

1111 alkaloid emetine as a promising agent for the induction and enhancement of drug-induced apoptosis in leukemia cells

MAREN MÖLLER¹, KERSTIN HERZER³, TILL WENGER^{2,4}, INGRID HERR² and MICHAEL WINK¹

¹Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Im Neuenheimer Feld 364;

²Molecular OncoSurgery, Department of Surgery, University and German Cancer Research Center, Im Neuenheimer Feld 365, 69120 Heidelberg; ³1st Department of Internal Medicine,

University of Mainz, Langenbeckstrasse 1, 55131 Mainz, Germany;

⁴Centre d'Immunologie de Marseille-Luminy, Marseille, France

Received January 8, 2007; Accepted February 20, 2007

Abstract. Emetine, a natural alkaloid from *Psychotria ipecacuanha*, has been used in phytomedicine to induce vomiting, and to treat cough and severe amoebiasis. Certain data suggest the induction of apoptosis by emetine in leukemia cells. Therefore, we examined the suitability of emetine for the sensitisation of leukemia cells to apoptosis induced by cisplatin. In response to emetine, we found a strong reduction in viability, an induction of apoptosis and caspase activity comparable to the cytotoxic effect of cisplatin. Moreover, emetine had an additive effect and increased cisplatin-induced apoptosis. Mechanistically, we demonstrate by DNA array analysis that emetine alone or together with cisplatin down-regulates several anti-survival genes and up-regulates several pro-apoptotic signalling molecules along with other effects on signalling. These data show that emetine is a strong inducer of apoptosis in leukemia cells and could be a suitable cytotoxic drug alone or in combination with other chemotherapeutics to sensitise leukemia cells to apoptosis.

Introduction

Ipecac [*Psychotria ipecacuanha* (BROT.) STOKES., synonym *Cephaelis ipecacuanha* (BROT.) A. RICH., Rubiaceae] is a traditional herbal medicine, which was introduced to western medicine over 300 years ago. Ipecac syrup has been used for decades as an emetic for the treatment of patients who ingested poisons, especially in dealing with intoxications in children. The pharmacological activity of ipecac syrup is

caused mainly by two isoquinoline alkaloids, emetine and cephaeline, having identical effects regarding the irritation of the respiratory tract (1). Nowadays, ipecac syrup is no longer recommended for the routine use in the management of poisoned patients (2) and recently a guideline on the use of ipecac syrup was published, stating that 'the circumstances in which ipecac-induced emesis is the appropriate or desired method of gastric decontamination are rare' (3). Moreover, at present there is a demand to remove ipecac from the over-the-counter category due to potential abuse especially in persons with eating disorders (4).

The alkaloid emetine and its derivative dehydroemetine have been used as an antibiotic in the treatment of severe amoebiasis, provoked by the protozoon, *Entamoeba histolytica*. However, due to severe side-effects such as cardiac damage and muscle weakness (5,6), emetine is today replaced by the chemotherapeutic, metronidazol. The cytotoxicity of emetine is due to the inhibition of protein biosynthesis in eukaryotic ribosomes (7,8) and the interaction with DNA (9). There are certain publications suggesting the induction of apoptosis by emetine in U937 (10,11), A549-S cells (12), and rat hepatocytes (13). Thus, emetine could be a suitable cytotoxic agent in cancer therapy, e.g. to overcome multidrug resistance or to take advantage of synergistic effects in order to minimize side-effects due to the high dosage of other cytotoxic agents. Evidence of emetine's activity against tumor cells first came to light in 1918 (14), and its use in phase I and II clinical cancer trials began over 30 years ago (15-19). To our knowledge, only one study was performed analyzing emetine in co-treatment with another chemotherapeutic. Here, patients with lung cancer were treated with 1.5 mg per kg emetine and 8 mg per kg cyclophosphamide, and 'each of these six diverse cases has shown a definite response to this combined therapy' (20). Nevertheless, emetine disappeared from the scene of cancer therapy by 1975.

In this study, new results were obtained suggesting the suitability of emetine for the co-treatment of leukemia patients. We investigated the combined effects of emetine and an alkylating substance, the very potent and well established chemotherapeutic, cisplatin. Emetine substantially reduced viability, induced apoptosis and activated caspases in a human

Correspondence to: Dr M. Wink, Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany
E-mail: wink@uni-hd.de

Key words: Jurkat T-cells, emetine, cisplatin, apoptosis, caspase 3, expression profiling

leukemia cell line. Moreover, emetine enhanced the cytotoxic effect of cisplatin when used in combination. Employing gene array analysis, we compared the effects on gene regulation of emetine or cisplatin alone as well as both substances together, and revealed new information on the underlying molecular mechanisms. Emetine itself induces the expression of several pro-apoptotic genes along with the inhibition of pro-survival factors and increases the pro-apoptotic gene expression of cisplatin when used in combination. Thus, our results suggest emetine to be a promising therapeutic agent for the treatment of leukemia cells.

Materials and methods

Materials. Culture media and supplements were obtained from Gibco (Invitrogen, Karlsruhe, Germany), fetal bovine serum (FBS) was purchased from Biochrome (Berlin, Germany), and fine chemicals and test compounds were provided by Sigma-Aldrich (Taufkirchen, Germany). Stock solutions of emetine were prepared with distilled water and cisplatin was dissolved in DMSO. Stocks were stored at -20°C and diluted 1:10 in cell culture medium before the addition to the cells.

Cell cultures. Jurkat T-cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in 75 cm^2 culture flasks (Greiner bio-one, Frickenhausen, Germany). Bcl-2 over-expressing leukemia cells (J16-bcl2) and caspase-8 deficient cells (Jurkat- Δcasp8) were obtained from Dr H. Walczak (DKFZ, Heidelberg) and were cultivated in the same way.

Cell viability assay. To determine cell viability cells were seeded in a density of 20,000 cells per well in 96-well plates, and incubated for 24 h before the addition of the drugs. Jurkat cells were incubated with or without the test compounds for 24 and 48 h, and analyzed by MTT assay according to Mosmann (21). In brief, MTT was added to each well (0.5 mg/ml) and incubation was continued for 4 h. MTT-formazan products that are an indicator of cell viability, were dissolved in 50% 2-propanol containing 0.02 N HCl, and absorbance was recorded at 560 nm in a plate reader (Wallac Victor Multilable Counter, Wallac, Freiburg, Germany). Cell viability was calculated relative to the untreated controls.

Flow cytometry analysis. Cell death was determined by a drop in the forward scatter (FSC)/side scatter (SSC) profile, and apoptosis was detected by the measurement of DNA fragmentation according to the method of Nicoletti *et al.* (22). Proliferating Jurkat T-cells were incubated with or without the test compounds for 24 or 48 h. After harvesting and centrifugation, the cells were suspended in PBS and analyzed subsequently using a flow cytometer (FACSCalibur, BD, Heidelberg, Germany). Alternatively, after washing with HBSS the cells were incubated in propidium iodide staining-buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 $\mu\text{g/ml}$

propidium iodide in HBSS) for several hours or overnight and then analyzed by flow cytometry (B). Data analysis was performed using CellQuest software.

Caspase activity assay. The activity of caspase-3 in Jurkat T-cells was analyzed using a fluorogenic caspase substrate as described by Umansky *et al.* (23). In brief, Jurkat cells were incubated with or without emetine (0.5 μM), cisplatin (7 μM), or both for 6, 8, 12 and 24 h. The cells were harvested, washed with ice-cold PBS and about 107 cells were re-suspended in 100 μl freshly prepared lysis buffer (10% saccharose, 100 mM HEPES, 1 mM EDTA, 0.4% CHAPS, 5 mM DTT, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin). Lysis was performed at room temperature for 15 min, the samples were centrifuged at maximum speed and the supernatant was stored at -20°C until further analysis. An aliquot was used for the determination of the protein concentration with the Bio-Rad Protein Assay (Bio-Rad Laboratories, München, Germany) in a plate reader Titertek Multiskan Plus MKII (Labsystems, Helsinki, Finland) at 595 nm. The samples were diluted to 2 mg/ml protein with lysis buffer. Ten microliters of the test solution were mixed with 20 μl substrate solution (129 μM Ac-DEVD-AMC in lysis buffer) and incubated at 37°C . Fluorescence was measured after 15, 30, and 60 min in a plate reader (Wallac Victor Multilable Counter), and four replicas were performed for each sample (355 nm Ex., 485 nm Em.). The time-dependent gradients of these three measurements were calculated, and the ratio of the curve slopes sample versus the control was determined. Resulting data are values for caspase activity.

RNA isolation and filter hybridization. Total RNA from 3×10^7 Jurkat cells was isolated with the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and purity of the RNA were checked via gel electrophoresis, the concentration was measured spectrophotometrically, and isolated RNA was stored at -80°C . RNA samples from Jurkat cells left untreated or treated for 24 h with cisplatin (7 μM), emetine (0.5 μM) or both, were hybridized to membranes spotted with 1,066 genes involved in apoptosis regulation, signaling and immune response (24). The list of genes is available upon request. Hybridization was repeated six times, and the relative difference $d(i)$ in gene expression with respect to the untreated control cells was calculated by SAM. Statistical analysis was performed to determine significant changes in gene expression with an $\text{FDR} \leq 5\%$ (25).

Semi-quantitative real-time PCR. Total RNA from Jurkat cells was extracted as described. RNA samples were treated with DNase (DNA-free, Ambion) according to the manufacturer's instructions. First strand cDNA synthesis was performed according to the protocol of the Reverse Transcription System (Promega Corporation, Madison, USA) with oligo(dT)₁₆ primers (Pharmacia Biotech, Uppsala, Sweden). Quantification of *DAXX* and *AKT1* m-RNA was achieved by real-time PCR using SYBR-Green I (2x) in a PCR-Mix containing 200 μM dNTP, 200 nM primer, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , 0.5% Triton X-100 and 1 U Taq-Polymerase with the Opticon 1 (MJ Research, Waltham,

SPANDIDOS PUBLICATIONS Primers for quantification of *DAXX* and *AKT1* mRNA relative to the housekeeping gene, β -Actin, via real-time PCR.

Gene	Product size accession no.	Primer	Sequence
β -Actin	285 bp (NM_001101)	β -Actin-5'	TCA TGA AGT GTG ACT TTA ACA TCC GT
		β -Actin-3'	CCT AGA AGC ATT TGC GGT GCA CGA TG
Akt1	285 bp (NM_005163)	Akt1-forward	ACC ATG AGA AGC TTT TTG AGC
		Akt1-reverse	TGA TCA TCT GGG CCG TGA AC
<i>DAXX</i>	285 bp (NM_001350)	<i>DAXX</i> -forward	TCA CCA TCG TTA CTG TCA GAA G
		<i>DAXX</i> -reverse	TCC CCA GTT GTG AGG AGA GAC

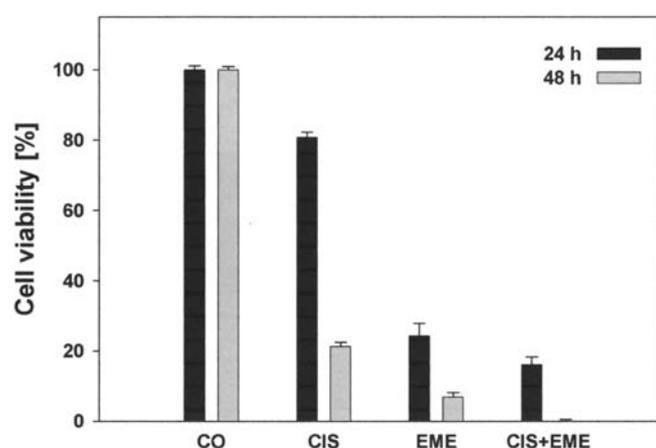


Figure 1. Reduction of cell viability by emetine. Jurkat cells were left either untreated (CO) or were treated with emetine (EME, 0.5 μ M) cisplatin (CIS, 7 μ M) or both agents together (EME + CIS). After 24- and 48-h incubation viability was measured by MTT-assay. Data obtained for the untreated control cells were set to 100%. Data are means \pm SEM of three independent experiments performed in four replicates (n=12).

USA). CT values were read at the moment of logarithmic amplification and relative quantification was obtained with the help of a standard curve. β -Actin was used as a housekeeping gene, and the relative expression of the target gene was set to 1 in the untreated cells. Data are means of four assays performed in duplicate. Primer sequences are given in Table I.

Statistical analysis. The effects of emetine or cisplatin alone or in combination were compared to the untreated controls; significance was assessed using an unpaired Student's t-test; a P-value <0.05 was regarded as significant.

Results

The human leukemic T-cell line, Jurkat, was incubated with emetine (0.5 μ M), cisplatin (7 μ M) or both together in order to determine the influence of emetine on the cytotoxic effect of cisplatin. The concentration of emetine corresponded to the ED₅₀ value that we have previously determined in our laboratory (data not shown).

After 24- and 48-h incubation, the viability of the Jurkat cells was analyzed with the MTT assay. After only 24 h emetine exhibited a substantial inhibition of cell growth and a reduced viability of about 20% relative to the control. In contrast, cell viability was 80% after treatment with cisplatin for the same amount of time. The application of both drugs together increased the cytotoxicity of emetine slightly but not significantly (Fig. 1). However, while the reduction in cell viability by emetine was still higher than by cisplatin after 48 h, the combined treatment with both substances caused a complete loss in cell viability.

Similar results were obtained by analyzing cell death by flow cytometry 24 and 48 h after treatment. Treatment of the cells with cisplatin or emetine alone was followed by a significant drop in the FSC/SSC profile (Fig. 2) indicating cell death. Moreover, the combined treatment with cisplatin and emetine led to enhanced cell death at both time-points. These data demonstrate that emetine alone is a potent cytotoxic agent in leukemia cells and the combination with cisplatin leads to a significant decrease in cell viability and increased cell death after incubation for two days.

To analyze the mode of cell death in depth, we determined cellular changes typical for apoptotic cells. The incubation of the Jurkat cells with emetine or cisplatin was followed by a significant increase in DNA-fragmentation 24 and 48 h after treatment (Fig. 3), as demonstrated by the increasing amount of cells with sub-G1 DNA content. Both agents together significantly increased apoptosis after 24 h while emetine only slightly increased the relatively high apoptosis rate of 70% induced by cisplatin after 48 h. These findings confirm that leukemia cells treated with cisplatin and emetine die by apoptosis and they also demonstrate that the loss of viability induced by emetine is at least partially due to the induction of apoptosis.

In order to gain information concerning pathways involved in apoptosis induction, we analyzed two additional cell lines via flow cytometry. In the J16-bcl2 Jurkat cell line, the intrinsic, mitochondrial pathway is blocked by the over-expression of the anti-apoptotic protein, bcl-2. Jurkat- Δ casp8 cells are deficient in the activation of the death receptor pathway due to the lack of the initiator, caspase-8. As can be seen clearly from Table II, there is almost no difference in cell death induction between the control cells and the cells deficient in caspase-8, whereas bcl-2 over-expressing cells

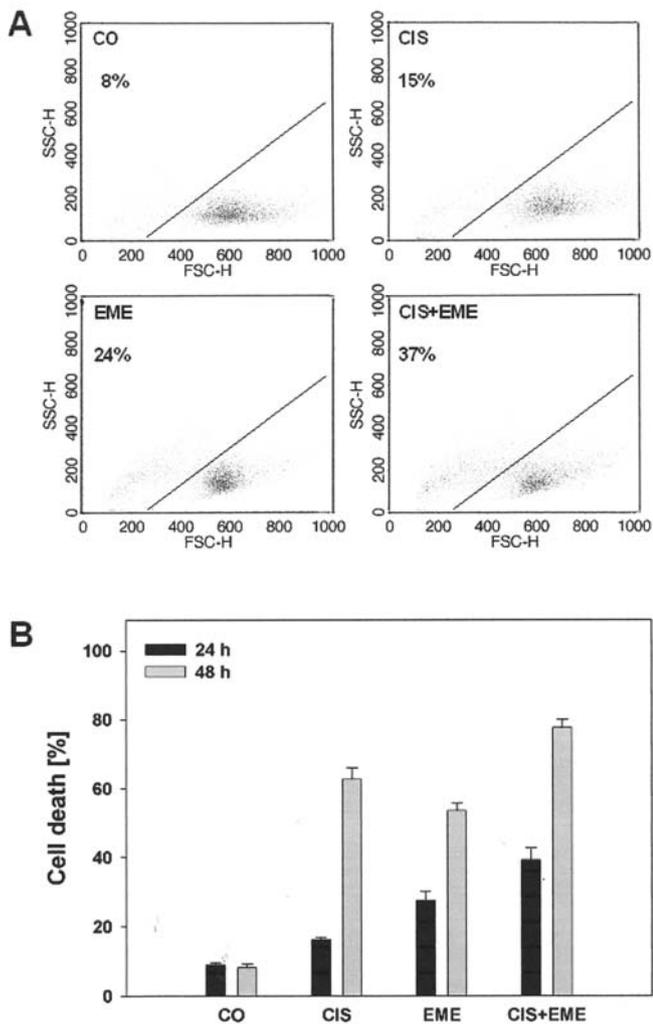


Figure 2. Emetine induces cell death and increases the cytotoxic effect of cisplatin. Jurkat cells were treated as described in Fig. 1. After incubation for 24 and 48 h cell death was examined by flow cytometry, analyzing forward and side scatter characteristics of the cells. Dead cells compared to viable cells are characterized by a higher side scatter (higher granularity) and a lower forward scatter (smaller size). (A) Dot-plots are representative results for Jurkat cells analyzed after treatment for 24 h. (B) Data are means \pm SEM of two independent assays analyzed in triplicate (n=6).

show a clear resistance to cisplatin and emetine-induced cell death, which was not influenced during co-treatment with both cytotoxic substances. Thus, the induction of apoptosis by both agents clearly depends on the activation of the intrinsic apoptosis pathway.

In order to determine other features of apoptosis induction by emetine, we treated Jurkat cells with emetine, cisplatin alone or in combination for 6, 8, 12 and 24 h and determined the activity of caspase-3. While emetine lead to an increased caspase activity as early as 6 h after treatment, cisplatin treatment induced a higher caspase activity 24 h after exposure. However, the combination of both drugs at 24 h dramatically increased caspase activity to a higher percentage than the one found for each agent alone (Fig. 4). These findings correlate well with the faster induction of cell death by emetine and further demonstrate that both agents induce typical apoptotic changes in leukemia cells.

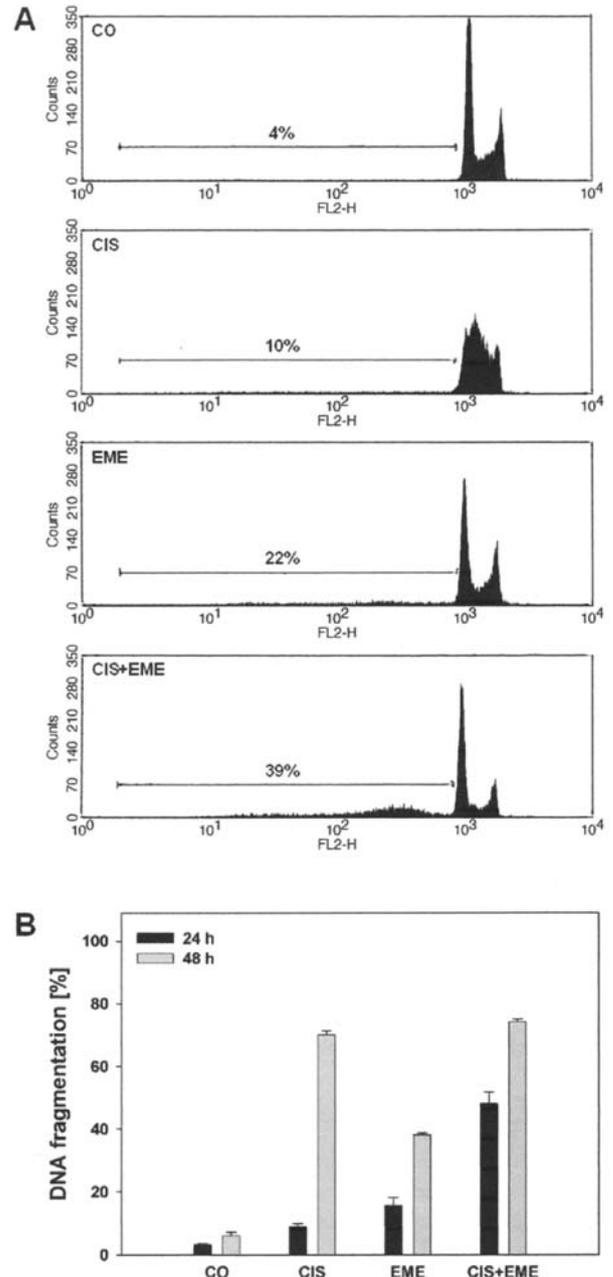


Figure 3. Emetine induces DNA fragmentation and increases the cytotoxic effect of cisplatin. Jurkat cells were treated as described in Fig. 1 and apoptosis was analyzed by staining the nuclear DNA with propidium iodide followed by the flow cytometric detection of the characteristic Sub-G1 peak. (A) Histogram-plots are representative results for Jurkat cells analyzed after treatment for 24 h. (B) Data are means \pm SEM of two independent assays analyzed in triplicate (n=6).

The mechanisms by which emetine influences apoptosis and proliferation are not well understood. Therefore, we examined Jurkat cells by DNA array analysis. Cells were exposed to emetine and cisplatin - either alone or in combination for 24 h. After controlling the quality of isolated RNA, the RNA was hybridized to nylon membranes containing specific PCR products of 1,066 cDNAs of human genes, involved in apoptosis, signaling and immune responses. Treatment with emetine in the presence or absence of cisplatin induced the cell type specific regulation of several

SPANDIDOS PUBLICATIONS Cell death (%) detected via forward/side scatter analysis after 24 and 48 h in three different cell lines.

Treatment	Jurkat-wt		J16-bcl2		Jurkat-Δcasp8	
	24 h	48 h	24 h	48 h	24 h	48 h
CO	11.4±1.2	8.5±1.1	13.7±3.2	7.7±1.1	9.8±0.5	5.7±0.5
CIS	25.6±4.5	57.4±6.7	16.7±3.5	21.9±3.2	24.8±1.9	61.5±3.1
EME	30.4±2.5	45.6±3.2	21.6±3	18.9±1.7	26.7±2.1	36.4±2.1
CIS + EME	48.5±4.2	78.6±2.9	23.9±3.5	24.1±2.9	37.7±2.1	68±4.4

Presented data are means ± SEM of three assays performed in triplicate. CO, untreated control; CIS, cisplatin; EME, emetine; CIS + EME, treatment with both substances.

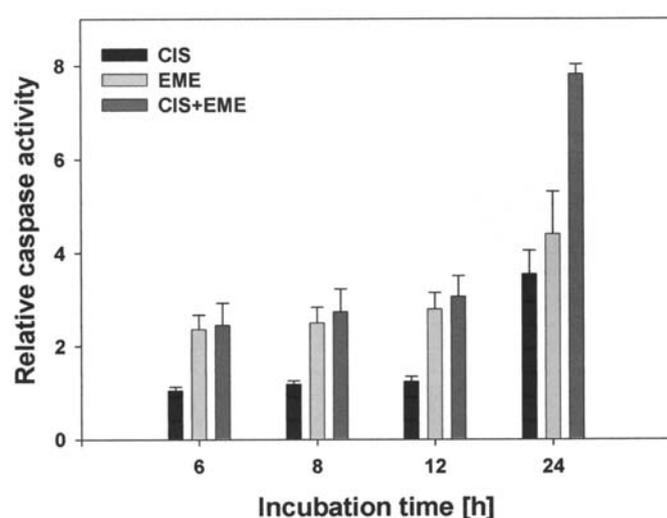


Figure 4. Emetine induces caspase activity and further enhances cisplatin-induced caspase activity. Jurkat cells were treated as described in Fig. 1 and the activity of caspase-3 was measured with a fluorogenic caspase-3 substrate. Cells were treated with emetine and/or cisplatin for 6, 8, 12, or 24 h. Cell extracts with defined protein concentrations were incubated with the fluorogenic substrate, fluorescence was detected after 15, 30, and 60 min, and the time-dependent gradients of fluorescence activity were calculated for each sample. In each case $R^2 > 0.99$. The relative caspase activity was determined as a ratio of the sample to the control. Results are expressed as means ± SEM of three assays.

sets of genes involved in signaling such as kinases, transcription factors, growth factors and others. Selected pro-apoptotic, anti-apoptotic and other genes involved in signaling are presented in Tables III and IV. The complete list of differentially regulated genes will be published within a doctoral thesis (26) and is available upon request. The observed regulation of members of the bcl-2 family, caspases, death adapter molecules, death receptors, death ligands and other death molecules in an anti-apoptotic and pro-apoptotic manner could be responsible for the cell type specific signaling. Altogether 230 out of 1,066 genes were differentially regulated compared to the untreated control cells. Thirty-six showed a correlated regulation for cisplatin and emetine (33 up, and 3 down), amongst them toll-like receptors 1, 2, and 4, tumor necrosis factor superfamily members, as well as some apoptosis relevant genes such as *AKT1* and *CASPASE-8*. Notably,

other members of the caspase family are differentially regulated: Cisplatin induced the up-regulation of *CASPASE-4* and *-7*, emetine led to the up-regulation of *CASPASE-9*. The effector-caspase-3 was up-regulated only in cells that were treated with both cisplatin and emetine. This effect could explain the additive caspase-3 activity after incubation for 24 h. Cells treated with cisplatin alone showed an up-regulation of 48 genes and a down-regulation of 67 genes. Emetine caused an up-regulation of 109 genes and a down-regulation of 16 genes. The combined treatment lead to an up-regulation of 120 genes and a down-regulation of only four genes. The regulation of gene expression mainly relates to the activity of emetine (>75%) while only about 3% can be ascribed to cisplatin activity. The complete data including fold induction and the accession numbers of the genes are available upon request.

In order to verify data obtained by the DNA array we determined the expression level of two differentially regulated genes involved in apoptosis signaling. Data are presented in Table V. *AKT1* is a serine/threonine kinase (protein kinase B) serving as a regulator for survival by inactivating caspase-9 and Bad (27). *DAXX* is a death domain associated protein that acts as a player in CD95-signalling and has anti-apoptotic properties as well (28). For *AKT1*, we showed a significant up-regulation on the mRNA level, but only for the combined treatment with emetine and cisplatin and not after treatment with each substance alone as expected from our filter experiments. Regarding the *DAXX* expression, a significant up-regulation was confirmed for treatment with emetine alone as well as in combination with cisplatin, supporting the data obtained by DNA array.

Discussion

Emetine, the main alkaloid of ipecac and a frequently used protein biosynthesis inhibitor in eukaryotic cells was studied as an anti-cancer agent over 30 years ago (NSC-33669). These trials did not lead to clinical application. In the present study, we investigated the cytotoxic and apoptosis inducing properties of emetine in the co-treatment with the well defined anti-cancer agent, cisplatin.

Emetine has been previously reported to induce apoptosis in different human cancer cell lines. This was confirmed by our results showing DNA fragmentation in Jurkat T-cells after incubation with 0.5 μM emetine for 24 h. This concen-

Table III. Expression of selected pro-apoptotic and anti-survival genes in Jurkat cells after treatment with emetine alone, cisplatin alone or in combination.

Gene	Function	Regulation		
		CIS	EME	CIS + EME
<i>BAK1</i>	BCL2-antagonist/killer 1	-	↑	↑
<i>BIK</i>	BCL2-interacting killer (apoptosis-inducing)	-	-	↑
<i>CASP3</i>	Caspase 3, apoptosis-related cysteine protease	-	-	↑
<i>CASP8</i>	Caspase 8, apoptosis-related cysteine protease	↑	↑	↑
<i>CASP9</i>	Caspase 9, apoptosis-related cysteine protease	-	↑	↑
<i>DAXX</i>	Death-associated protein 6	-	↑	↑
<i>GZMB</i>	Granzyme B	-	↑	↑
<i>TNFRSF6</i>	CD95/APO-1/Fas death receptor, tumor necrosis factor receptor superfamily, member 6	↑	↑	↑
<i>TNFRSF9</i>	CD137, tumor necrosis factor receptor superfamily, member 9	-	-	↑

CIS, cisplatin; EME, emetine; ↑, up-regulation; -, unchanged expression.

Table IV. Expression of selected anti-apoptotic and pro-survival genes in Jurkat cells after treatment with emetine, cisplatin alone or in combination.

Gene	Function	Regulation		
		CIS	EME	CIS + EME
<i>AKT1</i>	v-akt murine thymoma viral oncogene homolog 1	↑	↑	↑
<i>BCL2</i>	B-cell CLL/lymphoma 2	-	↓	↓
<i>EGFR</i>	Epidermal growth factor receptor	-	↓	↓
<i>EGR1</i>	Early growth response 1	↓	-	-
<i>MST1</i>	Macrophage stimulating 1 (hepatocyte growth factor-like)	-	↑	↑
<i>NOL3</i>	Nucleolar protein 3 (apoptosis repressor with CARD domain)	-	-	↑
<i>TNF</i>	Tumor necrosis factor (TNF superfamily, member 2); can act pro- as well as anti-apoptotic depending on the cellular context	↓	↓	-
<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b, osteoprotegerin; can act as a decoy receptor for TRAIL and is involved in RANKL/RANK signaling	↑	↑	↑
<i>TNFSF13</i>	Tumor necrosis factor (ligand) superfamily, member 13, APRIL, a proliferation-inducing ligand	-	↑	-

CIS, cisplatin; EME, emetine; ↑, up-regulation; ↓, down-regulation; -, unchanged expression.

Table V. Relative quantification of gene expression of the selected genes was performed via real-time PCR.

	<i>AKT1</i>	<i>DAXX</i>
CIS	1.21±0.15	1.65±0.29
EME	1.01±0.05	4.24±0.47 ^a
CIS + EME	3.44±0.67 ^a	8.47±2.19 ^a

CIS, cisplatin; EME, emetine. ^aIndicates significant changes compared to the untreated controls. Quantification was performed in duplicate and repeated four times, resented data are means ± SEM of 8 quantifications.

tration is lower than the concentrations reached when patients were treated for severe amoebiasis with 1 mg emetine per kg, with a maximal dose of 60 mg. Application of ipecac syrup to a poisoned adult leads to an intake of about 42 mg emetine, assuming a dose of 30 ml ipecac syrup and an alkaloid content of about 1.4 mg per ml (29). Blood levels of emetine are rarely available, and only one case report describing the intoxication of a 4-year-old child by the chronic administration of ipecac syrup stated an emetine concentration of about 1 μM (500 ng/ml) in the blood (30). In the course of the clinical trials with emetine as an anti-cancer agent the doses were even higher; a single intravenous dose of up to 1.5 mg/kg twice weekly was tolerated (15).

 SPANDIDOS PUBLICATIONS suggested by Watanabe *et al* (12), emetine induces via the mitochondrial pathway. We could confirm

this finding, showing that the deficiency in caspase-8 did not influence cell death in emetine-treated cells, whereas the over-expression of the anti-apoptotic protein, bcl-2, which plays a major role in mitochondria-mediated apoptosis pathways clearly leads to an increase in viability.

Corresponding facts are known for the alkylating cytostatic, cisplatin (31). However, the mechanisms by which both substances induce apoptosis are different. This becomes obvious not only by comparing the cell cycle distributions (Fig. 3A) but also by analyzing the gene expression patterns. Considering the cell cycle distribution, treatment with cisplatin and emetine had different consequences. Cisplatin treatment lead to a loss in G1 phase cells and an increase in M phase and G2 phase cells. In emetine-treated cells, the single or combination treatment with cisplatin lead to a cell cycle distribution which was very similar to the untreated control cells. It should be noted, that the combination of emetine and cisplatin did not cause the typical change in cell cycle distribution induced by cisplatin alone. Analyzing the gene expression patterns, it was remarkable that caspase-8, the caspase that is not required for apoptosis induction either by emetine or by cisplatin, was the only caspase that was up-regulated in the cisplatin-, emetine-, as well as the combination-treated cells.

In general, regarding the differential gene expression patterns 24 h after incubation with the cytotoxic substances, the correspondence between genes regulated by cisplatin and emetine was very low, supporting our suggestion that both substances activate different pathways. However, more detailed studies are essential in order to reveal the pathways that contribute to apoptosis induction in human tumor cells after incubation with both cisplatin and emetine.

In conclusion, our results indicate that emetine is a powerful inducer of apoptosis in human leukemia cells similar to the well established substance, cisplatin. With the concentrations used, the onset of apoptosis was earlier in those cells that were treated with emetine compared to the treatment with cisplatin. The combined treatment of cells with cisplatin and emetine enhanced apoptosis in a rather additive but synergistic fashion, cytotoxicity was not significantly influenced.

Whether or not emetine would be able to overcome drug resistance in leukemia cells remains to be shown, since resistance to bcl-2 over-expression was not effected by co-treatment.

Nevertheless, further analysis of emetine as a potential anti-cancer agent in leukemia, especially in combination with another cytostatic drug remains a promising topic for further studies.

Acknowledgements

The authors are grateful to Dr H. Walczak for providing the cell lines and Professor G. Kovács (Department of Molecular Oncology, Heidelberg University) for providing the Opticon 1 Real-time PCR system. This research was supported by a grant of the DFG (Graduiertenkolleg 388; fellowship to M.M.).

References

1. Boyd EM and Knight LM: The expectorant action of cephaeline, emetine and 2-dehydroemetine. *J Pharm Pharmacol* 16: 118-124, 1964.
2. American Academy of Clinical Toxicology, European Association of Poisons Centres and Clinical Toxicologists. Position paper: Ipecac syrup. *J Toxicol Clin Toxicol* 42: 133-143, 2004.
3. Manoguerra AS and Cobaugh DJ: Guideline on the use of ipecac syrup in the out-of-hospital management of ingested poisons. *Clin Toxicol* 43: 1-10, 2005.
4. Silber TJ: Ipecac syrup abuse, morbidity, and mortality: Isn't it time to repeal its over-the-counter status? *J Adolesc Health* 37: 256-260, 2005.
5. Bell DR: Treatment of amoebiasis. *Trop Doct* 3: 140-143, 1973.
6. Knight R: The chemotherapy of amoebiasis. *J Antimicrob Chemother* 6: 577-593, 1980.
7. Grollman AP: Structural basis for inhibition of protein synthesis by emetine and cycloheximide based on an analogy between ipecac alkaloids and glutarimide antibiotics. *Proc Natl Acad Sci USA* 56: 1867-1874, 1966.
8. Grollman AP and Huang MT: Inhibitors of protein synthesis in eukaryotes: tools in cell research. *Fed Proc* 32: 1673-1678, 1973.
9. Wink M, Schmeller T and Latz-Brüning B: Modes of action of allelochemical alkaloids: interaction with neuroreceptors, DNA, and other molecular targets. *J Chem Ecol* 24: 1881-1937, 1998.
10. Bicknell GR, Snowden RT and Cohen GM: Formation of high molecular mass DNA fragments is a marker of apoptosis in the human leukaemic cell line, U937. *J Cell Sci* 107: 2483-2489, 1994.
11. Kochi SK and Collier RJ: DNA fragmentation and cytolysis in U937 cells treated with diphtheria toxin or other inhibitors of protein synthesis. *Exp Cell Res* 208: 296-302, 1993.
12. Watanabe N, Iwamoto T, Dickinson DA, Iles KE and Forman HJ: Activation of the mitochondrial caspase cascade in the absence of protein synthesis does not require c-Jun N-terminal kinase. *Arch Biochem Biophys* 405: 231-240, 2002.
13. Meijerman I, Blom WM, de Bont HJ, Mulder GJ and Nagelkerke JF: Induction of apoptosis and changes in nuclear G-actin are mediated by different pathways: the effect of inhibitors of protein and RNA synthesis in isolated rat hepatocytes. *Toxicol Appl Pharmacol* 156: 46-55, 1999.
14. Lewisohn R: Action of emetin on malignant tumors. *JAMA* 70: 9-10, 1918.
15. Panettiere F and Coltman CA Jr: Experience with emetine hydrochloride (NSC 33669) as an antitumor agent. *Cancer* 27: 835-841, 1971.
16. Mastrangelo MJ, Grage TB, Bellet RE and Weiss AJ: A phase I study of emetine hydrochloride (NSC 33669) in solid tumors. *Cancer* 31: 1170-1175, 1973.
17. Siddiqui S, Firat D and Olshin S: Phase II study of emetine (NSC-33669) in the treatment of solid tumors. *Cancer Chemother Rep* 57: 423-428, 1973.
18. Moertel CG, Schutt AJ, Hahn RG and Reitemeier RJ: Treatment of advanced gastrointestinal cancer with emetine (NSC-33669). *Cancer Chemother Rep* 58: 229-232, 1974.
19. Kane RC, Cohen MH, Broder LE, Bull MI, Creaven PJ and Fossieck BE Jr: Phase I-II evaluation of emetine (NSC-33669) in the treatment of epidermoid bronchogenic carcinoma. *Cancer Chemother Rep* 59: 1171-1172, 1975.
20. Street EW: Cyclophosphamide plus emetine in lung cancer. *Lancet* 2: 381-382, 1972.
21. Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63, 1983.
22. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 139: 271-279, 1991.
23. Umansky V, Ratter F, Lampel S, Bucur M, Schirmmacher V and Ushmorov A: Inhibition of nitric-oxide-mediated apoptosis in Jurkat leukemia cells despite cytochrome c release. *Exp Cell Res* 265: 274-282, 2001.
24. Gressner O, Schilling T, Lorenz K, Schulze Schleithoff E, Koch A, Schulze-Bergkamen H, *et al*: TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. *EMBO J* 24: 2458-2471, 2005.

25. Okouoyo S, Herzer K, Ucur E, Mattern J, Krammer PH, Debatin KM, *et al*: Rescue of death receptor and mitochondrial apoptosis signaling in resistant human NSCLC *in vivo*. *Int J Cancer* 108: 580-587, 2004.
26. Möller M: Molecular pharmacology of the isoquinoline alkaloid emetine from *Psychotria ipecacuanha*, Rubiaceae. Heidelberg University, Heidelberg, 2006.
27. Siddik ZH: Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 22: 7265-7279, 2003.
28. Salomoni P and Khelifi AF: Daxx: death or survival protein? *Trends Cell Biol* 16: 97-104, 2006.
29. Pharmazeutisches Laboratorium. NRF - Neues Rezeptur-Formularium Rezepturhinweise: Ipecacuanha-Mixtur und brecherregender Ipecacuanha-Sirup, 1999.
30. Andersen JM, Keljo DJ and Argyle JC: Secretory diarrhea caused by ipecac poisoning. *J Pediatr Gastroenterol Nutr* 24: 612-615, 1997.
31. Koo MS, Kwo YG, Park JH, Choi WJ, Billiar TR and Kim YM: Signaling and function of caspase and c-jun N-terminal kinase in cisplatin-induced apoptosis. *Mol Cells* 13: 194-201, 2002.