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Inhibitors of arachidonic acid metabolism potentiate tumour necrosis factor-α-induced apoptosis in HL-60 cells

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Abstract

We investigated whether and how could various modulators of arachidonic acid metabolism affect apoptosis induced by tumour necrosis factor-α (TNF-α) in human myeloid leukaemia HL-60 cells. These included arachinonyltrifluoromethyl ketone ($AACOCF_3$; cytosolic phospholipase A_2 inhibitor), indomethacin (cyclooxygenase inhibitor), MK-886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid; 5-lipoxygenase-activating protein inhibitor), nordihydroguaiaretic acid (general lipoxygenase inhibitor), and arachidonic acid itself. Incubation of HL-60 cells with nordihydroguaiaretic acid resulted in apoptosis and it was characterised by mitochondria membrane depolarisation, release of cytochrome *c* from mitochondria into cytosol and activation of caspase-3. Indomethacin and nordihydroguaiaretic acid synergistically potentiated TNF-α-induced apoptosis, while arachidonic acid, $AACOCF_3$ and MK-886 did not modulate its effects. Furthermore, indomethacin potentiated apoptosis in cells treated with a differentiating agent, *all-trans* retinoic acid, which induces resistance to TNF-α. However, the observed effects were probably not associated either with the cyclooxygenase- or lipoxygenase-dependent activities of indomethacin and nordihydroguaiaretic acid, respectively. Since indomethacin may reportedly activate peroxisome proliferator-activated receptors (PPARs), the effects of specific ligands of PPARs on apoptosis were studied as well. It was found that selective PPARs ligands had no effects on TNF-α-induced apoptosis. The findings suggest that arachidonic acid metabolism does not play a key role in regulation of apoptosis induced by TNF-α in the present model. Nevertheless, our data raise the possibility that indomethacin could potentially be used to improve the treatment of human myeloid leukaemia.

Keywords: Retinoic acid, *all-trans*; TNF-α (tumor necrosis factor-α); Fas; Arachidonic acid; Lipoxygenase; Cyclooxygenase; PPAR (Peroxisome proliferator-activated receptor)

1. Introduction

Inappropriate cell survival has been linked to the development of various diseases, including human malignancies. It is controlled by programmed cell death (apoptosis), an active process that is critical for the homeostasis of tissues (Steller, 1995). Apoptosis is characterised by membrane blebbing, cytoplasmic, nuclear and chromatin condensation, activation of caspases, and DNA cleavage into multiples of intranucleosomal fragments (Kerr et al., 1972; Schulze-Osthoff et al., 1998). It can be induced by a number of stimuli, including the deprivation of survival factors, cell damaging stress, chemotherapy, and signals through death receptors (Jarpe et al., 1998). Death receptors, belonging to the tumour necrosis factor (TNF) receptor family, induce apoptosis through pathways that share many similarities, including recruitment of death domain-containing adapter proteins and activation of the caspase cascade via caspase-8 (Ashkenazi and Dixit, 1998; Jarpe et al., 1998; Schulze-Osthoff et al., 1998).

The action of the proinflammatory cytokine tumour necrosis factor-α (TNF-α) is mediated by its receptors, TNF receptor 1 (CD120a, p55 receptor) and TNF receptor 2 (CD120b, p75 receptor), the former belonging among death receptors. During recent years, four main groups of

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signalling molecules have been identified downstream of TNF receptor 1-caspases, phospholipases, mitogen activated protein kinases and the NF-κB signalling cascade (Wallach et al., 1999). TNF-α has been shown to induce release of arachidonic acid through activation of cytosolic phospholipase A_2; however, the role that this enzyme plays in mediating TNF-α-induced cell death has not been completely elucidated (Leslie, 1997; Wallach et al., 1999). Arachidonic acid release leads to the formation of a series of metabolites, including prostaglandins generated through cyclooxygenases, hydroxyeicosatetraenoic acids, lipoxins and leukotrienes generated through lipoxygenases and the products of the *P*450-monooxygenase system (Shimizu and Wolfe, 1990).

Thus, the arachidonic acid cascade generates a family of bioactive lipids that modulate diverse physiological and pathological responses, including tumour growth and promotion (Ara and Teicher, 1996). We and others have shown that arachidonic acid products, namely those of the 5-lipoxygenase pathway, play an important role in myelopoiesis and the regulation of proliferation and differentiation of human leukaemic cells (Hofmanová et al., 1998; Kozubík et al., 1997; Stenke et al., 1994). However, their role in the regulation of apoptosis in leukaemic cells is less clear.

It has been suggested that tumour growth, promotion and metastasis can be modulated by inhibition of production of arachidonic acid metabolites generated through lipoxygenase and cyclooxygenase pathways (Ara and Teicher, 1996). Recently, a number of both lipoxygenase and cyclooxygenase inhibitors, including non-steroidal anti-inflammatory drugs (NSAIDs), have been reported to induce apoptosis in various types of cancer cells, including cells of myeloid origin (Anderson et al., 1998; Bellosillo et al., 1998; Datta et al., 1999; Dittmann et al., 1998; Ghosh and Myers, 1998; Chan et al., 1998; Tang et al., 1996; Zhang et al., 2000). However, the role of various types of inhibitors of arachidonic acid metabolism is unclear, since it has been reported that they may even protect from apoptosis induced by TNF-α (Hepburn et al., 1987; Chang et al., 1992; O'Donnell et al., 1995).

In the present study, we investigated whether and how could various modulators of arachidonic acid metabolism affect apoptosis induced by TNF-α or an agonist antibody against Fas (another type of death receptor) in human myeloid leukaemia HL-60 cells. These included arachidonyltrifluoromethyl ketone ($AACOCF_3$; cytosolic phospholipase A_2 inhibitor), indomethacin (cyclooxygenase inhibitor), MK886 (3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid; 5-lipoxygenase-activating protein inhibitor), nordihydroguaiaretic acid (general lipoxygenase inhibitor when used at concentrations lower than those inhibiting cyclooxygenase), and arachidonic acid itself. It has been demonstrated that *all-trans* retinoic acid, an inducer of granulocytic differentiation in myeloid leukaemia cells, can efficiently inhibit TNF-α-induced apoptosis (Kikuchi et al., 1996; Vondráček et al., 2001). Therefore, we investigated the effects of indomethacin, which was found to significantly potentiate the TNF-α-induced programmed cell death, on suppression of apoptosis observed during *all-trans* retinoic acid-induced differentiation. Finally, since indomethacin was found to synergistically enhance TNF-α-induced apoptosis in relatively high concentrations in the present study and it has been reported to activate peroxisome proliferator-activated receptors (PPARs) (Lehmann et al., 1997), the effects of specific ligands of PPAR-α and -γ on apoptosis were studied as well.

2. Materials and methods

2.1. Cells

Human myeloid leukaemia HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml) and maintained in a humidified incubator at 37 °C in 5% CO_2. Exponentially growing cells were plated into Petri dishes at 2×10^5 cells per ml 1 h prior to application of inhibitors. For *all-trans* retinoic acid treatment, exponentially growing cells were seeded directly into medium supplemented with 1 μM *all-trans* retinoic acid.

2.2. Reagents

Human recombinant TNF-α, indomethacin (1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), nordihydroguaiaretic acid, arachidonic acid, RPMI-1640 medium, RNAse A, propidium iodide, secondary anti-murine IgG antibody conjugated with horse radish peroxidase and *all-trans* retinoic acid were purchased from Sigma (St. Louis, MO, USA). Wy-14,643 (4-chloro-6-[2,3-xylidino]-2-pyrimidinylthioacetic acid; PPAR-α ligand) and ciglitazone ((±)-5-[4-(1-methylcyclohexylmethoxy)-benzyl]thiazolidine-2,4-dione]; PPAR-γ ligand) were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). MK-886 was a kind gift from Merck (Canada). $AACOCF_3$ was from Calbiochem (San Diego, CA, USA). Anti-human Fas CH-11 antibody was from Immunotech (Marseille, France). Murine monoclonal anti-5-lipoxygenase and anti-caspase-3 antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Murine monoclonal anti-cytochrome *c* antibody was from PharMingen (San Diego, CA, USA). Rabbit polyclonal anti-cyclooxygenase-2 antibody and murine monoclonal anti-cytosolic phospholipase A_2 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-rabbit IgG antibody conjugated with horse radish peroxidase was purchased from

Cell Signaling Technology (Beverly, MA, USA). Fetal bovine serum was from PAN Systems (Nürnberg, Germany). 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was from Fluka (Buchs, Switzerland). MOWIOL® 40-88 was obtained from Aldrich (Milwaukee, WI, USA).

2.3. Induction and detection of apoptosis

Cells were incubated with selected inhibitors of arachidonic acid metabolism or PPAR ligands for 1 h prior to 16-h incubation with human recombinant TNF-α (0.001–10 ng/ml final concentration) or CH-11 mouse monoclonal antibody (100 ng/ml final concentration), which was reported to induce apoptosis by crosslinking Fas (Yonehara et al., 1989). Inhibitors of arachidonic acid metabolism were prepared as stock solutions in ethanol and stored at −20 °C. PPAR ligands and $AACOCF_3$ were dissolved in dimethyl sulfoxide and stored at −80 °C. Arachidonic acid was dissolved in ethanol and stored under nitrogen atmosphere at −80 °C. Following the incubation with apoptosis inducers, cells were harvested and prepared for DNA labelling with propidium iodide or DAPI, as follows.

For propidium iodide staining, cells were washed once with phosphate-buffered saline (PBS) and fixed in 70% cold ethanol. Fixed cells were washed twice with PBS and low molecular weight DNA was extracted with citric acid buffer (Gong et al., 1994). Cells were then resuspended in PBS containing 20 μg/ml propidium iodide and 5 Kunitz U/ml RNAse A and incubated for 30 min at room temperature. Cells were analysed using a FACS©Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A minimum of 15,000 events was collected per sample.

To verify the results of flow cytometry, nuclear morphology was examined by fluorescence microscopy. Cells (5×10^5) were resuspended with 50 μl of methanol containing 2 μg/ml DAPI (final concentration) and incubated for 30 min at room temperature. After incubation, cells were centrifuged, mixed with 20 μl of MOWIOL solution and mounted for counting with a fluorescence microscope. A minimum of 200 cells was counted per sample.

2.4. Western blot analysis

HL-60 cells were treated with selected compounds or their combination with TNF-α and CH-11 antibody for the time indicated. Cells were washed with PBS, cell pellets were lysed using sodium dodecyl sulfate (SDS) lysis buffer, and 30 μg of total protein per sample was separated on 12% (for detection of caspase-3 cleavage) or on 7.5% (for detection of 5-lipoxygenase, cyclooxygenase-2 and cytosolic phospholipase A_2 expression) polyacrylamide gel and transferred onto a polyvinyldifluoride membrane. After incubation with primary and secondary antibodies, detection was performed using the ECLPlus Western blotting detection system (Ammersham Pharmacia Biotech, Little Chalfont, UK).

For detection of cytochrome *c* release, cells were fractionated using the modified method described by Chen et al. (2000). Cells were twice washed with PBS and sonicated (3 × 5 s on ice) in buffer containing 20 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose and protease inhibitors. The

Table 1
Effects of inhibitors of arachidonic acid metabolism on TNF-α-induced apoptosis in HL-60 cells

Treatment	%Apoptotic cells			
	None	TNF-α (0.1 ng/ml)	TNF-α (1 ng/ml)	TNF-α (10 ng/ml)
None	10.0 ± 2.1	13.4 ± 3.6	23.1 ± 4.6	31.0 ± 7.5
AA (10 μM)	8.0 ± 1.2	7.0 ± 3.4	18.0 ± 7.1	24.5 ± 7.8
AA (20 μM)	7.5 ± 4.2	9.8 ± 4.6	20.8 ± 6.9	24.8 ± 4.9
AA (40 μM)	13.8 ± 7.5	19.5 ± 8.5	26.2 ± 10.2	31.5 ± 11.7
$AACOCF_3$ (10 μM)	8.3 ± 3.6	9.5 ± 5.7	20.3 ± 4.0	26.8 ± 3.8
$AACOCF_3$ (20 μM)	7.8 ± 3.0	10.3 ± 5.0	21.5 ± 6.4	28.5 ± 4.4
$AACOCF_3$ (40 μM)	7.3 ± 3.9	11.8 ± 4.4	21.8 ± 4.4	30.0 ± 3.9
MK-886 (1 μM)	13.3 ± 2.2	18.0 ± 3.6	27.8 ± 4.6	33.3 ± 7.3
MK-886 (5 μM)	14.0 ± 3.2	20.3 ± 3.4	28.0 ± 3.2	35.3 ± 5.0
MK-886 (10 μM)	14.5 ± 2.5	24.8 ± 6.4	31.8 ± 4.4	40.3 ± 3.8
NDGA (1 μM)	11.0 ± 4.1	13.4 ± 4.6	22.5 ± 3.7	31.3 ± 5.4
NDGA (5 μM)	21.8 ± 7.1[a]	21.3 ± 7.1[a]	34.0 ± 8.6[a]	38.8 ± 7.8[a]
NDGA (10 μM)	31.0 ± 6.9[a]	28.8 ± 3.4[a]	35.0 ± 6.7[a]	43.5 ± 6.8[a]
Indomethacin (10 μM)	12.0 ± 2.2	19.0 ± 5.3	32.3 ± 6.5	46.3 ± 8.8
Indomethacin (50 μM)	14.0 ± 3.7	28.8 ± 4.2[a]	40.3 ± 6.7[a]	51.8 ± 4.4[a]
Indomethacin (100 μM)	17.0 ± 2.9[a]	45.8 ± 3.3[a]	50.5 ± 7.6[a]	60.0 ± 7.3[a]

Cells were treated with TNF-α in the presence or absence of inhibitors in RPMI 1640 medium under standard cultivation conditions for 16 h. The percentage of apoptotic cells was determined by flow cytometry. All values represent means ± S.D. of at least three independent experiments. AA—arachidonic acid.

[a]A significant difference from the corresponding control group (without addition of inhibitors, $P < 0.05$). Differences were analysed using Mann–Whitney *U*-test and Kruskal–Wallis ANOVA.

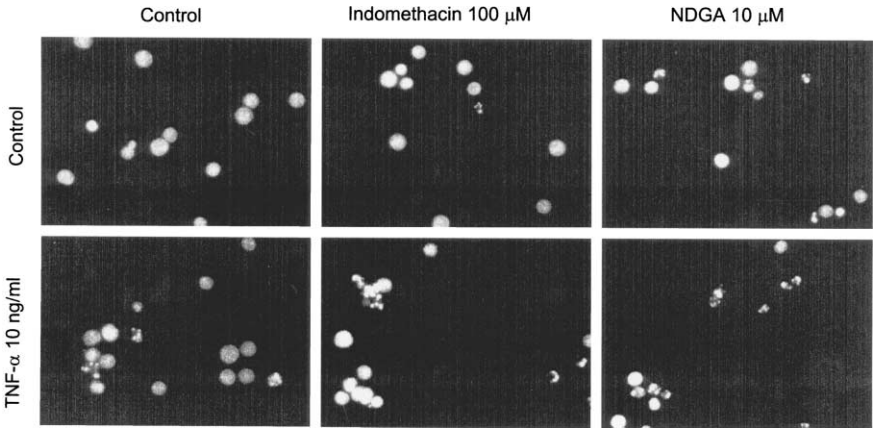

Fig. 1. Indomethacin and nordihydroguaiaretic acid (NDGA) potentiate TNF-α-induced apoptosis in HL-60 cells. Cells were stained with DAPI and observed by fluorescence microscopy. Results are representative of three independent experiments.

homogenates were centrifuged at $750 \times g$ for 5 min, and the supernatant was then centrifuged at $10{,}000 \times g$ for another 5 min. The mitochondria-containing pellet was designated P10 and the supernatant was subjected to further ultracentrifugation at $100{,}000 \times g$ for 60 min. The resulting supernatant represented the cytosolic fraction. Proteins in the supernatants were concentrated with 5–10% trichloroacetic acid. Both the supernatants and P10 were dissolved in Laemmli sample buffer (50 mM TRIS pH 6.8, 2% SDS, 10% glycerol). Following the quantification of proteins with Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA), all lysates were diluted to same protein concentration (1 mg/ml), and 1% β-mercaptoethanol and 0.1% bromophenol blue were added into lysates. Proteins were separated on polyacrylamide gel, transferred onto a polyvinyldifluoride membrane and probed with antibody against cytochrome *c* followed by incubation with a secondary antibody conjugated with horseradish peroxidase. Detection was performed using the ECLPlus Western blotting detection system.

2.5. Detection of mitochondrial membrane potential

The variation of mitochondrial transmembrane potential during nordihydroguaiaretic acid and indomethacin treatment was studied using tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes, Eugene, OR, USA) (Loew et al., 1994). Cells were washed twice with Hanks' balanced salt solution (HBSS), approximately 10^6 cells was resuspended in 100 nM of TMRE in HBSS and

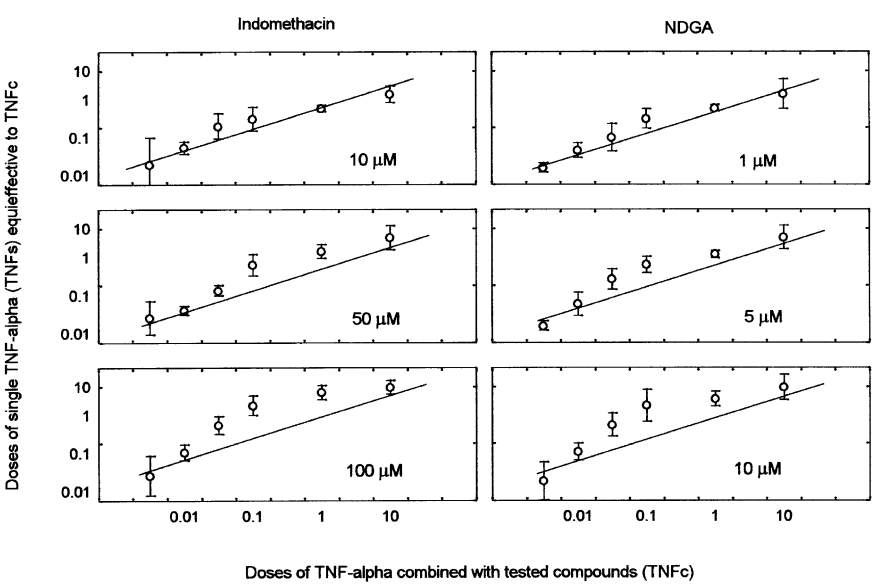

Fig. 2. Effects of combinations of TNF-α plus indomethacin or nordihydroguaiaretic acid (NDGA) on apoptosis of HL-60 cells. Cells were stained with propidium iodide and the percentage of cells with $subG_1$ DNA content was determined on a flow cytometer. Comparison of single TNF-α doses (TNFs) that are equieffective with the concentration of TNF-α combined with selected compound (TNFc). Ranges (I) represent 95% confidence intervals of respective mean value (○). No overlap of confidence interval with the diagonal additivity line indicates a significant potentiation of TNF-α induced apoptosis by a respective agent (synergy effect). Doses of TNF-α (ng/ml) were expressed in log units.

incubated for 20 min at room temperature in the dark. At the end of the incubation period, cells were washed with HBSS, resuspended in a total volume of 500 μl and analysed on FACS©Calibur flow cytometer (585/42 band pass filter). Data were evaluated as median of fluorescence of living cells gated by forward scatter versus side scatter.

2.6. Statistics

Data were expressed as means ± S.D. for at least three independent repeats and analysed by the nonparametric Mann–Whitney *U*-test and Kruskal–Wallis analysis of variance (ANOVA). To study interactive effects between TNF-α and effective compounds (nordihydroguaiaretic acid, indomethacin), an isobolographic approach based on the analysis of the equieffective quantities of agents in the mixture was used as described (Gessner, 1988). A P value of less than 0.05 was considered significant. All analyses were carried out using GraphPad Prism™ and MS-Excel™ software packages.

3. Results

3.1. Indomethacin and nordihydroguaiaretic acid significantly potentiate TNF-α-induced apoptosis

To examine effects of various inhibitors of arachidonic acid metabolism on sensitivity to TNF-α, changes in the $subG_1$ population in propidium iodide-stained cells, after extraction of low-molecular DNA with citric acid buffer, were studied. Table 1 gives a description of the observed effects. TNF-α induced apoptosis in HL-60 cells in a dose-dependent manner. Arachidonic acid, $AACOCF_3$ and MK-886 neither induced apoptosis in HL-60 cells nor potentiated the effects of TNF-α. On the other hand, nordihydroguaiaretic acid and indomethacin induced apoptosis when used at higher concentrations ($P < 0.05$). Both compounds were found to significantly potentiate induction of apoptosis by TNF-α ($P < 0.05$). The potentiation of TNF-α-mediated apoptosis by nordihydroguaiaretic acid and indomethacin was confirmed by morphological analysis of DAPI-stained cells, which revealed a significantly higher percentage of nuclei with typical apoptotic features in cells treated with either inhibitor plus TNF-α when compared to cells treated with TNF-α alone (Fig. 1).

Isobolographic analysis revealed that TNF-α combined with either indomethacin or nordihydroguaiaretic acid induced apoptosis in excess of a simple dose-additive manner. Nearly all of the tested concentrations of TNF-α combined with indomethacin (inhibitor concentrations, 50 and 100 μM) or NDGA (inhibitor concentrations, 5 and 10 μM) were significantly lower than the corresponding equieffective doses of single TNF-α (Fig. 2). Significant departures from zero interaction (dose-additive effect, the diagonal line in Fig. 2) were detected ($P < 0.05$), indicating a clear synergy in the action of TNF-α and the respective compounds.

3.2. Nordihydroguaiaretic acid induces mitochondrial depolarisation, cytochrome c release and caspase-3 cleavage in HL-60 cells

Since both nordihydroguaiaretic acid and indomethacin were found to induce cell death in HL-60 cells, we attempted to further characterise the mechanisms underlying

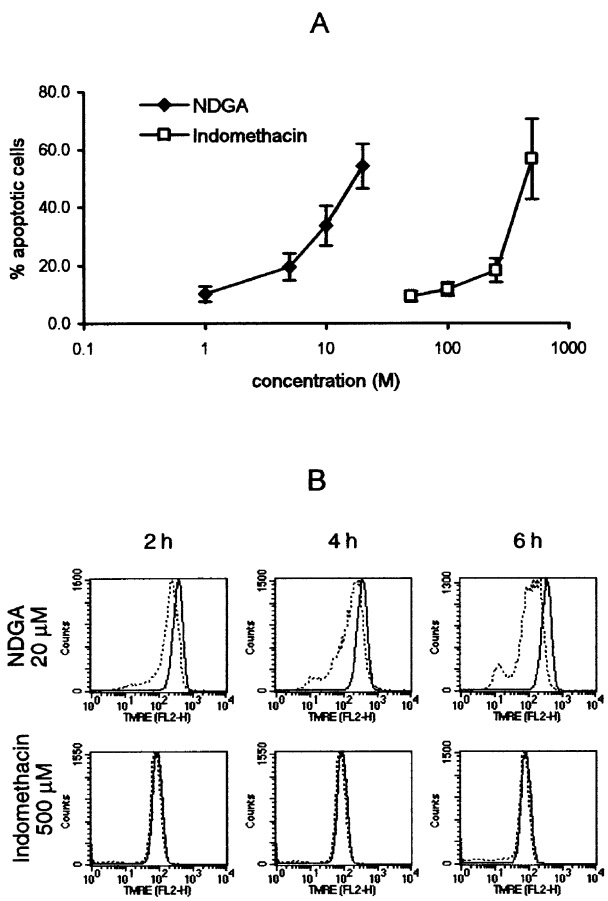

Fig. 3. Both indomethacin and nordihydroguaiaretic acid (NDGA) induce cell death in HL-60 cells in a dose-dependent manner, while only NDGA induces mitochondrial membrane depolarisation. (A) Cells were treated with indicated concentrations of indomethacin and NDGA, stained with propidium iodide and the percentage of cells with $subG_1$ DNA content was determined on a flow cytometer. Points represent means ± S.D. from three independent experiments. (B) Cells were incubated with 20 μM NDGA or 500 μM indomethacin for the time indicated, the cells were collected and stained with TMRE (100 nM) for 20 min and analysed on FACS® Calibur flow cytometer. The histograms show mitochondrial potential of living cells, gated on forward versus side scatter to exclude debris and dead cells. The results are representative of at least three independent experiments. The decrease in TMRE fluorescence demonstrates the loss of membrane potential in NDGA-treated group (dot lines), while no decrease is observable the vehicle-treated control group (full lines).

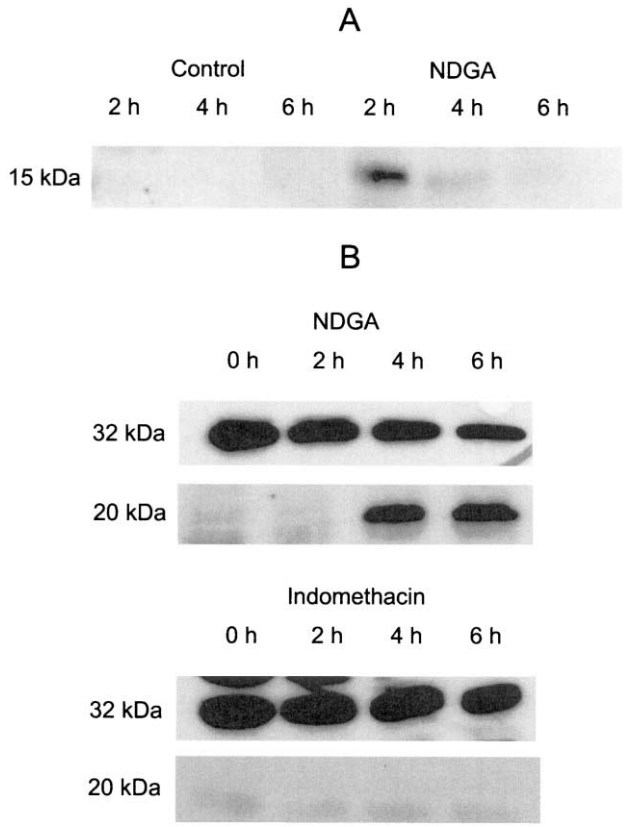

Fig. 4. Nordihydroguaiaretic acid (NDGA) induces cytochrome *c* release and caspase-3 activation. (A) Western blot analysis shows presence of cytochrome *c* in cytosolic fraction from NDGA-treated cells, indicating the release of cytochrome *c* from mitochondria. Cells were treated with 20 μM NDGA for the time indicated, fractionated, and Western blotting of cytosolic fraction was performed as described in Materials and methods. The Western blot is representative of two independent experiments. (B) Western blot analysis of caspase-3 cleavage upon NDGA and indomethacin treatment. Cells were treated with 20 μM NDGA or 500 μM indomethacin for the time indicated, lysed, and the lysate was used to perform Western blotting as described in Materials and methods; 30 μg total protein was loaded per lane. The results are representative of four independent experiments.

the apoptotic effects of these compounds. Mitochondrial membrane depolarisation and activation of caspase-3, cysteine protease play an important role in the process of apoptosis (Kroemer and Reed, 2000; Tyas et al., 2000). Compared to nordihydroguaiaretic acid, indomethacin was markedly cytotoxic only when used at very high concentrations (250 and 500 μM) (Fig. 3A). Nordihydroguaiaretic acid (20 μM) was found to induce loss of mitochondrial membrane potential (Fig. 3B) and cytochrome *c* release was detected already after 2 h of treatment (Fig. 4A). Incubation of HL-60 cells with nordihydroguaiaretic acid led to caspase-3 processing after 4 and 6 h of treatment, documented by the appearance of 20-kDa fragment (Fig. 4B). Contrary to that, we did not observe mitochondrial membrane depolarisation or caspase-3 cleavage in indomethacin-treated cells (Figs. 3B and 4B, respectively).

3.3. Selective inducers of PPARs do not alter TNF-α-induced apoptosis

Since NSAIDs have been shown to act as ligands of PPARs when present at relatively high concentrations, we investigated whether ligand-induced activation of these receptors could potentiate TNF-α-induced apoptosis. Two specific ligands for PPAR-α (Wy-14,643) and PPAR-γ (ciglitazone) were used in combination with TNF-α. However, neither of the compounds induced apoptosis or potentiated the effect of TNF-α in concentrations up to 50 μM (Table 2). The findings were confirmed by morphological analysis (data not shown, $n = 3$).

3.4. Combined treatment of HL-60 cells with both TNF-α and indomethacin can overcome all-trans retinoic acid-induced inhibition of apoptosis

In another set of experiments, the effects of combined treatment with indomethacin and TNF-α on apoptosis of cells treated with 1 μM *all-trans* retinoic acid were evaluated. The application of *all-trans* retinoic acid significantly inhibited TNF-α-induced apoptosis, as documented by a decrease in the subG$_1$ population (Fig. 5). However, indomethacin (100 μM) in combination with TNF-α was able to induce apoptosis to a similar extent as TNF-α

Table 2
Effects of PPAR ligands on TNF-α-induced apoptosis in HL-60 cells

Treatment	%Apoptotic cells			
	None	TNF-α (0.1 ng/ml)	TNF-α (1 ng/ml)	TNF-α (10 ng/ml)
None	9.25 ± 5.4	12.3 ± 8.1	31.0 ± 5.7	45.0 ± 7.4
Wy14,643 (10 μM)	9.0 ± 4.9	12.0 ± 5.5	27.8 ± 4.3	43.0 ± 7.9
Wy14,643 (50 μM)	12.0 ± 5.7	13.0 ± 5.7	30.5 ± 3.5	45.5 ± 12.0
Ciglitazone (10 μM)	9.3 ± 4.4	10.8 ± 5.7	22.8 ± 4.8	33.3 ± 6.6
Ciglitazone (50 μM)	14.5 ± 9.2	17.0 ± 9.9	24.0 ± 7.1	33.5 ± 5.1

Cells were treated with TNF-α in the presence or absence of PPAR ligands in RPMI 1640 medium under standard cultivation conditions for 16 h. The percentage of apoptotic cells was determined by flow cytometry. All values represent means ± S.D. of at least three independent experiments. [a]A significant difference from the corresponding control group (without addition of inhibitors, $P < 0.05$). Differences were analysed using Mann–Whitney *U*-test and Kruskal–Wallis ANOVA.

alone, although the combined effect was lower than in untreated cells (Fig. 5).

3.5. Indomethacin does not sensitise HL-60 cells to induction of apoptosis mediated by Fas

To further investigate the effect of indomethacin, we tested its effect on apoptosis mediated by Fas, a member of the TNF receptor superfamily. HL-60 cells were resistant to Fas-mediated apoptosis induced by Fas-crosslinking antibody at concentrations of 0.1–1 μg/ml (data not shown). The addition of indomethacin did not lead to sensitisation of cells towards CH-11 antibody as determined by flow cytometry (Fig. 6). These results were confirmed by fluorescence microscopy (data not shown, $n = 3$).

3.6. Nordihydroguaiaretic acid and indomethacin have no effect on expression of 5-lipoxygenase, cyclooxygenase-2 and cytosolic phospholipase A_2 in HL-60 cells

Since both cyclooxygenase- and lipoxygenase-independent effects have been suggested to be involved in nordihydroguaiaretic acid- and indomethacin-induced apoptosis (Biswal et al., 2000; Zhang et al., 2000), we studied effects of nordihydroguaiaretic acid and indomethacin on expression of 5-lipoxygenase, cyclooxygenase-2 and cytosolic phospholipase A_2. The inhibitors were applied to cells either alone or in combination with TNF-α (or CH-11 antibody in case of indomethacin). Undifferentiated HL-60 cells did not express 5-lipoxygenase protein and its expression was not induced by any of treatments (Fig. 7). This was confirmed by high performance liquid chromatography analysis, as we did not find detectable levels of 5-hydroxyeicosatetraenoic acid in an assay detecting 5-lipoxygenase activity (data not shown). None of the treatments significantly affected expression of cyclooxygenase-2 or cytosolic phospholipase A_2 in HL-60 cells (Fig. 7).

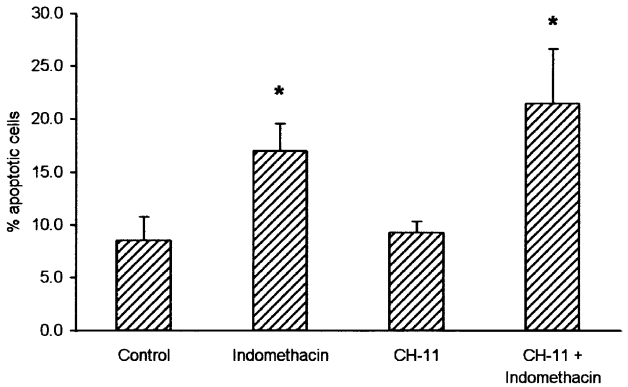

Fig. 6. Effects of combination of anti-human Fas murine monoclonal antibody CH-11 (100 ng/ml) with indomethacin (100 μM) on apoptosis of HL-60 cells. Cells were stained with propidium iodide and the percentage of cells with subG$_1$ DNA content was determined on a flow cytometer. Bars represent means ± S.D. from a minimum of three independent experiments. * Significant difference from the control group ($P < 0.05$) as determined by Mann–Whitney *U*-test.

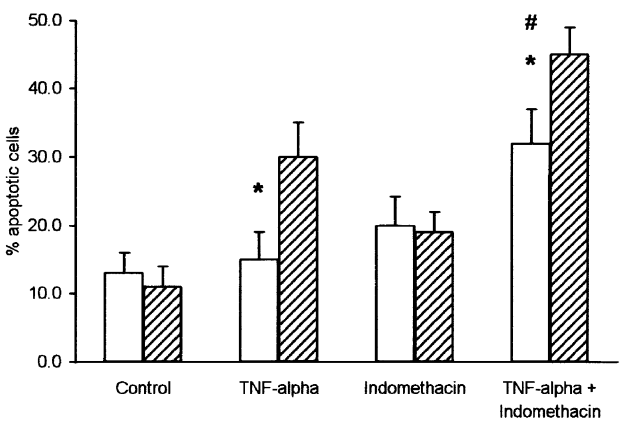

Fig. 5. Effects of combination of TNF-α (1 ng/ml) with indomethacin (100 μM) on apoptosis of untreated HL-60 cells (shaded bars) and cells treated with 1 μM *all-trans* retinoic acid (open bars). Cells were stained with propidium iodide and the percentage of cells with subG$_1$ DNA content was determined on a flow cytometer. Bars represent means ± S.D. from a minimum of three independent experiments. * Significant difference from the respective control group ($P < 0.05$), # Significant difference from the group treated with both TNF-α and *all-trans* retinoic acid ($P < 0.05$), as determined by Mann–Whitney *U*-test.

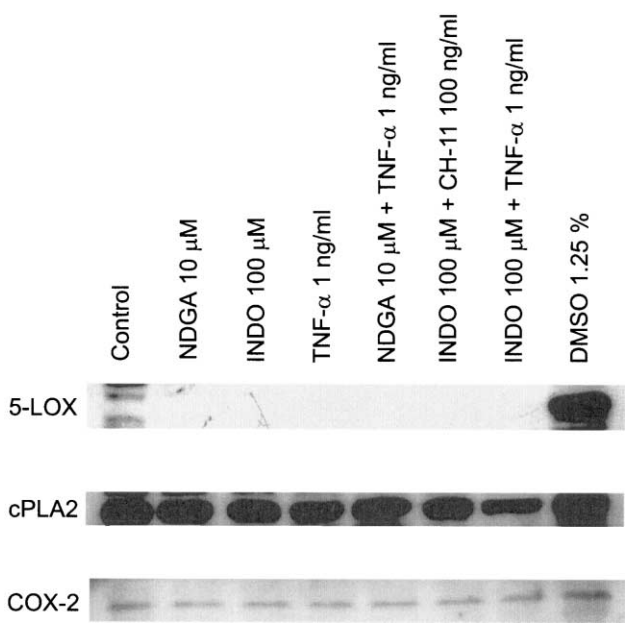

Fig. 7. Neither nordihydroguaiaretic acid (NDGA), nor indomethacin (INDO) induce 5-lipoxygenase (5-LOX) expression or affect the expression of cyclooxygenase-2 (COX-2) and cytosolic phospholipase A_2 (cPLA2) in HL-60 cells. Western blot analysis of protein expression was carried out as described in Materials and methods and the results are representative of at least three independent experiments. Cells were treated with inhibitors and/or TNF-α (or CH-11) for 16 h; 30 μg of total protein was loaded per lane. Differentiated HL-60 cells (incubated for 96 h with 1.25% dimethyl sulfoxide—DMSO) were used as a positive control for 5-LOX expression.

4. Discussion

Arachidonic acid has been reported to induce apoptosis in various cell types including myeloid leukaemia cell lines (Finstad et al., 1994, 1998; Rizzo et al., 1999; Surette et al., 1999) and it has been suggested to be an important mediator in regulation of sensitivity to TNF-α-induced apoptosis (De Valck et al., 1998; Hayakawa et al., 1993; Wissing et al., 1997; Wu et al., 1998). Various mechanisms, including lipid peroxidation and ceramide generation could play a role in arachidonic acid-induced programmed cell death (Jayadev et al., 1994). Contrary to that, it has been reported that arachidonic acid may even potentiate HL-60 proliferation in concentrations up to 80 μM (Liu and Levy, 1997), and that it may enhance tumour cell growth by promoting cell proliferation and by suppressing apoptosis (Tang et al., 1997). In the present study, arachidonic acid neither induced apoptosis nor modulated the effects of TNF-α. The discrepancies could be explained by the fact that cells are often incubated with high amounts of arachidonic acid (up 120 μM) for long time periods (up to 3 days in culture) in order to induce programmed cell death (Finstad et al., 1994, 1998), or by use of different cellular models.

Arachidonic acid has been suggested to participate in regulation of TNF-α-induced apoptosis, since overexpression of cytosolic phospholipase A_2, a principle enzyme responsible for TNF-α-induced arachidonic acid release, might increase sensitivity of cells to TNF-α cytotoxicity (Hayakawa et al., 1993), while inhibitors of cytosolic phospholipase A_2 block apoptosis induced by this cytokine (Wissing et al., 1997; Wu et al., 1998). However, we found that inhibition of arachidonic acid release by $AACOCF_3$ had no significant effects on TNF-α-induced cell death. Again, different cellular models could be partially responsible for the observed discrepancies, as some of the above-mentioned studies were performed in L929 murine fibrosarcoma cells. Moreover, inhibition of cytosolic phospholipase A_2 in some studies only partially blocked TNF-α-induced apoptosis. Neither indomethacin nor nordihydroguaiaretic acid, two compounds enhancing TNF-α-induced apoptosis, affected the expression of cytosolic phospholipase A_2. The findings suggest that arachidonic acid release does not play a key role in induction of programmed cell death by TNF-α in HL-60 cells.

The effects of various lipoxygenase and cyclooxygenase inhibitors on programmed cell death are unclear, since they have been reported both to induce and to reduce apoptosis in different cell types (Anderson et al., 1998; Bellosillo et al., 1998; Datta et al., 1999; Dittmann et al., 1998; Ghosh and Myers, 1998; Hepburn et al., 1987; Chang et al., 1992; O'Donnell et al., 1995). In the present study, nordihydroguaiaretic acid induced apoptosis in HL-60 cells and it synergistically potentiated the effects of TNF-α. Nordihydroguaiaretic acid has been suggested to induce apoptosis through glutathione depletion, lipid peroxidation and mitochondrial depolarisation (Biswal et al., 2000; Tang and Honn, 1997). Mitochondrial membrane permeabilisation, resulting in release of several proteins such as cytochrome *c* from the intermembrane space, is supposed to play a crucial role in most pathways leading to apoptosis (Kroemer and Reed, 2000). The loss of mitochondrial potential and activation of caspase-3, a cysteine protease playing an important role in the process of apoptosis, have been reported to be related in a number of cell types (Kroemer and Reed, 2000; Tyas et al., 2000). In the present study, nordihydroguaiaretic acid induced mitochondrial depolarisation, cytochrome *c* release and caspase-3 activation in HL-60 cells. Our results confirm previously reported activation of caspase-3 during nordihydroguaiaretic acid-induced apoptosis, although caspases other than caspase-3 could also participate in this process (Biswal et al., 2000; Tyas et al., 2000). However, the effects of nordihydroguaiaretic acid are probably not related to its 5-lipoxygenase inhibiting properties. This conclusion is based on the finding that: (i) another inhibitor of leukotriene production, MK-886, did not induce apoptosis and it had no significant effect on TNF-α-induced apoptosis in HL-60 cells; (ii) undifferentiated HL-60 cells have negligible 5-lipoxygenase expression and activity (Bennett et al., 1993). We have confirmed this and found that neither indomethacin nor nordihydroguaiaretic acid induce 5-lipoxygenase expression in HL-60 cells. We have shown previously that micromolar concentrations of MK-886 significantly potentiate differentiation of HL-60 cells induced by various differentiation inducers, without a significant increase in apoptosis (Hofmanová et al., 1998), although several authors have suggested that MK-886 is a potent inducer of apoptosis in leukaemia cell lines (Datta et al., 1999; Dittmann et al., 1998). Our hypothesis is further supported by the finding that nordihydroguaiaretic acid induces apoptosis in the murine hematopoietic cell line FL5.12 without 5-lipoxygenase protein (Biswal et al., 2000). Thus, 5-lipoxygenase products of arachidonic acid metabolism are probably not involved in the effects of nordihydroguaiaretic acid on TNF-α-induced programmed cell death in our model, while other modes of action of nordihydroguaiaretic acid, such as glutathione depletion, may sensitise HL-60 cells to apoptosis induced by this cytokine.

Indomethacin, a widely used NSAID, induced cell death in HL-60 cells and it synergistically potentiated the effects of TNF-α. Recently, effects of cyclooxygenase inhibitors on apoptosis received considerable attention due to potential beneficial effects of NSAIDs in reducing the risk of colon cancer. However, their beneficial effects may be mediated through both cyclooxygenase-dependent and -independent pathways (Paik et al., 2000). It has been documented that high concentrations of indomethacin induce programmed cell death in K562 cells, as well as in chronic myeloid leukaemia cells (Zhang et al., 2000), although others have reported that indomethacin does not induce

apoptosis when used at cyclooxygenase-inhibiting concentrations (Bellosillo et al., 1998). In the present study, high concentrations of indomethacin were found to induce cell death in HL-60 cells; however, we did not observe the loss of mitochondrial potential and caspase-3 cleavage that were typical for nordihydroguaiaretic acid-induced apoptosis. Indeed, it has been reported that the mitochondrial depolarisation and cytochrome *c* release is not always necessary for induction of apoptosis in HL-60 cells (Finucane et al., 1999; Li et al., 2000). Other effector caspases could be involved in programmed cell death induced by indomethacin. The mechanism of indomethacin-induced apoptosis and of its potentiation of TNF-α-induced cell death could be cyclooxygenase-independent, as its concentrations that inhibit cyclooxygenase in vitro are lower than those inducing apoptosis (Bellosillo et al., 1998; Zhang et al., 2000). This is further supported by our finding that two other NSAIDs, piroxicam and ibuprofen (data not shown), had no effect on TNF-α-induced apoptosis. Interestingly, it has been documented that prostaglandins do not reverse the effects of NSAIDs on programmed cell death (Hanif et al., 1996). NSAIDs have been shown both to induce cyclooxygenase-2 expression and to inhibit mitogen-induced cyclooxygenase-2 expression (Paik et al., 2000). We found that indomethacin does not affect cyclooxygenase-2 expression in HL-60 cells. These results seem to support our conclusion that arachidonic acid-independent mechanisms are involved in the observed effects of indomethacin.

Indomethacin, as well as other NSAIDs, has been reported to bind and activate PPAR-γ and PPAR-α (Lehmann et al., 1997). It has also been documented that PPAR-γ is expressed in human myeloid leukaemia cells, including HL-60 cells (Asou et al., 1999). Recently, a mechanism involving induction of negative interference with NF-κB anti-apoptotic signalling pathway by PPAR ligands has been reported to play a role in the apoptosis of human macrophages (Chinetti et al., 1998). Therefore, we have hypothesised that high concentrations of indomethacin that were found to effectively potentiate programmed cell death induced by TNF-α could act through activation of PPAR-γ. However, we found that ciglitazone, a selective ligand of PPAR-γ, did not potentiate TNF-α-induced apoptosis. No effects were found also for Wy-14,643, a specific PPAR-α ligand. These results suggest that the effects of indomethacin were not mediated by a PPAR-dependent mechanism.

The exact relationship between cell maturation and apoptosis is unclear. It has been reported that myeloid cells may become resistant to various apoptotic stimuli, such as death ligands, irradiation and cytostatics, during the process of differentiation induced by *all-trans* retinoic acid. (Ketley et al., 1997; Kikuchi et al., 1996). Recently, we have found that *all-trans* retinoic acid rapidly induces resistance to TNF-α in HL-60 cells (Vondráček et al., 2001). In an attempt to further characterise the effects of indomethacin, we found that combination of TNF-α with indomethacin induce apoptosis in HL-60 cells treated with *all-trans* retinoic acid.

Another important physiological mediator of apoptosis is the Fas/APO-1/CD95 receptor, a surface receptor belonging to the TNF receptor family. Compared to TNF-α, relatively little is known about the role of arachidonic acid in apoptosis mediated by Fas. It has been reported that, unlike TNF-α, anti-Fas antibody does not induce cytosolic phospholipase A_2 activity (De Valck et al., 1998). While some authors have concluded that arachidonic acid or its metabolites do not play a significant role in Fas-mediated apoptosis (Cifone et al., 1995; Enari et al., 1996), others have suggested that lipoxygenase could play a critical role in FasL-induced apoptosis (Wagenknecht et al., 1997). It has been reported that type VI Ca^{2+}-independent phospholipase A_2 mediates fatty acid release in Fas-stimulated U937 cells and may play a modifying, although not essential, role in the apoptotic cell death process (Atsumi et al., 1998). In the present study, indomethacin treatment did not sensitise HL-60 cells to Fas-mediated programmed cell death.

In conclusion, indomethacin, a widely used NSAID, and nordihydroguaiaretic acid, a general lipoxygenase inhibitor, may significantly potentiate apoptosis induced by TNF-α in a myeloid leukaemia cell line. This interaction has a synergistic character. Furthermore, this effect can be observed in cells treated with *all-trans* retinoic acid, a differentiation inducer used for treatment of acute myeloid leukaemia patients, which induces resistance to TNF-α-induced apoptosis. However, the effects of indomethacin and nordihydroguaiaretic acid are probably not associated with the cyclooxygenase- and lipoxygenase-dependent activities of these compounds. The precise mechanisms of action of both compounds deserve further investigation. Nevertheless, our data raise the possibility that indomethacin could potentially be used to improve the treatment of human myeloid leukaemia.

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