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**ESTABLISHMENT AND BIOCHEMICAL
CHARACTERIZATION OF A MULTIDRUG-RESISTANT
PROMYELOCYTIC LEUKEMIA CELL LINE HL-60/CDDP**

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Abstract

The present study reports on the establishment, validation and biochemical characterization of a novel chemoresistant variant of the HL-60 acute myeloid leukemia (AML) cell line developed at the Laboratory of Experimental Chemotherapy at the Faculty of Pharmacy of MU-Sofia. Selection of the chemoresistant strain (HL-60/CDDP) has been carried out by prolonged serial exposures of the parent chemosensitive HL-60 cell line to gradually increasing concentrations of the metal complex. In its final variant, the resultant HL-60/CDDP test system showed significantly altered chemosensitivity profiles to the Pt(II)-based drugs cisplatin, carboplatin, oxaliplatin and was maintained in an RPMI-1640 medium containing 25 μ M cisplatin. The promyelocytic cells also showed diminished susceptibility to differentiation therapy with arsenic trioxide As₂O₃. The emergence of the multiresistant phenotype is suggested to be most likely due to common biochemical properties of the screened compounds, including their strong affinity and high reactivity with sulfhydryl SH-groups. The presented study is focused on elucidating the multidrug resistance patterns in the newly established leukemic model that can be used as a potential test system for exploring the drug-resistance reversal capacity of various drugs and experimental compounds.

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Key words: AML, promyelocytic leukemia, cisplatin resistance, multidrug resistance, glutathione, antioxidant enzymes, efflux pumps

Introduction. The drug resistance phenomenon is associated with lacking or significantly reduced drug sensitivity in tumour cells and can occur primarily (before treatment), or secondarily when acquired in the course of treatment [1,2]. Multiple drug resistance (MDR) is defined as reduced or absent chemosensitivity to antineoplastic agents that lack structural or mechanistic similarities and often results in treatment failure [3]. An MDR phenotype can arise by various mechanisms but is prominently developed on a “pre-target” level as a result of an active efflux or increased inactivation of the xenobiotic drugs [2,4]. For example, resistance to electrophilic drugs such as alkylating and platinating agents is often related to changes in glutathione homeostasis and sequestration of their bioactive forms. As glutathione-S-transferase (GST) substrates, platinum complexes are subjected to GSH conjugation and further detoxification by the unilateral efflux transporter MRP1 [3,4]. The triad factors (GSH, GST and MRP1) and their conjoined effects constitute one of the main paradigms in cisplatin resistance, that is related to cellular pharmacokinetics rather than DNA adducts repair [5]. Given the clinical importance of both intrinsic and acquired resistance to antitumour chemotherapy, the development and validation of an adequate in vitro test system for high-throughput evaluation of new cytotoxic agents or potential MDR modulators is of particular interest.

Experimental design. The parental chemosensitive HL-60 cell line was cultured in RPMI-1640 medium in the presence of gradually increasing concentrations of cisplatin, with an initial inhibitory concentration of 500 nM (0–7 days), that was continuously increased each subsequent week as follows: by 500 nM (weeks 2–4), 1 µM (weeks 5–7), 2.5 µM (weeks 8–15). A resistance phenotype was achieved allowing for the unperturbed proliferation of the AML cells at a cisplatin concentration as high as 25 µM. The newly established subline has been sustained through cell cultivation in a growth medium containing 25 µM of the metal complex.

A standard MTT assay was used for the comparative chemosensitivity studies of both the parental (HL-60) and resistant leukemia cell lines (HL-60/CDDP) to platinating agents of different generations and As₂O₃. To avoid possible synergistic suppression of cell viability and proliferation by the CDDP complex contained in the medium, resistant cells were transferred to a cisplatin-free RPMI-1640 medium 5 days prior to the experiments.

To investigate the mechanistic traits of the observed resistance phenotype, a series of multi-endpoint experiments were conducted tracing the expression and functional status of several antioxidant factors that contribute to the so-called “pre-target” resistance to platinating agents. Cellular levels of reduced glutathione (GSH), the activities of its related enzymes glutathione-S-transferase (GST), glu-

tathione peroxidase (GPx) and glutathione reductase (GR), as well as the expression levels of the glutathione S-conjugate carrier MRP-1 were measured and compared in both in vitro leukemic models.

Results. Comparative drug sensitivity studies of HL-60/CDDP and HL-60. Both cell lines were treated for 72 h with serial dilutions of the clinically used anticancer drugs cisplatin, carboplatin and oxaliplatin, as well as As₂O₃, following which the cytotoxic effects were assessed using the MTT assay (Fig. 1). As indicated by the obtained data, HL-60 cells originating from acute myeloid leukemia are highly sensitive to the inhibitory effect of platinum antineoplastic agents and arsenic trioxide, whereas in the resistant variant HL-60/CDDP a significant shift to the right of all dose-response curves was observed. A comparative analysis of the estimated half-inhibitory concentrations (IC₅₀) of the tested compounds against the chemosensitive vs. the resistant HL-60 variant was used to define the corresponding resistance indices (RI) that are valuable parameters in chemosensitivity quantification (Table 1). The modified HL-60/CDDP cells demonstrated an almost identical degree of resistance toward the prototype drug cisplatin and its second generation analogue carboplatin with RI values of 19.3 and 20, respectively. The third generation representative oxaliplatin also failed to overcome the reduced chemosensitivity of HL-60/CDDP to platinum complexes, but the estimated resistance index (8.6) was about twice lower than the ones for cisplatin and carboplatin. Of the As₂O₃ treated probes, HL-60/CDDP cells were 2.5 times less sensitive as compared to the original variant HL-60, indicating that the resistance phenotype is not limited to platinum anticancer drugs and is most likely determined by broad spectrum mechanisms associated with multiple drug resistance.

T a b l e 1

In vitro cytotoxicity [IC₅₀ (μM) ± SD] of the tested drugs against HL-60 cells with different chemosensitivity and the correspondent resistance indices, defined as the ratio between the IC₅₀ estimates in the chemoresistant (HL-60/CDDP) and the chemosensitive (HL-60) variants

compound/cell line	HL-60	HL-60/CDDP	RI
cisplatin	7.0 ± 1.2	135.2** ± 9.3	19.3
carboplatin	11.4 ± 0.9	229.3** ± 11.4	20.1
oxaliplatin	5.2 ± 0.6	44.95* ± 3.6	8.6
As ₂ O ₃	1.99 ± 0.4	5.07* ± 0.2	2.55

RI = IC₅₀ (HL-60/CDDP): IC₅₀ (HL-60); **p* ≤ 0.05; ***p* ≤ 0.01

Plausible mechanisms governing the HL-60/CDDP resistant phenotype. The biochemical characterization of the newly established HL-60/CDDP MDR leukemic variant was centred on the functional activity of the glutathione-

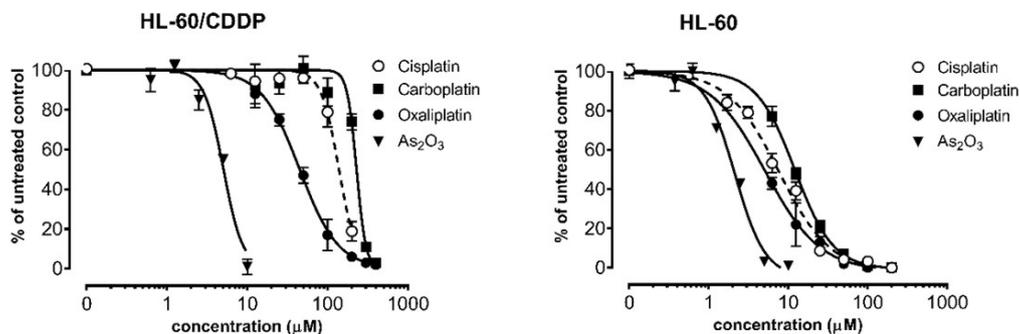


Fig. 1. Comparative cytotoxicity of clinically used platinum-based drugs and As_2O_3 in the original chemosensitive (HL-60) and its newly established resistant variant (HL-60/CDDP)

dependent antioxidant enzyme systems that have been implicated in resistance to platinum-based therapy. The levels of reduced glutathione (GSH) and the activity of several related enzymes: glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) have been measured in both HL-60 and HL-60/CDDP cells. The data presented in Table 2 suggest a nearly two-fold increase in the activities of the GSH-associated enzymes and seven-fold higher GSH levels in the chemoresistant as compared to the chemosensitive variant of the screened AML cell lines.

T a b l e 2

Biochemical characteristics of the HL-60 and HL-60/CDDP cell line subtypes

Parameter/enzymatic activity	HL-60	HL-60/CDDP	Δ (in folds)
Levels of reduced glutathione (nmol/ 10^6 cells)	1.8 ± 0.60	$12.8 \pm 1.90^{**}$	\uparrow 7.1
Glutathione peroxidase (GPx) ($\mu\text{mol}/\text{min}/\text{mg}$)	0.477 ± 0.13	$0.849 \pm 0.06^*$	\uparrow 1.8
Glutathione reductase (GR) ($\mu\text{mol}/\text{min}/\text{mg}$)	0.206 ± 0.03	$0.361 \pm 0.05^*$	\uparrow 1.75
Glutathione-S-transferase (GST) ($\mu\text{mol}/\text{min}/\text{mg}$)	1.5 ± 0.3	$2.8 \pm 0.6^*$	\uparrow 1.9

* $p \leq 0.05$; ** $p \leq 0.01$ against HL-60 (Student *t*-test)

MRP1 expression was also monitored as it is a major component of the mercapturic acid pathway for biotransformation and elimination of electrophilic compounds. In contrast to the HL-60 cells, in which no changes in the expression profile of the efflux transporter were detected, the resistant model showed significant induction of the carrier protein (Fig. 2).

Discussion. To gain insights into the mechanistic aspects of the MDR phenomenon and uncover possible future prospects for chemosensitivity restoration, an in-depth biochemical characterization was performed on both HL-60/CDDP and HL-60 cells in a comparative manner. As can be seen from the presented data, the emergence of acquired resistance as a defense mechanism to the gradual

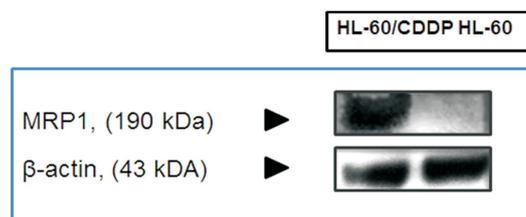


Fig. 2. Electropherogram derived from the conducted Western blot experiment. Protein expression levels were measured by densitometric scanning of the band signals and normalized against β -actin. The observed enhancement in MRP1 expression is specific to the newly established resistant cell line (HL-60/CDDP)

exposure to cisplatin is multifactorial and mediated by complementary pleiotropic cellular mechanisms that neutralize the action of the screened anticancer drugs. A main feature of the resistance phenotype is the alteration in glutathione homeostasis leading to an extensive depletion of the platinum species prior to reaching their DNA target.

Glutathione (GSH) is the major non-protein thiol in mammalian cells. It is a tripeptide (γ -glutamyl-cysteinyl-glycine) where the cysteine SH group undergoes cyclic changes from reduced to oxidized form. As a key player in cellular redox homeostasis, GSH performs a variety of functions including inactivating free radicals and electrophilic xenobiotics [6]. As demonstrated by many research groups, cisplatin-induced resistance in malignant cells often correlates with a substantial increase in cellular glutathione levels [7,8]. Studies on other resistant cell lines have also shown increased γ -glutamylcysteine synthetase activity [6,9].

Resistance to cisplatin in the glutathione pathway can occur by several other mechanisms as well. Conjugation of cisplatin to GSH is primal and determined by the drug's higher binding affinity for the cysteine thiol group of the tripeptide than for the nucleophilic sites of its pharmacological target (nucleotides). Each molecule of the drug reacts with two GSH moieties, and the so formed adducts are stable and pharmacologically inactive [10].

The formation of GSH conjugates with electrophilic substrates is preceded by the activation of the thiolate group and can occur spontaneously or under the catalytic action of glutathione-S-transferases (GST). These enzymes are a family of proteins divided on the basis of homology in their amino acid sequence into seven classes – α (GSTA), μ (GSTM), π (GSTP), τ (GSTT), σ , ε and κ (GSTK). Each class, regardless of its comparative homology, exhibits significant differences in its substrate specificity [6]. Increased activity and/or cellular levels of glutathione-S-transferase (including mRNA for this isoenzyme) have been shown to correlate with decreased susceptibility of malignant cells to cisplatin [11], which

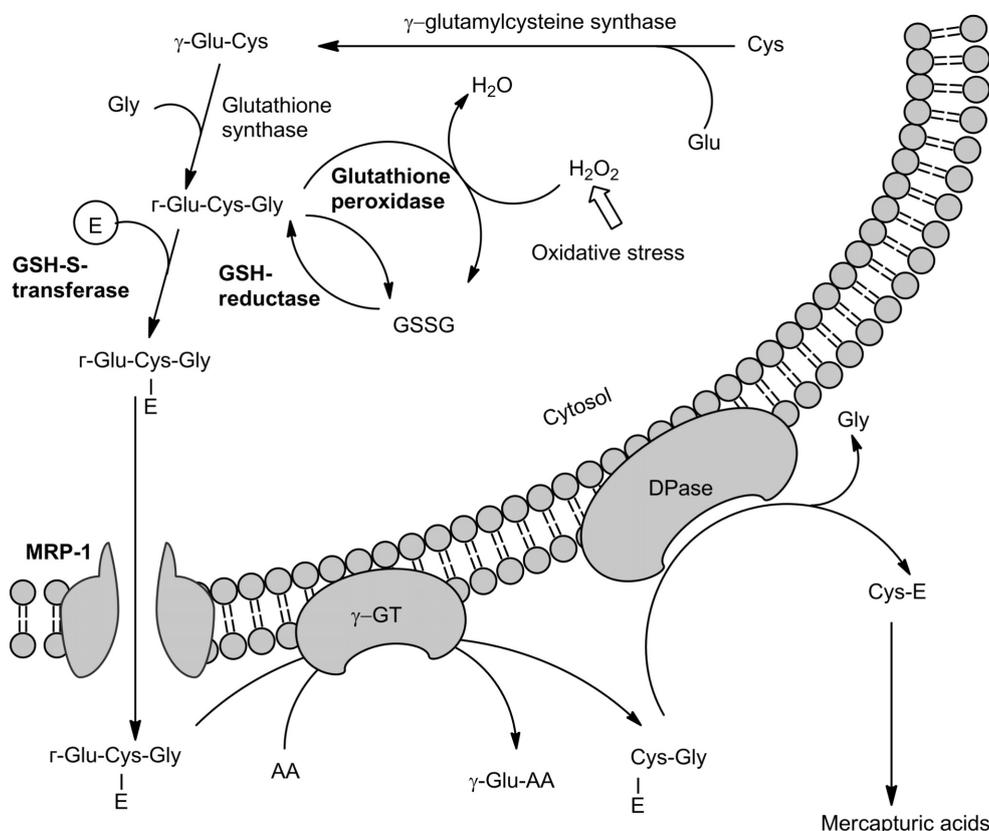


Fig. 3. Hypothetical model of multidrug-resistance patterns in HL-60/CDDP. This cellular test system is characterized by induced activity of the GSH-associated antioxidant defense system, as well as increased expression of the MRP1 transporter, which mediates unilateral efflux of glutathione-conjugated xenobiotics. Given that both metal complexes and metalloids show tropism to thiol groups owing to their electrophilic properties, the established mechanisms most likely govern cellular cross-resistant phenotype to both platinum anticancer drugs and As_2O_3

corresponds to our findings of the induced glutathione-S-transferase activity in the newly established HL-60/CDDP leukemic sub-line.

Glutathione conjugates of electrophilic xenobiotics are extensively removed from cells by various pumps, including MRP1 (multidrug-resistance-associated-protein-1, ABCC1), which has been associated with cisplatin resistance in some types of cervical cancer [4]. On this basis, elevation in MRP1 levels in HL-60/CDDP compared to its original chemosensitive variant, is a major driver to the multiresistant phenotype in this particular AML model. The biochemical characteristics of the newly developed leukemia strain are summarized in Fig. 3.

The MDR phenomenon in the newly developed HL-60/CDDP cell line can be linked to some cellular kinetic aspects of arsenic's pharmacology. For example,

overexpression of MRP1 in various tumour cell lines has been shown to correlate with their low responsiveness to As₂O₃-based differentiation chemotherapy [12]. Adversely, melanoma cells that do not synthesize GSH are characterized by higher sensitivity to the arsenic drug As₂O₃[1]. Despite their differences in pharmacodynamics, both platinum and arsenic derivatives pose similar biochemical characteristics accounting for their high affinity for nucleophilic molecules such as glutathione and other low molecular weight thiols.

Conclusion. Continuous exposure of the chemosensitive HL-60 cells to subtoxic gradually increasing concentrations of cisplatin results in upregulation of the GSH antioxidant enzyme systems and induction of the associated MRP1 efflux pump. This constellation of synergistically acting mechanisms drastically reduces cell chemosensitivity to platinum-based drugs and As₂O₃ and indicates the potential of using this in vitro test system as a validated preclinical model of resistance. On this basis, HL-60/CDDP has been successfully used by our research group [4,13–15] as a test system in a number of oncopharmacological studies on drug susceptibility and the molecular patterns of collateral sensitivity in this particular MDR phenotype.

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