Promising anti-leukemic activity of atorvastatin

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Received November 13, 2012; Accepted February 4, 2013

DOI: 10.3892/or.2013.2301

Abstract. There is a current need for novel therapeutic strategies for the treatment of chronic lymphocytic leukemia (CLL), a still incurable hematological cancer involving mainly deregulated apoptosis. The purpose of the present study was to determine ex vivo the effect of the synthetic statin, atorvastatin, a known cholesterol-lowering drug, on peripheral blood mononuclear cells obtained from CLL patients. Using flow cytometry, we investigated the viability and induction of apoptosis in leukemic cells exposed to statin by the Vybrant Apoptosis Assay kit #4, compared with untreated control cells. We also examined the expression levels of apoptosis-regulatory proteins (Mcl-1, Bcl-2 and Bax), as well as products of the expression/proteolysis of lamin B, poly(ADP-ribose)polymerase-1 (PARP-1) and p27Kip1 by western blot analysis. Moreover, the number of sub-G1 cells and DNA fragmentation in atorvastatin-treated leukemic cells were examined by flow cytometry and agarose gel electrophoresis, respectively. The obtained results indicated that CLL cells ex vivo were extremely sensitive to atorvastatin. The cytotoxic effect of this statin was caused by the induction of apoptosis in the leukemic cells. The induction of apoptosis in the drug-treated model cells was confirmed by the reduction or proteolysis of apoptotic markers, such as PARP-1, lamin B and p27Kip1, the increase in the number of sub-G1 cells and DNA ladder formation. During atorvastatin-triggered apoptosis, changes in the expression levels of mitochondrial outer membrane permeability regulatory proteins of the Bcl-2 family were also observed. Ex vivo promising data indicate the strong cytotoxic and pro-apoptotic potential of atorvastatin against leukemic cells, but not normal cells. The obtained data suggest that atorvastatin be considered as a therapeutic option for the treatment of CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent type of adult leukemia in western countries. The disease is heterogeneous as regards prognosis and clinical outcome and usually affects people over the age of 60. The median age at diagnosis is 72 years (1). Although significant progress has recently been made in the treatment of CLL, the disease remains incurable (2,3).

The limited efficacy of CLL anti-leukemic therapy may be associated with the particular nature of this type of cancer, which is characterized by the accumulation of apoptosis-defective leukemic cells in the blood, bone marrow and lymph nodes of patients. Transformed cells circulating in the peripheral blood of individuals who suffer from CLL are arrested in the G0/G1 phase, whereas bone marrow, lymph nodes and lymph nodules in peripheral lymphoid tissue may serve as reservoirs of cells which divide and supply blood with the accumulating pool of cells (4,5). Thus, effective therapeutic approaches should be directed toward resting cells in peripheral blood as well as toward the proliferating pool of cells in the germinal centers of lymphocytes.

Statins are well-known drugs commonly used in the treatment of hypercholesterolemia (6). They block the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to L-mevalonic acid by the competitive inhibition of HMG-CoA reductase, a key enzyme in the cholesterol biosynthesis pathway. Statins simultaneously inhibit the synthesis of all intermediates downstream of mevalonate, i.e., farnesyl pyrophosphate or geranyl pyrophosphate. Consistently, they prevent protein prenylation leading to the modulation of their cellular localization and function. The statin-mediated inhibition of small G protein modification is thought to be responsible for the cholesterol level-independent anti-proliferative activity of these drugs in a variety of human cancer cell lines (7). Additionally, these drugs can induce apoptosis in rapidly proliferating cells, as well as in cells which do not proliferate; however, the mechanism behind this activity remains unclear (8-10). Taking these facts into consideration, statins may prove particularly useful in the treatment of CLL. Moreover, many years of clinical...
experience with statins have confirmed their safety and low toxicity against normal cells (11).

In the present study, we evaluated ex vivo the anti-leukemic potential of atorvastatin in peripheral blood mononuclear cells (PBMCs) isolated from previously untreated CLL patients. Additionally, the cytotoxicity of the tested drug was also examined in mononuclear cells isolated from the blood of 4 healthy volunteers. Atorvastatin is a synthetic open-ring compound that does not require β-lactone ring hydrolysis for its activity (12). It has shown pro-apoptotic potential in a large number of cancer cell lines (11,13-16). However, the pro-apoptotic potential of atorvastatin in primary CLL cells has yet not been established.

Materials and methods

Patients. Mononuclear cells were obtained from the peripheral blood of 15 untreated CLL patients (3 females and 12 males) with different stages of the disease (I-IV), classified according to the staging system described in the study by Rai et al (17). The median age of the patients at the time of the study was 62 years (range, 51 to 80 years), and the median leukocytosis, 182.67×10^3/μl (range, 20×10^3 to 600×10^3/μl). All patients enrolled in the study required anti-leukemic therapy.

CLL was diagnosed on the basis of standard clinical and immunological criteria (17). This study was approved by the Local Ethics Committee of the Medical University of Łódź (no. RNN/143/10/KE) and all patients gave their written consent prior to enrollment.

Additionally, the peripheral cells from the blood of 4 healthy volunteers (1 male and 3 females, aged 23-65 years) with normal leukocytosis were isolated to compare the activity of atorvastatin in normal and primary tumor cells.

Isolation of mononuclear cells. PBMCs were isolated from peripheral blood samples (collected into EDTA as the anticoagulant) obtained from CLL patients or healthy donors by Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA). The CLL or control cell pellets were then resuspended in phosphate-buffered saline (PBS) and divided as required for the planned experiments.

Cell culture and drug treatment. The model cell samples were resuspended in RPMI-1640 medium with 10% fetal calf serum supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin to a final concentration of 2×10^6 cells/ml and incubated with atorvastatin (LKT Laboratories, Inc.) at concentrations of 5, 10, 25, 50, 100 and 150 μM. The cells were incubated with this statin (or without, controls) as well as with 0.15% DMSO (vehicle controls) for 24 and 48 h at 37°C in an atmosphere of 5% CO₂.

Cell viability and apoptosis determination. To evaluate the viability of leukemic (11 samples) and normal mononuclear cells (4 samples), as well as the percentage of apoptotic cells in the model cells exposed to atorvastatin, the Vybrant Apoptosis Assay kit #4 from Invitrogen Molecular Probes (Eugene, OR, USA) was used. The mononuclear cell population was gated on the basis of forward scatter (FSC) and side scatter (SSC) parameters. The percentage of viable cells was determined after 24 and 48 h of incubation with atorvastatin and quantified using the LSR II Flow Cytometer (Becton-Dickinson, San Jose, CA, USA). The number of viable cells was quantified in 4 experiments by a colorimetric MTT assay based on MTT reduction, as previously described (18) or propidium iodide (PI) staining only (data not shown).

DNA content analysis. The DNA content in the PBMCs from the blood of 8 CLL patients, as well as from healthy volunteers, was estimated after 48-h incubation with/without atorvastatin. Briefly, 1×10^6 cells were fixed with 70% ethanol and incubated at -20°C for 2 h. Subsequently, the cells were incubated in the presence of RNase A (at a final concentration in PBS of 0.5 mg/ml) and PI (at a final concentration in PBS of 0.01 mg/ml) for 30 min, at 37°C, in the dark. The fluorescence of PI was then measured by flow cytometry (FACScalibur; Becton-Dickinson) and the number of sub-G1 cells was evaluated on the basis of FL-3 histograms using CellQuest Pro software (Becton-Dickinson). Ten thousand events were examined for each analysis.

DNA fragmentation analysis. The DNA fragmentation was assessed by agarose gel electrophoresis performed according the procedure described in the study by Bellossillo et al (19), with slight modifications. Briefly, 6×10^5 CLL cells (after washing with PBS) were lysed and treated with proteinase K (0.2 mg/ml) in a buffer containing 5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100 for about 12 h at 37°C. DNA was extracted twice with buffered phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol at -20°C, overnight. The DNA precipitates were washed twice with 75% ethanol, dissolved in triple-distilled water, and digested with RNase A (1 mg/ml) for 2 h at 37°C. Finally, the DNA samples were electrophoresed by standard agarose gel (2.0%) electrophoresis. Etiđium bromide was used for DNA visualization under ultraviolet light.

Protein separation and immunoblot assay. Leukemic and normal PBMCs were lysed and prepared for western blot analysis as previously described (18). Protein determination in the cell lysates was performed according to the method described by Lowry et al (20). Approximately 50 μg of protein was loaded per each lane and the proteins were separated by SDS-PAGE into 8% and 12.5% slab gels, depending on the molecular weights of the analyzed proteins. The proteins were then transferred onto Immobilon-P membranes according to the method described by Towbin et al (21) and stained reversibly with 0.05% Ponceau S solution to confirm their equal loading. Subsequently, the membranes were incubated in the presence of 5% non-fat dry milk in TBST buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] for 1 h at room temperature with successive incubation with appropriate antibodies, overnight. The following antibody dilutions were used: anti-poly(ADP-ribose) polymerase-1 (PARP-1; 1:5,000), anti-lamin B (1:2,000) and anti-caspase-9 (1:5,000) from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-actin (1:1,000) from Abcam (Cambridge, UK). After being washed 3 times in TBST buffer, the membranes were incubated with secondary antiserum conjugated with alkaline phosphatase (Sigma-Aldrich) for 2 h and washed 3 times again. The antigen-antibody complexes were visualized following incubation of the membranes with a phosphatase substrate solution.
Approximately 50% (46.7±10.4) reduction in CLL cell viability was observed at 24 h of cell incubation with the statin (data not shown). By contrast, the leukemic cells were sensitive to all tested drug concentrations. The effect was already indicated no significant effect of 0.15% DMSO on model cell viability. A further increase in the drug concentration (100 µM) decreased the viability of the CLL cells by ~70% compared to the controls and the vehicle controls (13.8 vs. 87.24 and 86.12%, respectively). The decrease in CLL cell viability was primarily caused by the induction of apoptosis (Fig. 1B). However, the higher the concentration of atorvastatin used, the greater the increase in the dead cell population. The Vybrant Apoptosis Assay kit #4 does not distinguish between necrotic and late apoptotic cells. Therefore, the DNA content was estimated to better determine the apoptotic cell numbers in the population of drug-exposed CLL cells. Since the majority of viable leukemic cells do not proliferate, on DNA content histograms, they are viewed as a diploid cell population following PI staining. By contrast, apoptotic cells are characterized by the presence of oligonucleosomal DNA fragments leaking from the cells after their fixation (22). Hence, they are revealed during cytometrical tests as the cells with a diminished DNA content (sub-G1/hypodiploid cells) in comparison with viable cells. The estimation of DNA content in the cells obtained from the blood of 8 CLL patients confirmed the induction of apoptosis in the cells treated with atorvastatin (Fig. 2). The exposure of CLL cells to 50 µM atorvastatin increased the sub-G1 cell population from ~15% (control) to >55%. Of note, the increase in the concentration of atorvastatin to ≥100 µM led to a decrease in this population. These data, together with the results obtained from the Vybrant Apoptosis Assay kit #4, suggest that higher concentrations of atorvastatin induce cell death mainly through necrosis.

On the basis of CLL cell viability, the doses of 50 and 100 µM atorvastatin were selected for further experiments with the use of normal mononuclear cells. In contrast to the leukemic cells, normal cells were considerably resistant to 50 µM atorvastatin, which caused ~50% decrease in leukemic cell viability after 48 h of incubation (Fig. 3). Furthermore, the viability and apoptosis of mononuclear cells from the blood of

Figure 1. Effect of atorvastatin (5-150 µM) on (A) cell viability and (B) induction of apoptosis in CLL cells after 48 h of incubation. The viability and apoptotic cell numbers were measured using the Vybrant Apoptosis Assay kit #4. The obtained results represent the means ± SD of 11 independent experiments. In case of leukemic cell samples exposed to 100 and 150 µM atorvastatin, the cell viability/apoptotic cell numbers were analyzed twice. Ctr (control cells), CLL cells incubated in culture medium without drugs or DMSO; Ctr* (vehicle control), CLL cells incubated in culture medium with 0.15% DMSO. *P≤0.05, significant difference compared to corresponding control cells.

Figure 2. Effect of atorvastatin (5-150 µM) on DNA content in CLL cells. The DNA content in drug-exposed leukemic cells was assessed by flow cytometry following the staining of the cells with propidium iodide. The presented results are the means ± SD of 8 independent experiments. In the case of leukemic cells exposed to 100 and 150 µM atorvastatin, the DNA content was analyzed twice. Ctr (control cells), CLL cells incubated in culture medium without drugs or DMSO; Ctr* (vehicle control), CLL cells incubated in culture medium with 0.15% DMSO. *P≤0.05, significant difference compared to the corresponding control cells.

**Results**

**Cell viability and apoptosis induction.** The Vybrant Apoptosis Assay kit #4 was used to select the dose of atorvastatin that caused ~50% decrease in leukemic cell viability in comparison to the cells incubated with culture medium only (control). Examinations were performed on the cell samples obtained from 11 CLL patients. The viability of CLL cells was determined following a 24-h (data not shown) and 48-h exposure to atorvastatin at increasing concentrations (5, 10, 25 and 50 µM) (Fig. 1A). In 2 cases, the exposure of the cells to 100 and 150 µM of atorvastatin was also examined. Additionally, the effect of 0.15% DMSO (used as a solvent for atorvastatin) on CLL cell viability was examined. The obtained results indicated no significant effect of 0.15% DMSO on model cell viability. By contrast, the leukemic cells were sensitive to all tested drug concentrations. The effect was already observed at 24 h of cell incubation with the statin (data not shown). Approximately 50% (46.7±10.4) reduction in CLL cell viability in comparison with the control cells was observed following the 48-h exposure of the cells to 50 µM atorvastatin. A further increase in the drug concentration (100 µM) decreased the viability of the CLL cells by ~70% compared to the controls and the vehicle controls (13.8 vs. 87.24 and 86.12%, respectively). The decrease in CLL cell viability was primarily caused by the induction of apoptosis (Fig. 1B).
4 healthy volunteers was only slightly influenced by 100 µM atorvastatin.

The cell viability results were partially confirmed by sub-G1 cell population analyses. In normal mononuclear cells, the DNA content was assessed following exposure to 50 and 100 µM (in 2 cases) atorvastatin for 48 h (Fig. 4). The tested drug concentration (50 µM) caused an increase in the sub-G1 cell population of normal PBMCs, on average, only by ~10% in comparison to the controls, i.e., the cells which were not exposed to atorvastatin (controls, Ctr) or exposed to 0.15% DMSO (vehicle control, Ctrv).

Figure 4. Effect of atorvastatin (50 and 100 µM) on DNA content in normal PBMCs from the blood of healthy donors assessed by flow cytometry after 48 h of drug incubation. The obtained results represent the means ± SD of 4 independent experiments. Ctr (control cells), PBMCs incubated in culture medium without drugs or DMSO; Ctrv (vehicle control), PBMCs incubated in culture medium with 0.15% DMSO.

DNA fragmentation. To confirm the pro-apoptotic activity of atorvastatin in leukemic cells, analysis of DNA fragmentation was performed. Fig. 5 illustrates the representative results of DNA fragmentation analysis for PBMCs from the blood of an exemplary CLL patient and treated ex vivo with atorvastatin. As illustrated, the exposure of leukemic cells to 25 and 50 µM atorvastatin resulted in DNA degradation to the fragments visible as a DNA ladder, following electrophoretic separation on agarose gels. DNA laddering is a known feature of apoptosis. Notably, the dose of 50 and 100 µM atorvastatin did not induce DNA fragmentation in normal PBMCs (data not shown).

Expression of apoptosis-related proteins. Issues regarding the induction of apoptosis caused by atorvastatin were addressed by comparative analysis of selected nuclear proteins, as well as regulatory Bcl-2 family member expression (Fig. 6).

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The western blot analysis results of lamin B (67 kDa) and PARP1 (116 kDa), known markers of apoptosis, revealed that atorvastatin effectively induced apoptosis in CLL cells following treatment with the statin. The representative results, shown in Fig. 6A, confirmed the cleavage of the native forms of the nuclear proteins accompanied by an appearance of their 44- and 85-kDa proteolytic products, respectively. Moreover, immunoblot analysis revealed the proteolytic cleavage of the cell cycle inhibitory protein precursor, p27Kip1, to a 23-kDa product in CLL cells incubated with atorvastatin at all tested drug concentrations. It is worth mentioning, however, that the proteolytic level of these proteins varied noticeably between samples from different CLL patients, depending on their individual susceptibility to the used drug.
anti-apoptotic protein, Bcl-2 (26 kDa), decreased, while the pro-apoptotic protein, Bax (23 kDa), demonstrated a stable expression following CLL cell incubation with atorvastatin at a dose of up to 50 µM. The increase in the expression of the latter protein was observed only in the lysates from the cells treated with the highest studied drug concentration.

By contrast, even the highest tested concentration of atorvastatin did not alter the expression of examined apoptosis-related proteins in the mononuclear cells obtained from healthy volunteers (data not shown).

Discussion

It has been well-established that statins exert potent anticancer activity in a large number of cancer cell lines (11,12,15,23). Little is known, however, about the activity of these drugs in CLL cells. Taking into consideration the pleiotropic mechanisms of their action, as well as the nature of the disease, i.e., the accumulation of quiescent cells in the blood accompanied by the presence of a proliferating pool of cells in the bone marrow and lymphatic organs, it seems that statins should be highly efficient in the treatment of this hematological cancer. Recently, the anti-leukemic potential of simvastatin against CLL cells used in combination with the purine analogs, fludarabine and cladribine, was demonstrated in the study by Podhorecka et al. (24). The examined concentration of simvastatin was 10 µM. The synergism of this drug with conventional chemotherapeutics was revealed. Additionally, no significant effect of simvastatin was observed on normal cells.

In this study, we evaluated the cytotoxic and pro-apoptotic potential of another statin (atorvastatin) in primary tumor cells obtained from the blood of CLL patients prior to therapy. CLL cells are characterized by their strong resistance to apoptosis (4,5). This lack of sensitivity to death-inducing stimuli is mainly associated with the deregulation of certain signaling pathways [mitogen-activated protein kinase (MAPK) or protein kinase B (PKB) pathways] and the overexpression of a number of pro-survival molecules [Bcl-2, Mcl-1, survivin and inhibitor of apoptosis (IAP) proteins] in the transformed cells (25-30). For this reason, it was of high importance to examine the influence of increasing doses of atorvastatin on normal and leukemic cells. Our results revealed an increased cytoxic activity of atorvastatin alone against PBMCs from CLL patients with different clinical stages of the disease. The susceptibility of CLL cells obtained from different patients was diverse, although it was not dependent on the stage of leukemia. Atorvastatin at the dose of 50 µM decreased the viability of the tested leukemic cells, on average by ~50% in comparison to the control cells. Importantly, the same dose of the drug did not visibly affect the viability of normal cells. Moreover, a 2-fold higher concentration of atorvastatin (100 µM), which caused extensive necrosis in leukemic cells, caused only a slight decline in the viable cell number in the population of PBMCs obtained from the blood of healthy volunteers. By contrast, Salman et al. (31) reported a small, but significant decrease in normal PBMC apoptosis following exposure to atorvastatin at the concentration of 50 µM, which is confusing, while taking into consideration the fact that the significant influence of the drug has been limited only to early apoptotic cells.

The results of our DNA fragmentation analysis and DNA content in the drug-treated cells indicated the selective pro-apoptotic potential of atorvastatin in leukemic, but not in normal cells. The pro-apoptotic activity of this statin was confirmed by western blot analysis of PARP-1 and lamin B expression. It is widely accepted that the degradation of lamin B and PARP-1 into 44- and 85-kDa fragments results from the proteolytic activity of caspase-6, as well as caspase-3 and -7, respectively (32,33). The proteolysis of lamin B and PARP-1 was observed in the leukemic cells following their incubation with atorvastatin. In addition, we investigated the impact of this statin on the expression level of the cyclin-dependent kinase inhibitor, p27, survivin, Mcl-1, Bcl-2, and Bax, (separated on 12.5% polyacrylamide gels) by western blot analysis. The obtained results from a selected CLL patient are presented. Ctrl, CLL cells incubated in culture medium with 0.15% DMSO. Poncet staining was used as a loading control.

Figure 6. Ex vivo changes in the expression level of selected apoptosis-related proteins in CLL cell samples exposed to atorvastatin (10, 25, 50 and 100 µM) for 48 h. Protein lysates (50 µg) from atorvastatin-treated CLL cells. After electrophoretic separation and transfer, proteins immobilized on Immobilon-P membranes were analyzed for the expression of (A) lamin B and PARP-1, (separated on 8% polyacrylamide gels) and (B) apoptosis-regulated proteins, i.e., p27kip1, Mcl-1, Bcl-2 and Bax, (separated on 12.5% polyacrylamide gels) by western blot analysis. The obtained results from a selected CLL patient are presented.
of anti-apoptotic proteins that regulate the function of pore-forming proteins of the Bcl-2 family (Bax or Bak) (37). Bax molecules assemble the channels in mitochondrial membranes in response to various apoptotic stimuli and enable pro-apoptotic factors to be released from the mitochondria to the cytosol (37,38). Under physiological conditions, when the pro- and anti-apoptotic members of Bcl-2 family retain a dynamic balance in the cells, Bax can be bound by its counter partners, Bcl-2 and Mcl-1 (37). This interaction leads to the inhibition of mitochondrial membrane permeability and inhibits apoptosis. The expression of Mcl-1 and Bcl-2 proteins is known to be elevated in CLL cells (26,28). Of note, Mcl-1 has been reported to be capable of hampering apoptosis in hematopoietic cells to a higher extent than Bcl-2. Mcl-1 overexpression in leukemic cells is related to chemoresistance and progression of the disease. We, as well as others have previously confirmed the overexpression of anti-apoptotic Bcl-2 family proteins in CLL cells in vivo, which is considered to be one of the reasons of their resistance towards apoptosis, as well as changes in the Bax/Bcl-2 ratio in response to chemotherapy (27,28).

In this study, we revealed that the exposure of CLL cells to atorvastatin ex vivo disturbs the dynamic balance between pro- and anti-apoptotic proteins of Bcl-2 family via the diminution of Bcl-2 and Mcl-1 expression level without altering Bax expression.

Atorvastatin, similar to other statins, beyond its cholesterol-reducing properties, has also demonstrated pro-apoptotic properties against many types of cancer cells. The anticancer potential of statins has encouraged their use in the treatment of cancer. It has been suggested that these drugs may be useful in combination with other therapeutic agents (24,39). For this reason, further studies are required to establish the potential benefits of statin alone/statin-combined therapy for different types of cancer, including CLL.

In conclusion, atorvastatin induces the mitochondrial pathway of apoptosis in leukemic PBMCs by affecting the cellular concentration of anti-apoptotic proteins that are deregulated in CLL cells, i.e., Mcl-1 and Bcl-2. Moreover, it triggers alterations in drug-exposed leukemic cells, resulting in the proteolysis of the cell cycle-related proteins, p27Kip1, lamin B and PARP-1. Importantly, the pro-apoptotic potential of atorvastatin is limited specifically to leukemic cells. Normal PBMCs from healthy volunteers do not exhibit susceptibility to this statin, even at very high concentrations. The obtained results suggest that atorvastatin may be use for the treatment of CLL, possibly in conjunction with other chemotherapeutic agents.

Acknowledgements

The present study was supported in part by a grant from the University of Lodz (No. 545/479).

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