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P-glycoprotein (PGP), and not lung resistance-related protein (LRP), is a negative prognostic factor in secondary leukemias

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Abstract

Background and Objective. **In cell lines, there is an ongoing debate about the role of the lung resistance-related protein (LRP) whereas the role played by P-glycoprotein (PGP) in determining a multidrug resistance is well known. The aim of this study was to evaluate the frequency and the role of a PGP and an LRP overexpression in affecting the intracellular daunorubicin accumulation (IDA) and in predicting the therapy outcome on a subset of overt secondary acute non lymphocytic leukemias (ANLL). An adjunctive point was to evaluate the efficacy of the reversal agent SDZ PSC 833 (PSC) in counteracting impaired IDA.**

Design and Methods. **By flow cytometry, PGP and LRP expression and the IDA were evaluated on 54 overt secondary ANLL. PGP and LRP overexpressions were respectively defined by an MRK-16 mean fluorescence index (MFI) ≥ 6 (PGP^+) and by an LRP-56 MFI ≥ 5 i.e. by MRK-16 and LRP-56 MFIs higher than the one observed in normal leukocytes. The blasts' IDA was studied after a two-hour incubation in 1000 ng/mL daunorubicin in the presence or in the absence of the MDR reversal agent SDZ PSC 833 (PSC) 1.6 µmol.**

Results. **A PGP overexpression was detected in 40/54 (74%) cases while an LRP overexpression was observed on 33/54 (61%) cases. No differences were found in terms of PGP and LRP expressions between ANLL developing after chemo/radiotherapy (therapy-related ANLL) or evolving from a myelodysplastic syndrome (MDS-related ANLL). Compared to the PGP^-, the PGP^+ cases showed a significantly lower mean IDA (DNR NMFI 196±46 vs. 267±53, p < 0.001). The co-incubation of DNR with the PSC significantly increased only the mean IDA of the PGP^+ cases, that grew from a DNR NMFI of 196±46 to a DNR NMFI of 284±67 (p < 0.0001). With respect to normal leukocytes, even the PGP- cases had an impaired IDA suggesting that other mechanisms, including an LRP overexpression, could affect the IDA. A strongly negative correlation was observed between PGP overexpression and therapy outcome, in fact, 8/10 (80%) PGP^- but only 2/27 (7%) PGP^+ patients obtained complete remission (p = 0.0002). Moreover, 7/33 (21%) cases showing an impaired IDA (NMFI < 280) but 4/4 (100%) with NMFI > 280 had complete remission (p=0.006). No correlation was found between therapy response and LRP or CD34 expression.**

Interpretation and Conclusions. **This data suggests that an important role in determining therapy outcome is played by PGP in *secondary* leukemias. Even if the LRP is frequently overexpressed in *secondary* leukemias and is likely to contribute to the reduction of the intracellular drug accumulation, the role played by LRP in determining the therapy-outcome has still to be cleared.**

Key words: P-glycoprotein, lung resistance-related protein, secondary leukemias, chemotherapy, prognosis

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Resistance to chemotherapy still remains a major obstacle limiting the cure of the *de novo* acute non lymphoblastic leukemias (ANLL). The treatment of ANLL is usually based on a combination of cytosine arabinoside (ARA-C) and anthracyclines. These regimens enable obtainment of complete remission in more than 70% of patients, but the initial remissions are rarely sustained. Most of the patients relapse, becoming refractory to further therapy and after 5 years less than 25% of the patients survive.[1] Compared to the *de novo* ANLL, acute leukemias which develop from a myelodysplastic syndrome or after chemotherapy and/or radiotherapy regimens to treat hematological or solid malignancies (*secondary* leukemia), represent a subset of the disease with a particular poor prognosis.[2-5] Only 15-44% of the patients achieve complete remission, often with a short duration (5-23 months). Moreover, secondary leukemias give high death rate during induction therapy, and considering the low rate of durable remission achievable, intensive regimens are often likely to shorten rather than prolong survival.[3-5] The efforts to define the factors able to identify the patients which can take advantage of intensive

chemotherapy have not been successful.[4] Even if resistance of chemotherapy is probably multifactorial, in the past years P-glycoprotein (PGP) has been hypothesized to have an important role.[6-11]

P-glycoprotein is a 170-kd transmembrane protein, encoded by the MDR-1 gene, located in humans on the long arm of chromosome 7.[12] PGP acts as an ATP dependent efflux pump, able to pump drugs out of the cell.[13] A wide range of lipophilic compounds are substrates of PGP, including several anticancer agents, mainly anthracyclines, epipodophyllotoxins and vinca alkaloids.[14] PGP is physiologically expressed in most of the normal tissues even if at different levels. A high level of PGP is observed in liver, kidney, intestine and hemopoietic stem cells.[15-20] The physiological role of PGP in these tissues is not well established, but knockout gene experiments in mice suggest that it could play a defensive role against xenobiotics.[21] In an experimental setting a direct relationship between the protein amount on the membrane, the reduction of the intracellular drug concentration and resistance to cytotoxic drug has been demonstrated.[13]

In the past years, several studies have been performed in attempt to determine its clinical implications. In particular, in *de novo* ANLL, PGP seems to negatively correlate with treatment outcome.[6-11] More recently other proteins able to confer resistance to many structurally and functionally unrelated compounds were identified: the multidrug resistance-associated protein (MRP) and the lung resistance-related protein (LRP). MRP belongs to the ATP-binding cassette family of transporter proteins and, like PGP, is supposed to mediate drug resistance by acting as a drug efflux pump.[22] LRP, also known as the major vault protein, is supposed to provoke resistance by diverting cytostatic drugs from their intracellular target through a different mechanism.[22,23] Vaults are, in fact, complex and highly conserved structures, which are mostly localized in the cytoplasm and in a small fraction in the nuclear membrane. Their physiological function is not completely clear, but they seem to be implicated in the intracellular vesicular traffic, possibly detoxifying the cells by compartmentalizing toxic agents in the cytoplasm. The aims of this study were to verify the incidence of PGP and LRP overexpression in secondary leukemias, their biological implication on the intracellular daunorubicin accumulation (IDA), and their relationship with the therapy response. Another point was to evaluate if the contemporary screening of the two MDR related proteins could help in selecting a group of patients with particularly poor prognosis that could take advantage of more intensive therapy protocols, including reversal agents.

Materials and Methods

Patients

Fifty-four consecutive patients with secondary acute non lymphocytic leukemia admitted at the Division of Hematology, University of Udine, between 1994 and 96 were entered in the study: 16/54 (30%) developed acute leukemia after chemo/radiotherapy for solid or hematological malignancies (therapy-related ANLL) and 38/54 (70%) had a progression after a period of documented myelodysplastic syndrome (6 months at least) (MDS-related ANLL). The median age of patients was 56 years (range 25-72). The karyotype could be evaluated in 26 patients: in 8 of them it was normal, 14 had a 7 or 5 monosomy (3 isolated and 9 associated with complex abnormalities), 1 had the 9;22 translocation, 1 the 7;11 translocation, 1 a monosomy of the chromosome 1, 1 a trisomy of chromosome 2.

After informed consent, 37/54 (68%) patients received the same chemotherapy regimen, consisting in a first course with standard dose cytosine arabinoside and idarubicin (3+7), a second course with high dose cytosine arabinoside and idarubicin and a consolidation course including mitoxantrone, etoposide, cytosine arabinoside (MEC). Response to treatment was defined as follows: death during induction (DDI, death during or after the first induction therapy with aplastic hypocellular marrow, i.e. without evidence of leukemia); complete remission (CR, cellular marrow with less than 5% of blast cells, a neutrophil count > 1.5×10^6/L, platelet count > 100×10^6/L and no evidence of leukemia in other sites) and resistance (cellular marrow with more than 5% blast cells or evidence of leukemia in other sites after at least two courses of chemotherapy). Seventeen out of 54 patients, all aged over 65, did not enter the protocol for clinical reasons (renal or heart failure, 10 patients; refusal, 3 patients; poor performance status, 4 patients).

Normal peripheral and marrow leukocytes

Peripheral blood and marrow samples were obtained after informed consent from 10 healthy volunteers and from 5 bone marrow healthy donors during harvest procedures. The mononuclear fraction was separated on Ficoll gradient, and tested for PGP and LRP expression and for IDA.

Blast cells

Blast cells of the 54 patients with overt secondary acute leukemia, that entered the study were collected from bone marrow during diagnostic procedures. Bone marrow samples were separated on Ficoll, washed twice and tested for PGP and LRP expression and for intracellular daunorubicin accumulation. In all the samples, more than 70% of the cells were blasts. Moreover, to avoid the possible contamination with residual normal leukocytes, analysis was performed after gating blast cells by using scatter parameters. Notwithstanding cell quality was subsequently checked by using a CD3 antibody, which always reacted in all the tested cases with less than 2% of the gated cells.

Cell lines

The T lymphoblastic CCRF-CEM cell line and its PGP overexpressing subline CEM-VLB,[24] and the SW1573 cell line and its LRP overexpressing subline SW1573/2R120[25] were used to check the activity of the specific anti-PGP and anti-LRP antibodies.

PGP and LRP expression

PGP expression was tested in normal peripheral, in normal marrow leukocytes and in blast cells as previously described.[16,26,27] Briefly, after Ficoll sedimentation, 1×10^6 cells of the mononuclear fraction was fixed in PLP (periodate lysine paraformaldehyde) 15' at 4°C, washed twice in PBS and incubated 15' at R.T. with the MRK-16 antibody (Kamiya) at final concentration of 2 µg/mL and with 50 µL of saponin 0.02% (Sigma). After two washes, cells were incubated with 2.5 µL of FITC goat anti-mouse (DAKO), washed twice and analyzed by a flow cytometer (FACScan, Becton Dickinson). LRP expression was performed by using the LRP-56 antibody (Kamiya), according to the manufacturer's indication. In brief, cells were permeabilized by the Facs Lysing solution (Becton Dickinson) for 10', washed twice and incubated with 10 µL of the primary antibody in a PBS-saponin solution (final concentration 1 µg/mL), for 1 hour at 4°C. After two washes, cells were incubated with the secondary antibody as described above, washed and analyzed. Negative controls were prepared for each test by using irrelevant isotypic antibodies. Acquisition and analysis were performed using the Lysis II software (Becton Dickinson). The percentage of positive cells and the intensity of their reaction expressed as the mean fluorescence index (MFI = the ratio between the mean fluorescence of the test sample and that of its isotypic control) were calculated. This method, as well the D value, obtained by the Kolmogorov Smirnov test, defined at the Memphis workshop[28] gives an indirect measure of the amount of protein in the tested cells.

CD34 expression

CD34 expression was evaluated in all the blast samples by using the HPCA-2 PE monoclonal antibody (BD, Bruxelles, Belgium). In brief, after Ficoll separation, 1×10^6 mononuclear cells were incubated with 10 µL of the antibody at room temperature in the dark for 15'. After two washes at least 10000 events were acquired and analyzed by using the Lysis II software. Samples with more than 10% of cells reacting with the antibody were considered $CD34^+$ cases.

Intracellular daunorubicin accumulation

As previously described,[16] 2.5×10^6 blast cells obtained as above, were incubated for two hours at 37°C with 5% CO_2 in medium with daunorubicin (Pharmacia, Italy) 1 µg/mL alone or in presence of the reversal agent SDZ PSC 833 (PSC) 1.6 µM (kindly provided by Sandoz, Basel, Switzerland). At the end of the incubation cells were washed twice in cold PBS, kept in ice and immediately analyzed. Appropriate controls with cells alone or with cells plus PSC 1.6 µM were prepared in each experiment. Daunorubicin accumulation was expressed as the normalized mean fluorescence index (NMFI) according to the method of Luk and Tannock,[29] in which the fluorescence intensity of daunorubicin was corrected with the FSC value to minimize volume modifications due the H_2O exchange through the cell membrane during DNR uptake.

Statistical analysis

Statistical analysis was performed with the Winstat software. Yates' corrected chi-square test, Wilcoxon matched pair test and Mann Withney U test were employed as needed.

Results

Normal leukocytes and cell lines

PGP and LRP expression in normal peripheral and marrow leukocytes and in the MDR cell line systems is shown in Table 1. A high PGP (MFI = 28) and LRP overexpression (MFI = 16.1) was observed in the resistant sublines CEM-VLB and SW1573/2R120, respectively, in over 70% of the cells. On the contrary, only a low expression of PGP and LRP has been shown in the parental CCRF-CEM and SW1573 cell lines, even if the same percentage of cells was stained. The majority (more than 80%) of normal leukocytes reacted with both the antibodies but the intensity of the reaction was always low, only slightly exceeding the one of the parental, non-MDR sublines (CCRF-CEM and SW 1573). An example of the reactivity of the anti-PGP and anti-LRP antibodies in cell lines and in normal peripheral leukocytes is shown in Figure 1. In leukemic patients, to discriminate between non-overexpressing and overexpressing cases only the intensity of the reaction was taken into considera-

Table 1. PGP and LRP expression in normal peripheral leukocytes, in normal bone marrow leukocytes and in MDR and non MDR cell lines.

	MRK-16 MFI median (range)	*LRP-56 MFI median (range)*
Normal peripheral leukocytes	4.8 (2.2-5.5)	3.4 (2.0-4.7)
Normal marrow leukocytes	4.7 (2.5-5.8)	3.7 (2.0-4.9)
CCRF-CEM	4.5 (3.0-5.5)	1.5 (1.2-2.6)
CEM-VLB	28.0 (24.1-30.2)	2.0 (1.3-2.8)
SW 1573	3.5 (2.0-5.0)	2.8 (2.5-4.5)
SW1573/2R120	4.2 (2.3-4.7)	16.1 (12.0-18.1)

MFI (Mean Fluorescence Index) is the ratio between the mean fluorescence intensity of the test sample and its isotypic control.

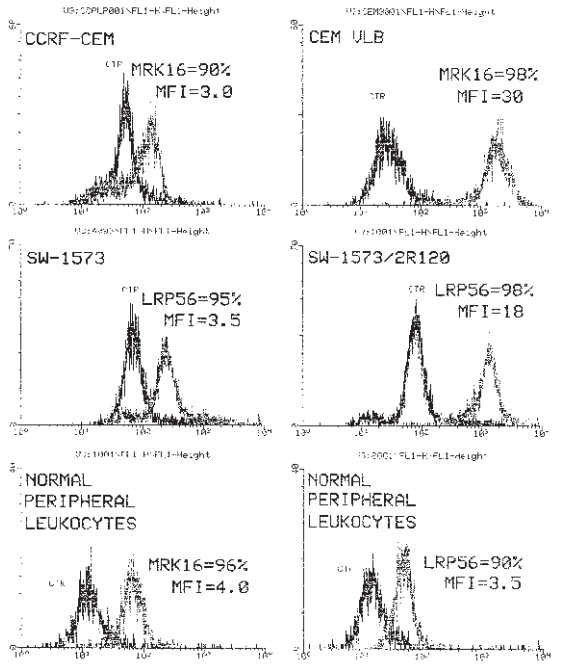

Figure 1. Reactivity of the MRK-16 and of the LRP-56 antibodies in normal leukocytes and in MDR cell lines (MFI = mean fluorescence index).

tion without considering the percentage of positive cells. The cut off was established at the maximum mean fluorescence index (MFI) observed in normal cells and in non-MDR cell lines: MRK-16 MFI ≥ 6 for PGP and LRP-56 MFI ≥ 5 for LRP overexpression.

PGP, LRP, and CD34 expression in blast cells

Pgp expression was evaluated in 54 patients with secondary acute leukemia by flow cytometry using the MRK-16 antibody. In each sample only blast cells were analyzed as described above. More than 80% of the blast cells reacted with the MRK-16 antibody, but compared to normal leukocytes a much wider intensity of the reaction was observed (MRK-16 MFI range 2.7-12.5). Forty out of 54 (74%) patients had an MFI equal or higher than 6 and were considered as *overexpressing PGP* (PGP^+); 14/54 (26%) showed an MFI less than 6 and were considered as *non-overexpressing PGP* (PGP^-). PGP resulted overexpressed in 13/16 (81%) therapy-related ANLL and in 27/38 (71%) MDS-related ANLL (p = 0.51, Yates corrected chi square test). *LRP expression* was evaluated by the LRP-56 monoclonal antibody. Thirty-three out of 54 (61%) patients showed an LRP-56 MFI ≥ 5 and so, they were considered as LRP^+. No differences were seen between MDS or therapy-related cases (22/38 vs. 11/16 respectively, p = 0.55, Yates corrected chi-square test), Table 2. A simultaneous PGP and LRP overexpression was frequently found: 24/54 (44%) were in fact PGP^+/LRP^+, whereas 16/54 (30%) were only PGP (PGP^+/LRP^-), 9/54 (17%) only LRP (PGP^-/LRP^+) and 5/54 (9%) evolved from a previous myelodysplastic syndrome, overexpressed neither PGP nor LRP (PGP^-/LRP^-). The CD34 antigen was expressed in 29/54 (55%) cases, 10 (38%) after chemotherapy and 19 (35%) after myelodysplastic syndrome (p = 0.96, Yates corrected chi square test).

Table 2. PGP and LRP expression in 54 patients of secondary ANLL: 38 MDS- and 16 therapy-related cases.

MDR phenotype	*MDS-related ANLL*	*Therapy-related ANLL*	
PGP^-	11/38 (29%)	3/16 (19%)	p = 0.51*
PGP^+	27/38 (71%)	13/16 (81%)	
LRP^-	16/38 (42%)	5/16 (31%)	p = 0.55*
LRP^+	22/38 (58%)	11/16 (69%)	

**Fisher's exact test.*

Table 3. Flow cytometric evaluation of the intracellular daunorubicin accumulation (IDA) with or without the reversal agent SDZ PSC 833 (PSC) 1.6 µM.

	DNR (NMFI)	*DNR + PSC (NMFI)*	
PGP^-			
mean	267±53	276±55	p = 0.06*
median (range)	269 (187-382)	266 (188-414)	
PGP^+			
mean	196±46	284±67	p < 0.0001*
median (range)	186 (102-269)	275 (196-420)	
	p < 0.001°		

**Wilcoxon matched pair test; °Mann Withney U test. NMFI = normalized mean fluorescence index; DNR = daunorubicin.*

Intracellular daunorubicin accumulation

The intracellular daunorubicin accumulation (IDA) after a two-hour incubation was tested in normal peripheral and marrow leukocytes, and in all of the 54 leukemic patients. In peripheral blood and in normal marrow leukocytes the mean intracellular daunorubicin accumulation expressed as the DNR NMFI was 345±61. In the leukemic cases, the IDA was significantly lower in PGP^+ compared to PGP^- patients (NMFI = 196±46 vs. 267±53, p < 0.001, Mann Whitney U test). However, with respect to normal leukocytes, even in PGP^- patients the DNR intracellular content was always reduced (NMFI = 267±53 vs. 345±61, p < 0.001). In all the PGP^+ patients the reversal agent PSC always increased the IDA more

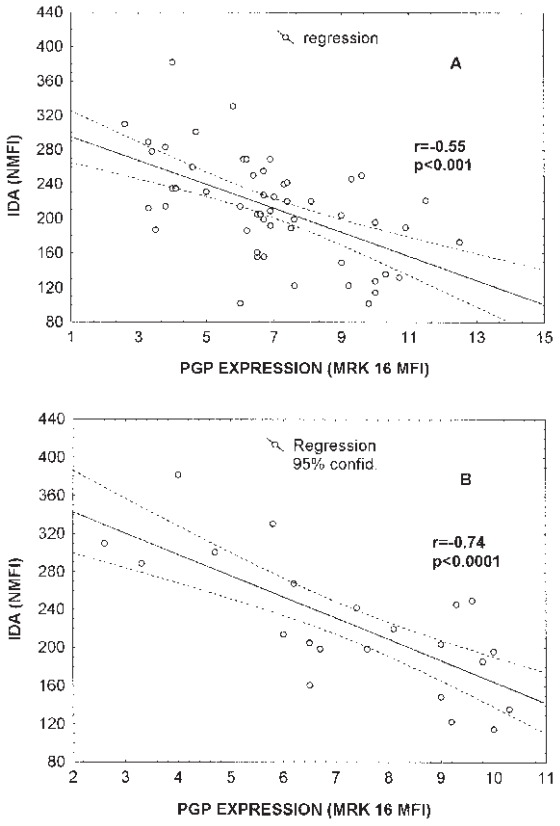

Figure 2. Correlation between the PGP expression (MRK-16 MFI) and the intracellular daunorubicin accumulation (IDA) in all the 54 cases of secondary ANLL (A) and excluding the LRP coexpressing cases (B).
MFI = mean fluorescence index; NMFI = normalized mean fluorescence index.

than 25% respect to baseline values (range 25-150%), without significant differences between only PGP or PGP$^+$/LRP$^+$ cases. The mean IDA rose from a NMFI of 196±46 in absence of PSC to an NMFI of 284±67 in presence of PSC ($p < 0.0001$, Wilcoxon matched pair test). On the contrary, PSC did not significantly affect the intracellular DNR accumulation in the PGP$^-$ cases, even if LRP were overexpressed. The maximum increase was 8% over the baseline value (mean NMFI of 267±53 without, and 276±55 with PSC, $p = 0.06$) (Table 3). A linear correlation was found between PGP overexpression and IDA ($r = -0.55$, $p < 0.001$). This correlation became even stronger leaving out all the LRP co-expressing cases ($r = -0.74$, $p < 0.0001$), Figure 2. On the contrary, LRP overexpression did not correlate with IDA ($R = -0.12$, $p = 0.47$).

PGP, LRP, IDA, CD34 expression and response to therapy

Thirty seven out of 54 patients started the same chemotherapy protocol and the relationship between PGP, LRP and CD34 expression, IDA and the treatment response could be evaluated (Table 4). One patient (overexpressing both PGP and LRP and showing an impaired IDA) died during induction. Ten out of 37 (27%) patients achieved complete remission with a significant difference between PGP$^-$ or PGP$^+$ cases: in fact, only 2/27 (8%) PGP$^+$, but 8/10 (80%) PGP$^-$ achieved complete remission ($p < 0.0001$, Fisher's exact test). No difference in response rates were found based on the LRP expression: in fact, complete remission was obtained in 5/12 (42%) LRP$^-$ and in 5/25 (20%) LRP$^+$ patients ($p = 0.35$, Fisher's exact test). Nevertheless, it must be noted that while 3/3 PGP$^-$/LRP$^-$ evaluable cases obtained complete remission 17/17 PGP$^+$/LRP$^+$ failed to respond to therapy. An impaired IDA significantly affected complete remission, which was reached in only 7/33 (21%) patients with reduced IDA (NMFI < 280) vs 4/4 (100%) with DNR NMFI > 280 ($p=0.003$). Complete remission was obtained in 6/29 (20%) CD34$^+$ and 4/8 (50%) CD34$^-$ cases ($p = 0.22$, Fisher's exact test). The combination of CD34 with PGP or LRP expression did not seem to worsen the therapy outcome. No correlation between complete remission, PGP, LRP overexpression, IDA and other hematological or clinical features, such as age, sex, number of leukocytes or FAB classification were found. Karyotype was unfortunately available in a too small number of patients (only 24/37 evaluable cases) to draw correlation between cytogenetic and the therapy outcome. However, complete remission was achieved in 4/7 patients with normal karyotype and in only 1/12 patients with unfavorable cytogenetic abnormalities, such –7/7q–, –5/5q– or complex alterations, which are frequently detected in myelodysplastic syndromes and in secondary leukemias.

Discussion

In *de novo* acute leukemias, factors affecting remission are quite well characterized. Recently, several authors have investigated the incidence of the mdr-1 gene product in *de novo* ANLL, demonstrating a strong negative correlation between Pgp expression and therapy outcome: a reduction of complete remission rate and a shortening of survival was observed in PGP$^+$ cases.[6-11] Moreover, several authors have suggested that the association between CD34 positivity and PGP overexpression enables the identification of a poorer prognosis cohort of patients.[6] Secondary acute leukemias are usually considered a group of malignancies with extremely poor prognosis: the patients are often resistant to induction therapy and complete remission, if achieved, is rarely sustained.[2-5] However, only sporadic observations have been reported on the prognostic significance of PGP.[6,30-33]

In this paper, the expression of PGP, LRP, CD34 and the IDA were studied in 54 consecutive patients with an overt secondary leukemia developed from an antecedent myelodysplastic syndrome or after radio/chemotherapy for solid or hematological malignancies, admitted for treatment at the Division of Hematology of the

Table 4. Relationship between PGP and LRP expression. Intracellular daunorubicin accumulation (IDA) and response to therapy in 37 cases of secondary ANLL.

	Complete remission	*Primary resistance*	*p**
PGP⁻	8/10 (80%)	2/10 (20%)	0.0001*
PGP⁺	2/27 (7%)	24/27 (89%)	
LRP⁻	5/12 (42%)	5/12 (56%)	0.35*
LRP⁺	5/25 (20%)	19/25 (78%)	
DNR > 280	4/4 (100%)	0/4	0.006*
DNR < 280	7/33 (21%)	25/33 (76%)	

*One patient, both PGP and LRP overexpressing and with low DNR uptake, DDI. *Yates corrected chi square test.*

Udine University between 1994 and 1996. PGP was detected by flow cytometry, using MRK-16 antibody, in fixed and permeabilized blast cells. Even if this antibody is able to recognize an external epitope on the cell membrane, previous studies have reported that the binding site of the antibody can be masked, and needs to be revealed by a permeabilizing or a neuraminidase treatment.[26,34] As previously described, these methods were able to unmask the epitope recognized by the antibody, revealing even small amounts of PGP protein on all the cells. Low levels of the protein were detected in normal peripheral and marrow leukocytes, as well as in blast cells.

The detection of small amounts of PGP on normal blood cells is not so surprising. It is well known, in fact, that PGP has a physiological role in the cell and that its expression can be modulated by environmental factors. Besides, in a previous work, low levels of the MDR-1 gene transcript (comparable to the one found in sensitive subline LOVO 109 and CCRF-CEM) were detectable by Northern blot analysis in the buffy coat of normal blood donors.[26] For this reason, in leukemic patients, overexpressing and non-overexpressing cases were distinguished considering the intensity of the reaction rather than the percentage of cells reacting with the MRK-16 antibody, which was comparable in all the tested cases.

As previously discussed,[35] the mean fluorescence index, exactly as the D value obtained by the Kolmogorov Smirnov statistic,[28] gives an indirect measure of the amount of protein present in the tested cells, and can be applied on fixed and permeabilized cells. The maximum MFI observed in the normal peripheral or marrow cells was chosen as a cut-off point to discriminate between overexpressing and non-overexpressing cases.[16,26,27] By this method, an overexpression of PGP could be detected in 74% of the patients (40/54), 13/16 (81%) after chemotherapy and 27/38 (71%) after myelodysplastic syndromes. The high frequency of PGP overexpression in secondary leukemias was consistent with the observations of Campos *et al.*,[6] Guerci et al.,[30] Lepelley *et al.*,[31] Sato *et al.*,[32] and Leith *et al*.[36] Lepelley *et al*.[31] studied PGP expression in low and high risk myelodysplastic syndromes, demonstrating that a high percentage of positive cells was already detectable in cases with a high probability of leukemic evolution, whereas the low risk cases only rarely contained PGP⁺ cells. On the basis of this observation, they suggested that the occurrence of PGP expression could be a late event in disease progression. In myelodysplastic syndromes, Sonneveld *et al.*[33] and List *et al*.[37] demonstrated an association between PGP and the expression of immature markers, such as CD34 and Tdt, and that these cells were responsible for disease progression.

In our series of overt secondary acute ANLL, we could demonstrate neither a different proportion of CD34⁺ blast cells in PGP positive and negative cases nor an association between CD34 expression and therapy outcome. It is well known that in cell lines, other proteins have been supposed to cause an MDR. Among these, the LRP, bearing a striking identity with the major vault protein mVp-α, was recently identified in a drug selected lung cancer cell line lacking a PGP overexpression but showing impaired intracellular drug accumulation and an atypical MDR.[38] Data on LRP ability in provoking a MDR is not complete, however, transfection experiments with full length LRP cDNA suggested that the LRP transfection itself was insufficient to cause an MDR.[38,39] The incidence of a high LRP expression in acute leukemias has been mainly investigated by using immunohistochemical methods. In this work, the expression of LRP in blast cells of secondary leukemias was tested by flow cytometry using the LRP-56 antibody. The same method for PGP was employed to discriminate overexpressing and non overexpressing cases. An overexpression of LRP was found in 61% of the tested cases without significant differences between cases arising from an antecedent hematological disorder or cytotoxic therapy. This data is consistent with List *et al.*[40] who, employing an immunohistochemical method, found an LRP overexpression in 48% of the secondary leukemias tested. In our study, 44% of cases (24/54) co-expressed the two proteins while only 5/54 patients did not overexpress either PGP or LRP. A biological implication of PGP but also of LRP overexpression is suggested by the *in vitro* evaluation of the IDA. Compared to normal leukocytes, an impaired IDA (DNR NMFI < 280) was found in not only all the PGP⁺ cases, but also in LRP⁺ ones. On the contrary, PSC did not increase the DNR uptake in PGP⁻ cases. The lower DNR uptake observed on the LRP overexpressing cases was already shown in leukemic patients MRP and PGP non overexpressing and, as previously reported,[35] it is likely that it could contribute to justify the weak correlation found between the MRK-16 MFI and the DNR accumulation ($r = -0.55$). In fact,

if all the LRP overexpressing cases were excluded from the analysis, the correlation between PGP expression and IDA would become much stronger (r = –0.74), confirming the relationship between PGP amount and daunorubicin accumulation already found by us in chronic myeloid leukemias[16] and more recently by Broxterman *et al.*[41] in acute leukemias. The reversal agent PSC always significantly increased the IDA, without differences between the only PGP^+ and PGP^+/LRP^+ cases.

The prognostic significance of a PGP overexpression is quite well known in *de novo* ANLL.[7-11,36,42] Our data confirms the negative prognostic role of PGP overexpression and of a low IDA also in secondary leukemias: in fact, only 8% of the PGP^+ patients, and only 21% of the patients with impaired IDA entered complete remission. The role of LRP overexpression in a clinical setting is still unclear. List *et al.*[40] investigated LRP in 69 cases of leukemia, including not only *secondary,* but also *de novo* leukemias and chronic myeloid leukemias in blastic phase, and found a negative correlation between the protein overexpression and the therapy outcome. Borg *et al.*[43] found a significant difference in remission rate between LRP^- and LRP^+ in 72 patients with ANLL at presentation. te Boeckhorst *et al.*[44] drew the same conclusion from the study of the LRP expression in 35 untreated ANLL.

On the contrary, Sonneveld *et al.*[45] analyzed the PGP and LRP expression in a group of untreated ANLL, concluding that, differently from PGP, LRP has no predictive value for response to chemotherapy. In our *secondary* leukemias an isolated LRP overexpression did not prevent the achievement of complete remission. However, it must be understood that none of the patients who had contemporary PGP and LRP overexpression obtained complete remission. Like Sato *et al.*[32] and Campos *et al.*[6] we did not find correlations between therapy outcome and clinical features at the onset of the disease. Karyotype was available in only 24 evaluable cases, so that statistical analysis was not performed. The remission rate appeared to be conditioned only by PGP overexpression and was significantly lower than in primary acute leukemias.

In conclusion, this data suggests that an important role in determining therapy outcome is played by PGP also in secondary leukemias. LRP is frequently overexpressed in secondary leukemias and probably contributes in reducing the amount of the drugs able to target the nucleous. However, its role in determining a decrease of IDA and the prognosis has still to be cleared. Further studies in larger series of cases are required to better understand the weight of LRP in clinical multidrug resistance and to identify molecules able to counteract its function.

Contributions and Acknowledgments

DD and MM were the principal investigators, designed the study, analyzed the data and wrote the paper. PM, AM, and DR carried out the flow cytometric assay. AE, AC, AG, RS and SG followed the patients clinically. RJS and MB contributed to the writing of the paper and gave the final approval of the version to be published.

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Disclosures

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