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Carboxyamidotriazole-Orotate Inhibits the Growth of Imatinib-Resistant Chronic Myeloid Leukaemia Cells and Modulates Exosomes-Stimulated Angiogenesis

Chiara Corrado[1☯], Anna Maria Flugy[1☯], Simona Taverna[1☯], Stefania Raimondo[1], Giuliana Guggino[1], Rashida Karmali[2], Giacomo De Leo[1¶], Riccardo Alessandro[1*¶]

1 Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Sezione di Biologia e Genetica, Università di Palermo, Italy, **2** Tactical Therapeutics Inc., New York, New York, United States of America

Abstract

The Bcr/Abl kinase has been targeted for the treatment of chronic myelogenous leukaemia (CML) by imatinib mesylate. While imatinib has been extremely effective for chronic phase CML, blast crisis CML are often resistant. New therapeutic options are therefore needed for this fatal disease. Although more common in solid tumors, increased microvessel density was also reported in chronic myelogenous leukaemia and was associated with a significant increase of angiogenic factors, suggesting that vascularity in hematologic malignancies is a controlled process and may play a role in the leukaemogenic process thus representing an alternative therapeutic target. Carboxyamidotriazole-orotate (CTO) is the orotate salt form of carboxyamidotriazole (CAI), an orally bioavailable signal transduction inhibitor that *in vitro* has been shown to possess antileukaemic activities. CTO, which has a reduced toxicity, increased oral bioavailability and stronger efficacy when compared to the parental compound, was tested in this study for its ability to affect imatinib-resistant CML tumor growth in a xenograft model. The active cross talk between endothelial cells and leukemic cells in the bone marrow involving exosomes plays an important role in modulating the process of neovascularization in CML. We have thus investigated the effects of CTO on exosome-stimulated angiogenesis. Our results indicate that CTO may be effective in targeting both cancer cell growth and the tumor microenvironment, thus suggesting a potential therapeutic utility for CTO in leukaemia patients.

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* E-mail: riccardo.alessandro@unipa.it

☯ These authors contributed equally to this work.

¶ These authors also contributed equally to this work.

Introduction

Chronic myeloid leukaemia is characterized by the Philadelphia (Ph) chromosome encoding the chimeric Bcr–Abl oncoprotein with a constitutive tyrosine kinase activity that drives disease pathogenesis by stimulating a number of downstream signalling cascades [1,2]. Whereas CML can be effectively treated, during chronic phase (CP), with tyrosine kinase inhibitors (TKIs) such as imatinib [3], the acquisition of imatinib resistance, mainly due to point mutations, causes disease progression (blast crisis) that can be fatal within months. To circumvent resistance, more potent TKIs, such as nilotinib and dasatinib, have been recently approved [4]. However, these compounds do not have therapeutic activity against all imatinib-resistant mutants of Bcr-Abl and therefore, a long-term tolerability problem has emerged [5]. Combination strategies of imatinib with drugs that target downstream signalling molecules have shown some success in the treatment of the imatinib-resistant cells in *in vitro* settings and in mouse models but have not been studied in clinical trials yet [6,7]. Therefore, there is an urgent need for new anticancer agents and combinations that can improve responses and survival rates for CML.

Carboxyamidotriazole-orotate (CTO) is the orotate salt form of carboxyamidotriazole (CAI), an orally bioavailable small molecule that was recently shown to decrease *in vitro* cell viability and to augment apoptosis in three different imatinib-resistant CML cell lines through the down-regulation of Bcr-Abl protein, inhibition of tyrosine phosphorylation of Bcr-Abl, STAT5, CrkL, as well as inhibition of ERK1/2 phosphorylation [8,9]. CTO shows a reduced toxicity, increased oral bioavailability and achieves higher plasma concentrations and stronger efficacy when compared to the parental compound [10].

We have recently demonstrated that LAMA84 CML cells release exosomes and that the addition of those microvesicles to HUVEC affects several steps of *in vitro* angiogenesis including motility, cytokine production, cell adhesion, and cell signalling as well as *in vivo* angiogenesis in nude mice [11]. A number of studies have recently described exosomes as new players in modulating the tumor microenvironment, promoting angiogenesis and tumor

development [12]; furthermore, neovascularization is known to exert an important role in the progression of chronic myeloid leukaemia and may represent a valid alternative target for therapy.

Taking these data into account, the aims of our study were (i) to test if CTO is able to inhibit *in vivo* the growth of imatinib-resistant CML cells and (ii) to investigate the ability of CTO to affect tumor microenvironment by modulating exosome-stimulated angiogenesis *in vitro* and *in vivo*. Our results indicate that administration of CTO to a CML xenograft model in NOD/SCID mice may increase survival and that CTO reduces in a dose- and time-dependent fashion exosomes-stimulated angiogenic process. Further work is necessary to demonstrate in a clinical setting, the possible use of CTO as an alternative therapeutical option in the treatment of imatinib-resistant forms of chronic myelogenous leukaemia.

Materials and Methods

Ethic Statement

All animal experiments were conducted in full compliance with Universita' di Palermo and Italian Legislation for Animal Care and Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi (DiBiMef) review board has approved this study.

Cell Culture and Reagents

Imatinib resistant LAMA84 and K562 cells (LAMA84R and K562R) were kindly provided by Dr. P. Vigneri, Università di Catania [13]. Cells were cultured in RPMI 1640 medium (Euroclone, UK) supplemented with 10% fetal bovine serum (Euroclone, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone, UK) and 1 µM of imatinib to maintain the resistance. HUVEC were obtained from Lonza and grown in Endothelial Growth Medium (EGM) according to supplier's information (Clonetics, Verviers, Belgium). Imatinib mesylate (Selleck chemicals, Houston, TX, USA) was prepared as a 1 mM stock solution in sterile phosphate-buffered saline (PBS); CAI orotate (CTO) from Tactical Therapeutics Inc, New York, USA was solubilized in DMSO at 0.1 M for *in vitro* assay. For *in vivo* assay CTO was prepared at 50 mg/ml in 80% PEG-100, sonicated, aliquoted and kept at −20°C; imatinib was dissolved in PBS at a concentration of 2 mg/ml. All other reagents were purchased from Sigma (St. Louis, MO), if not cited otherwise.

Proliferation Assay (MTT Assay)

Methyl-thiazol-tetrazolium (MTT) assay was done as previously described [8], cells were plated in triplicate or quadruplicate at 1.5×10^4 per well and exposed to escalating doses of CTO for up to 4 days. Means and standard deviations generated from 3 to 4 independent experiments are reported as the percentage of growth. Cell proliferation curves were derived from these data by using Microsoft Excel software.

Western Blot

Total protein cell lysates or exosome lysates were obtained and analyzed by SDS-PAGE followed by Western blotting as previously described [8]. Antibodies used in the experiments were: c-Abl, phospho-Abl, phospho-CrkL, Erk 1/2, phospho Erk 1/2, Hsc70, β-actin (all from Cell Signalling Technology, MA, USA); anti-CrkL, VCAM1-FITC, ICAM1-FITC and anti-CD63 (all from Santa Cruz Biotechnology, CA, USA).

CML Mouse Xenograft

Male NOD/SCID mice four-to-five week old were purchased from Charles River (Charles River Laboratories International, Inc, MA, USA) and acclimated for a week prior to experimentation. Mice received filtered water and sterilized diet *ad libitum*. Animals were observed daily and clinical signs were noted.

Each mouse was inoculated subcutaneously (sc) in the right flank with viable single cells (1×10^7) suspended in 0.2 ml of PBS. The day of injection was considered as Day 0. On Day 7, when tumors were palpable, mice were randomly assigned to groups of ten and were treated with imatinib administered intraperitoneally (i.p) (50 mg/Kg, three days a week for two rounds) or with its vehicle (PBS) in combination either with CTO 342 mg/kg (Q1D×5 for two rounds) or with CTO 513 mg/kg (Q1D×5 for two rounds) or with their vehicle (80% PEG-100). All mice received both p.o. and i.p. doses of the vehicle to control for morbidity associated with the treatment. Tumor xenografts were measured and the mice were weighed three times a week starting on Day 7. Tumor volume was determined by calliper by using the following formula: $L\times W^2/2 = mm^3$ where L and W are the longest and shortest perpendicular measurements in *millimeters*, respectively. The same formula was used to calculate tumor weights assuming that $1\ mm^3 = 1$ mg. Due to Università di Palermo rules and Italian legislation, animals were euthanized when sc tumor xenografts reached 4000 mg in weight.

Exosome Isolation and Characterization

Exosomes produced by LAMA84R cells during a 24 h culture period, were isolated from conditioned culture medium by different centrifugations as described previously [11]. Exosome protein content was determined by the Bradford assay (Pierce, Rockford, IL, USA). The activity of acetylcholinesterase, an exosome marker protein, was determined as described by Savina et al [14]. Briefly a total of 10 µg of exosomes or 10 µg of total cell lysate in 100 µl of PBS were resuspended in a solution of 1.25 mM acetylthiocoline and 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) in a final volume of 1 ml. The incubation was carried out in cuvettes at 37°C and the change in absorbance at 412 nm was followed at different time points (from 0 to 180 min).

Isolated exosomes were observed with a scanning electron microscope. They were fixed with 2% glutaraldehyde in PBS for 10 min, attached onto stubs, coated with gold in a sputterer (Sputter Coater 150A, Edwards, UK) and observed using a field emission scanning electron microscope (FEGESEM QUANTA 200 FEI) at a working voltage of 30 kV.

RNA Interference

Small interfering RNAs (siRNA) targeting IL8 or scramble siRNA were purchased from Dharmacon (ON-TARGET plus SMART pool, Human, Dharmacon inc. CO, USA) and used to transfect HUVEC by employing oligofectamine (Invitrogen, UK), according to the suggested protocol. Briefly, 85% confluent cells were incubated with a mix of oligofectamine- Opti-MEM containing 75 pmol of siRNA or equal volume of PBS (CN). After 24 h, the conditioned medium was collected for ELISA assay and cells were lysed for RNA extraction. Knockdown efficiency was determined by Real-time PCR and ELISA quantitation of IL8 release.

RNA Extraction and Real-time PCR

HUVEC were grown to confluence in 12-well plates, pre-treated or not with CTO (5 and 10 µM) for 24 h or 48 h and incubated for 6 h with exosomes (20 and 50 µg/ml). To investigate efficacy of IL8 silencing, HUVEC were grown to confluence in 6-well plates, transfected with siRNAs, incubated for 6 h with 50 µg/ml of exosomes and then lysed to extract RNA. For all experiments, IL8, VCAM1, ICAM1 transcript levels were

measured by reverse transcription (RT) and *Taq*Man real-time quantitative polymerase chain reaction (RQ-PCR) and were analyzed as previously described [11]. The following primers were used: IL8 Hs00174103 m1, VCAM1 Hs00174239 m1, ICAM1 Hs00277001 m1 and GAPDH Hs99999905 m1 (Applied Biosystems, Foster City, CA, USA) used as internal controls.

Flow Cytometry

Expression of HUVEC cell surface VCAM1 and ICAM1 was determined by flow cytometry analysis. HUVEC were pre-treated or not with 10 μM CTO for 24 h and incubated over night with 50 μg/ml of LAMA84R-exosomes in a low serum medium (EGM:RPMI, 1:9). 5×10^5 cells were washed in PBS and incubated with 0.5 μg anti VCAM1-FITC or ICAM1-FITC (Santa Cruz Biotechnology, CA, USA) for 15 min at 4°C according to manufacturer's recommendations. Isotype-matched irrelevant antibodies were used as a negative control. Viable cells were gated by forward and side scatter and analysis was performed on 100,000 acquired events for each sample. Samples were analyzed on a FACS Calibur with the use of the CellQuest software (BD Biosciences, NJ, USA).

Elisa

HUVEC conditioned medium (CM) was collected from cells pre-treated or not for 24 h with 10 μM CTO and then stimulated for 6 h with indicated treatments; HUVEC CM was also collected from cells after 24 h of transfection with siRNA (scramble or IL8) and 6 h of incubation with 50 μg/ml of exosomes; CM aliquots were centrifuged to remove cellular debris and afterwards IL8 protein concentrations were quantified using an ELISA kit (R&D Systems, MN, USA), according to manufacturer's protocol. IL8 was also measured directly in LAMA84R exosomes.

Migration Assay

Migration assays were performed in Transwell chemotaxis chambers assay (NeuroProbe, Cabin John, MD, USA) [8]. Briefly, HUVEC (2×10^6/ ml) were suspended in serum-free RPMI 1640 medium supplemented with 0.1% BSA with or without CTO (1, 5, 10 μM), in transwell chemotaxis chamber equipped with 8 μm pore filters and exposed to chemoattractants with exosomes (20–50 μg/ml), 10 ng/ml of recombinant IL8 or neutralizing antibodies anti IL8 (5 μg/ml) (R&D system, MN, USA) as indicated. To evaluate the migration ability of HUVEC transfected with siRNA, endothelial cells were suspended in RPMI 1640 medium supplemented with 0.1% BSA with or without 10 μM CTO, were exposed to chemoattractants with 50 μg/ml of exosomes and were processed as above. Filters were removed after 6 h, were fixed in methanol and were stained with Diff-Quick (Medion Diagnostics GmbH, Dudingen, Switzerland). Each test group was tested in three independent experiments; the number of migrating cells in five high-power fields per well were counted at 400X magnification.

Adhesion Assay

Adhesion assays were performed as previously described [11]. Briefly, HUVEC were pre-treated or not with 5–10 μM of CTO for 24 h and HUVEC monolayer was incubated for 6 h with indicated conditions, as described in the results. After treatment, cells were washed with PBS and CML cells were added for 2 h at 37°C. Adherent cells were stained with hematoxylin/eosin, each test group was assayed in triplicate; five high power (400X) fields were counted for each condition.

Tube Formation of HUVEC on Matrigel

Matrigel was used to test the effects of exosomes on *in vitro* vascular tube formation as described [11,15]. 50 μg/ml of exosomes were added to HUVEC plated on Matrigel in low serum medium and 10 μM CTO. For HUVEC transfected with siRNA the same experiment were performed after 24 h of transfection. Cells were incubated for 6 h and then evaluated by phase-contrast microscopy and photographed. The length of the cables was measured manually with the IMAGE-J software (http://rsbweb.nih.gov/ij/) [16].

Matrigel Plug Assay

Groups of six NOD/SCID mice (4 weeks) (Charles River) were injected subcutaneously with 400 μL of Matrigel (BD Biosciences Pharmingen, San Diego, CA, USA) as follows: respectively, animals in group 1 and 2 were injected with matrigel plus PBS (PBS) or plus 100 μg LAMA84R-derived exosomes (Exo), and were treated with 80% PEG-100 and PBS. Animals in group 3-4-5 were injected with matrigel plus 100 μg LAMA84R-derived exosomes and were treated either with CTO 513 mg/Kg/inj and imatinib vehicle (group 3, Exo + CTO) or with CTO 513 mg/Kg/inj and imatinib 50 mg/kg (group 4, Exo + IM + CTO) or with CTO vehicle and imatinib 50 mg/kg (group 5, Exo + IM). Animals in group 6 and 7 were injected with matrigel plus 50 ng of recombinant IL8 and were respectively treated either with vehicles of CTO and imatinib (Rec IL8) or with CTO 513 mg/Kg/inj and imatinib vehicle (Rec IL8+ CTO), The degree of vascularization was evaluated by determination of hemoglobin content using the Drabkin method (Drabkin's reagent kit) [17].

Statistics

Data were expressed as means ± SEMs of the indicated number of experiments. Statistical analysis was performed by using a paired samples *t* test. Differences were considered to be significant when P values were smaller than 0.05.

Results

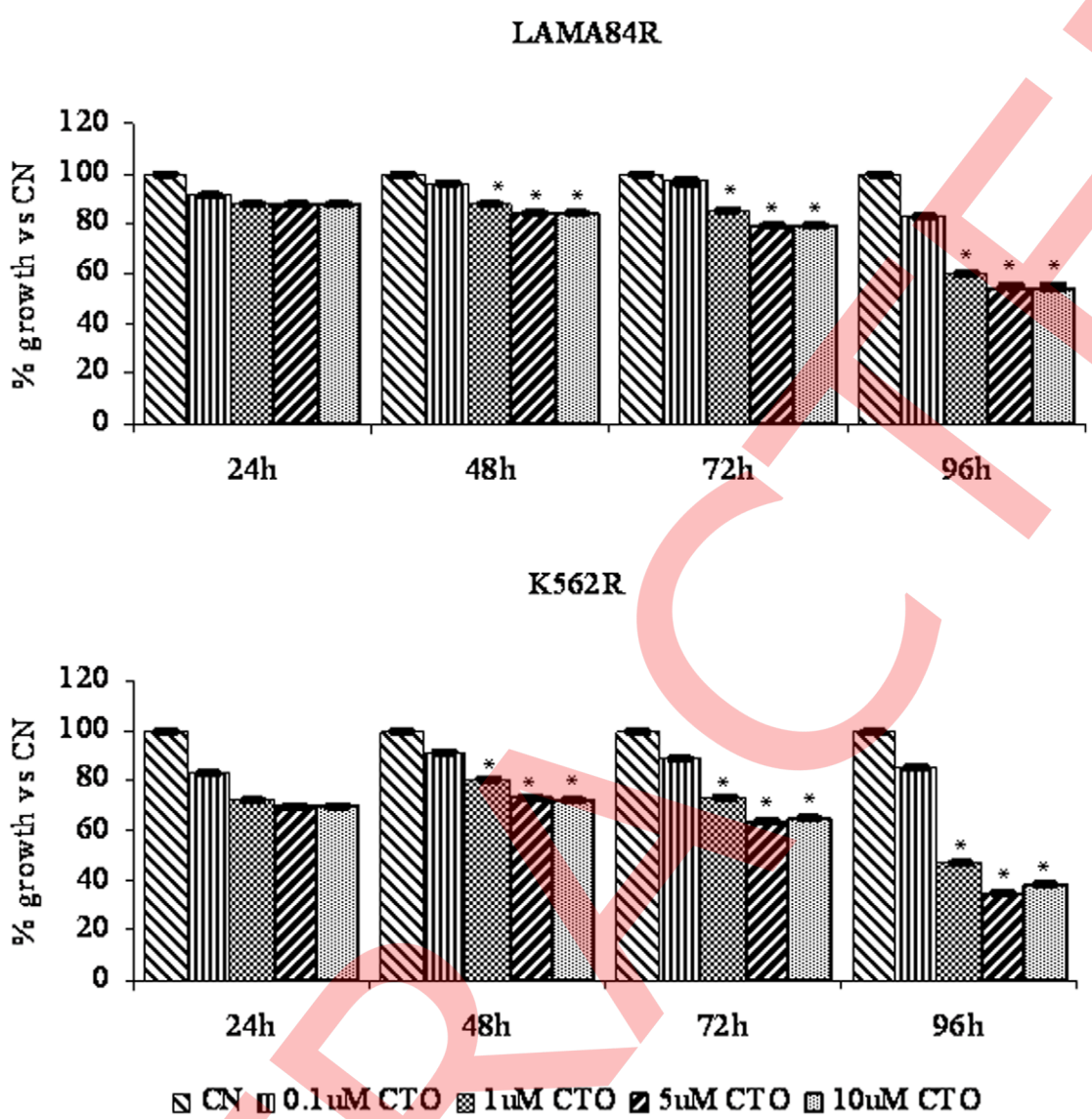
Effects of CTO on Growth of Human CML Cells and Bcr-Abl Mediated Tyrosine Phosphorylation

MTT assays were performed to determine the antiproliferative effects of CTO on LAMA84R and on K562R cells. Data presented in figure 1 (panel a) show results of 4 days treatment. CTO inhibits cell growth of LAMA84R and K562R in the low micromolar range in a dose dependent fashion ($p<0.001$). The results herein show a 50% growth reduction of the CML lines with 5 μM CTO at 96 h time point. In order to correlate the antiproliferative effects of CTO on CML cells with the Bcr-Abl activity, cells were incubated with increasing concentrations of CTO, were harvested and were subjected to immunoblotting with antibodies against phosphorylated Bcr-Abl and CrkL. As shown in figure 1 (panel b), a dose-dependent inhibition of both total and phosphorylated Bcr-Abl levels was observed after 72 and 96 h of drug exposure. Consistent with this conclusion, CTO inhibits the phosphorylation of a selected target of Bcr-Abl kinase; tyrosine phosphorylation of CrkL was reduced by 5 μM CTO treatment.

Effects of CTO on Tumor Xenograft Growth

On the basis of the *in vitro* growth and Bcr-Abl signalling inhibitory effects of CTO, we further examined the antineoplastic effect of CTO on LAMA84R using a xenograft CML tumor model. Otherwise CTO–treated mice seemed healthy and did not

a



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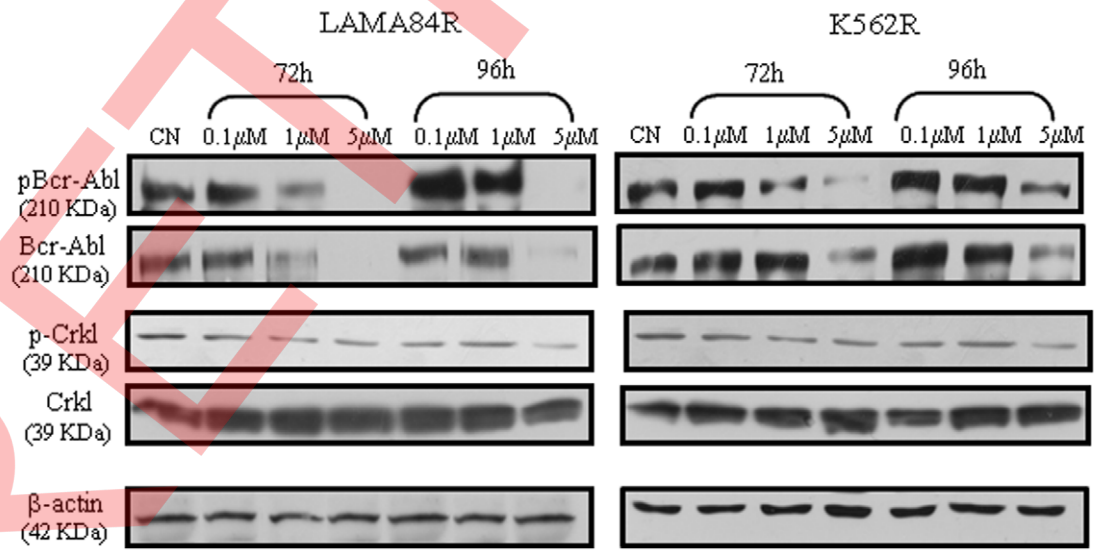

Figure 1. CTO inhibits cell proliferation of LAMA84R and K562R cell lines. (a) Cell growth was measured by MTT assay after 24, 48, 72, 96 h of treatment with increasing doses of CTO (0.1-1-5-10 μM). The values were plotted as a percentage of the control (cells treated with DMSO). Each point represents the mean ± SD for three independent experiments. *p≤0.001. (b) CTO treatment decreases Bcr–Abl expression, inhibits Bcr–Abl tyrosine phosphorylation and its downstream substrate CrkL on LAMA84R and K562R cell lines. These cell lines were treated with increasing doses of CTO (0.1–5 μM) or DMSO (CN) for 72 h and 96 h; afterwards protein lysates were subjected to western blot analysis as described in Material and Methods using anti-phospho-Abl, anti-cAbl, anti-phospho-CrkL and anti-CrkL antibodies. Blots were then stripped and subsequently reprobed with antibody against β-actin to ensure equal loading.
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exhibit any signs of distress during the drug treatment. For these analyses, mice receiving either CTO alone or in combination with imatinib were treated, as described in Material and Methods, until day 26 of treatment regimen. Animals were then maintained until tumor weight reached 4000 mg.

Tumor weight curve analysis (figure 2) showed that mice bearing LAMA84R tumor- reached on average the 4000 mg weight after 26 d in the following subsequent groups: CTO 342 mg/Kg plus imatinib; CTO 342 mg/Kg plus PBS and imatinib plus PEG. By contrast, CTO 513 mg/Kg group resulted in a slight longer period of time to reach the 4000 mg weight (33 d) compared with the control group (PBS plus PEG). The combination of CTO 513 mg/Kg plus imatinib slowed tumor growth to a greater extent than the control group ($P<0.01$), attaining the experimental end point after 40 d.

Exosome Vesiscles Released by LAMA84R Cells

LAMA84R cells release exosomes into the culture medium during a 24 h period as demonstrated by their characteristic shape and diameter (70 nm ±10) observed with scanning electron microscope (figure 3a) and by the presence of Hsc70 and CD63 proteins (figure 3b). Furthermore, acetylcholinesterase activity, a characteristic enzyme localized in exosomes, was found associated with the exosome fraction (Figure 3c).

CTO Inhibits the Exosome-stimulated Increase of Cell-cell Adhesion Molecules and IL8 Expression in HUVEC

To determine the potential effects of CTO on exosome-mediated induction of genes associated with angiogenesis, we evaluated by TaqMan PCR analysis the mRNA levels of cell-cell adhesion molecules and cytokines after adding the drug to exosome-stimulated HUVEC. Figure 4 panel I shows that LAMA84R-derived exosomes added to HUVEC monolayer caused, compared to control, a dose-dependent increase of VCAM1 (a), ICAM1 (b) and IL8 (c) mRNA expression. Increase of mRNA production was statistically significant and reached approximately a 24-, 10-, 60-fold induction respectively after 6 h stimulation of the endothelial monolayer with 50 μg/ml of exosomes. Treatment of endothelial cells with exosomes together with increasing doses of CTO caused a dose-dependent inhibition of VCAM1, ICAM1 and IL8 mRNA levels. Figure 4 panel II shows that a comparable effect to the one of LAMA84R exosomes

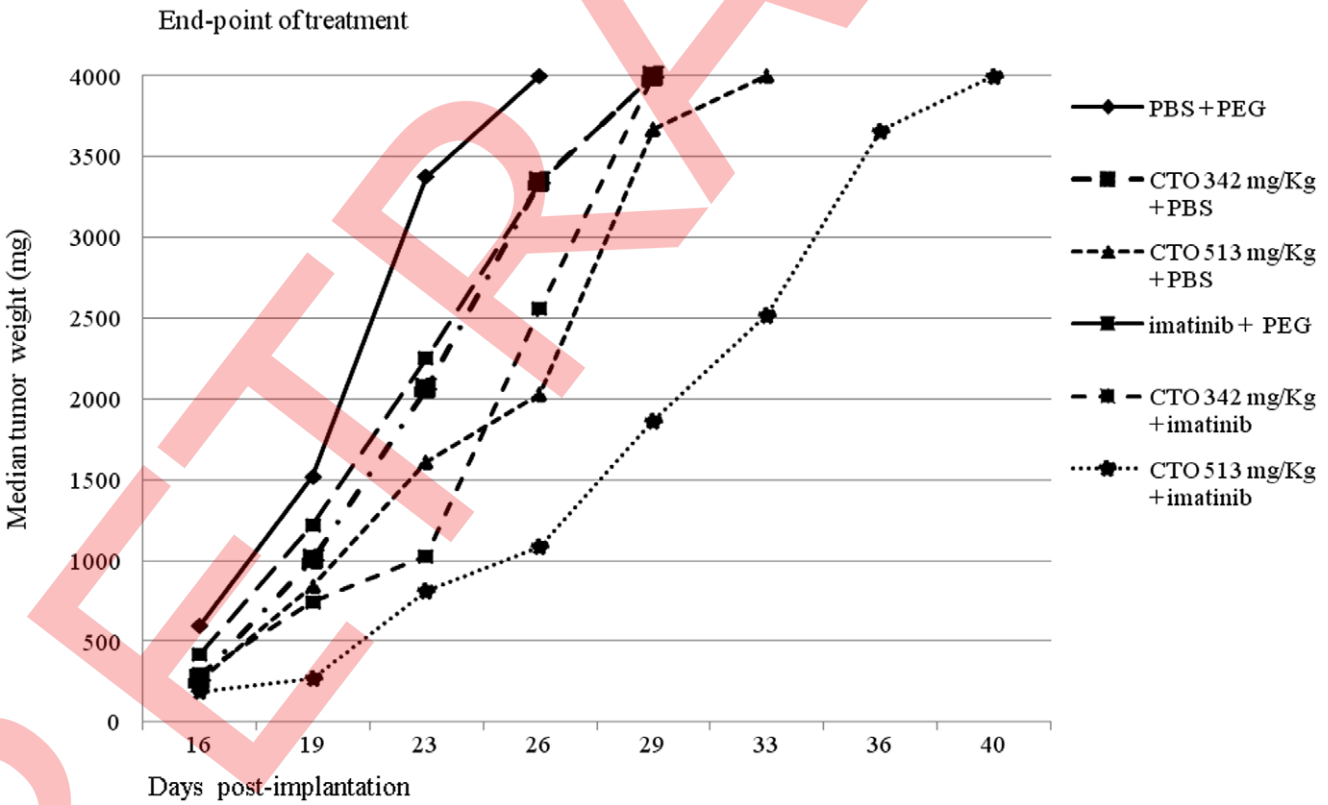

Figure 2. Antitumor activity of CTO on human CML xenografts. LAMA84R cells were injected subcutaneously in NOD/SCID mice as described. After palpable tumor formation, mice were treated as described in Material and Methods. Comparison of the median tumor weight was used as index of the antitumor efficacy of the compounds.
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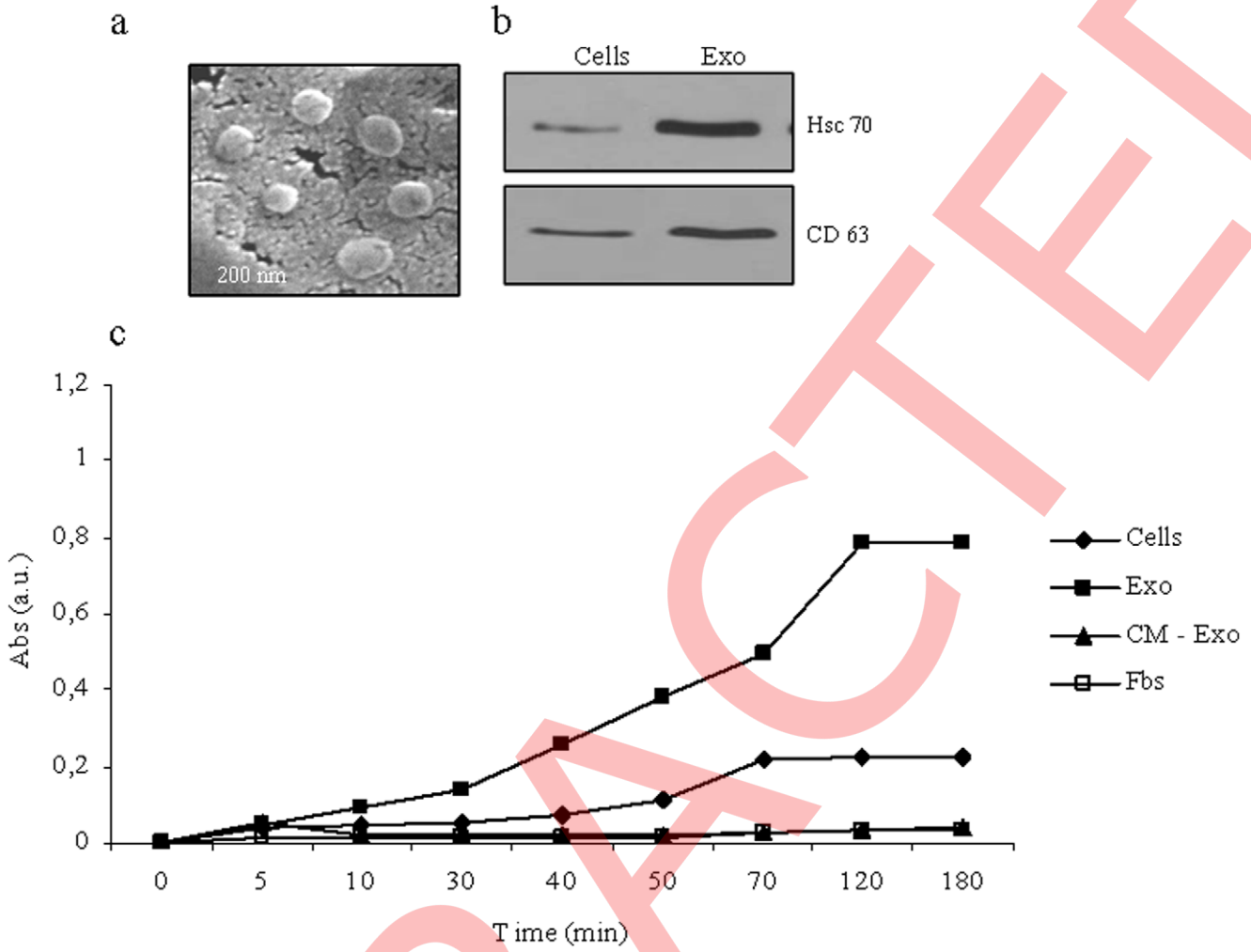

Figure 3. LAMA84R exosomes characterization. (a) Exosomes released by LAMA84R cells observed with a scanning electron microscope. (b) Detection of Hsc70 and CD63 in 30 µg of cell lysate (lane 1) and 30 µg of exosomes lysate (lane 2). (c) Acetylcholinesterase assay. The activity of acetylcholinesterase, an exosome-specific protein marker, was determined in 10 µg either of total cell lysate (Cells) or of Exosomes (Exo); exosome-deprived conditioned medium (CM-Exo) and exosome-deprived Fbs (Fbs–Exo) were used as negative controls. Figure 3a is excluded from this article's CC-BY license. See the accompanying retraction notice for more information.
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on the mRNA induction was observed when endothelial cells (EC) were incubated with 10 ng/ml of recombinant IL8; adding 10 µM CTO or neutralizing anti-IL8 antibody revert the increase in VCAM1 (a), ICAM1 (b) and IL8 (c) mRNA expression.

FACS analysis confirmed that incubation of HUVEC with LAMA84R exosomes resulted in detection of VCAM1 (figure 5a) and ICAM1 (figure 5b) on the surface of HUVEC which was blunted by treatment of cells with 10 µM CTO. ELISA assay demonstrated the increasing release of IL8 into HUVEC conditioned medium after treatment with CML exosomes (figure 5c). Small amount of IL8 was also found in LAMA84R exosomes (41 pg/50 µg exosomes). Transfection of HUVEC with IL8 siRNA caused, as expected, a striking reduction in both IL8 mRNA levels and cytokine release in conditioned medium compared to EC transfected with scramble siRNA (figure S1 and b).

CTO Inhibits the Adhesion of CML Cells to HUVEC Monolayer

While leukaemia progresses, cancer cells adhere to endothelial cells in order to infiltrate and colonize extramedullary sites. Figure 6a shows that the increase of adhesion of LAMA84R cells (arrows) to HUVEC monolayer was inhibited by pre-treatment of EC with 10 µM CTO. Accordingly, figure 6b shows that pre-treatment of HUVEC with 50 µg/ml of LAMA84R exosomes induces an 80 fold increase of CML cells adhesion to EC; CTO inhibits CML cells adhesion in a dose dependent manner. Figure 6c shows the inhibitory effects of both a neutralizing anti-IL8 antibody and CTO on IL8-stimulated adhesion of CML cells to EC.

Effects of CTO on Exosomes-stimulated Migration of Endothelial Cells

We examined the effect of CTO treatment on exosome-stimulated EC motility by Boyden chamber assay. Our results showed that adding a range of concentrations of CTO (1–10µM) to the upper well of the chamber caused, after 6 h, a dose-dependent inhibition of LAMA84R exosome-stimulated endothelial cell migration (figure 7a). Figure 7b shows the inhibitory effects of a neutralizing anti-IL8 antibody and CTO on IL8-stimulated motility of EC cells. Furthermore, we used EC transfected with IL8 siRNA to demonstrate the role of IL8 in exosome-stimulated

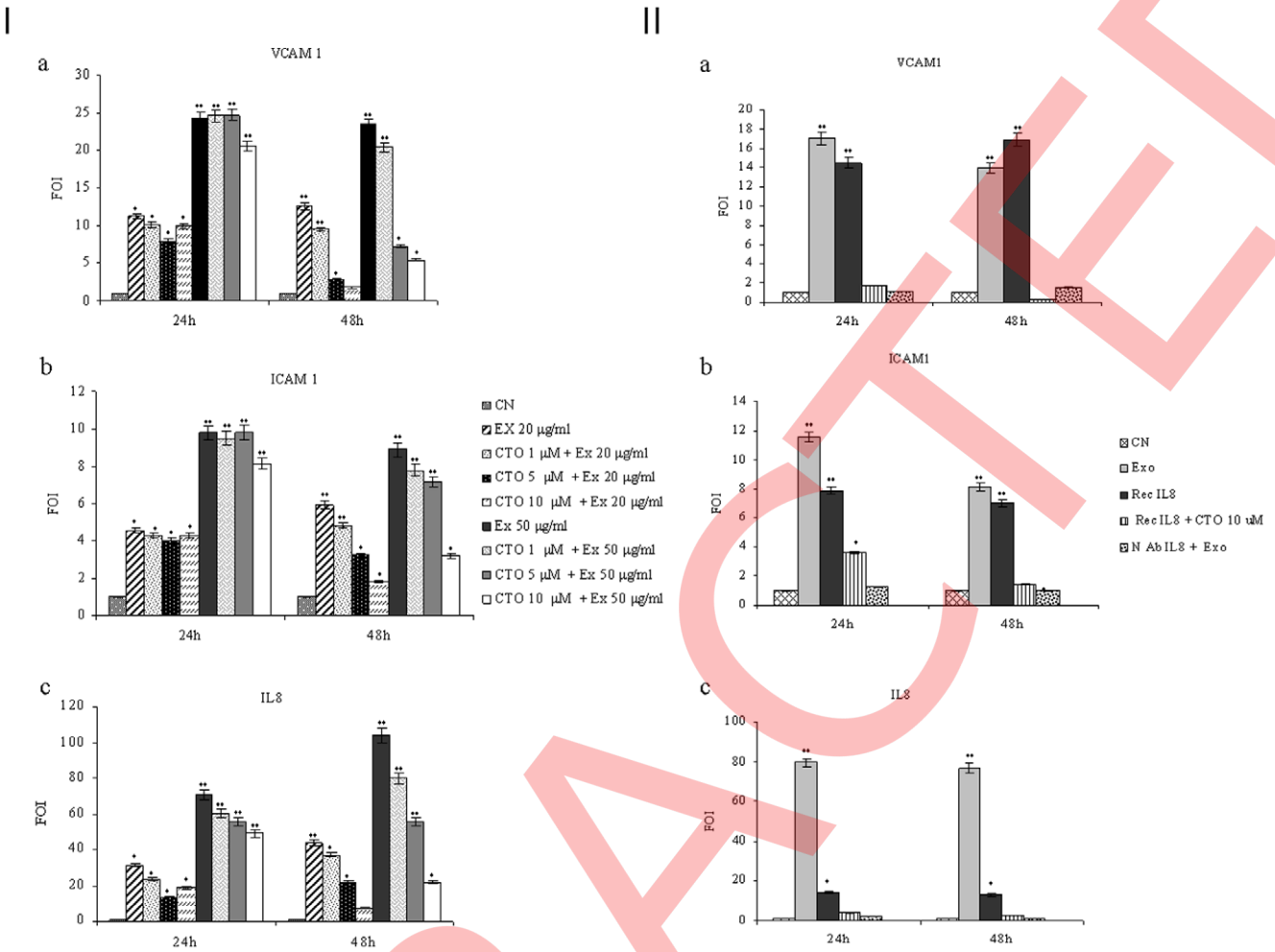

Figure 4. Effects of CTO on cell adhesion molecules and cytokines mRNA expression. (I) CTO reverts the effects of CML exosome treatment on VCAM1, ICAM1 and IL8 mRNA expression in HUVEC cells. VCAM1 (a), ICAM1(b) and IL8 (c) mRNA expression increased in a dose dependent manner after adding exosomes (Exo) to endothelial cell monolayer. CTO (1-5-10 µM) reverts these effects in a time- and dose dependent manner. (II) VCAM1, ICAM1 and IL8 mRNA expression in HUVEC treated for 6 h either with low serum medium (CN), or with 50 µg/ml exosomes (Exo), or with 10 ng/ml of recombinant IL8 (Rec IL8) with or without CTO 10 µM, or with 50 µg/ml exosomes plus 10 µg/ml of a neutralizing anti-IL8 antibody (N Ab IL8). Values are representative for three independent experiments. *p≤0.05; **p≤0.01.
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migration. Our results show that exosome-induced HUVEC migration decreased when IL8 was silenced compared to cells transfected with scramble siRNA control (Figure S2a).

CTO Treatment Inhibits *in vitro* and *in vivo* Exosome-mediated Angiogenesis

We evaluated the properties of CTO to inhibit exosome-stimulated angiogenesis by using *in vitro* and *in vivo* angiogenesis models. Angiogenesis is a very complex process involving several kinds of cells; tube formation of endothelial cells is one of the key steps of angiogenesis. Therefore, firstly we showed that, compared to cells maintained in low serum medium, LAMA84R exosomes stimulate *in vitro* tube formation as tested by Matrigel assay to a similar extent of addition of recombinant IL8 used as positive control (figure 8a). Addition of a neutralizing anti-IL8 antibody or 10 µM CTO, inhibited exosome-induced tube formation (figure 8a). These results were confirmed by the measurement of the length of tubular connections that showed a more than threefold increase in cellular projections interconnecting HUVEC after treatment with LAMA84R exosomes, or recombinant IL8 compared to control. Adding CTO or neutralizing anti-IL8 antibody caused a dramatic inhibition of exosome-mediated effects on tube formation (figure 8b). LAMA84R exosomes do not induce the tube formation on IL8-silenced endothelial cells (Figure S2b).

Then we evaluated *in vivo* antiangiogenic effect of CTO on a mouse Matrigel plug model. An initial sign of the levels of activity of CTO as inhibitor of the exosome-stimulated angiogenesis was visually assessed because of the color difference in the vascularized plugs when compared with the controls. The reddish color of LAMA84R-exosomes (Exo) or recombinant IL8 (Rec IL8) containing plugs in the vehicle control-treated mice reflected the development of a dense neovascularization. In contrast, the pale color of the plugs removed from CTO–treated mice indicated inhibition of exosome-stimulated vascularization over a 4 weeks period (figure 9a). Drabkin's assay was used to measure haemoglobin content in the plugs as a marker of vascularity (figure 9b).

Next we examined whether LAMA84R exosomes stimulate phosphorylation of signalling proteins, particularly Akt and Erk 1/2, which are the principal mediators of cell proliferation, survival,

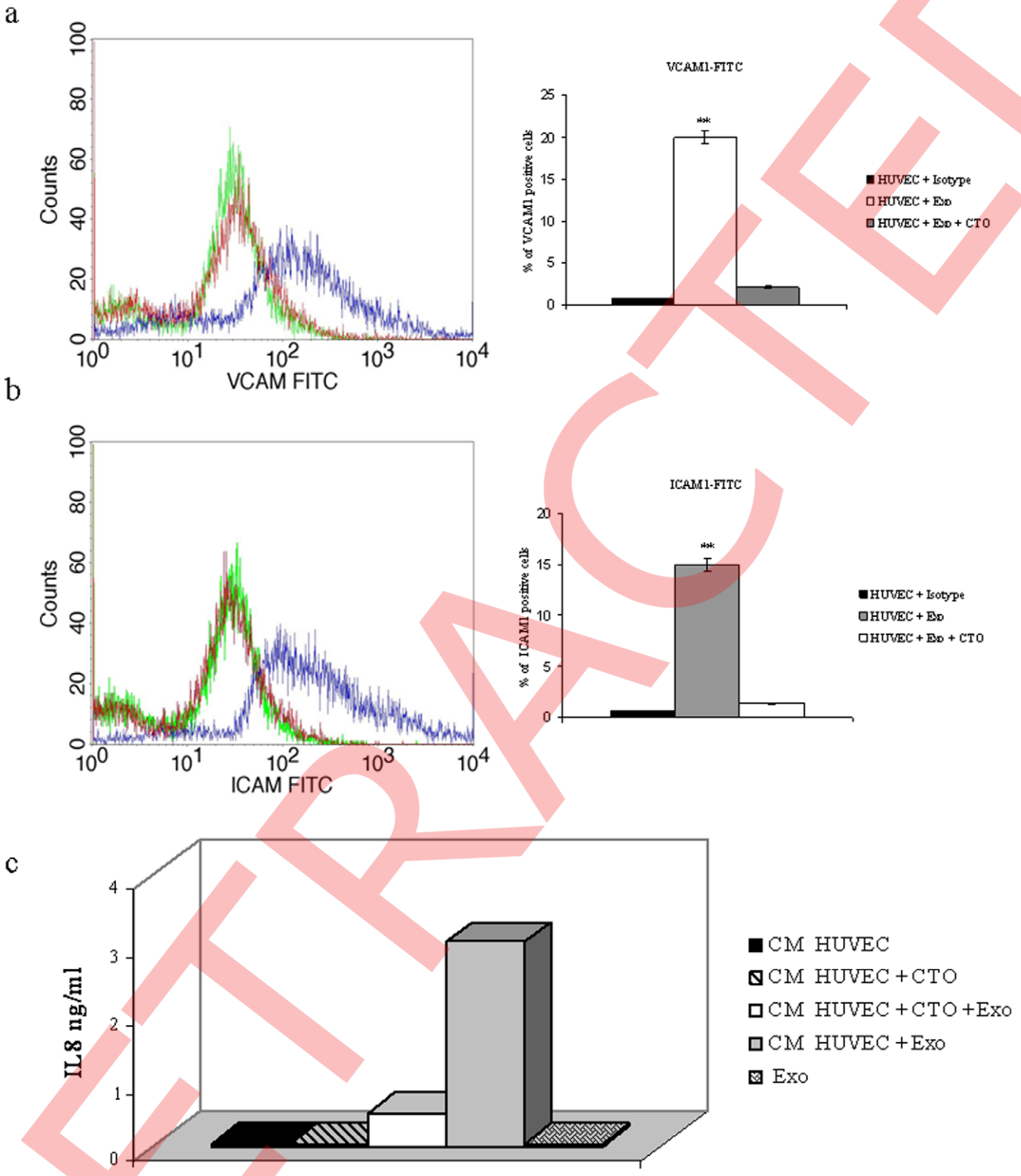

Figure 5. Effects of CTO on cell adhesion molecules and cytokines production. CTO inhibits the exosomes-stimulated increase of VCAM1, ICAM1 expression and IL8 secretion on HUVEC cells. Representative overlay histograms showing an increase of surface expression of VCAM1 (a) and ICAM1 (b) on HUVEC treated with 50 μg/ml of LAMA84R exosomes (blue line) compared to HUVEC treated with 50 μg/ml of LAMA84R exosomes plus 10 μM CTO (red line) or untreated HUVEC, as control (green line). (c) ELISA for IL8 release by HUVEC. Aliquots of conditioned medium (CM) from cells were collected after 6 h of stimulation either with 50 μg/ml of exosomes (CM HUVEC + Exo) or with 50 μg/ml of exosomes plus 10 μM CTO (CM HUVEC + CTO + Exo); low serum medium (CM HUVEC) or low serum medium plus 10 μM CTO (CM HUVEC + CTO) were used as negative controls. The amount of IL8 in 50 μg/ml of exosomes was also quantified.
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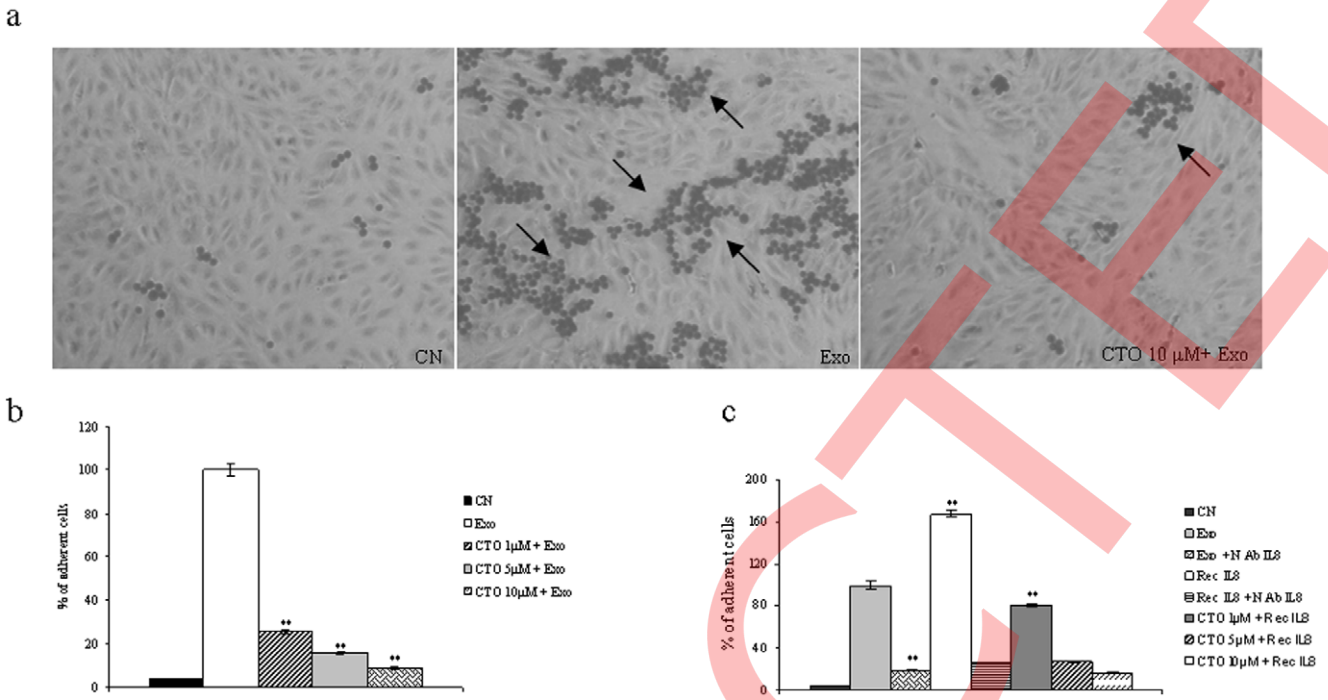

Figure 6. CTO inhibits the adhesion of LAMA84R cells to exosome-treated HUVEC monolayer. (a) Phase contrast micrographs showing the adhesion of LAMA84R cells (arrows) on HUVEC monolayer treated with 50 μg/ml of exosomes (Exo) and with 50 μg/ml of exosomes after pre-treatment of 24 h with 10 μM CTO. (b) Adhesion of LAMA84R cells to endothelial cell monolayer treated for 6 h with: 50 μg/ml of exosomes (Exo) and exosomes plus increasing doses of CTO (1–10 μM); (c) Adhesion of LAMA84R cells to endothelial cell monolayer treated for 6 h with: 50 μg/ml of exosomes (Exo), 50 μg/ml of exosomes plus 10 μg/ml of a neutralizing anti-IL8 antibody (N Ab IL8), 10 ng/ml of recombinant IL8 (Rec IL8), 10 ng/ml of recombinant IL8 plus 10 μg/ml of a neutralizing anti-IL8 antibody and 10 ng/ml of recombinant IL8 with increasing doses of CTO (1–10 μM). Values are representative for three independent experiments. *p≤0.05; **p≤0.01.
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and chemotaxis in endothelial cells and if CTO was able to modulate these pathways. Figure 10 shows that over night addition of 50 μg/ml LAMA84R exosomes or of 10 ng/ml IL8 to endothelial cells trigger the phosphorylation of both the signalling molecules; this result is consistent and extends our previous findings [11] suggesting that microvesicles are able to interact directly with target cells and act as a signalling molecule. The enhanced Erk 1/2 and Akt phosphorylation was reduced by treatment of endothelial monolayer with 10 μM CTO (figure 10).

Discussion

In the present study we tested the anti-tumor effects of carboxyamidotriazole-orotate *in vitro* and in xenograft model of imatinib-resistant human CML, furthermore we evaluated the

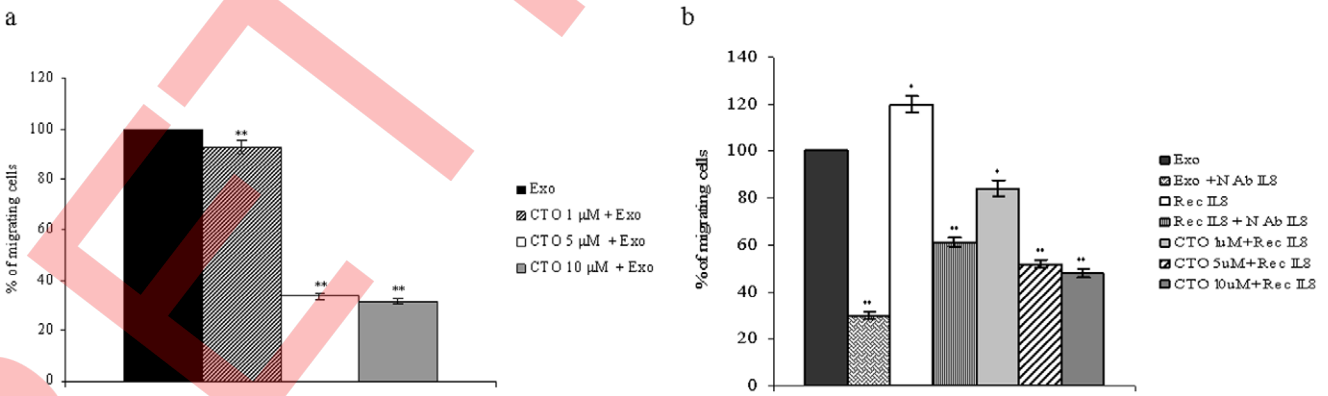

Figure 7. CTO inhibits the effects of LAMA84R exosomes on HUVEC migration. (a) Effects on migration of CTO-treated endothelial cells using 50 μg/ml of exosomes as chemoattractant. (b) 50 μg/ml of exosomes (Exo) with or without 10 μg/ml of neutralizing anti-IL8 antibody (N Ab IL8), or 10 ng/ml of recombinant IL8 (Rec IL8) with or without neutralizing anti-IL8 antibody were added as chemoattractants to the bottom wells., Motility of endothelial cells with or without increasing doses of CTO (1–10 μM) was evaluated as described in Material and Methods. *p≤0.05; **p≤0.01.
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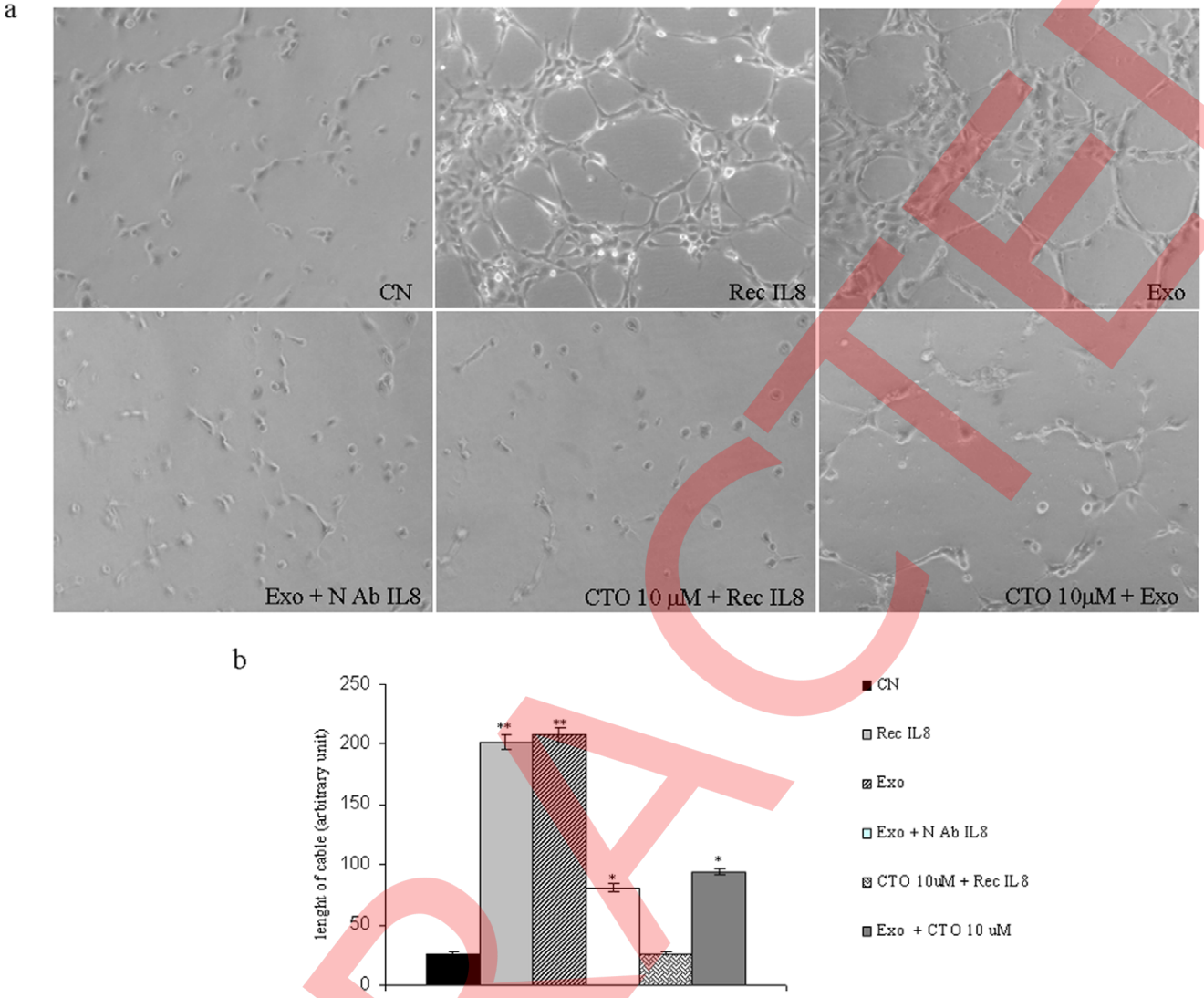

Figure 8. *in vitro* inhibition of exosome-stimulated angiogenesis by CTO. (a) Phase contrast micrographs showing the effects of LAMA84R exosomes and CTO treatment on endothelial network formation (matrigel assay). Few cables are observed when HUVEC are plated in low serum medium (CN). Addition to HUVEC cells of 10 ng/ml of recombinant IL8 (Rec IL8) or 50 μg/ml of LAMA84R exosomes (Exo) induces the formation of capillary-like structures. No tube formation is observed when HUVEC are plated in the presence of 50 μg/ml of exosomes plus neutralizing anti-IL8 antibody (Exo + N Ab IL8). CTO inhibits the effects of recombinant IL8 (10 μM CTO + Rec IL8) or exosomes (Exo +10 μM CTO) on tube formation by HUVEC on matrigel. (b) Histograms showing the quantitative analysis of the cables length by Image J software.
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ability of CTO to inhibit CML exosome-stimulated angiogenesis both *in vitro* and *in vivo* models. Imatinib treatment has been remarkably successful in reducing the tumor burden and suppressing the progression of patients in the chronic phase of CML, however, a lower efficacy has been observed during the accelerated phase of the disease, mainly due to the evolution of clinical resistance. Second generation inhibitors such as dasatinib or nilotinib, do not show activity against all point mutations responsible for imatinib resistance. Among patients with CML who developed imatinib resistance, Bcr-Abl mutations was reported just in 31% to 42% of cases, suggesting mechanisms of disease progression that are Bcr-Abl-independent [18]. New approaches reling on the use of compounds targeting either pathways downstream of Bcr-Abl activation or events that contribute or modulate leukaemia progression are necessary. We have previously demonstrated that CAI, an inhibitor of calcium-mediated signal transduction [19], decreases cell viability and induces apoptosis of imatinib-resistant CML cells, reducing both total and phosphorylated Bcr-Abl [8]; furthermore, we showed that CAI activity against imatinib-resistant CML cells was due to the ability to increase intracellular reactive oxygen species [9]. Although CAI has been tested in phase II clinical trial for treatment of solid tumours, its use in clinical settings has been hampered by the limited solubility, toxicity, or evident clinical benefits [20].

CTO is the orotate salt form of carboxyamidotriazole showing a reduced toxicity, increased oral bioavailability and stronger efficacy when compared to the parental compound [10].

One of the initial findings of our study was that CTO caused in both K562R and LAMA84R cells, inhibition of proliferation concomitant to Bcr-Abl down-regulation, dephosphorylation and decrease in tyrosine phosphorylation of CrkL, more rapidly than

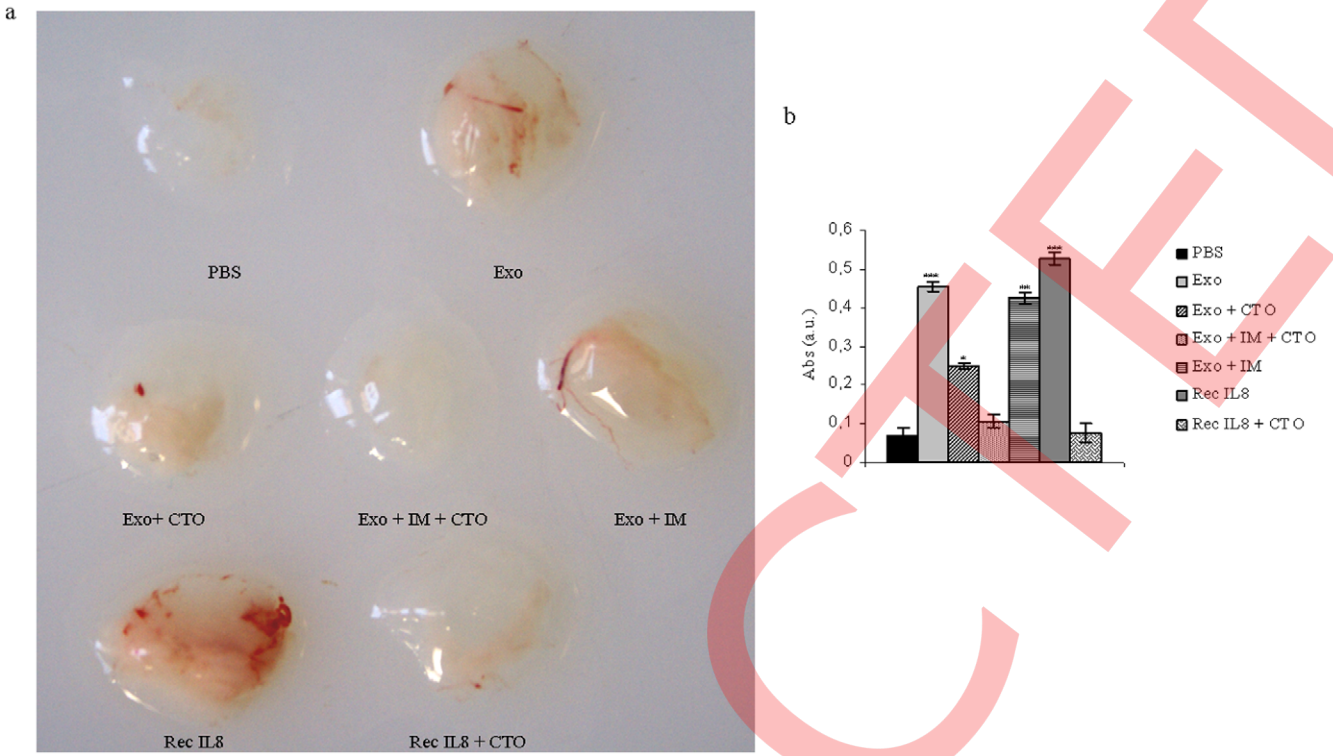

Figure 9. CTO treatment inhibits exosome-stimulated angiogenesis *in vivo*. NOD/SCID mice treated with CTO show a decreased exosome-stimulated angiogenesis in matrigel plug assay. (a) Matrigel plugs implanted subcutaneously in mice and containing: PBS as negative control,or 100 μg of LAMA84R exosomes (Exo) or 50 ng of recombinant IL8 (Rec IL8). Mice were treated with vehicles or drugs (CTO and/or imatinib) as described in Material and Methods and plugs were removed after 4 weeks. (b) Evaluation of haemoglobin concentration in the matrigel plugs by Drabkin assay. *p≤0.05; **p≤0.01, ***p≤0.001.
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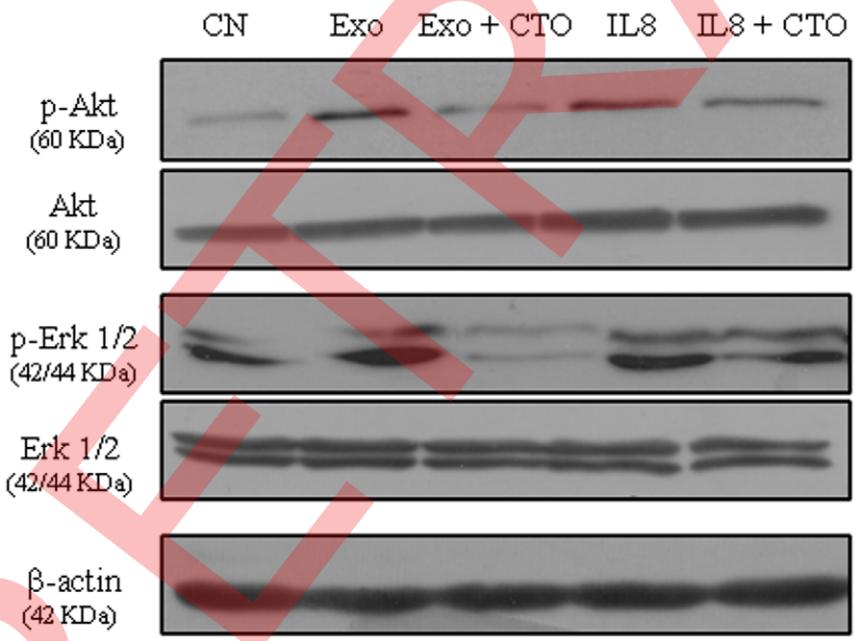

Figure 10. CTO inhibits Akt and Erk 1/2 phosphorylation in exosomes-stimulated HUVEC. HUVEC were incubated with 50 μg/ml of exosomes with or without 10 μM CTO or with 10 ng/ml of IL8 with or without 10 μM CTO for 1 h (pErk 1/2 and Erk 1/2) or over night (pAkt and Akt). After the treatments, protein lysates were subjected to western blot analysis as described in Material and Methods using anti-phospho-Akt, anti- Akt, anti-phospho-Erk, anti-Erk antibodies. Blots were then stripped and subsequently reprobed with antibody against β-actin to ensure equal loading.
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the parental compound. Furthermore, it showed to be more active than CAI on a molar basis [8]. Rapid reduction of Bcr-Abl protein coupled with kinase inactivation, as seen with CTO, can be particularly advantageous because of the multiple Bcr-Abl domains that mediate protein interactions triggering different signalling pathways responsible for cell proliferation, adhesion, and inhibition of apoptosis [21]. The inhibitory effects of CTO against CML cells in culture, is mirrored by its activity against CML xenografts in NOD/SCID model. Tumor growth retardation in mice treated with CTO 513 mg/kg plus imatinib was evident suggesting that CTO was acting in this model as an antileukaemic agent. Recently, there are increasing data showing that angiogenesis plays an important role in the development and progression of chronic myeloid leukaemia [22,23]. The bone marrow of patients with CML exhibit marked neovascularization and increased number of endothelial cells [24]; the cross-talk between tumor cells and endothelial cells leads to enhanced tumor growth, metastasis and altered response to anti-cancer therapy [25]. Recently, a number of studies have recently described exosomes as new players in modulating tumor microenvironment, promoting angiogenesis and tumor progression [12]. Our group and other collaborators have shown that exosomes released by imatinib-sensitive LAMA84 [11] and K562 CML cells [26] have a potential to influence *in vitro* and *in vivo* angiogenesis by affecting directly endothelial cells properties. One of the findings of the present study was the confirmation, by morphological and biochemical analysis, that LAMA84R CML cells secrete exosomes and that these vesiscles are able to modulate angiogenesis *in vitro* and *in vivo*.

These findings drove us to investigate if CTO could target both tumor cells and the tumor microenvironment. Therefore, we focused on the inhibitory effects of CTO on *in vitro* selected functional steps of angiogenesis as well as on *in vivo* angiogenesis in NOD/SCID mice. Our *in vitro* studies with HUVEC demonstrated that CTO inhibits exosome stimulated motility, cytokines and cell-adhesion molecules (ICAM1 and VCAM1) expression of endothelial cells; moreover CTO inhibits exosomes activated signalling pathways and capillary-like structure formation. The matrigel plug assay that mimics physiological neo-angiogenesis, was used as *in vivo* model; our study showed that CTO drastically decreased exosome-stimulated angiogenesis. To investigate on the possible molecular mechanisms of the CTO-mediated antiangiogenic effect, we examined whether CTO inhibited the activation of intracellular signalling pathways involved in endothelial cell activation. Treatment of the EC with CTO blocked significantly the exosome-induced phosphorylation of signalling proteins, particularly Akt and Erk 1/2, which are the principal mediators of cell proliferation, survival, and chemotaxis in endothelial cells [27]. Kinase-dependent and kinase-independent mechanisms are known to contribute to the abnormal adhesion and migration of CML progenitors, thus the effect of CTO on both endothelial cells and leukemic cells may concomitantly inhibit adhesion of leukaemia cells to vascular endothelium and conditions that favour leukostasis and tissue infiltration. IL8 is a member of the CXC family of chemokines, a potent proangiogenic factor [28], and its plasma levels are found significantly higher in patients affected by chronic myelogenous leukaemia [29]. Interestingly we showed, through the use of IL8 neutralizing antibodies and short interfering RNAs, that IL8 was in part responsible for the effects of LAMA84R exosomes on EC activation; furthermore, treatment of EC with CTO inhibited the IL8-stimulated angiogenic phenotype. It is conceivable to hypothesize that IL8 secreted by EC stimulated with CML exosomes, may modulate both myeloid malignant cells and endothelial cells, thus generating a paracrine machinery between hematopoietic malignant cells and newly generated endothelium. In this tumor microenvironment, CTO could inhibit the angiogenic process through blocking the exosome-mediated crosstalk, thus causing the interruption of a reciprocal stimulatory loop between leukemic and endothelial cells.

Other groups have pointed their attention on the close relationship between exosome production and tumor microenvironment modulation; Hood and collaborators demonstrated that exosomes released by melanoma cells modulate both angiogenic and immunological cytokine signalling, thus serving as paracrine nanocarriers that might prepare distal sites for the arrest of metastatic cells [30]. In this context, the inhibition of either exosomes shedding or modulation of their function has been proposed as worthwhile approach to cancer therapy. Al-Nedawi et al. showed that the treatment of A431 tumor xenografts with Diannexin, which inhibits the uptake of the A431 (human squamous cell carcinoma cell line)-derived microvesicles into endothelial cells, led to a reduction of tumor growth rate and microvascular density [31].

As far as we are aware, this is the first study that demonstrates the inhibitory effect of an anticancer drug on angiogenesis stimulated by exosomes released from drug-resistant cancer cells; collectively, our findings generate a rationale for investigating clinical efficacy of molecules such as CTO that are endowed with antitumor and antiangiogenic properties.

Supporting Information

Figure S1 IL8 siRNA inhibits IL8 mRNA expression and cytokine release from HUVEC. IL8 mRNA expression levels (a) or IL8 protein release in conditioned medium (b) were evaluated in HUVEC transfected either with oligofectamine (CN), or with scramble siRNA or with IL8 siRNA. HUVEC transfected were treated or not for 6 h with 50 μg of LAMA84R exosomes (Exo).
(TIF)

Figure S2 IL8 siRNA inhibits the effects of LAMA84R exosomes on migration and tube formation capabilities of HUVEC. (a) Addition of exosomes to the bottom wells of Boyden chamber increases the migration of either HUVEC and HUVEC transfected with scramble siRNA while concomitant CTO treatment reverts this effect. On the contrary exosomes have not significative effects on migration of IL8-silenced HUVEC.(b) Phase contrast micrographs showing the effects of LAMA84R exosomes and CTO treatment on endothelial network formation after silencing of HUVEC with IL8 siRNA (matrigel assay). Exosomes (Exo) induce formation of capillary-like structures on HUVEC transfected with scramble siRNA compared to control cells (siRNA scramble). No tube formation is observed when exosomes stimulated EC were silenced for IL 8 mRNA expression with short interfering RNAs.
(TIF)

Author Contributions

Conceived and designed the experiments: CC ST AF GDL RA. Performed the experiments: CC AF ST SR GG. Analyzed the data: CC AF ST SR GG RA. Contributed reagents/materials/analysis tools: RK. Wrote the paper: CC AF ST RA.

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