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14. ABSTRACT: Numerous studies demonstrate that non-steroidal anti-inflammatory drugs (NSAIDs) are effective in chemoprevention or treatment of cancer. Nevertheless, the mechanisms underlying the antineoplastic actions of NSAIDs remain poorly understood. We started deciphering now the mechanisms by which NSAIDs induce programmed cell death and growth arrest in cancer. In this report we show that induction of the pro-apoptotic cytokine melanoma differentiation associated gene-7/Interleukin-24 (MDA-7/IL-24) and the expression of growth arrest and DNA damage inducible (GADD) 45 α and γ by several NSAIDs is an essential step for G2/M growth arrest and apoptosis induction of cancer cells and inhibition of tumor growth in vivo. MDA-7/IL24 dependent upregulation of GADD45 α and γ expression is sufficient for cancer cell apoptosis, since inhibition of GADD45 α and γ by small interfering RNA abrogates apoptosis and growth arrest induction by the NSAID, blocks JNK activation and restores CDC2 kinase activity. Our results establish MDA-7/IL-24 and GADD45 α and γ as critical mediators of apoptosis and growth arrest in response to NSAIDs in cancer cells.					
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Introduction

An increasing number of studies have demonstrated that some non-steroidal anti-inflammatory drugs (NSAIDs) such as Sulindac sulfide, at clinically tolerable concentrations, are effective in the treatment of several types of cancer. Sulindac sulfide has been shown to reduce CaP proliferation and induce CaP apoptosis in vitro and in vivo. Nevertheless, the mechanism of apoptosis induction is poorly understood and more studies are needed to fully elucidate the molecular and biochemical pathways of Sulindac-induced apoptosis. Our notion is that by dissecting the molecular mechanisms of CaP apoptosis induction by Sulindac sulfide and of a whole panel of NSAIDs with potential anti-prostate cancer activities we may be able to rationally design a combination of several NSAIDs with distinct target specificities that should act synergistically and, thus, more effectively against CaP. Our goal is to systematically decipher the pathways that are involved in apoptosis induction by Sulindac sulfide with particular emphasis on the role of GADD45 α and γ , IL-24 and JNK kinase. Our hypothesis is that the detailed pathways will provide a multitude of novel entry points for targeted drug development, towards drugs specific for CaP apoptosis induction without the adverse reactions of current NSAIDs.

Body

Based on the approved Statement of Work the following research accomplishments are associated with each task outlined below:

Aim 1. Determine the role of the GADD45 family, JNK and other apoptosis and cell cycle related proteins in Sulindac mediated apoptosis of CaP (1-18 months)

Multiple NSAIDs are potent inducers of apoptosis in prostate cancer cells. A broad panel of NSAIDs was tested for their abilities to induce apoptosis in cancer cells. The concentrations for all NSAIDs drugs used in this study were selected carefully and comparable to the achievable plasma concentrations (1-3). However, some drug concentrations exceeded the physiologically achievable doses (1-3). Apoptosis was measured 24 and 48 hours after treatment of DU145 prostate cancer cells with this set of NSAIDs, revealing that a variety of, but not all NSAIDs induced apoptosis in DU145 cells. Strong inducers of apoptosis included Sulindac sulfide, Finasteride, Diclofenac, Flufenamic acid, Flurbiprofen, Sulindac sulfone and NS-398 when compared with the solvent controls, whereas treatment with Aspirin, Celecoxib, Acetaminophen, Ibuprofen, Naproxen, Meloxicam and Ebselen resulted only in marginal apoptosis induction (see Fig. 1 in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Sulindac sulfide was the strongest inducer of apoptosis in prostate cancer cells. Sulindac sulfide treatment also induced G2/M growth arrest in prostate cancer cell lines when compared to control by increasing the fraction of cells in the G2/M phase by 2fold (2,3).

It has been established that Sulindac sulfide reaches peak plasma concentrations of 30-50 μ M (2,3), coming down to a steady state plasma concentration of 5-10 μ M (4). We, therefore, decide to evaluate whether the sulindac sulfide steady plasma concentration achievable in patients (4) is still able to induce apoptosis in cancer cells. Prostate cancer cells were treated with 5, 10, 25 and 50 μ M of Sulindac sulfide and apoptosis was measured 24 hours post-treatment. We demonstrate that even low concentrations as 5-10 μ M Sulindac sulfide are sufficient to induce apoptosis in cancer cells (see supplementary Figure 4A in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

Our transcriptional profiling experiments, moreover, demonstrated a strong upregulation of GADD45 α up to 16-fold by Sulindac sulfide (see supplementary Table 1 in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). The GADD45

gene family encodes three structurally highly related growth arrest- and DNA damage-inducible proteins, GADD45 α , β and γ (5) that play a role in the G2/M checkpoint in response to DNA damage (6). To evaluate whether regulation of GADD45 genes is involved in NSAID-mediated apoptosis, expression of GADD45 family members was measured by real time PCR in the different cancer cell lines treated with Sulindac sulfide (see Fig. 4A in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Whereas GADD45 β expression was not significantly affected by Sulindac sulfide in any of the cell lines, the drug induced GADD45 α and γ expression 1.5-27-fold in the various cancer cell types, indicating that GADD45 α and γ expression is consistently regulated by Sulindac sulfide (see in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section Fig. 4A). Interestingly, the correlation between induction of GADD45 α and γ was significant (Correlation coefficient 0.85; p value=0.0001).

Differences in mRNA expression of the GADD45 family members were corroborated on the protein level by Western blot analysis using protein extracts from DU145 and PC-3 cells treated with Sulindac sulfide for 24 hours. GADD45 α and γ , but not β protein expression were induced in Sulindac sulfide treated cancer cells (see Fig. 4B in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

a) Determine whether JNK kinase activation by Sulindac is GADD45 α , β or γ dependent – (Months 1-4)

The importance of GADD45 α and γ for NSAID-induced JNK activation was evaluated in DU145 and PC-3 cells infected with the GADD45 α or γ siRNA lentiviruses or the control lentivirus and treated with Sulindac sulfide for 24 hours. siRNA mediated inhibition of Sulindac sulfide-mediated upregulation of GADD45 α and γ expression drastically reduced JNK activation in both cell lines, correlating with the inhibition of apoptosis induction (see Fig. 6D in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

To establish the relevance of JNK activation in mediating apoptosis in cancer cells, apoptosis was measured in protein extracts obtained from DU145 and PC-3 cells treated with 50 μ M of Sulindac sulfide or DMSO in the absence or presence of a specific JNK inhibitor, JNKII. Compared to the control, apoptosis of Sulindac sulfide treated cells was reduced by more than 56% in DU145 cells and 40% in PC-3 cells in JNK II treated cells, but inhibition of JNK did not fully abolish apoptosis induction (see Fig. 6E in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These results suggest that JNK contributes to, but is not absolutely essential for Sulindac sulfide-mediated apoptosis.

b) Determine whether inhibition of GADD45 family genes and JNK affects cell cycle, proliferation and/or apoptosis in CaP treated with Sulindac –(Months 1-8)

NSAID-mediated GADD45 α and γ induction is essential for CDK1/cdc2 kinase activation and growth arrest. Our transcriptional profiling analysis demonstrated that in concordance with the observed G2/M cell cycle arrest induced by Sulindac sulfide, several genes involved in the G2/M checkpoint and CDK1/cdc2 kinase regulation are downregulated by Sulindac sulfide including cdc25C, Cdk1/cdc2, cyclin B1, and cyclin B2, whereas the cyclin dependent kinase inhibitor p21 is upregulated (see supplementary Table 1 in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Western blot analysis of protein extracts from DU145 and PC-3 cells treated with Sulindac sulfide for 24 hours corroborated the expression changes on the protein level. Protein expression of cdc-25C, cyclin B1, and cyclin B2 decreased and p21 protein expression increased in response to Sulindac sulfide (see Fig. 5a in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). GADD45 α has been shown to inhibit the kinase activity of the Cdc2/CyclinB1 complex (7) and progression from the G2 to the M phase of the cell cycle (8). To determine the role of GADD45 α

and GADD45 γ in NSAID-induced G2/M arrest, we infected DU145 prostate cancer cells with lentiviruses encoding siRNAs for GADD45 α , GADD45 γ or GFP. An *in vitro* Cdc2 kinase assay was performed with whole cell lysates from these infected cells after treatment with Sulindac sulfide or DMSO for 24 hours using histone H1 as the Cdc2 substrate. Active Cdc2 directly correlates with enhanced phosphorylation of histone H1. A significant level of phosphorylated histone H1 in untreated cells infected with the control lentivirus indicated significant Cdc2 activity in proliferating DU145 cells (see Fig. 5B in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Cdc2 activity was substantially decreased 24 hours after Sulindac sulfide treatment correlating with enhanced GADD45 α and γ expression and G2/M arrest. However, inhibition of GADD45 α and γ expression in DU145 cells by the siRNA lentiviruses restored Cdc2 kinase activity as seen by increased histone H1 phosphorylation (see Fig. 5B in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These data suggest that Sulindac sulfide induced G2/M cell cycle arrest is due to a combination of decreased expression of several G2/M transition cell cycle regulators and MDA-7/IL-24 induced GADD45 α and GADD45 γ upregulation that leads to inhibition of Cdc2 activity.

To elucidate the functional relevance of GADD45 α and γ for NSAID-mediated apoptosis, we measured apoptosis induction by Sulindac sulfide in GADD45 α and γ knockdown cells. siRNA mediated inhibition of Sulindac sulfide induced upregulation of GADD45 α or γ expression almost completely abrogated apoptosis induction (see Fig. 6A in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section), clearly demonstrating the absolute requirement of MDA-7/IL-24-dependent GADD45 α and γ upregulation for apoptosis induction by NSAIDs.

Since we and others had shown that c-jun N-terminal kinase (JNK) activation plays a role in apoptosis induction in cancer cells and GADD45 α and γ interact with the upstream kinase of JNK, MEKK4, and activate JNK (5), we evaluated the relevance of JNK for NSAID-mediated apoptosis. JNK kinase activity was tested in protein extracts obtained from DU145 and PC-3 cells treated with Sulindac sulfide or DMSO for 24 hours by an *in vitro* kinase assay. Western blot analysis revealed very little JNK activity in untreated control cells and a strong increase in JNK activity in both cell lines upon treatment with Sulindac sulfide (see Fig. 6B in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

c) Determine whether inhibition or overexpression of GADD45 family genes affects tumor formation in SCID mice treated with Sulindac – (Months 4-18)

To determine whether NSAIDs reduce tumor growth *in vivo* and to evaluate whether their effects may be dependent on induction of GADD45 family genes, prostate cancer cells stably infected with LV-siRNA GFP or LV-siRNA GADD45 α and γ as well as uninfected cells were orthotopically implanted into the prostate of SCID mice. The mice were randomly divided into two groups and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200ppm Sulindac sulfide. Two months later the animals were examined for tumor formation and tumor weight. All mice developed tumors indicating that this particular dose of Sulindac sulfide did not prevent tumor formation. As seen in Figure 3E, infection of DU145 cells with the LV-siRNA GFP virus did not affect tumor growth in the control diet group, since implantation of uninfected DU145 cells showed a similar pattern of tumor growth and tumor weight when compared with the LV-siRNA GFP group (see Fig. 3E in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Sulindac sulfide treatment reduced the average tumor weight in the LV-siRNA GFP group by 38% when compared to the control diet confirming its anti-tumor efficacy (see Fig. 3E in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

Although tumor weight increase in the Sulindac sulfide treated LV-siRNA GADD45 α γ group compared with the Sulindac sulfide treated LV-siRNA GFP group (0.487g versus 0.38g), this result was not statistically significant ($p=0.0638$).

However, the same experiments were done using prostate cancer cells stably infected with LV-siRNA GFP or LV-siRNA IL-24. Surprisingly, the blockage of IL-24 expression by siRNA interference in the LV-siRNA IL24 group strongly enhanced tumor growth demonstrating that the low endogenous basal IL-24 expression acts as a tumor suppressor (p value =0.010) (see Fig. 3E in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Tumor weight markedly increased by 60% in the LV-siRNA IL24 group fed with the control diet when compared with the LV-siRNA GFP group (0.969g versus 0.623g; p value 0.010).

Sulindac sulfide treatment also reduced tumor growth in the LV-siRNA IL24 group to some extent; however, tumor weight was still 75% higher than the Sulindac-treated LV-siRNA GFP group (p value = 0.024) (see Fig. 3E in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These results indicate that NSAID-mediated IL-24 induction plays a critical role in tumor growth and also indicate a tumor suppressor activity of IL-24.

Aim 2. Elucidate the precise role of IL-24 in Sulindac mediated apoptosis of CaP (12-24).

a) Evaluate whether IL-24 is mediating Sulindac triggered apoptosis by RNA interference experiments. - (Months 12-24)

The pro-apoptotic cytokine MDA-7/IL-24 is the critical mediator of NSAID induced apoptosis and growth arrest in cancer cells and inhibition of tumor growth in vivo. To elucidate the detailed molecular mechanisms underlying NSAIDs-mediated cell cycle arrest and apoptosis in cancer cells, we performed oligonucleotide microarray-based transcriptional profiling of DU145 and PC-3 cells treated with 50 μ M of Sulindac sulfide vs. DMSO. Detailed bioinformatic analysis revealed that Sulindac sulfide does not trigger indiscriminate transcriptional shutdown of cancer cells, but induces distinct patterns of gene expression changes for a wide range of transcripts related to apoptosis and cell cycle (see supplementary Table 1 in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section) that were consistent across the two cell lines, further confirming their apparent relevance for the cell cycle and cell death effects of Sulindac sulfide.

Particularly striking and unanticipated was the dramatic upregulation of the pro-apoptotic cytokine interleukin 24, also named melanoma differentiation associated gene-7 (IL-24 or MDA-7) (9,10). MDA-7/IL-24 was by far the highest upregulated gene in both cell lines (140-fold in PC-3 and 722-fold in DU145). MDA-7/IL-24 has been shown to be a novel tumor suppressor gene (10,11). At low presumably physiological concentrations, MDA-7/IL-24 functions predominantly as a cytokine involved in immune regulation (10,11). However, when overexpressed at supra-physiological levels using an adenovirus vector, MDA-7/IL-24 shows cancer-cell specific growth inhibitory properties without negatively affecting normal cells (10-12). Furthermore, elevated endogenous MDA-7/IL-24 expression correlates with enhanced apoptosis and prolonged overall survival of patients with small cell lung cancer, further supporting the anti-cancer role of MDA-7/IL-24 (13).

To evaluate the functional relevance of MDA-7/IL-24 induction for NSAIDs-mediated apoptosis, we measured mRNA expression levels of MDA-7/IL-24 in response to Sulindac sulfide in the same cancer cell lines tested above for apoptosis induction by Sulindac sulfide. Real time PCR analysis demonstrated that Sulindac sulfide induces MDA-7/IL-24 expression in a variety of cancer types, up to 124-fold in DU145 cells and ~10-20-fold in various other cancer cell lines (see Fig. 3a in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Induction of MDA-7/IL-24 by Sulindac sulfide correlated with the ability of

this drug to induce extensive apoptosis in these cell lines (see Fig. 2A in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These results indicate that Sulindac sulfide mediated induction of MDA-7/IL-24 expression is a common pathway in various types of cancer cells that respond to this NSAID by undergoing apoptosis and strongly suggest that MDA-7/IL-24 plays a critical role in apoptosis induction.

To evaluate the relationship between MDA-7/IL-24 induction and apoptosis induction by various NSAIDs, we measured mRNA expression levels of MDA-7/IL-24 in response to different NSAIDs in prostate cancer cells. Real time PCR analysis demonstrated that induction of MDA-7/IL-24 is common to NSAIDs that induce apoptosis in cancer cells, since multiple, structurally unrelated NSAIDs strongly induced MDA-7/IL-24 expression in DU145 prostate carcinoma cells (see Fig. 3b in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section) in strong correlation with NSAID-mediated apoptosis induction (correlation coefficient 0.91; $p < .0001$). NSAIDs that strongly enhanced apoptosis (see Fig. 1 in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section) dramatically induced MDA-7/IL-24 expression (see Fig. 3b in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section), whereas NSAIDs that only marginally induced apoptosis (Fig. 1) did not significantly enhance MDA-7/IL-24 expression (see Fig. 3b in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These data suggest a common mechanism for structurally unrelated NSAIDs in apoptosis induction.

We and others have shown (10-12) that overexpression of MDA-7/IL-24 following infection with an adenovirus carrying the MDA-7/IL-24 gene induces apoptosis and inhibits cell proliferation in cancer cells (14) (see supplementary Fig. 5A and B in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). To determine whether induction of growth arrest and apoptosis in cancer cells by NSAIDs is dependent on MDA-7/IL-24 upregulation, we generated siRNA oligonucleotides and a lentivirus encoding this siRNA against MDA-7/IL-24. The specificity of the MDA-7/IL-24 siRNA oligos was validated as described in supplementary methods and supplementary Fig. 5C in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section. Infection with the MDA-7/IL-24 siRNA lentivirus reduced apoptosis induced by multiple NSAIDs by 90% (see Fig. 3C in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section) and reversed for a large part the G2/M cell cycle arrest (see Fig. 3D in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These results demonstrate that MDA-7/IL-24 may play an important role in tumor cell survival and, for the first time, implicate MDA-7/IL-24 as an essential mediator of NSAID action in cancer cells.

MDA-7/IL-24 regulates and induces GADD45 α and γ without affecting GADD45 β expression (14,15) (also see supplementary Fig. 6 in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). Furthermore, our data shows that upregulation of GADD45 α and γ strongly correlated with the ability of Sulindac sulfide to induce MDA-7/IL-24 expression (Correlation coefficient 0.63; p value=0.016 and correlation coefficient 0.69; p value= 0.0068, respectively) (see Fig. 3A in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

To evaluate whether NSAID-mediated induction of GADD45 α and γ expression is dependent on MDA-7/IL-24 upregulation, we transfected prostate cancer cells with MDA-7/IL-24 siRNA oligonucleotides and measured GADD45 expression 24 hours after treatment with Sulindac sulfide by real time PCR. Interference with MDA-7/IL-24 expression almost completely blocked Sulindac sulfide-mediated induction of GADD45 α and γ gene expression without affecting GADD45 β expression (see Fig. 4C in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section). These data most vividly demonstrate that GADD45 α and γ induction by Sulindac sulfide is mediated via MDA-7/IL-24 upregulation.

MDA-7/IL-24-dependent GADD45 α and γ induction and JNK activation are critical for NSAID-mediated apoptosis induction in cancer cells. JNK activation by Sulindac sulfide was at least partially dependent on MDA-7/IL-24 induction, since JNK activity in Sulindac sulfide treated MDA-7/IL-24 $^{-/-}$ cells was reduced by 62%, but not completely abolished when compared to MDA-7/IL-24 $^{+/+}$ cells (see Fig. 6C in the manuscript Zerbini *et al.* Cancer Research 66: 11922-31, 2006 in the appendices section).

Key Research Accomplishments

- We report the discovery of a novel biological pathway involving MDA-7/IL-24 and the GADD45 gene family that are targeted by a set of NSAIDs in prostate cancer and whose activation directly correlates with the efficacy of NSAIDs to induce cancer cell death.
- We demonstrate that multiple classes of structurally unrelated NSAIDs induce apoptosis and growth arrest via induction of MDA-7/IL-24 expression that leads to GADD45 α and γ expression and JNK activation and G2/M cell cycle arrest due to GADD45 α and γ dependent inhibition of Cdc2 activity.
- Our discovery of specific high level induction of MDA-7/IL-24 in different types of cancer cells including prostate cancer by a variety of NSAIDs provides a new entry point to enhance MDA-7/IL-24 levels in cancer cells on a systemic level.
- Our in vivo orthotopic tumor model further support the notion that MDA-7/IL-24 is indeed a tumor suppressor gene. It also suggests that MDA-24/IL24 is not the unique pathway targeted.
- Our data provide strong evidence that multiple NSAIDs induce cancer cell death through MDA-7/IL-24-mediated upregulation of GADD45 α and γ , irrespective of their ability to block COX-2. For example, Finasteride, a selective 5-alpha-reductase inhibitor, is not known to inhibit COX-2, strongly induces MDA-7/IL-24 expression and apoptosis, whereas the potent selective COX-2 inhibitor Celecoxib is significantly less effective in inducing MDA-7/IL-24 and apoptosis. On the other hand, the non-selective cation channel blocker flufenamic acid and the non-selective COX-1 and COX-2 inhibitors Sulindac sulfide and Diclofenac are very potent inducers of MDA-7/IL-24 and apoptosis. Thus, the pro-apoptotic anti-cancer activity of several divergent classes of drugs evaluated here appears not to be due to their effects on their supposed targets, but due to the off-target induction of MDA-7/IL-24.

Reportable Outcomes

Zerbini LF, Czibere A, Wang Y, Correa RG, Out H, Joseph M, Takayasu Y, Silver M, Gu X, Li L, Sarkar D, Zhou JR, Fisher PB and Libermann TA. A novel pathway involving MDA-7/IL-24 mediates NSAID induced apoptosis of cancer cells. **Cancer Research** 66: 11922-31. 2006.

Conclusion

The ability of NSAIDs to induce apoptosis appears to depend on their abilities to induce MDA-7/IL-24 expression and enhance GADD45 α and γ expression. Thus, apoptosis and growth arrest induction of cancer cells as a result of enhanced MDA-7/IL-24 expression appears to be a common pathway for multiple classes of drugs. These results also provide a rationale to screen small molecule libraries, natural compound libraries and chemically modified NSAIDs for selective inducers of MDA-7/IL-24 expression in cancer cells in order to obtain more effective anti-cancer drugs.

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Appendices

See attached manuscript

A Novel Pathway Involving Melanoma Differentiation Associated Gene-7/Interleukin-24 Mediates Nonsteroidal Anti-inflammatory Drug-Induced Apoptosis and Growth Arrest of Cancer Cells

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Abstract

Numerous studies show that nonsteroidal anti-inflammatory drugs (NSAIDs) are effective in chemoprevention or treatment of cancer. Nevertheless, the mechanisms underlying these antineoplastic effects remain poorly understood. Here, we report that induction of the cancer-specific proapoptotic cytokine melanoma differentiation associated gene-7/interleukin-24 (*MDA-7/IL-24*) by several NSAIDs is an essential step for induction of apoptosis and G₂-M growth arrest in cancer cells *in vitro* and inhibition of tumor growth *in vivo*. We also show that *MDA-7/IL-24*-dependent up-regulation of growth arrest and DNA damage inducible 45 α (*GADD45 α*) and *GADD45 γ* gene expression is sufficient for cancer cell apoptosis via c-Jun NH₂-terminal kinase (JNK) activation and growth arrest induction through inhibition of Cdc2-cyclin B checkpoint kinase. Knockdown of *GADD45 α* and *GADD45 γ* transcription by small interfering RNA abrogates apoptosis and growth arrest induction by the NSAID treatment, blocks JNK activation, and restores Cdc2-cyclin B kinase activity. Our results establish *MDA-7/IL-24* and *GADD45 α* and *GADD45 γ* as critical mediators of apoptosis and growth arrest in response to NSAIDs in cancer cells. (Cancer Res 2006; 66(24): 11922-31)

Introduction

Various studies indicate that nonsteroidal anti-inflammatory drugs (NSAIDs), at clinically relevant concentrations, may be effective in prevention and treatment of common cancers (1, 2). Epidemiologic studies have suggested that regular use of certain NSAIDs reduces the risk of colorectal, breast, and ovarian cancer, and the number of precancerous colorectal polyps (3–5). The detailed molecular mechanisms by which NSAIDs inhibit neoplastic growth are, however, poorly understood and likely involve many

off-target and divergent activities among different NSAIDs. Additionally, current clinical trials are evaluating a range of NSAIDs for a variety of cancers without any clear vision of the best way to use them.

Preclinical efficacy studies in animal models and cancer cell lines show strong antineoplastic effects of NSAIDs *in vivo* and *in vitro* (6, 7). The chemopreventive and antitumorigenic effects of NSAIDs are, to a large part, attributed to their apoptosis-inducing potential and may involve cyclooxygenase-2 (COX-2) inhibition (8–11). Nevertheless, the relevance of COX-2 inhibition for apoptosis induction is not entirely clear, because apoptosis induction by NSAIDs does not always correlate with their abilities to inhibit COX-2. Chemical modifications of NSAIDs that select for NSAIDs with enhanced proapoptotic activity reveal that structural requirements necessary for inhibition of cell growth and apoptosis induction in cancer cells can be distinct from those effecting COX-2 inhibition (12–15).

Because COX-2 inhibition does not seem to be the only antineoplastic, proapoptotic pathway targeted by NSAIDs in cancer cells, it is essential to unravel the molecular processes involved in apoptosis induction by these agents (16–18). Understanding these mechanisms will help to design drugs that are more specifically targeted against cancer, and, indeed, recent efforts show that chemical modifications of NSAIDs enable the selection of more efficient inducers of cancer cell apoptosis with enhanced growth inhibitory properties.

The objectives of our study were to obtain a comprehensive view of NSAID-mediated apoptosis in cancer cells and to decipher the precise molecular mechanisms of action by surveying and comparing a complete set of NSAIDs for their efficacies to induce apoptosis and growth arrest in cancer cells. We describe here a novel pathway by which NSAIDs induce apoptosis and growth arrest in cancer cells. We show that induction of the proapoptotic cytokine melanoma differentiation associated gene-7/interleukin-24 (*MDA-7/IL-24*) that mediates induction of growth arrest and DNA damage inducible 45 α (*GADD45 α*) and *GADD45 γ* expression (18–21) is sufficient for NSAID-induced cancer cell apoptosis and growth arrest. *MDA-7/IL-24* overexpression is currently used in clinical trials, and identification of drugs that are most efficient in *MDA-7/IL-24* induction may significantly enhance the antineoplastic effect of this novel cytokine.

Materials and Methods

Cell culture. The prostate cancer cell lines LNCaP, DU145, and PC-3; renal cancer cell lines Caki, UOK, A704, ACHN, and A498; stomach cancer cell lines Kato, SNU1, SNU16, NCI, and AGS1; breast cancer cell lines MDA231, MDA453, MDA435, SKBR3, and MCF-7; and the HEK 293 cell line

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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were obtained from American Type Culture Collection (Rockville, MD). The F-12 foreskin fibroblast cell line was kindly provided by Dr. Steven Goldring (Beth Israel Deaconess Medical Center), and the CW19 and CW22 prostate cancer cell lines were kindly provided by Dr. Steven P. Balk (Beth Israel Deaconess Medical Center). The MS-1 endothelial cell line was kindly provided by Peter Oettgen (Beth Israel Deaconess Medical Center). A704, ACHN A498, and DU145 cells were grown in MEM (Life Technologies, Carlsbad, CA); CW19, CW22, MDA231, MCF-7, SKBR3, MDA453, MDA435, UOK, MS-1, F-12, and HEK 293 were grown in DMEM (Life Technologies); LNCaP, SNU1, SNU16, Kato, and NCI were grown in RPMI medium (Life Technologies); Caki cells were grown in McCoy's 5A medium (Life Technologies); and AGS1 and PC-3 cells were grown in HAMS F-12 medium (BioWhittaker, Walkersville, MD). The medium was supplemented with 10% fetal bovine serum (FBS), 50 units penicillin/mL, and 50 µg streptomycin/mL (all from Life Technologies). The cells were maintained in a 5% CO₂-humidified incubator at 37°C.

Reagents. Sulindac sulfide, sulindac sulfone, ibuprofen, aspirin, acetaminophen, and naproxen were obtained from Sigma-Aldrich (St. Louis, MO). Meloxicam, celecoxib, diclofenac, finasteride, and flufenamic acid were obtained from LKT Laboratories (St. Paul, MN). NS-398, ebselen, and flurbiprofen were purchased from Calbiochem (San Diego, CA). The drugs were dissolved in DMSO or ethanol. Cancer cells were treated in their particular medium for 24 hours. The final concentration for each compound were as follows: 50 µmol/L sulindac sulfide, 5 mmol/L aspirin, 200 µmol/L ibuprofen, 50 µmol/L sulindac sulfone, 1 mmol/L acetaminophen, 200 µmol/L naproxen, 200 µmol/L NS-398, 50 µmol/L celecoxib, 40 µmol/L diclofenac, 50 µmol/L finasteride, 200 µmol/L flufenamic acid, 10 µmol/L meloxicam, 50 µmol/L ebselen, and 20 nmol/L flurbiprofen. For the controls, cells were treated with an equal amount of DMSO or ethanol, which was <0.1% of the final concentration.

Real-time PCR. Total RNA was harvested using QIAshredder (Qiagen, Valencia, CA) and RNeasy Mini kit (Qiagen). Real-time PCR was done as described (20). cDNAs were generated from 2 µg of total RNA using Ready-to-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Amplifications of 0.1 µg cDNA were carried out using SYBR Green I-based real-time PCR on the MJ Research DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Inc., Waltham, MA). All PCR mixtures contained PCR buffer [final concentration 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 2 mmol/L MgCl₂, and 0.1% Triton X-100], 250 µmol/L deoxynucleotide triphosphate (Roche, Indianapolis, IN), 0.5 µmol/L of each PCR primer, 0.5× SYBR Green I (Molecular Probes, Eugene, OR), 5% DMSO, and 1 unit Taq DNA polymerase (Promega, Madison, WI) with 2 µL cDNA in a 25 µL final volume reaction mix. The samples were loaded into wells of low-profile, 96-well microplates. After an initial denaturation step of 60 seconds at 94°C, conditions for cycling were 40 cycles of 30 seconds at 94°C, 30 seconds at 52°C, and 1 minute at 72°C. Then, the fluorescence signal was measured right after incubation for 5 seconds at 79°C that follows the extension step, which eliminates possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify the specificity of the PCR product. For each run, serial dilutions of human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) plasmids were used as standards for quantitative measurement of the amount of amplified DNA. Also, for normalization of each sample, hGAPDH primers were used to measure the amount of hGAPDH cDNA. All samples were run in triplicates and the data were presented as gene-to-GAPDH ratio. The sequences of the primers are as follows: for *GADD45α*, sense 5'-GCC-TGTGAGTGAGTGCAGAA-3'; antisense 5'-ATCTCTGTCGTCGTCCTCGT-3'; for *GADD45β*, sense 5'-TCGGATTTTGC AATTTCTCC-3'; antisense 5'-GGAT-GAGCGTGAAGTGGATT-3'; for *GADD45γ*, sense 5'-CTGCATGAGTTGCTG-CTGTC-3'; antisense 5'-TTCGAAATGAGGATGCAGTG-3'; for *MDA-7/IL-24*, sense 5'-CAAAGCCTGTGGACTTTAGCC-3'; antisense 5'-GAATAGCAGAAA-CCGCCTGTG-3'; and for *hGAPDH*, sense 5'-CAAAGTTGTCATGGATGACC-3'; antisense 5'-CCATGGAGAAGGCTGGGG-3'.

Western blot analysis. Whole-cell lysates were prepared in lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium PPi, 1 mmol/L β-glycerolphosphate, 1 mmol/L Na₃VO₄, 1 µg/mL leupeptin, and 1 mmol/L

phenylmethylsulfonyl fluoride] of treated and control cells. One hundred micrograms of protein were electrophoresed in a 10% SDS-polyacrylamide gel. Proteins were electroblotted onto polyvinylidene difluoride membranes in a 50 mmol/L Tris base, 20% methanol, and 40 mmol/L glycine electrophoresis buffer. Membranes were incubated in 5% nonfat dry milk in TBST (60 mmol/L Tris-base, 120 mmol/L NaCl, 0.2% Tween 20) for 1 hour. Blots were probed with primary antibody overnight at 4°C in 2% bovine serum albumin in TBST, and then incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA) in 5% dry milk in TBST for 1 hour at room temperature. Bound antibodies were detected by chemiluminescence with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) and were visualized by autoradiography. The primary antibodies used for Western blot analysis were anti-CDC25C (Cell Signaling Technology), anti-cyclin B1 (EMD Biosciences, San Diego, CA), anti-cyclin B2 (Santa Cruz Biotechnology), anti-phospho c-Jun (Cell Signaling Technology), anti-p21 (Cell Signaling Technology), anti-GADD45α (Santa Cruz Biotechnology), anti-GADD45β (Santa Cruz Biotechnology), and anti-GADD45γ (Santa Cruz Biotechnology). The MDA-7/IL-24 antibody was kindly provided by Sunil Chada (Introgen, Inc.).

Kinase assays. Cdc2 kinase assay was done using histone H1 as the Cdc2 substrate. Five hundred micrograms of the cell lysate were immunoprecipitated using 2 µg agarose-conjugated anti-Cdc2 monoclonal antibody (Santa Cruz Biotechnology) overnight at 4°C. The beads were washed twice with lysis buffer and twice with kinase buffer [25 mmol/L Tris (pH 7.5), 5 mmol/L β-glycerolphosphate, 2 mmol/L DTT, 0.11 mmol/L Na₃VO₄, 10 mmol/L MgCl₂] and subjected to the kinase assays. The beads were suspended in 50 µL of kinase buffer supplemented with 200 µmol/L ATP and 30 µL Cdc2 substrate cocktail [Upstate; 2 mg/mL histone H1 in 20 mmol/L MOPS (pH 7.2), 25 mmol/L β-glycerolphosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT], and incubated for 30 minutes at 30°C. The reactions were terminated by adding 25 µL of 3× SDS sample buffer, and proteins were resolved by SDS-10% PAGE and probed with phospho-histone H1 antibody (Upstate). Active Cdc2 directly correlates with enhanced phosphorylation of histone H1. c-Jun NH₂-terminal kinase (JNK) kinase activity was measured by using the stress-activated protein kinase (SAPK)/JNK assay kit (Cell Signaling Technology) according to the manufacturer's protocol and as described in the supporting text.

Apoptotic assays. Apoptosis was assayed by using the Apoptotic Cell Death Detection ELISA (Roche) and/or the Cell Death Detection (Nuclear Matrix Protein, San Diego, CA) ELISA (EMD Biosciences) according to the manufacturer's protocol.

Animals, diets, and orthotopic implantation of DU145 tumor cells. Eight-week-old male severe combined immunodeficient (SCID)-beige mice were purchased from Taconic (Germantown, NY) and housed in a pathogen-free environment. Immediately before implantation, DU145 cells infected with lentiviral vector small interfering RNA green fluorescent protein (LV-siRNA GFP) or LV-siRNA MDA-7/IL-24 or uninfected cells were trypsinized and resuspended in MEM with 10% FBS. Cell viability was determined by trypan blue exclusion. Then, a single cell suspension with >90% viability was used for implantation. A transverse incision was made in the lower abdomen, and the bladder and seminal vesicles were delivered through the incision to expose the dorsal prostate. DU145 cells (2 × 10⁶ in 50 µL) were carefully injected under the prostatic capsule via a 30-gauge needle as described previously (20). Proper inoculation of cell suspension was indicated by blebbing under the prostate capsule. The incision was closed using a running suture of 5-0 silk. All procedures with animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center according to NIH guidelines. The mice were randomly divided into two groups (*n* = 8 per group) and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200 ppm sulindac sulfide. The diets were prepared by Research Diets, Inc. (New Brunswick, NJ). Body weight and food intake were measured weekly. Six weeks after cancer cell implantation, phlebotomy was done by accessing the retro-orbital venous plexus to obtain 150 µL of blood from each mouse. Serum IL-6 level was measured by ELISA to estimate the tumor-take rate and tumor size. At the end of the experiment (8 weeks), animals were sacrificed

and tumors were carefully dissected and weighed. Lymph nodes and lungs were collected to determine metastases.

siRNA oligonucleotides and transfections. The oligonucleotides for the three GADD45 family members have been described (20). The sense-strand sequence for each siRNA (Dharmacon, Chicago, IL) is described (a complementary oligonucleotide was synthesized for each): MDA-7/IL-24 siRNA 5'-AACTTTGTTCTCATCGTGTC-3'. RNA duplexes (50 $\mu\text{mol/L}$) were transfected into cells using TKO transfection reagent (Mirus, Madison, WI) and tested for specificity and efficiency (see Supplementary Fig. S5C).

Adenovirus constructs. The adenoviruses encoding β -galactosidase (Ad5-CMV β -gal) and MDA-7/IL-24 (Ad5-MDA-7/IL-24) genes were described previously (20, 21).

siRNA lentiviral vectors. The lentiviruses encoding siRNA against the three GADD45 family members have been described (20). The LV-siRNA GFP construct (control) was kindly donated by Dr. Oded Singer (Salk Institute for Biological Studies). The lentivirus encoding siRNA against MDA-7/IL-24 gene was cloned using Advantage 2 PCR kit (Clontech, Mountain View, CA), and the virus was generated by using a previously described methodology (20). The following siRNA oligonucleotides were used: 5'-CTGTCTAGACAAAACTTTGTCTCATCGTGTCATCTCTTGAATGACACGATGAGAACAAAGGGGATCTGTGGTCTCATACA-3' for MDA-7/IL-24.

Production of lentiviral vectors and infections. Vesicular stomatitis virus G envelope protein-pseudotyped lentiviruses were prepared and purified as described (20). The specificity of all lentivirus vectors was tested (see supporting text).

Microarray analysis. Total RNA was harvested from cells treated with 50 $\mu\text{mol/L}$ sulindac sulfide or DMSO 24 hours posttreatment using QIAshredder (Qiagen) and RNeasy Mini kit (Qiagen) and converted into cRNA according to manufacturer's instructions (Affymetrix, Santa Clara, CA). Experiments were done in duplicates. cRNAs were hybridized to the HG-U133A gene array (Affymetrix), washed, and scanned according to the manufacturer's instructions (Affymetrix). Scanned array images were analyzed by dChip, where model-based gene expression values were obtained using a smoothing-spline normalization method (22) to compare two groups of samples aiming to identify genes enriched in a given phenotype. If 90% lower confidence bound of the fold change between the two groups was >1.2 , the corresponding gene was considered to be differentially expressed (22). Lower confidence bound is a stringent estimate of fold change and has been shown to be the better-ranking statistic (23). It has been suggested that a criterion of selecting genes that have a lower confidence bound above 1.2 most likely corresponds to genes with an "actual" fold change of at least 3 in gene expression (22–24).

Cell cycle analysis. Prostate cancer cells were treated with NSAIDs or DMSO as described above. After 24 hours of treatment, the medium was replaced by serum-containing medium, and cells were allowed to grow for another 24 hours. The cells were trypsinized, washed twice with cold PBS containing 2% FBS, and fixed in 70% ethanol for 60 minutes at 4°C. The cells were then washed twice with PBS and stained with 200 μL propidium iodide stock solution (50 $\mu\text{g/mL}$ propidium iodide, 3.8 mmol/L sodium triphosphate in PBS) supplemented with 50 μL RNase A (10 $\mu\text{g/mL}$) for 3 hours at 4°C and then analyzed with a FACScan cell sorter (Becton Dickinson, Franklin Lakes, NJ). Ten thousand cells were collected and the cell cycle profiles were calculated using the Cellquest Software.

Results

Multiple NSAIDs are potent inducers of apoptosis in prostate cancer cells. A broad panel of NSAIDs was tested for their abilities to induce apoptosis in cancer cells. The concentrations for all NSAIDs used in this study were selected to reflect achievable plasma concentrations (25–40). However, some drug concentrations exceeded the physiologically achievable doses (25–40). Apoptosis was measured 24 and 48 hours after treatment of DU145 prostate cancer cells with this set of NSAIDs, revealing that a variety of, but not all, NSAIDs induced apoptosis in DU145 cells.

Strong inducers of apoptosis included sulindac sulfide, finasteride, diclofenac, flufenamic acid, flurbiprofen, sulindac sulfone, and NS-398 compared with solvent controls, whereas treatment with aspirin, celecoxib, acetaminophen, ibuprofen, naproxen, meloxicam, and ebselen resulted in only marginal apoptosis induction (Fig. 1). Sulindac sulfide was the strongest inducer of apoptosis in DU145 cells and it was one of the top three apoptotic inducers in a variety of cancer cells (data not shown).

NSAIDs induce G₂-M growth arrest and apoptosis in cancer cells. Sulindac sulfide and sulindac sulfone are the two major metabolites of sulindac sulfoxide (Clinoril; Merck, Whitehouse Station, NJ). Whereas sulindac sulfide is a COX-2 inhibitor, the sulfone compound is considered not to block COX-2. Sulindac sulfide inhibits proliferation and suppresses growth of various types of cancers in xenograft mouse models (refs. 41, 42; Supplementary Fig. S1A and B). The role of NSAIDs, such as sulindac sulfide, in growth arrest remains less well characterized. Cell cycle analysis of DU145 and PC-3 cells treated with 50 $\mu\text{mol/L}$ sulindac sulfide for 24 hours revealed that sulindac sulfide induced G₂-M growth arrest in both prostate cancer cell lines when compared with control (Supplementary Fig. S2) by increasing the fraction of cells in the G₂-M phase by 2-fold (25–27).

To evaluate whether sulindac sulfide induces apoptosis in cancer cells, prostate, breast, renal, and stomach cancer cell lines, as well as untransformed cells, were treated with sulindac sulfide for 24 hours. Sulindac sulfide induced apoptosis in most prostate cancer cell lines and in almost all breast, renal, and stomach cancer cell lines except the A704 renal cancer cell line (Fig. 2). In contrast, untransformed cells, such as the MS-1 endothelial and F12 foreskin fibroblast cells, were not affected by sulindac sulfide, demonstrating broad-range specificity for cancer cells (Fig. 2). Moreover, a time course experiment showed that sulindac sulfide started to induce programmed cell death as early as 12 hours posttreatment (Supplementary Fig. S3A).

It has been established that sulindac sulfide reaches peak plasma concentrations of 30 to 50 $\mu\text{mol/L}$ (25–27), coming down to a steady-state plasma concentration of 5 to 10 $\mu\text{mol/L}$ (43). We,

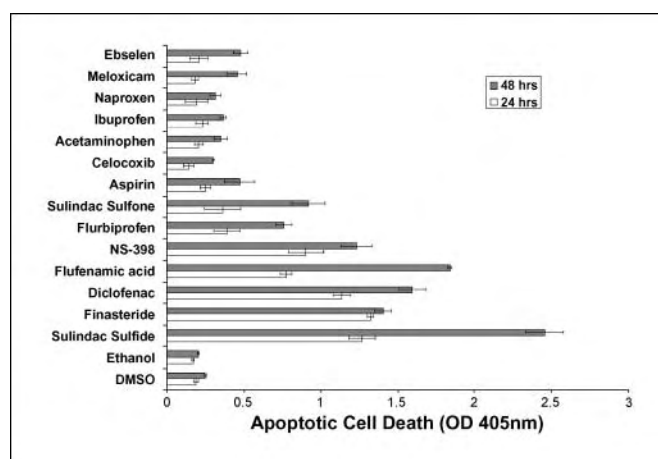


Figure 1. Multiple NSAIDs induce apoptosis in cancer cells. Apoptosis assay of DU145 prostate cancer cells after treatment with 50 $\mu\text{mol/L}$ sulindac sulfide, 5 mmol/L aspirin, 200 $\mu\text{mol/L}$ ibuprofen, 200 $\mu\text{mol/L}$ sulindac sulfone, 1 mmol/L acetaminophen, 200 $\mu\text{mol/L}$ naproxen, 200 $\mu\text{mol/L}$ NS-398, 50 $\mu\text{mol/L}$ celecoxib, 200 $\mu\text{mol/L}$ diclofenac, 50 $\mu\text{mol/L}$ finasteride, 200 $\mu\text{mol/L}$ flufenamic acid, 40 $\mu\text{mol/L}$ meloxicam, 50 $\mu\text{mol/L}$ ebselen, and 20 nmol/L flurbiprofen or DMSO. Columns, mean of triplicate independent experiments for each treatment; bars, SD. OD, absorbance.

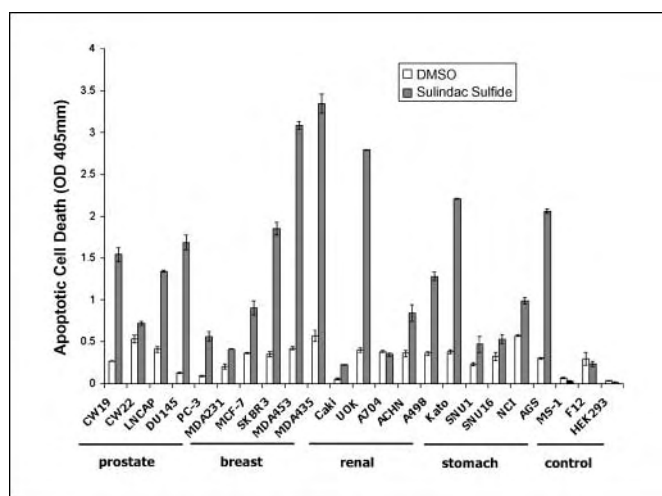


Figure 2. The NSAID sulindac sulfide induces apoptosis in cancer cells. Apoptosis assay of prostate, breast, renal, and stomach cancer cells. Cells were treated with 50 $\mu\text{mol/L}$ sulindac sulfide or DMSO. Apoptosis was measured 24 hours posttreatment. Columns, mean of triplicate independent experiments for each treatment; bars, SD.

therefore, decided to evaluate whether the sulindac sulfide steady plasma concentration achievable in patients (43) was still able to induce apoptosis in cancer cells. Prostate cancer cells were treated with 5, 10, 25, and 50 $\mu\text{mol/L}$ sulindac sulfide, and apoptosis was measured 24 hours posttreatment. We show that even low concentrations (5–10 $\mu\text{mol/L}$) of sulindac sulfide are sufficient to induce apoptosis in cancer cells (Supplementary Fig. S4A).

The proapoptotic cytokine MDA-7/IL-24 is the critical mediator of NSAID-induced apoptosis and growth arrest in cancer cells and inhibition of tumor growth *in vivo*. To elucidate the detailed molecular mechanisms underlying NSAID-mediated cell cycle arrest and apoptosis in cancer cells, we did oligonucleotide microarray-based transcriptional profiling of DU145 and PC-3 cells treated with 50 $\mu\text{mol/L}$ sulindac sulfide versus DMSO. Detailed bioinformatic analysis revealed that sulindac sulfide does not trigger indiscriminate transcriptional shutdown of cancer cells, but induces distinct patterns of gene expression changes for a wide range of transcripts related to apoptosis and cell cycle (Supplementary Table S1⁵) that were consistent across the two cell lines, further confirming their apparent relevance for the cell cycle and cell death effects of sulindac sulfide.

Particularly striking and unanticipated was the dramatic up-regulation of the proapoptotic cytokine IL-24, also named MDA-7 (18, 19). *MDA-7/IL-24* was by far the highest up-regulated gene in both cell lines (140-fold in PC-3 and 722-fold in DU145). *MDA-7/IL-24* has been shown to be a novel tumor-suppressor gene (19, 44). At low, presumably physiologic concentrations, *MDA-7/IL-24* functions predominantly as a cytokine involved in immunoregulation (19, 44). However, when overexpressed at supraphysiologic levels using an adenovirus vector, *MDA-7/IL-24* shows cancer cell-specific growth inhibitory properties without negatively affecting normal cells (19, 21, 44, 45). Furthermore, elevated endogenous *MDA-7/IL-24* expression correlates with enhanced apoptosis and prolonged overall survival of patients with small-cell lung cancer,

further supporting the anticancer role of *MDA-7/IL-24* (46). To evaluate the functional relevance of *MDA-7/IL-24* induction for NSAID-mediated apoptosis, we measured mRNA expression levels of *MDA-7/IL-24* in response to sulindac sulfide in the same cancer cell lines tested above for apoptosis induction by sulindac sulfide. Real-time PCR analysis showed that sulindac sulfide induces *MDA-7/IL-24* expression in a variety of cancer types, up to 124-fold in DU145 cells and ~10- to 20-fold in various other cancer cell lines (Fig. 3A). Induction of *MDA-7/IL-24* by sulindac sulfide correlated with the ability of this drug to induce extensive apoptosis in these cell lines (Fig. 2A). Furthermore, sulindac sulfide induced *MDA-7/IL-24* gene expression as early as 8 hours posttreatment, before induction of programmed cell death at 12 hours posttreatment (Supplementary Fig. S3A and B).

These results indicate that sulindac sulfide-mediated induction of *MDA-7/IL-24* expression is a common pathway in various types of cancer cells that respond to this NSAID by undergoing apoptosis and strongly suggest that *MDA-7/IL-24* may play a critical role in this apoptosis induction.

To evaluate the relationship between *MDA-7/IL-24* induction and apoptosis induction by various NSAIDs, we measured mRNA expression levels of *MDA-7/IL-24* in response to different NSAIDs in prostate cancer cells. Real-time PCR analysis showed that induction of *MDA-7/IL-24* is common to NSAIDs that induce apoptosis in cancer cells, because multiple, structurally unrelated NSAIDs strongly induced *MDA-7/IL-24* expression in DU145 prostate carcinoma cells (Fig. 3B) in strong correlation with NSAID-mediated apoptosis induction (correlation coefficient, 0.91; $P < 0.0001$). NSAIDs that strongly enhanced apoptosis (Fig. 1) dramatically induced *MDA-7/IL-24* expression (Fig. 3B), whereas NSAIDs that only marginally induced apoptosis (Fig. 1) did not significantly enhance *MDA-7/IL-24* expression (Fig. 3B). These data suggest a common mechanism for structurally unrelated NSAIDs in targeting *MDA-7/IL-24* induction and apoptosis induction.

We and others have shown (19, 21, 44, 45) that overexpression of *MDA-7/IL-24* following infection with an adenovirus carrying the *MDA-7/IL-24* gene induces apoptosis and inhibits cell proliferation in cancer cells (Supplementary Fig. S5A and B). To determine whether induction of growth arrest and apoptosis in cancer cells by NSAIDs is dependent on *MDA-7/IL-24* up-regulation, we generated siRNA oligonucleotides and a lentivirus encoding this siRNA against *MDA-7/IL-24*. The specificity of the *MDA-7/IL-24* siRNA oligonucleotides was validated as described in Supplementary Methods and Supplementary Fig. S5C. Infection with the LV-siRNA *MDA-7/IL-24* reduced apoptosis induction by multiple NSAIDs by 90% (Fig. 3C) and reversed to a large part the G₂-M cell cycle arrest (Fig. 3D). These results show that *MDA-7/IL-24* may play an important role in tumor cell survival and, for the first time, implicate *MDA-7/IL-24* as an essential mediator of NSAID action in cancer cells.

To determine whether NSAIDs reduce tumor growth *in vivo* and to evaluate whether their effects may be dependent on induction of *MDA-7/IL-24*, prostate cancer cells stably infected with LV-siRNA GFP or LV-siRNA *MDA-7/IL-24*, as well as uninfected cells, were orthotopically implanted into the prostate of SCID mice. The mice were randomly divided into two groups and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200 ppm sulindac sulfide. Two months later, the animals were examined for tumor formation and tumor weight. All mice developed tumors, indicating that this particular dose of sulindac sulfide did not prevent tumor formation. As seen in Fig. 3E, infection of DU145 cells with the

⁵ www.bidmcgenomics.org/SulindacSulfide.

LV-siRNA GFP virus did not affect tumor growth in the control diet group, because implantation of uninfected DU145 cells showed a similar pattern of tumor growth and tumor weight when compared with the LV-siRNA GFP group (Fig. 3E). Surprisingly, the blockage of MDA-7/IL-24 expression by siRNA interference in the LV-siRNA MDA-7/IL-24 group strongly enhanced tumor growth, demonstrating that the low endogenous basal MDA-7/IL-24 expression acts as a tumor suppressor ($P = 0.010$; Fig. 3E). Tumor weight markedly increased by 60% in the LV-siRNA MDA-7/IL-24 group fed with the

control diet when compared with the LV-siRNA GFP group (0.969 versus 0.623 g; $P = 0.010$).

Sulindac sulfide treatment reduced the average tumor weight in the LV-siRNA GFP group by 38% when compared with the control diet, confirming its antitumor efficacy (Fig. 3E). Sulindac sulfide treatment also reduced tumor growth in the LV-siRNA MDA-7/IL-24 group to some extent; however, tumor weight was still 75% higher than in the sulindac-treated LV-siRNA GFP group ($P = 0.024$; Fig. 3E). These results indicate that NSAID-mediated MDA-7/IL-24

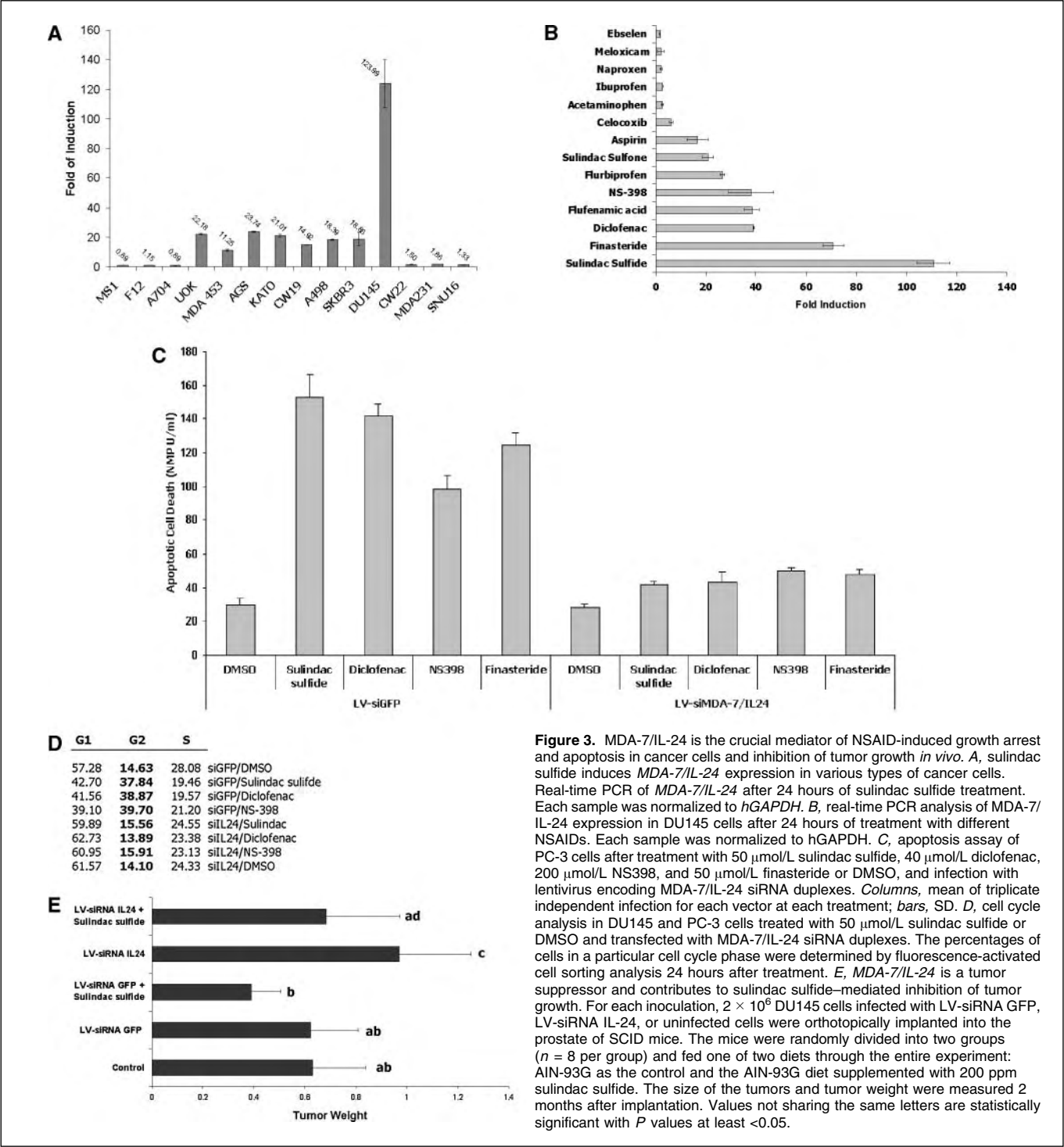


Figure 3. MDA-7/IL-24 is the crucial mediator of NSAID-induced growth arrest and apoptosis in cancer cells and inhibition of tumor growth *in vivo*. **A**, sulindac sulfide induces *MDA-7/IL-24* expression in various types of cancer cells. Real-time PCR of *MDA-7/IL-24* after 24 hours of sulindac sulfide treatment. Each sample was normalized to *hGAPDH*. **B**, real-time PCR analysis of *MDA-7/IL-24* expression in DU145 cells after 24 hours of treatment with different NSAIDs. Each sample was normalized to *hGAPDH*. **C**, apoptosis assay of PC-3 cells after treatment with 50 μ mol/L sulindac sulfide, 40 μ mol/L diclofenac, 200 μ mol/L NS398, and 50 μ mol/L finasteride or DMSO, and infection with lentivirus encoding MDA-7/IL-24 siRNA duplexes. Columns, mean of triplicate independent infection for each vector at each treatment; bars, SD. **D**, cell cycle analysis in DU145 and PC-3 cells treated with 50 μ mol/L sulindac sulfide or DMSO and transfected with MDA-7/IL-24 siRNA duplexes. The percentages of cells in a particular cell cycle phase were determined by fluorescence-activated cell sorting analysis 24 hours after treatment. **E**, *MDA-7/IL-24* is a tumor suppressor and contributes to sulindac sulfide-mediated inhibition of tumor growth. For each inoculation, 2×10^6 DU145 cells infected with LV-siRNA GFP, LV-siRNA IL-24, or uninfected cells were orthotopically implanted into the prostate of SCID mice. The mice were randomly divided into two groups ($n = 8$ per group) and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200 ppm sulindac sulfide. The size of the tumors and tumor weight were measured 2 months after implantation. Values not sharing the same letters are statistically significant with P values at least <0.05 .

induction plays a critical role in tumor growth and also indicate a tumor-suppressor activity of MDA-7/IL-24.

Induction of the *GADD45α* and *GADD45γ* genes is tightly regulated by MDA-7/IL-24 in NSAID-treated cancer cells. Our transcriptional profiling experiments, moreover, showed a strong up-regulation of *GADD45α* up to 16-fold by sulindac sulfide (Supplementary Table S1). MDA-7/IL-24 regulates and induces *GADD45α* and *GADD45γ* without affecting *GADD45β* expression (refs. 20, 21; Supplementary Fig. S6), and we have previously shown that *GADD45α* and *GADD45γ* up-regulation upon inhibition of nuclear factor- κ B (NF- κ B) is critical for induction of apoptosis in cancer cells (20). The *GADD45* gene family encodes three structurally highly related growth arrest- and DNA damage-inducible proteins, *GADD45α*, *GADD45β*, and *GADD45γ* (47), which play a role in the G₂-M checkpoint in response to DNA damage (48).

To evaluate whether regulation of *GADD45* genes is involved in NSAID-mediated apoptosis and whether *GADD45α* and *GADD45γ* regulation is a result of MDA-7/IL-24 induction by NSAIDs, expression of *GADD45* family members was measured by real-time PCR in the different cancer cell lines treated with sulindac sulfide (Fig. 4A). Whereas *GADD45β* expression was not significantly affected by sulindac sulfide in any of the cell lines, the drug induced *GADD45α* and *GADD45γ* expression 1.5- to 27-fold in various cancer cell types, indicating that *GADD45α* and *GADD45γ* expression is consistently regulated by sulindac sulfide (Fig. 4A). Furthermore, up-regulation of *GADD45α* and *GADD45γ* strongly correlated with the ability of sulindac sulfide to induce MDA-7/IL-24 expression (correlation coefficient, 0.63; $P = 0.016$; correlation coefficient, 0.69; $P = 0.0068$, respectively; Fig. 3A). Interestingly, the correlation between induction of *GADD45α* and *GADD45γ* was also significant (correlation coefficient, 0.85; $P = 0.0001$).

Differences in mRNA expression of the *GADD45* family members were corroborated on the protein level by Western blot analysis using protein extracts from DU145 and PC-3 cells treated with sulindac sulfide for 24 hours. *GADD45α* and *GADD45γ*, but not *GADD45β* protein expression, were induced in sulindac sulfide-treated cancer cells (Fig. 4B).

To evaluate whether NSAID-mediated induction of *GADD45α* and *GADD45γ* expression is dependent on MDA-7/IL-24 up-regulation, we transfected prostate cancer cells with MDA-7/IL-24 siRNA oligonucleotides and measured *GADD45* expression 24 hours after treatment with sulindac sulfide by real-time PCR. Interference with MDA-7/IL-24 expression almost completely blocked sulindac sulfide-mediated induction of *GADD45α* and *GADD45γ* gene expression without affecting *GADD45β* expression (Fig. 4C). These data most vividly show that *GADD45α* and *GADD45γ* induction by sulindac sulfide is mediated via MDA-7/IL-24 up-regulation.

Inhibition of NF- κ B also has been shown to induce apoptosis due to up-regulation of *GADD45α* and *GADD45γ* (20). Surprisingly, treatment of prostate cancer cells with sulindac sulfide had no effect on the NF- κ B signaling pathways (Supplementary Fig. S7A and S7B).

NSAID-mediated *GADD45α* and *GADD45γ* induction is essential for Cdc2 kinase activation and growth arrest. Our transcriptional profiling analysis showed that, in concordance with the observed G₂-M cell cycle arrest induced by sulindac sulfide, several genes involved in the G₂-M checkpoint and Cdc2 kinase regulation are down-regulated by sulindac sulfide, including Cdc25C, Cdc2, cyclin B1, and cyclin B2, whereas the cyclin-dependent kinase inhibitor p21 is up-regulated (Supplementary Table S1). Western blot analysis of protein extracts from DU145 and PC-3 cells treated with

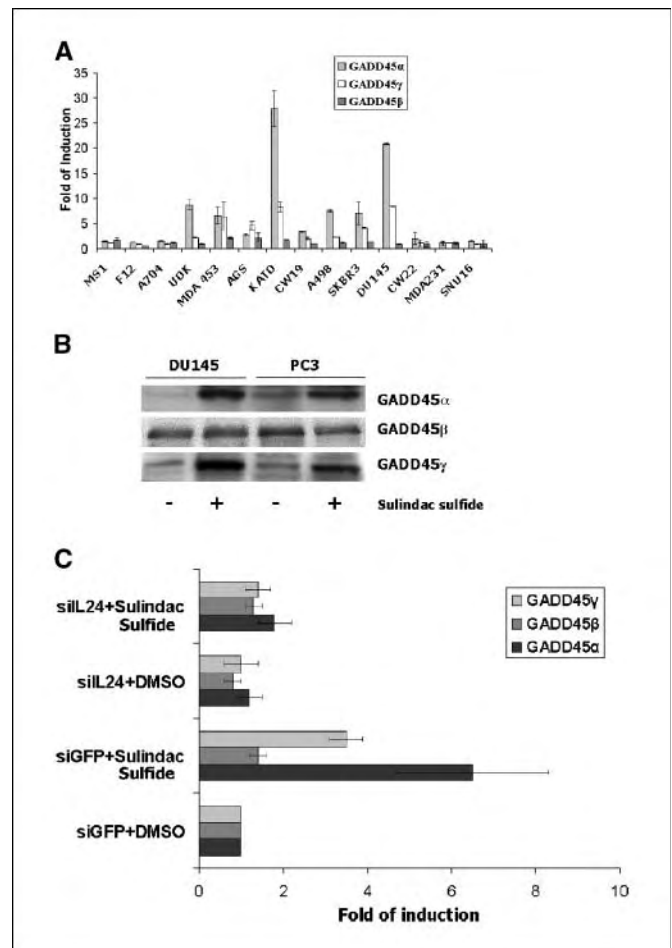


Figure 4. Expression of the *GADD45* family members is tightly regulated by NSAIDs through induction of MDA-7/IL-24. **A**, real-time PCR analysis of *GADD45α*, *GADD45β*, and *GADD45γ* expression after treatment with 50 μ M/L sulindac sulfide or DMSO in prostate, breast, renal, and stomach cancer cell lines. Each sample was normalized to *hGAPDH*. **B**, Western blot analysis of *GADD45* family members in response to sulindac sulfide treatment. Protein extracts were obtained 24 hours after treatment of prostate cancer cells with 50 μ M/L sulindac sulfide or DMSO. **C**, real-time PCR analysis of *GADD45α*, *GADD45β*, and *GADD45γ* expression after treatment of DU145 and PC-3 cells with 50 μ M/L sulindac sulfide or DMSO and transfection of MDA-7/IL-24 siRNA duplex (50 nmol/L) in prostate cancer cell lines. Each sample was normalized to *hGAPDH*.

sulindac sulfide for 24 hours corroborated the expression changes on the protein level. Protein expression of Cdc25C, cyclin B1, and cyclin B2 decreased, and p21 protein expression increased, in response to sulindac sulfide (Fig. 5A). *GADD45α* has been shown to inhibit the kinase activity of the Cdc2-cyclin B complex (49) and progression from the G₂ to the M phase of the cell cycle (50). To determine the role of *GADD45α* and *GADD45γ* in NSAID-induced G₂-M arrest, we infected DU145 prostate cancer cells with lentiviruses encoding siRNAs for *GADD45α*, *GADD45γ*, or *GFP*. An *in vitro* Cdc2 kinase assay was done with whole-cell lysates from these infected cells after treatment with sulindac sulfide or DMSO for 24 hours using histone H1 as the Cdc2 substrate. Active Cdc2 directly correlates with enhanced phosphorylation of histone H1. A significant level of phosphorylated histone H1 in untreated cells infected with the control lentivirus indicated significant Cdc2 activity in proliferating DU145 cells (Fig. 5B). Cdc2 activity was substantially decreased 24 hours after sulindac sulfide treatment correlating with enhanced

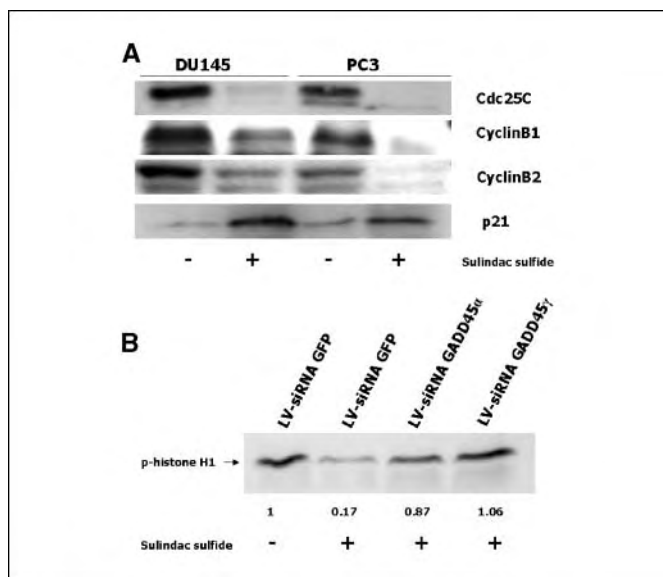


Figure 5. Sulindac sulfide-induced GADD45 expression is essential for growth arrest induction by altering the Cdc2-cyclin B checkpoint. **A**, regulation of genes involved in the Cdc2-cyclin B signaling pathway by sulindac sulfide. Western blot analysis of Cdc25C, cyclin B1, cyclin B2, and p21 proteins in DU145 and PC-3 cells treated for 24 hours with 50 $\mu\text{mol/L}$ sulindac sulfide or DMSO. **B**, inhibition of Cdc2 activation in response to sulindac sulfide treatment is due to induction of GADD45 α and GADD45 γ . Phosphorylation of the Cdc2 substrate histone H1 by the Cdc2-cyclin B complex in PC-3 cells 24 hours after treatment with 50 $\mu\text{mol/L}$ sulindac sulfide and infections with GADD45 α and GADD45 γ LV-siRNAs.

GADD45 α and GADD45 γ expression and G₂-M arrest. However, inhibition of GADD45 α and GADD45 γ expression in DU145 cells by the LV-siRNAs restored Cdc2 kinase activity as seen by increased histone H1 phosphorylation (Fig. 5B). These data suggest that sulindac sulfide-induced G₂-M cell cycle arrest is due to a combination of decreased expression of several G₂-M transition cell cycle regulators and MDA-7/IL-24-induced GADD45 α and GADD45 γ up-regulation that leads to inhibition of Cdc2 activity.

MDA-7/IL-24-dependent GADD45 α and GADD45 γ induction and JNK activation are critical for NSAID-mediated apoptosis induction in cancer cells. To elucidate the functional relevance of GADD45 α and GADD45 γ for NSAID-mediated apoptosis, we measured apoptosis induction by sulindac sulfide in GADD45 α and GADD45 γ knockdown cells. siRNA-mediated inhibition of sulindac sulfide induced up-regulation of GADD45 α or GADD45 γ expression and almost completely abrogated apoptosis induction (Fig. 6A), clearly demonstrating the absolute requirement of MDA-7/IL-24-dependent GADD45 α and GADD45 γ up-regulation for apoptosis induction by NSAIDs.

Because we and others had shown that JNK activation plays a role in apoptosis induction in cancer cells and GADD45 α and GADD45 γ interact with the upstream kinase of JNK, mitogen-activated protein kinase kinase 4, and activate JNK (47), we evaluated the relevance of JNK for NSAID-mediated apoptosis. JNK kinase activity was tested in protein extracts obtained from DU145 and PC-3 cells treated with sulindac sulfide or DMSO for 24 hours by an *in vitro* kinase assay. Western blot analysis revealed very little JNK activity in untreated control cells and a strong increase in JNK activity in both cell lines upon treatment with sulindac sulfide (Fig. 6B). JNK activation by sulindac sulfide was at least partially dependent on MDA-7/IL-24 induction because JNK activity in sulindac sulfide-treated MDA-7/IL-24^{-/-} cells was reduced by

62%, but not completely abolished when compared with MDA-7/IL-24^{+/+} cells (Fig. 6C). The importance of GADD45 α and GADD45 γ for NSAID-induced JNK activation was evaluated in DU145 and PC-3 cells infected with the GADD45 α or GADD45 γ siRNA lentiviruses or the control lentivirus and treated with sulindac sulfide for 24 hours. Inhibition of sulindac sulfide-mediated up-regulation of GADD45 α and GADD45 γ expression by siRNA drastically reduced JNK activation in both cell lines, correlating with the inhibition of apoptosis induction (Fig. 6D).

To establish the relevance of JNK activation in mediating apoptosis in cancer cells, apoptosis was measured in protein extracts obtained from DU145 and PC-3 cells treated with 50 $\mu\text{mol/L}$ sulindac sulfide or DMSO in the absence or presence of a specific JNK inhibitor, JNKII. Compared with controls, apoptosis of sulindac sulfide-treated cells was reduced by >56% in DU145 cells and 40% in PC-3 cells in JNKII-treated cells, but inhibition of JNK did not fully abolish apoptosis induction (Fig. 6E). These results suggest that JNK contributes to, but is not absolutely essential for, sulindac sulfide-mediated apoptosis.

Discussion

Dissection of the biological and biochemical pathways targeted by NSAIDs will provide ample opportunities to screen for chemically modified NSAIDs or for new compounds that are more effective and specific in destroying cancer cells. Furthermore, NSAID treatments have been reported to induce changes in gene expression in a variety of cancer cells affecting multiple target genes (51–54). We report here the discovery of a novel biological pathway involving MDA-7/IL-24 and the GADD45 gene family that are targeted by a set of NSAIDs in a number of cancer types and whose activation directly correlates with the efficacy of NSAIDs to induce cancer cell death. We show that multiple classes of structurally unrelated NSAIDs induce apoptosis and growth arrest via induction of MDA-7/IL-24 expression with consecutive GADD45 α and GADD45 γ induction, leading to JNK activation and GADD45 α - and GADD45 γ -dependent inhibition of Cdc2 activity (Fig. 6F).

Several studies using an adenovirus encoding the MDA-7/IL-24 gene show its profound and selective anticancer activity *in vitro* and in animal models (19, 21, 44, 45, 55). However, transient expression, frequent adverse immune reactions, and limitations to local delivery may restrict the use of adenoviral delivery of MDA-7/IL-24. This potential problem is partly ameliorated by the potent “bystander antitumor” activity of MDA-7/IL-24 mediated by its cytokine properties (19, 44, 56, 57). In these contexts, our discovery of specific high-level induction of MDA-7/IL-24 in different types of cancer cells, by a variety of NSAIDs, provides a new entry point to enhance MDA-7/IL-24 levels in cancer cells on a systemic level that should be significantly more effective than adenoviral delivery. Additionally, recent studies correlate increased levels of endogenous MDA-7/IL-24 with a favorable prognosis in lung adenocarcinoma and a significantly higher incidence of apoptotic tumor cells (46) and treatment of human lung tumor xenografts in nude mice with Ad-MDA-7/IL-24 plus sulindac suppressed growth more efficiently than Ad-MDA-7/IL-24 or sulindac alone (58). Therefore, our findings that NSAIDs induce high levels of MDA-7/IL-24 in various types of cancers and use the potent proapoptotic activity of MDA-7/IL-24 to induce apoptosis and cell cycle arrest in cancer cells strongly support the notion that therapeutic strategies that lead to enhanced MDA-7/IL-24 expression in cancer cells will have a significant effect on cancer patient survival.

Our *in vivo* orthotopic tumor model provides further support of the hypothesis that MDA-7/IL-24 is indeed a tumor-suppressor gene. Blocking MDA-7/IL-24 expression by siRNA interference not only reduced the ability of sulindac sulfide to inhibit tumor growth *in vivo*, but also enhanced tumor growth in the LV-siRNA MDA-7/IL-24 group. However, sulindac sulfide, to some extent, still inhibited tumor growth in animals receiving LV-siRNA MDA-7/IL-

24. This partial *in vivo* effect of sulindac sulfide on tumor growth following injection with tumor cells expressing the MDA-7/IL-24 siRNA could be explained by several mechanisms: Although MDA-7/IL-24 expression was inhibited in the tumor cells, systemic sulindac sulfide exposure in the mice led to MDA-7/IL-24 expression in mouse-derived cells or tissues that affected the implanted tumor cells and partially reversed the effect of the

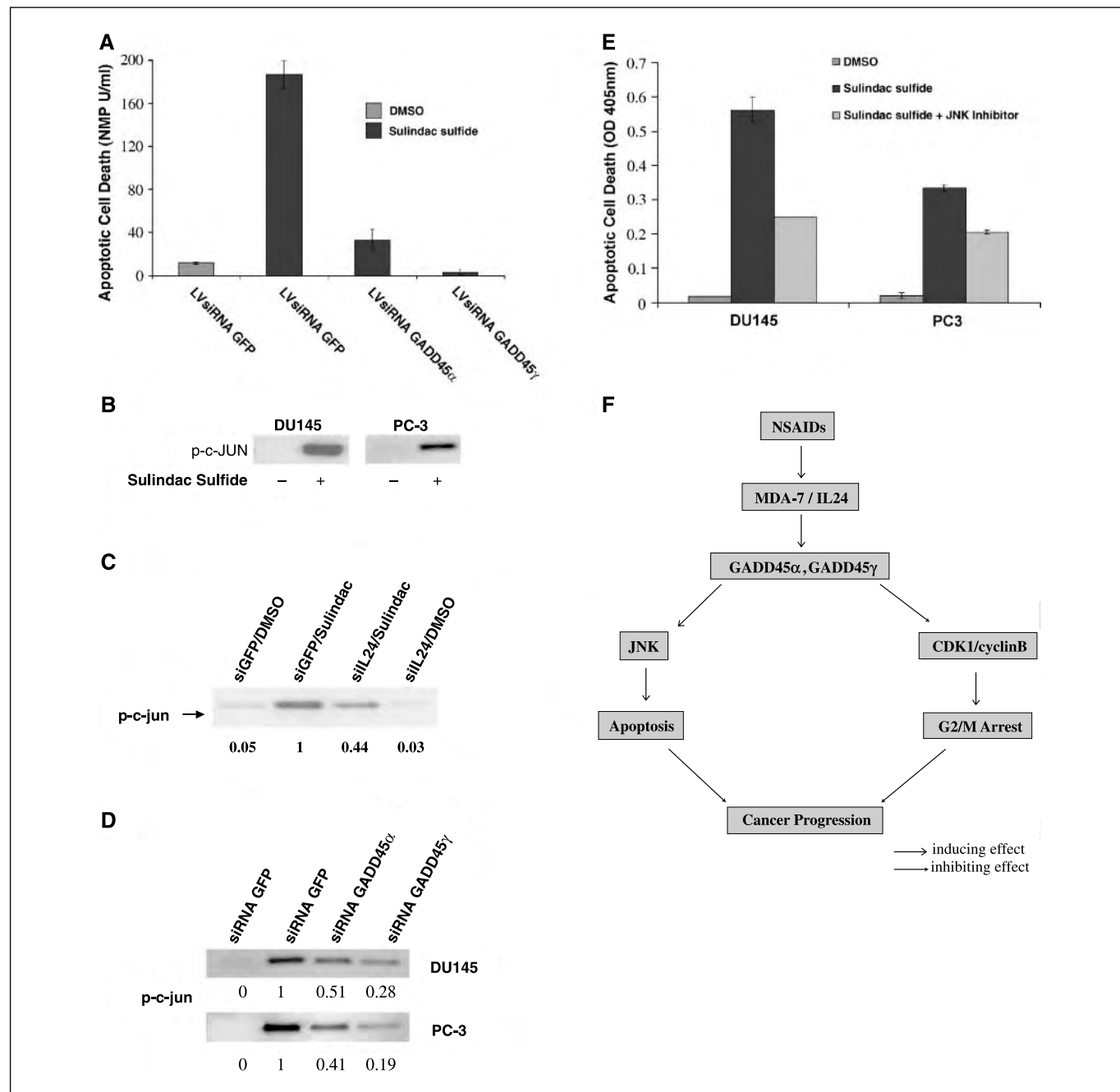


Figure 6. Induction of GADD45 expression by NSAIDs is essential for JNK activation and apoptosis induction. **A**, apoptosis assay of PC-3 cells after treatment with 50 μ mol/L sulindac sulfide or DMSO and infection with GADD45 α and GADD45 γ LV-siRNAs. Columns, mean of triplicate independent infections for each vector at each treatment; bars, SD. **B**, induction of JNK activation by sulindac sulfide. JNK kinase was analyzed in cell lysates from DU145 and PC-3 cells treated with 50 μ mol/L sulindac sulfide or DMSO using the SAPK/JNK assay kit (Cell Signaling). **C**, kinase assay showing inhibition of JNK kinase activity by MDA-7/IL-24 siRNA (50 μ mol/L) in prostate cancer cells treated with 50 μ mol/L sulindac sulfide or DMSO. **D**, kinase assay showing inhibition of JNK kinase activity by GADD45 siRNA duplexes (50 μ mol/L) in prostate cancer cells treated with 50 μ mol/L sulindac sulfide or DMSO. **E**, apoptosis of prostate cancer cells 24 hours after treatment with 50 μ mol/L sulindac sulfide or DMSO and treatment with the JNK inhibitor JNKII SP600125 (100 nmol/L; Calbiochem). Columns, mean of triplicate independent treatments; bars, SD. **F**, schematic presentation of NSAID signaling pathway in cancer.

MDA-7/IL-24 siRNA; it is also likely that MDA-24/IL-24 is not the sole pathway targeted by sulindac sulfide and the drug could act through multiple pathways to induce programmed cell death in cancer cells, including indirect antitumor effects on stromal and vascular cells.

Additionally, our data provide strong evidence that multiple NSAIDs induce cancer cell death through MDA-7/IL-24-mediated up-regulation of GADD45 α and GADD45 γ , irrespective of their ability to block COX-2. For example, finasteride, a selective 5- α -reductase inhibitor (59), is not known to inhibit COX-2 but strongly induces MDA-7/IL-24 expression and apoptosis, whereas the potent selective COX-2 inhibitor celecoxib is significantly less effective in inducing MDA-7/IL-24 and apoptosis. On the other hand, the nonselective cation channel blocker flufenamic acid and the nonselective COX-1 and COX-2 inhibitors sulindac sulfide and diclofenac are very potent inducers of MDA-7/IL-24 and apoptosis. Thus, the proapoptotic anticancer activity of several divergent classes of drugs evaluated here seems not to be due primarily to their effects on their supposed targets, but due to the off-target induction of MDA-7/IL-24.

Inhibition of NF- κ B in the same cell lines tested here induced apoptosis without inducing MDA-7/IL-24, although up-regulation of GADD45 α and GADD45 γ via repression of c-myc expression was as essential for apoptosis induction as in response to NSAIDs (20) and multiple MDA-7/IL-24-dependent and MDA-7/IL-24-

independent pathways seem to merge into GADD45 α and GADD45 γ . Although current studies indicate that members of the GADD45 family appear infrequently mutated in cancer, reduced GADD45 expression due to gene and/or promoter methylation have been frequently observed in several types of human cancer (60, 61).

In conclusion, the ability of NSAIDs to induce apoptosis seems to depend on their abilities to induce MDA-7/IL-24 expression and enhance GADD45 α and GADD45 γ expression. Thus, apoptosis and growth arrest induction of cancer cells as a result of enhanced MDA-7/IL-24 expression seems to be a common pathway for multiple classes of drugs. These results also provide a rationale to screen small-molecule libraries, natural compound libraries, and chemically modified NSAIDs for selective inducers of MDA-7/IL-24 expression in cancer cells to obtain more effective anticancer drugs.

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