JNK (Cell Signaling, Beverly, MA) over night at 4°C followed by the detection steps described above.

Caspase-3 colorimetric assay

In addition to the antibody-based detection of cleaved caspase-3, a colorimetric assay based on quantification of protease activity was performed according to the manufacturers instructions (BD ApoAlert™ Caspase Colorimetric Assay Kit; BD Biosciences). Briefly, 2×10^6 cells were

seeded on 6 cm dishes (in duplicate cell plates) and after 24 h treated with 500 μ M Exisulind, 125 μ M Sulindac Sulfide, 50 ng/ml Staurosporin (a known apoptosis inducer; Sigma) or 0.2% DMSO. After 24 h, cells were collected by trypsinization and resuspended in cell lysis buffer. After precipitation of the cellular debris, the remaining supernatant was incubated with a caspase-3 substrate for 1 h at 37°C. The colorimetric assay uses the spectrophotometric detection of the chromophore *p*-nitroaniline (pNA) after its cleavage by caspases from the labeled caspase-specific substrates. Samples were read at 405 nm in a microplate reader. The reading-values of the uninduced control were subtracted from its corresponding induced sample and Units of caspase-3 activity were calculated using a pNA (supplied in the kit) calibration curve.

List of abbreviations

MPNST: malignant peripheral nerve sheath tumor; NF1: neurofibromatosis type 1; FAP: familial adenomatous polyposis; NSAID: nonsteroidal anti-inflammatory drug; COX: cyclooxygenase; PDE: phosphodiesterase; PKG: protein kinase G; JNK: C-jun N-terminal protein kinase; MEKK: mitogen activated protein kinase kinase; PARP: poly (ADP-ribose) polymerase; phospho-ERK1/2: phosphorylated extracellular signal-regulated kinase 1 and 2; NIH: national institute of health; DMEM: Dulbecco's modified eagle medium; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; BrdU: 5-Bromo-2'-deoxyuridine; bFGF: basic fibroblastic growth factor; PBS: phosphate buffered saline; XTT: sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; TUNEL: terminal deoxynucleotidyltransferase dUTP nick end labelling; PI: propidium iodide; TBS: Tris-buffered normal saline; EGF: epidermal growth factor; DTT: dithiothreitol; PMSF: phenylmethylsulfonyl fluoride; DHA; docosahexaenoic acid; SAPK: stress-activated protein kinase; h: hours.

Authors' contributions

SF carried out all experimental techniques and drafted the manuscript. AK and LK participated in the design of the study and revised the manuscript. FF provided expertise in western blotting techniques and experimental design. RF resected the tumors used for primary cell culture. VM conceived of the study, participated in its coordination, and attended the NF1 patients who donated the tumors. All authors read and approved the final manuscript.

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References

- Gutmann DH, Collins FS: **Neurofibromatosis type 1**. In: *The metabolic and molecular bases of inherited disease* Edited by: Scriver CR, Beaudet AL, Sly WS, Valle D. New York: McGraw-Hill; 1995:76-96.
- Evans DG, Baser ME, McLaughran J, Sharif S, Howard E, Moran A: **Malignant peripheral nerve sheath tumours in neurofibromatosis 1**. *J Med Genet* 2002, **39**:311-314.
- Huson SM: **Recent developments in the diagnosis and management of neurofibromatosis**. *Arch Dis Child* 1989, **64**:745-749.
- Riccardi VM: **Neurofibromatosis: Phenotype, Natural History and Pathogenesis**. *The Johns Hopkins University Press, Baltimore* 1992.
- Viskochil D, Buchberg AM, Xu G, Stevens J, Wolff RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White R, O'Connell P: **Deletions and translocations interrupt a cloned gene at the neurofibromatosis type 1 locus**. *Cell* 1990, **62**:187-192.
- Cichowski K, Jacks T: **NF1 tumor suppressor gene function: narrowing the GAP**. *Cell* 2001, **104**:593-604.
- Legius E, Dierick H, Wu R, Hall BK, Marynen P, Cassiman JJ, Glover TW: **TP53 mutations are frequent in malignant NF1 tumors**. *Genes Chromosomes Cancer* 1994, **10**:250-255.
- Berner JM, Sorlie T, Mertens J, Henriksen J, Sater G, Mandahl N, Brogger A, Myklebost O, Lothe RA: **Chromosome band 9p21 is frequently altered in malignant peripheral nerve sheath tumors: Studies of CDKN2A and other genes of the pRB pathway**. *Genes Chromosomes Cancer* 1999, **26**:151-160.
- Ferner RE, Gutmann DH: **International consensus statement on malignant peripheral nerve sheath tumors in neurofibromatosis 1**. *Cancer Res* 2002, **62**:1573-1577.
- Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ: **Treatment of colonic and rectal adenomas with Sulindac in familial adenomatous polyposis**. *N Engl J Med* 1993, **328**:1313-1316.
- Piazza GA, Rahm AL, Krutzsch M, Sperl G, Paranka NS, Gross PH, Brendel K, Burt RW, Alberts DS, Pamukcu R: **Antineoplastic drugs Sulindac Sulfide and Sulfone inhibit cell growth by inducing apoptosis**. *Cancer Res* 1995, **55**:3110-3116.
- Han EK, Arber N, Yamamoto H, Lim JT, Delohery T, Pamukcu R, Piazza GA, Xing WQ, Weinstein IB: **Effects of Sulindac and its metabolites on growth and apoptosis in human mammary epithelial and breast carcinoma cell lines**. *Breast Cancer Res Treat* 1998, **48**:195-203.
- Lim JT, Piazza GA, Han EK, Delohery TM, Li H, Finn TS, Buttyan R, Yamamoto H, Sperl GJ, Brendel K, Gross PH, Pamukcu R, Weinstein IB: **Sulindac derivatives inhibit growth and induce apoptosis in human prostate cancer cell lines**. *Biochem Pharmacol* 1999, **58**:1097-1107.
- Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr, Chou TC: **Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines**. *Cancer Res* 1999, **59**:6178-6184.
- Piazza GA, Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T, Bogert C, Guillen JM, Brendel K, Gross PH, Sperl G, Ritchie J, Burt RW, Ellsworth L, Ahnen DJ, Pamukcu R: **Sulindac Sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels**. *Cancer Res* 1997, **57**:2909-2915.
- Thompson HJ, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS, Pamukcu R, Ahnen DJ: **Sulfone metabolite of Sulindac inhibits mammary carcinogenesis**. *Cancer Res* 1997, **57**:267-271.
- Malkinson AM, Koski KM, Dwyer-Nield LD, Rice PL, Rioux N, Castonguay A, Ahnen J, Thompson H, Pamukcu R, Piazza GA: **Inhibition of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone-induced lung tumor formation by FGN-1 (Sulindac Sulfone)**. *Carcinogenesis (Lond)* 1998, **19**:1353-1356.
- Piazza GA, Rahm AK, Finn TS, Fryer BH, Li H, Stoumen AL, Pamukcu R, Ahnen DJ: **Apoptosis primarily accounts for the growth-inhibitory properties of Sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest, and p53 induction**. *Cancer Res* 1997, **57**:2452-2459.
- Goluboff ET: **Exisulind, a selective apoptotic antineoplastic drug**. *Expert Opin Investig Drugs* 2001, **10**:1875-1882.
- Thompson WJ, Piazza GA, Li H, Liu L, Fetter J, Zhu B, Sperl G, Ahnen D, Pamukcu R: **Exisulind induction of apoptosis involves guanosine 3',5'-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin**. *Cancer Res* 2000, **60**:3338-3342.

21. Soh JW, Mao Y, Kim MG, Pamukcu R, Li H, Piazza GA, Thompson WJ, Weinstein IB: **Cyclic GMP mediates apoptosis induced by Sulindac derivatives via activation of c-Jun NH2-terminal kinase I.** *Clin Cancer Res* 2000, **6**:4136-4141.
22. Soh JW, Mao Y, Liu L, Thompson WJ, Pamukcu R, Weinstein IB: **Protein kinase G activates the JNK1 pathway via phosphorylation of MEKK1.** *J Biol Chem* 2001, **276**:16406-16410.
23. Patel T, Gores GJ, Kaufmann SH: **The role of proteases during apoptosis.** *FASEB J* 1996, **10**:587-597.
24. Herrmann C, Block C, Geisen C, Haas K, Weber C, Winde G, Moroy T, Müller O: **Sulindac Sulfide inhibits Ras signaling.** *Oncogene* 1998, **17**:1769-1776.
25. Rice PL, Goldberg RJ, Ray EC, Driggers LJ, Ahnen DJ: **Inhibition of extracellular signal-regulated kinase 1/2 phosphorylation and induction of apoptosis by Sulindac metabolites.** *Cancer Res* 2001, **61**:1541-1547.
26. Van Stolk R, Stoner G, Hayton WL, Chan K, DeYoung B, Kresty L, Kemmenoe BH, Elson P, Rybicki L, Church J, Provencher K, McLain D, Hawk E, Fryer B, Kelloff G, Ganapathi R, Budd GT: **Phase I trial of exisulind (Sulindac Sulfone, FGN-1) as a chemopreventive agent in patients with familial adenomatous polyposis.** *Clin Cancer Res* 2000, **6**:78-89.
27. Bunn PA Jr, Chan DC, Earle K, Zhao TL, Helfrich B, Kelly K, Piazza G, Whitehead CM, Pamukcu R, Thompson W, Alila H: **Preclinical and clinical studies of docetaxel and exisulind in the treatment of human lung cancer.** *Semin Oncol* 2002, **29**:87-94.
28. Chan DC, Earle KA, Zhao TL, Helfrich B, Zeng C, Baron A, Whitehead CM, Piazza G, Pamukcu R, Thompson WJ, Alila H, Nelson P, Bunn PA Jr: **Exisulind in combination with docetaxel inhibits growth and metastasis of human lung cancer and prolongs survival in athymic nude rats with orthotopic lung tumors.** *Clin Cancer Res* 2002, **8**:904-912.
29. Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS, Downward J: **Aberrant regulation of ras proteins in malignant tumour cells from type I neurofibromatosis patients.** *Nature* 1992, **356**:713-715.
30. DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR: **Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type I) neurofibromatosis.** *Cell* 1992, **69**:265-273.
31. Guha A, Lau N, Huvar I, Gutmann D, Provias J, Pawson T, Boss G: **Ras-GTP levels are elevated in human NF1 peripheral nerve tumors.** *Oncogene* 1996, **12**:507-513.
32. Li H, Velasco-Miguel S, Vass WC, Parada LF, DeClue JE: **Epidermal growth factor receptor signaling pathways are associated with tumorigenesis in the NF1:p53 mouse tumor model.** *Cancer Res* 2002, **62**:4507-4513.
33. Vivo C, Liu W: **Broadus VC c-Jun N-terminal kinase contributes to apoptotic synergy induced by tumor necrosis factor-related apoptosis-inducing ligand plus DNA damage in chemoresistant, p53 inactive mesothelioma cells.** *J Biol Chem* 2003, **278**:25461-25467.
34. Subbaramaiah K, Zakim D, Weksler BB, Dannenberg AJ: **Inhibition of cyclooxygenase: a novel approach to cancer prevention.** *Proc Soc Exp Biol Med* 1997, **216**:201-210.
35. Frahm S, Mautner VF, Brems H, Legius E, Debiec-Richter M, Friedrich RE, Knöfel WT, Peiper M, Kluwe L: **Genetic and phenotypic characterization of tumor cells derived from malignant peripheral nerve sheath tumors of neurofibromatosis I patients.** *Neurobiol Dis* 2004 in press.

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