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Anticancer Drug-mediated Induction of Multidrug Resistance-associated Genes and Protein Kinase C Isozymes in the T-Lymphoblastoid Cell Line CCRF-CEM and in Blasts from Patients with Acute Lymphoblastic Leukemias

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The major determinants mediating drug resistance in acute lymphoblastic leukemias (ALL) unresponsive to chemotherapy, are still unclear. For example, it is still unknown whether selection or induction processes are responsible for drug resistance here or whether protein kinase C (PKC) isozymes contribute to the resistant phenotype. Therefore, inducibility of resistance factors or PKC isozymes genes was examined in CCRF-CEM cells treated with diverse anticancer drugs—adriamycin, camptothecin, etoposide or vincristine—at sublethal concentrations for 24 h. *MDR1*, *MRP1*, *LRP* and *PKC* isozyme α, β_1, β_2, ε, ι, η, θ, ζ gene expression was determined by cDNA-PCR. We found significant dose-dependent, mostly combined, induction of the *MDR1*, *MRP1* and *LRP* genes. Significantly enhanced gene expression of the majority of *PKC* isozyme genes was found after treatment with camptothecin. PKCζ was upregulated throughout by each anticancer drug applied in this setting. A series of selected CCRF-CEM-derived multidrug resistance (MDR) sublines also showed enhanced expression of the PKC isozymes compared to the parental cell line. *MDR1* and *PKC*η gene expression levels were correlated highly significantly. Blasts from two patients with ALL during the first week of monotherapy with steroids revealed combined induction of the MDR1, multidrug resistance-associated protein 1 (MRP1), lung cancer resistance-related protein (LRP) and most PKC isozymes, predominantly PKCζ. Another patient with T-ALL, who failed to respond to four months of intensive chemotherapy, showed an enhanced *MRP1* gene expression combined with markedly overexpression of PKCη and PKCθ. Furthermore, the camptothecin and etoposide-mediated induction of resistance factors in the CCRF-CEM cell line could be suppressed by staurosporine, a rather unspecific inhibitor of protein kinases. However, selective inhibitors of PKC isozymes (bisindolylmaleimide GÖ 6850, indolocarbazole GÖ 6976) produced no significant effects here. Therefore, the PKC isozymes η, θ and ζ are of interest as potential targets to overcome drug resistance in ALL.

Key words: Acute lymphoblastic leukemia — Anticancer drugs — Gene induction — MDR — Protein kinase C

The mechanisms of failure of anticancer chemotherapy are still poorly understood. Several factors functionally involved in the multidrug resistance (MDR) phenotype of cell lines selected *in vitro* have been identified, such as the MDR1/P-glycoprotein (MDR1),[1)] the multidrug resistance associated protein (MRP1)[2)] or the lung cancer resistance-related protein (LRP).[3)] MDR cell lines selected *in vitro* over long periods of time mostly show stably a high expression of one of the MDR factors, often due to gene amplification. In the clinic, enhanced expression of these factors appears to be caused by upregulation of mRNA steady-state levels rather independently of gene copy numbers.[4–8, 9)] However, a series of recent reports point to a quick response in expression at least of *P-gp* genes after treatment of rodent or human cells with various xenobiotics, such as carcinogens, cocarcinogens,[7, 10, 11)] antineoplastic drugs,[12–16)] differentiating agents,[17, 18)] protein kinase C (PKC) activators,[19)] or other stresses, e.g., heat shock.[20, 21)] The phenomenon that anticancer drugs transported by the human P-glycoprotein (P-gp) induce the expression of this drug transporter gene at drug concentrations not affecting cell vitality might be especially relevant to the clinical situation. Some influence of PKC on P-gp associated MDR, either via phosphorylation of P-gp or/and via modulation of *MDR1* gene expression was indicated by the work of several groups, and inhibition of PKC has been discussed as a new approach for overcoming MDR in cancer

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chemotherapy.[14, 22–24] The bisindolylmaleimide GF 109203X (identical with GÖ 6850)[25] shows highly selective inhibition of many PKC isozymes *in vitro* (IC_{50}: 30–200 n*M*), except for the atypical isoform PKCζ (IC_{50}: 6 μ*M*), while other kinases are affected only at distinctly higher concentrations. The indolocarbazole compound GÖ 6976 is an even more selective PKC inhibitor, since only the Ca^{2+}-dependent PKC isozymes are blocked,[26] together with PKCη (IC_{50}=0.6 μ*M*). As PKC isoforms are suggested to represent a part of a stress response activating for example the *MDR1* gene, PKC inhibitors might be able to attenuate the activation of drug resistance-associated genes.[27] Following recent results that MDR in the clinic might be caused by multiple factors and that distinct PKC isozymes are associated with various resistance factors[7, 9] we investigated several selected MDR cell lines for gene expression of resistance factors (MDR1, MRP1 and LRP) and PKC isozymes. Furthermore, we tested several anticancer agents for the ability to induce gene expression of these resistance factors and PKC isoforms, and examined whether PKC inhibitors attenuate gene induction. In addition, we investigated the expression pattern of these genes in blasts from acute lymphoblastic leukemia (ALL) patients during first-line treatment with steroids, and in one case nonresponding to intensive chemotherapy. The results indicate that particular PKC isoforms (PKCη, θ, ζ) may at least partially abrogate the emergence of multiple drug resistance.

MATERIALS AND METHODS

Leukemic cells and cell lines The parental human T-lymphoblastoid cell line CCRF-CEM was obtained from the American Type Culture Collection, Rockville, MD (ATCC CCL 119). The cell lines CCRF VCR100, CCRF VCR1000, CCRF ACTD400 and CCRF ADR5000 are corresponding resistant cell lines, selected and permanently cultured in the presence of 100 or 1000 ng/ml of vincristine (VCR), 400 ng/ml of actinomycin D (ACTD) or 5000 ng/ml of adriamycin (ADR).[28] The cell lines were routinely checked for the absence of mycoplasma infection with a mycoplasma detection kit (Boehringer, Mannheim, Germany).

Blasts from patients (1 common ALL, 1 T-ALL) with remarkable, initial high blast count numbers (300 000–500 000/μl) were obtained daily from peripheral blood aspirates during first-week induction therapy with steroids, which is given as an initial monotherapy in this time interval according to the medium risk regimen of the BFM-ALL-95 treatment protocol.[29] Both patients responded well to steroids and to further combinatorial chemotherapy and have remained in continuous complete remission for more than three years. Furthermore, we investigated blasts from a T-ALL patient nonresponding to three months of intensive chemotherapy according to the high risk regimen of the BFM-ALL-95 treatment protocol.[29] This patient received combinatorial chemotherapy including ADR, asparaginase, cyclophosphamide, cytosine-arabinoside, dexamethasone, etoposide, methotrexate, prednisolone, 6-mercaptopurine, VCR and vindesine before blasts were newly drawn from bone marrow aspirates in a final stage of progression. This patient died from progressive disease four months after initial diagnosis.

Mononuclear cells were isolated by the standard Ficoll-Hypaque technique (Lymphoprep, Nycomed, Oslo/Norway), washed twice with phosphate-buffered saline (PBS), immediately lysed with guanidinium isothiocyanate or frozen in the presence of 7% dimethylsulfoxide (DMSO) and 50% fetal calf serum (FCS) under controlled conditions, and stored in liquid nitrogen. An aliquot of mononuclear cells isolated was tested for its content of blasts. Probes with less than 95% of blasts were excluded from further analysis. Cell count and determination of vital cells were performed using a Neubauer hemacytometer chamber and staining with trypan blue.

Drugs GF 109203X (GÖ 6850), GÖ 6976 and staurosporine (STAU)[30, 31] were purchased from Calbiochem (Bad Soden, Germany). Camptothecin (CAM) and ACTD were obtained from Sigma (Deisenhofen, Germany). The other drugs were taken as ready-prepared solutions for clinical use: ADR ("Adriblastin," Pharmacia & Upjohn, Erlangen, Germany), ETO (etoposide, "Vepesid," Bristol-Myers Squibbs, Troisdorf, Germany) and VCR ("Vincristin Liquid," Lilly, Bad Homburg, Germany). All other chemicals, supplies and tissue culture media were of the purest grade available, purchased from commercial sources.

Cell cycle analysis At the end of induction experiments 100 000 cells were fixed in 500 μl of 70% ethanol at 4°C overnight and than stained in 500 μl of a solution containing 5 mg/ml propidium iodide and 10 mg/ml RNAse. Cells (20 000) from each experiment were measured in a flow cytometer (FACS-Calibur, Becton Dickinson, Heidelberg, Germany). Histograms were analyzed with Cell Quest software (Becton Dickinson). Experiments were carried out in triplicate. The distribution of cell cycle phases after cytostatic drug treatment is shown in Fig. 5.

Induction experiments To analyze the induction of the genes of interest we treated CCRF-CEM cells in strictly the same manner. Cells were seeded into 6-well plates at a cell density of 1×10^6/ml for 24 h with or without drugs as indicated. An 8 h treatment showed variable results (data not shown). We used the following drug concentrations: 10, 30 and 100 ng/ml ADR; 3, 10 and 30 ng/ml CAM; 300, 1000 and 3000 ng/ml ETO and 3, 10 and 30 ng/ml VCR. Cells showed a viability greater than 90% after 24 h incubation with these compounds. This seems to be an important prerequisite to avoid measuring short-term selection effects instead of induced gene expression. After

the times indicated, cells were washed twice with PBS and immediately lysed with a 4 *M* solution of guanidinium isothiocyanate according to Chirgwin *et al*.[32]

Treatment with protein kinase inhibitors To test the potential of various protein kinase inhibitors to suppress gene induction of the resistance factors we preincubated cells for 4 h with 30 n*M* STAU, 1 μM GÖ 6976 or 5 μM GÖ 6850 before adding the cytostatic agents for an additional 24 h. In these experiments cDNA-PCR was performed exclusively for *MDR1*, *MRP1* and *LRP* gene expression (Fig. 8). The viability of cells after coincubation was in the range from 60 to 90%.

cDNA-PCR gene expression analysis The preparation of total cellular RNA, the synthesis of cDNA using random hexanucleotide primers and RAV2 reverse transcriptase, and the cDNA-PCR using MDR1, MRP1, LRP, PKCα, β_1, β_2, ε, ι, η, θ, ζ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific amplimers were performed as recently described.[6, 9] The conditions for PCR though, were modified. We used "Ampli Taq Gold" DNA polymerase (Perkin Elmer, Applied Biosystems, Weiterstadt, Germany), and took 45 μl of each PCR product for polyacrylamide gel electrophoresis (Fig. 1). PCR was carried out in the exponential range throughout. Therefore we used the following cycle numbers: GAPDH, 18; MDR1, 27; MRP1, 24; LRP, 26; PKCα, 27; PKCβ_1, 29; PKCβ_2, 24; PKCε, 27; PKCι, 24; PKCη, 24; PKCθ, 24; PKCζ, 29. PCR reaction mixtures of the reference gene *GAPDH* and the genes of interest were combined and precipitated with ethanol and 1/10 volume of 3 *M* sodium acetate, pH 5.2. The precipitate was dissolved in loading buffer at 60°C/10 min. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. Signals of interest were directly digitalized using the CS1-video imager (Cybertech, Berlin, Germany) and densitometrically analyzed by the "WINCAM" software (Cybertech). The specific signals were normalized to the internal standard GAPDH. In numerous experiments we tested γ-actin and β_2-microglobulin as further controls to rule out the possibility that alterations of gene expression of the internal standard by cytostatic agents might affect the relative gene expression. However, results from experiments using γ-actin and β_2-microglobulin corresponded favorably with those for GAPDH (data not shown). Furthermore, experiments with cell lines were carried out in triplicate throughout. We isolated RNA of cells from each experiment and determined gene expression levels in triplicate for each single RNA. Mean values±SD were there-

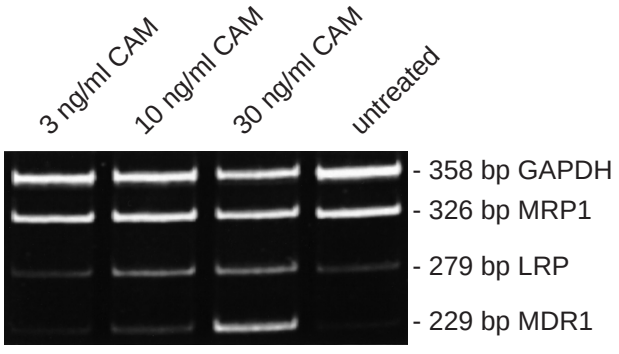

Fig. 1. Separation of cDNA-PCR products (MDR1, MRP1, LRP) by polyacrylamide gel electrophoresis. Cellular RNA was isolated from the T-lymphoblastoid cell line CCRF-CEM after a 24 h treatment with different concentrations of camptothecin (CAM) as indicated.

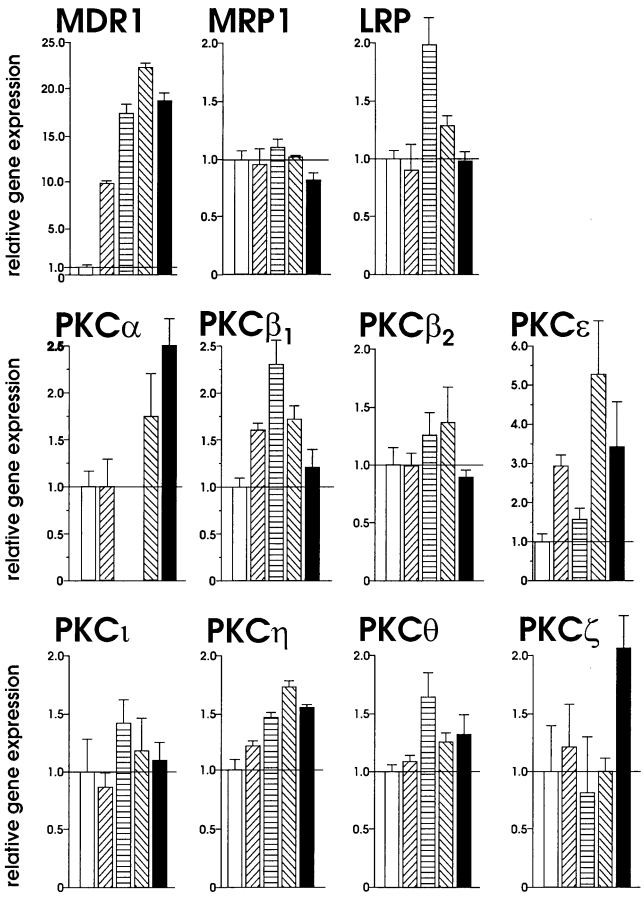

Fig. 2. *MDR1, MRP1, LRP, PKCα, β_1, β_2, ε, ι, η, θ, ζ* gene expression in the T-lymphoblastoid cell line CCRF-CEM and corresponding resistant cell lines CCRF VCR100, CCRF VCR1000, CCRF ACTD400 and CCRF ADR5000 selected and permanently cultured in the presence of 100 or 1000 ng/ml of vincristine (VCR), 400 ng/ml of actinomycin D (ACTD) or 5000 ng/ml of adriamycin (ADR). □ CCRF-CEM, ▨ CCRF VCR100, ▤ CCRF VCR1000, ▧ CCRF ACTD400, ■ CCRF ADR5000.

fore calculated from an overall of 9 PCR reactions. Finally, values were referred to the untreated control which was arbitrarily set at 1.0.

Statistics We performed variance analyses using JMP statistic software (SAS, Heidelberg, Germany) to determine if a factor (cytostatic agent or PKC inhibitor) exhibited a significant ($P<0.05$) effect on variables (expression of gene of interest).

RESULTS

Expression analysis of multidrug resistance associated genes (*MDR1*, *MRP1*, *LRP*) and *PKC* isozymes (α, β_1, β_2, ε, ζ, η, θ, ι) in a series of VCR, ACTD or ADR-selected CCRF-CEM T-lymphoblastoid cell lines compared to the parental cell line revealed enhanced expression of the *MDR1* and the majority of *PKC* isozyme genes (Fig. 2). A highly significant correlation between level of drug resistance, *MDR1* gene expression and *PKC*η was seen. For PKCη, in the meantime, quantitative TaqMan PCR was established. The data correspond well with those from our semiquantitative PCR approach (Gekeler *et al.*, data not shown). Interestingly, PKCα was not detectable in the CCRF subline VCR1000 (Fig. 2) but is overexpressed in CCRF ADR5000, which corresponds well with PKCα protein levels previously determined by western blot analysis.[33)]

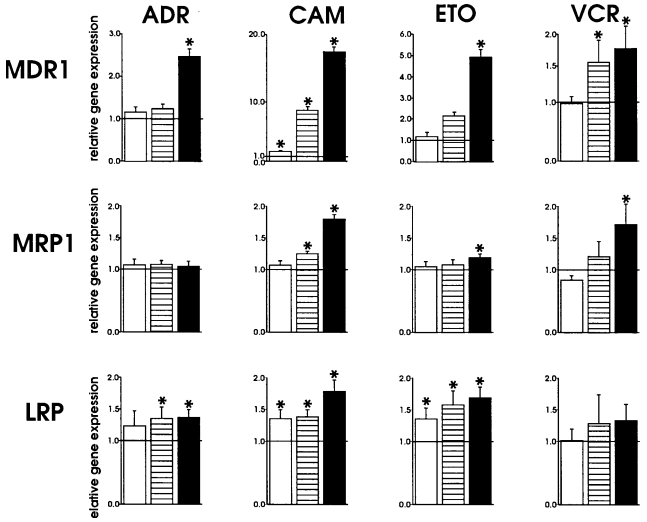

Fig. 3. *MDR1*, *MRP1* and *LRP* gene expression in the cell line CCRF-CEM after 24 h incubation with adriamycin (ADR, □ 10, ▤ 30, ■ 100 ng/ml), camptothecin (CAM, □ 3, ▤ 10, ■ 30 ng/ml), etoposide (ETO, □ 300, ▤ 1000, ■ 3000 ng/ml) or vincristine (VCR, □ 3, ▤ 10, ■ 30 ng/ml). Signal intensities of the untreated control were arbitrarily set at 1.0. Asterisks represent significant effects ($P<0.05$) gained from three experiments.

A 24 h incubation of CCRF-CEM with ADR, CAM, ETO or VCR revealed significant dose-dependent and mostly combined induction of the *MDR1*, *MRP1* and *LRP* genes (Fig. 3). No significant effects were seen for MRP1 after treatment with ADR or LRP after treatment with VCR (Fig. 3).

The majority of *PKC* isozyme genes were found to be highly induced when CAM was used, while only PKCβ_1, but interestingly not PKCβ_2, was downregulated. PKCζ was generally upregulated by each anticancer drug applied in this setting (Fig. 4). Cell cycle analysis revealed an

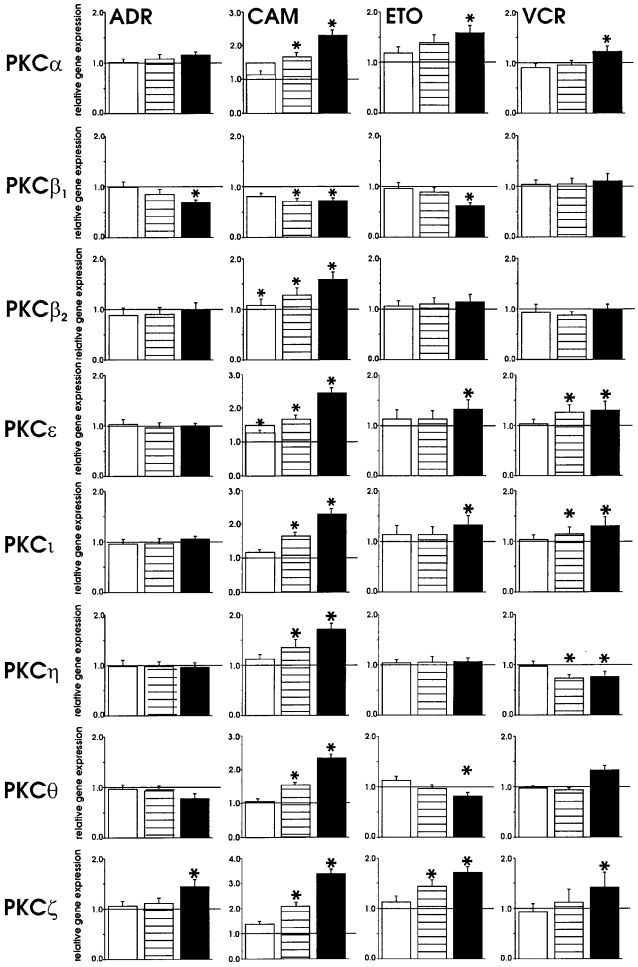

Fig. 4. *PKC*α, β_1, β_2, ε, ι, η, θ, ζ gene expression in the cell line CCRF-CEM after 24 h incubation with adriamycin (ADR, □ 10, ▤ 30, ■ 100 ng/ml), camptothecin (CAM, □ 3, ▤ 10, ■ 30 ng/ml), etoposide (ETO, □ 300, ▤ 1000, ■ 3000 ng/ml) or vincristine (VCR, □ 3, ▤ 10, ■ 30 ng/ml). Signal intensities of the untreated control were arbitrarily set at 1.0. Asterisks represent significant effects ($P<0.05$) gained from three experiments.

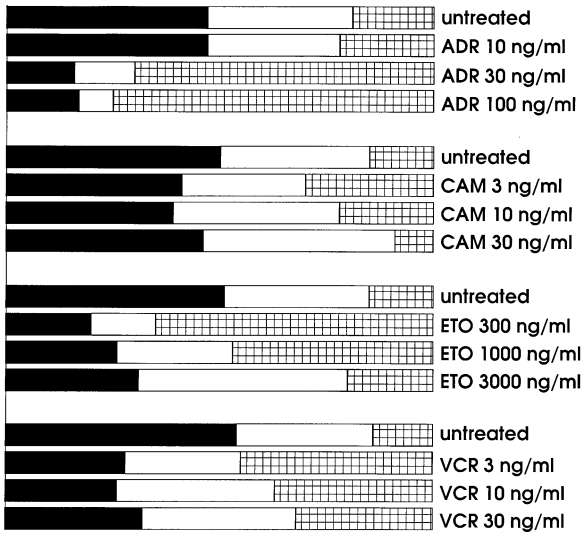

Fig. 5. Cell cycle distribution in the cell line CCRF-CEM after 24 h incubation with ADR, CAM, ETO or VCR, respectively, using concentrations as indicated. ■ G0/G1, □ S, ▦ G2/M.

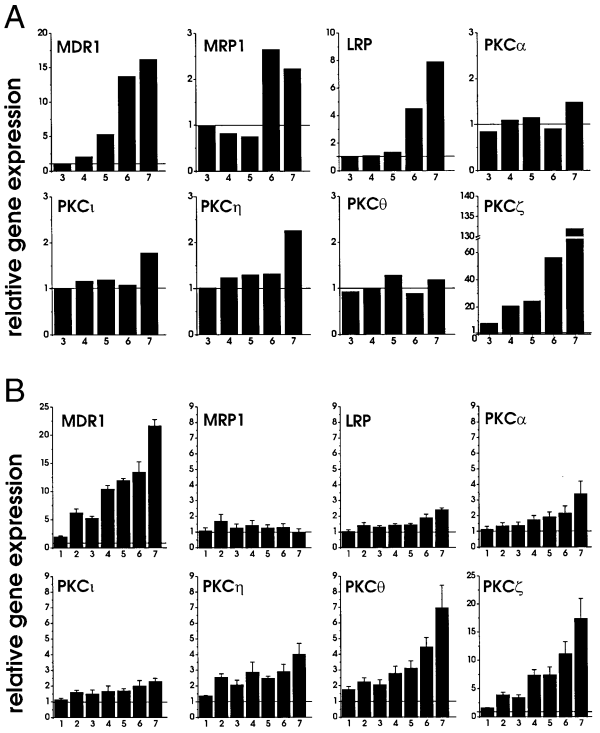

Fig. 6. *MDR1*, *MRP1*, *LRP*, *PKCα, ι, η, θ, ζ* gene expression in blasts from two ALL patients (A, T-ALL; B, common ALL) during the course of initial induction therapy with prednisolone. Signal intensities of primary untreated cells at diagnosis were arbitrarily set at 1.0.

enhancement of cells in S and/or G2/M. However, a correlation of distinct cell cycle distribution and gene expression pattern was only seen when CAM or ETO was used. Exposure to these cytostatics in a range of sublethal concentrations caused a shift to reduced number of cells in the G2/M-phase and enhanced number of cells in the S-phase of the cell cycle after 24 h. This was found to be correlated with marked induction of resistance factors and PKC isozymes (Fig. 5).

Furthermore, blasts from two ALL patients during first-line therapy with steroids revealed a combined induction of resistance factors, predominantly MDR1, and various PKC isozymes, preferentially PKCζ (Fig. 6).

Blasts of a patient with T-ALL, non responding to four months of intensive chemotherapy, drawn in a final stage of the disease, showed an enhanced *MRP1* but not *MDR1* or *LRP* gene expression combined with marked overexpression of the PKCη and θ isozymes (Fig. 7).

The induction of the resistance factor genes (*MDR1*, *MRP1* and *LRP*) in the CCRF-CEM cell line could significantly be suppressed by STAU, a rather unspecific inhibitor of protein kinases inhibiting most of the PKC isozymes. Specific inhibitors of mainly calcium-dependent PKCα, $\beta_{1,2}$, η isozymes (indolocarbazole GÖ 6976) and also of PKCε, δ, η or θ (bisindolylmaleimide GÖ 6850) produced no significant effects here (Fig. 8).

DISCUSSION

Gene expression analysis at the mRNA level by cDNA-PCR reveals the relative activity of genes, not of gene products. Thus, our work only gives circumstantial evidence for functional overexpression of drug transporters or

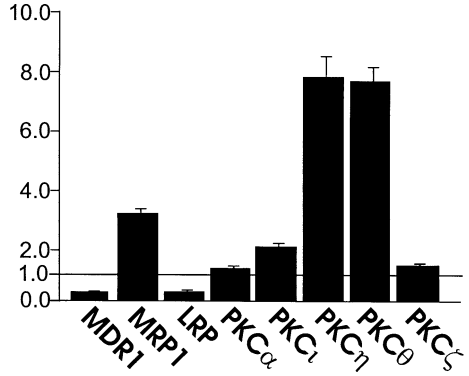

Fig. 7. *MDR1*, *MRP1*, *LRP*, *PKCα, ι, η, θ, ζ* gene expression in blasts from a patient with a T-ALL, non responding to intensive treatment with different antineoplastic agents. Signal intensities of primary untreated cells at diagnosis were arbitrarily set at 1.0.

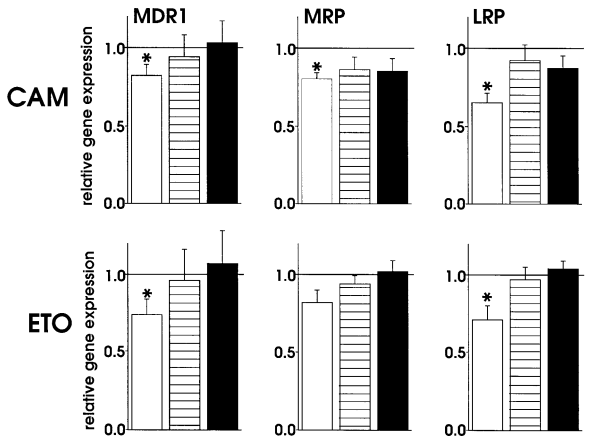

Fig. 8. *MDR1*, *MRP1* and *LRP* gene expression in the T-lymphoblastoid cell line CCRF-CEM after 24 h with 30 ng/ml camptothecin (CAM) or 3000 ng/ml etoposide (ETO) supplemented after a 4 h preincubation with □ 30 n*M* STAU, ▤ 1 μM GÖ 6976 or ■ 5 μM GÖ 6850, respectively. Signal intensities of the untreated control were arbitrarily set at 1.0. Asterisks represent significant effects gained from three experiments.

PKC isozymes. Nonetheless, a recent report states that PKC expression is mainly regulated at the transcriptional level in hematopoietic cells.[34] We ourselves found a very good correlation between PKCα mRNA levels monitored by cDNA-PCR and the PKCα protein content estimated by western immunoblotting in preparations of a series of cell lines of different origin,[33] pointing to the value of cDNA-PCR measurements. Our study based on gene expression data should therefore be considered as evaluative to find possible targets to overcome drug resistance, and will require confirmation at the functional level in future work.

DNA intercalators such as ADR, and topoisomerase II inhibitors such as ADR and ETO, as well as the tubulin-affecting compound VCR, are known to be involved in P-gp or MRP1-associated MDR phenotypes, but the topoisomerase I inhibitor CAM is not.[35, 36] However, the antineoplastic agents used here generally show significant and dose-dependent induction of the *MDR* genes in CCRF-CEM cells after 24 h (Fig. 3). CAM was found most effective in inducing the *MDR1*, *MRP1* and *LRP* genes. The fact that we used sublethal concentrations of cytostatics for a short time resulted in cell viability greater than 90% at the end of each experiment, so the effects found by cDNA-PCR should not be due to short-term selection of cells showing enhanced gene expression beforehand.

Furthermore, cell cycle analysis showed that marked induction of resistance factors and PKC isozymes after incubation with CAM and ETO is correlated with a reduced number of cells in G2/M-phase and an enhanced number of cells in the S-phase. If genes in fact are inducible to a higher extent during S-phase or if enhanced activity of, for example, distinct PKC isozymes might cause this shift is presently unclear.

Furthermore, experiments with the protein kinase inhibitor STAU revealed a significant suppression of induction of *MDR* genes mediated by CAM or ETO, though GÖ 6976 or GÖ 6850 alone produced non-significant effects here. Moreover, no significant effects were caused by any of these substances including STAU on ADR or VCR-mediated *MDR1*, *MRP1* or *LRP* gene induction (data not shown).

The findings suggest that not only the *MDR1*, but also the *MRP1* and *LRP* genes are inducible by various antineoplastic agents in the cell line CCRF-CEM. The effective suppression of induction by the nonspecific protein kinase inhibitor STAU, but not by the rather specific PKC inhibitors GÖ 6976 or GÖ 6850 in the experiments using CAM or ETO, where resistance factors and PKC isozymes were remarkably induced, indicates that protein kinases are involved in the complex stress response. The data do not support the involvement of PKC or particular PKC isozymes. Although GÖ 6850 and GÖ 6976 might be ineffective in inhibiting a PKC such as PKCζ under the conditions applied, our observation that ADR or VCR-mediated *MDR1* gene induction cannot be suppressed either by STAU or by GÖ 6850 or GÖ 6976 points to mechanisms different from ETO- or CAM-mediated ones. Furthermore, antagonistic effects between different PKC isozymes have to be taken into account. Interestingly, it was recently shown that LRP mRNA can be induced by TPA or cytarabine (Ara C). Furthermore, LRP induction by phorbol 12-myristate 13-acetate (TPA) can be prevented with a bisindolylmaleimide PKC inhibitor, but induction by Ara C cannot. The authors noted that the *LRP* gene seems to be activated by different mechanisms, some of which involve PKC.[37] If mechanisms beside PKC might contribute to induction of resistance factors, this would explain the difference between the action of VCR and ADR compared to CAM and ETO.

A major goal in the field of cancer chemotherapy is to define the mechanisms underlying transcriptional regulation of drug resistance genes in an attempt to specify targets for therapeutic intervention. Our analysis points to distinct PKC isozymes as possible targets. PKCζ, in particular, was found to be inducible in CCRF-CEM by four different cytostatics and upregulated in two patients during first-line therapy with steroids.

Enhanced expression of PKCη and θ was detectable in highly resistant leukemic blasts of a T-ALL patient and, furthermore, in various selected CCRF-CEM sublines correlating to the grade of resistance and *MDR1* gene expression. Furthermore, an earlier study on resistance factors and PKC isozymes in primary and relapsed states of acute

myelogenous leukemias (AML) revealed enhanced *MDR1*, *MRP* and *PKCθ* gene expression levels in relapses of AML compared to the primary forms and significant correlations between *MDR1* or *MRP1* and *PKCη* or *PKCθ* gene expression.[38] In summary, we suggest that PKC isozymes η, θ and ζ should be considered as possible targets to overcome drug resistance in ALL.

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