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Aldehyde Dehydrogenase Involvement in a Variant of the Brown Norway Rat Acute Myelocytic Leukaemia (BNML) that Acquired Cyclophosphamide Resistance *In vivo*

C.J. de Groot, A.C.M. Martens and A. Hagenbeek

The development of drug resistance is an important factor contributing to failure of chemotherapy in cancer patients. Cyclophosphamide (CP) is a cytostatic drug widely used in the treatment of haematological malignancies and solid tumours. Because CP requires bioactivation to become cytotoxic, an *in vivo* approach was chosen to generate a subline of the Brown Norway rat acute myelocytic leukaemia (BNML/CPR) highly resistant to CP to serve as a model to investigate the molecular mechanism(s) of cyclophosphamide resistance. The role of the CP-detoxifying enzyme aldehyde dehydrogenase (ALDH) in the molecular mechanism of CP resistance in this subline of the BNML has been investigated. Compared to the parent BNML cell line, the BNML/CPR cell line displayed an approximately 6-fold higher level of ALDH enzyme activity. Pretreatment of leukaemic rats with the ALDH inhibitor disulfiram resulted in a restoration of CP sensitivity of animals carrying the BNML/CPR cells. Furthermore, *in vitro* incubation of BNML/CPR cells with disulfiram prior to incubation with the activated CP derivative mafosfamide resulted in an extra 2–3 log cell kill as indicated by the survival time of rats which were injected with disulfiram pretreated BNML/CPR cells compared to non-pretreated BNML/CPR cells. Data on the glutathione S-transferases (GSTs) isozyme profiles of cytoplasmic liver and spleen extracts of BNML- and BNML/CPR-carrying leukaemic rats indicated that the total GST enzyme amount was lower in BNML/CPR cells than in parent BNML cells. Furthermore, the BNML/CPR subline proved to be sensitive to phosphoramide mustard, both *in vivo* and *in vitro*.

Key words: cyclophosphamide resistance; aldehyde dehydrogenase (ALDH), disulfiram; glutathione S-transferase; BN rat acute myelocytic leukaemia (BNML)
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INTRODUCTION

THE DEVELOPMENT of resistance to chemotherapeutic agents is considered to be a major cause of treatment failure in cancer patients. The mechanisms by which drug resistance develops in cancer patients are poorly understood. The alkylating agent cyclophosphamide (CP) is commonly used in the treatment of haematological malignancies such as acute lymphocytic leukaemia, multiple myeloma, Hodgkin's disease, non-Hodgkin's lymphoma, and in a variety of solid tumours. Furthermore, CP is incorporated in many conditioning regimens prior to bone marrow transplantation [1]. Its *in vitro* active metabolite 4-hydroperoxy-cyclophosphamide is frequently used for purging purposes in case of autologous bone marrow transplant for acute leukaemia [2]. Obviously, given these clinical applications, studies on the mechanism(s) of CP resistance are highly relevant.

The Brown Norway rat acute myelocytic leukaemia (BNML) has been recognised as a realistic model for human acute myelocytic leukaemia (AML), both for the development of new diagnostic tools as well as a preclinical model from which new clinical treatment strategies can be derived [3–5]. The parent BNML cell line does not grow *in vitro*. CP itself is not active *in vitro*; it has to be activated first to yield 4-hydroxy-CP/aldophosphamide intermediates which by further metabolism yield the alkylating agent phosphoramide mustard (PM) [6]. To mimic the clinical situation and to be able to study all possible aspects of the molecular mechanism(s) of CP resistance, including pharmacodynamics, activation and de-activation (detoxification), we have developed a subline of the well-characterised BNML, highly resistant to CP by repeated *in vivo* treatment with CP. After 15 CP injections of 100 mg/kg each, during seven subsequent *in vivo* passages, a variant resistant to CP developed as became apparent from the absence of an increase in life span after CP-treatment compared to non-CP-treated leukaemic controls [7].

A limited number of animal and human tumours and cell lines resistant to CP have been isolated or developed and (partly) characterised [8–13]. Several mechanisms of drug resistance have been described in acute leukaemia, both in animal and human tumours and cell lines (for reviews, see [14, 15]). Cellular detoxification and increased DNA repair have been shown to be involved in resistance to alkylating agents such as CP, busulphan

Correspondence to A.C.M. Martens.
The authors are at the Institute of Haematology, Eramus University Rotterdam, RM Eel 393, P.O. Box 1738, 3000 DR Rotterdam; and A. Hagenbeek is also at the Department of Haemato-Oncology, The Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands.
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[16] and nitrosoureas [17]. Increased expression of the detoxification pathways involving ALDH and glutathione (GSH) and glutathione-dependent enzymes, such as glutathione S-transferases (GSTs) (reviewed in [18, 19] have been shown to be associated with CP resistance. Activated CP (aldophosphamide) can be inactivated to the non-toxic carboxyphosphamide catalysed by ALDH [20, 21] or through the action of GSH or glutathione-dependent enzymes, to 4-mercapto-CP [6, 10].

Cell lines resistant to CP have been shown to exhibit increased ALDH activity [13, 21–23] or elevated GSH levels [24] and increased GST α, μ or π isozyme activity [10, 25]. Cross-resistance to alkylating agents other than CP, and the effect of specific inhibitors of the detoxifying pathways on the cytotoxicity of CP in this resistant rat AML cell line can reveal the molecular mechanism involved in CP resistance.

Disulfiram (DSF) is a specific inhibitor of the cytosolic enzyme ALDH [22]. Inhibition by DSF of the ALDH-catalysed detoxification route results in elevated levels of PM. In this way, sensitivity of the tumour cells can be restored [21, 22].

Here we report on the experimental evidence that in an *in vivo* developed subline of a rat acute myelocytic leukaemia, which is highly resistant to CP, an increased ALDH enzyme level plays a significant role in the mechanism of CP resistance. Moreover, inhibition of ALDH by DSF treatment *in vivo* can fully reverse CP resistance, while incubation *in vitro* with DSF leads to a partial restoration of sensitivity to the *in vitro* active mafosfamide. Cross-resistance with the alkylating product of CP metabolism, i.e. PM, was also investigated. Similar to the CP sensitive parental line BNML/S, the BNML/CPR cell line proved to be sensitive to phosphoramide mustard, both *in vivo* and *in vitro*. The clinical implications of these findings to circumvent specific drug resistance are discussed.

MATERIALS AND METHODS

Animals

Rats used were the SPF inbred Brown Norway (BN) strain BNBi/Rij, produced in the breeding colony of the TNO Medical Biological Laboratory, (Rijswijk, The Netherlands). Male rats, 13–16 weeks of age were used (mean body weight 260 g).

Cell lines

The experiments were performed with the CP-sensitive BNML/S parent line and with the CP-resistant variant BNML/CPR, described in detail elsewhere [7].

Treatment of animals

Unless otherwise stated, CP and DSF (dissolved in DMSO) were given intraperitoneally (i.p.), while phosphoramide mustard (PM) was injected intravenously (i.v.). PM was kindly provided by Drs P. Hilgard and J. Pohl from Asta Werke A.G. (Bielefeld, Germany). Because slight differences in the growth kinetics between the parent BNML/S and the CP-resistant BNML/CPR were observed in earlier studies [7], animals were not treated on the same day because the tumour load at the time of treatment would not be identical. To ensure a comparable tumour load, animals that received 10^6 BNML/S cells i.v. were treated on day 14 or day 16, and animals receiving 10^6 BNML/CPR cells were treated on day 10 or day 12, respectively.

Preparation of cell suspensions

Cell suspensions were made from the spleen of leukaemic animals. Part of the spleen was minced with scissors and gently pressed through a nylon sieve with a spatula to obtain a monocellular suspension in Hanks' hepes buffered balanced salt solution.

After staining the samples with Türks' solution and counting in a Bürker-type haemocytometer, the cell suspensions were adjusted to the required concentration for injection into animals.

The survival time assay

This assay is based on the observed linear relationship in the BNML model between the number of injected cells and the survival time of the animals [4, 5]. From this relationship, the effect of anti-leukaemic treatment can be derived by measuring the prolongation of the survival time, i.e. 4 days increase in life span (ILS) corresponds with a 10-fold reduction in tumour load, referred to as 1 log cell kill (LCK) for the BNML/S parent line, while for the BNML/CPR variant, having slightly different growth characteristics, an ILS of 3 days corresponds with 1 LCK. The accuracy of the assay is in the order of 1 log cell kill.

In vitro *incubations*

Bone marrow cells or spleen cells were incubated at 37°C at a concentration of 1–2 $\times$ 10^6 cells/ml in Hank's medium supplemented with 5% fetal calf serum. After incubation with the inhibitory substance DSF [dissolved in 95% ethanol (v/v) or DMSO] and/or drugs (mafosfamide or PM, both dissolved in medium), cells were pelleted (5 min, 1500 rpm), washed and suspended at the required concentration for i.v. injection into recipient animals. The *in vitro* active mafosfamide (-lysine salt, i.e. ASTA-Z-7654) was kindly provided by Drs P. Hilgard and J. Pohl (Asta Werke A.G.).

Determination of ALDH enzyme activity

Tissues (spleen, bone marrow, liver) were homogenised and fractionated to a cytosolic and a mitochondrial fraction, essentially as described in [26, 27]. Alternatively, spleen cell lysates were made by subjecting the cells to three cycles of freezing and thawing, as described by Hilton [21]. ALDH enzyme activity was determined fluorometrically (excitation wavelength 360 nm; emission wavelength 455 nm) by monitoring the production of NADH using propionaldehyde as substrate [21, 26, 27]. Protein content of the lysates was determined by the Bradford method [28], using the Bio-Rad protein dye reagent with bovine serum albumin as a protein standard.

Determination of GST enzyme activity and protein

The total GST enzyme activities, using 1-chloro-2,4-dinitrobenzene as substrate, in cell-free cytoplasmic extracts of the different rat tissues, were determined using a reversed phase HPLC separation method [29].

RESULTS

ALDH enzyme activity

To be able to correlate ALDH enzyme activity levels directly with CP resistance, enzyme activity levels in different fractions of rat tissues were determined (Table 1). The cytosolic fraction of CP-resistant spleen and bone marrow cells taken from rats at fully developed leukaemic state revealed several-fold higher ALDH activity levels than their CP-sensitive counterparts. Since in many extracts of spleens (and bone marrow) of rats carrying BNML/S cells, the cytosolic ALDH activity level was below our detection limit (approx. 0.1 nmol/min/mg protein), the factor of increase in ALDH activity in the CP-resistant cells cannot be given precisely, but it seems to be on average in the order of at least 6- to 7-fold (Table 1). Cytosolic ALDH activity

Table 1. Aldehyde dehydrogenase (ALDH) enzyme activity levels in cytosolic tissue extracts of rats carrying the cyclophosphamide-resistant BNML line compared to the BNML/S parent line and normal BN rats

Source	ALDH activity (nmol/min/mg protein) Spleen	Bone marrow	Liver
Normal BN	0.13–0.36	<0.1	0.68
BNML/S (parent)	<0.1–0.38	<0.1	0.45
BNML/CPR	0.36–1.39	1.94	0.53

The data represent a range of two to six independent experiments or the mean value of duplicate measurements of one to two tissue preparations in each individual experiment. The lower detection limit of ALDH enzyme activity was estimated to be 0.1 nmol/min/mg protein.

in liver extracts was far less divergent (0.53 for BNML/CPR-carrying rats versus 0.45 for BNML/S-carrying rats, while normal BN rats have an average cytosolic activity of 0.68 nmol/min/mg protein), possibly because of a lower fraction of leukaemic cells in the liver of leukaemic rats. In the latter case, the endogenous normal cytosolic liver ALDH masks the ALDH activity of the leukaemic cells.

Alternatively, the mitochondrial fraction of the tissues tested, which contains the mitochondrially localised ALDH2 enzyme [30], displayed lower ALDH activity levels in those carrying the CP-resistant cells compared to the parent sensitive cells. The mitochondrial fraction of the spleen of rats carrying the BNML/CPR line contained 84% of the ALDH activity of the corresponding fraction found in BNML/S carrying rats, while this value was 67% for mitochondrial fractions of the liver, respectively (data not shown).

The addition of 100 μM of pyrazole to the incubation mixture (to inhibit alcohol dehydrogenases) had no significant effect on ALDH enzyme activity determinations. Furthermore, the inclusion of protease inhibitors in cytoplasmic and mitochondrial extracts just before storage at −80°C had no detectable effect on enzyme activity levels (data not shown).

Effect of pretreatment with disulfiram

In vivo. To investigate the role of ALDH in the mechanism of CP resistance in the BNML/CPR cell line, leukaemic rats were pretreated with the ALDH inhibitor, DSF. A significant increase in the survival time of the BNML/CPR-carrying rats (reflecting an increase in log cell kill, LCK) was observed (Table 2). Treatment with DSF alone led to a 0.1–0.3 LCK, while injection of CP only (100 mg/kg) resulted in 0.3–1.5 LCK. No toxic deaths were observed in these BNML/CPR control groups. Pretreatment with DSF (25–100 mg/kg) followed by CP (100 mg/kg) did lead to a LCK ranging from 4.3 to 6.5 in BNML/CPR-carrying rats, indicating a cytostatic effect of CP completely comparable with its effect on the sensitive parent line (Table 2). In some groups injected with DSF and CP, lethal toxicity occurred within the first week after treatment. However, for most combinations of DSF and CP, a sufficient number of rats at risk of dying from leukaemia was left to draw meaningful conclusions.

In the BNML/S groups, similar combinations of DSF and CP were tested (Table 2).

Considerable toxic side-effects were seen in the DSF (100 mg/kg) control group. After employing lower dosages of CP (30 and 50 mg/kg) in combination with DSF pretreatment at the highest dose (100 mg/kg), lethal toxicity was still observed (Table 2). For comparison, the effect of a low dose of CP only (30 mg/kg i.p) on the BNML/S line was determined. As can be seen in Table 2, the pretreatment with DSF of the rats carrying BNML/S cells had hardly any additive cell-killing effect to that of CP treatment alone, although the high toxic death rate, especially in the case of the highest DSF dose used, hampered the investigation of the effects of ALDH inhibition with respect to their expected increased sensitivity to CP. Furthermore, the effective cytoreductive effect of CP alone (100 mg/kg) on the parent line (3.6–5.2 LCK) might mask a minor additional cytostatic CP effect by DSF pretreatment.

In vitro. In Table 3, the effect of incubation with DSF on the cytotoxic efficacy of mafosfamide, the *in vitro* active analogue of CP, on both cell lines is shown. Since there was no significant difference between the survival times of rats injected with cells that were treated with the solvents DMSO or ethanol alone, or with DSF alone (data not shown), these four groups (five rats/group) were pooled. Survival times were compared to the corresponding control group and differences in survival times calculated as LCK. In this particular experiment, there was a considerable cytotoxic effect resulting from mafosfamide (100 μM) incubation on the CPR cells (1.0 LCK, Table 3). A cell kill measured in the BNML/S cell line at 50 and 100 μM was observed in the BNML/CPR line only at 500 μM. Fifteen- to 150-fold differences in sensitivity to mafosfamide between the BNML/S and BNML/CPR cell lines were observed (Table 3, LCK values at 50 and 100 μM of mafosfamide).

As can be inferred from Table 3, preincubation of the CP-resistant BNML cells with the ALDH inhibitor DSF led to a 20-fold increase in the cytotoxic effect of mafosfamide [i.e. a difference of 1.3 LCK derived from a LCK of 2.5 after DSF plus mafosfamide, corrected for the 0.2 LCK from incubation with DSF only, versus a LCK of 1.0 for mafosfamide only (100 μM)]. In addition, with the CP-sensitive parent cell line, a pronounced increase in cell kill was observed. The value of 2.2 LCK by incubation *in vitro* of the BNML/S cells with 100 μM of mafosfamide might be a relatively low value, considering the cytotoxic activity of the lower concentrations tested in a separate experiment (20 and 50 μM leading to a LCK of 0.5 and 2.2, respectively; Table 3). This might be due to inter-experimental variations. The slightly higher cytotoxic activity of DSF alone (0.4 versus 0.2 LCK for the BNML/CPR cells not significantly different, however) in combination with the high sensitivity for mafosfamide of the BNML/S cells might explain the high cytotoxic effect of the combination treatment (LCK of more than 5), leaving very low numbers of surviving BNML/S cells.

Sensitivity to PM in vivo *and* in vitro

If increased levels of ALDH are solely responsible for the CP resistance of the BNML/CPR cell line, it is to be expected that this cell line should be sensitive to the alkylating product of CP metabolism, i.e. PM. Therefore, we investigated the sensitivity of the CP-resistant cell line to PM in comparison with the sensitive parent line, both *in vivo* and *in vitro*. The results of the *in vivo* experiments are shown in Figure 1. Up to a 4.2 LCK could be obtained with a PM dose of 120 mg/kg body weight in rats carrying the CPR cells, comparable to the 3.5 LCK obtained by the same dose in rats carrying the sensitive cell line. However, at lower doses of PM, the rats carrying the BNML/S cells displayed a slightly higher sensitivity to PM compared with those carrying the CP-resistant cells.

In Figure 2, the effects of incubation of CP-resistant and

Table 2. Effect of pretreatment with disulfiram (DSF) on the anti-leukaemic effect of cyclophosphamide (CP) in vivo

	DSF (mg/kg)	CP (mg/kg)	Day of treatment*	Number of rats	Cause of death: Leukaemia	Cause of death: Toxicity	Log cell kill
BNML/CPR cell line							
DSF+CP	25	100	10	8	4	4	5.0
	50	100	10	8	6	2	5.3
	100	50†	12	10	5	5	2.3
	100	100	10/12	18	6	12‡	6.5, 4.3
DSF control	25	—	10	8	8	0	0.2
	50	—	10	8	8	0	0.3
	100	—	10/12	18	18	0	0.1
CP control	—	100	10/12	24	24	0	1.5, 0.3
BNML/S cell line							
DSF+CP	25	100	14	8	7	1	4.0
	50	100	14	8	4	4	5.1
	100	100	14	8	3	5§	5.6
	100	50	16	8	1	7	2.1
	100	30	16	8	1	7	1.1
DSF control	25	—	14	8	8	0	0
	50	—	14	8	8	0	0
	100	—	14/16	16	9	7	0
CP control	—	100	14/16	24	24	0	3.6, 5.2
	—	50	16	4	4	0	2.4
	—	30	16	4	3	1	1.1

For the BNML/CPR cells, 1 log cell kill corresponds with an increase of life span 3 days compared to non-treated leukaemic controls, while for the BNML/S cells, 1 LCK corresponds with an increased life span of 4 days. Leukaemia as cause of death was judged from the presence of an enlarged spleen and liver, while in case of toxicity (mostly deaths early after treatment, the rats dying before the control group due to aplasia or urotoxicity) these organs were not enlarged. LCK values are calculated based on leukaemic deaths only. * Days after injection of leukaemic cells into hosts. CP was given 4–4.5 h after DSF. † 50 mg/kg CP alone had no cytotoxic effect on BNML/CPR cells. ‡ Spleen weights of two rats that died at day 18 (6 days after treatment): 0.24 and 0.45 g (2–3 g at the start of the treatment). § One rat died 16 wks after injection of BNML/S cells; cause of death: aplasia.

Table 3. Effect of pretreatment with disulfiram on the anti-leukaemic effect of mafosfamide in vitro

	MAFOS dose (μM)	Log cell kill
BNML/CPR		
DSF + MAFOS	100	2.5
DSF control	—	0.2
MAFOS control	50	0*
	100	1.0
	200	1.3*
	500	2.7*
BNML/S		
DSF + MAFOS	100	5–7†*
DSF control	0	0.4
MAFOS control	20	0.5*
	50	2.2*
	100	2.2

* The effect of these concentrations of mafosfamide on both cell lines was determined in a separate experiment. In this incubation group, two of nine rats did not develop leukaemia, while seven of nine died of leukaemia with a survival time corresponding to 1–100 leukaemic cells surviving (indicated by a log cell kill of 5–7†). MAFOS, mafosfamide; DSF, disulfiram (10 μM). For details on incubations, see Materials and Methods.

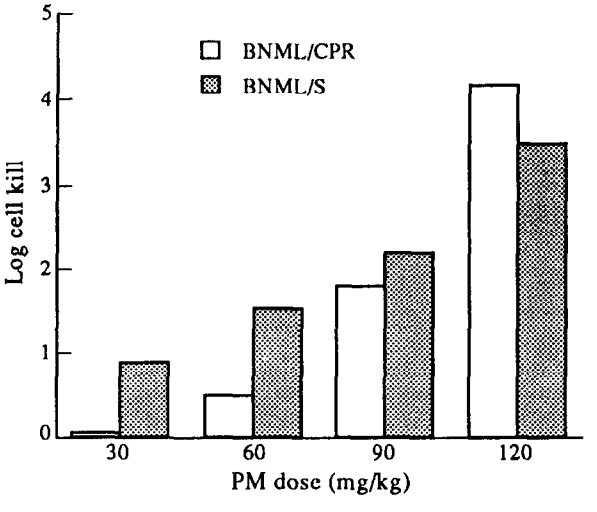

Figure 1. Comparison of the response of the BNML/CPR and BNML/S line to treatment with phosphoramide mustard (PM) *in vivo*. Injected i.v. on day 11 (60–120 mg/kg; five rats/group) or day 12 (30 or 60 mg/kg; $n = 16$ and 8, respectively). The log cell kill values are calculated from the increase in life span (ILS) of treated animals compared to non-treated leukaemic animals. The mean values of three independent experiments are depicted. With treatment with PM at doses higher than 30 mg/kg, 8 h after drug treatment 10^8 isologous bone marrow cells were infused to avoid marrow failure.

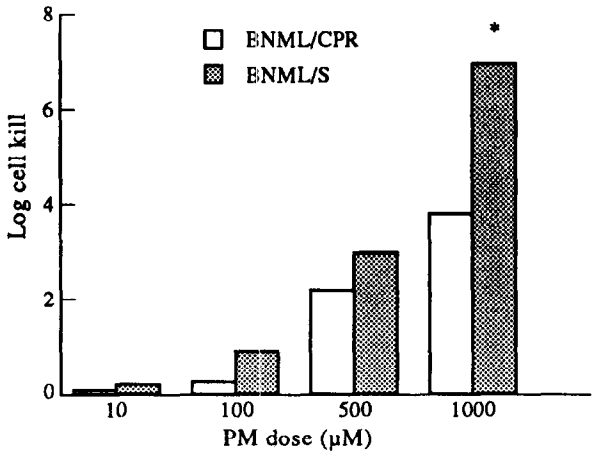

Figure 2. Comparison of the response of the BNML/CPR and BNML/S line to treatment with phosphoramide mustard (PM) *in vitro*. After incubation of the cells with the drug, the cells were washed and injected i.v. (10^7 cells/rat) into recipient animals (five to 10 rats/group). The log cell kill values are calculated from the increase in life span. *No animals died of leukaemia (observation period of 63 days).

-sensitive cells *in vitro* with PM on cell survival is depicted. As shown for the *in vivo* treatment (Figure 1), the CP-resistant BNML cells were sensitive to incubation with PM *in vitro* (Figure 2): a LCK of 2.2 for the BNML/CPR cells versus 3.0 for the BNML/S cells at a PM concentration of 500 μM is observed. However, *in vitro*, the BNML/S cells seem to be more sensitive to PM than the CP-resistant cells, although in nearly all cases the difference in LCK values was within the limits of the sensitivity of the survival time assay, being approximately 1 LCK (Figure 2).

GST isozyme activity

Determination of the total GST enzyme activity and protein level of liver and spleen extracts of BNML/S and BNML/CPR carrying endstage leukaemic rats revealed that, in liver, the total GST protein content of BNML/CPR-carrying rats is considerably lower than in BNML/S-carrying rats (approximately 24 μg GST/mg protein for BNML/CPR rats versus approximately 76 μg GST/mg protein for BNML/S rats). The latter value is comparable to the amount of GST protein in normal BN rat liver (approximately 89 μg/mg protein). Although a lower number of spleen extracts of both groups has been analysed thus far, here also the BNML/CPR extracts contained less GST protein than the BNML/S extracts (2.5 and 3.1 μg/mg protein, respectively).

DISCUSSION

Since CP is often used in a "cocktail" together with several other cytostatic drugs, it is not clear at present to what extent acquired (and natural) CP resistance plays a role in the non-responsiveness of drug-resistant human tumours. Knowledge on the molecular mechanism(s) of CP resistance will hopefully provide us with the tools to detect CP-resistant tumour cells at an early stage of the disease, and this may improve the response rates of cancer chemotherapy by choosing another cytostatic drug or by combination of CP with specific inhibitors that can reverse CP resistance.

Two mechanisms of drug resistance involving CP detoxification have been implicated in experimental CP-resistant cell lines. One concerns increased levels of GSH and GST enzymes [10], while in the other increased expression of cytosolic ALDH has been demonstrated [13, 21, 22]. Both systems can detoxify the CP metabolites 4-hydroxy-CP and aldophosphamide [6, 22].

In this paper, we have demonstrated that in a subline of the BNML made resistant to CP *in vivo*, increased levels of ALDH activity are detected (Table 1). The expression of the ALDH1 gene, coding for the cytosolic ALDH enzyme involved in CP detoxification [23], seems to be regulated primarily at the transcriptional level, since northern blot analysis revealed a higher ALDH1 mRNA level in RNA preparations from CP-resistant BNML cells than from BNML/S cells (unpublished observations; [31]). This elevated ALDH level in CP-resistant BNML cells is thought to be responsible for the observed CP resistance, i.e. as a result of increased detoxification of the "activated" CP intermediates yielding the non-toxic carboxyphosphamide, only lower concentrations of the cytotoxic PM can be reached. Since this is an intrinsic property of the BNML/CPR cells, the resistant phenotype is also displayed *in vitro*, i.e. the BNML/CPR cells are cross-resistant to mafosfamide. Unlike the CP-resistant tumour cell line described by Teicher and associates [12], in which pharmacokinetics seemed to be the major mechanism of drug resistance observed only *in vivo*, the *in vivo* developed CP-resistant BNML cell line described here is resistant both *in vivo* and *in vitro* (compared with [13]). Inhibition of ALDH with a specific inhibitor like DSF could block this detoxification pathway, thereby elevating the PM concentration and reversing (partially) the CP resistance of the BNML/CPR cells (Tables 2, 3). The conclusion that CP displayed a cytotoxic activity on the CPR cells after DSF injection could be drawn, not only from the increased LCK, but also from the fact that the weight of the spleen of the rats that died 6 days after CP treatment from toxicity was less than 0.5 g, having weighed 2–3 g at the start of the treatment (Table 2).

The observation that DSF in itself is more toxic to rats carrying CP-sensitive BNML cells (Table 2) and that the combination of DSF followed by CP treatment is also more toxic to the parent line (Table 2) might be explained by increased toxic side-effects of DSF in the rats carrying the BNML/S cells, because of their lower levels of ALDH enzyme (Table 1). Furthermore, proliferating haematopoietic stem cells and committed progenitor cells of the different haematopoietic lineages also contain significant levels of NAD^+-dependent ALDH which is inhibited by DSF [32, 33]. It is hypothesised that in the case of BNML/S carrying animals, the higher concentration of free DSF, not bound to ALDH enzyme molecules, inhibits the ALDH of normal haematopoietic cells and other vital cells in the animal leading to increased lethal toxicity.

The detoxification of PM by SH-containing compounds is not included in most schemes of CP metabolism [6]. However, to explain the observed cross-resistance to PM of a CP-resistant Yoshida sarcoma cell line, McGown and Fox [10] concluded that GSH and GST enzymes can also inactivate PM. We have shown that, above a certain threshold, the BNML/CPR cells are as sensitive to PM as their sensitive counterparts (Figures 1, 2). In a recent paper by Lee [34], indeed detoxification of PM by conjugation with GSH, giving the inactive product GS-PM, is included in the scheme of CP metabolism. This seems to be a non-enzyme catalysed reaction, however.

Since the total amount of GST protein is even lower in BNML/CPR cells than in BNML/S cells (see Results), involvement of increased GST enzyme expression in this CP-resistance model is not very likely.

The observation that the BNML/CPR cell line is equally sensitive to the endproduct of CP metabolism, i.e. PM, as

the parental BNML/S line, in combination with the observed increased ALDH level in CP-resistant cells and the complete reversal of CP sensitivity of the BNML/CPR cells by ALDH inhibition *in vivo*, support the idea that increased ALDH expression is the major determinant of CP resistance in this particular cell line. However, from a theoretical point of view, other mechanisms of drug resistance, such as changes in pharmacokinetic factors or increased DNA repair, cannot be ruled out to play a (minor) role in this CP-resistant rat leukaemic cell line as well.

The molecular mechanism underlying the observed enhanced ALDH gene expression is still unclear. It might be due to increased transcription which can be caused by gene amplification, by mutations that upregulate the transcription rate or by mutations that stabilise the mRNA or by combinations of these mechanisms. Furthermore, several post-transcriptional regulatory mechanisms can be operative, of which (de)methylation is a likely candidate [36]. In case of drug-resistant cell lines, gene amplification is a frequently observed phenomenon. However, the number of cases in which gene amplification in cancer patients resistant to chemotherapeutic agents has been reported is only limited [37].

Both by conventional karyotyping and by flow karyotyping, the chromosomal aberrations of the BNML/S and the BNML/CPR cells have been documented ([7, 38] compared with [39]). The CP-resistant variant is characterised by a specific $2p^+q^+$ marker chromosome, i.e. an elongation of the long arm of the $2p^+$ chromosome, which is one of the chromosomal aberrations characteristic for the BNML/S cell line [38]. Currently, it is not known whether this extra piece of DNA on the $2p^+$ chromosome is the result of DNA amplification, or of one or more translocations. By performing the polymerase chain reaction (PCR) using rat ALDH-specific primers on flow-sorted chromosomes of both CP-resistant and -sensitive BNML cells, we are currently establishing the chromosomal localisation of the ALDH1 gene in these cells, and investigating whether the observed enhanced ALDH gene expression is caused by gene amplification or, for instance, by a translocation which might have placed the ALDH1 gene under control of a stronger or differently regulated promoter in the CP-resistant BNML cell line. Like the group of Radin and associates using a murine CP-resistant cell line [31], we could not detect differences in the genomic structure of the ALDH gene in CP-sensitive and -resistant BNML cells, using a partial human ALDH1 cDNA probe [30] (unpublished observations). The molecular mechanism underlying increased expression of NAD^+-dependent ALDH in CP-resistant cell lines needs further investigation. If overexpression of ALDH is indeed a phenomenon which occurs clinically in CP-resistant tumours, then specific inhibition of ALDH might reverse the susceptibility of drug-resistant cancer cells to CP.

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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