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Matrix metalloproteinase inhibitors prevent the release and proteolytic activity of monocyte/macrophage-derived microparticles

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Abstract

Background: The role of monocyte/macrophage-derived microparticles (MPs) in the pathophysiology of cancer and chronic inflammatory diseases has been reported; nevertheless, the mechanism underlying microparticles release is currently unclear. The aim of the current study was to investigate whether matrix metalloproteinase (MMP) inhibitors can prevent MP shedding from stimulated human monocyte/macrophage.

Methods: Microparticles were obtained by isolated peripheral blood mononuclear cells after stimulation with the calcium ionophore, A23187. MP shedding, intracellular calcium concentration, analysis of RhoA expression, and proteolytic activities of isolated MPs were assessed in the absence or presence of MMP inhibitors.

Results: We demonstrated that MMP inhibitors remarkably prevented MP shedding in a concentration-dependent manner with IC_{50} values in the nano- to micromolar range. Such an effect was related to their ability to reduce the intracellular Ca^{2+} levels induced by the calcium ionophore and the consequent translocation of RhoA from cytosol to membrane. Furthermore, MMP inhibitors could inhibit the proteolytic activity of cell-derived MPs.

Conclusions: The current study provide evidence that MMP inhibitors can prevent MPs shedding from stimulated human monocyte/macrophage and the proteolytic activity of released MPs. Finally, the most active compound tested might represent the lead compound of a new class of molecules with therapeutic potential in cancer and chronic inflammatory diseases.

Key words: microparticles; matrix metalloproteinases; mononuclear cells.

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1. Introduction

Exosomes and ectosomes are extracellular vesicles (EVs) involved in intercellular communication. Ectosomes (also known as microvesicles or microparticles) are small membrane bound vesicles released from the cell under homeostatic conditions and cellular activation that play a key role in the pathophysiology of several chronic diseases [1,2]. For example, human monocytes were found to shed ectosomes (also termed microparticles, MPs) with procoagulant activity [1,2], whereas tissue factor-bearing circulating MPs have been recognized as important players to the pathogenesis of disseminated intravascular dissemination in acute myeloid leukemia [3]. As far as inflammation is concerned, evidence obtained during cell-cell communication studies demonstrated that endothelial cell activation induced by polymorphonuclear cells was at least partially mediated by MPs [4]. In line with this notion are data showing that MP numbers are increased in blood and other fluids during inflammatory conditions with high levels found in synovial fluid of inflamed joints of patients with rheumatoid arthritis [5]. As a matter of fact, monocytes play a pivotal role in the pathogenesis of rheumatoid arthritis and immune cell MPs has been reported to induce cytokine expression in synovial fibroblast [6]. Furthermore, monocyte-derived MPs were reported to up-regulate inflammatory mediator synthesis in both human airways epithelial [7] and bronchial smooth muscle cells [8] and to promote an autocrine activation of human monocyte/macrophages [9]. Most of our understanding on the mechanism by which EVs are assembled derived from studies carried out on exosomes, while only recently have the research efforts focused on ectosomes [10].

The Ras homolog gene family member A (RhoA) is a small GTPase protein that has been associated with cytoskeleton rearrangements [11] and biogenesis of apoptotic exosome-like vesicles in bone marrow-derived macrophages [12]. Furthermore, the role of Ca2+ in the regulation of actin cytoskeleton remodeling and cell migration has also been demonstrated [13].

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play a key role in various pathophysiological processes [14]. Noteworthy, MMPs have been detected in exosomes and some of them have been shown to be proteolytically active [11]. Although some evidence is reported that metalloproteinase activation can stimulate exosome releases [15], it is currently unknown whether metalloproteinase inhibitors can directly modulate MP shedding and, if they do, the underlying mechanism.
Findings of the current study demonstrated that synthetic metalloproteinase inhibitors can reduce the release of MPs from stimulated human mononuclear cells by an off-target mechanism. Furthermore, these compounds inhibited the protease activity of isolated MPs.

2. Materials and methods

2.1 Reagents and MMP inhibitors

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, dulbecco phosphate buffered saline (PBS), fetal bovine serum (FBS), dextran, ficoll–hystopaque, calcium ionophore A23187 were obtained from Sigma Aldrich (Milan, Italy). The Zymuphen MP-Activity kit was obtained from Hyphen BioMed (Neuville-sur-Oise, France). Fluorogenic substrate FS-6 (Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2) was purchased from Calbiochem (Merck Millipore). MMP inhibitors ARP100, EN140 and LP109 (Figure 1) have been synthesized, as previously described [16-18].

2.2 Peripheral blood mononuclear cells (PBMC) isolation and culture

PBMC were isolated from fresh buffy coats obtained from the local blood bank with minor modifications, as described [19]. The procedure was approved by the local ethics committee in accordance with the Declaration of Helsinki. Due to the strictly blind nature of the procedure, we were not informed whether some of the blood bank donors were taking medications or had some abnormal laboratory data. Briefly, a fresh buffy coat was mixed gently with an equal volume of 2.5% Dextran T500, and left for 40 min for erythrocyte

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sedimentation. Ten mL of leukocyte-rich supernatant was layered over 5 mL of Ficoll–Hystopaque and centrifuged for 30 min at 350 ×g at 4 °C. The PBMC-rich ring was washed twice in PBS. PBMC were then resuspended in RPMI/1% penicillin and streptomycin/1% L-glutamine and allowed to adhere for 30 min at 37 °C on 24-well plates (2 × 10^6 cells/well). The cells were then washed two times with pre-warmed PBS, resuspended in RPMI 1% penicillin and streptomycin, 1% L-glutamine, 5% fetal bovine serum at 37 °C for 18 h at 37°C on 24-well plates (10^6 cells/well) or onto 8-well chamber slides (25x10^4).

2.3 MP generation and purification

Upon overnight incubation PBMC were washed twice with pre-warmed PBS and stimulated with calcium ionophore A23187 at 12 µM for 15 min after a 30 min of pre-incubation with three different MMP inhibitors (ARP100, EN140 and LP109) or control buffer. At the end of the treatment period the conditioned medium was collected and cleared by centrifugation at 14,000g for 5 min at 4°C to remove dead cells and cell fragments. In experiments designed to investigate the "MMP activity" on MPs, conditioned medium was further submitted to centrifugation (16,000×g for 1h at 4°C) and the pellet obtained was suspended in 100 µl PBS.

2.4 Measurement of MP in solid phase

MP were detected in the conditioned medium of PBMC using the Zymuphen MP-activity kit according to the manufacturer's instructions and expressed as phosphatidylserine (PS) equivalents. Briefly, the assay is based on the property of annexin-V, immobilized onto plastic wells, to bind PS. PBMC supernatant was added to the wells and, after extensive washing, captured MP were detected by the addition of activated factor V, FXa, Ca^{2+} and prothrombin. Under the conditions used, the rate of thrombin formation is limited by PS availability and is therefore proportional to MP concentration. A chromogenic substrate was finally added to quantify thrombin concentration with a microplate reader (iMark™ Microplate

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Absorbance Reader, Bio-Rad, Milan, Italy). Known amounts of PS were used to obtain a standard curve [20].

2.5 Measurement of intracellular calcium concentration

Molecular Probes Fluo-4 NW Calcium Assay kit was used to measure the changes in the intracellular calcium concentration ([Ca2+]i) of mononuclear cells. Briefly, prewashed mononuclear cells on 96-multiwell plate (1 x 10^6/well) were loaded with 100 µl of the dye loading solution containing Fluo-4 NW dye and Probenecid according to the manufacturer's instructions. The 96-well plate was incubated at 37 °C for 30-45 min in the dark and the calcium ionophore, A23187, was added to the cells in the presence or in the absence of EN140 or ARP100 or LP109. The changes in Fluo-4 NW fluorescence were measured by the Wallac 1420 Victor 2 (PerkinElmer, Milan, Italy) at λ_{ex} 494 nm and λ_{em} 516 nm. Calcium mobilization was recorded continuously over time up to 110 sec and analyzed by the Wallac 1420 Software version 3 (PerkinElmer Life and Analitical Sciences, Wallac, Milan, Italy). The increase in $[Ca^{2+}]_i$ fluorescence (expressed as relative fluorescence units, RFU) was calculated as the difference between the mean stimulation fluorescence value and the mean baseline fluorescence observed (delta RFU).

2.6 Microscopic analysis of RhoA expression

PBMC grown in chamber slides were washed twice with pre-warmed PBS and stimulated with calcium ionophore A23187 at 12 µM for 15 min after a 30 min of pre-incubation with three different MMP inhibitors ARP100 (1 µM) EN140 (10 µM) and LP109 (10 µM). Then, cells were washed with PBS, fixed with 1% formalin/PBS for 15 min at 4 °C and analyzed for RhoA expression.

To control a possible RhoA activation, we evaluated its cellular distribution by immunofluorescence analysis. Indeed, as concerns the Rho proteins, they are GTP-bound

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and located at membrane level when they are activated, while they are GDP-bound and localized at cytoplasm level when they are inactivated [21].

Slides with fixed cells were treated for 10 min with 0.2% triton-X100/PBS and, after 1 h in blocking solution (BS: 0.1% Tween, 0.25% bovine serum albumin in PBS), they were incubated overnight at 4°C with mouse anti RhoA antibody (1B3-4A10, Thermo Scientific,Waltham, Massachusetts, USA), diluted 1:100 in BS. Slides were then washed three times in BS and incubated for 90 min in the dark with relative fluorescent secondary antibody diluted 1:250 in BS (Alexa Fluor® 488 anti-mouse, Life Technologies Italia, Monza, MB, Italia). After washes, samples were finally mounted with PBS-glycerol solution. All steps were performed at room temperature unless otherwise specified. Negative controls for the specificity of secondary antibody were performed omitting the primary antibody. The samples were observed with a confocal laser scanning microscope (TC SSP8 Leica Microsystems, Mannheim, Germany) at 63x magnification using a 488-nm excitation wavelength laser.

2.7 Assessment of MMP-dependent proteolytic activity of MPs

We used a fluorometric assay to assess the proteolytic activities of MPs isolated from human mononuclear cells stimulated or not with A23187. We pre-warmed isolated MPs and 10 μM fluorogenic substrate FS-6, a known substrate for MMPs [22], in separate aliquots of buffer (25 mM Tris/HCl, pH 8.0, containing 2.5 μM $ZnCl_2$, and 3 mM $CaCl_2$) at 37°C for 30 min. These aliquots were mixed to initiate the enzymatic reaction in a final volume of 100 μL within wells of a 96-well plate at 37°C. Cleavage of the substrate (final concentration 5 μM) was kinetically monitored at 325 nm excitation and 400 nm emission every 5 min for 2 h using a Molecular Devices SpectraMax Gemini XPS plate reader. Experiments were carried out in the presence or absence (control) of different concentrations of test compounds. Data were analyzed using GraphPad software to calculate the slopes (deltaF/deltaT) of the linear portion of the time-response curves.

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2.8 Data analysis and statistics

Data were presented as mean ± standard deviation (SD) from at least three independent experiments. Data were analyzed by using SoftMax Pro software (version 5.4.3, Molecular Devices, Sunnyvale, CA) and Prism 7 (GraphPad Software, Inc.). The level of statistical significance was $p < 0.05$ (Student t-test or one-way ANOVA followed by Dunnetts's multiple comparison test).

3. Results

3.1 inhibition of MP generation

Cells exposed to A23187 produced MPs, reaching 3 times the values from control (Figure 2). ARP100 significantly ($p < 0.05$) reduced MP release in a concentration-dependent manner with an IC_{50} of 0.03 ± 0.02 μM. The maximum effect (90% reduction of MPs release, as compared to control) was reached at 0.5 μM and maintained at the lowest concentration tested (i.e., 10 μM) (Figure 2). The two broad-spectrum MMP inhibitors, EN140 and LP109, also dose-dependently inhibited the MP release induced by A23187, but with IC_{50} values much higher than ARP100 (i.e., 2.6 ± 1.2 and 1.7 ± 0.3 μM, respectively). There was a trend towards a maximum effect greater for LP109 than EN140 (80 and 58% reduction of MPs release, respectively) (Figure 2).

3.2 Prevention of changes in the intracellular calcium concentration $[Ca^{2+}]_i$

Experiments were carried out to assess whether test compounds could modulate the $[Ca^{2+}]_i$ in mononuclear cells. As expected, stimulation with the calcium ionophore, A23187, significantly increased $[Ca^{2+}]_i$ by 6-fold, as compared to control. Figure 3 clearly demonstrated that ARP100 at 1 μM, and LP109 and EN140 at 10 μM, (i.e., concentrations proven to maximally reduced MPs release) completely prevented the A23187-induced $[Ca2+]_i$ increase (Figure 3).

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3.3 Immunofluorescence microscopy of RhoA

Treatment of cells with A23187 promoted translocation of RhoA from cytosol to plasma membrane (Figure 4). Pretreatment with test compounds (i.e., 1 μM ARP100, and 10 μM LP109 or EN140) was found to prevent A23187—induced membrane translocation of RhoA (Figure 4).

3.4 Effects on the proteolytic activity of released MPs

The ability of isolated MPs to cleave the fluorogenic substrate, FS-6, i.e., a synthetic substrate commonly used for studying MMP activity [22], is shown in Figure 5. The same experiment conduced on the original monocytes, without stimulation with A23187, showed a flat curve with a low increase of fluorescence over time (data not shown). The addition of ARP100 to isolated MPs produced a concentration-dependent downward and a rightward shift in the time-response curve (Figure 5). At the maximum concentration tested (i.e., 100 μM), the slope (deltaF/deltaT) of the linear portion of the curves were significantly reduced after treatment with ARP100 compared to untreated MPs (18.9 ± 4.9 *vs.* 25.1 ± 4.2; $p < 0.05$). A similar effect on the kinetic profile of the enzymatic reaction was observed when EN140 and LP109 were tested in the same concentration range of ARP100 (Figure 5). As for ARP100, a significant reduction of the slope was observed for both EN140 (16.9 ± 5.7 *vs.* 22.1 ± 5.6; $p < 0.05$) and LP109 (14.5 ± 4 *vs.* 19 ± 4.7; $p < 0.05$) at the maximum concentrations tested.

4. Discussion

In the current study, we demonstrated that MMP inhibitors, at clinically achievable concentrations, could prevent the MP shedding from stimulated human mononuclear cells in a concentration-dependent manner. Three previously developed hydroxamate-based inhibitors were chosen, two broad-spectrum (EN140 and LP109) and one selective MMP-2

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inhibitor (ARP100) [17,18]. Compounds had different potency with ARP100 being the most active one (IC_{50} about 100-fold lower than the others). Interestingly, previous in vitro fluorometric assays on isolated MMPs have shown that EN140 [18] and LP109 [17] had greater inhibitory potencies than ARP100 (Table 1), suggesting that the ability of MMP inhibitors to prevent MP shedding could be unrelated to their main mechanism of action. To test this hypothesis, we investigated whether metalloproteinase inhibitors could affect changes in $[Ca^{2+}]_i$, i.e., a fundamental process during membrane remodeling and shedding of MPs [23,24]. Our experiments clearly demonstrated that ARP100 prevented $[Ca^{2+}]_i$ increases induced in stimulated mononuclear cells by calcium ionophore, at concentrations 10-fold lower than EN140 and LP109. ARP100 was also the most active compound in the inhibition of RhoA translocation from cytosol to cell membrane, a calcium—mediated mechanism associated with cytoskeleton reorganization which ultimately results in the release of MPs into the extracellular space [25]. Differences in potency between ARP100 and EN140 or LP109 in $[Ca^{2+}]_i$ and RhoA experiments reflected those obtained in the MP release test suggesting that reduction of MP shedding by metalloproteinase inhibitors occurred through an off-target mechanism. In line with this notion, there is evidence that mechanisms of ectosome formation include Ca^{2+}-activated processes and the presence of lipidic anchors of proteins that may contribute to membrane curvature, including RhoA [10]. Overall, our findings suggest that metalloproteinase inhibitors could inhibit MP shedding from stimulated mononuclear cells mainly through preventing the $[Ca^{2+}]_i$ increases and the consequent translocation of RhoA from cytosol to plasma membrane. The off-target ability of metalloproteinase inhibitors to remarkably reduce the release of MPs in the nanomolar to micromolar range opens the opportunity to develop a new class of molecules with therapeutic potential in those diseases where both MPs and MMPs are important triggering elements. For example, it has been demonstrated that MP shedding from tumor cell may reduce immune surveillance [26] and MMP activity was found to be involved in extracellular matrix rearrangement during tumor development [27]. In line with this notion, MMP-2 has

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been proven to be involved in tumor invasiveness by platelet-derived MPs [28] and in vascular matrix remodeling by endothelial cell-derived MPs [29]. Furthermore, exposure of human macrophages to tobacco smoke extract has proven to induce the release of proteolytic MMP14-expressing MPs, which may play a role in matrix destruction and disease development [30]. Finally, macrophage-derived MPs promote activation of rheumatoid arthritis synovial fibroblasts and induction of MMPs, thus reinforcing the importance of MPs and MMPs in disease pathogenesis [6].

Another important evidence emerged from the current study is the ability of test compounds to inhibit the proteolytic activity of isolated monocytes/macrophages-derived MPs in a concentration-dependent manner. Such an effect occurred in the micromolar range with no substantial differences among compounds. This property appears to be relevant since ADAMs have been implicated in proteolysis on the exosome surface [14] and the production of ADAM10- and ADAM17-positive MVs from smoke-exposed neutrophils has been associated to a potential higher risk to develop abdominal aortic aneurysm [31].

In conclusion, findings of the current study identify ARP100 as a potential lead compound of a new class of molecules with dual pharmacological activities (i.e., MMP inhibition and prevention of MP shedding) which deserve further investigation to confirm their therapeutic potential in important chronic diseases.

Declaration of Interest statement

None.

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Figure legends

Figure 1. Structures of tested MMP inhibitors.

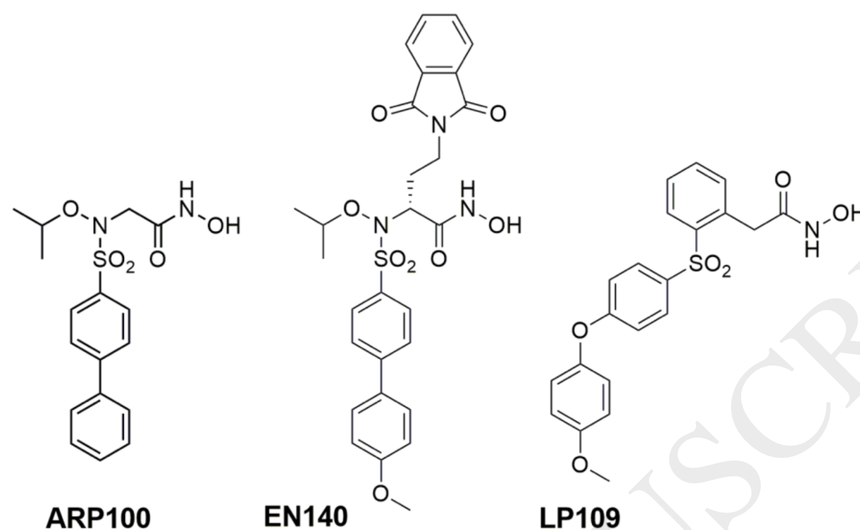

Figure 2. Effects of test compounds on the generation of human monocyte/macrophage-derived microparticles. A23187: calcium ionophore. Data presented as mean ± SD of at least three independent experiments. *$p < 0.05$, as compared to control (Student's t-test or one-way ANOVA followed by Dunnetts's test for multiple comparison).

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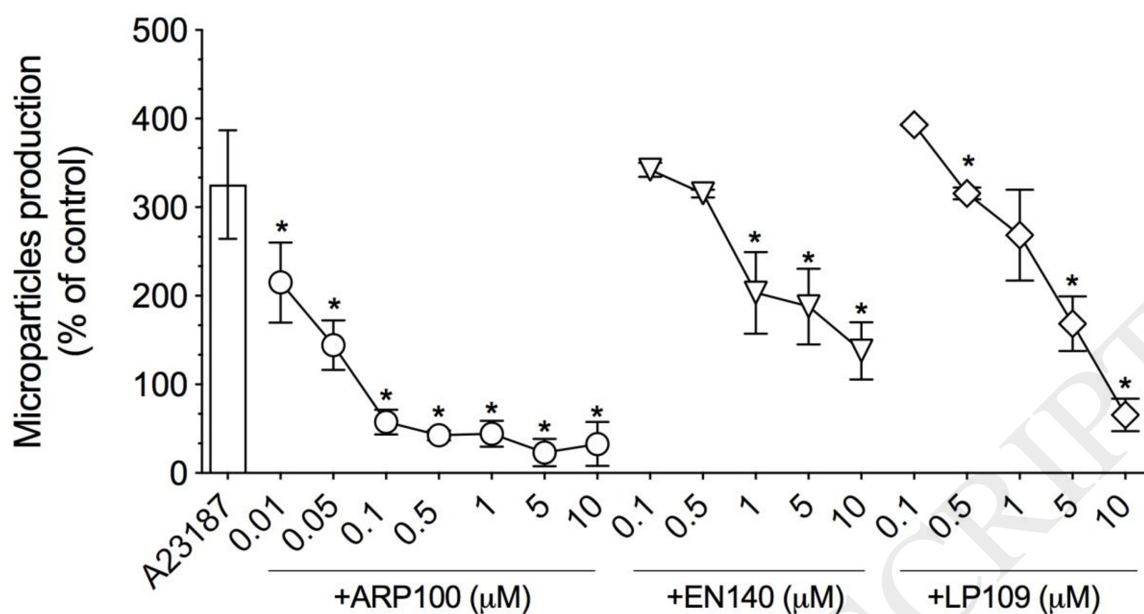

Figure 3. Effect of test compounds on intracellular calcium concentration. Data presented as mean ± SD of at least three independent experiments. $*p < 0.05$, as compared to control (one-way ANOVA followed by Dunnetts's test for multiple comparison).

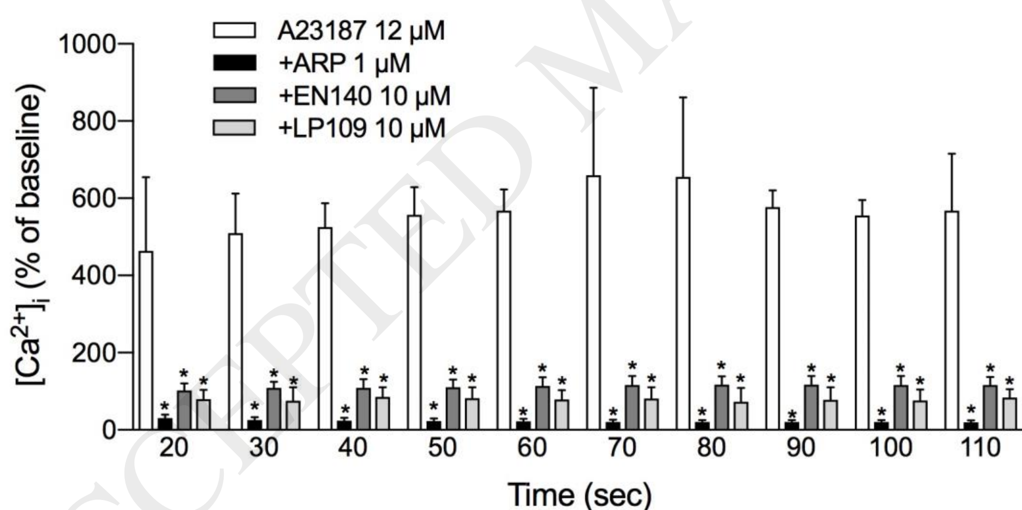

Figure 4. Representative images of human PBMC cells immunostained for RhoA (green). Cells were grown in control buffer alone (A), in the presence of calcium ionophore, alone (B) or with 10 μM LP109 (C), 10 μM EN140 (D) and 1 μM ARP100 (E). Scale bar: 10 μm.

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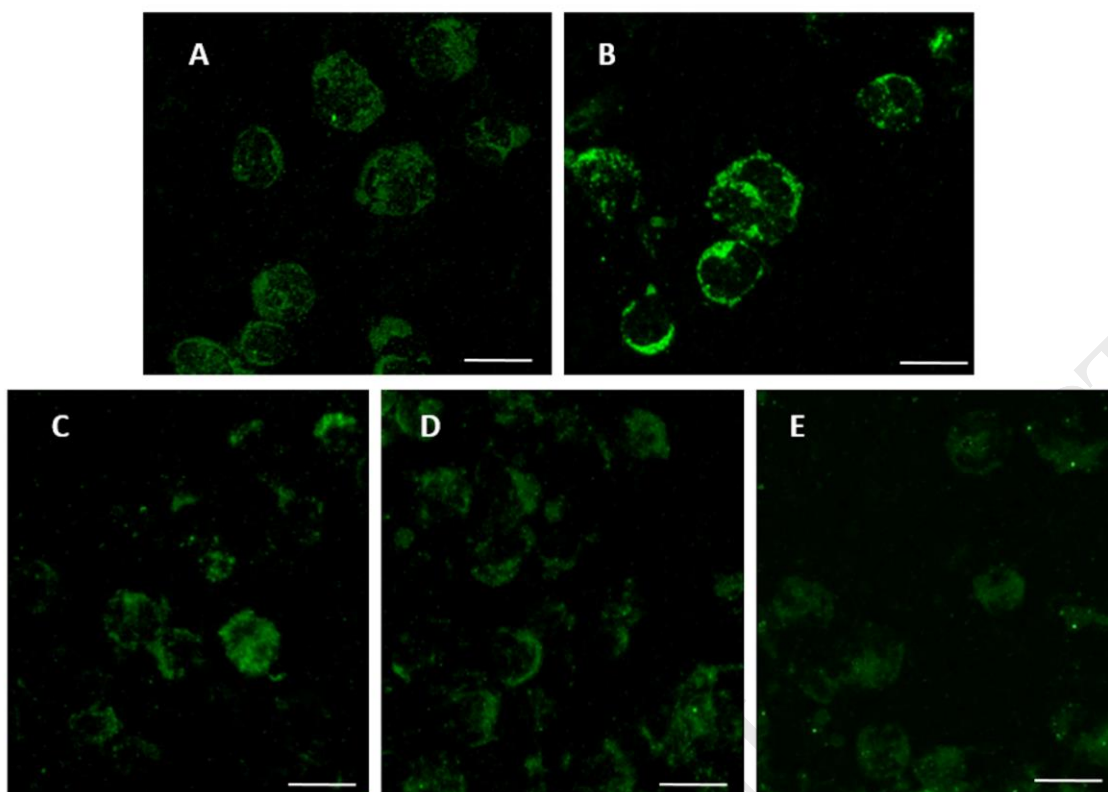
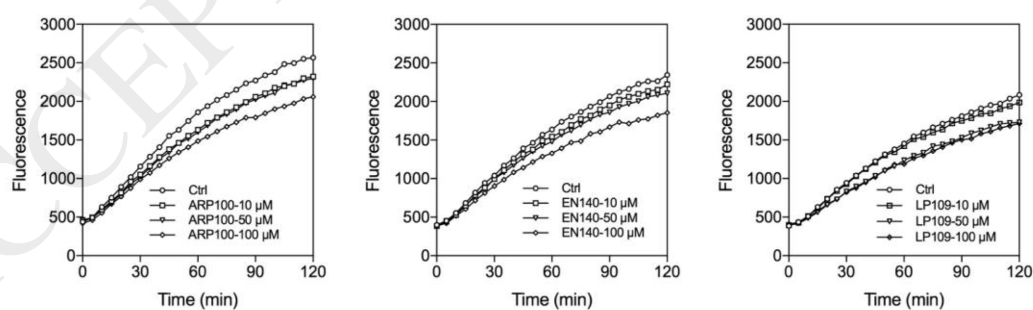


Figure 5. Proteolytic activity of MPs derived from human mononuclear cells. Fluorometric assay was carried out by using fluorogenic substrate FS-6, an artificial substrate for MMPs. Control (Ctrl) refers to untreated MPs (i.e., isolated MPs in the absence of MMP inhibitors).



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Tables

Table 1. *In vitro*[a] inhibitory activity (IC_{50}, nM) of ARP100, EN140 and LP109 on human recombinant metalloproteinases (MMPs) measured by a fluorometric assay, as previously reported [17,18].

Compd	(IC_{50}, nM)								Ref.
	MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-13	MMP-14	ADAM-17	
ARP100	12000	12	5900	260	200	45	2300	130,000	[32]
EN140	170	0.67	55	1.7	0.43	0.19	3.9	212,000	[18]
LP109	3500	3.5	21	5.1	0.8	4.1	33	19,000	[17]

[a]Enzymatic data are mean values for three independent experiments performed in duplicate. SD was generally within ± 10%. MMP: matrix metalloproteinase.

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