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Inhibitors of lipoxygenase metabolism exert synergistic effects with retinoic acid on differentiation of human leukemia HL-60 cells

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Abstract

The interaction between drugs suppressing the production of arachidonic acid metabolites and inducers of granulocytic differentiation, i.e., *all-trans* retinoic acid and dimethyl sulphoxide (DMSO) was investigated using the human myeloid leukemia HL-60 cell line. The experiments were designed as a complete factorial combination of treatments and used chemiluminescence as a marker of cell oxidative burst (level of differentiation). It was clearly demonstrated that two structurally different inhibitors of 5-lipoxygenase metabolism, i.e., 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid (MK-886) and esculetin, significantly potentiated the HL-60 cell differentiation induced by retinoic acid or DMSO. Detailed mathematical evaluation of the results revealed the synergistic character of the interaction. The most significant effects were achieved with a combination of 5-lipoxygenase inhibitors and low doses of retinoic acid. These results were confirmed by analysis of cell morphology and expression of cell surface antigen CD 11b after treatment of the cells with selected concentrations of agents. In contrast to those on differentiation, no additional effects of MK-886 or esculetin on cell proliferation (cell number and cell cycle parameters) and apoptosis were observed. An inhibitor of cyclooxygenases, indomethacin, affected neither cell proliferation nor differentiation of cells. The results implied that either modulation of 5-lipoxygenase metabolism or a certain type of imbalance in arachidonic acid metabolism could modulate the effects of retinoic acid or DMSO on myeloid cell differentiation.

Keywords: Arachidonic acid; Granulocyte; Dimethyl sulfoxide; Oxidative burst; Chemiluminescence; Cell cycle

1. Introduction

Recent approaches in differentiation therapy have received increasing attention due to a remarkable activity of the vitamin A derivative, *all-trans* retinoic acid. Retinoic acid inhibits the clonal growth of fresh leukemic cells and cell lines from patients with acute myelogenous leukemia in vitro (Ferrero et al., 1992; Kizaki et al., 1996). Clinical studies have shown that a high proportion of patients with acute promyelocytic leukemia achieve complete remission after treatment with high doses of retinoic acid (Huang et al., 1988; Tallman, 1996). Nevertheless, the general applicability of retinoic acid and a number of other retinoids is limited due to indiscriminate systemic side-effects (Frankel et al., 1992). There are antineoplastic strategies searching for more effective combinations of various agents that efficiently allow for dose reduction. A fertile area for exploration includes combinations of retinoids and other putative differentiating agents such as interferons and vitamin D_3 (Hellström et al., 1988).

During the differentiation of hematopoietic cells, some characteristic changes in membrane lipids occur which reflect the appearance of specific cellular function. However, our knowledge of the biological roles of the various lipid components is still incomplete. Numerous studies have demonstrated that polyunsaturated fatty acids, particularly arachidonic acid (20:4,$n-6$) liberated from membrane phospholipids, and its metabolites, eicosanoids, act as modulators and/or messengers of signals from the extracellular environment to the nucleus and the genome (Di Marzo, 1995). In addition, recent studies have indicated that polyunsaturated fatty acids have pronounced effects on gene expression, leading to changes in metabolism, cell growth and differentiation (Jump et al., 1996).

The immortal human myeloid leukemia cell line, HL-60, serves as an in vitro model which has been extensively

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used to gain insight into the processes of myeloid cell differentiation and their control mechanisms. The cell line has amplified *myc* and activated *ras* oncogenes, it is p53 negative and does not contain the characteristic t(15;17) translocation seen in acute promyelocytic leukemia (Dalton et al., 1988). These cells are bipotent, i.e., they can be induced to differentiate either into granulocytes on exposure to retinoic acid (RA) or dimethyl sulphoxide (DMSO) (Breitman et al., 1980; Collins et al., 1978), or into monocytes/macrophage-like cells with various other agents (Collins, 1987). The functions of differentiated HL-60 cells are partly dependent on the production of eicosanoids. These metabolites are produced from arachidonic acid through several oxidative pathways catalysed by (1) cyclooxygenases 1 and 2, producing prostaglandins, prostacyclins and thromboxanes, (2) a P450-monooxygenase system, producing epoxides, diols and monohydroxy-acids, and (3) lipoxygenases, producing hydroxy acids and mainly leukotrienes through the 5-lipoxygenase pathway. The HL-60 cell model is often used to study the regulation of 5-lipoxygenase expression and leukotriene production during myelopoiesis. It was shown that there is a correlation between granulocytic cell differentiation on the one hand and increased expression of 5-lipoxygenase-activating protein and 5-lipoxygenase mRNAs, 5-lipoxygenase-activating and 5-lipoxygenase proteins, 5-lipoxygenase activity and production of leukotrienes on the other (Kargman and Rouzer, 1989; Bennett et al., 1993). However, it has not yet been clearly documented that these changes may reflect only the functional state of more differentiated (or mature) cells, or that they are integral to the differentiation process.

It has been suggested that there are differences in lipoxygenase pathway dependence of normal vs. leukemic cells (Miller et al., 1989). It has been demonstrated by ourselves and others that an intact lipoxygenase pathway of arachidonic acid metabolism is necessary for normal granulocyte-macrophage progenitor cell proliferation and differentiation (Miller et al., 1986; Stenke et al., 1993; Kozubík et al., 1994), and also that the growth of cells of myeloid leukemia origin is partially dependent on an intact lipoxygenase pathway (Snyder et al., 1989; Ondrey et al., 1989; Hofmanová et al., 1996). Moreover, we have reported that 5-lipoxygenase inhibitors potentiate the effects of transforming growth factor β_1 (TGF-β_1) on HL-60 cell differentiation induced by retinoic acid or DMSO (Kozubík

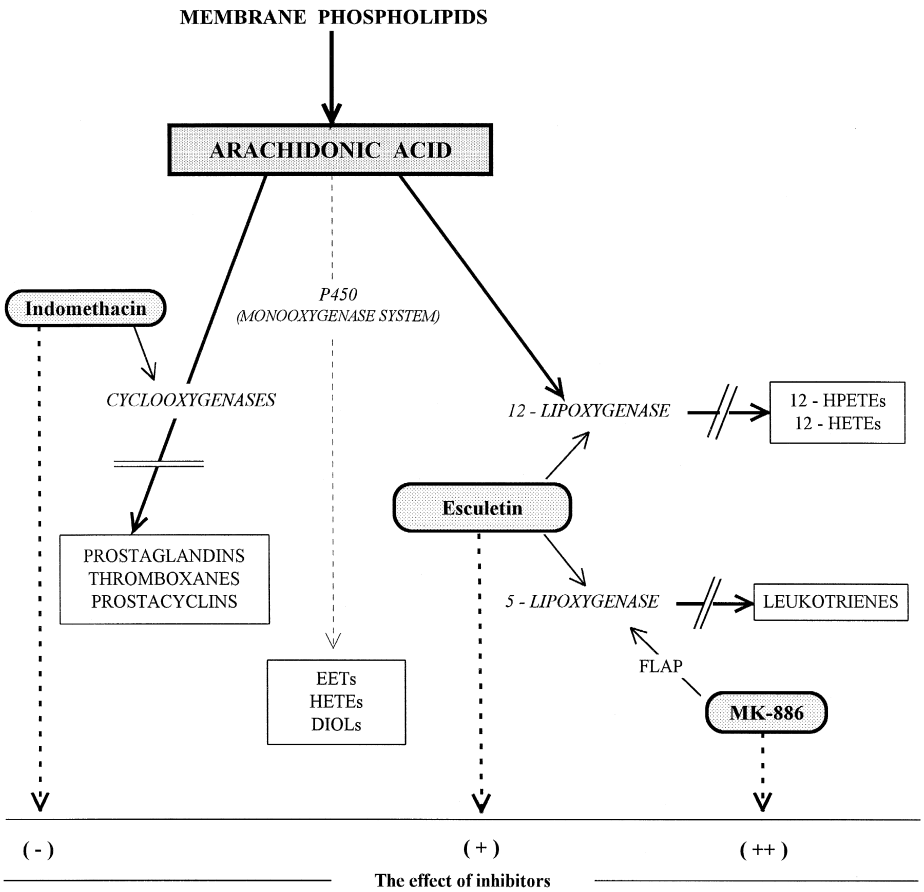

Fig. 1. Formation of arachidonic acid metabolites by lipoxygenases, cyclooxygenases and the P450-monooxygenase system and the mechanism of action of specific inhibitors of selected metabolic pathways. The effects of inhibitors on HL-60 cell differentiation induced by retinoic acid or DMSO in the experiments presented are shown schematically under the figure: (−) no effect; (+) the level of potentiation. HPETEs = hydroperoxy acids; HETEs = monohydroxy acids; EETs = epoxy-eicosatrienoic acids; FLAP = 5-lipoxygenase activating protein.

et al., 1997). Thus, manipulating the lipoxygenase pathway may lead to further understanding of the leukemic process and regulation of differentiation, and may provide strategies for the induction of differentiation in leukemic cells.

We now investigate the proliferation and differentiation of HL-60 cells induced to the granulocytic pathway either by retinoic acid or DMSO under conditions of arachidonic acid metabolism selectively modulated by inhibitors of its specific oxidative pathways as shown in Fig. 1. We provide evidence for synergistic potentiation of retinoic acid or DMSO induced differentiation of HL-60 cells by inhibitors of 5-lipoxygenase metabolism.

2. Material and methods

2.1. Cell culture and culture conditions

Human leukemia HL-60 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK) and were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum and 50 μg/ml gentamycin (Sebak, Germany) in a humidified incubator at 37°C in a controlled 5% CO_2 atmosphere. Stock cultures were maintained in 75 cm^2 flasks (Nunc) and recultured every 3 days at an initial density of 0.2×10^6 cells/ml. HL-60 cells in the maximal range of 20 passages were used for this study.

2.2. Inhibitors of arachidonic acid metabolism

Esculetin: 6,7-dihydroxycoumarin, an inhibitor of 5- and 12-lipoxygenases, does not inhibit cyclooxygenase; indomethacin: (1-[*p*-chlorobenzoyl]-5-methoxy-2-methyl-indole-3-acetic acid), an inhibitor of cyclooxygenase-1 and 2 (both agents from Sigma, Germany); MK-886: 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl propanoic acid (a kind gift from Merck, Canada), a specific inhibitor of 5-lipoxygenase activating protein, which has been proposed to function as an anchoring protein for 5-lipoxygenase at the membrane (Vickers, 1995). It prevents leukotriene formation in vivo and in intact cells in vitro. The drugs were diluted in absolute ethanol and added to the culture medium. The final concentration of ethanol did not exceed 0.1%, a concentration which did not significantly influence any of the parameters tested.

2.3. Cell growth and differentiation

For differentiation studies, exponentially growing cells were plated onto polystyrene Petri dishes (60 mm diameter, Nunc) at a density of 0.2×10^6 cells/ml and grown in the absence (control) or in the presence of retinoic acid or DMSO (Sigma). Inhibitors of eicosanoid synthesis were added to the cells 1 h before the addition of differentiation inducers. Following 96 h of incubation, the cells were counted with a Coulter Counter (model ZM; Coulter Electronics, UK) and assayed for differentiation using detection of cell oxidative burst by luminol-dependent chemiluminescence (O'Keefe et al., 1991), morphological analysis and determination of cell surface differentiation markers (CD). Cell viability was determined with the trypan blue (0.2%) exclusion assay.

2.3.1. Chemiluminescence

Cells, 10^6 from each experimental group, were harvested and resuspended in a serum-free cultivation medium. The cells were induced using zymosan opsonized by human serum. Luminol (5×10^{-4} M)-dependent chemiluminescence was measured for 60 min (20 cycles) at 37°C with an LKB Wallac 1251 luminometer (Pharmacia, Finland).

2.3.2. Cell morphology

The cells were placed on glass slides utilizing a cytocentrifuge. After staining with May-Grünwald/Giemsa-Romanowski, morphological assessment was made via light microscopy.

2.3.3. Cell surface markers

CD 11b and CD 14 antigens were detected by two-colour immunofluorescence, using commercially available reagents. In brief, 10^6 cells were incubated simultaneously with fluorescein isothiocyanate-conjugated CD 11b and phycoerythrin-conjugated CD 14 monoclonal antibodies (Immunotech, France) in phosphate buffered saline (PBS) with 1% fetal calf serum and 0.1% sodium azide for 30 min at 4°C. After washing, at least 10^4 cells were analysed by flow cytometry (Coulter EPICS XL, argon ion laser, 488 nm for excitation).

2.4. Cell cycle analysis

Following 24, 48 and 96 h of incubation, the cells were cooled in ice, washed with cold PBS, and stained with propidium iodide in Vindelov's solution (Vindelov, 1977) for 30 min at 37°C. Fluorescence (DNA content) was measured with a Coulter EPICS XL apparatus. The 1.5–2 $\times 10^4$ cells analysed in each sample served to determine the percentage of cells in each phase of the cell cycle, using standard software. Four independent experiments were performed.

2.5. Experimental design and data analysis

2.5.1. Chemiluminescence response

The experiments were designed as a complete factorial combination of DMSO (0–1.25%) or retinoic acid (0–5 μM) with (1) MK-886 (0–5 μM), (2) esculetin (0–25 μM), and (3) indomethacin (0–25 μM). Dose–response curves for single factors were evaluated for the concentration range of DMSO: 0–1.65%; retinoic acid: 0–20 μM; MK-886: 0–15 μM; esculetin and indomethacin: 0–25

μM. All experimental variations were repeated independently four to six times in order to reach a sufficiently low coefficient of variance (4–8%). All the results are presented as percent of peak value of the chemiluminescence signal in control samples.

2.5.2. Analysis of variance (ANOVA) and polynomial regression models

The results were analysed using two-way ANOVA followed by the Newman–Keuls multiple-range test (verified normality and homogeneity of variance) (Zar, 1984). The ANOVA models assessed the effects of single factors and their interactions as well. The null hypothesis for an interaction between two combined factors was that the response of the chemiluminescence signal did not differ among the specific levels of one factor depending upon the particular level of the second factor. In order to assess the quantitative contribution of interactive and additive components to the total inhibition effect, a general approach was applied for investigating the regression model from two-way experimental data (Anderson and McLean, 1974). Dose–response models were constructed by fitting log-transformed data to polynomials and evaluated applying the correlation between the observed and predicted data and the lack-of-fit statistics. In order to quantify the dose–response relationship in a standard way, EC_{50} and maximum reached effects were estimated from fitted polynomial regression models. Variability measures that supplied the EC_{50} and maximum effect values allowed pairwise comparison of experimental combinations of the agents by *t*-test.

2.5.3. Interaction index approach

Significant departures from zero interaction (additive effect) between the combined agents were detected using an interaction index approach that provided a safe indication of synergism irrespective of the type of effect caused by single agents (according to Berenbaum, 1981, modified). The combined doses of DMSO or retinoic acid (RA) with MK-886, esculetin or indomethacin which produced some effect ($DMSO_C$, RA_C, $MK\text{-}886_C$, $ESCUL_C$ and $INDO_C$) were compared with equieffective doses of single factors ($DMSO_E$, RA_E, $MK\text{-}886_E$, $ESCUL_E$ and $INDO_E$). The higher the value of an equieffective dose (d_E) as compared with the dose of the same agent involved in a specified combination (d_C), the higher the synergistic character of the interaction effect. The values of d_E including standard errors were extracted from dose–response models for single agents, which also allowed direct comparison with d_C values using a one-tailed *t*-test.

The statistical analyses were performed using Statistical Programs for Social Sciences Version 4.0 (SPSS/PC + ™, 1990). The minimum significance level for rejection of the tested hypotheses was taken as $P = 0.05$ in all tests.

2.5.4. Cell proliferation, cell morphology and cell surface antigen expression

These experiments were designed as selected treatments of cells with the relevant agents (see Section 3) and were repeated independently four to seven times. The statistical significance of differences in cell numbers was calculated by one-way ANOVA and Bonferroni corrected *P* value for multiple comparisons. The relative frequencies of cells in specific cell cycle phases and the results of morphological and CD 11b expression analyses were compared using the two-sample binomial test.

3. Results

Proliferation and differentiation were studied after combined treatment of the HL-60 cells with three inhibitors of arachidonic acid metabolic pathways or with inducers of granulocytic differentiation, i.e., retinoic acid or DMSO. The precise evaluation of experiments designed as a complete factorial combination of treatments was performed using peak values of the chemiluminescence signal as a parameter reflecting oxidative burst of cells, i.e., the functional stage of differentiation.

3.1. Effects of single agents on chemiluminescence response

Fig. 2 gives a description of the effects of a wide range of concentrations of retinoic acid, DMSO and MK-886 on chemiluminescence responsibility of HL-60 cells after 96 h of incubation. It is apparent that the applied agents varied considerably in their dose–response characteristics. While there was a relatively steep increase in the chemiluminescence signal as a reaction to retinoic acid treatment (without a significant shoulder), some threshold concentration was necessary for a significant stimulatory effect in the case of DMSO (approx. 0.8%). The curves continued increasing only with a limited range of applied concentrations (retinoic acid: 0–8 μM; dmso: 0–1.45%; MK-886: 0–15 μM). In the case of esculetin and indomethacin, no significant changes in the chemiluminescence signal were detected as compared with the control (data not shown). The main factor that complicated estimation of the effects at the higher doses applied was viability of the cells. Cell viability at lower doses of the applied drugs ranged between 85–95%, but it decreased significantly at doses higher than 1.8% of DMSO, 15 μM of retinoic acid, 15 μM of MK-886, 20 μM of esculetin and 25 μM of indomethacin.

3.2. Effects of combinations of retinoic acid or DMSO with MK-886 on chemiluminescence response—polynomial regression model

Although the same experimental design was applied for the three inhibitors, more detailed mathematical analyses are presented only for the 5-lipoxygenase activating pro-

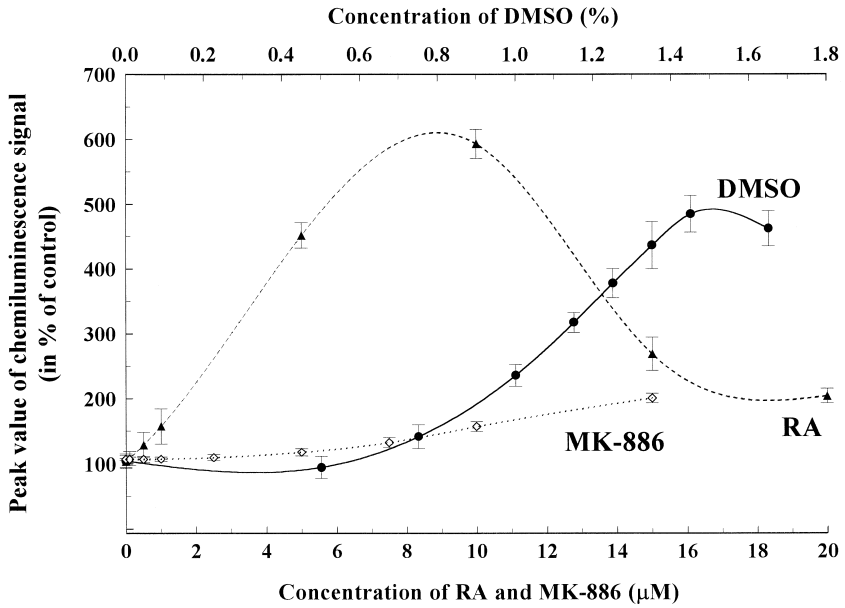

Fig. 2. Effects of single agents on chemiluminescence response of HL-60 cells. Cells (2×10^5/ml) were cultured with various concentrations of retinoic acid, DMSO or MK-886 for 96 h. Luminol-dependent chemiluminescence was measured in 10^6 cells from each experimental group in two parallels. Dose–response curves describe the peak of chemiluminescence (in % of non-treated control; transformed to natural logarithm for statistical evaluation). The data represent mean values from 4–6 independent experiments with standard errors.

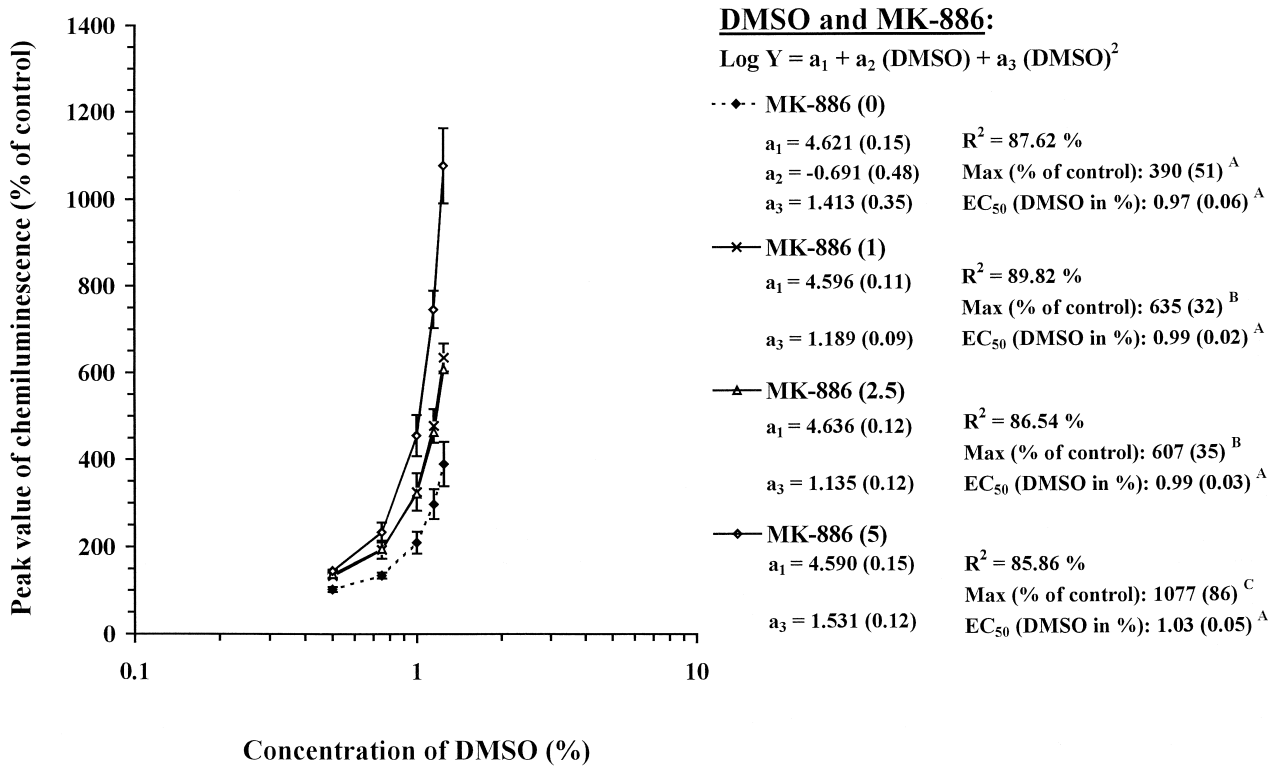

Fig. 3. Effects of combination of MK-886 and retinoic acid on the chemiluminescence response of HL-60 cells. The peak of the chemiluminescence signal (in % of non-treated control; transformed to natural logarithm for statistical evaluation) in dose–response models based on RA as an independent variable (concentration values are on log scale) combined with different concentrations of MK-886. The lines represent mean values from 4–8 independent experiments with standard errors. a_1, a_2, a_3, estimated parameters of constructed regression models. In parentheses: standard errors. R^2: coefficient of determinance; MAX—maximum effect reached; EC_{50} and MAX estimates are given with standard errors (in parentheses). EC_{50} and MAX estimated for different curves followed by the same letter are not significantly different ($P < 0.05$; pairwise comparison by *t*-test).

tein inhibitor, MK-886, because of its very significant interaction with both retinoic acid and DMSO, and because of its specificity for the 5-lipoxygenase pathway.

The effect of fixed doses of MK-886 on the shape of the dose–response curves of retinoic acid and DMSO was studied using doses with no significant effects on cell viability. Evaluation of the data based on polynomial regression revealed a synergistic character of the interactions. The application of MK-886 with retinoic acid increased significantly the slope of the dose–response curves, an effect which was reflected by the values of the linear term in polynomial models and by the significantly decreased EC_{50} values in comparison that for retinoic acid applied alone (Fig. 3). The synergistic pattern of the interaction effect was less pronounced in the case of combinations that involved a 5 μM dose of retinoic acid, where only the highest doses of MK-886 significantly increased the effect. The decreased potentiation of higher doses of retinoic acid by MK-886 was reflected in the negative second-order terms of polynomial models describing the combined effects.

The application of MK-886 with DMSO significantly decreased the shoulder and increased the final slope of the dose–response curve in comparison with DMSO added alone (Fig. 4). The dose–response relationships for the effect of DMSO in combination with MK-886 corresponded to second-order polynomials without the linear term, which was incorporated as significant only in the model for DMSO alone. Although the addition of MK-886 at low and middle-range doses did not change the EC_{50} values of the resulting curves, the effect of a combination of higher doses of MK-886 and DMSO reached a level that exceeded the maximum possible effect as predicted from dose–response curves for single factors (> 900% of the control). Generally, as extracted from ANOVA models, the interaction effect in the experiment with inhibitors and retinoic acid accounted for a higher portion of the total variance (significant at $P = 0.0001$) than it did with DMSO experiments (significant at $P = 0.0132$).

3.3. Effects of combinations of retinoic acid or DMSO with MK-886, esculetin or indomethacin— interaction index approach

The results described above documented the synergistic character of the cell response to the combined treatment using MK-886 with retinoic acid or DMSO. However, as expected, the significance level of synergism depended strongly on the character of the dose–response curves for single agents and on the combined range of concentrations.

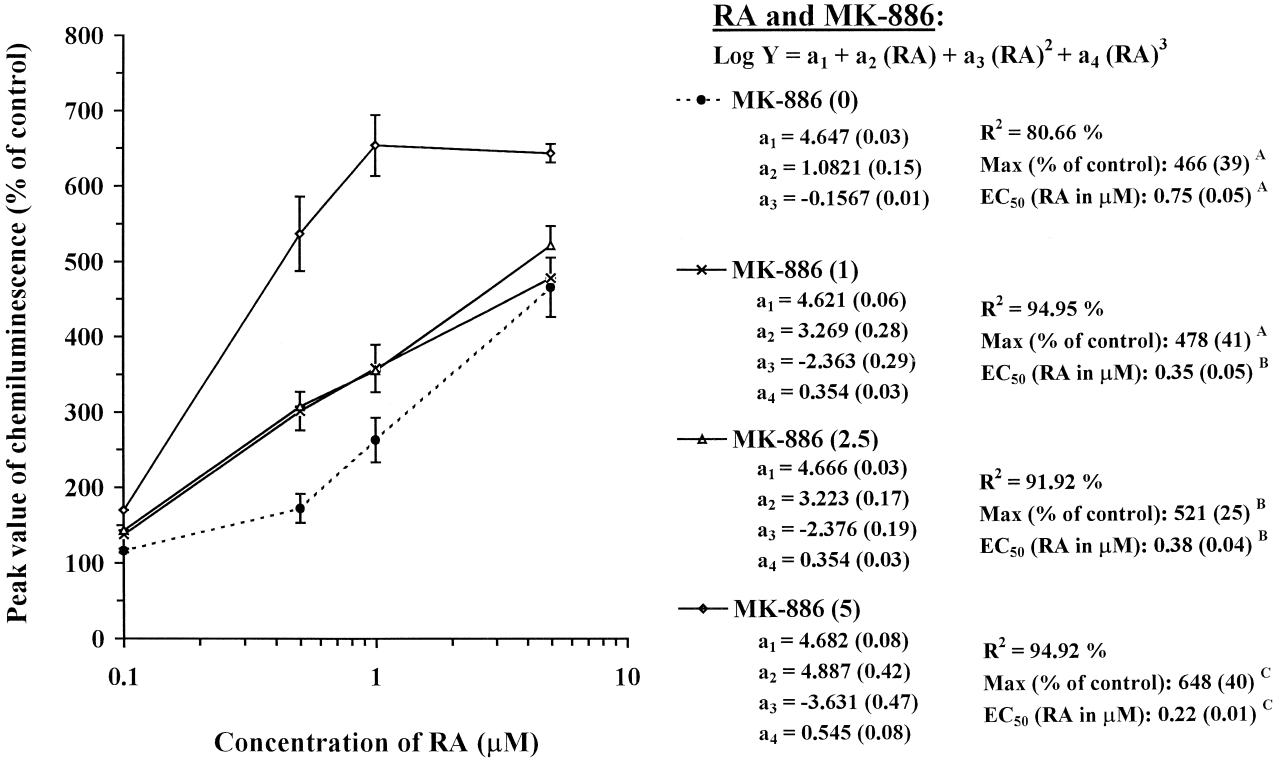

Fig. 4. Effects of combination of MK-886 and DMSO on the chemiluminescence response of HL-60 cells. The peak of the chemiluminescence signal (in % of non-treated control; transformed to natural logarithm) in dose–response models based on DMSO as an independent variable (concentration values are on log scale) combined with different concentration levels of MK-886. The lines represent mean values from 4–8 independent experiments with standard errors. a_1, a_2, a_3, a_4, estimated parameters of constructed regression models. In parentheses: standard errors. R^2: coefficient of determinance; MAX—maximum effect reached; EC_{50} and MAX estimates are shown with standard errors (in parentheses). EC_{50} and MAX estimated for different curves followed by the same letter are not significantly different ($P < 0.05$; pairwise comparison by *t*-test).

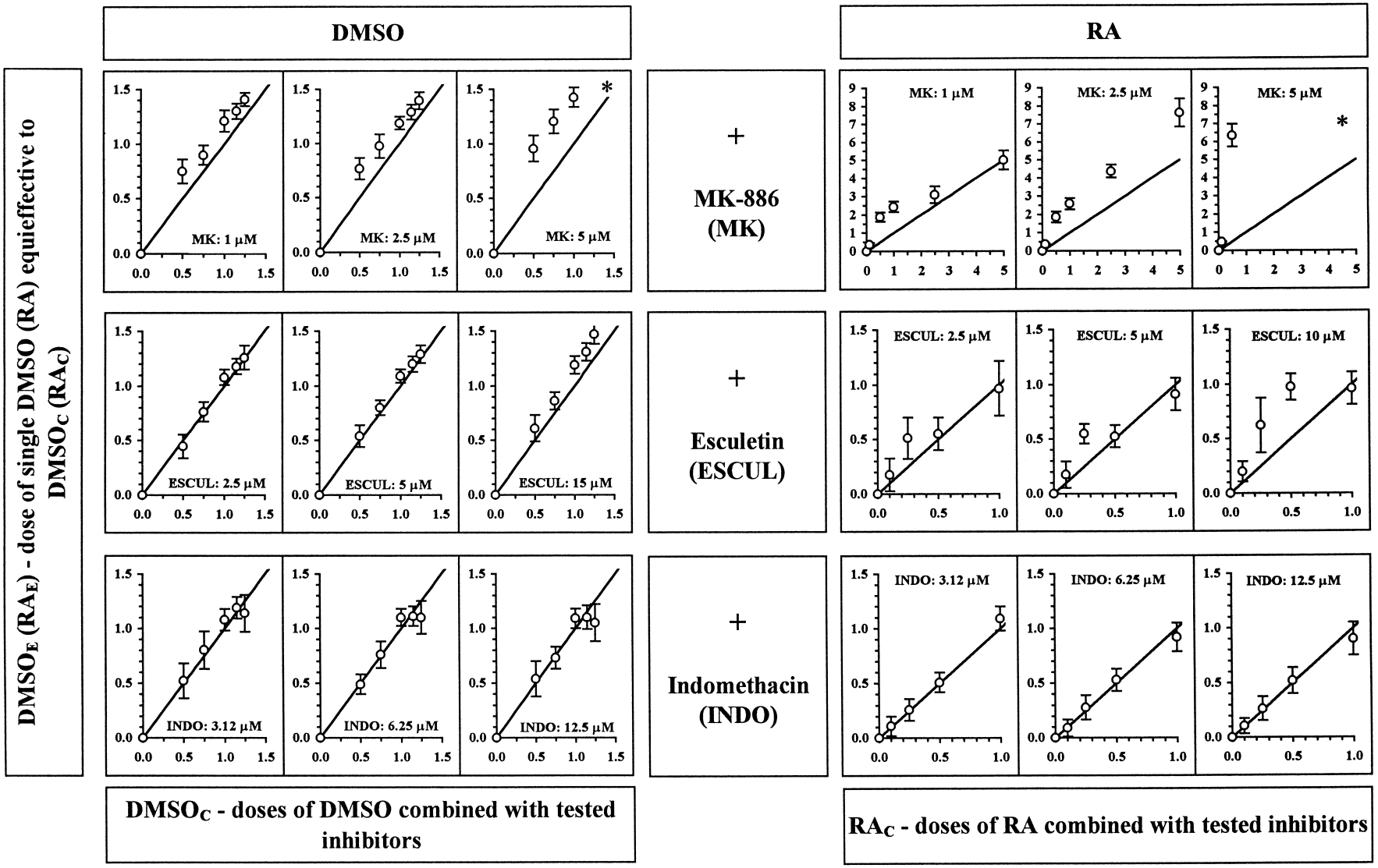

Fig. 5. Effects of combinations of retinoic acid or DMSO with inhibitors of AA metabolism on the chemiluminescence response of HL-60 cells. Statistical comparison of retinoic acid or DMSO doses (RA_E, $DMSO_E$) that are equieffective with the concentrations (RA_C, $DMSO_C$) involved in selected combinations with MK-886, esculetin or indomethacin. The effectiveness of retinoic acid or DMSO as single agents and in combination with an inhibitor did not differ significantly for combinations where 95% confidence limits for RA_E or $DMSO_E$ values involved the diagonal additivity line ($P = 0.05$). I: 95% confidence limits for mean RA_E or $DMSO_E$ values: ○. ∗ The effect of combinations led to a stimulation significantly exceeding the level predictable from dose–response curves of retinoic acid or DMSO as single factors.

Therefore, an alternative approach was necessary for comparative evaluation of the potentiation effects of MK-886, esculetin and indomethacin. Important experimental points, i.e., doses of RA_C or $DMSO_C$ combined with selected doses of MK-886, esculetin or indomethacin, were compared on an equieffective basis with doses of RA_E or $DMSO_E$ (Fig. 5). Doses of MK-886 equieffective to the concentrations involved in combinations with retinoic acid or DMSO could not be estimated because nearly all the combined effects exceeded the potential maximum of this inhibitor if applied alone. Similarly, indomethacin and esculetin as single agents with no significant effect on the chemiluminescence signal did not allow equieffective comparison to any combination with retinoic acid or DMSO. Therefore, the degree of synergy in the effect of any experimental combination had to be assessed on the basis of equieffective comparison made for only one of the constituents, i.e., retinoic acid or DMSO. Nearly all of the tested concentrations of retinoic acid combined with MK-886 (RA_C) were significantly lower than the corresponding equieffective doses of RA_E, except for higher retinoic acid if combined with lower doses of MK-886 ($< 2\ \mu M$), where the effects were additive. Generally, the potentiation effect of MK-886 decreased in combinations with higher doses of retinoic acid ($> 3\ \mu M$). The doses of DMSO ($DMSO_E$) estimated as equieffective to those in combinations with MK-886 were significantly higher ($P < 0.05$) than the doses involved in combinations ($DMSO_C$). Relatively more significant differences ($P < 0.01$–0.001) were observed in the case of higher doses of DMSO, particularly when it was combined with higher doses of MK-886 ($> 3\ \mu M$). It must be emphasized that the combination of the highest doses of MK-886 with higher doses of either retinoic acid or DMSO led to a stimulation that significantly exceeded the maximum level predictable, not only for MK-886 alone but even for retinoic acid or DMSO as single factors (Fig. 5; marked with $*$).

When compared with MK-886, esculetin had qualitatively the same pattern of interactions, i.e., increased potentiation of lower doses of retinoic acid and of higher doses of DMSO (Fig. 5). However, the potentiation due to the application of esculetin was much less significant than the effect of MK-886, and significant synergy ($P < 0.05$) was proved only for higher doses of esculetin ($> 10\ \mu M$). As with combinations with MK-886, the interaction of esculetin with retinoic acid led to a quantitatively higher stimulation of the cell response than did combination with DMSO. No significant potentiation of retinoic acid and DMSO was reached when these agents were combined with a wide range of indomethacin doses.

3.4. Cell morphology and expression of cell surface antigens

Based on chemiluminescence data, the potentiation ability of MK-886 on the retinoic acid- or DMSO-induced differentiation of HL-60 cells was verified by morphological estimation of the differentiation stage of the treated cells and by detection of cell surface antigens (Table 1). In these experiments we used only the most effective concentration of MK-886, i.e., 5 μM, and concentrations of retinoic acid or DMSO that are routinely used in differentiation experiments, i.e., 1 μM and 1.25%, respectively. After combined treatment with MK-886 and retinoic acid or DMSO, the cells shifted to more mature stages than with retinoic acid or DMSO as single factors. A combination of MK-886 with DMSO apparently shifted the cells from promyelocytes to myelocytes/metamyelocytes. Because of the marked differentiating effect of retinoic acid alone, there was a less pronounced shift of cells from the myelocytic to the metamyelocytic stage with the combined treatment with MK-886. Combined treatment of cells with MK-886 and retinoic acid or DMSO did not significantly affect the percentage of apoptotic cells in comparison with treatment with retinoic acid or DMSO alone.

In comparison with the cells treated only with retinoic acid or DMSO, the amount of CD 11b positive cells increased significantly after combined treatment with MK-886 and retinoic acid or DMSO (Table 1). The monocytic CD 14 antigen was not expressed, irrespective of the type of treatment. Flow cytometric diagrams of CD 11b and CD

Table 1
Differentiation of HL-60 cells following 96 h treatment with DMSO (1.25%), retinoic acid (RA, 1 μM), MK-886 (5μM) and their combinations

Parameter / Treatment	Cell morphology (%)				CD 11b (% of expression)
	Promyelocytes	Myelocytes	Metamyelocytes	Apoptotic	
Control (non-treated)	90.4 (2.6)^{A}	8.2 (2.6)^{A}	0^{A}	1.8 (0.4)^{A}	0.85 (0.21)^{A}
DMSO	64.1 (2.9)^{B}	29.7 (2.9)^{B}	3.3 (0.5)^{AB}	3.1 (0.7)^{A}	22.78 (3.58)^{B}
RA	5.0 (1.9)^{C}	59.6 (5.2)^{C}	21.3 (3.7)^{C}	14.1 (2.9)^{B}	30.28 (6.20)^{B}
MK-886	92.9 (2.5)^{A}	8.3 (2.7)^{A}	0^{A}	1.4 (0.2)^{A}	1.65 (0.46)^{A}
MK-886/DMSO	32.7 (5.1)^{D}	57.0 (5.3)^{C}	6.6 (2.1)^{B}	3.7 (1.0)^{A}	44.30 (6.51)^{C}
MK-886/RA	8.7 (1.2)^{C}	46.5 (8.7)^{C}	31.2 (6.2)^{C}	15.4 (4.0)^{B}	43.95 (7.27)^{C}

The numbers are the means of five independent experiments. For morphological analysis, 200 cells per group/experiment were evaluated.
For CD 11b expression, 10^4 cells per group/experiment were analysed by flow cytometry.
Standard errors are given in parentheses.
The numbers within a single column followed by the same letter are not significantly different (binomial test, $P < 0.05$).

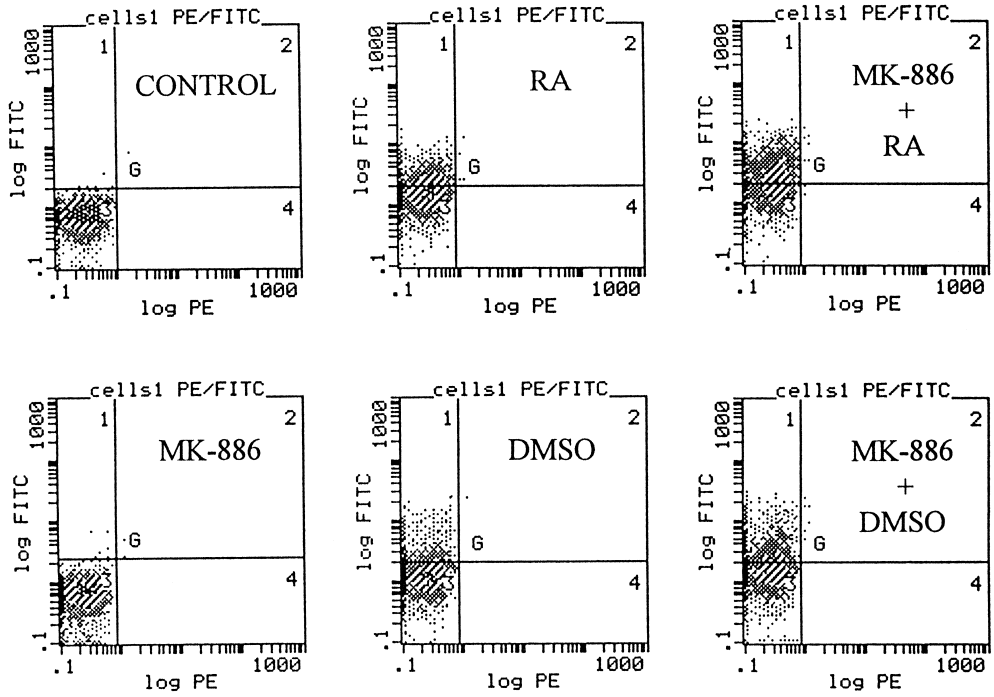

Fig. 6. Effects of MK-886, retinoic acid, DMSO and their combinations on CD 11b and CD 14 expression. Representative results of flow cytometric analysis of CD 11b (FITC labelled) and CD 14 (PE labelled) expression after 96 h treatment of HL-60 cells with 1 μM retinoic acid, 1.25% DMSO, and their combinations with 5 μM MK-886.

14 expression after treatment with individual agents or their combination from the representative experiment are shown in Fig. 6.

3.5. Cell number and cell cycle analysis

Studies of cell proliferation showed a significant decrease in cell number due to treatment with retinoic acid (1 μM) and DMSO (1.25%) in comparison with the control non-treated population after 96 h of incubation. Simultaneously, there was an increased amount of cells in G_0/G_1 and a decreased amount of cells in the S phase after this treatment (no significant effects were observed up to 48 h). On the other hand, treatment of the cells with MK-886 did not significantly change either cell number or cell cycle parameters. The combined treatment with DMSO or retinoic acid and MK-886 had no significant additional effects (in comparison with retinoic acid or DMSO alone) on the parameters studied (Table 2).

Similar results, i.e., potentiation of CD 11b expression and more differentiated cells as determined by morphological evaluation, without changes in proliferation parameters, were observed after the treatment with a combination of

Table 2
Cell number and cell cycle analysis of HL-60 cells following treatment with DMSO (1.25%), retinoic acid (RA, 1 μM), MK-886 (5μM) and their combinations

Parameter / Treatment	Time							
	48 h				96 h			
	Cell number[b]	Cell cycle phase (% of cells)[a]			Cell number[b]	Cell cycle phase (% of cells)[b]		
	($\times 10^5$)	G_0/G_1	S	G_2/M	($\times 10^5$)	G_0/G_1	S	G_2/M
Control	7.57 (0.26)^A	53.3 (3.9)	24.5 (5.7)	22.2 (1.7)	25.73 (1.89)^A	59.6 (2.6)^A	24.3 (2.3)^A	15.4 (0.8)^{AB}
DMSO	6.59 (0.27)^B	60.1 (3.5)	18.5 (3.6)	21.4 (0.1)	15.99 (1.74)^{BC}	72.6 (2.8)^B	8.7 (2.2)^B	17.6 (2.4)^{AB}
RA	7.12 (0.49)^A	58.1 (6.3)	19.8 (4.8)	22.5 (2.1)	13.95 (1.62)^C	65.6 (2.2)^C	14.8 (0.9)^C	19.4 (3.0)^A
MK-886	6.88 (0.38)^A	55.1 (4.0)	21.7 (3.0)	23.2 (1.0)	24.61 (1.36)^A	61.6 (2.9)^C	24.5 (2.9)^A	11.5 (0.4)^B
MK-886/DMSO	6.29 (0.55)^B	58.6 (4.8)	17.6 (1.2)	23.8 (3.6)	17.41 (2.33)^B	72.0 (2.9)^B	10.1 (1.8)^B	17.1 (2.4)^{AB}
MK-886/RA	7.00 (0.55)^A	59.4 (8.9)	18.7 (4.6)	21.9 (4.3)	16.94 (0.89)^B	64.6 (3.5)^C	15.4 (1.5)^C	18.7 (2.5)^A

The numbers are the means of seven (cell number) or four (cell cycle analysis) independent experiments ($1.5–2 \times 10^4$ cells per group/experiment). Standard errors are given in parentheses.

[a] No significant differences between experimental groups were observed (binomial test, $P < 0.05$).

[b] The numbers within a single column followed by the same letter are not significantly different (binomial test, $P < 0.05$).

retinoic acid (0.5 μM) or DMSO (1.25%) and esculetin (10 or 15 μM, respectively) (data not shown).

4. Discussion

Our results demonstrated a synergistic interaction of the 5-lipoxygenase activating protein inhibitor, MK-886, and inducers of granulocytic differentiation, retinoic acid and DMSO, in human leukemia HL-60 cells. Similar results (but with less synergy) were obtained with the lipoxygenase inhibitor, esculetin. No effects were observed with the cyclooxygenase inhibitor, indomethacin (for a scheme, see Fig. 1). These effects were clearly demonstrated by the ability of cells to produce reactive oxygen species (oxidative burst) as measured by chemiluminescence. This parameter reflects well the functional state of differentiation of HL-60 cells, and correlates well with the widely used nitroblue tetrazolium reduction test (Kozubík et al., 1997; O'Keefe et al., 1991). Significant synergism for the interaction of the agents was shown by the changed character of dose–response relationships (polynomial regression models based on two-way ANOVA models) as well as by the results of an equieffective comparison of doses of retinoic acid or DMSO as single factors and as constituents of mixtures (Berenbaum, 1981). Comparison of the doses of single agents equieffective with the combined concentrations provides a safe indication of synergism irrespective of the type of dose–response curve. Furthermore, this index approach allowed evaluation of important experimental combinations regardless of the character of the interaction in the other parts of the dose–response curves, specifically with respect to the concentrations actually combined.

In summary, the character of the interactions between the agents tested was not necessarily the same over the whole range of doses. In the case of combination of the highest doses of MK-886 tested with either retinoic acid or DMSO, we can clearly detect the significant synergy directly from dose–response analyses. More specifically, the effect of retinoic acid was potentiated more strongly by MK-886 in the lower range of concentrations (retinoic acid: 0–2.5 μM), while the opposite pattern was revealed in the case of DMSO, i.e., more pronounced potentiation by MK-886 if combined with higher doses of DMSO ($>1\%$). This different character of the kinetics of potentiation with retinoic acid and DMSO could be explained by assuming different mechanisms for their effects. Retinoic acid represents a physiological stimulus and exerts its activity by interaction with specific nuclear receptors which act as homo and heterodimeric transcription regulators (Kizaki et al., 1996). DMSO is a non-physiological polar–planar compound, and numerous mechanisms for the induction of myeloid maturation have been suggested, such as membrane property alteration or direct interaction with hydrogen bonds within the chromatin (Antoun et al., 1991; Ponton et al., 1996). Retinoic acid- and DMSO-treated cells differ in several aspects. Intensive studies of Yen et al. (1987) demonstrated that retinoic acid- and DMSO-induced pathways diverge in the early events needed in the cellular program to affect phenotypic differentiation. It has been shown that retinoic acid could be a more complete and effective inducer of granulocytic differentiation than DMSO, particularly regarding the morphology (Dufer et al., 1989). It was also reported that DMSO and retinoic acid did not induce the same membrane phenotypic changes during maturation of HL-60 cells, and that this maturation was incomplete and partially defective (Brackman et al., 1995). The results of our experiments exploring cell morphology and CD 11b expression after the combined treatment with inhibitors of 5-lipoxygenase metabolism and retinoic acid (or DMSO) confirmed a shift of HL-60 cells toward more differentiated stages. No cells with a fully mature morphological phenotype were seen.

The mechanisms of interaction between the differentiation events induced by retinoic acid or DMSO and the effects of 5-lipoxygenase activating protein and 5-lipoxygenase inhibitors in our experiments are not known. Our results showed that while both retinoic acid and DMSO treatment of cells caused a time-dependent decrease in cell number and growth arrest, the combined treatment with MK-886 and retinoic acid or DMSO, which significantly potentiated cell differentiation, did not additionally affect cell proliferation parameters. This is in agreement with reports suggesting that proliferation and differentiation of HL-60 cells involve different regulatory mechanisms (Yen et al., 1987; Liu and Levy, 1997).

It was reported that treatment with either retinoic acid or DMSO ultimately leads to changes in the gene expression program of HL-60 cells which are similar to but not fully parallel with those of normal myeloid cell differentiation. In addition to the changes in immediate early gene expression such as c-*myc*, c-*myb*, c-*fos*, the metabolic cascade induced by retinoic acid or DMSO depends on the function of some other genes coding particularly for components of the prototypic signal transduction pathways (Collins, 1987; Lübbert et al., 1991). Differentiation of HL-60 cells is further accompanied by induction of p21, WAF1/CIP1 expression (Jiang et al., 1994), alteration of activity of specific enzymes and phosphorylation of specific proteins (Lübbert et al., 1991), or by expression of specific cell surface molecules (Brackman et al., 1995).

It is now universally accepted that arachidonic acid and eicosanoids, apart from functioning as second messengers themselves (Peppelenbosch et al., 1993), play a key role in the fine tuning of signal transduction, both by modulating the other major intracellular metabolic signalling pathways and by being targets for their action, thereby creating several possibilities for interaction (Di Marzo, 1995). Moreover, it was demonstrated that 5-lipoxygenase may have a previously unrecognized role in tyrosine kinase signalling distinct from its catalysis of lipid mediator

formation (Lepley and Fitzpatrick, 1994). In our previous study it was demonstrated that eicosanoid inhibitors enhance synergistically the antiproliferative effects of TGF-β_1 on CCL64 mink lung epithelial cells (Kozubík et al., 1996). It is clear from a number of reports that 5-lipoxygenase metabolism is closely connected with myeloid cell differentiation (Kargman and Rouzer, 1989; Bennett et al., 1993; Ponton et al., 1996). We can suppose that the changes in 5-lipoxygenase activity and/or leukotriene production caused by selective inhibitors can modulate some specific events leading to cell differentiation after treatment with retinoic acid or DMSO. In that case our results would confirm the suggestion that arachidonic acid metabolism, and particularly its 5-lipoxygenase pathway, might represent one of the ways by which cells are able to control their responsiveness to differentiation stimuli (Miller et al., 1990).

In addition to direct interference of the 5-lipoxygenase pathway with the differentiation events, we should mention the other reasons for the effects observed. It is possible that suppression of 5-lipoxygenase caused an imbalance in arachidonic acid metabolism, and that subsequently the cells contain more free arachidonic acid or more alternative arachidonic acid metabolites, for example prostaglandins, are produced. It was shown that exogenous arachidonic acid can have differentiating effects or can potentiate retinoic acid effects on HL-60 cells (Finstad et al., 1994). Also, exogenous prostaglandin E was shown to promote HL-60 cell monocytic differentiation (Kawase et al., 1995). Finally we would like to mention that the effects observed could reflect a pharmacological property of MK-886 not connected with arachidonic acid metabolism. However, this is unlikely because the structurally different 5-lipoxygenase inhibitor, esculetin, had a similar effect, i.e., potentiation of induced differentiation.

In any case, the precise mechanisms of the differentiation effects observed in our experiments need further study. In spite of this uncertainty associated with the mechanisms of the effects, our results could be important from the practical point of view. In vivo differentiating therapy of acute promyelocytic leukemias frequently requires high and relatively toxic doses of retinoic acid (Frankel et al., 1992; Tallman, 1996). Many inhibitors of lipoxygenase metabolism are used clinically, mainly as anti inflammatory and anti asthmatic drugs (Musser and Kreft, 1992). Our results showing a synergistic interaction of retinoic acid with 5-lipoxygenase inhibitors imply the possibility of using these compounds in combination with lower doses of RA to achieve more potent differentiating capacities without detrimental side-effects.

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