# FLOW-CYTOMETRIC STUDY OF MULTIDRUG RESISTANCE AND APOPTOSIS IN PATIENTS WITH MALIGN HEMOPATIES

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***Abstract:*** *We aimed to studying the level of apoptosis and multidrug resistance in our patients with malign hemopathies and at studying the possibility of influencing these, by using a statin and the verapamil. The therapy with statin and verapamil was generally well tolerated, without major side effects.*

***Keywords:*** *drug resistance, malign hemopathies, flow cytometry*

***Rezumat:*** *Ne-am propus să studiem nivelul apoptozei şi prezenţa rezistenţei multimedicamentoase la pacienţii noştri cu hemopatii maligne şi să studiem posibilitatea de a le influenţa folosind o statină şi Verapamilul. Terapia pacienţilor noştri cu Simvastatin şi cu Verapamil a fost în general bine tolerată, fără efecte adverse majore.*

***Cuvinte cheie:*** *rezistenţă medicamentoasă, hemopatii maligne, flowcytometrie*

## INTRODUCTION

Besides the hypolipemiant effect, by blocking the cholesterol biosynthesis, statins have immunomodulatory, anti-inflammatory, antiangiogenic and antiproliferative effects, through the reduction of proteins isoprenilation, involved in the transduction of the cellular signal, such as Ras and RhoA (1).

Farnesyltransferase catalyses the transfer of farnesyl groups towards the terminal cysteine of the proteic sublayer, that is Ras proteine – signal-transductor G protein. It requires the alteration of prenyl lipids and of membranes for the signals transduction. The alteration involves the covalent addition, either of farnesyl or of geranylgeranyl, a process catalyzed by Farnesyltransferase, respectively by geranylgeranyltransferase. The last process is inhibited by the farnesyltransferase inhibitors. Both processes are inhibited by lovastin, through the inhibition of the mevalonate production that deprives the isoprenoids cells (2). Simvastatin, another competitive inhibitor of 3-hydroxi-3-metilglutaril coenzyme A reductase, has antineoplasic effects, too. It was proved that the B clonal lymphocyte exposure coming from the patients with chronic lymphatic leukaemia to simvastatin significantly decrease their viability through the induction of apoptosis (3).

An important objective in the malign hemopaties therapy is the achievement of the reversion of the multidrug resistance through the P glycoprotein (P-gp) modulation. Such non-toxic modulators of the P glycoprotein are: verapamil, cyclosporine A and PSC 833, which were studied in acute leukaemia and in the multiple myeloma (4).

We aimed at studying the level of apoptosis and multidrug resistance in our patients with malign hemopathies and at studying the possibility of influencing these, by using a statin and the verapamil.

## MATERIAL AND METHOD

We accomplished a transversal study that included all the patients (33) hospitalized in the Hematology Section of the County Clinical Emergency Hospital of Sibiu, diagnosticated with chronic lymphatic leukaemia, non-hodgkin malign lymphomas with peripheral discharge, multiple myeloma and acute leukaemia, whose clinical situation allowed taking biological samples and the administration of drugs and who gave their consent for participating in this study. The following biological samples were taken from all patients in the morning, a jeun: hemoleucogramme, glycaemia, creatininemia, ALAT, ASAT, bilirubinemia, cholesterolemia and triglyceridemia. Moreover, a sample of the peripheral venous blood was taken in an EDTA test tube, except for those patients with multiple myeloma, cases in which medullar juice was taken (through medullar puncture aspiration), from which annexin V (a marker of apoptosis) and P glycoprotein (a marker of the multidrug resistance) were dosed. Dosing the annexin V and P glycoprotein was made with the help of a flow cytometre, CYTOMICS FC500, which may recognize 4 different fluorochromes. The protocol for annexin V for the patients with chronic lymphoproliferation (except for the patients with multiple myeloma) was the following: annexin V-FITC, PIPE, CD20-ECD, CD5-PC5, and for those with multiple myeloma: annexin V-FITC, PIPE, CD45-ECD, CD38-PC5. The protocol for the

establishment of the P glycoprotein in the patients with chronic lymphoproliferation (except for the patients with multiple myeloma) was: CD45-FITC, CD243-PE, CD20-ECD, CD5-PC5, while in the patients with multiple myeloma: CD45-FITC, DE243-PE, CD38-PC5. The patients were treated with verapamil 40 mg x 2 /day, for three days if they were hypertensive and did not register any counter indication to this drug (the sub-batch V), and the rest of them with simvastatin 120 mg / day, if they did not register any counter indication for statin (sub-batch S). Three days after, they were recalled and annexin V, P glycoprotein and the biochemical tests were dosed again. The possible side effects were noted and they were supervised until they have disappeared. Multidrug resistance and the level of the neoplasic cells were analysed according to diagnosis, period of time from the establishment of the diagnosis, age and gender. The effect of verapamil and simvastatin were subsequently analysed regarding the induction of apoptosis and the P glycoprotein expression. The results were statistically analysed, by using the SPSS programme. Testing the equality of the samples pairs averages was made with Paired-Samples T Test – a procedure that is applied in the case of the dependent samples (it compares the averages for one single group observed in different moments). Interpretation: if $p \leq 0,01$, it may be established with a precision of 99%, that the average of the different pairs significantly differs from 0; if $p > 0,05$, it cannot be said that the average of the pairs differences significantly differs from 0.

## RESULTS

The total batch included 33 patients with the average age between 64,82 +/- 10,63 years. The distribution per gender was the following: 14 women and 18 men. Their diagnoses were; chronic lymphatic leukaemia– 15 patients, non-hodgkin malign lymphoma – 9 patients, refractory anemia with excess of blasts in transformation – 2 patients and multiple myeloma – 7 patients. The average length of survival after the diagnosis was of 27,70+/-26,78 months. On average, they made 1,48+/-1,3 therapeutic lines. Out of the total number, 8 patients were treated with verapamil and 23 with simvastatin; 2 patients registered counter indications to both drugs; in these two cases, only the initial parameters were established. In the case of 2 patients, the sample for establishing the annexin was damaged and in the case of one patient, that for testing the p glycoprotein.

The patients of the sub-batch treated with verapamil (V) were aged below 66,25+/-7,5, the average length of survival from the date of the diagnosis was of 24,63+/-18,65 months; they were submitted to an average number of 1,75+/-1,17 therapeutic lines and had the following diagnoses: chronic lymphatic leukaemia – 6 patients, non-hodgkin malign lymphoma – 1 patient, and multiple myeloma – 1 patient.

The patients of the sub-batch treated with simvastatin (S) had the average age of 65,78+/-10,91 years; the average length of survival from the data of diagnosis was of 28,09+/-30,39 months; they were submitted to an average number of 1,17+/-1,11 therapeutic lines and had the following diagnoses: chronic lymphatic leukaemia – 8 patients, non-hodgkin malign lymphoma – 8 patients, refractory anemia with excess of blasts in transformation – 1 patient and multiple myeloma – 6 patients.

The average values of the hemoleucogramme parameters are listed in the table below:

**Table I: Results of the hemoleucogramme**

| BATCH | | Hb | Ht | L | Tr |
|---|---|---|---|---|---|
| Without treatment | Number | 2 | 2 | 2 | 2 |
| | Average | 10.150 | 30.5000 | 21305.00 | 32250.00 |
| | Standard deviation | 5.869 | 17.6777 | 26438.72 | 44901.28 |
| S | Number | 23 | 23 | 23 | 23 |
| | Average | 12.383 | 38.0022 | 19267.83 | 175434.78 |
| | Standard deviation | 1.385 | 3.7904 | 25199.58 | 86630.26 |
| V | Number | 8 | 8 | 8 | 8 |
| | Average | 11.800 | 36.8375 | 30525.00 | 209875.00 |
| | Standard deviation | 1.859 | 5.6442 | 33733.51 | 97780.27 |
| Total | Number | 33 | 33 | 33 | 33 |
| | Average | 12.106 | 37.2652 | 22120.30 | 175106.06 |
| | Standard deviation | 1.862 | 5.4688 | 27035.08 | 94296.39 |
| p Fisher test | | 0,237 | 0,173 | 0,612 | 0,053 |

**Table II: Results of the biochemical tests in the entire analysed batch and the statistic interpretation of the observed variations**

| Variable | | N | Average | Standard deviation | T Test | p |
|---|---|---|---|---|---|---|
| LOTUL TOTAL | | | | | | |
| Pair 1 | CHOLESTEROL1 | 31 | 197.03 | 48.01 | 4.481 | 0.000** |
| | CHOLESTEROL2 | 31 | 174.74 | 53.32 | | |
| Pair 2 | TRIGLYCERIDE1 | 31 | 148.16 | 134.65 | 0.576 | 0.569 |
| | TRIGLYCERIDE2 | 31 | 137.84 | 99.54 | | |
| Pair 3 | CREATININE1 | 31 | 1.0065 | 0.3184 | 2.461 | 0.020* |
| | CREATININE2 | 31 | 0.8935 | 0.2634 | | |
| Pair 4 | ASAT1 | 31 | 20.81 | 8.92 | -2.049 | 0.049* |
| | ASAT2 | 31 | 22.58 | 9.98 | | |
| Pair 5 | ALAT1 | 31 | 25.19 | 12.42 | 1.744 | 0.091 |
| | ALAT2 | 31 | 22.71 | 13.22 | | |
| Pair 6 | GLYCEMIA1 | 31 | 107.10 | 39.30 | -0.399 | 0.693 |
| | GLYCEMIA2 | 31 | 109.48 | 34.76 | | |
| Pair 7 | LIPIDEMIA1 | 31 | 688.81 | 191.20 | 2.782 | 0.009** |
| | LIPIDEMIA2 | 31 | 627.26 | 193.01 | | |

A decrease of cholesterolemia, lipidemia and creatininemia can be observed in the entire batch.

Cholesterolemia and lipidemia significantly decreased after three days of simvastatin treatment. Moreover, a reduction of creatininemia was recorded in the sub-batch S, as well. ASAT increase in this sub-batch is at the limit of the statistic significance and does not exceed the normal values, while ALAT registered an

insignificant reduction. Early and late apoptosis significantly increased after the simvastatin treatment, but the expression of P glycoprotein did not change after the therapy. Regarding the sub batch treated with verapamil, there could be noticed a slight increase of glycaemia, but 2 patients did not observe the indications.

**Table III: The Results of the biochemical tests, of annexin dosage (early apoptosis –EP and late apoptosis – LT) and P glycoprotein (PGP) in the two sub-batches.**

| Variable | | N | Average | Standard deviation | T Test | p |
|---|---|---|---|---|---|---|
| SUB-BATCH S | | | | | | |
| Pair 1 | CHOLESTEROL1 | 23 | 192.17 | 41.95 | 6.081 | 0.000 ** |
| | CHOLESTEROL2 | 23 | 166.83 | 45.91 | | |
| Pair 2 | TRIGLYCERIDE1 | 23 | 150.13 | 151.35 | 0.730 | 0.473 |
| | TRIGLYCERIDE2 | 23 | 132.74 | 107.47 | | |
| Pair 3 | CREATININE1 | 23 | 1.0009 | 0.3144 | 3.005 | 0.007 ** |
| | CREATININE2 | 23 | .8578 | 0.2413 | | |
| Pair 4 | ASAT1 | 23 | 21.35 | 10.08 | -2.075 | 0.050 * |
| | ASAT2 | 23 | 23.57 | 11.22 | | |
| Pair 5 | ALAT1 | 23 | 26.09 | 14.08 | 1.224 | 0.234 |
| | ALAT2 | 23 | 23.87 | 14.53 | | |
| Pair 6 | GLYCEMIA1 | 23 | 109.48 | 40.87 | 0.284 | 0.779 |
| | GLYCEMIA2 | 23 | 107.30 | 31.70 | | |
| Pair 7 | LIPIDEMIA1 | 23 | 671.96 | 193.96 | 2.612 | 0.016 * |
| | LIPIDEMIA2 | 23 | 597.74 | 189.17 | | |
| Pair 8 | AP1 | 20 | 0.1030 | 0.1154 | -3.055 | 0.007 ** |
| | AP2 | 20 | 1.0230 | 1.3596 | | |
| Pair 9 | AT1 | 19 | 6.158E-02 | 4.018E-02 | -3.423 | 0.003 ** |
| | AT2 | 19 | 0.3758 | 0.3899 | | |
| Pair 10 | GPP1 | 21 | 0.1405 | 0.2095 | 1.436 | 0.167 |
| | GPP2 | 21 | 6.524E-02 | 0.1524 | | |
| SUB-BATC V | | | | | | |
| Pair 1 | CHOLESTEROL1 | 8 | 211.00 | 63.62 | 0.873 | 0.411 |
| | CHOLESTEROL2 | 8 | 197.50 | 69.02 | | |
| Pair 2 | TRIGLYCERIDE1 | 8 | 142.50 | 75.26 | -0.881 | 0.408 |
| | TRIGLYCERIDE2 | 8 | 152.50 | 76.39 | | |
| Pair 3 | CREATININE1 | 8 | 1.0225 | 0.3516 | 0.230 | 0.825 |
| | CREATININE2 | 8 | 0.9963 | 0.3130 | | |
| Pair 4 | ASAT1 | 8 | 19.25 | 4.27 | -0.370 | 0.722 |
| | ASAT2 | 8 | 19.75 | 4.40 | | |
| Pair 5 | ALAT1 | 8 | 22.63 | 5.26 | 1.642 | 0.145 |
| | ALAT2 | 8 | 19.38 | 8.23 | | |
| Pair 6 | GLYCEMIA1 | 8 | 100.25 | 35.98 | -2.700 | 0.031 * |
| | GLYCEMIA2 | 8 | 115.75 | 44.28 | | |
| Pair 7 | LIPIDEMIA1 | 8 | 737.25 | 186.60 | 1.050 | 0.329 |
| | LIPIDEMIA2 | 8 | 712.13 | 189.99 | | |
| Pair 8 | AP1 | 8 | 0.1100 | 0.1322 | -2.001 | 0.085 |
| | AP2 | 8 | 1.6938 | 2.3561 | | |
| Pair 9 | AT1 | 8 | 6.375E-02 | 5.579E-02 | -2.108 | 0.073 |
| | AT2 | 8 | 0.7125 | 0.8521 | | |
| Pair 10 | GPP1 | 8 | 0.1275 | 0.1644 | 0.822 | 0.438 |
| | GPP2 | 8 | 6.125E-02 | 0.1212 | | |

It may observed that only the difference between the early initial apoptosis and that after the treatment, of the patients of the batch V is correlated inversely to the age of the patients.

Simvastatin and verapamil were generally well tolerated. Only one patient, treated with simvastatin, presented soft stools, but she has had such signs previously, too, in the absence of statin, so the relation with this drug is still disputable. There was another patient who complained about dizziness, but she was known with vertebrobasilar circulatory insufficiency.

**Table IV: The analysis of the correlation between apoptosis and age, the survival after the diagnosis and the number of therapeutic lines**

| VARIABLE | | AGE | SURVIVAL AFTER THE DIAGNOSIS | NO. OF THERAPEUTIC LINES |
|---|---|---|---|---|
| STATINS BATCH | | | | |
| DIF early apoptosis | N | 21 | 21 | 21 |
| | Pearson Correlation | 0.316 | 0.260 | 0.134 |
| | p | 0.163 | 0.255 | 0.561 |
| DIF late apoptosis | N | 21 | 21 | 21 |
| | Pearson Correlation | 0.071 | 0.200 | -0.009 |
| | p | 0.759 | 0.384 | 0.969 |
| VERAPAMIL BATCH | | | | |
| DIF early apoptosis | N | 8 | 8 | 8 |
| | Pearson Correlation | -0.763 | -0.217 | 0.007 |
| | p | 0.028* | 0.605 | 0.987 |
| DIF late apoptosis | N | 8 | 8 | 8 |
| | Pearson Correlation | -0.199 | 0.140 | 0.321 |
| | p | 0.637 | 0.740 | 0.438 |

## DISCUSSIONS

Most of the patients with multiple myeloma respond to chemotherapy initially, with the decrease of the number of the medullar plasmocytes and the reduction of the level of monoclonal immunoglobulin, but in most of the cases, the disease reoccurs and becomes refractory to treatment (5).

One of the statins – Lovastatin – was proved to be an attractive option, whose target is Ras, due to the fact that it may inhibit the process of isoprenilation in myelomatous cell lines. Moreover, it was proved that Lovastatin induces apoptosis through the inhibition of gernylgeranylation (2, 6), rather than of the farnesylation (2), possible through the regulation of Mcl-1 (2, 6), which is a critical factor of survival for the myelomatous cells. It also may lead to the reversion of the drug resistance mediated through the cellular adhesion in the studied cellular lines (2).

Ras protein, whose farnesylation is inhibited by statins, registers mutations in the multiple myeloma, frequently.

Statins also inhibit the production of IL-6, a key cytokine in the multiple myeloma. That is why, it is considered that they may have antiproliferative and/or pro-apoptotic properties in this malign hemopathy. The exposure of the myelomatous cell lines to simvastatin brought about the decrease of their viability, as a result of the monthly cycle arrest and of the cellular death. Calcium cytoplasmic levels were significantly reduced in all studied cellular lines and the secretion of IL-6 of the U266 cells was cancelled by the simvastatin treatment (7). In vitro, statins inhibit apoptosis in the myelomatous and lymphomatous cells in a dose and time-dependent manner (8).

In another study made in vitro, the majority of the examined myelomatous cells (12 out of 13) presented an inhibition of the increase due to simvastatin, inhibition that was dose-dependent. Simvastatin combined with other therapeutic agents such as: ATRA or dexamethasone has additional effects on the increase. The myelomatous cells treated with simvastatin present an inactivation of different mechanisms of MAP-kinase type, such as: ERK1/2, MEK1/2, JNK and p38. Based on these findings, it is considered that statins can be an option for their use in clinics, in the maintenance treatment of the patients with multiple myeloma (9). Multicentric studies are in development regarding the Bcl-2 oligonucleotide antisens and with large doses of simvastatin, in combination with chemotherapy in the pre-treated patients with multiple myeloma (6).

Together with dexamethasone and doxorubicin, stains have chemostabilizing effect. In a recent study, 28 patients with multiple myeloma or lymphoma were treated with simvastatin for 7 days, followed by VAD (for the multiple myeloma) and CHOP (for the patients with lymphoma). The tolerated maximum dose of Simvastatin was of 15 mg/kg day. Most frequent reported side effects were: fatigue, gastrointestinal disorders and neutropenic fever. Dose-limited toxicity was the neutropenic septicaemia and the gastrointestinal effects of stage 3 (8).

Leukemic and myelomatous cells exposure to toxic minimal concentrations of 7-hidroxistaurosporin (UCN-01) and varied statins (lovastatin, simvastatin or fluvastatin) dramatically increase the mitochondrial dysfunction and the activation of caspases and apoptosis. Comparable effects were also noticed in other leukemic and myelomatous cell lines, as in the blasts of the primary myeloid acute leukaemia, but not as in the normal hematopoietic cells. UCN-01 lethality increase by lovastatin was associated to the perturbation of the prenylation and Ras activation. These events were significantly attenuated by farnesyl pyrophosphate and not by geranylgeranyl pyrophosphate, involving perturbations of farnesylation rather than of the gernylgeranylation in synergic interactions. The co-exposure to statins and UCN-01 brought about the inactivation of ERK1/2 and Akt, accompanied by JNK activation (10).

In a recent study, it was noticed that some of the natural statins (lovastatin, simvastatin) and some of the synthetic ones (cerivastatin and atorvastatin) exercise cytotoxic effects on the T, B tumoral cells and on the myelomatous ones, promoting the apoptosis. Cerivastatin promptly activated the cellular death even in the cellular lines resistant to doxorubicin, while pravastatin, a hydrophilic compound, did not have any effect on proliferation or apoptosis. The mechanism of statins-induced apoptosis in these cellular lines was probably regulated through the alteration of Ras or RhoA prenylation. Statins-induced apoptosis involved mitochondria, whose membranary potential was reduced and the cytosolic release of secondary activator of caspases derived from mitochondria. The mechanism of apoptosis was caspases-dependent, due to the fact that caspases 9,3 and 8 were efficiently activated. Thus, it was proved that statins, in association with the conventional therapy, could be used as trigger agents of apoptosis in these tumours (1).

Regarding our study, statins therapy led to a significant increase of the early and late apoptosis of the analysed malign tumours. The fact that verapamil influenced neither apoptosis nor the multidrug resistance, may be related to the dosed used. In future, a larger dose would be indicated, as well as a retard product under strict control of the individual tolerance to that drug,

Among the medullar myelomatous cells, P-gp was absent or registered low levels in the patients with untreated multiple myeloma, but it was expressed in large levels in the case of the patients previously treated with chemotherapy. Regarding the previously untreated patients, the majority of lymphocytes B / plasmocytes that express P-gp are located in the mononuclear cells of the peripheral blood and not in the bone marrow, so that the B peripheral lymphocytes could be the predominant set of tumoral cells resistant to chemotherapy. From the flow cytometry point of view, it was proved that P-gp is functional in these cells and may be efficiently blocked by veramil or ciclosporin A (5).

Extending the life of the monoclonal lymphocytes of the chronic lymphatic leukaemia with B cells is due to the programmed cellular death. There are proofs that P-gp plays an important part in regulating the apoptosis induced by varied stimuli. A group of Polish authors noticed that there is an inverse relation between P-gp expression and the apoptotic index, 24 hours after the cultivation of cells from the patients with B chronic lymphatic leukaemia, suggesting that P-gp expression plays a quite protective part on the survival of these cells in vitro. The apoptotic index was larger in women as against the men (11).

P-gp may be modulated with blockers of the calcium channels (12). Thus, verapamil may block the multidrug resistance (13). In an in vitro study, verapamil produced the cellular death, dose and time-dependent of the cells of the chronic lymphatic leukaemia B, through apoptosis, evidenced through the annexin positiveness and condensation of the cells treated with verapamil. A significant effect was obtained with at least 4 microM. Verapamil increased effectively the toxicity of cytosporin,

chlorambucil, 2-clorodeoxiadenozinei, cisplatin, fludarabin, prednisolon, adriamycin and vincristin. It results that the blockers of the calcium channels increase the P-gp-independent anticancerous drugs. The initial signals of calcium depletion and anticancerous drugs facilitate the cellular death (12).

The therapy of our patients with simvastatin and with verapamil was in general well tolerated, without major side effects.

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