Mechanisms of nordihydroguaiaretic acid-induced growth inhibition and apoptosis in human cancer cells

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Arachidonic acid and its metabolites are important second messengers in the signal transduction pathways induced by receptor tyrosine kinases and G protein coupled receptors (Di Marzo, 1995; Rozengurt, 1998). 5-hydrocicosatetraenoic acid (5-HETE), the major 5-lipoxygenase metabolite of arachidonic acid, has been implicated as a growth promoting factor for various human cancer cells including prostate, lung and pancreatic cancer cells (Avis et al., 1993), it has been used extensively to examine the role of lipoxigenases in human cancer cell lines. However, nordihydroguaiaretic acid inhibits growth and induces apoptosis of these cancer cells in vitro and in vivo. Potential mechanisms mediating these effects of nordihydroguaiaretic acid were examined. Nordihydroguaiaretic acid had no inhibitory effect on growth and survival signals such as tyrosine phosphorylation of the epidermal growth factor receptor or basal and growth factor-stimulated activities of extracellular signal-regulated kinase 1/2, p70S6k and AKT but selectively inhibited expression of cyclin D1 in the cancer cells. In addition, treatment with nordihydroguaiaretic acid lead to a disruption of the filamentous actin cytoskeleton in human pancreatic and cervical cancer cells which was accompanied by the activation of Jun-NH2-terminal kinase and p38mapk. Similar effects were obtained by treatment of the cancer cells with cytochalasin D. These results suggest that nordihydroguaiaretic acid induces anoikis-like apoptosis as a result of disruption of the actin cytoskeleton in vivo.

Keywords: NDGA; apoptosis; pancreatic cancer; cervical cancer; JNK; actin cytoskeleton

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Arachidonic acid and its metabolites are important second messengers in the signal transduction pathways induced by receptor tyrosine kinases and G protein coupled receptors (Di Marzo, 1995; Rozengurt, 1998). 5-hydrocicosatetraenoic acid (5-HETE), the major 5-lipoxygenase metabolite of arachidonic acid, has been implicated as a growth promoting factor for various human cancer cells including prostate, lung and pancreatic cancer cells (Avis et al., 1993; Ghosh and Myers, 1998; Ding et al., 1999a). The resinous plant exudate nordihydroguaiaretic acid (NDGA) inhibits lipoxigenases including 5-lipoxygenases (Van Wauwe and Goossens, 1983; Chang et al., 1984). Although nordihydroguaiaretic acid (NDGA) may target other kinases (Rondeau et al., 1990; Domin et al., 1993), it has been used extensively to examine the role of the lipoxigenase pathway in the action of growth factors and cytokines (Haliday et al., 1991; Peppelenbosch et al., 1992). Indeed, NDGA blocks lipoxigenase production, inhibits growth and induces apoptosis of human lung cancer cells (Avis et al., 1996; Ghosh and Myers, 1998). In pancreatic cancer cells, NDGA inhibits thymidine incorporation and anchorage-dependent proliferation and induces apoptosis in vitro (Ding et al., 1999a,b). However, the potential mechanisms mediating these effects have not been examined. In addition, it is still unclear whether NDGA can block growth of various tumours in vivo. This is an important question as numerous compounds inhibit cancer cell growth in vitro but fail to be effective in vivo.

Apoptosis can be induced by many different events such as direct damage to the cell or its DNA or by the removal of survival signals provided by growth factors, cell-cell contacts and the extracellular matrix. The Raf-MEK-ERK cascade is one of the major signalling pathways promoting cell survival (Parrizas et al., 1997; Kurada and White, 1998; Anderson and Tolkovsky, 1999; Bonni et al., 1999). However, Ras-dependent cell survival is likely to require additional downstream effectors such as the PI3-kinase-AKT signaling pathway (Datta et al., 1999). There is a controversy regarding the contribution of other mitogen-activated protein kinases (MAPKs) such as Jun-NH2 terminal kinase (JNKs) and p38mapk to cell survival or apoptosis. Under certain circumstances, activation of JNKs contributes to proliferation. However, in other model systems, activation of JNKs and p38mapk (also known as stress activated protein kinases) can mediate apoptosis (Xia et al., 1995; Chen et al., 1996; Verheij, 1996; Zanke et al., 1996; Bossy-Wetzel et al., 1997; Behrens et al., 1999; Tournier et al., 2000). A similar role for stress activated protein kinases has been proposed for anoikis, the induction of apoptosis in epithelial cells by disruption of cell-cell and cell-matrix contacts (Frisch and Francis, 1994; Frisch et al., 1996).

Here we demonstrate that NDGA markedly inhibits growth and induces apoptosis of human pancreatic and cervical cancer cells in vitro and in vivo. NDGA did not prevent constitutive phosphorylation of p70S6K in these cells which regulates autonomous

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anchorage-dependent and -independent proliferation of tumour cells (Grewe et al, 1999). In addition, NDGA did not inhibit major survival pathways such as tyrosine phosphorylation of the EGF, or TGFR-induced activation of the ERK cascade and Akt. However, treatment of cells with NDGA leads to activation of JNKs as indicated in the figure legends. Controls received bated with TGFβ.

For ERK assays serum-starved SW 850 and C4-I cells were incubated with NDGA as indicated in the figure legends and lysed in SDS -PAGE sample buffer. Proteins were separated by SDS -PAGE followed by autoradiography. For JNK and p38 MAPK cells were washed with PBS and lysed in 20 mM HEPES pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na3VO4, 10% glycerol, 1% Triton X-100, 2 µM leupeptin, 0.5 µM AEBSF, 5 µg ml -1 aprotinin, 0.1 µg ml -1 okadaic acid. After 5 min on ice, the lysate was clarified and immunoprecipitated with the respective antibodies. The immunoprecipitates were washed thrice each in lysis buffer and finally in assay buffer (JNK: 20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM MgCl2, 1 mM DTT, 0.1% Triton X-100; p38: 20 mM MOPS, pH 7, 1 mM EDTA, 5% glycerol, 0.1% β-mercaptoethanol, 0.01% Brij 35, 0.1 µg ml -1 okadaic acid). Kinase reactions contained 20 µl kinase buffer with 1 µg of either ATF2- or cJun-GST fusion protein and Mg-ATP mixes as follows: JNK: 7.5 µl 50 mM MgCl2, 100 µM ATP, 2 µCi [γ-32P]ATP, p38 MAPK, 6 µl 60 mM Mg acetate, 300 µM ATP and 2 µCi [γ-32P]ATP. After 20 min at 30°C, the reactions were stopped by addition of 5 × SDS-PAGE sample buffer and further analysed as described above. GST-cJun and GST-ATF2 fusion proteins were prepared essentially as described (Seufferlein et al, 1999).

p70 euk mobility shift assays

Activation of p70 euk by mitogens can be determined by the appearance of slower migrating forms in SDS-PAGE due to phosphorylation of p70 euk on Thr 389 and Thr 405 which are not phosphorylated in quiescent cells (Ferrari and Thomas, 1994). For p70 euk mobility shift assays cells were treated as indicated in the figure legends and lysed in SDS-PAGE sample buffer. Samples were further analysed by SDS-PAGE and Western blotting with a specific anti-p70 euk antibody.

In situ detection of apoptotic cells

DNA fragmentation was measured by catalytically incorporating fluorescein-12-dUTP at the 3'-OH DNA ends using the enzyme TdT according to the principle of the TUNEL assay. To detect apoptosis in tumour xenografts paraffin-embedded tissue sections of the tumours were deparaffinised and rehydrated through graded ethanol washes, fixed in 4% methanol-formaldehyde, treated with 20 µg ml -1 protease K solution for 8–10 min, fixed again and further processed according to the manufacturer’s protocol.

Tumour growth in athymic mice

1.5 × 10⁶ SW 850 or 2 × 10⁶ C4-I cells were inoculated subcutaneously into both flanks of 4–6 week old female athymic NMRI/nu-nu mice and the mice were maintained in a pathogen-free environment. The animals were observed daily for tumour development. When measurable tumours were established (> 15 mm³), the animals were randomised into two groups of six animals. One group received 750 µg NDGA (corresponding to about 90 µM...
NDGA (w/v) in 0.1 ml of a solution containing 90% sterile H₂O and 10% ethanol i.p. 5× per week. The control animals received an equivalent amount of solvent i.p. Tumour size was measured twice weekly and mice were killed after 3 weeks of treatment. Growth curves for xenografts were determined by externally measuring the length, height and width of the tumours, and the volume was calculated according to the following equation: volume=\((\text{length} \times \text{height} \times \text{width}) \times 0.5\). To examine statistical significance an univariate Students t-test was performed.

Materials

NDGA was obtained from FLUKA/Sigma-Aldrich (Deisenhofen, Germany). Antibodies against p70S6K, p27kip1, cyclin D1, cyclin E, JNK1/2, p38\(^{\text{GAPD}}\), and the EGFR were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). The phospho-specific anti-p38\(^{\text{GAPD}}\) and anti-AKT antibodies were from New England Biolabs (Schwalbach/Taunus, Germany). Protein A sepharose was obtained from Boehringer Mannheim (Mannheim, Germany). The apoptosis detection system fluorescein was from Promega (Mannheim, Germany). Oregon-green labelled phalloidin was obtained from Molecular Probes (Leiden, Netherlands). MBP was from Sigma (Deisenhofen, Germany). All other reagents were of the purest grade available.

RESULTS

NDGA delays growth of tumours established in athymic mice

To establish whether NDGA could indeed inhibit tumour growth in vivo we first examined a panel of various epithelial cancer cell lines for their ability to consistently induce xenograft tumours in nude mice. Among these tumour cell lines, SW 850 human pancreatic cancer cells and C4-I human cervical cancer cells induced tumour xenografts in athymic mice most consistently and were therefore used for all subsequent experiments (data not shown). Tumours were established by subcutaneous injection of SW 850 and C4-I cells to both flanks of athymic mice. When the tumour volume was 15 mm\(^3\), mice were treated with five times per week i.p. injections of NDGA or diluent for 3 weeks. As shown in Figure 1A, treatment with NDGA delayed the growth of both SW 850 and C4-I tumours by about 50%. The compound was very well tolerated by the animals. No side effects or behavioural abnormalities were observed during the course of treatment. There were no marked differences in body weight of the animals treated with NDGA or with solvent during the course of treatment despite the fact that the animals treated with solvent had bigger tumours. In animals injected with SW 850 cells, body weight in the control group was 21.5±1.8 g at the beginning and 24±1.3 g at the end of the treatment. In the NDGA group body weight of the animals was 23.3±1.2 g at the beginning and 24.3±1.4 g at the end of the treatment. The corresponding figures in the animals injected with C4-I cells were 27.5±2.1 g and 29.8±1.7 g in the control group and 27.5±2 g and 29.2±2.1 g in the NDGA group, respectively.

NDGA inhibits anchorage-independent growth of SW 850 and C4-I cancer cells

In addition to its effect on tumour growth in vivo we examined the effect of NDGA on colony formation of SW 850 and C4-I cells in soft agar, a useful in vitro-test to judge the efficacy of a compound as a potential anticancer agent (Carney et al., 1980). NDGA potently inhibited colony formation in response to both 0.5% FBS and 10% FBS, which induced maximum clonogenic growth in SW 850 and C4-I cells (Figure 1B). Thus, NDGA is a potent inhibitor of anchorage-independent growth in SW 850 and C4-I cells even in the presence of maximum stimulatory concentrations of FBS.

NDGA has previously been reported to inhibit thymidine incorporation and anchorage-dependent proliferation in human pancreatic cancer cells (Ding et al., 1999a). We confirmed these observations in SW 850 and also in C4-I cervical cancer cells (data not shown). Interestingly, we observed that as early as 8 h after treatment with NDGA, cells started to detach from the tissue culture dishes making it difficult to determine whether the reduction in thymidine incorporation and cell numbers observed in response to NDGA were due to inhibition of DNA synthesis rather than cell detachment.

Figure 1 (A) NDGA delays growth of xenograft tumours established in athymic mice: A: 1.5×10\(^6\) SW 850 or 2×10\(^3\) C4-I cells were inoculated subcutaneously into both flanks of 4–6 week old female athymic NMR/Nu-nu mice and the mice were maintained in a pathogen-free environment. When measurable tumours were established (>15 mm\(^3\)), the animals were randomised into two groups of six animals and treated as described in Materials and Methods. Growth curves for xenografts were determined by externally measuring the length, height and width of the tumours at the days indicated. The tumour volume was calculated according to the following equation: volume=\((\text{length} \times \text{height} \times \text{width}) \times 0.5\). Values are the means ± SE (n=6). Mice were sacrificed after 3 weeks of treatment with 750 μg NDGA 5× per week i.p. * Indicates a statistically significant difference in tumour size between the NDGA group and the control group (P<0.05). (B) NDGA inhibits colony growth in SW 850 and C4-I cells. (A) Single cell suspensions of SW 850 (left panel), or C4-I cells (right panel) were plated at a density of 3×10\(^3\) cells/dish in agarose medium containing RPMI and 0.5% (left) or 10% FBS (right) and various concentrations of NDGA as indicated. Colonies were counted after 2 weeks of incubation. In all cases, a representative of two independent experiments each performed in triplicates is shown.
NDGA induces apoptosis of SW 850 and C4-I cancer cells in vitro and in vivo

NDGA has been reported to induce apoptosis in certain cancer cells in vitro. Indeed, NDGA induced apoptosis in SW 850 pancreatic cancer cells and also in C4-I human cervical cancer cells as judged by TUNEL assays (Figure 2A). Upon treatment of cells with 25 μM NDGA for 12 h, the proportion of cells exhibiting fragmented DNA markedly increased from 2 to 34% in SW 850 and from 0 to 26% in C4-I cells. The apoptosis-inducing effect of NDGA was first detectable after 3 h of incubation and reached a maximum after 12–16 h of incubation (data not shown). To examine whether NDGA induced similar molecular mechanisms in the pancreatic and cervical xenograft tumours in vivo, paraffin-embedded tissue sections of tumours obtained from animals treated with NDGA or with solvent were examined by in situ-fluorescence TUNEL assays. Treatment of mice with NDGA led to a marked increase in the number of apoptotic cells in tumours established from SW 850 and C4-I cells (Figure 2B). Thus, NDGA induces apoptosis in pancreatic and cervical tumours in vitro and in vivo.

NDGA does not inhibit growth and survival signals in SW 850 and C4-I cells

Next, we were interested in potential mechanisms mediating the growth inhibitory effect of NDGA in human pancreatic and cervical cancer cells. NDGA has been shown to inhibit tyrosine phosphorylation of certain receptor tyrosine kinases (Domin et al, 1993). The EGFR and its respective ligands such as TGF-α trigger growth and prevent apoptosis by autocrine and/or paracrine mechanisms in many cancer cell lines (Korc et al, 1992; Bonni et al, 1999; Seufferlein et al, 1999). To examine whether NDGA could inhibit tyrosine phosphorylation of the EGFR, serum starved SW 850 and C4-I cells were treated with TGF-α in the absence or presence of NDGA and autophosphorylation of the EGFR was determined by anti-Tyr(P) Western blotting. NDGA had no effect on TGF-α-induced tyrosine phosphorylation of the EGFR in SW 850 and C4-I cells, respectively (Figure 3A). Similar data were obtained for insulin-like growth factor I-induced phosphorylation of the IGF-1 receptor (data not shown).

Activity of the serine-threonine kinase p70S6K is important for progression from the G1 to the S phase of the cell cycle (Chou and Blenis, 1995). We have recently demonstrated that the FRAP-p70S6K pathway is constitutively active in human pancreatic cancer cells and regulates autonomous growth of these cells (Grewel et al, 1999). As shown in Figure 3B, NDGA did not substantially inhibit basal phosphorylation of p70S6K. NDGA treatment of SW 850 cells also did not markedly interfere with further phosphorylation of p70S6K in response to TGF-α. In contrast, treatment of cells with the selective inhibitor of the FRAP-p70S6K pathway, rapamycin (Brown et al, 1995), prevented both, basal and TGF-α-stimulated phosphorylation of p70S6K (Figure 3B). Similar results were obtained in the C4-I cell line (data not shown).

NDGA inhibits expression of cyclin D1 in SW 850 and C4-I human cancer cells

Thus, inhibition of tumour cell growth by NDGA could not be explained by inhibition of the major growth promoting intracellular signalling pathways in response to NDGA. Therefore, we reasoned that this compound could directly affect the regulation of the cell cycle machinery. In particular, cyclin D1 is a major regu-
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Figure 3

(A) TGFα-induced tyrosine phosphorylation of the EGFR in SW 850 and C4-I cells is not affected by NDGA treatment of cells. Serum starved SW 850 (left) or C4-I cells (right) were treated for 60 min with 25 μM NDGA (+). Control cells received an equivalent amount of solvent (−). Cells were subsequently treated with 50 ng ml⁻¹ TGFα (TGFα, +) or an equivalent amount of solvent (−) for 10 min. Cells were then lysed and tyrosine phosphorylation of the EGFR was further analysed as described in Materials and Methods. (B) NDGA does not inhibit constitutive and TGFα-stimulated phosphorylation of p70S6K in SW 850 cells. Serum-starved cultures of SW 850 cells were treated for 15 min with 50 ng ml⁻¹ TGFα in the absence (−) or presence of 30 μM NDGA (NDGA, +) or 20 ng ml⁻¹ rapamycin (Rapa, +) or received an equivalent amount of solvent (−). p70S6K mobility shift assays were performed as described in Materials and Methods. (C) NDGA does not inhibit basal and TGFα-stimulated activation of Akt. Serum-starved cultures of C4-I cells were treated for 15 min with 50 ng ml⁻¹ TGFα in the absence (−) or presence of 30 μM NDGA (+) or 20 μM LY294002 (+) or received an equivalent amount of solvent (−). Phosphorylation of Akt at Ser⁴³¹ was determined using an activation-specific antibody as described in Materials and Methods. (D) NDGA does not inhibit basal and TGFα-stimulated activation of ERK1/2. Serum-starved cultures of C4-I cells were treated for 15 min with 50 ng ml⁻¹ TGFα (T) in the absence (−) or presence of 25 μM NDGA (N) or 15 μM PD 098059 (PD) or received an equivalent amount of solvent (−). ERK1/2 immune complex kinase assays were performed as described in Materials and Methods. (E) NDGA inhibits constitutive cyclin D1 expression in SW 850 and C4-I cancer cells: Subconfluent cultures of SW 850 and C4-I cells were treated with 25 μM NDGA (N) for 18 h. Control cells received an equivalent amount of solvent (−). Cells were lysed and further analysed by Western blotting with either anti-cyclin D1, anti-cyclin E or anti-p27kip1 antibodies as indicated by an arrow. The results shown in each case are representative of at least three independent experiments.

Figure 4

NDGA and cytochalasin D induce depolymerisation of the actin cytoskeleton in SW 850 and C4-I cells

The actin cytoskeleton in concert with adhesion molecules controls cell-cell and cell-substrate interactions and participates in transmembrane signalling. Upon treatment of cells with NDGA we observed substantial cell detachment. This could be a consequence of the induction of apoptosis, but also due to a more direct effect of NDGA on the cytoskeleton in pancreatic and cervical cancer cells. To examine the effect of NDGA on the actin cytoskeleton in SW 850 and C4-I cells, actin was analysed by immunofluorescence using Oregon-green-labelled phalloidin after treatment of cells with NDGA or solvent. As shown in Figure 4 (top panels), control cells exhibited a well developed actin cytoskeleton with a plasma membrane and actin stress fibres crossing the cells. Strikingly, within 30 min of exposure to 25 μM NDGA, actin stress fibers disappeared and the number of mikrospires was markedly reduced. A maximum effect of NDGA on the actin cytoskeleton was observed after 60 min of incubation (Figure 4, middle panels) and clearly preceded cell detachment and the induction of apoptosis in response to NDGA (data not shown). Interestingly, cortical actin filaments were less affected by the NDGA treatment (Figure 4, middle panels). The effect of NDGA on the filamentous actin cytoskeleton was comparable to that of cytochalasin D. However, in addition to the disruption of actin stress fibres, cytochalasin D

latory of proliferation at the level of the cell cycle (Sherr, 1993) and is overexpressed in many cancers (Weinstein, 1996). In pancreatic cancer, overexpression of cyclin D1 is associated with increased aggressiveness of these tumours (Kornmann et al, 1998). We have previously shown that cyclin D1 and E but also cyclin-dependent kinase inhibitors such as p27kip1 are constitutively expressed in human pancreatic cancer cells (Grewe et al, 1999). As shown in Figure 3E, cyclin D1, cyclin E and p27kip1 were also constitutively expressed in C4-I human cervical cancer cells. Incubation of cells with NDGA resulted in a marked reduction in the expression of cyclin D1 in both cell lines whereas the levels of expression of cyclin E and p27kip1 remained unchanged. These results suggest that the inhibition of proliferation in response to NDGA in SW 850 and C4-I cells could, at least in part, be mediated by inhibition of cyclin D1 expression.
also disrupted the circumferential actin filament network (Figure 4, bottom panels). The effect of cytochalasin D on the actin cytoskeleton in SW 850 and C4-I cells was first visible at 0.3 μM and reached a maximum at 2 μM cytochalasin D. At these concentrations, cytochalasin D also induced apoptosis in both cell lines (data not shown). These data suggest that NDGA by inhibiting the organisation of actin stress fibers in SW 850 and C4-I cells interferes with cell-matrix interaction and induces anokias.

**NDGA and cytochalasin D activate stress-activated protein kinases in SW 850 and C4-I cells**

Anoikis by detachment of epithelial cells from their matrix is associated with the activation of Jun-NH₂-terminal kinases (Frisch et al., 1996). Treatment of SW 850 and C4-I cells with cytochalasin D also induced JNK1/2 activation in a concentration dependent manner reaching a maximum at 2.4 μM cytochalasin D in both cell lines (Figure 5A and data not shown). Interestingly, NDGA also induced a dose dependent activation of JNK1/2 in both cell lines with a maximum effect at 25 μM (Figure 5B, top panel and data not shown). In both cell lines, GST-cJun phosphorylation in response to NDGA was about 60% of that in response to 1 mM mannitol, a potent osmotic stressor (Kyriakis and Avruch, 1996) (Figure 5B, bottom panels). Furthermore, NDGA induced phospho-JNK immunoreactivity exclusively in the nucleus of SW 850 and C4-I cells treated with NDGA providing additional evidence that NDGA is a strong activator of JNKS (Cavigelli et al., 1995; Sanchez-Perez et al., 1998; data not shown).

Figure 5C shows the kinetics of NDGA-induced JNK1/2 activation. Activation of JNK1/2 in response to NDGA was detectable as early as 10 min following treatment of SW 850 and C4-I cells with 25 μM NDGA; a maximum effect was obtained after 60 min of incubation.

NDGA did not affect the activation of the non-stress related members of the MAPK family ERK1/2 in cancer cell lines examined. In marked contrast, the results in Figure 6A demonstrate that NDGA activates the stress related p38MAPK in immune complex kinase assays reaching 60 and 50% of mannitol-induced GST-ATF2 phosphorylation in SW 850 and C4-I cells, respectively. Activation of p38MAPK occurs upon dual phosphorylation at Thr180 and Tyr182 (Han et al., 1994). Using an antibody which specifically detects phosphorylation of the kinase at these two residues, p38MAPK phosphorylation was first detectable at about 2 μM NDGA reaching a maximum at 15 μM NDGA in C4-I and SW 850 cells (Figure 6B, top panel and data not shown). p38MAPK phosphorylation could be detected as early as 5 min after incubation with NDGA reaching a maximum after 30 min of incubation (Figure 6B, bottom panel and data not shown). Thus, NDGA selectively activates the stress-activated protein kinases of the MAPK family.

**DISCUSSION**

A better understanding of the biology of tumours could greatly improve our current concepts of cancer therapy. Arachidonic acid and its metabolites such as 5-HETE have been implicated as growth promoting factors for various human cancers (Avis et al., 1996; Ghosh and Myers, 1998; Ding et al., 1999a). Here, we demonstrate that the resinous plant exsudate NDGA, a 5-lipoxygenase inhibitor, markedly inhibits anchorage-independent growth of human pancreatic and cervical cancer cells in serum as well as growth of xenograft tumours established from these cells in athymic and JNK1/2-immune complex kinase assays were performed as described in Materials and Methods. A typical result in SW 850 cells was subjected to scanning densitometry. Data are expressed as per cent of maximal JNK 1/2 activation obtained after 60 min of incubation.
NDGA markedely induced apoptosis in SW 850 and C4-I cells in vitro and also in xenograft tumours established in athymic mice. This suggests that similar mechanisms mediate the effects of NDGA in vitro and in vivo. Again, the induction of apoptosis by NDGA was not due to inhibition of Ras-dependent survival pathways including the antiapoptotic kinase AKT. Instead, our data suggest that NDGA induces anoikis by disrupting the filamentous actin cytoskeleton in human pancreatic and cervical cancer cells. The effect of NDGA on actin stress fibres was comparable to that of cytochalasin D. In contrast to cytochalasin D, the effect of NDGA on the actin cytoskeleton appears to be cell type specific: NDGA could affect stress fibres in fibroblasts (Hirata et al., 1984; Chong et al., 1987), but failed to do so in leukocytes (Shalit et al., 1987) or keratinocytes (Coutant et al., 1997).

Anoikis is accompanied by induction of stress activated protein kinases (Frish et al., 1996; Khwaja and Downward, 1997). Indeed, cytochalasin D potently induced activation of JNK1/2 in both cell lines. NDGA similarly activated JNK1/2 and p38^mapk but had no effect on ERK1/2 activation in SW 850 and C4-I cells. Again, this effect of NDGA is likely to be cell type specific: No effect of NDGA on JNK activation could be demonstrated in vascular smooth muscle cells (Madamanchi et al., 1998), Hela cells and HL 60 cells (Hii et al., 1998). There is some controversy regarding the possible role of stress activated protein kinases as mediators of apoptosis. For example, there is evidence that these kinases can mediate apoptosis under certain circumstances (Behrens et al., 1999; Tournier et al., 2000) including anoikis (Frish et al., 1996). However, it has been questioned whether JNK activation occurring 30 min after suspending the cells by trypsinisation can contribute to anoikis (Khajia and Downward, 1997). The sequence of events presented in this manuscript is different: We show that disruption of the actin cytoskeleton by NDGA is associated with activation of JNK and p38^mapk and that both events precede cell detachment. The early activation of JNKs and p38^mapk prior to cell detachment could contribute to anoikis. Our finding that similar concentrations of NDGA are required for disruption of actin stress fibres, JNK activation and the induction of apoptosis further supports our conclusion that these events could be related. However, the precise contribution of JNK1/2 and p38^mapk activation to NDGA-induced cytoskeletal disruption and apoptosis requires further examination.

NDGA has been widely used as a specific lipoxygenase inhibitor. The precise relationship between the inhibitory effect of NDGA on lipoxygenases and the signalling events induced by NDGA described in this paper is not clear. Ding et al. (1999a) described an inhibitory effect of NDGA on basal and 5-HETE-induced DNA synthesis in pancreatic cancer cells at concentrations comparable to those used in our experiments. This could suggest that some of the signalling events are related to the lipoxygenase inhibitory action of NDGA. However, the activation of JNKs and p38^mapk in response to NDGA seems to be cell type specific, whereas NDGA will inhibit lipoxygenases in virtually all cells. Thus, either the consequences of lipoxygenase inhibition are cell type specific or NDGA induces additional effects in a cell-type specific manner which are independent from its lipoxygenase inhibitory actions. Therefore, given the activation of multiple signalling events by NDGA, this compound should not be used anymore as a ‘specific’ lipoxygenase inhibitor without the examination of additional pathways.

The effect of NDGA on tumour growth observed in vivo was moderate but there was no toxicity detectable in the animals at the concentration used. The fact that NDGA seems to be effective against diverse and often incurable tumour types suggests that a more detailed analysis of the effects of NDGA on tumour growth in vivo is urgently required. Moreover, NDGA could provide a lead compound for the development of novel therapeutics in pancreatic and cervical cancer.

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**Figure 6** NDGA selectively stimulates p38^mapk activation in SW 850 and C4-I cells. (A) Top: SW 850 and C4-I cells were incubated with 25 μM NDGA (N) or 1 mM mannitol (M) for 60 min. Control cells received an equivalent amount of solvent (—). Cells were further analysed by p38^mapk immune complex kinase assays using a GST-ATF2 fusion protein as substrate. (A) Bottom panel: Typical results of p38^mapk immune complex kinase assays in SW 850 (left) and C4-I (right) cells were subjected to scanning densitometry. Data are expressed as per cent of maximal GST-ATF2 phosphorylation (% of maximum) induced by 1 μM PDBu. (B) Top panel: Serum starved SW 850 cells were incubated with various concentrations of NDGA for 60 min and further analysed by Western blotting using a phosphospecific anti-p38^mapk antibody as described in Materials and Methods. (B) Bottom panel: Serum starved C4-I cells were incubated with 25 μM NDGA for various times and further analysed by Western blotting using a phosphospecific anti-p38^mapk antibody as described in Materials and Methods.

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**Experimental Therapeutics**
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