

Lovastatin Enhances *in Vitro* Radiation-Induced Apoptosis of Rat B-cell Lymphoma Cells

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Our previous demonstration of an antimetastatic effect of lovastatin, both in rat sarcoma and lymphoma tumor-models, as well as the fact that lovastatin and radiation are able to stop the cell cycle in different phases, suggested the feasibility of a combined treatment. We studied the effect of the *in vitro* combined treatment of a B-cell rat lymphoma (L-TACB) with lovastatin and irradiation. The results herein obtained provide new information about the role of statins as radiosensitizers. The antitumor effect of the combined treatment was higher than that elicited by either treatment alone. This effect could be a consequence, at least in part, of an enhanced apoptosis.

Key Words: Lovastatin, Apoptosis, Lymphoma, Antitumor effect, Radioresistance

The importance of cholesterol in cell viability and growth resides not only in its function as a main component of cell membranes and as a precursor of steroid hormones, vitamins and bile acids, but also in its participation in intracellular signal transduction pathways involved in the regulation of the cell cycle (1,2).

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the synthesis of mevalonate (MV) from HMG-CoA, which constitutes the rate-limiting step in the biosynthesis of cholesterol (3). It has already been shown that products of the MV pathway play a critical role in the progress of cell cycle and cell survival (3,4,5). Several experimental observations showed that human (6,7) and animal (8,9) tumor tissues upregulate HMG-CoA reductase. Those findings suggested that tumor growth could be restrained by interfering with the availability or synthesis of cholesterol, turning this biosynthesis pathway into a target for pharmacological intervention.

Lovastatin (LOV), a drug introduced in the 80's for the treatment of hypercholesterolemia (10), competitively inhibits HMG-CoA reductase, blocking the synthesis of MV and, hence, of farnesyl residues. The antitumoral and/or antimetastatic effect of Lovastatin was demonstrated both *in vitro* (11-18) and *in vivo* (9,19-22) in different experimental models.

One of the processes affected by inhibition of the synthesis of farnesyl moieties by LOV is anchorage of the p21^{ras} protein to the cell membrane. This pathway, that allows the protein to act as a signal transducer, requires a post-translational modification involving the transference of farnesol, a 15-carbon isoprenoid lipid, from farnesyl diphosphate to a cysteine residue in the Ras protein (23). On the other hand, alterations in *ras* oncogene expression have been associated with increased cellular resistance to ionizing radiation. Miller et al. have demonstrated that elevation in *ras* gene expression can lead to increased radioresistance of human tumor cells and that anchorage of p21^{ras} to the cell membrane is essential to maintain the radioresistant phenotype (24). They also found that elevation of *ras* expression in human cells is associated with a decrease in radiation-induced micronuclei (25).

The aforementioned information together with the knowledge that LOV and radiation are able to stop cell cycle in different phases (G₁ and G₂, respectively) (26,27), suggested the feasibility of a combined therapeutic intervention. We studied the effect of the *in vitro* combined treatment of rat B-cell lymphoma L-TACB with LOV and radiation. The results showed a higher antitumor effect for the combined treatment than that obtained by each single treatment. This

could be a consequence of the enhanced tumor cell apoptosis generated by the combined treatment.

Materials and Methods

Animals. Adult male and female inbred IIM e/Fm rats (from here on *e* rats) (28) from the breeding facilities of the Instituto de Genética Experimental, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Argentina, were used. Animals were fed with a commercial chow and water *ad libitum* and were maintained in a 12 h light/dark cycle. All the experiments were done during the first half of the light cycle. The animals were treated in accordance with the guidelines issued by the Canadian Council on Animal Care (29).

Tumor. L-TACB is a poorly differentiated B-cell lymphoma that histologically resembles a Burkitt's type lymphoma, which arose spontaneously in an *e* rat (30). It is maintained by serial subcutaneous (s.c.) grafting by trocar of 1mm³ tumor fragments (approximately 10⁶ cells) in syngeneic rats. When L-TACB is injected s.c., lymph nodes are the exclusive site of metastatic growth.

Drug. Lovastatin (LOV) was kindly provided by the Instituto SIDUS S.A. (Buenos Aires, Argentina) in the inactive lactone form. It was converted to the active form by dissolving 480 mg of lactone in 12.5 ml of 96° ethanol, adding 18 ml of 0.1 M NaOH, heating at 50 °C for 2 hours, neutralizing the solution with 0.1 M HCL to pH 7 and adjusting with distilled water to a volume of 60 ml. Aliquots of this stock solution (20 mM) were stored frozen at -20°C until use.

Irradiation. The irradiation was performed on a Theratron 80 Co-60 unit (AECL Atomic Energy of Canada) at a dose rate of approximately 1.15 Gy/min at 56 cm source to skin distance (SSD). In order to determine the dose absorbed by our system while it was exposed to ionizing radiation, the Fricke and Morse dosimeter chemical system was used. The standard dosimeter solution is composed of ferrous sulphate (1mol FeSO₄ in 400 mol H₂SO₄). Upon exposure to radiation, the oxidation of ferrous ions is directly proportional to the absorbed dose (31). Under the conditions described, the dose was equivalent to 9 Gy.

Effect of LOV and radiation on L-TACB cells viability. L-TACB cells, obtained by mechanical disruption

of a 14 days growth s.c. primary tumor, were suspended in RPMI-1640 culture medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Natocor, Córdoba, Argentina). Cell suspensions with more than 95% viability were seeded in 96-well flat bottomed microplates (Corning Costar) in the proportion of 1 x 10⁵ L-TACB cells per well in 200 µl culture medium. Four groups were established to test the effect of the different treatments on the viability of lymphoma cells: 1) **Control**: without treatment, 2) **LOV**: treated with 20 µM Lovastatin, 3) **Rad**: treated with 9 Gy irradiation, and 4) **LOV + Rad**: treated with 20 µM Lovastatin and 9 Gy irradiation. All measurements were performed in quadruplicate. After 72 hours incubation at 37°C in a 5% CO₂ humidified atmosphere, the cell viability was determined with the MTS/PMS colorimetric assay (Cell Titer 96™ Aqueous non-radioactive cell proliferation kit, Promega Corp, Madison, WI, USA), according to the manufacturer instructions. Briefly, 40µl of the combined MTS (tetrazolium salt)/PMS (electron coupling reagent) [20:1] were added into each well. The plates were incubated for 4 hours at 37°C in a humidified 5% CO₂ atmosphere. In this assay, dehydrogenase enzymes found in metabolically active cells accomplish the conversion of MTS into the aqueous soluble formazan. The quantity of formazan product, as measured by the absorbance at 490 nm, is directly proportional to the number of live cells in culture.

Apoptosis assays. The induction of apoptosis by the different treatments was determined in 5 ml of L-TACB cells suspension (2.5x10⁶ cells in RPMI-1640 supplemented with 10% FBS) seeded in 25 cm² culture flasks, which were exposed to: 1) No further treatment (**Control**), 2) 20 µM Lovastatin (**LOV**), 3) 9 Gy irradiation (**Rad**), or 4) 20 µM Lovastatin + 9 Gy irradiation (**LOV + Rad**). Apoptosis was evaluated with the following assays:

Annexin V-FITC: After 12 hrs treatment, the cells were collected and stained with a conjugate of Annexin V and fluorescein isothiocyanate (Annexin V-FITC Apoptosis Detection Kit, Oncogene Research Products, Boston, USA) according to the protocol provided. The assay is based on the observation that early apoptotic cells translocate phosphatidyl serine from the inner to the outer face of the plasma membrane. The reaction of Annexin V with the phospholipid results in green fluorescence on the cell surface, while necrotic cells, that have lost cell membrane integrity, show a red fluorescent nucleus stained by propidium iodide. The quantification of early apoptotic cells was performed

in a fluorescence microscope, counting 300 cells in each group.

DNA ladder assay: Tumor cells were incubated for 24hrs under the four treatment modalities previously explained, collected and lysed by incubation for 8 hours at 60°C in 50 mM Tris-ClH, pH 8, 10 mM EDTA and 100 µg/ml proteinase K. The fragmented DNA was obtained by phenol extraction and precipitation with sodium acetate and ethanol, and then resuspended in distilled water, the absorbance being determined at 260 nm. Ten µg of DNA from each group was analyzed by electrophoresis on 2% agarose gels visualized by ethidium bromide staining.

TUNEL staining: Cells treated for 24 hrs as already explained, were centrifuged and the pellet obtained was fixed in 10% buffer formalin for 24 hrs and embedded in paraffin. Apoptotic cells were identified in the sections using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-peroxidase nick end labeling (TUNEL) method (Apoptag® Peroxidase *in situ* Apoptosis Detection Kit, for immunoperoxidase staining, Intergen Company, New York, USA) following the manufacturer instructions. The apoptotic cell percentage was calculated by counting the total number of cells and the cells with characteristic features of apoptosis and positive for TUNEL staining, observed per HPF (high power field, 1000X) in 10 fields per group.

Statistical analysis. To estimate the effect of the treatments on L-TACB cells viability, the statistical significance of the differences in absorbance among groups was assessed by a one-way analysis of variance (ANOVA); comparisons between treatment pairs were done with Bonferroni's test. Variables expressed as percentage were analyzed by means of the χ^2 non-parametric test. Differences were considered significant if $p < 0.05$.

Results

To determine whether addition of LOV could increase the cytotoxicity achieved with irradiation of L-TACB cells, the effect of single or combined treatments on cell viability was studied. Figure 1 shows the effect of the different treatments on L-TACB cells viability after 72 hrs culture. Both LOV and Rad induced a significant decrease in cell viability (37% and 23%, respectively) as compared with the control ($p < 0.001$ and $p < 0.05$, respectively). The combined treatment also differed from the control ($p < 0.001$), leading to a

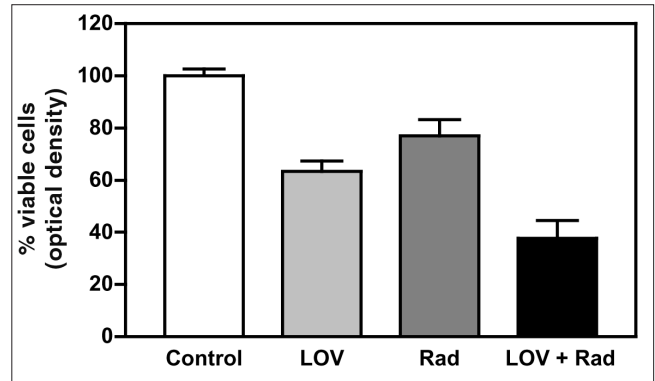


Fig. 1 - Mean viability of L-TACB cells ± S.E. The data are presented as the fraction of the optical density of the control wells and plotted as a percentage. Significance of the differences between groups: Control *versus* LOV, $p < 0.001$; Control *versus* Rad, $p < 0.05$; LOV *versus* Rad, n.s.; Control *versus* LOV + Rad, $p < 0.0001$; LOV *versus* LOV + Rad, $p < 0.05$; Rad *versus* LOV + Rad, $p < 0.001$. Significance is based on a one way ANOVA of the optical density data from five replicates of each group.

62% reduction of viability. Interestingly, cell viability after treatment with LOV + Rad was significantly lower than that achieved by either single treatment ($p < 0.05$ and $p < 0.001$, respectively), resulting in an additive interaction.

Annexin V-FITC estimates of early apoptosis are displayed in Figure 2. Twelve hours after treatment,

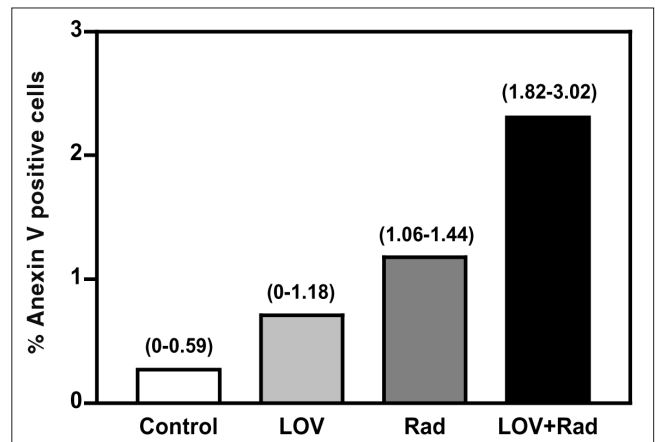


Fig. 2 - Early apoptotic cell death expressed as percentage of positive cells for Annexin V[median (range)]: effect of the different treatments after 12 hours culture. Significance of the differences between groups, based on the chi-squared test: Control *versus* LOV, n.s.; Control *versus* Rad, $p = 0.017$; LOV *versus* Rad, $p < 0.001$; Control *versus* LOV + Rad, $p < 0.0001$; LOV *versus* LOV + Rad, $p < 0.0001$; Rad *versus* LOV + Rad, $p = 0.045$.

comparison with the control showed that Rad produced a significant increase in the percentage of apoptotic cells ($p < 0.025$), while LOV presented no differences ($p > 0.05$). Treatment with both LOV and Rad almost doubled the percentage of apoptotic cells observed with Rad alone, this increase being significantly different from the control, LOV and Rad groups ($p < 0.01$). In two other experiments done 24 and 48 h after treatment, the differences in the % of Annexin V positive cells among experimental groups were similar to those obtained after 12 h (data not shown).

Figure 3 shows the effect of the different treatments on DNA fragmentation after 24 hrs culture, as assessed by electrophoresis on agarose gels. DNA fragmentation was not detectable in the control group (lane 1). On the contrary, the typical DNA ladder could be ob-

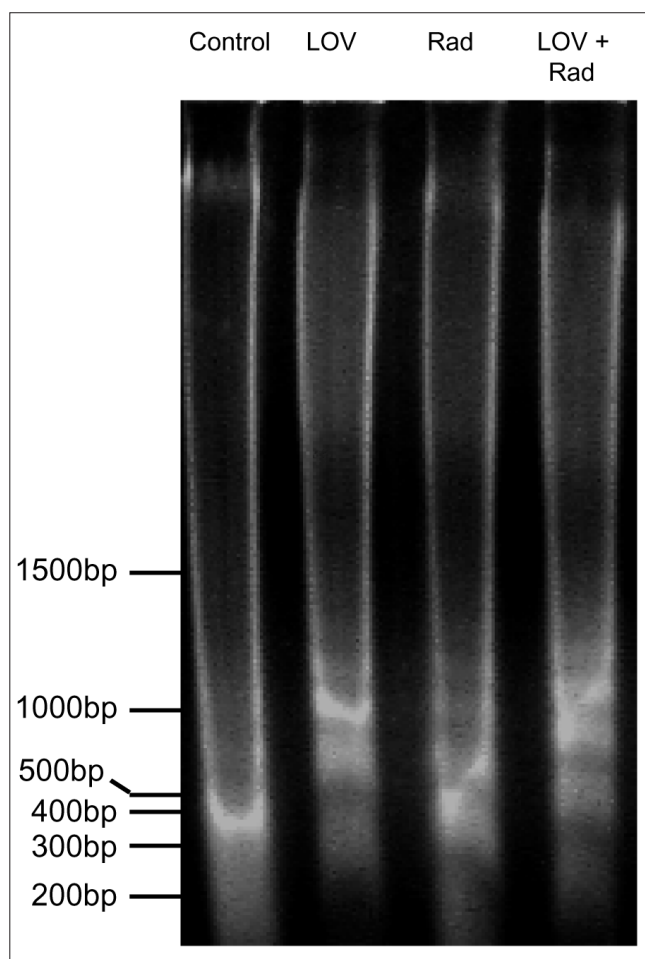


Fig. 3 - Effect of the different treatments on DNA fragmentation of L-TACB cells after 24 h incubation under different treatment conditions. *Lane 1:* control; *Lane 2:* 20 μ M LOV; *Lane 3:* 9 Gy irradiation; *Lane 4:* LOV 20 μ M and 9 Gy irradiation.

served in LOV (lane 2), Rad (lane 3) and LOV + Rad (lane 4) treated groups. The combined treatment demonstrated a noticeable increase in DNA fragmentation when compared to LOV or Rad alone.

Quantification of late apoptosis by measuring the percentage of TUNEL positive cells gave similar results to those obtained with Annexin-V and DNA ladder (Fig.4). While cells treated with LOV did not show differences with the control cells, the group treated with Rad showed a significant increase in the percentage of apoptotic cells ($p < 0.001$) when compared to the control group. In agreement with the two other methods of apoptosis evaluation, the combined treatment (LOV + Rad) caused the highest percentage of apoptotic cells, differing significantly from those measured in control cells ($p < 0.0001$), cells treated with LOV ($p < 0.0001$) or with Rad ($p = 0.01$).

Discussion

One of the great progresses in cancer treatment during the XXth century was the development and use of chemotherapy and radiotherapy. These treatments have demonstrated antitumor efficacy through their damaging action on cellular DNA, which prevents proliferation and, frequently, driving the cell to its death. Since

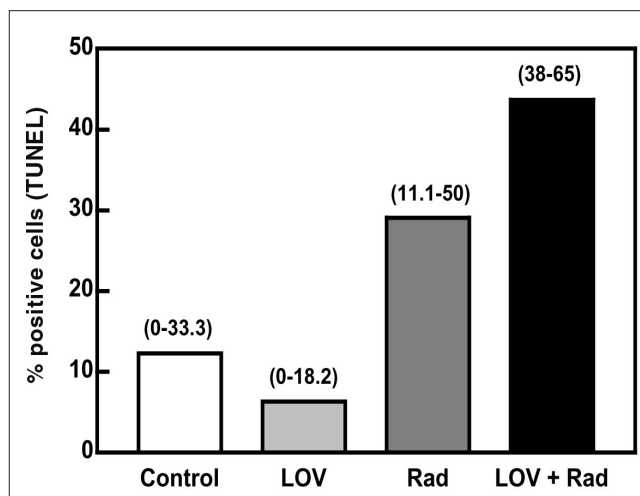


Fig. 4 - Apoptosis of L-TACB cells treated with the different modalities, as determined by TUNEL staining. The data are presented as the percentage of the TUNEL positive cells [median (range)]. Significance of the differences between groups, based on the chi-squared test: Control *versus* LOV, n.s.; Control *versus* Rad, $p < 0.01$; LOV *versus* Rad, $p < 0.0001$; Control *versus* LOV + Rad, $p < 0.0001$; LOV *versus* LOV + Rad, $p < 0.0001$; Rad *versus* LOV + Rad, $p = 0.01$.

only a fraction of the tumor cells undergo damage and death, high drug or radiation doses are required to achieve noticeable clinical results. However, this is usually associated with high toxicity for the patients. Hence, one of the current goals in cancer therapy is to reduce the appearance of side effects while retaining the antitumoral capacity obtained with higher doses. As we have already demonstrated *in vivo*, both in sarcoma and lymphoma rat tumor models (19,20), LOV exerts antitumoral and antimetastatic effects, and is devoid of toxicity at the dose and schedule used.

The lack of toxicity of our treatment protocol, the knowledge that p21^{ras} farnesylation was inhibited by LOV in an *in vivo* model (23), and the fact that alterations in *ras* oncogene expression are often associated with increased cellular resistance to ionizing radiation (24), confirmed the rationale of our proposal. Our intended aim was to set the foundations for designing a combined treatment protocol for lymphomas that, through the additive or synergistic effect of its components, could achieve the same antitumoral benefits obtained by higher radiation doses, with the advantage of having less or no toxicity at all.

Our results show that the *in vitro* co-treatment of L-TACB cells with radiation and the hypocholesterolemic drug lovastatin was significantly more effective in reducing cell viability than radiation or lovastatin alone. A more effective response was to be expected because radiation and LOV may stop the cell cycle in different phases (13,32). Interestingly, both treatments, when combined, acted in an additive manner, thus allowing a response similar to that of higher radiation doses. To our knowledge, the only papers showing results after treatment with LOV plus radiation in other tumor systems were those obtained *in vitro* by Miller (24) and Fritz (33) and that developed *in vivo* in a Phase I clinical trial by Larner et al (34).

Noteworthy is the high radioresistance of the L-TACB cells, 77% of which remained alive, after 9 Gy irradiation. Nevertheless, this result should not be surprising because of the wide spectrum of radiation sensitivity displayed by lymphoma cell lines (35).

Since induction of apoptosis is one of the mechanisms likely to be involved in the antitumoral effect of the combined treatment, the process was evaluated by three independent methods. The loss of the asymmetric distribution of phospholipids, with the exposure of phosphatidylserine (PS) on the apical surface of the cytoplasmic membrane, is one of the first changes that can be detected during the development of the apoptotic cycle. Annexin-V, is a PS binding protein commonly used for monitoring the earliest stages of apop-

osis (36). Though the detected numbers of Annexin-V positive cells were low in our model, the combined treatment induced a seven fold increase when compared to the control ($p < 0.0001$). Interestingly, apoptosis induced by LOV + Rad was significantly higher than that induced by each treatment alone, using the same doses. These results were confirmed when a later phase of apoptosis was evaluated.

DNA fragmentation is an important biological hallmark of apoptosis in many cells and tissues. The evaluation of fragmented DNA of apoptotic cells by the classical DNA ladder obtained after separation on an agarose gel yielded results in agreement to those obtained with Annexin-V. The combined treatment was the one which showed the highest intensity of DNA fragmentation profile.

An additional method for examining apoptosis via DNA fragmentation, far more sensitive than the agarose gels, is the *in situ* staining of DNA strand breaks detected by the TUNEL assay. The results obtained with this method agreed with the other two, the treatment with LOV + Rad being the one that showed the highest percentage of apoptosis with values significantly higher than those induced by either treatment alone.

It is noteworthy that in the Annexin-V and TUNEL assays, LOV, without producing apoptosis on its own, enhanced radiation-induced apoptosis to greater additive levels.

As we have previously demonstrated, the *in vivo* administration of LOV can inhibit membrane anchorage of the p21^{ras} protein in L-TACB tumor cells (37), a step necessary for p21^{ras} to fulfill its role as a signal transducer. We can speculate that *in vitro* treatment of L-TACB cells with LOV also inhibits p21^{ras} membrane anchorage. This could, in part, explain the exacerbation of the apoptosis induced by radiation when the cells are co-treated with LOV. There is a wide spectrum of processes boosted by the activation of p21^{ras} that are directly related to progression to malignancy (38-41). Interestingly, the pharmacological action of LOV on cancer cells is observed even in those cells with nonmutated *ras* oncogenes (17). Our finding, as well as another showing the association between *ras* expression and radioresistance (24), underline the importance of developing therapies directed to diminish the expression of the *ras* oncogene.

The results herein obtained, provide new information about the role of statins as radiosensitizers in a lymphoma tumor-model. Moreover, their therapeutic use in combination with radiotherapy could fulfill the ever-present aim of cancer treatment; namely, to

achieve better results with very low or no toxicity at all. The confirmation of these results by *in vivo* experiments, which is our next goal, will shorten the gap between the experimental and the clinical setting and will likely provide the foundation needed to begin Phase I clinical trials for lymphoma treatment.

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