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Molecular and Cellular Effects of Hexadecylphosphocholine (Miltefosine) in Human Myeloid Leukaemic Cell Lines

T. Beckers, R. Voegeli and P. Hilgard

The molecular and cellular effects of the anti-neoplastic alkylphospholipid hexadecylphosphocholine (Miltefosine, MIL) on parameters associated with growth and differentiation of human myeloid leukaemic cell lines U937, KG1 and KG1a were investigated. On a cellular level, MIL has dose-dependent differentiation-inducing, growth-promoting and cytotoxic activities exemplified by induction of respiratory burst activity, stimulation of interleukin-3 (IL-3)/granulocyte–macrophage colony stimulating factor (GM-CSF)-dependent growth of the KG1 cell line in soft agar culture, inhibition of cellular net growth and finally cell death. By northern blot analysis, transcription of functional receptors for IL-3, GM-CSF, G-CSF and FcRI were studied. It was shown that MIL has stimulatory activity on IL-3 and GM-CSF receptor gene transcription. In addition, the transcription of proliferation- and differentiation-associated proteins, namely histone subtypes, c-*myc* and NF-κB p50, were studied. MIL suppressed c-*myc* and enhanced NF-κB p50 transcription in the U937 cell line, comparable to the well-characterised differentiation-inducing phorbolester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We conclude that the interaction of MIL with its molecular target(s) in myeloid cells induces molecular and cellular effects associated with induction of differentiation, distinct from its cytotoxic activity.

Key words: anti-neoplastic phospholipids, myeloid differentiation, CSF receptors, gene transcription
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INTRODUCTION

ETHERPHOSPHOLIPIDS AND lysophospholipids are naturally occurring derivatives of membrane phospholipids with interesting biological characteristics. For example, the etherphospholipid 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor, PAF) causes platelet aggregation and dilatation of blood vessels [1]. Lysophosphatidic acid (L-α-lysophosphatidic acid, LPA), as the simplest natural phospholipid, is a potent mitogen for quiescent fibroblasts [2]. 2-lysophosphatidylcholine and analogues have stimulatory effects on the immune system, as exemplified by enhanced macrophage phagocytosis [3].

These biological effects were the rationale for chemical synthesis of compounds with a metabolism different from the naturally occurring ether- and lysophospholipids. Numerous derivatives of these compounds were subsequently synthesised, and recently the alkylphospholipid hexadecylphosphocholine (Miltefosine, MIL) was identified as a potent experimental anticancer agent [4–6]. Miltefosine solution (Miltex®) is used

clinically for the topical treatment of skin metastases of human mammary carcinoma.

Apart from its anti-neoplastic activity, MIL can induce effects associated with differentiation of myeloid leukaemic cell lines [7–9] and adenocarcinoma cells in dimethylbenzanthracene (DMBA)-induced mammary carcinoma [10]. Another possible aspect of the differentiation-inducing activity of MIL is the induction of leucocytosis in rats, and during initial clinical studies with systemic treatment in humans [4, 11]. The significance of this phenomenon is not clear, but may be related to the synergistic effects of MIL on the colony-stimulating factor (CSF)-dependent growth of haematopoietic cells [12, 13] or cytokine expression in human mononuclear cells [14].

The intention of our present study was to analyse the effects of MIL on parameters associated with proliferation and cellular differentiation in the human leukaemic myeloid cell lines KG1, KG1a and U937. These cell lines are well characterised to differentiate, with stimuli such as phorbolester TPA or tumour necrosis factor-α (TNFα), to monocytes/macrophages [15].

MATERIALS AND METHODS

Cell culture

The human cell line U937 (ATCC CRL 1593) was cultured at 37°C, 5% CO_2 in RPMI-1640 medium supplemented with 10% v:v fetal calf serum, penicillin/streptomycin and glutamine. The cell lines KG1 (ATCC CCL 246) and KG1a (ATCC CCL 246.1) were cultured in IMDM supplemented with 20% v:v fetal calf serum, penicillin/streptomycin and glutamine and kept at 37°C, 5% CO_2. All cell lines were established from ATCC cryo cultures. Cells were grown at densities between 1×10^5 and 1.5×10^6 cells/ml for a maximum of 35 passages. Only cells from growing cultures, with ≤5% Trypan blue-positive cells, at densities from 0.5 to 1×10^6 cells/ml were harvested, resuspended in fresh culture medium and incubated with the test compounds. Phorbolester TPA (Sigma, St Louis, Missouri, U.S.A.) was added from a stock solution (10 mM in dimethylsulfoxide, DMSO) to culture medium at 10 and 100 nM final concentration. The final concentration of the solvent DMSO alone (0.1–1% v:v) has been shown to have no significant effect on myeloid cell lines [31]. Hexadecylphosphocholine (MIL; D-18506—synthesised by ASTA Medica AG, Frankfurt/Main, Germany) was added from a stock solution (20 mM in phosphate buffered saline, PBS) at the indicated concentrations.

Cytotoxicity assays

The effect of MIL on metabolic activity of myeloid cell lines after incubation for 24 h in liquid culture was quantified by the MTT assay [16]. Metabolic reduction of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] (Sigma) leads to formation of MTT-formazan. The assay was performed essentially as described [17]. EC_{50} values were calculated from dose–response curves by regression analysis. Cell number and viability were determined by Trypan blue dye exclusion using a Neubauer chamber. Cells were resuspended in Trypan blue solution (Boehringer Mannheim, Mannheim, Germany) and counted immediately by phase contrast microscopy.

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Nitrobluetetrazolium (NBT) reduction assay

The reduction of yellow NBT to a black–blue formazan precipitate by cellular superoxide [18] was quantified colorimetrically by a modified method [7]. Cells were incubated with TPA or MIL for 48 h in suspension culture at 5×10^5 cells/ml (0 h) at the indicated concentrations. They were harvested by centrifugation, if necessary after detaching adherent cells with PBS/1 mM ethylenediaminetetraacetate (EDTA), and analysed for cell viability by Trypan blue dye exculsion. After resuspending 3×10^6 (viable and Trypan blue-positive) cells in 1 ml culture medium, an equal volume of NBT solution [0.2% w:v NBT (Sigma), 1.67 μM TPA in PBS] was added. After incubation at 37°C for 1.5–2 h, cells were harvested by centrifugation, the supernatant discharged and the cell pellet washed with PBS. Finally, the cells were lysed in 0.5 ml lysis buffer [1.6% v:v Triton X-100 (Sigma) in PBS] under rapid agitation for 1 h at room temperature. Cellular debris was removed by centrifugation and the supernatant was analysed for absorption at 540 nm using a Perkin-Elmer lambda 2 spectrophotometer. In all experiments, formazan formation was estimated in triplicate (mean ± S.D.) and analysed in parallel by microscopy using a Neubauer chamber. It was corrected for toxic effects to obtain the NBT reduction of 3×10^6 viable cells using the equation: measured $Abs_{540} \times 100/\%$ viable cells. Since the total amount of formazan varies between experiments, NBT reduction is expressed as $\%Abs_{540}$ using the equation: $Abs_{540} \times 100/Abs_{540}$ of untreated cells. For example, after lysis of untreated cells the NBT reduction is quantified by $Abs_{540} = 0.03 \pm 0.001$. After lysis of cells treated with 45 μM MIL, NBT reduction is quantified by $Abs_{540} = 0.224 \pm 0.0065$ (747 ± 22%). After correction for 90.3% viable cells, the calculated NBT reduction is $Abs_{540} = 0.248 \pm 0.0075$ (827 ± 25%) for 3×10^6 viable cells.

Colony formation assay

The *in vitro* assay for colony formation in soft agar of KG1 and KG1a cells was performed according to the method of Hamburger and Salmon [19] with some modifications. Cells from growing cultures were collected by centrifugation, resuspended in IMDM containing 20% v:v fetal calf serum, penicillin/streptomycin, glutamine and solidified with 0.3% w:v agar (Difco) in the presence of different concentrations of IL-3 ($\sim 2–5 \times 10^7$ U/mg, concentration 100 μg/ml) and GM-CSF ($\sim 3 \times 10^7$ U/mg, concentration 1 μg/ml; Genzyme, Cambridge, Massachusetts, U.S.A.). PBS or MIL in different concentrations as indicated were added on top of the agar. For each concentration, five 8.5-cm^2 culture dishes with 5000 cells/dish were cultured at 37°C, 95% humidity and 5% CO_2. After 14 days incubation, colonies of more than 50 cells were counted using a video camera-based colony counter.

Northern blot analysis

For isolation of total RNA, cells were collected by centrifugation and lysed in GTC buffer (4 M guanidinium thiocyanate, 0.5% w:v sodium lauryl sarcosinate, 285 mM β-mercaptoethanol, 25 mM sodium citrate pH 7). After shearing nuclear DNA by Ultra-Turrax homogeniser (IKA T25) treatment, the homogenate was put on top of a CsCl cushion (5.7 M CsCl, 25 mM sodium acetate, 1 mM EDTA pH 8) and centrifuged for ≥12 h at 35 000 rpm in a swinging bucket Beckman SW55 rotor at room temperature. Following this centrifugation step, the supernatant was discharged, the bottom of the tube cut off and the RNA pellet dissolved in diethylpyrocarbonate (DEPC)-

treated water. The RNA was precipitated with ethanol and quantified by measurement of absorption at 258 nm.

The northern blot analysis was performed essentially as described previously [20] with 10 μg denatured total RNA in sample buffer separated on a formaldehyde-containing agarose gel [1% w:v agarose, 40 mM 3-(*N*-morpholino)propanesulfonic acid, 10 mM sodiumacetate, 1 mM EDTA, 0.74% v:v formaldehyde] and transferred to Hybond N^+ membrane (Amersham Buchler, Braunschweig, Germany) by capillary elution. The following human cDNA probes, labelled by random priming using the Megaprime DNA labelling system (Amersham Buchler), were used for hybridisation: IL-3Rα—1.46 kBp XhoI fragment (clone DUK-1 [21]), GM-CSF-Rα—1.25 kBp XhoI fragment (clone KH125 [22]), IL-3/GM-CSF-Rβ—0.28, 0.3 and 1.57 kBp PstI fragments (clone KH97 [23]). G-CSF-R—1.37 and 0.98 kBp *Bam*HI fragments (clone HQ3 [24]), FcRI/CD64—1.5 kBp XbaI fragment (clone p135 [25]), c-*myc*—0.45 kBp PstI fragment [26], NF-κB p50—≈1.9 kBp *Eco*RI/XbaI fragment (clone 9, [27]), histone H2A.1—0.4 kbp XbaI/SacI fragment (Donecke, unpublished; Genbank Acc # M60752), histone H1°—0.6 kBp Hind3/BamHI fragment [28], histone H3.3—0.44 kBp Hind3/BamHI fragment [29], β-actin—1.1 kBp EcoRI fragment (ATCC 65128, clone HHC189), GAPDH—1.3 kBp EcoRI fragment (clone KS321 [30]). The cDNA genes for human histone H2A.1, H3.3 and H1° were isolated by PCR with specific oligonucleotide primer, subcloned and verified by DNA sequence analysis (Beckers, unpublished).

RESULTS

Sensitivity of human myeloid leukaemic cell lines to MIL treatment

The sensitivity of the human myeloid leukaemic cell lines U937, KG1 and KG1a to treatment with MIL was quantified by the MTT assay [16]. As shown in Table 1, the sensitivities of KG1 and KG1a cells were similar with EC_{50} values of 75.8 and 71.0 μM, respectively. In comparison, U937 cells were more sensitive to MIL treatment with an EC_{50} value of 30.1 μM.

NBT reduction, anti-proliferative activity and cytotoxicity of MIL in KG1 and KG1a cells

To evaluate the concentration-dependent cellular effects of MIL on KG1 and KG1a cells, we estimated cell growth/viability and induction of differentiation as quantified by the ability to reduce NBT. The reduction of NBT to formazan is dependent on an intact respiratory burst, a property of mature macrophages and granulocytes [18].

Table 1. Sensitivity of human myeloid leukaemia cell lines to treatment with MIL

Cell line	Leukaemia	EC_{50} (μM)
U937	Human histiocytic lymphoma	30.1 ± 5.0 (4)
KG1	Human acute myelogenous leukaemia (bone marrow)	75.8 ± 16.9 (3)
KG1a	Human acute myelogenous leukaemia (bone marrow)	71.0 ± 22.6 (2)

The effect of MIL on metabolic activity of myeloid cell lines was quantified by the MTT assay. EC_{50} values were calculated by analysis of dose–response curves, representing the mean ± S.D. of multiple experiments (number in parentheses).

As shown in Figure 1, the reduction of NBT and the anti-proliferative effect on KG1 (Figure 1a, b) and KG1a (Figure 1c, d) cells were both dependent on the MIL concentration. Significant effects were detectable at concentrations from 9 to 90 μM. At higher concentrations of MIL or longer incubation time, mainly toxic effects (exemplified by cell lysis and >40% Trypan blue-positive cells) were dominant in this experimental setting (data not shown). Negligible net cell growth was observed with ≥80% viable cells during 2 days of suspension culture in medium with 45–90 μM MIL. The induction of NBT reduction as a marker for mature myeloid cells was correlated with the anti-proliferative effect of MIL. The percentage of adherent KG1 cells, typically <2% in untreated cultures, was not elevated by treatment with MIL (data not shown).

Treatment of KG1 and KG1a cells with 100 nM TPA for 72 h showed that the KG1a cell line was TPA-resistant as previously described [31]. Whereas KG1 had a strongly decreased growth (13.8% proliferation rate), adherence to culture dish (>90% adherent cells, detached only by trypsin/EDTA treatment) and NBT reduction (relative increase 369%), 100 nM TPA had no effect on growth, NBT reduction and adherence of KG1a cells (≈80% weakly adherent cells, detachable by shaking, comparable to untreated cells).

Effects on transcription of receptor genes in myeloid cell lines

The effects of MIL on transcription of CSF- and IgG Fc-receptor genes were studied by northern blot analysis. As shown in Figure 2, MIL raised the transcription of the genes for IL-3 R α-chain in KG1, KG1a, U937 cells; GM-CSF R α-chain in U937 cells; and their common β-chain in KG1, U937 cells. These effects are qualitatively, but not quantitatively comparable to those seen in phorbolester-treated cells. There was no significant effect of TPA or MIL on the transcription level of G-CSF R. In contrast, HL60 cells treated with 10 nM TPA (differentiation to monocyte/macrophage-like cells) or 1.25% DMSO (differentiation to neutrophilic granulocyte-like cells) showed reduced and enhanced G-CSF R transcription, respectively (data not shown). Whereas TPA induced transcription of FcRI/CD64 in KG1 cells, there was no effect of MIL. Despite the insensitivity of KG1a cells to TPA, the IL-3 Rα mRNA was elevated by treatment with TPA. In contrast to the parental cell line KG1, the βc mRNA was undetectable in KG1a cells. As a control, stripped Hybond N^+ blotting membrane was rehybridised with human β-actin probe, showing equal amounts of mRNA in all samples.

Concentration-dependent stimulation of KG1 cell colony formation by MIL

The KG1 cell line depends on CSFs for colony growth in semi-solid medium. As shown in Figure 2, MIL increased the low basal transcription level of receptors for IL-3 (α- and β-subunit) and GM-CSF (β-subunit). Therefore, MIL might have synergistic stimulatory effects on the IL-3/GM-CSF-dependent colony growth of these cells. The results of different experiments are summarised in Figure 3. The colony formation of KG1 cells was dose-dependently increased by IL-3 or GM-CSF as seen in Figure 3a. The cloning efficiency was not enhanced by incubation with both cytokines, even at their optimal concentrations of 0.5 ng/ml GM-CSF and 5 ng/ml IL-3 (data not shown). As shown in Figure 3b, 3.16 μM MIL by itself stimulated the colony growth of KG1 cells without added CSF. This effect was critical with respect to the concentration of MIL, since 10 and 31.6 μM were inhibitory, decreasing the colony number below

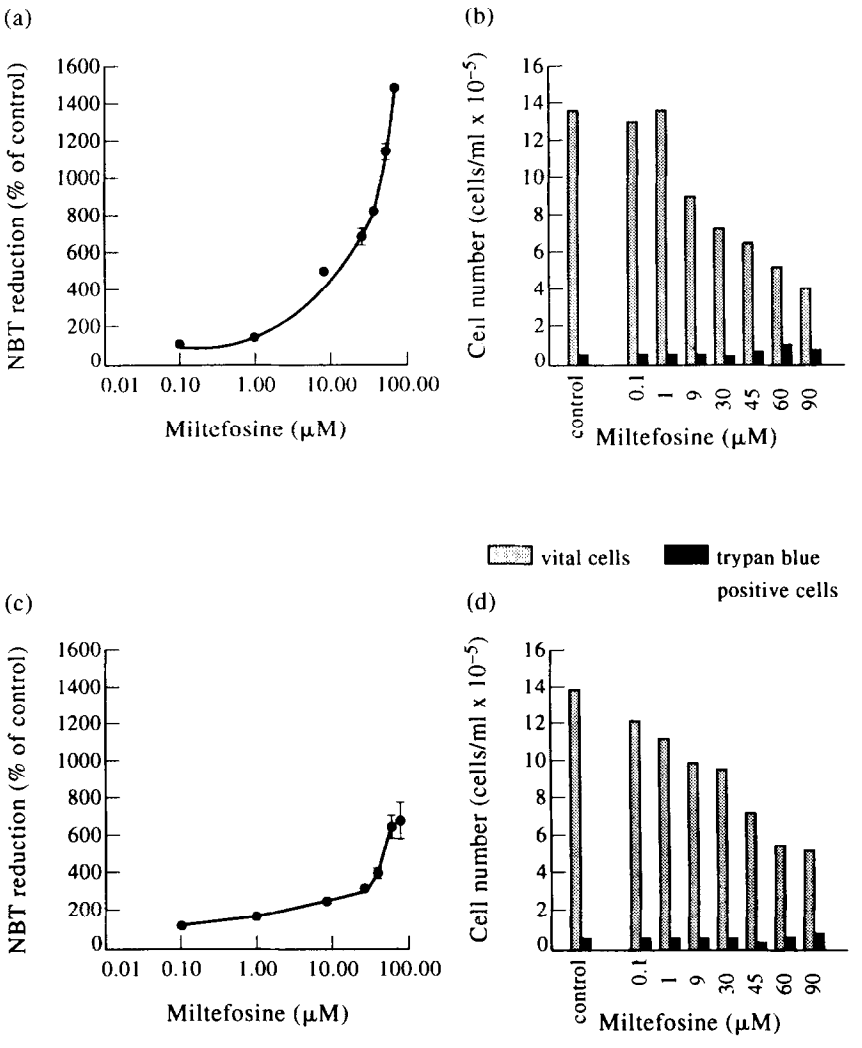

Figure 1. Dose–response relationship for KG1 and KG1a cells treated with MIL. KG1 (a, b) and KG1a (c, d) cells (5×10^5 cells/ml, time 0 h) were incubated with different MIL concentrations in suspension culture for 48 h before analysis. The NBT reduction was determined for 3×10^6 cells (total cell number) in triplicate (mean ± S.D.) for each MIL concentration (a–c). It was corrected for toxic effects of MIL to obtain the NBT reduction of 3×10^6 viable cells. The NBT reduction is shown as % Abs_{540} in relation to Abs_{540} of untreated cells (a, c). Trypan blue-positive and viable cell numbers were determined using a Neubauer chamber (b, d). The experiment was performed in duplicate with similar results.

control values. The stimulatory effect became more pronounced with the addition of IL-3 or GM-CSF at optimal concentrations (Figure 3c). In comparison to cultures treated with 0.5 ng/ml GM-CSF, the cloning efficiency was doubled to 15% in cultures with 0.5 ng/ml GM-CSF and 3.16 μM MIL. Again, concentrations >10 μM MIL were inhibitory to colony growth of KG1 cells. The growth of KG1a cells in semi-solid medium was negligible and there was no effect of IL-3 or GM-CSF. MIL had only a weak stimulatory activity on KG1a colony growth compared to the experiments described above (data not shown).

Effects on transcription of nuclear proteins in myeloid cell lines

For further analysis of proliferation- and differentiation-associated effects on gene transcription induced by MIL, we analysed the expression of nuclear proteins, namely histone subtypes H1°, H2A.1, H3.3, the proto-oncogene c-*myc* and NF-κB p50, a c-rel proto-oncogene homologue.

As shown in Figure 4, MIL had distinct effects on the transcription of H2A.1, c-*myc* and NF-κB p50 in U937 cells. The reduction of H2A.1 mRNA reflects the growth arrest of MIL-treated U937 cells. The transcriptional induction of NF-κB p50 and suppression of c-*myc* by 25 μM MIL was correlated with U937 differentiation along the monocyte/macrophage lineage. We found no effect on histone subtype H1° in this cell line, neither by 10 nM TPA nor 25 μM MIL.

For KG1 cells, treatment with 10 nM/100 nM TPA induced the transcription of histone H1°, whereas 45 μM MIL had no effect on the low basal transcription level of this differentiation-associated histone subtype. There was no H1° mRNA detectable in KG1a cells under these experimental conditions. The transcription of H2A.1 was proliferation-independent in KG1 and KG1a cells, since treatment with 45 μM MIL resulted in growth arrest (see Figure 1b, d). The high basal transcription of histone subtype H3.3 was not influenced in KG1, KG1a and U937 cell lines. As a control, stripped Hybond N^+ blotting membrane was rehybridised with human GAPDH probe showing equal amounts of mRNA in all samples.

DISCUSSION

The molecular mechanism(s) responsible for the growth-inhibitory and differentiation-inducing effects of alkylphospho-

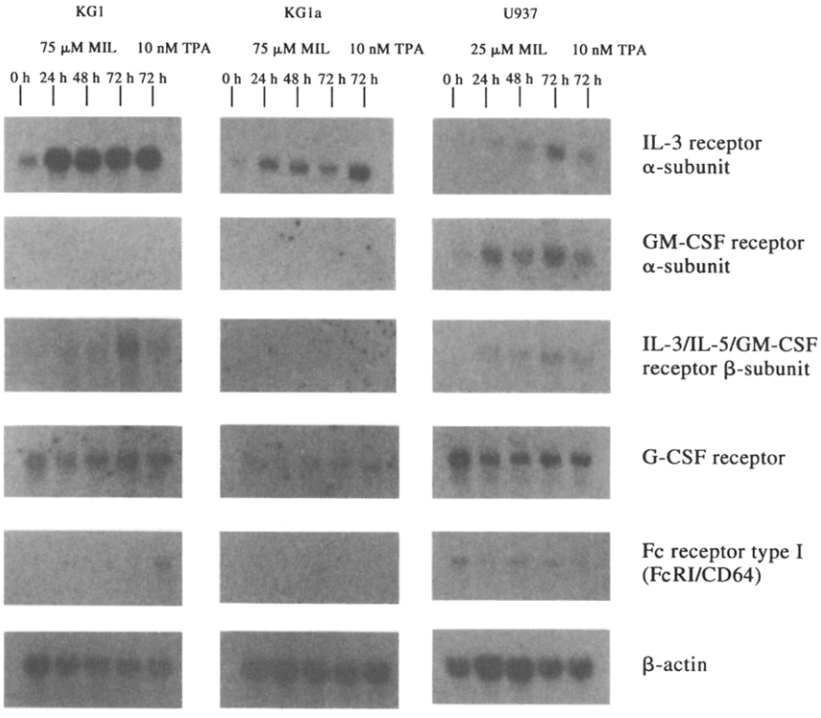

Figure 2. Analysis of receptor gene transcription in myeloid cell lines. Myeloid cell lines were incubated in suspension culture with 75 μM MIL (KG1, KG1a), 25 μM MIL (U937) or 10 nM TPA (KG1, KG1a, U937) for 0, 24, 48 and 72 h before isolation of total RNA. Transcription of IL-3Rα, GM-CSF-Rα, IL-3/IL-5/GM-CSF-Rβ, G-CSF-R, FcRI and β-actin (control) was analysed by northern blotting with the corresponding cDNA gene probes. In general, cell viability was ≥80% after 72 h incubation with MIL or TPA.

lipids is not fully understood. Until now protein kinase C (PKC), CTP:phosphocholine cytidylyltransferase, phospholipase C (phosphoinositidase C) and Na,K-ATPase have been described as putative targets [32–35]. So far, there is no clear evidence that targeting these membrane associated enzymes causes the anti-tumour or haematopoietic actions of MIL *in vitro* and *in vivo*.

Several groups have reported that differentiation parameters of human myeloid leukaemic cell lines are influenced by treatment with MIL [7–9, 34] or other phospholipid derivatives [36, 37]. There are several studies describing the action of MIL on haemopoiesis. A synergistic effect of low MIL concentrations has been described on the G-CSF- and GM-CSF-induced colony growth *in vitro* of human haematopoietic progenitor cells [12]. In rats treated with high doses of MIL, the white blood cell count has been shown to be increased, mainly due to an increase in mature granulocytes [4]. Leucocyte and platelet counts were elevated by systemic treatment with MIL in clinical studies [11]. In addition, there is evidence for growth-enhancing synergistic effects of MIL with IL-3 and GM-CSF *in vivo* and *in vitro* on murine clonogenic haematopoietic progenitor cells [13].

It may be speculated that cytotoxicity and effects on haematopoiesis/differentiation are two separate activities of alkylphospholipids. Therefore, we studied the effects of MIL on human myeloid leukaemic cell lines KG1, KG1a and U937. These cell lines are well established as differentiation models [15], and there are reports describing the effects of phospholipid derivatives [7, 8, 36, 37]. The dose–response relationship showed that the anti-proliferative and differentiation-inducing activity (quantified by respiratory burst activity) of MIL was strictly dose-dependent in KG1 and KG1a cells. The anti-proliferative effect of MIL correlated with cellular respiratory burst activity as quantified by NBT reduction. The KG1a cell line is described as a phorbolester resistant subline of KG1, arrested at a very young myeloblast stage [31, 38]. The molecular basis for this phorbolester resistance is unknown, since KG1 and KG1a have a similar density of high-affinity [^{3}H]phorbol-12,13-dibutyrate binding sites [39]. In our experiments, there were only marginal differences between MIL-treated KG1 and KG1a cells in terms of the NBT reduction and proliferation, indicating that MIL might overcome the differentiation block in the KG1a cell line. One might speculate that there is a target(s) other than PKC.

CSFs are not only proliferative stimuli in granulocyte–macrophage progenitor cells, they also control differentiation commitment, initiation of maturation and functional activity of the mature cells finally produced [40]. Similarly they influence growth and differentiation of leukaemic myeloid cell lines [41, 42]. The cDNA of receptors for human GM-CSF, IL-3 and G-CSF have been cloned [21–24]. The functional receptors for IL-3 and GM-CSF are heterodimeric with different α-subunits, but a common β-subunit (βc [21]). One hypothesis is that the haematopoietic/differentiation-inducing effects of MIL might be explained by induction of CSF secretion [14] and/or effect on receptor expression. As shown by northern blot analysis, MIL increased the low basal transcription of IL-3 Rα-subunit in KG1, KG1a and U937 cells, GM-CSF Rα-subunit in U937 cells and βc-subunit in KG1 and U937 cells. There was no significant effect on G-CSF R transcription in any of the cell lines. The effects were qualitatively comparable to that of cells treated with 10 nM TPA, so it seems unlikely that there was a MIL-specific mode of action. In those cases where no signal was detectable, it cannot be excluded that very low transcriptional levels remained undetected. It is known that differentiation-inducing compounds increase CSF receptor expression in leukaemic cells [22, 43]. Therefore, the increased CSF receptor gene transcription has to be interpreted as a parameter of myeloid

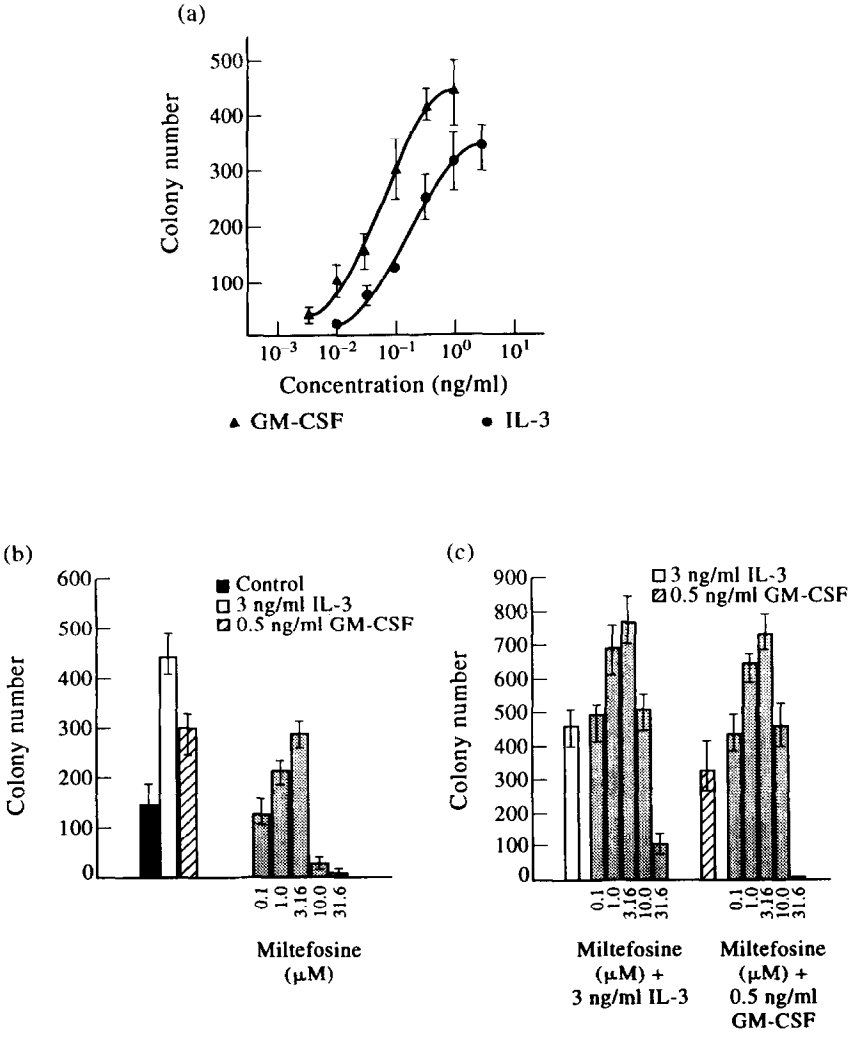

Figure 3. Effect of MIL on colony formation of KG1 cells. KG1 cells (5×10^3 cells/dish) were cultured in semi-solid medium for 14 days and analysed for colony formation. The colony formation was largely dependent on human recombinant IL-3 or GM-CSF. The dose–response relationship is shown in (a). The influence of different MIL concentrations on colony formation with and without 3 ng/ml IL-3 or 0.5 ng/ml GM-CSF is shown in (c) and (b), respectively. The colony number was calculated as mean ± S.D. (n = 5). Experiments were performed in duplicate with similar results.

cell differentiation. A protein kinase C activator (phorbolester TPA) and a putative PKC inhibitor (MIL) have comparable influence on CSF-R gene transcription, although there are subtle quantitative differences. Receptor binding assays may be able to show whether the increased transcription is reflected by higher expression of functional, high-affinity receptors on the cell surface. The transcription of human high-affinity receptor for IgG-Fc (FcRI/CD64) was analysed, since mature monocytes/macrophages are phagocytically active cells with Fc-receptor expression [31]. There was no significant effect of MIL on the transcription of FcRI, despite an inducing effect of TPA in KG1 cells.

As mentioned before, the colony growth of KG1 is dependent on CSFs [38]. We tested IL-3 and GM-CSF and found a dose-dependent stimulation of KG1 colony growth, but no effect on KG1a cell growth in soft agar. A small amount of KG1 cells (≤2%) formed colonies without added CSF, which might be caused by growth in medium containing 20% fetal calf serum, stimulating a subpopulation to grow in soft agar. MIL has a dose-dependent stimulatory activity on KG1 colony growth, which is highest in cultures with optimal IL-3 or GM-CSF concentrations. It is possible that MIL acts as a "CSF-like activity" and/or it enhances the sensitivity of immature myeloid cells to IL-3/GM-CSF by increasing the expression of functional IL-3/GM-CSF receptors. Further studies have to be performed to elucidate this stimulatory effect.

Many different genes are involved in the regulation of cellular proliferation and differentiation or its dysregulation in tumorigenesis. We analysed the transcription of proto-oncogenes c-*myc* and NF-κB p50 (c-rel homologue) and histone subtypes H1°, H3.3 and H2A.1. Histone subtypes H1° and H3.3 are histone proteins with enhanced expression in differentiated cells [44, 45], whereas core histone H2A.1 is replication-associated, only expressed in dividing, proliferating cells [46]. The histone subtype H1° was induced by TPA in KG1 cells, whereas MIL had no effect. This shows that MIL had distinct transcriptional effects, different from TPA in this cellular system. In contrast to Hochhuth and associates [7], we found no change in the H1° mRNA level in U937 cells, neither by TPA or MIL treatment. The effects on H2A.1 and c-*myc* transcription are, however, comparable to their data [7, 8]. Recently, it was reported that histone subtype H3.3 expression is induced in differentiating

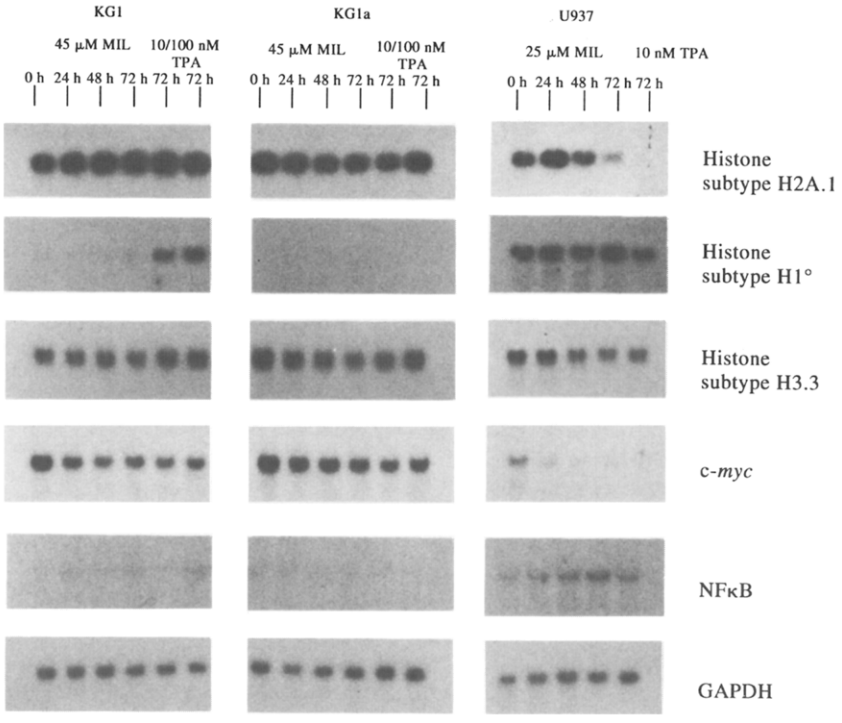

Figure 4. Analysis of gene transcription of differentiation-associated nuclear proteins in myeloid cell lines. Myeloid cell lines were incubated in suspension culture with 45 μM MIL (KG1, KG1a), 25 μM MIL (U937), 10/100 nM TPA (KG1, KG1a) or 10 nM U937 for 0, 24, 48 and 72 h before isolation of total RNA. Transcription of histone subtypes H2A.1, H1γ and H3.3, c-*myc*, NF-κB p50 and GAPDH (control) was analysed by northern blotting with the corresponding cDNA gene probes. In general, cell viability was ≥80% after 72 h incubation with MIL or TPA.

cells [45]. We found a high basal transcription of histone H3.3 in human leukaemic cell lines, which is not changed by TPA or MIL. Therefore, leukaemic myeloid cells must represent a certain state of differentiation with high histone H3.3 level or the expression is coupled to differentiation only in certain cellular systems. The same is true for the replication-associated histone H2A.1 [46] which is highly expressed in growth arrested KG1 or KG1a cells.

The transcription of c-*myc* and NF-κB p50 in U937 cells is suppressed and induced, respectively. There is no qualitative difference between TPA and MIL. Therefore, the transcriptional changes might be signs for a differentiation stimulus to U937 cells. Differentiating cells reduce c-*myc* transcription [47], whereas NF-κB p50 is induced [27]. Both proteins are very important in the control of proliferation and differentiation. The c-*myc* proto-oncogene codes for an evolutionary conserved nuclear phosphoprotein, ubiquitously expressed in somatic cells, that complexes with the max-protein and functions as a transcription factor [47]. Nuclear factor κB (NF-κB), as a pleiotropic transcription factor, is involved in the expression of various viral and cellular genes (for review see [48]). It is regulated at the protein level by complexing with its inhibitor iκB and, in addition, on a transcriptional level [27]. The translocation of active NF-κB p50/p65 complex to the nucleus and the induction of NF-κB p50 mRNA is induced by many stimuli, e.g. phorbolester, TNFα, IL-1, lectins and lipopolysaccharide [48]. In this regard, MIL is another stimulus for NF-κB p50 transcription, like TPA or TNFα.

The molecular and cellular effects of MIL induced in human leukaemic myeloid cell lines as presented in this paper can be summarised as follows: (i) MIL has dose-dependent differentiation-inducing, growth promoting and cytotoxic activities; (ii) MIL has stimulatory activity on IL-3 R and GM-CSF R gene transcription, (iii) c-*myc* and NF-κB p50 transcription is affected comparably to phorbolester TPA, acting as a differentiation stimulus; (iv) beside its putative inhibition of PKC, MIL has (an)other, so far unknown molecular target(s) in myeloid cells; (v) the interaction of MIL with its molecular target(s) gives a pattern of molecular and cellular effects that are associated with differentiation but clearly different from inducers such as phorbolester or growth factors.

Future studies using transfected model cell lines with gene-regulatory elements fused to reporter genes for screening of miltefosine derivatives are in preparation, and will help to elucidate the mode of action and structure–function relationships of alkylphospholipids.

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