Everolimus Inhibits Monocyte/Macrophage Migration in Vitro and Their Accumulation in Carotid Lesions of Cholesterol-Fed Rabbits

Roberta Baetta, Agnese Granata, Monica Canavesi, Nicola Ferri, Lorenzo Arnaboldi, Stefano Bellosta, Pascal Pfister,1 and Alberto Corsini

Department of Pharmacological Sciences, University of Milan, Milan, Italy (R.B., A.G., M.C., N.F., L.A., S.B., A.C.); and Novartis Pharma AG, Basel, Switzerland (P.P.)

Received July 29, 2008; accepted November 19, 2008

ABSTRACT

Monocytes/macrophages recruited into the arterial wall during atherogenesis are crucial in the initiation and progression of atherosclerosis and play a fundamental role in the destabilization process that is the main causal event of acute coronary syndromes. In the present study, we investigated the effect of the mammalian target of rapamycin inhibitor everolimus on macrophage accumulation within carotid lesions elicited by perivascular collar placement in cholesterol-fed rabbits. Everolimus (1.5 mg/kg given 1 day before collaring followed by 1 mg/kg/day for 14 days, administered by oral gavage) markedly decreased lesion macrophage content as compared with vehicle control (−65%; p < 0.01). This effect was associated with a reduction in intimal thickening and occurred in the absence of changes in plasma cholesterol concentrations. To gain insights on the potential mechanism(s) underlying this effect, we investigated the influence of everolimus on chemotactic monocyte/monocytant-induced migration of human monocytes in vitro. Pretreatment with therapeutic concentrations of everolimus (10 nM) significantly lowered monocyte chemotaxis in response to various chemotactic factors (i.e., monocyte chemoattractant protein-1/CCL2, fractalkine/CX3CL1, interleukin-8/CXCL8, complement fragment 5a, or N-formyl-Met-Leu-Phe) without inducing monocyte cell death. These results suggest that everolimus may favorably influence the atherosclerotic process by affecting the recruitment of monocytes into early lesions.

Monocyte recruitment into the arterial wall is crucial in the initiation of atherosclerosis, leading to fatty streak formation (Ross, 1999; Hansson and Libby, 2006). Moreover, macrophages are an essential component of unstable atherosclerotic plaques and play a central role in the destabilization process that is the main causal event of acute coronary syndromes (Hansson and Libby, 2006). Therefore, monocyte/macrophage functions represent an important therapeutic target for the prevention and treatment of atherosclerosis and related diseases.

A recent class of drugs with both immunosuppressive and antiproliferative properties is represented by the mammalian target of rapamycin (mTOR) inhibitors such as rapamycin (sirolimus), everolimus, temsirolimus, and deforolimus (Tsang et al., 2007). Upon binding to the cytosolic immunophilin FK-binding protein 12, these drugs inhibit mTOR, a conserved serine/threonine kinase that acts as a central integrator of various extracellular and intracellular signals (Wullschleger et al., 2006). Inhibition of mTOR abrogates the antiproliferative properties is represented by the mammalian target of rapamycin (mTOR) inhibitors such as rapamycin (sirolimus), everolimus, temsirolimus, and deforolimus (Tsang et al., 2007). Upon binding to the cytosolic immunophilin FK-binding protein 12, these drugs inhibit mTOR, a conserved serine/threonine kinase that acts as a central integrator of various extracellular and intracellular signals (Wullschleger et al., 2006). Inhibition of mTOR abrogates the activation of the mTOR substrates p70 ribosomal S6 kinase 1 and initiation factor 4E binding protein 1 elicited by a diversity of mitogenic stimuli, resulting in the arrest of growth factor-driven proliferation of both immune and nonimmune cells (Wullschleger et al., 2006).

Everolimus is a hydroxyethyl ether derivative of rapamycin developed for oral administration (Schuler et al., 1997).

ABBREVIATIONS: mTOR, mammalian target of rapamycin; SMC, smooth muscle cell; CCL2 (MCP-1), monocyte chemoattractant protein-1; fMLP, N-formyl-Met-Leu-Phe; rh, recombinant human; CX3CL1, fractalkine; CsA, complement fragment 5a; CXCL8 (IL-8), interleukin-8; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HC, high cholesterol; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; l/M, intima-media area ratio; DMEM, Dulbecco’s modified Eagle’s medium; PI, propidium iodide; TC, total cholesterol.
The drug became available in Europe in 2003/2004 for immunosuppression in renal and heart transplantation, based on clinical trials showing its efficacy in preventing graft rejection (Eisen et al., 2003; Webster et al., 2006; Viganò et al., 2007). Oral everolimus may also protect against transplant vasculopathy (Eisen et al., 2003; Webster et al., 2006; Viganò et al., 2007) and limit coronary artery intimal thickening in cardiac transplant recipients (Eisen et al., 2003). Furthermore, everolimus-eluting stents have been shown to prevent in-stent restenosis (Tsuchiya et al., 2006). Moreover, data obtained in transplant vasculopathy and in-stent restenosis animal models showed the ability of systemic everolimus treatments to reduce intimal thickening of aortic orthotopic transplants in rats (Cole et al., 1998), to prevent neointima formation of carotid allografts in apolipoprotein E-deficient mice (Matsumoto et al., 2003), to decrease in-stent neointimal growth in iliac arteries of normocholesterolemic rabbits (Farb et al., 2002), and to prevent the development of atherosclerotic plaques at the aortic root and at the brachiocephalic artery in LDLR(−/−) mice (Mueller et al., 2008).

Regarding the effect on lesion cell composition, stent-based delivery of everolimus has been shown to selectively clear macrophages in established aortic plaques from cholesterol-fed rabbits, without affecting the amount of lesion SMCs (Verhey et al., 2007). In contrast, the beneficial effect of everolimus on atherosclerotic lesion development in LDLR(−/−) mice was not accompanied by a decrease of the relative amount of lesional macrophages (Mueller et al., 2008). Thus, the effect of everolimus on the macrophage content of experimental atherosclerotic lesions is still uncertain and needs further investigations. In this perspective, we investigated the effect of oral everolimus on the macrophage content of carotid lesions induced in cholesterol-fed rabbits by perivascular collar placement, a well-established method of arterial manipulation in which site-controlled atherosclerotic-like lesions develop in the presence of an anatomically intact endothelium and in the absence of medial injury, interruption of blood flow, or thrombus formation (Booth et al., 1989; Donetti et al., 2002). In addition, to gain insights on the potential mechanism(s) underlying the effects observed in vivo, we investigated the effect of everolimus on chemoattractant-induced migration and viability of human monocytes in vitro.

Materials and Methods

Reagents

The enzymatic colorimetric kit for cholesterol quantification was purchased from DASIT (Milan, Italy). MCP-1 (CCL2), DMLP, rh-fractalkine (CX3CL1), rh-complement C5a, Triton X-100, propidium iodide, anti-α-smooth muscle actin monoclonal antibody, and 3,3′-diaminobenzidine were purchased from Sigma-Aldrich (St. Louis, MO). rhIL-8 (CXCL8) was obtained from R&D Systems (Minneapolis, MN). RAM11 monoclonal antibody was purchased from Dako Denmark A/S (Glostrup, Denmark). Vectastain ABC Elite kit was obtained from Vector Laboratories (Burlingame, CA). Diff-Quik staining reagents were from Medion Diagnostic International Inc. (Miami, FL). Lactate dehydrogenase (LDH) cytotoxicity detection kit was purchased from Takara (Kyoto, Japan), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) colorimetric assay reagents were obtained from Millipore (Billerica, MA).

Drug

Everolimus [40-O-(2-hydroxyethyl)-rapamycin] was supplied by Novartis Pharma AG (Basel, Switzerland) (Schuler et al., 1997). For in vivo application, it was formulated at 2% (w/v) in a microemulsion preconcentrate that was diluted to the appropriate concentration with 5% (w/v) glucose solution just before administration by oral gavage. The 2% microemulsion was stored refrigerated (2–8°C) under light protection. For in vitro work, everolimus was dissolved at 10 mg/M in absolute ethanol, and the stock solution was stored light-protected at −20°C.

In Vivo Experiments

The studies were performed in accordance with the principles stated in the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). The animal research protocols were approved by the responsible public authorities as required by the Italian Law in accordance with European Union regulations.

Experimental Protocols

The schematic diagrams of the experimental protocols are presented in Fig. 1, A and B. All the rabbits appeared to be healthy throughout the studies. There was no significant difference in water and food consumption between control and everolimus-treated rabbits.

Protocol 1: Dose-Finding Study. A preliminary pharmacokinetic study was performed to identify the optimal oral dose of everolimus for the subsequent pharmacodynamic evaluation. Twelve male New Zealand White rabbits (Charles River Laboratories, Inc., Wilmington, MA) were divided into four groups receiving, respectively everolimus at a dose of 0.75, 1, and 1.5 mg/kg/day or vehicle (5% glucose solution) in a volume of 2 ml/kg body weight. Rabbits were assigned randomly to each of the treatment groups and received everolimus or vehicle for 5 consecutive days by oral gavage. During the study, animals were provided ad libitum with standard rabbit chow (Laboratorio Dottori Piccioni, Milan, Italy) and water. At various time points, blood samples were drawn from the ear vein. Whole-blood everolimus concentrations were determined by Novartis Pharma AG using high-performance liquid chromatography-tandem mass spectroscopy.

Protocol 2: Effect of Everolimus on Intimal Lesions Induced by Perivascular Collar Placement in the Carotid Artery of Cholesterol-Fed Rabbits. Twenty-eight rabbits were allowed to acclimatize for 1 week, after which they were shifted from standard rabbit chow to a 1% cholesterol-rich diet (HC diet; Laboratorio Dottori Piccioni). The daily amount of HC diet was restricted to 120 g, whereas water was provided ad libitum. Three weeks after the beginning of the HC diet rabbits underwent cholesterol screening to assess their response to cholesterol feeding. One day before collar ing, rabbits were evenly distributed into control animals and animals receiving everolimus (n = 14 per group), according to body weight and plasma total cholesterol concentrations. Treatment with everolimus was performed by oral gavage and consisted of a 1.5 mg/kg loading dose, given 1 day before collar ing, followed by 1 mg/kg/day everolimus for 14 days. Control animals were gavaged with a matching volume of vehicle (2 ml/kg body weight).

Animal behavior and food intake were monitored daily. The weight of the animals was controlled weekly to adjust the dose of drug. To monitor the response to HC diet exposure, blood samples were drawn from the ear central artery at the beginning of the study (baseline), after 3 weeks on HC diet (intermediate), at the time of collar surgery, and at sacrifice. Plasma total cholesterol levels were measured in triplicate by standard colorimetric assays (cholesterol oxidase method).

Perivascular Carotid Collar Placement

Rabbits were anesthetized by intramuscular injection of xylazine (5 mg/kg, Rompun; Bayer, Milan, Italy) and ketamine (35 mg/kg, Ketavet; Farmaceutici Gellini Spa, Aprilia, Italy). A midline neck incision was made to surgically expose both carotid arteries, and a nonocclusive silastic collar was positioned around the right common carotid artery, as previously described (Baetta et al., 2002). The contralateral carotid artery was sham-operated by surgical dissection from surrounding tis-
Effect of Everolimus on Monocyte/Macrophage Recruitment

PLASMA CHOLESTEROL PROFILE

FPLC Analysis of Plasma Cholesterol Profiles

Histology and Immunohistochemistry

In Vitro Experiments

Human Monocytes

Monocyte Chemotaxis Bioassay

Fig. 1. Schematic diagrams of in vivo studies. A, experimental protocol 1. Rabbits (n = 12) were divided into four groups receiving everolimus (0.75, 1, or 1.5 mg/kg/day, respectively) or a matching volume of vehicle for 5 consecutive days by oral gavage. Blood samples were drawn at the indicated time points for evaluation of whole-blood everolimus concentrations. B, experimental protocol 2. Twenty-eight days after the beginning of a 1% cholesterol-rich diet (HC), rabbits (n = 28) underwent carotid collar surgery. Treatment with everolimus consisted of a 1.5 mg/kg loading dose, given 1 day before collaring, followed by 1 mg/kg/day everolimus for 14 days; control animals were gavaged with a matching volume of vehicle solution.

Fig. 2. Whole-blood everolimus concentrations. Rabbits were treated as shown in Fig. 1A. Everolimus content in the whole blood was determined by high-performance liquid chromatography-tandem mass spectrometry.
Plasma total cholesterol (TC) levels throughout the study were comprised between 3 and 8 ng/ml (Starling et al., 2004; Lorber et al., 2006). Monocyte concentrations that better matched the therapeutic concentration measured over the course of the dose-finding study are reported in Table 1. As depicted in Fig. 3, the HC diet caused an accumulation of cholesterol mainly in the very low-density lipoprotein fraction (FPLC fractions 6–16), and the pharmacological treatment did not significantly alter the FPLC cholesterol profile.

**Everolimus Reduced Carotid Intimal Thickening and Lesion Macrophage Accumulation without Affecting SMC Content.** Representative microphotographs of H&E-stained sections of collared carotids from control and everolimus-treated animals are shown in Fig. 4, and quantitative results from histomorphometric analysis are reported in Table 2. Collared carotids of the control group showed an evident intimal thickening that was less marked in carotids from everolimus-treated rabbits (Fig. 4, top), which exhibited a lower intimal area and a decreased I/M (Table 2). No difference in medial area was detected between the two experimental groups (Table 2).

**In Vitro Experiments**

To explain the observation that everolimus treatment reduced macrophage accumulation in the carotid lesions, we

![Fig. 3. Effect of everolimus on cholesterol profile. Rabbits were treated as shown in Fig. 1B. Plasma samples obtained at sacrifice from HC rabbits treated or not with everolimus were pooled, fractionated by FPLC, and the cholesterol concentration of each fraction was determined as described under Materials and Methods. The average cholesterol content of each FPLC fraction from each treatment group is shown. Fractions 6 to 16, very low-density lipoprotein; fractions 17 to 30, intermediate-density lipoprotein/low-density lipoprotein; fractions 31 to 40, high-density lipoprotein. Red, control (vehicle-treated rabbits); blue, everolimus-treated group.](image-url)
hypothesized that the drug might affect monocyte chemotaxis and tested this hypothesis by in vitro experiments on monocyte migration.

**Everolimus Inhibits Monocyte Chemotaxis in Response to Different Chemotactic Factors.** First, we investigated the effect of everolimus on MCP-1 (CCL2)-mediated monocyte chemotaxis in vitro and found that overnight pretreatment of monocytes with clinically relevant concentrations of everolimus (0.1–100 nM) (Starling et al., 2004; Lorber et al., 2005) abolished MCP-1 elicited migration of monocytes through the Boyden chamber filter (Fig. 5A).

To assess whether this effect was specific to the MCP-1/CCR2 pathway or everolimus might interfere also with the response to other chemotactic factors, we next investigated the effect of everolimus on monocyte migration induced by other chemotactic factors, such as fragment (CX3CL1), interleukin-8 (CXCL8), the complement fragment C5a, and fMLP (N-formyl-Met-Leu-Phe). We found that everolimus inhibited the migration of monocytes induced by all these chemotactic factors (Fig. 5B), thus suggesting a broad antichemotactic effect of the drug.

**The Inhibitory Effect of Everolimus on Monocyte Chemotaxis Is Not Associated with Induction of Cell Death.** To rule out the possibility that inhibition of monocyte

---

**TABLE 2**

Effect of everolimus on intimal thickening, macrophage accumulation, and SMC content of collar-induced rabbit carotid arterial lesions

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Everolimus (1 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimal area (μm²)</td>
<td>114,900 ± 22,170</td>
<td>70,970 ± 12,660</td>
</tr>
<tr>
<td>Medial area (μm²)</td>
<td>369,500 ± 25,800</td>
<td>358,300 ± 10,650</td>
</tr>
<tr>
<td>I/M</td>
<td>0.296 ± 0.035</td>
<td>0.190 ± 0.030*</td>
</tr>
<tr>
<td>RAM11-positive area (%)</td>
<td>14.4 ± 2.7</td>
<td>5.0 ± 1.1**</td>
</tr>
<tr>
<td>α-Actin-positive area (%)</td>
<td>29.3 ± 2.2</td>
<td>31.4 ± 1.5</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. control.

**Fig. 4.** Effect of everolimus on intimal thickening, macrophage accumulation, and SMC content of rabbit carotid artery lesions 14 days after collar placement. Rabbits were treated as shown in Fig. 1B. Formalin-fixed, paraffin-embedded sections of collared carotids retrieved at sacrifice from control (n = 12) and everolimus-treated (n = 14) animals were stained with H&E (top) or immunostained with RAM11 antibody (middle, brown, macrophages (MΦ)) and anti-α-smooth muscle actin antibody (bottom, brown, SMC).

**Fig. 5.** Effect of everolimus on monocyte chemotaxis. Human monocytes were incubated overnight in the presence or the absence of everolimus at the indicated concentrations. Cell migration in response to chemotactic factors (10 nM) was determined by the Boyden chamber chemotaxis assay, as described under Materials and Methods. The number of cells migrating in the presence of chemottractant was taken as 100%, and the values of cells migrating after pretreatment with everolimus were calculated relative to this. A, effect of different concentrations of everolimus on MCP-1/CCL2-induced monocyte migration. The average cell number/field in stimulated controls was 135 ± 35. Results are representative of three independent experiments performed in triplicate. B, effect of 10 nM everolimus on monocyte migration in response to the different chemotactants (10 nM). The increase in cell migration in stimulated controls as compared with unstimulated control (basal) was as follows: CCL2, 1.9-fold; CX3CL1, 2.3-fold; CXCL8, 1.7-fold; C5a, 3-fold; and fMLP, 3-fold, p < 0.01 for all chemotactants. All conditions were assayed in triplicate and individual experiments were performed at least twice.
chemotaxis by everolimus treatment was because of induction of cell death, we evaluated the effect of the drug on monocyte viability. We found that everolimus treatment neither increased the release of the cytoplasmic enzyme LDH compared with control monocytes (Fig. 6A) nor reduced the ability of monocytes to metabolize MTT yielding the blue formazan product, a process that requires active mitochondria and occurs only in living cells (Fig. 6B). Furthermore, we found that everolimus treatment did not induce the appearance of hypodiploid cells (average percentage of sub-G0/G1 cells: 3.69 versus 4.20% for control and 100 nM everolimus, respectively). Overall, these findings indicate that everolimus did not cause monocyte cell death under the experimental conditions of the present study.

Discussion

In this study, we showed that systemic administration of the mTOR inhibitor everolimus to cholesterol-fed rabbits strongly reduced macrophage accumulation within carotid lesions induced by perivascular collar placement, without affecting lesion SMC content. This effect was accompanied by a reduction of intimal thickening and occurred in the absence of changes in plasma cholesterol.

The results of the present study extend previous findings showing potential antiatherosclerotic effects of everolimus (Cole et al., 1998; Farb et al., 2002; Matsumoto et al., 2003; Verheye et al., 2007; Mueller et al., 2008). In particular, our results are in agreement with recent data from Verheye et al. (2007), who showed a marked reduction of macrophage content in established aortic plaques of cholesterol-fed rabbits after stent-based delivery of everolimus. Thus, everolimus, administered either locally or systemically, can decrease macrophage content of both early and established atherosclerotic lesions. Yet, in contrast to these findings, the beneficial effect of subcutaneous everolimus treatment on atherosclerotic lesion development in LDLR(−/−) mice recently reported by Mueller et al. (2008) was not accompanied by a decrease of the relative amount of lesional macrophages; on the contrary, it was associated with the presence of lesions that were smaller and less complicated but predominantly macrophage-filled. Overall, therefore, preclinical data suggest that the effect of everolimus on the macrophage component of the evolving atheroma is complex and might be dependent on several experimental factors, including the animal species, the nature and stage of the arterial lesion, and the treatment modalities.

To gain insights on the potential mechanism(s) mediating the effect of everolimus on lesional macrophage content, we investigated the influence of everolimus on chemoattractant-induced migration of human monocytes in vitro. We found that treatment with clinically relevant concentrations of everolimus (10 nM) decreased monocyte chemotaxis in response to various chemotactic factors, including MCP-1, fractalkine, IL-8, C5a, and fMLP. This finding is in agreement with evidence that indicate the involvement of the mTOR signaling pathway in the regulation of leukocyte migration in response to a variety of mediators (Gomez-Cambronero et al., 2005; Pakala et al., 2005; Fox et al., 2007; Murooka et al., 2008), suggesting that disruption of mTOR signaling by mTOR inhibitors may hamper the ability of leukocytes to migrate in response to inflammatory stimuli. Alternatively, as proposed by Verheye et al. (2007), mTOR inhibition by everolimus may induce macrophage clearance via autophagy, a recently discovered mechanism mediating cell death in mammalian cells (Rubinsztein et al., 2007). The reduction of monocyte chemotaxis reported in our study, however, was not associated with reduction of cell viability by everolimus, most likely because the concentrations used in our experiments (0.1–100 nM) were significantly lower compared with the concentration at which everolimus has been shown to induce macrophage autophagy (10 μM) (Verheye et al., 2007). In this regard, it is interesting to note that both the systemic everolimus concentrations measured in our in vivo kinetic study and the everolimus through concentrations in transplanted patients (Starling et al., 2004; Lorber et al., 2005) are within this range. Thus, we propose that everolimus may possess a significant antimacrophage activity, mediated by at least two potentially independent mechanisms, namely the inhibition of monocyte recruitment into the arterial wall and the induction of macrophage death by autophagy (Verheye et al., 2007). In particular, inhibition of monocyte recruitment is effective in vitro at drug concentrations that are in the range of the therapeutic levels achieved in kidney and heart transplanted patient treated with...
systemic everolimus for the prophylaxis of organ rejection, whereas induction of autophagy seems to require concentrations of everolimus that greatly exceed therapeutic concentrations but are potentially achievable locally by stent-based everolimus delivery (Schomig et al., 2005). The molecular mechanism(s) mediating the antichemotactic action of everolimus reported in this study will require further investigation. Results indicate that the effect is not specific to selected chemotactant receptor pathways, suggesting that inhibition occurs downstream of ligand-receptor interaction. In line with this hypothesis, one possible mechanistic explanation can be delineated based on the recent findings from Fox et al. (2007). The authors showed that macrophage adherence promoted signaling through P-selectin glycoprotein ligand-1/Akt/mTOR that resulted in synthesis of catalytically active Rho-associated kinase-1, a serine/threonine kinase that plays a critical role in physiological functions of macrophages, and that inhibition of mTOR by rapamycin abolished Rho-associated kinase-1 synthesis in macrophages, resulting in a partial inhibition of chemotaxis and phagocytosis (Fox et al., 2007). In addition, it is worthwhile to mention the recent hypothesis from Murooka et al. (2008), who proposed that mTOR inhibition might reduce the chemotaxis of immune cells by hampering the initiation of mRNA translation through mTOR/initiation factor 4E binding protein 1, a crucial step of the migratory response to chemotactic factors. However, the complex mode of action of mTOR inhibitors and the continuous advances in the understanding of mTOR signaling make it necessary to consider several different hypotheses when dealing with the molecular basis underlying the pharmacological effects of such drugs.

In summary, mTOR signaling has recently emerged as a key process in many human diseases, including cancer, diabetes, obesity, and cardiovascular and neurological diseases (Tsang et al., 2007). As a consequence, new clinical indications for mTOR inhibitors are emerging, beyond their immunosuppressive activity (Tsang et al., 2007). Although the systematic administration of immunosuppressive drugs is currently not an option for treating atherosclerosis, the findings of the present study, along with previous data in different animal models, indicate that everolimus may favorably influence the inflammatory response that occurs during experimental atherosclerosis. In particular, everolimus seems able to target both the recruitment of monocytes into the nascent lesion and the survival of macrophages in mature pre-existing plaques. These findings cannot be extrapolated to humans. Thus, clinical trials are required to gain insights on the potential antiatherosclerotic properties of everolimus.

Acknowledgments
We thank Dr. Francesca Silva and Dr. Lucia Sordo for technical assistance with the in vivo work; Patrizia Ubolbi for technical assistance with FPLC analysis; Dr. Barbara Viviani for helpful advice about cytotoxicity assays; and Dr. Walter Schuler, Dr. Richard Dorent, and Dr. Bettina Ulbricht for kind support, fruitful discussion, and critical review of the manuscript.

References


Address correspondence to: Roberta Baetta, Department of Pharmacolog-ical Sciences, University of Milan, Via Balzaretti 9, 20133 Milan, Italy. E-mail: roberta.baetta@unimi.it