Chronic myelogenous leukaemia-derived exosomes mediate autocrine and paracrine interplays within the tumour microenvironment: a role for IL8

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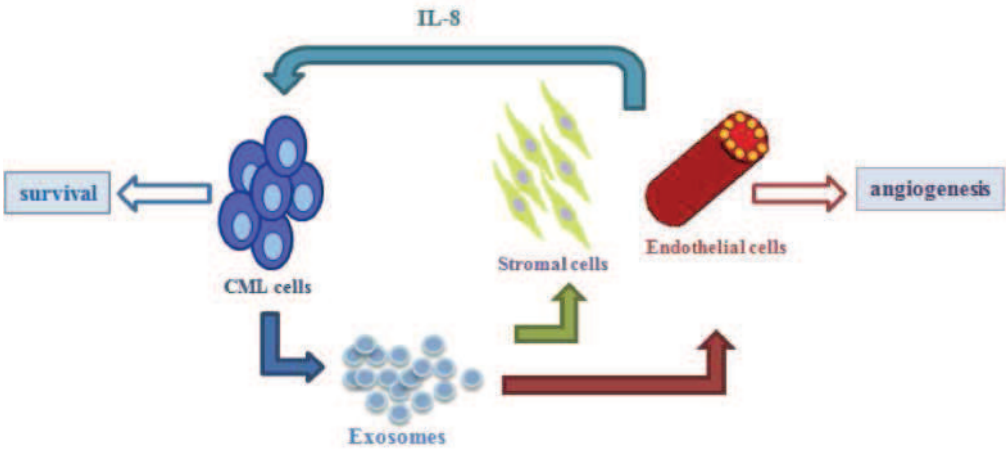

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La scienza non fa veri progressi se non quando una verità nuova trova un ambiente pronto ad accoglierla.

Science does not make real progresses except when a new truth is in an environment ready to welcome it

Pëtr Kropotkin

To my family

Index

Abstract

Chronic Myelogenous Leukaemia (CML) is a clonal hematopoietic stem cell disorder in which leukaemic cells display a reciprocal t(9:22) translocation that results in the formation of the chimeric BCR-ABL oncoprotein with a constitutive tyrosine kinase activity. Imatinib mesylate (IM) is a selective well-tolerated inhibitor of the BCR-ABL tyrosine kinase that has significantly improved the prognosis of patients with chronic phase CML. Despite this remarkable progress, a major problem associated with the administration of imatinib is acquired resistance. Therefore, there is an urgent need for new anticancer agents and combinations that could improve responses and survival rates for CML. Recently, a considerable interest in the cancer field has focused on the role of the microenvironment in regulating the growth, survival and drug-resistance of leukaemic cells. Exosomes are small vesicles of 40-100 nm diameter that are initially formed within the endosomal compartment and are secreted when a multivesicular body (MVB) fuses with the plasma membrane. Exosomes released by cancer cells constitute an important part of the tumour microenvironment as they can transfer various messages to target cells thus affecting disease, pointing out the role of vesicles as new actors in the crosstalk between cancer and normal cells in the tumour microenvironment. The overall aim of this thesis is to evaluate the role of IM-sensitive and -resistant CML-derived exosomes in the modulation of tumour microenvironment and to test the ability of a new compounds in interfering in this crosstalk. Our data show that carboxyamidotriazole-orotate (CTO) is able to inhibit both *in vitro* and *in vivo* the growth of imatinib-resistant CML cells and to affect tumour microenvironment by modulating exosome-stimulated angiogenesis. CTO may be effective in targeting both cancer cell growth and the tumour microenvironment, thus suggesting a potential therapeutic utility in the treatment of leukaemia patients (chapter 1).We also report that leukaemic and stromal cells establish a bi-directional crosstalk: CML-derived exosomes induce the production of the proangiogenic cytokine IL8 in stromal cells thus sustaining the survival of CML cells in a paracrine way (chapter 2).

Sommario

La Leucemia Mieloide Cronica (LMC) è una neoplasia ematologica causata dalla traslocazione reciproca t(9:22) con conseguente formazione dell'oncoproteina BCR-ABL con attività tirosin-chinasica costitutiva. L'Imatinib mesilato (IM) è un inibitore selettivo dell'oncoproteina che ha migliorato significativamente la prognosi dei pazienti affetti da LMC. Nonostante il successo terapeutico, un grave problema connesso con la somministrazione di imatinib è lo sviluppo di resistenza farmacologica. E' quindi necessario lo sviluppo di nuovi agenti antineoplastici che abbiano come *target* non soltanto le cellule tumorali ma anche il microambiente che le circonda. Recentemente, infatti, l'attenzione dei ricercatori è volta allo studio del ruolo del microambiente tumorale nella regolazione della crescita, della sopravvivenza e della risposta ai farmaci delle cellule leucemiche. Gli esosomi sono piccole vescicole di diametro compreso tra 40-100 nm, rilasciati da molti tipi cellular comprese le cellule neoplastiche. Gli esosomi costituiscono una parte importante del microambiente tumorale, trasferiscono messaggi alle cellule bersaglio, influenzando così la progressione neoplastica. Lo scopo di questa tesi è valutare il ruolo degli esosomi rilasciati da cellule di LMC, sensibili e resistenti all'Imatinib, nella modulazione del microambiente tumorale e di testare la capacità di un nuovi composti di interferire in questo *crosstalk*. I nostri dati mostrano che il carbossiamidotriazolo-orotato (CTO) è in grado di inibire, sia *in vitro* che *in vivo,* la crescita delle cellule leucemiche imatinib-resistenti e contemporaneamente di inibire i meccanismi angiogenici stimolati dagli esosomi.Il CTO può dunque essere efficace sia nell'inibizione della crescita delle cellule tumorali che nella modulazione del microambiente tumorale, suggerendo così una sua potenziale utilità terapeutica nel trattamento dei pazienti affetti da LMC (capitolo 1). I nostri dati, inoltre, mostrano che le cellule leucemiche e le cellule stromali stabiliscono un *crosstalk* bi-direzionale: gli esosomi di LMC inducono la produzione della citochina proangiogenica IL8 in cellule stromali, determinando pertanto, mediante un meccanismo paracrino, la sopravvivenza delle cellule leucemiche (capitolo 2).

Introduction

Chronic Myelogenous Leukaemia: general aspects and molecular biology of the disease

Chronic myelogenous leukaemia (CML) is a myeloproliferative neoplasm associated with a chromosomal translocation that gives rise to the Philadelphia (Ph) chromosome containing the fusion bcr-abl gene. The term "myelogenous leukaemia" was coined in 1978 by Neumann, who deduced that the disease originated from the bone marrow [1]; only the discovery, in 1960, of the Philadelphia chromosome allowed a better understanding of the pathogenesis of the disease. Thirteen years later it was discovered that the Ph chromosome results from an unbalanced chromosomal translocation between the long arm of chromosome 9 and the long arm of chromosome 22 [2](figure 1). The translocation t (9; 22) (q34; q11), present in all patients with (CML), in 25% of adults and 5% of children with acute lymphoid leukaemia (LAL), determines the fusion of the bcr (breakpoint cluster region) gene, located on chromosome 22, with the abl gene (abelson tyrosine kinase), located on chromosome 9, and the subsequent formation of the fusion oncogene bcr-abl coding for an aberrant tyrosine kinase with a constitutive activity[3].

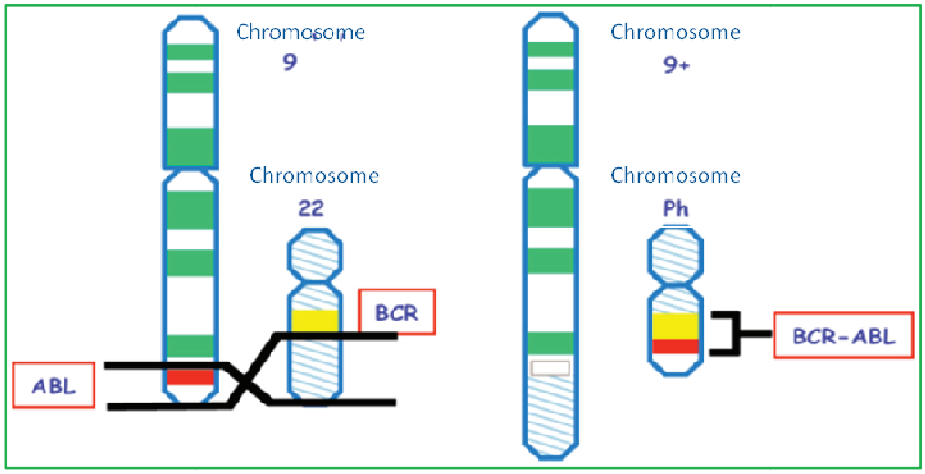


Figure 1. The t(9;22)(q34;q11) reciprocal translocation: The normal chromosomes 9 and 22 carry the c-abl and c-bcr genes, respectively. The translocation results in the formation of a shortened chromosome 22 (the Philadelphia chromosome) carrying the bcr-abl hybrid gene.

It has been shown that breaking points in three different sites of the bcr gene (M, m or μ) produce three distinct proteins of 210, 230 and 190 kDa (p210BCR-ABL, p230BCR-ABLand p190BCR-ABL), which influence the biological and clinical

phenotype associated with the expression of BCR-ABL[4]. P190BCR-ABL *in vitro* stimulates the expansion of lymphoid cells [5] and, for this reason, it is mainly expressed in acute, Ph+, lymphoid leukaemia.The two forms of BCR-ABL, ABL-p190BCR and p230BCR-ABL, however, were occasionally found in patients affected by chronic myelogenous leukaemia[4].

Chronic myelogenousleukaemia is a cancer in which the different clinical stages coincide with the genetic progression of the disease. Clinically, CML can be divided into three phases (fig. 3): chronic phase, accelerated phase and blast crisis. The chronic phase is characterized by the expansion of myelogenous progenitor cells with apparently normal differentiation. The chronic phase may progress in accelerated phase characterized by an increase in frequency of progenitor cells rather than differentiated cells. Several years later, this is followed by the acute leukaemic progression phase called blast crisis.

In order to better understand the role of p210 BCR-ABL oncoprotein in the pathogenesis of CML, it is important to describe the structure and function of the two physiologically expressed proteins, ABL and BCR.

The endogenous c-ABL protein is encoded by a gene of 12 exons, whose transcription, through alternative splicing mechanisms, may give rise to different messengers RNA. In all cases, the resulting protein is a tyrosine kinase of 145 kDa [6] involved in the regulation of the cell cycle, in the response to genotoxic stress and apoptosis [7]. The N-terminal portion (NH_2) of the protein contains three homologous domains to Src: the SH1 domain has tyrosine kinase activity, while SH2 and SH3 domains have a role in the interaction with other proteins. The central portion of ABL, thanks to the presence of many regions rich in proline, bind SH3 domains of other proteins as Crkl, while the C-terminal portion (COOH) contains a DNA binding domain, a domain of binding to actin and a nuclear localization signal [8]. BCR protein is encoded by a gene of twenty-three exons whose transcript generates two different mRNAs coding for two proteins of 130 and 160 kDa. The protein BCR is normally localized both in the nucleus and in the cytoplasm. The N-terminal region (NH_2) contains a oligomerization domain, which is required for

activation of the ABL kinase in BCR-ABL, a serine/threonine kinase domain and domains homologous to Src (SH2). The central portion contains domains that promote the exchange of GDP for GTP with proteins that activate transcription factors such as NF-kB. Finally, the C-terminal region (COOH) presents a domain with GTPase activity on Rac, a small GTPase of the Ras family [7].

The clinical manifestations of CML involve massive expansion of myelogenous progenitor cells without differentiation block. BCR-ABL kinase drives the pathogenesis of BCR-ABL positive leukaemia through the phosphorylation and activation of a broad range of downstream substrates that play critical roles in cellular signal transduction and transformation, and thus representing potential therapeutic targets (figure 2). The oncogenic capabilities of BCR-ABL have been demonstrated in mouse models in which BCR-ABL expression is sufficient to cause the disease [9]. The critical pathways elicited by the constitutively active ABL tyrosine kinase include Ras-MAPK (Erk), PI3K, and transcription factors Myc and Stat5. Ras activation in BCR-ABL expressing cells is mediated by the interaction with the adaptor signalling molecules Grb2, Shc, Sos and Dok[10]. The sum of these interactions results in the favouring of Ras in its active GTP-bound form; this, in turn, leads to the activation of Raf-1 serine/threonine kinase activity with the subsequent activation of the MEK pathway, resulting in pro-mitotic transcriptional regulation[11].The phosphatidylinositol-3 kinase (PI-3K) is another important pathway activated by ectopic expression of oncogenic ABL proteins[12]. BCR-ABL interacts indirectly with the p85 regulatory subunit of PI-3K via various docking proteins including GRB-2/Gab2 and c-cbl. Activation of the PI-3K pathway triggers an Akt-dependent cascade that has a critical role in BCR-ABL transformation[13], by regulating proliferation, survival, the cell cycle and apoptosis [13].Finally, it was highlighted in different BCR-ABL positive cell lines, constitutive phosphorylation of the transcription factors Stat1 and Stat5, by Jak. Stat5 directed target of BCR-ABL, is responsible for activating the antiapoptotic Bcl-xl protein and inactivating the proapoptotic Bad protein. The role of Stat5 in neoplastic transformation mediated by

BCR-ABL was demonstrated through studies on mutants defective for this transcription factor [14].

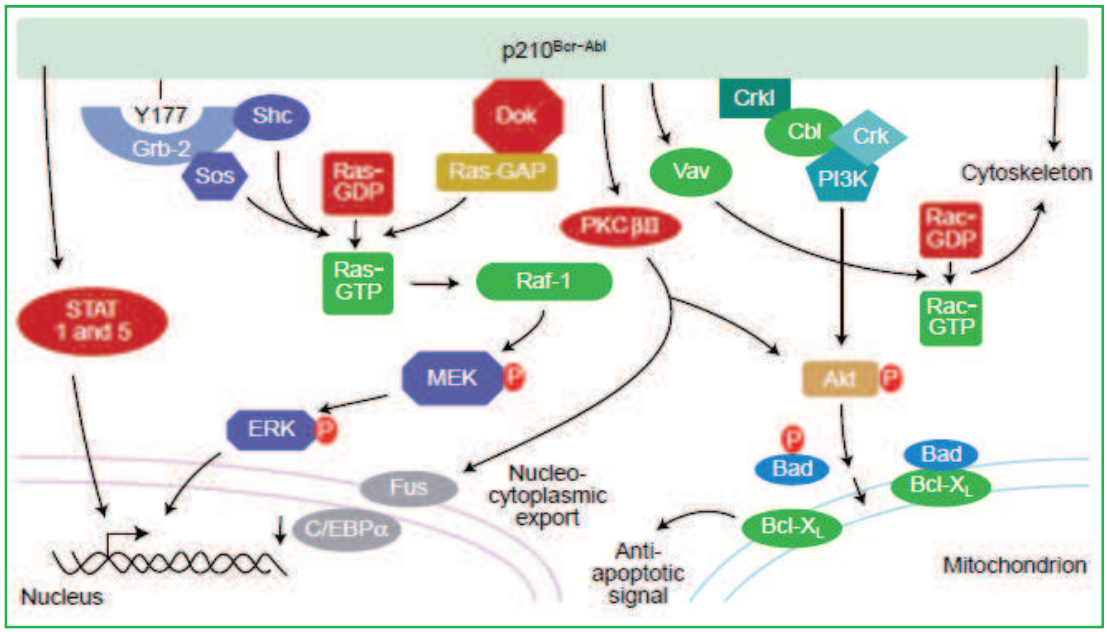


Figure 2. Schematic representation of BCR-ABL- mediated signal trasduction pathways: BCR-ABL protein tyrosine kinase mediates an array of effects on signal transduction pathways affecting cell survival, proliferation, adhesion and genetic stability (Adapted from: Smith DL et al. Molecular pathogenesis of chronic myelogenous leukaemia.Expert Rev Mol Med. 2003).

Imatinib: pharmacological resistance and alternative strategies

The prognosis of Philadelphia positive (Ph+) chronic myelogenous leukaemia has been revolutionized since the discovery of the pathogenetic role of BCR-ABL and the development of tyrosine kinase inhibitors (TKIs), such as Imatinib mesylate. Before Imatinib, CML was the most common reason for hematopoietic stem cell transplantation (HSCT) [15]. Since the introduction of Imatinib, a small organic molecule synthesized for the purpose of protein kinase inhibition, the number of patients undergoing transplantation has decreased dramatically. Imatinib occupies the interface between the N-terminal and C-terminal subdomains of the ABL kinase domain from one side to the other. Its binding site overlaps with the ATP binding pocket and therefore acts as an ATP-competitive inhibitor,blocking and stabilizing the oncoprotein in its inactive form[16; 17]. This binding prevents the transfer of phosphate group of ATP, inhibiting the constitutive activity of BCR-ABL and then, the activation of downstream signaling pathways (figure 3). BCR-ABL inhibition is

associated with decreased cell proliferation and induction of apoptosis; this causes the return to normal hematopoiesis.

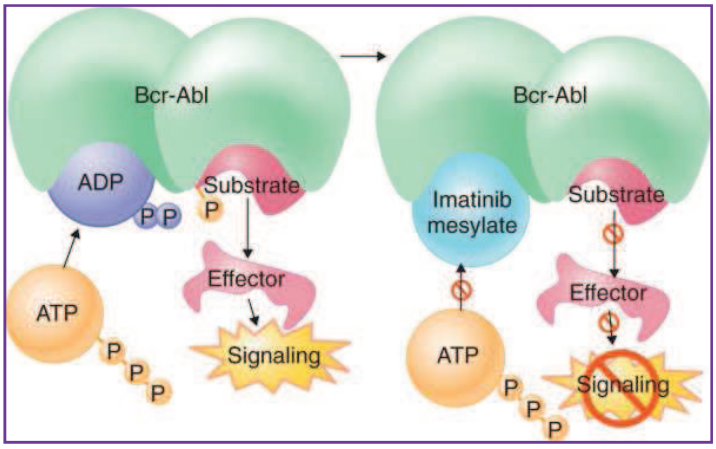


Figure 3. Mechanism of action of Imatinib:by occupying the ATP binding pocket of the ABL kinase domain, Imatinib prevents substrate phosphorylation and downstream activation of signals, thus inhibiting the leukemogenic effects of BCR-ABL (Adapted from: Kantarjian H, O'Brien S. The chronic leukaemias. Goldman: Cecil Medicine, 2007).

Despite the fact that Imatinib is an effective agent for treating CML, its therapeutic potential is limited due to the development of pharmacological resistance (figure 4). Resistance to Imatinib is divided into two categories: primary (innate) resistance and secondary resistance (also referred to as "acquired resistance"). Patients with primary resistance display a lack of response from the start of therapy, whereas patients with secondary resistance achieve a degree of response for a variable length of time before the development of resistance. Amplification of the bcr-abl gene or emergence of point mutations in BCR-ABL are the most frequent causes of resistance [18]. Although mutations outside the ABL kinase domain have been observed, the best studied mechanism is related to mutations in this domain; mutations may be located in different regions such as at the Imatinib-binding site, at the ATP-binding site, in the activation loop, etc.[18]. Currently, approximately 100 different BCR-ABL kinase domain mutations have been described in Imatinib resistant CML patients[19].The T315I mutation, the first to be described [20], is caused by the substitution of threonine in position 315 to isoleucine, resulting in a steric hindrance

that interferes with the ability of Imatinib to bind the kinase domain of c-ABL. Currently, the resistance due to the T315I mutation is one of the most critical aspects of the treatment of CML since even new generation inhibitors, such as nilotinib and dasatinib, are ineffective in the presence of this mutation. To overcome the resistance observed with Imatinib treatment, other selective BCR-ABL tyrosine kinase inhibitors (TKIs) have been developed[21; 22].

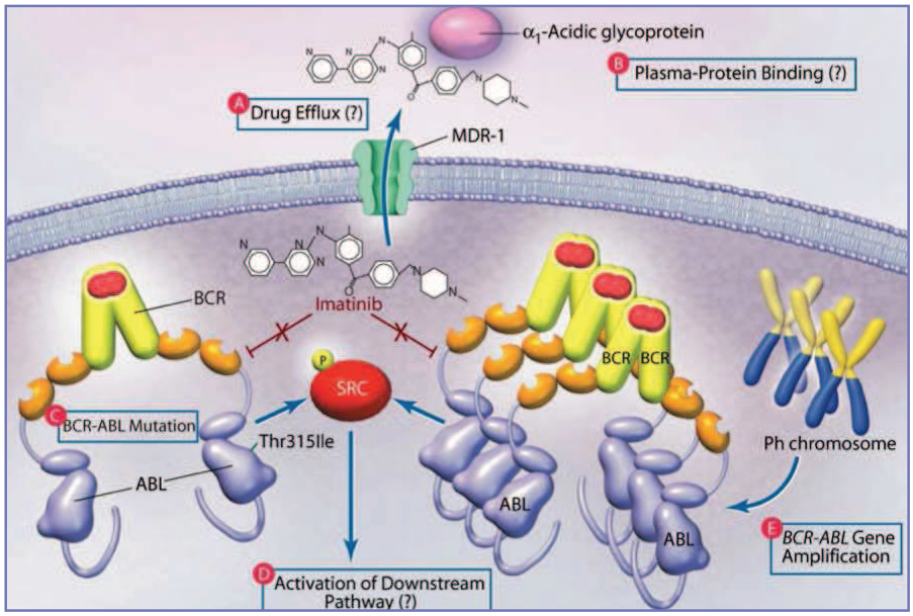


Figure 4. Schematic representation of different mechanisms for Imatinib resistance.(Adapted from: Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med. 2005).

Despite the development of second-generation of TKIs, a minority of CML patients in chronic phase and a substantial proportion of patients in advanced phase are either initially refractory to TKIs or eventually develop resistance [19]. Recent studies from our laboratory have shown that addition of carboxyamidotriazole (CAI), an inhibitor of calcium-mediated signal transduction [23], to Imatinib-resistant human CML cells induces a marked decrease in cell viability and augmented apoptosis, events associated with down-regulation of BCR-ABL protein, inhibition of tyrosine phosphorylation of BCR-ABL, STAT5, CrkL, as well as inhibition of ERK1/2 phosphorylation [24]. Moreover we showed that the molecular mechanism by which CAI is inhibiting CML cell proliferation is through an increase of reactive oxygen species that in turn modulate BCR-ABL levels, tyrosine phosphorylation of oncoprotein, downstream signalling and apoptosis[25].

Development of BCR-ABL tyrosine kinase inhibitors (TKIs) such as Imatinib, dasatinib, and nilotinib, have improved outcomes for patients diagnosed with chronic myelogenous leukaemia, however, the challenge of overcoming resistance to TKI therapy persists in the management of CML. There is no doubt that certain BCR-ABL mutations contribute to the development of resistance in patients with CML. However, TKI-resistant CML is more complex than indicated by the presence of mutations alone, and BCR-ABL independent mechanisms of resistance also have a considerable clinical impact. In the last years there are increasing evidences supporting the role of environmental factors in the escape of tumour cell from cytotoxic agent.

Role of bone marrow microenvironment in CML

Recently, a considerable interest in the cancer field has focused on tumour microenvironment (TME) and how the interaction between malignant cells and normal host cells affects cancer progression. Physiologically, the stromal environment consists of a dynamic framework of extracellular matrix components (collagen, fibronectin, proteoglycans etc) intermingled with immune cells, fibroblasts and vascular cells. The different components of the stroma include adipocytes, reticular cells, macrophages, endothelial cells, smooth muscle cells and mesenchymal stromal cells (MSC) [26]. A first important function of the stromal cells is the production and deposition of a complex extracellular matrix composed of hyaluronic acid, fibronectin, collagen type I and type IV, laminin and glycosaminoglycans. It provides a structural scaffold to stromal elements but also acts as a reservoir of many growth factors, cytokines, and enzymes that create a stromal niche within the bone marrow[27]. In recent years there has been a growing interest in the role of mesenchymal stem cells (MSC) in cancer progression. The term "mesenchymal" refers to their great plasticity, since they are able to differentiate into various cell types, including osteoblasts, adipocytes, myocytes, chondroblasts, neurons, astrocytes, endothelial cells and pulmonary epithelial cells [28; 29]. Mesenchymal

stromal cells also secrete factors such as M-CSF, Flt-3 l, SCF, IL-6, IL-7, IL-11, IL-12, IL-14, IL-15 and are considered the main source of the homeostatic chemokine CXCL12 [30; 31].

The importance of tumour microenvironment for cancer progression is recently becoming widely recognized. Several data indicate that during cancer progression, tumour stroma has not only a supportive role, but is also a leading player in the modulation of carcinoma development. Tumours actively recruit various bone marrow-derived cell types to the tumour microenvironment where these cells facilitate primary tumour progression and metastasis (figure 5)[32].

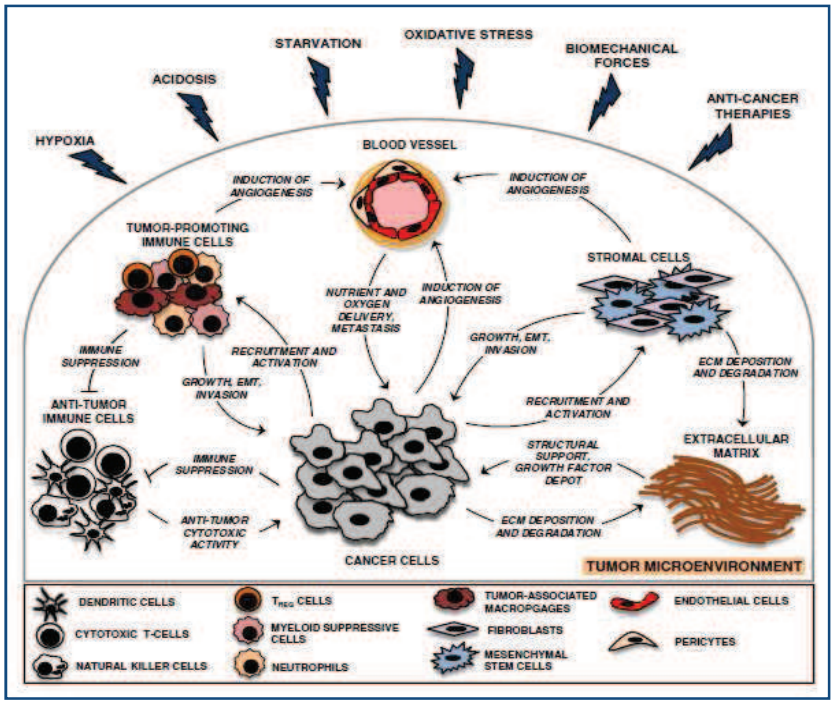


Figure 5. Heterotypic cellular interactions in the tumour microenvironment:cancer cells establish a bi-directional crosstalk with the different components of the tumour microenvironment, thus promoting its growth, survival and resistance to chemotherapies (Adapted from: Kucharzewska P, Belting M. Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress. J Extracell Vesicles, 2013.)

The nature of the relationship between MSC and tumour cells appears dual. Primary and metastatic tumours attract MSC in their microenvironment where they become tumour-associated fibroblasts (CAFs), affect tumour cell survival and angiogenesis, and have an immunomodulatory function; vice versa in the bone marrow MSC attract tumour cells and contribute to a microenvironment that promotes osteolysis,

tumour growth, survival, and drug resistance. The mechanisms of communication between tumour cells and the TME are complex but fall into two main categories, contact-dependent mechanisms that involve cell–cell and cell–ECM adhesion molecules and contact-independent mechanisms in which soluble molecules such as growth factors, chemokines and cytokines, and soluble subcellular organelles including microvesicles and exosomes play an essential role. Ultimately, these interactions activate, via juxtacrine and paracrine mechanisms, several signaling pathways in malignant cells and in nonmalignant stromal cells that can inhibit or favor tumour progression [33]. A new class of cancer therapies that targets this pathological communication interface between tumour cells and host cells is currently under development.

The tumour vasculature is an important component of the tumour microenvironment.Tumours are endowed with angiogenic-inducing capability, and their growth, invasion and metastasis are angiogenesis dependent. The development of the tumour vasculature is regarded to derive from both the pre-existing vessels via endothelial cell migration and proliferation (angiogenesis) and recruitment of progenitor cells from bone marrow (vasculogenesis)[34; 35]. Several studies have shown that the dynamic crosstalk between endothelial cells and other stromal cells substantially contribute to tumour angiogenesis [36].

Astimulating aspect in the field of CML biology is the role of the microenvironment in regulating the behaviour of leukaemic cells.Bone marrow (BM) stromal cells are the source of signals such as cytokines, growth factors and ECM molecules that can modulate leukaemia cell growth, survival and response to drug. Dysfunction of the bone marrow niche may contribute to leukemogenesis by supplying abundant growth factors that promote proliferation and/or inhibit apoptosis [37]. For example, Bhatia and colleagues showed that alterations in the CML BM microenvironment, due to the presence of malignant stromal macrophages, might contribute to the selective expansion of leukaemic progenitors and suppression of normal hematopoiesis [38]. In bone marrow, leukaemia cells seed in hematopoietic microenvironment, which produces a number of soluble and insoluble cytokines,

growth factors, adhesive molecules, chemotactic factors and receptors, such as CXCL12/CXCR4 axis, one of the key elements in the modulation of hematopoietic stem cell homing. CXC chemokine ligand 12 (CXCL12), a member of the CXC subfamily previously called stromal cell-derived factor-1a, is a chemokine produced by stromal cells that acts through its cognate receptor CXCR4[39]. CXCL12 functions both as a chemoattractant and a modulator of cellular growth/survival[40]. G protein coupled CXCR4, which is expressed on the membrane of normal and malignant hematopoietic cells, mediates chemotaxis and has a key role in the homing of these cells to the BM microenvironment[41]. It has been demonstrated that BCR-ABL may inhibit SDF1a-induced migration and signaling and therefore interfere with blast migration to bone marrow stromal cells [42]. Taken together, these results suggest that cancer cells require CXCR4 signaling to maintain engraftment and tumour survival in the bone marrow microenvironment, and that this axis can be targeted to overcome *de novo* drug resistance in hematologic malignancies.

Many studies have shown that bone marrow stromal cells or extracellular matrix may influence the response of leukaemic cells to chemotherapy and small molecule-targeted therapies. Meads and colleagues have reported that ECM molecules of the BM such as fibronectin, osteopontin and vitronectin may play a role in the acquisition of drug-resistance in several hematopoietic cancers including CML [43]. Data from Hazlehurst's group have demonstrated that culturing CML cells in bone marrow-derived conditioned medium was sufficient to cause resistance to Imatinib[44]. In turn, Imatinib treatment increases CXCR4-mediated migration of CML cell lines to BM mesenchymal stromal cells and results in increased cell cycle arrest and survival of quiescent cells[42].

In addition, during CML progression, bone marrow shows an increased vascularity that represents an independent, adverse prognostic feature [45; 46]. Increased bone marrow vascularity is part of the pathophysiology of human chronic myelogenous leukaemia[45]. One of the well-known clinical manifestations of CML is splenomegaly. The extramedullary growth of leukaemic cells in spleen is analogous to metastatic tumour growth in distant tissues, where metastatic organs prepare the

microenvironment for coming tumour cells. Expansive growth of CML progenitor cells in CML spleen is considered to be one of the extremes of organ specific metastasis. Growth factors such as VEGF, transforming growth factor beta (TGFβ) and tumour necrosis factor alpha (TNFα) are required for the establishment of the metastatic microenvironment [47]. mRNA levels of VEGF, placental growth factor (PlGF) and IL-6 are upregulated in CML bone marrow and anti-PlGF antibody is reported to prolong survival of CML mice and inhibit lung metastasis [48; 49]. Bone marrow and circulating VEGF levels are elevated significantly in patients with CML[45]and are associated with reduced complete response (CR) rates, disease free survival (post-CR), and overall survival. Other angiogenic molecules such as bFGF, IL-8 and HGF are secreted by CML cells and affect the endothelial phenotype [50].

Interleukin 8

Interleukin-8 (IL-8), alternatively known as CXCL8, is a proinflammatory CXC chemokine frequently coexpressed with VEGF in tumours, where they are reciprocally upregulated and cooperate to induce angiogenesis. IL-8 expression has been shown to be regulated by a number of different factors including inflammatory signals (e.g., tumour necrosis factor α, IL-1β), chemical and environmental stresses [51]. The biological effects of IL-8 are mediated through the binding of IL-8 to two cell-surface G protein–coupled receptors, termed CXCR1 and CXCR2.

IL-8 is widely expressed in tumour, stroma, and endothelial cells [52; 53]. Its autocrine and paracrine function has been shown to play an important role in angiogenesis, tumour growth, and metastasis [54; 55]. The increased synthesis and secretion of IL-8 from tumour cells has wider significance to the tumour microenvironment given the characterized expression of CXCR1 and CXCR2 receptors on cancer cells, endothelial cells, and neutrophils/tumour- associated macrophages. After stimulation of CXCR1 and CXCR2 receptors with IL-8, a wide range of signaling pathways is activated (figure 6). IL-8 signaling promotes activation of the primary effectors phosphatidyl-inositol-3-kinase or phospholipase C,

promoting the activation of Akt, PKC, calcium mobilization and/or MAPK signaling cascades. These signaling pathways have been shown to promote protein translation and regulate the activity of a range of transcription factors involved in cell survival, proliferation, invasion and angiogenesis.

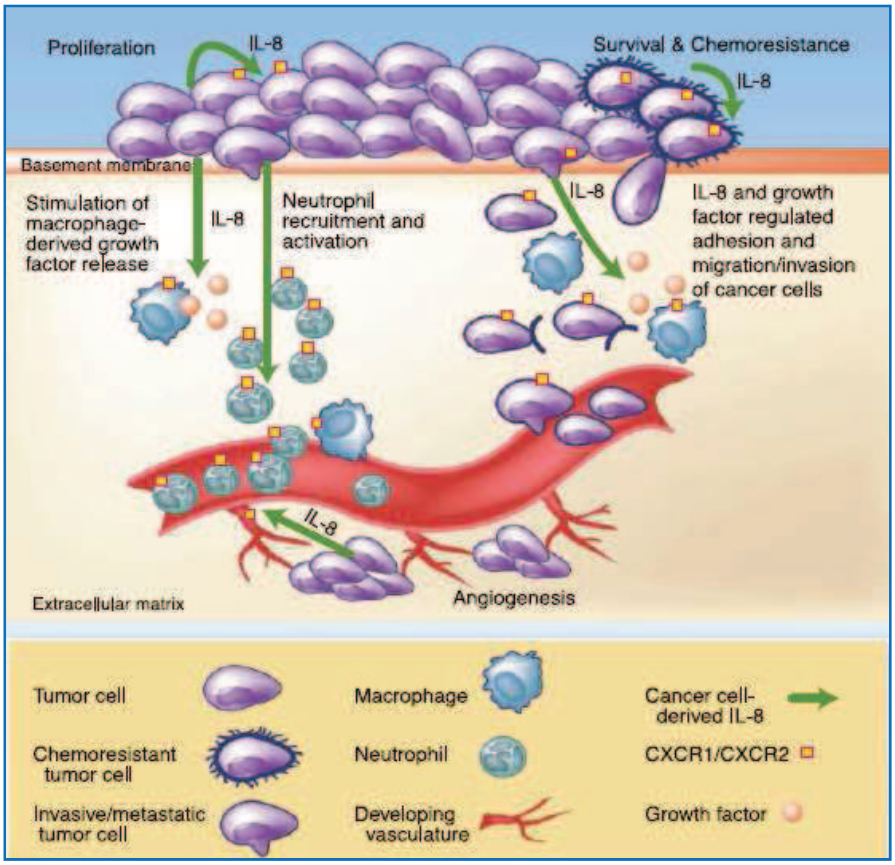


Figura 6. The role of IL-8 signaling in tumour microenvironment:IL-8 signaling promotes angiogenic responses in endothelial cells, increases proliferation and survival of endothelial and cancer cells, and potentiates the migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumour site. (Adapted from: Waugh DJ, Wilson C. The interleukin-8 pathway in cancer.Clin Cancer Res. 2008)

The expression of IL-8 receptors on cancer cells, endothelial cells, neutrophils, and tumour-associated macrophages suggests that the secretion of IL-8 from cancer cells may have a profound effect on the tumour microenvironment. Li and colleagues observed that IL-8 stimulates endothelial cell proliferation and capillary tube organization in a concentration-dependent manner, which can be blocked by neutralizing anti-IL-8 Abs, suggesting that IL-8 promotes angiogenesis by directly interacting with endothelial cells [56]. In human gliomas, IL-8 is espressed at high

levels both *in vitro* and *in vivo*, and it is crucial to glial tumour neovascularity and progression [57]. There is significant support for targeting IL-8 signaling in numerous solid tumours (gastric, pancreatic, melanome, ovarian and prostate) but little is known on the role of IL-8 in the biology of CML. We have previously reported that exosomes released from CML cells activate, in endothelial cells, signal transduction pathways leading to the release of IL-8 that ultimately causes, by an autocrine mechanisms, the activation of an angiogenic phenotype; moreover, blocking with anti IL-8 neutralizing antibodies the interaction of the cytokine with endothelial cell surface, causes the inhibition of angiogenic process [58].

Exosomes: biogenesis, composition, release and uptake of exosomes by target cells

Cells are known to deliver proteins and molecules between the intracellular organelles via membrane vesicles containing definite receptors to ensure traffic specificity; the results accumulated over the last ten years have demonstrated that a heterogeneous group of vesicles are also released from the cell surface and used as intercellular signalosomes in information exchange, even over a long distance [59]. These membranous vescicles, released by a variety of cells and generally termed extracellular vescicles (EVs), can be divided into three main classes: exosomes, microvescicles and apoptotic bodies. Among these, exosomes have recently received most of the attention. While exosomes are vescicles of endocytic origin and homogeneous in shape and size (40–100 nm), apoptotic bodies, released by cells undergoing programmed cell death, and microvescicles, are derived directly from the plasma membrane and show variable size (50–500 nm for apoptotic bodies, 100–1000 nm for microvescicles) and shape (figure 7). EVs have been originally also named on the basis of their tissue origin; for example, prostasomes are vesicles secreted by prostate epithelial cells and secreted into the seminal fluid, argosomes are exosome-like vesicles isolated from *Drosophilamelanogaster* and oncosomes, vescicles released by cancer cells [60].

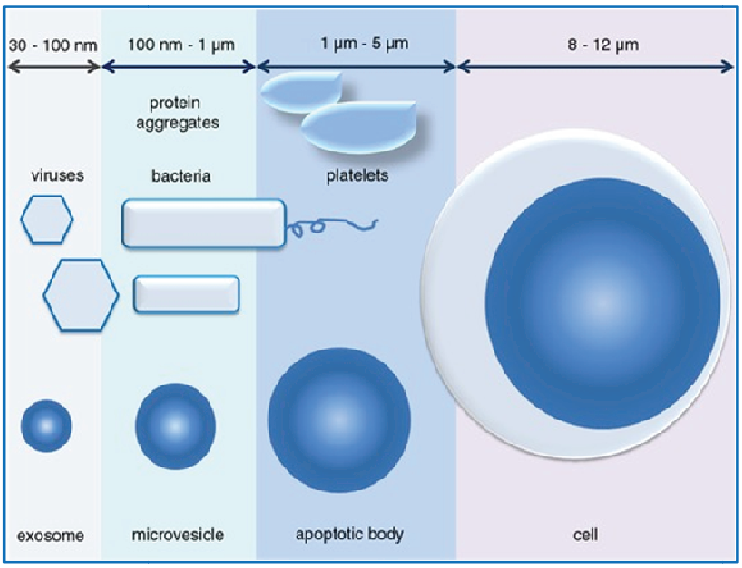


Figure 7. Sizes of different types of membrane vesicles. (Adapted from: György B et. al.Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles.Cell Mol Life Sci. 2011)

Exosome secretion can be constitutive or inducible depending on the cell type. Savina and colleagues showed that the release of exosomes from erythroleukaemia K562 cells was markedly enhanced by MON treatment, a Na+/H+ exchanger that induces changes in intracellular calcium (Ca2+) implying a requirement for Ca2+ in this process[61]. Levine's group demonstrated that mouse embryo fibroblasts with a wild-type p53 gene produced exosomes after DNA damage but isogenic MEFs with no p53 genes (from knockout mice) failed to produce exosomes after the same genotoxic stress. A p53-regulated gene product, TSAP6, was shown to be involved in exosome production thus alerting adjacent cells and the immune system of these events [62]. Exosomes are thought to be somewhat unique in their protein and lipid composition, providing additional traits for their identification (figure 8). Due to their endosomal origin, all exosomes contain membrane transport and fusion proteins (GTPases, Annexins, flotillin), tetraspannins (CD9, CD63, CD81, CD82), heat shock proteins (Hsc70, Hsp 90), proteins involved in multivesicular body biogenesis (Alix, TSG101), as well as lipid-related proteins and phospholipases[63]. Besides proteins, exosomes are enriched in certain raft-associated lipids such as cholesterol (primarily B lymphocytes), ceramide (implicated in the differentiation of exosomes from lysosomes) other sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains [64].

In the last few years exosomes have been reported as containing not only proteins and lipids but also nucleic acids such as DNA, RNA, miRNA and long non coding RNA [65; 66]. RNA-containing exosomes may represent an alternative pathway of cellular communication with significant implications in the modulation of cell phenotypes. The first experimental indication of the presence of mRNA and miRNA in exosomes from mouse and human mast cell lines was reported by Valadi and co-workers [65]; they showed that exosome mRNAs were functional, could be transferred in target cells and translated into proteins.

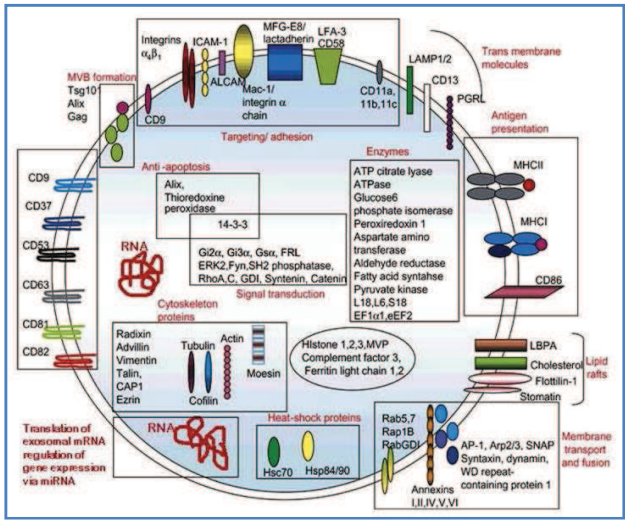


Figure 8. Protein, lipid and RNA composition of exosomes:(Adapted from: Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes. Nat Rev Immunol. 2005)

Once released in the extracellular space, exosomes interact with target cells inducing, according to the delivered molecules, a modulation of the phenotype toward a differentiated or activated state. How exosomes may interact with a target cell is not yet fully known and several mechanisms have been hypothesized [59; 67; 68](figure9). For example, exosomes can fuse with the target cell resulting in the non-selective transfer of proteins and RNA from exosome to the recipient cell. Parolini showed that exosome uptake from melanoma cells increase at a low pH and that this mechanism is dependent on the presence of sphingomyelin/ganglioside GM3. Moreover, they showed that fusion efficiency is higher with exosomes released from metastatic cells compared to those derived from primary tumours or normal cells [69]. Montecalvo and colleagues showed that dendritic cells (DCs) communicate with

neighboring DCs through exosome-shuttle miRNAs, this cell-cell interaction was mediated via a "2-step event" consisting of exosome hemifusion with the cell membrane followed by the complete fusion of the exosomes with the limiting membrane of the phagosome; interestingly, they also observed that exosome content could vary according to the maturation of the parental DCs thus reinforcing the notion of an accurate selectivity in molecules sorted in exosomal cargos [70]. Exosomal membrane proteins can interact with the target cell in a juxtacrine fashion, acting as a ligand for cell surface receptors. Segura and colleagues studied the interaction between exosomes released by bone marrow-derived dendritic cells and CD8+ dendritic recipients cells showing that exosomes interact with DCs through a specific saturable receptor. Furthermore, they showed that exosomes secreted by mature DCs, bearing both functional MHC class II-peptide complexes and high amounts of ICAM-1 molecules, are captured and presented *in vivo* to non migrating murine CD8+ DCs through specific interactions with LFA-1, the naturally occurring counter-receptor of ICAM-1 [71]. It is well established that rafts play an important role in signal transduction providing a well organized microenvironment, where the phosphorylation state of the resident proteins can be modified by local kinases and phosphatases, resulting in downstream signaling. Calzolari and colleagues demonstrated for the first time that activation of transferrin receptor 2 (TfR2) induces the activation of ERK1/2 and p38 MAPK pathways, supporting the hypothesis that TfR2 may function as a signaling receptor. In particular they showed that TfR2 is a new raft component sorted in exosomes and the localization of TfR2 in lipid rafts is essential for its signaling. Through the exosomal pathway TfR2 could act as an intercellular messenger, carrying a message about cell iron status [72]. Another example of receptor-ligand interaction mediated by exosomes on target cells is reported by Clayton and colleagues: using a panel of tumour cell lines, they showed that tumour exosomes may suppress a key tumour cell recognition pathway involving NKG2D, a receptor found in NK, NKT, CD8+ and gammadelta T cells. In particular, they demonstrated that tumour exosomes carry NKG2D ligand, such as MICA and MICB, triggering a selective down regulation of cell surface NKG2D.

These effects mediate suppression of lymphocyte functions, even in the presence of inflammatory cytokines such as IL-15, and consequently may mediate immune evasion in cancer [73]. In another study, the interaction between exosomes and the cell surface of target cells was mediated by fibronectin,an extracellular matrix component; B-lymphocytes also release exosomes that show high levels of integrin receptors on their membranes. The addition of these exosomes to TNF-alpha-activated fibroblasts triggered integrin-dependent changes in cytosolic calcium, measured by single cell imaging thus demonstrating not only that these receptors are fully functional but also that they may represent a novel mode of delivering adhesion signals over long distances[74]. Finally, a recent paper by Feng and colleagues showed that in phagocytic cells, exosomes could be internalized efficiently via phagocytosis [45]. Their experimental data demonstrated that exosomes adhered easily to the cell surface of non-phagocytic cells but could not be internalized, as they could be removed by trypsinization or with extensive acid washing. In contrast, the same treatment did not remove exosomes from phagocytes, as they were already inside the cell [68].

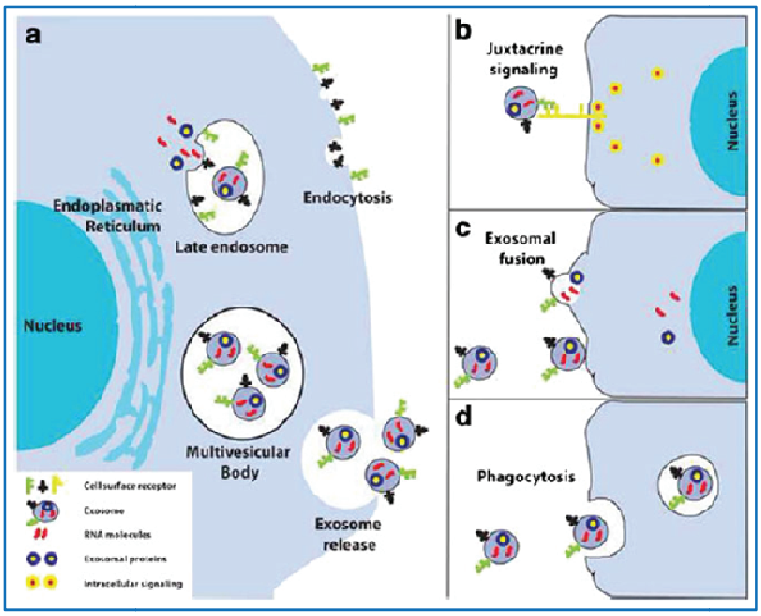


Figure 9. Schematic representation of exosomes biogenesis, release and uptake by target cells. (Adapted from Kahlert C, Kalluri R. Exosomes in tumour microenvironment influence cancer progression and metastasis.J. Mol. Med. 2013).

Biological role of exosomes in cell phisiology and pathology

Exosomes are now considered as an integral part of the intercellular microenvironment and may act as regulators of cell-to-cell communication. They may act as immune modulators with immunosuppressive or immune-activating effects by delivering proteins or nucleic acid to recipient cells. Exosomes, once released, may locally affect the behavior of target cells or enter in a biological fluid thus reaching distant sites. Exosomes have a wide range of functions, depending on cell origin and on the physiological state of the originating cell.

Exosomes are found in body fluids including plasma, urine, saliva, synovial fluid, breast milk, bronchoalveolar lavage fluid and epididymal fluid [75; 76; 77; 78], supporting their *in vivo* role in physiological processes. Most of the studies have focused on the immunomodulatory abilities of these exosomes. Since 1996, it has known that immune system cells release exosomes [79]; at first, immunologists supposed that exosomes might be extracellular organelles important to intercellular communication, with a potential role in immune-modulation. Currently, it is well known that exosomes contain several molecules used to vehicle messages between immune cells or between immune and target cells, and thus have immunosuppressive or immune-activating effects on different steps of the immune response.

Exosomes have been proposed as being a way of intercellular communication in the normal physiology of the nervous system; several reports have demonstrated the release of exosomes by astrocytes or microglial neurons [80]. Recently, Lachenal and collaborators demonstrated that exosomes, released by differentiated neurons, are regulated by synaptic glutamergic activity and might thus be part of normal synaptic physiology [81]. Exosomes may also play a key role in neuronal communication during neurodegenerative diseases, such as Alzheimer's or Parkinson's diseases; these pathologies are characterized by the accumulation of misfolded proteins; recent studies have shown that the misfolded protein incorporation into exosomes protects them from degradation and also facilitates their delivery over long distances [82].

Another emerging field is the role of exosomes in the disease progression of some autoimmune disorders such as Rheumatoid Arthritis (RA). Altered apoptosis sensitivity of fibroblast-like synoviocytes and T lymphocytes, leading to both synovial hyperplasia and chronic inflammation, has been extensively demonstrated in patients with rheumatoid arthritis (RA). Exosomes produced by synovial fibroblasts obtained from patients with RA contain a membrane form of TNF-α that, by inducing AKT and NF-κB, renders these cells resistant to apoptosis [83].

Role of exosomes in tumourigenesis

The evolution of a clinically significant invasive carcinoma requires an active collaboration of different actors found at the tumour-host interface: malignant epithelial cells, extracellular matrix, cancer-associated fibroblasts, inflammatory immune cells and normal mesenchymal cells [84].

A variety of cytokines, growth factors, adhesion molecules and extracellular matrix proteins are secreted by both tumour and non-tumour cells, mediating cell-to-cell communication within the tumour microenvironment and providing a suitable niche for cancer cell growth and survival. A wealth of information has been obtained on the role of these molecules and cell types in promoting cancer invasion and metastasis. Recently, exosomes have been considered as new vehicles of these molecules into the tumour microenvironment and, for this reason, data is beginning to accumulate on their role as new actors in the crosstalk between cancer and normal cells in the tumour microenvironment (figure10). Exosomes may function in an autocrine or paracrine manner to promote tumour-induced immune suppression, angiogenesis or premetastatic niche formation [85; 86].

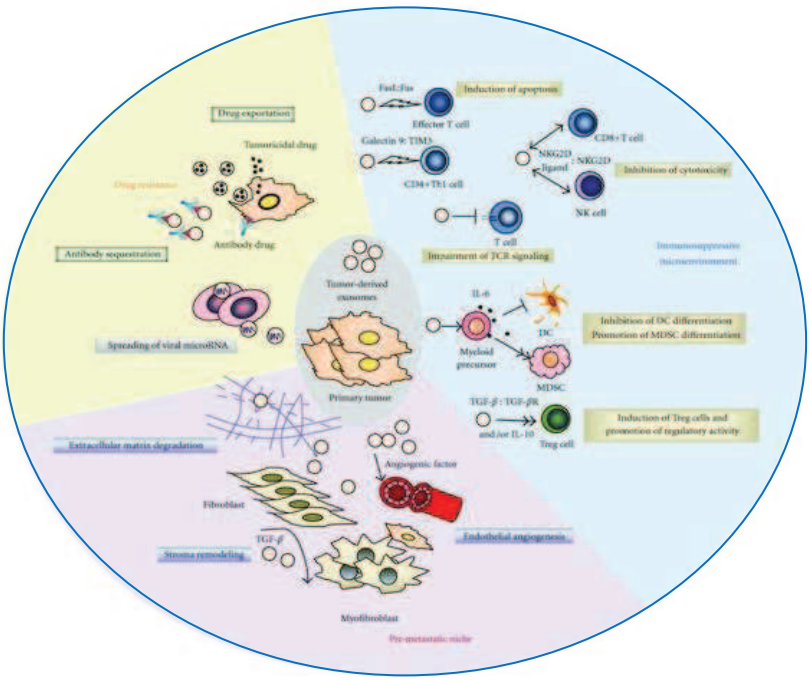


Figure 10: The protumourigenic role of cancer-derived exosomes: exosome-mediated exchange of signalling proteins and genetic material induces a microenvironment favorable for tumour growth and progression. (Adapted from: Yang C, Robbins PD. The roles of tumour derived exosomes in cancer pathogenesis.Clin Dev Immunol. 2011)

Luga and collaborators have recently shown that cancer associated fibroblasts (CAFs) secrete exosomes that are able to promote, in an autocrine fashion, breast cancer cell protrusive activity and motility via Wnt-planar cell polarity (PCP) signaling. In particular, their results reveal a complex intercellular communication pathway, whereby CAF-secreted exosomes are internalized, modified and loaded with Wnt11 into breast cancer cells; exosomes are then released to activate an autocrine PCP signaling in breast cancer cells, thus promoting cancer cell migration and the acquisition of invasive behavior [87]. Bone marrow derived human mesenchymal stromal/stem cells (MSC), adjacent to primary tumour cells, have been shown to affect cancer progression by providing a favourable microenvironment and therefore promoting tumour growth, invasion and also metastasis through a process called premetastatic niche formation [88; 89; 90]. Recently, in fact, Zhu and colleagues have demonstrated that MSC release exosomes able to strongly trigger VEGF- and CXCR4-mediated pathways in tumour cells through the activation of Erk 1/2 and p38

MAPK kinases, thus resulting in an enhanced angiogenesis and tumour growth *in vivo* [91]. On the other hand, exosomes released by cancer cells could affect tumour growth, invasion and metastatic niche formation, angiogenesis and resistance to chemotherapy. Peinado and colleagues have recently shown that cancer-derived exosomes modulate the crosstalk between malignant cells and the bone marrow microenvironment; they reported, for the first time, that metastatic melanoma cells release exosomes that are able to "educate" bone marrow progenitors thereby inducing their mobilization which supports tumour vasculogenesis, invasion and metastasis, through the activation of the MET receptor tyrosine kinase. They identified that the MET-activated signaling proteins are expressed in highly metastatic melanoma derived-exosomes and that the transfer of the exosomal receptor tyrosine kinase MET from tumour derived-exosomes to bone marrow progenitor cells promotes the metastic process *in vivo*. These results suggest that bone marrow cells retain the educated phenotype after engraftment into a new host [85]. In lung cancer, Croce's research group has reported that tumour derived-exosomes are enriched in particular miRNAs (miR-21, miR-27 and miR-29-a), that not only can be used as molecular markers of this cancer histotype but also act as paracrine agonists of the Toll like receptor (TLR) family in immune cells thus triggering a TLR-mediated prometastatic inflammatory response that induces tumour growth as well as the formation of secondary colonies at the metastatic site [92]. Several studies have revealed the role of cancer derived-exosomes in activating signal transduction pathways involved in cancer cell proliferation and survival. For example, Demory Beckler and colleagues detected KRAS in exosomes released by colon cancer cells and showed that the mutated KRAS can alter the signals induced by cells via exosomes, leading to a growth advantage in the recipient non-transformed wild-type KRAS-expressing cells [93]. Qu *et al.* have demonstrated that gastric cancer derived-exosomes promote, through an autocrine mechanism, the proliferation of tumour cells by PI3K/Akt and MAPK/ERK activation [94].

Although most of the studies on cancer exosomes have been done on solid tumours, little data has been produced for hematopoietic malignancies such as leukaemia. Our

group has reported that human Chronic Myelogenous Leukaemia (CML) cell lines such as LAMA84 and Imatinib-resistant LAMA84 cells as well as patients' leukaemic blasts, release exosomes that directly affect endothelial cells thus modulating the process of neovascularisation. Specifically, the stimulation of vascular endothelial cells (HUVEC) with LAMA84-exosomes activate signal transduction pathways leading to the release of IL-8 and the induction of an angiogenic phenotype *in vitro* and *in vivo*[58; 95]. Moreover, Mineo and colleagues have shown that exosomes, released from K562 CML cells, are internalized by HUVEC during tubular differentiation on Matrigel and move within and between nanotubular structures, connecting the remodeling endothelial cells; this exosome-induced angiogenic activity was mediated by Src kinase [96]. Exosomal miRNAs may have an important role in leukaemia-endothelial cell communication; miR-92-a was detected in K562 derived-exosomes and has been demonstrated as having a role in enhancing endothelial cell migration and tube formation [97]. Recent data from Kurre's group examined the role of exosomes released by Acute Myelogenous Leukaemia cells in modulating cell signalling in the bone marrow microenvironment. They showed that both primary AML blasts and AML cell lines released exosomes enriched in microRNA relevant to AML pathogenesis. In particular, they reported that the enrichment of miR-150 in AML derived-exosomes modified transcriptional responses and protein secretion in recipient cells; exosome transfer to Ba/F3 progenitor cells was, in fact, associated with a loss of CXCR4 surface expression and a consequent decrease in cell migration toward SDF-1α [98]. Hypoxia plays an important role in human tumour progression from *in situ* to metastatic cancers. Growing tumours are forced to survive in a poorly vascularised microenvironment characterized by hypoxia. To survive and grow in this hypoxic environment, tumour cells use adaptive mechanisms to promote proliferation, become resistant to apoptosis, induce angiogenesis, evade immune attack and migrate to less hypoxic areas; these mechanisms are mediated by the Hypoxia Inducible Factor (HIF) family of transcription factors[99; 100]. Hypoxic tumour cells have been shown to release more exosomes to promote their own survival, angiogenesis and invasion; breast cancer

cells exposed to a hypoxic environment enhance the production of exosomes in part due to HIF induction as shown using the HIF hydroxylase inhibitor dimethyloxalylglycine and siRNA interference [101]. Other significant data were obtained through a quantitative proteomics study carried out on the A431 human epidermoid carcinoma. In this work, the authors reported that under hypoxia, cells secreted higher levels of proteins involved in angiogenesis, focal adhesion formation, extracellular matrix-receptor interaction and immune cell recruitment in comparison to cells undergoing normoxic and reoxygenation conditions. They found that more than 50% of these secreted proteins, predominantly classified as cytoplasmic and membrane proteins, were localized in exosomes [102]. A recent paper from Tadokoro and colleagues has shown that hypoxic leukaemic cells release exosomal miRNA, which promote an angiogenic phenotype through the inhibition of the receptor tyrosine kinase ligand Ephrin-A3. In particolar they consider that miR-210 is the principal exosomal miRNA secreted at high level from hypoxic cells and that it is able to down-regulate EFNA3, and thereby, enhance tube formation of HUVECs[103].

Exosomes may indirectly contribute to tumour progression and metastasis development by interfering with the action of therapeutic agents, possibly through the transfer of mRNAs, miRNAs and/or proteins involved in drug resistance that drive the phenotypic changes of recipient cells. Exosomes may carry proteins involved in multidrug resistance, such us the P-glycoprotein or alternatively sequester the chemotherapeutic agents thereby decreasing their intracellular amount. Safei and coworkers demonstrated that cisplatinum-resistant ovarian cancer cells actively expelled anticancer drugs by enhancing the release of exosomes. Moreover, they reported that exosomes released by cisplatinum-resistant cells were enriched in cisplatinum and expressed higher levels of the transporters MRP2, ATP7A and ATP7B in comparison to sensitive cells [104]. Further studies showed that, in ovarian cancer cells, resistance to cisplatinum is also associated with an increased secretion of annexin A3, a member of the Ca^{2+} and phospholipid-binding annexin family, which prevents the uptake or accumulation of platinum in cells [105]. Electron microscopy

observations have shown that annexin A3 detected in culture medium was localized in exosomes, revealing yet another exosome-mediated mechanism that affects a drug's action [106]. Recently, Battke and colleagues have shown that exosomes can hamper the action of anticancer therapies by interfering with antibody-based drugs. The authors demonstrated that breast cancer cell lines over expressing HER2, release exosomes expressing a full-length HER2 molecule that is able to bind, both *in vitro* and *in vivo*, to the HER2 antibody, Trastuzumab, resulting in a reduced amount of antibodies available for the antibody-dependent cytotoxicity[107]. Data discussed in this section shows the key role of exosomes in modulating the tumour microenvironment: exosomes are not simply vehicles of molecules targeted into recipient cells, but also induce phenotypic changes in neighboring cells by activating specific cell signaling pathways, leading to cancer progression.

Exosomes as biomarkers and their therapeutical applications

Exosomes, due to their cellular origin, their role in both physiologic and pathologic conditions and to recent technological developments that allow their selective capture, characterization and manipulation, are expected to significantly change many areas of clinical science. It has been largely demonstrated that biological fluids carry exosomes and because of their specific protein, lipid and RNA content, exosomes could represent a basis on which can be developed new biomarkers and targeted therapies.For example, Rabinowits and colleagues showed the significant difference in total exosome and miRNA levels between lung cancer patients and controls, suggesting that circulating exosomal miRNAs might be useful as a screening test for lung adenocarcinoma [108]. Moreover, Taylor and colleagues showed that microRNA signatures of tumour-derived exosomes can be used as diagnostic biomarkers of ovarian cancer [109]. Recent proteomic studies have indicated that exosomes from cancer patients show specific protein patterns, thus indicating that the protein composition of exosomes might be useful for the early detection of various cancers. For example, a proteomic analysis of urinary exosomes

allowed the identification of eight proteins that were potential bladder cancer biomarkers [110]. Liang and collaborators performed a high quality proteomic study of ovarian cancer-derived exosomes. The gene ontology (GO) analysis results revealed that exosomes may carry tissue specific proteins, which may provide some new biomarkers for the diagnosis of ovarian cancer[111]. Through the same approach, Chen and colleagues have recently identified differentially expressed proteins in urinary exosomes as novel candidate biomarkers for the discrimination of low-grade and high-grade bladder cancer[66].

Interest in using exosomes in therapeutic approaches is an even more recent development. Several recent studies have demonstrated that exosomes have a specific cell tropism, according to their characteristics, which can be used to target them to specific tissues and/or organs. The use of exosomes as a drug delivery system is now an emerging field; recent studies have shown that exosomes may be used to encapsulate and protect exogenous oligonucleotides for their delivery to target cells. They therefore may be valuable for the delivery of siRNA and microRNA regulatory molecules. For example, Ohno *et* colleagues have recently reported that modified exosomes,engineered to express EGF on their surface, can efficiently deliver microRNA (miRNA) to epidermal growth factor receptor (EGFR)- expressing breast cancer cells [112].

Aims

Aims

By elucidating the role of the tumour microenvironment in the pathogenesis of hemaetologic tumours, recent studies have provided the framework for identifying and validating novel therapies that target both leukaemic cells and cells of the surrounding microenvironment. Recently, a number of studies have described exosomes as new players in modulating tumour microenvironment, promoting angiogenesis and tumour progression. Since we have previously reported that CML cells, LAMA84, released exosomes able to induce angiogenic phenotype[58], we want to evaluate if the imatinib resistant cell line,LAMA84, release exosomes and then to test the ability of Carboxyamidotriazole Orotate (CTO) to target both cancer cells and the tumour microenvironment. Furthermore, a better understanding of the role of exosomes in mediating the crosstalk between leukaemic and bone marrow stromal cells could improve future studies aimed to target tumour microenvironment.

More specifically, the aims of this thesis were to:

- Test if CTO is able to inhibit *in vitro* and *in vivo* the growth of imatinib-resistant CML cells and to investigate the ability of CTO to affect tumour microenvironment by modulating exosome-stimulated angiogenesis *in vitro* and *in vivo* (chapter 1).

- Test if the release of exosomes from CML cells can modulate the tumour microenvironment through the secretion of both soluble and insoluble molecules from stromal cells, thus establishing paracrine interplay among leukaemia cells and stromal cells (chapter 2).

Chapter 1

Carboxyamidotriazole-orotate inhibits the growth of Imatinib-resistant chronic myelogenous leukaemia cells and modulates exosomes-stimulated angiogenesis

Introduction

Imatinib mesylate (IM) is a selective well-tolerated inhibitor of the BCR-ABL tyrosine kinase that has significantly improved the prognosis of patients with chronic phase CML. Despite this remarkable progress, a major problem associated with the administration of imatinib is acquired resistance. Therefore, there is an urgent need for new anticancer agents and combinations that could improve responses and survival rates for CML. Recent studies from our laboratory have shown that addition of Carboxyamidotriazole (CAI), an inhibitor of calcium-mediated signal transduction, to imatinib resistant human CML cells induces a marked decrease in cell viability and augmented apoptosis, events associated with downregulation of BCR-ABL protein and inhibition of tyrosine phosphorylation of BCR-ABL, STAT5, CrkL[24; 25]. Carboxyamidotriazole Orotate (CTO) is the orotic acid salt of CAI that has been developed at Tactical Therapeutics. CTO possesses increased solubility, higher bioavailability and stronger efficacy compared to the parental compound. CTO enters the bloodstream faster than CAI, and achieves higher plasma concentrations while maintaining a similar elimination half-life. Pharmacokinetics data indicate that CTO may enable smaller dosages of CAI for inhibition of tumour cell proliferation, with reduced toxicity [113]. The combination of CTO and chemotherapy was found to produce significantly greater antitumour effects than chemotherapy alone in glioblastoma and colon xenograft mouse models [114].

Microenvironment-targeted treatment has gained increasing attention in hemato-oncology. Targeting the bone marrow (BM) environment may provide alternative opportunities to improve the efficacy of anti-CML treatment for TKI-refractory or intolerant cases. The aim of this project is to evaluate the *in vitro* effect of CTO on Im-resistant CML cell lines, LAMA84R and K562R and then to test its effects on tumour xenograft growth. Furthermore, since exosomes have a role in cell-to-cell communication and tumour-stroma interaction, thus potentially affecting cancer progression and response to therapy, we isolate, characterize and investigate the angiogenic potential of exosomes released by Im-resistant CML cells, LAMA84 and then test the effect of CTO on exosome-stimulated angiogenesis.

Matherial 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. Paolo Vigneri, Università di Catania [115]. 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 1mM 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.

Proliferation assay (MTT assay)

Methyl-thiazol-tetrazolium (MTT) assay was done as previously described [24], cells were plated in triplicate or quadruplicate at 1.5 x 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 [24]. 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 tumours were palpable, mice were randomly assigned to groups of ten and were treated with imatinib administered intraperitoneally (i.p) (50mg/Kg, three days a week for two rounds) or with its vehicle (PBS) in combination either with CTO 342 mg/kg (Q1Dx5 for two rounds) or with CTO 513 mg/kg (Q1Dx5 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. Tumour xenografts were measured and the mice were weighed three times a week starting on Day 7. Tumour 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 tumour weights assuming that 1 mm^3 = 1 mg. Due to Università di Palermo rules and Italian legislation, animals were euthanized when sc tumour xenografts reached 4000 mg in weight.

Exosome isolation and characterization

Exosomes produced by LAMA84R cells during a 24h culture period, were isolated from conditioned culture medium by different centrifugations as described previously[58]. 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 [61]. 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 24h, 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 24h or 48h and incubated for 6h 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 6h 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 [116]. The following primers were used: IL8 Hs00174103m1, VCAM1 Hs00174239m1, ICAM1 Hs00277001m1 and GAPDH Hs99999905m1 (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 24h and incubated over night with 50 μg/ml of LAMA84R-exosomes in a low serum medium (EGM:RPMI, 1:9). 5 x 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 24h with 10 μM CTO and then stimulated for 6h with indicated treatments; HUVEC CM was also collected from cells after 24h of transfection with siRNA (scramble or IL8) and 6h 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) [24]. Briefly, HUVEC (2 x 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 6h, 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 [116]. Briefly, HUVEC were pre-treated or not with 5-10 μM of CTO for 24h and HUVEC monolayer was incubated for 6h with indicated conditions, as described in the results. After treatment, cells were washed with PBS and CML cells were added for 2h 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 [116; 117]. 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 24h 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/) [118].

Matrigel plug assay

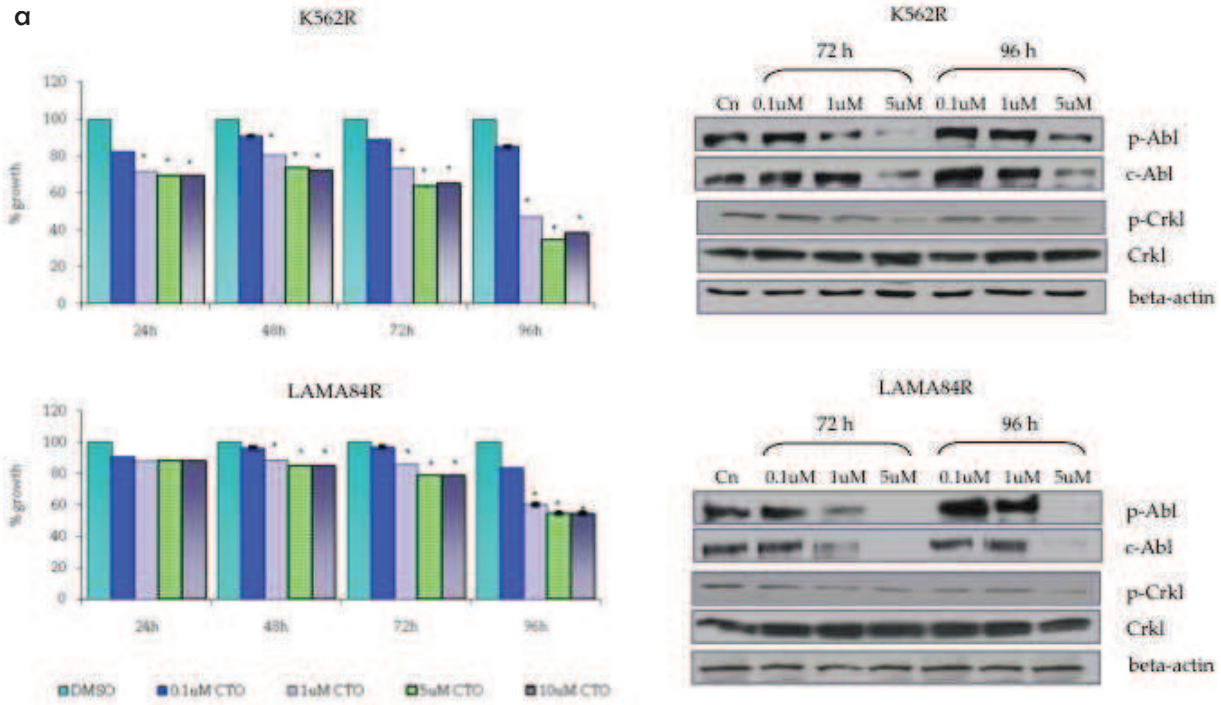
Groups of six NOD/SCID mice (4 weeks) (Charles River) were injected subcutaneously with 400 μL of Matrigel (BD Biosciences Pharmingen, San Diego, CA, USA) as follows: animals in group 1 and 2 were injected with matrigel plus PBS (PBS) or plus 100 μg LAMA84R-derived exosomes (Exo), and were treated with 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 50ng of recombinant IL8 and were respectively treated either with vehicles of CTO and imatinib (Rec IL8) or with CTO 513mg/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)[119].

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 K562R cells. Data presented in figure1a show that CTO inhibits cell growth of LAMA84R and K562R in the low micromolar range in a dose dependent fashion. The results herein show a 50% growth reduction of the CML lines with 5 μM CTO at 96h 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 1b, a dose-dependent inhibition of both total and phosphorylated BCR-ABL levels was observed after 72 and 96h 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.

Figure 1

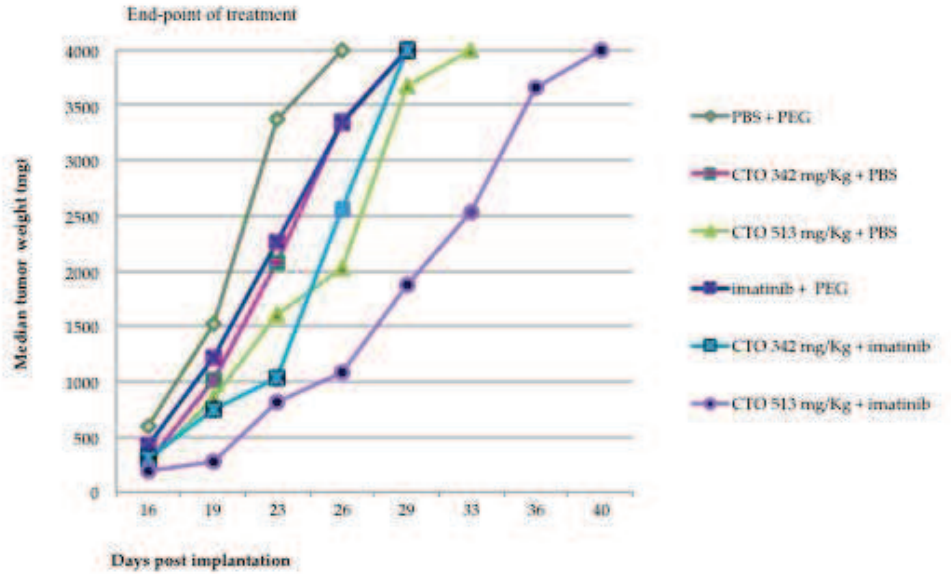


CTO inhibits cell proliferation of LAMA84R and K562R cell lines and inhibits BCR-ABL mediated tyrosine phosphorylation(a) Cell growth was measured by MTT assay after 24, 48, 72, 96h of treatment with increasing doses of CTO (0.1-1-5-10 μM). The values were plotted as a percentage of the control (cells treated with DMSO). Each point represents the mean ± SD for three independent experiments. *p ≤ 0.001. (b) cell lines were treated with increasing doses of CTO (0.1-5 μM) or DMSO (CN) for 72h and 96h; afterwards protein lysates were subjected to western blot analysis using anti-phospho-Abl, anti-cAbl, anti-phospho-CrkL and anti-CrkL antibodies.

Effects of CTO on tumour 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 tumour model. Otherwise CTO–treated mice seemed healthy and did not exhibit any signs of distress during the drug treatment. For these analyses, mice receiving either CTO alone or in combination with imatinib were treated until day 26 of treatment regimen. Animals were then maintained until tumour weight reached 4000 mg. Tumour weight curve analysis (figure 2) showed that mice bearing LAMA84R tumours 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 tumour growth to a greater extent than the control group ($P < 0.01$), attaining the experimental end point after 40 d.

Figure 2

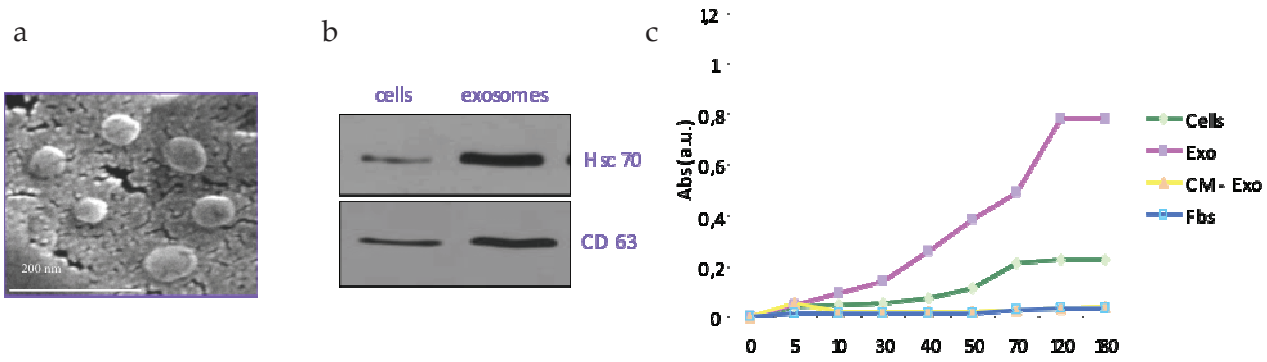


Antitumour activity of CTO on human CML xenografts: LAMA84R cells were injected subcutaneously in NOD/SCID mice as described. After palpable tumour formation, mice were treated with imatinib administered intraperitoneally (i.p) or with its vehicle (PBS) in combination either with CTO 342 mg/kg or with CTO 513 mg/kg or with their vehicle (80% PEG-100). Comparison of the median tumor weight was used as index of the antitumor efficacy of the compounds.

LAMA84R cells release exosomes

LAMA84R cells release exosomes into the culture medium during a 24h period as demonstrated by their characteristic shape and diameter (70 nm ± 10), by the presence of Hsc70 and CD63 proteins and by the presence of acetylcholinesterase, a characteristic enzyme localized in exosomes (figure3).

Figure 3



LAMA84R exosome characterization through specific marker: (a) scanning electron microscopy of exosomes released in the culture medium during a 24h period: (b) western blot analysis for Hsc70 and CD63 in cell and exosome protein lysate; (c) acetylcolinesterase assay: acetylcolinesterase, a characteristic enzyme localized in exosomes, is associated with exosome fraction.

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 4a shows that LAMA84R-derived exosomes added to HUVEC monolayer caused, compared to control, a dose-dependent increase of VCAM1, ICAM1, and IL8 mRNA expression. 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 4b shows that a comparable effect to the one of LAMA84R exosomes on the mRNA induction was observed when endothelial cells were incubated with 10 ng/ml of recombinant IL8; adding 10μM CTO or neutralizing anti-IL8 antibody revert the increase in VCAM1, ICAM1 and IL8 mRNA expression.

FACS analysis confirmed that incubation of HUVEC with LAMA84R exosomes resulted in detection of VCAM1 and ICAM1 on the surface of HUVEC, which was blunted by treatment of cells with CTO (figure 4c). ELISA assay demonstrated the increasing release of IL8 into HUVEC conditioned medium after treatment with CML exosomes (figure 4d).

Figure 4a

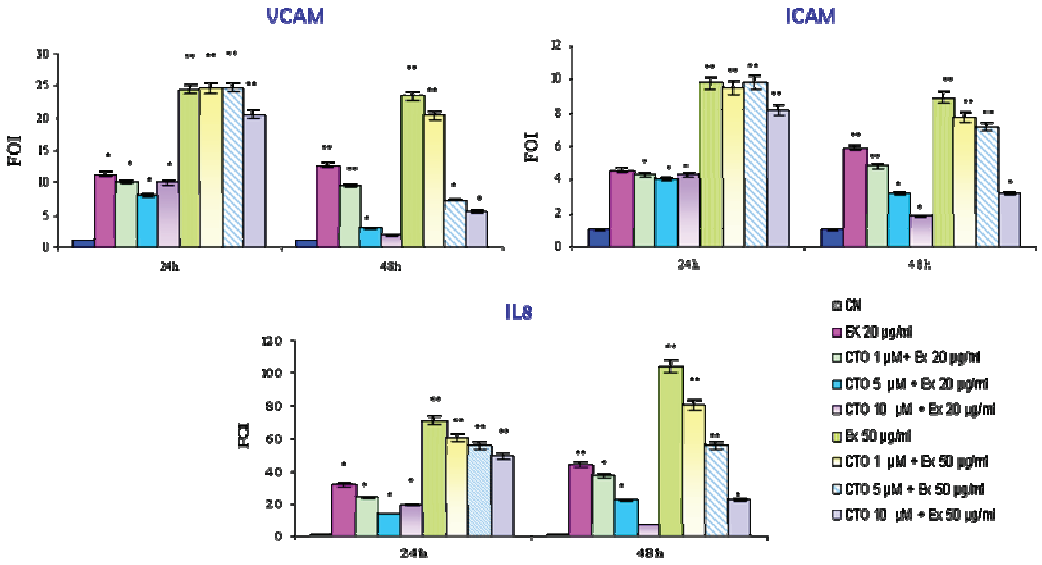


Figure 4b

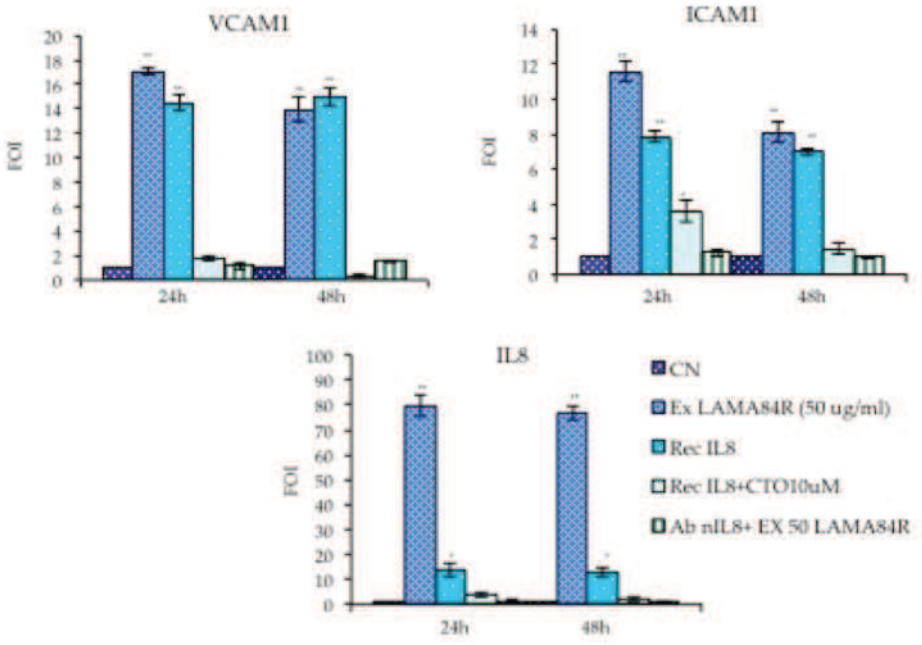


Figure 4c

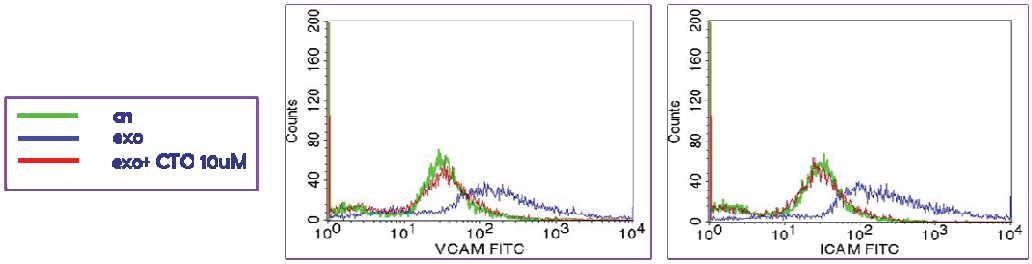
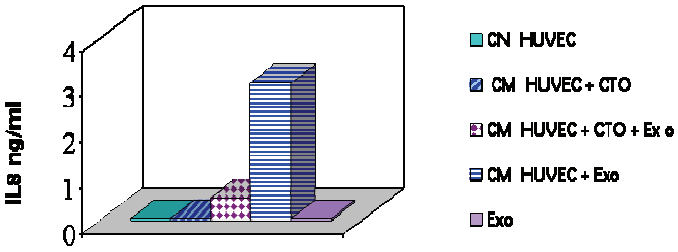


Figure 4d



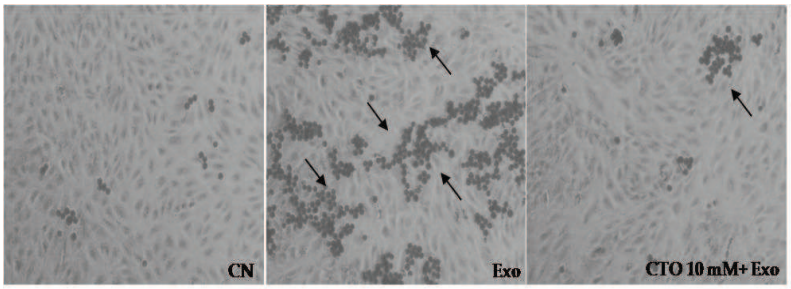
CTO inhibits the exosome-stimulated increase of cell-cell adhesion molecules and IL8 expression in Huvec: (a) 50µg of LAMA84R-derived exosomes added to Huvec monolayer for 6h increase the mRNA expression of VCAM1, ICAM1 and IL8 compared to control; pre-treatment with increasing doses of CTO is able to revert the effects of CML exosomes. *p ≤ 0.05, **p ≤ 0.01(b) VCAM1, ICAM1 and IL8 mRNA expression in Huvec treated for 6 h either with low serum medium (CN), or with 50µg/ml exosomes, or with 10ng/ml of recombinant IL8 (RecIL8) with or without CTO 10µM, or with 50 µg/ml exosomes plus 10µg/ml of a neutralizing anti-IL8 antibody (N ab IL8). *p ≤ 0.05, **p ≤ 0.01 (c) flow cytometry analysis to determine the expression of VACAM1 and ICAM1 on Huvec cell surface after treatment with 50µg of LAMA84R: exosomes 50 µg/ml of LAMA84R exosomes (blue line) compared to Huvec treated with 50 µg/ml of LAMA84R exosomes plus 10 µM CTO (red) and untreated Huvec, as control (green). (d) ELISA for IL8 release by Huvec, obtained after 6h of stimulation with: low serum medium (CM Huvec), CM Huvec plus 10 µM CTO, CM Huvec plus 10 µM CTO plus 50 µg/ml of exosomes and CM Huvec plus 50 µg/ml of exosomes.

CTO inhibits the adhesion of CML cells to HUVEC monolayer

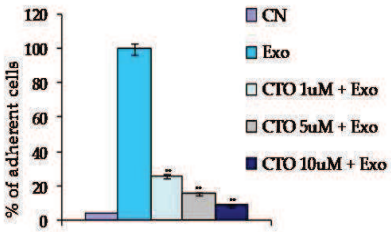
While leukaemia progresses, cancer cells adhere to endothelial cells in order to infiltrate and colonize extramedullary sites. Figure 5 shows that the increase of adhesion of LAMA84R cells (arrows) to HUVEC monolayer was inhibited by pre-treatment of EC with CTO.

Figure 5

a



b



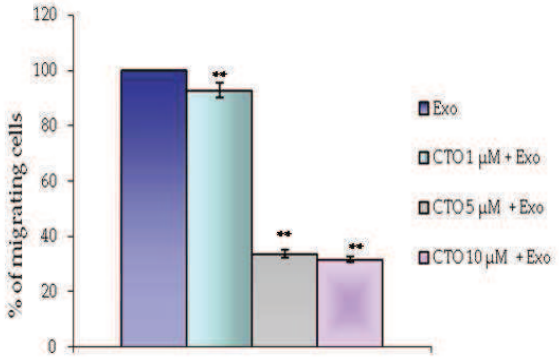
CTO inhibits the adhesion of CML cells to Huvec monolayer: (a) the increase of adhesion of LAMA84R cells (arrow) after 6h of stimulation with 50 µg/ml of exosomes is inhibited after pre-treatment of 24h with 10 µM CTO. (b) Treatment of Huvec monolayer with 50 µg/ml of LAMA84R exosomes induce an increase of CML cell adhesion to endothelial cells; CTO inhibits CML cells adhesion in a dose- and time-dependent manner. **p ≤ 0.01.

Effects of CTO on exosomes-stimulated migration of endothelial cells

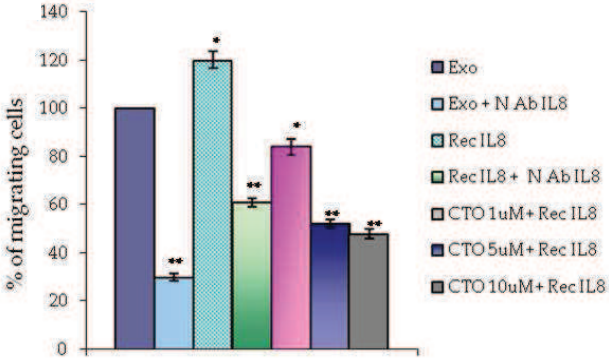
We examined the effect of CTO treatment on exosome-stimulated EC motility by Boyden chamber assay. Our results showed that adding a range of concentrations of CTO (1–10 µM) to the upper well of the chamber caused, after 6h, a dose-dependent inhibition of LAMA84R exosome-stimulated endothelial cell migration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

Figure 6

a



b

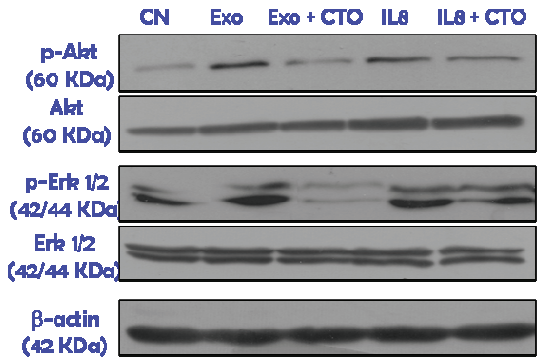


CTO inhibits exosome-stimulated migration of endothelial cells: (a) motility of endothelial cells with or without increasing doses of CTO (1-10 µM) was evaluated using as chemoattractant 50 µg/ml of exosomes (Exo). (b) 50 µg/ml of exosomes (Exo) with or without 10 µg/ml of neutralizing anti-IL8 antibody (N AbIL8), 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. *p ≤ 0.05 **p ≤ 0.01.

Effects of CTO on Akt and Erk 1/2 phosphorylation in exosomes-stimulated HUVEC

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, and chemotaxis in endothelial cells and if CTO was able to modulate these pathways. Figure 7 shows that over night addition of LAMA84R exosomes or of 10 ng/ml IL8 to endothelial cells trigger the phosphorylation of both the signalling molecules, 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 CTO.

Figure 7



CTO inhibits Akt and Erk1/2 phosphorylation: overnight addition of 50 µg/ml LAMA84R exosomes or 10 ng/ml IL8 to endothelial cells triggers phosphorylation of Akt and Erk ½; treatment of Huvec with 10 uM CTO reduces these effects.

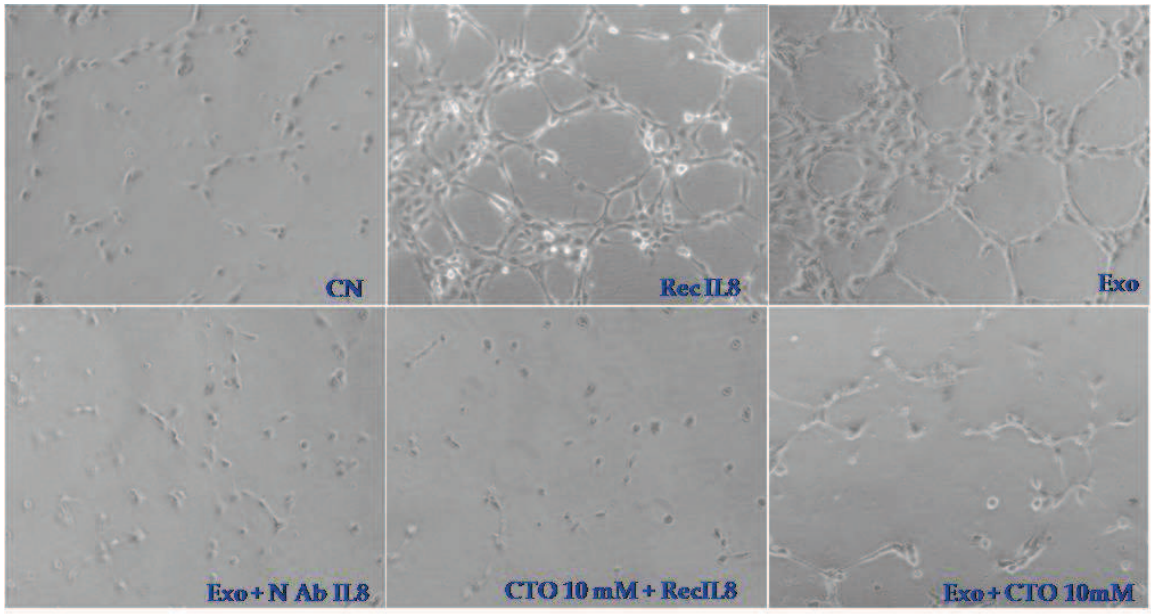
CTO treatment inhibits *in vitro* and *in vivo* exosome-mediated angiogenesis

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 cells; the ability of endothelial cells to form tubes is a key step 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 1a). Quantitative analysis of

tubular connections showed a more than three fold increase in cellular projections interconnecting HUVEC after treatment with LAMA84R exosomes, or recombinant IL8 compared to control. Addition of CTO or neutralizing anti-IL8 antibody caused a dramatic inhibition of exosome-mediated effects on tube formation (figure 8b).

Figure 8

a



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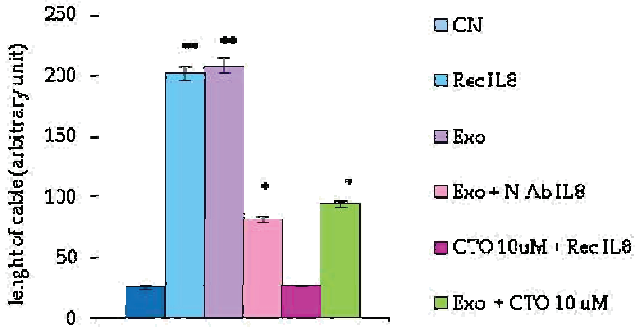


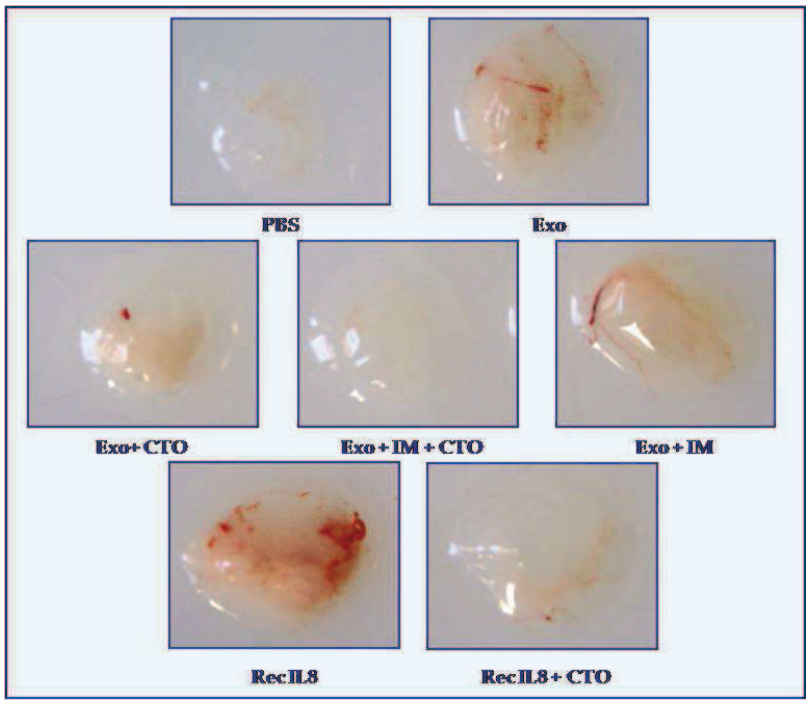
CTO inhibits the effects of LAMA84R exosomes on *in vitro* angiogenesis: (a) Phase contrast micrographs showing the effects of LAMA84R exosomes and CTO (10μM) treatment on endothelial network formation (matrigel assay). Addition to HUVEC of 10 ng/ml of recombinant IL8 (Rec IL8) or 50 μg/ml of LAMA84R exosomes (Exo) induces the formation of capillary-like structures. CTO inhibits the effects of recombinant IL8 (CTO + Rec IL8) or exosomes (Exo + CTO) on tube formation by HUVEC; neutralizing anti IL8 antibody reverts the effects of exosomes (Exo + N Ab IL8). (b) Histograms showing the quantitative analysis of the cables length by Image J software.**p ≤ 0.05,**p ≤ 0.01.

Then we evaluated *in vivo* the antiangiogenic effect of CTO on a mouse Matrigel plug model. As shown in figure 9a, plugs containing LAMA84R-exosomes (Exo) or recombinant IL8 (Rec IL8) are more vascularised in mice treated with PBS alone than in implants removed from CTO-treated mice. This suggests that CTO is able to

inhibit exosome-stimulated vascularisation over a 4 weeks period. Drabkin's assay was used to measure haemoglobin content in the plugs as a marker of vascularity (figure 9b).

Figure 9

a



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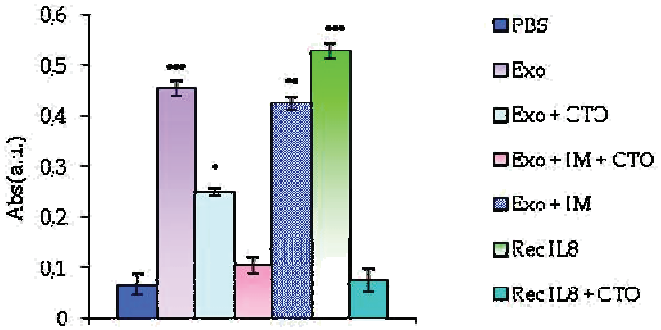


CTO inhibits the effects of LAMA84R exosomes on *in vivo* angiogenesis:(a) NOD/SCID mice treated with CTO show a decreased exosome-stimulated angiogenesis in matrigel plug assay; matrigel plugs implanted subcutaneously in NOD/SCID mice and containing: PBS as negative control, 100 µg of LAMA84R exosomes (Exo) and 50 ng of recombinant IL8 (Rec IL8). The mice were treated with vehicles (PBS and/or PEG) or drugs (CTO and/or imatinib) and plugs were removed after 4 weeks. (b) Evaluation of haemoglobin concentration in the matrigel plugs by Drabkin's assay. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$

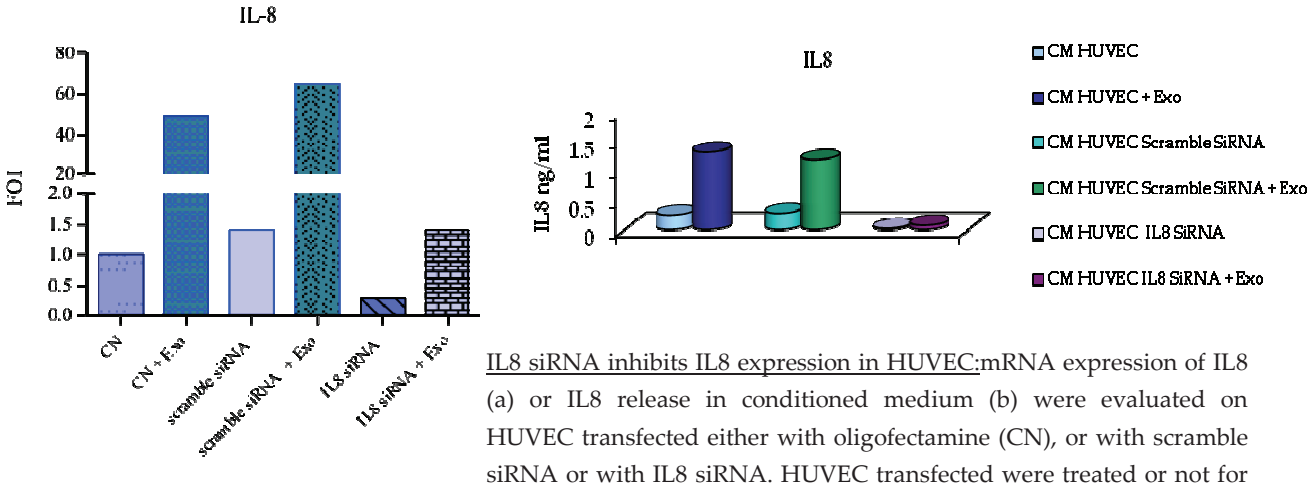
IL8 siRNA inhibits IL8 mRNA expression and cytokine release from HUVEC

To better understand the role of IL8, released after exosomes stimulation, in mediating the crosstalk between endothelial and CML cells, we silenced IL8 expression in HUVEC.

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. After HUVEC transfection and 6 h of incubation with 50 µg/ml of exosomes, HUVEC conditioned medium was collected. 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 endothelial cells transfected with scramble siRNA (figure 10 a and b).

Figure 10

a b



IL8 siRNA inhibits IL8 expression in HUVEC:mRNA expression of IL8 (a) or IL8 release in conditioned medium (b) were evaluated on HUVEC transfected either with oligofectamine (CN), or with scramble siRNA or with IL8 siRNA. HUVEC transfected were treated or not for 6h with 50 µg of LAMA84R exosomes (Exo).

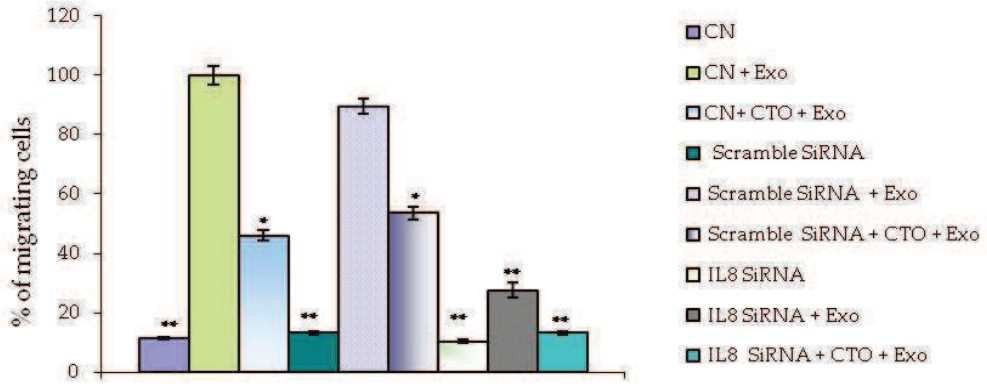
IL8 siRNA inhibits the effects of LAMA84R exosomes on migration and tube formation capabilities of HUVEC

We have previously reported that addition of exosomes to the bottom wells of Boyden chamber increases the migration of HUVEC. The same result is obtained when we trasfected HUVEC with scramble siRNA, while concomitant CTO treatment reverts this effect. Our results show that exosome-induced HUVEC migration decreased when IL8 was silenced (figure 11a). Moreover, matrigel assay

shows that LAMA84R exosomes did not induce the tube formation on IL8-silenced endothelial cells (figure 11b).

Figure 11

a



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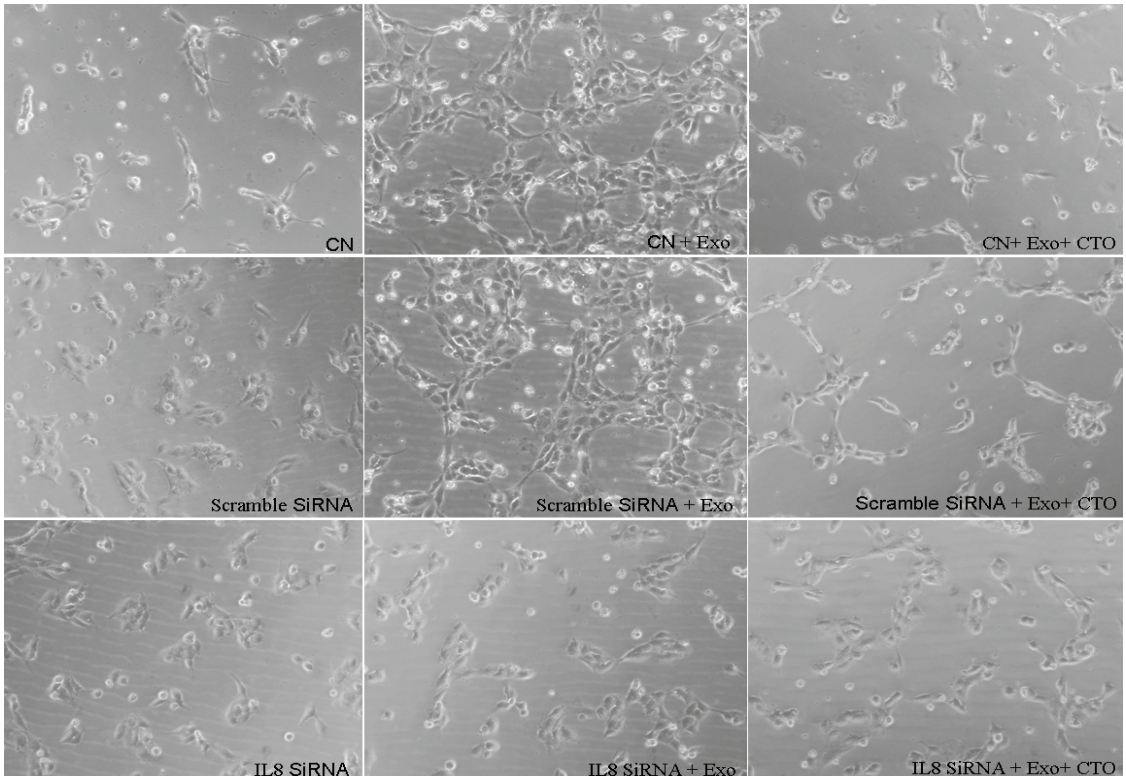


IL8 silencing inhibits exosome-stimulated migration of LAMA84R and *in vitro* angiogenesis: (a) IL8 silencing in HUVEC reduce the migration capability of cells; LAMA84R exosomes partially restore the migration of HUVEC and co-treatment with exosome-CTO reverts these effect. *$p \leq 0.05$; **$p \leq 0.01$. (b) 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 endothelial cells were silenced for IL8 mRNA expression with short interfering RNAs.

Conclusions

Whereas CML can be effectively treated, during chronic phase, with tyrosine kinase inhibitors (TKIs) such as imatinib, the acquisition of imatinib resistance causes disease progression (blast crisis) that can be fatal within months. New approaches reling on the use of compounds targeting either patways downstream of BCR-ABL activation or events that contribute or modulate leukaemia progression are necessary. In the present study we tested the anti-tumour effects of carboxyamidotriazole-orotate *in vitro* and in a xenograft model of imatinib-resistant human CML. 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.Furthermore, the inhibitory effects of CTO against CML cells was validated in a CML xenograft model. Recently, there are increasing data showing that angiogenesis plays an important role in the development and progression of chronic myelogenous leukaemia. 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 led us to investigate if CTO could target both tumour cells and the tumour 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. 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 generate a paracrine machinery between hematopoietic malignant cells and newly generated endothelium. In this tumour microenvironment, CTO could inhibit the angiogenic process through blocking the exosome-mediated crosstalk, thus causing the interruption of a reciprocal stimulatory loop between leukaemic and endothelial cells.

Chapter 2

Exosomes mediate paracrine interplay between Chronic Myelogenous Leukaemia and stromal cells: a role for interleukin 8

Introduction

In the last years the understanding of tumour cell–stroma interactions has increased and pathways involved have been better characterized. Mesenchymal stromal cells (MSC), the dominant cell population in BM, is known to regulate normal hematopoiesis by providing adhesion sites and secreted growth factors; it has been proposed that altered crosstalk between MSC and CML cells may affect leukaemia cell survival and resistance to chemotherapy[113; 114]. As a consequence, new therapeutical approaches interfering either with the recruitment of stromal cells into the tumour microenvironment, with tumour cell–stromal interaction, or with specific pathways activated by the tumour microenvironment, have been tested[120]. Since exosomes are released by CML cells within tumour microenvironment, stromal cells could "interpret" exosome variations to mutually balance their behaviour. Data concerning the effect of exosomes in CML-stromal cell interactions are, to my knowledge, up to now missing. The proposal of the second part of my PhD project is to better investigate if the release of exosomes from CML cells can modulate the tumour microenvironment (the secretion of both soluble and insoluble molecules) through paracrine interplay among leukaemia cells and stromal cells. IL-8 is widely expressed in tumour, stroma, and endothelial cells [52; 53]. Its autocrine and paracrine function has been shown to play an important role in angiogenesis, tumour growth, and metastasis[54; 55]. The expression of IL-8 receptors on cancer cells, endothelial cells, neutrophils, and tumour-associated macrophages suggests that the secretion of IL-8 from cancer cells may have a profound effect on the tumour microenvironment.

Our hypothesis is that LAMA84-derived exosomes are able of activating bone marrow stromal cells (HS5), which in turn release interleukin 8 acting as an *in vitro* and *in vivo* pro survival actor for chronic myelogenous leukaemia cells.

Matherials and Methods

Ethic Statement

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

Cell lines and cell culture

Chronic myelogenous leukaemia cell line,LAMA84, was obtained by DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 medium (Euroclone, UK). Bone marrow-derived stromal cell line, HS5, was obtained by ATCC (Manassas, VA, USA) and cultured in DMEM high glucose (Euroclone, UK). Both cell lines were cultured in media supplemented with 10% fetal bovine serum (Euroclone, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Euroclone, UK).

Exosome preparation

LAMA84-derived exosomes were isolated as previously described [58]. Briefly, LAMA84 were grown for 24h in RPMI supplemented with 10% exosomes-depleted FBS. Conditioned medium was collected and centrifugatedat 300 g x 5 min, 3000 g x 15 min, 10000 gx 30 min. Conditioned medium was filtered (0,22μm filter) and was ultracentrifuged in a fixed angle rotor at 100000 g x 1h45min. Exosome pellet was washed and then resuspended in PBS. Exosome protein content was determined by the Bradford assay (Pierce, Rockford, IL, USA).

Adhesion assay

Adhesion assays were performed as previously described [58]. Briefly, HS5 monolayer was pretreated for 48 or 72h with 50 μg/ml of LAMA84-derived exosomes or with exosome-deprived medium, as negative control, in a 24-well plate. After treatment, stromal monolayer was washed with PBS and CML cells ($0.5x10^6$ cells/well/ml of RPMI 0% FBS) were left to adhere for 3.5h at 37°C. In another set of

experiments, LAMA84 cells were stimulated for 48h with 5 or 10 ng/ml of recombinant IL8 (r-IL8) (R&D Systems, MN, USA) in presence or not of 1000nM of CXCR1 and CXCR2 inhibitors (SB225002) (Cayman Chemical, Michigan, USA). Adhered cells were stained with haematoxylin eosin, each test group was assayed in triplicate; 15 high power (400X) fields were counted for each condition.

Migration assay

Migration assays were performed in transwell chemotaxis chambers with 3 μm pore filters (NeuroProbe, Cabin John, MD, USA). Specifically, LAMA84 (0.5x106cells/ well) were resuspended in 500 μl of RPMI 1% FBS with or without SB225002 (600-1000-1200nM), and exposed, as chemoattractant, to conditioned medium of HS5 previously treated with LAMA84-exosomes (50 μg/ml) for 6, 24, 48 or 72h. After 18h at 37°C, filters were removed, fixed in methanol and 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 400Xmagnification.

RNA extraction and realtime PCR

HS5 were grown in 12-well plates and pretreated or not with 50 μg/ml of LAMA-84 exosomes for 24, 48 and 72h. RNA was extracted using the commercially available illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA). RT-QPCR was performed in 48-well plates using the using the Step-One Real-Time PCR system (Applied Biosystem, Foster City, CA, USA).Taqman gene expression assays for GAPDH (Hs99999905_m1) control and for IL-8(Hs00174103_m1) were obtained from Applied Biosystems (Foster City, CA, USA). All experiments were performed in duplicates for each data point. Relative changes in gene expression between controls and treated samples were determined using the ΔΔCt method.

Elisa

HS5 conditioned medium was collected from cells pretreated or not with 50 μg/ml of LAMA84-exosomes for 6, 24, 48, 72h. Conditioned medium aliquots were centrifuged to remove cellular debris and then IL8 protein concentration was quantified using the ELISA kit (R&D Systems, MN, USA), according to manufacturer's protocol.

RT PCR

Conventional PCRwas performed using cDNA from LAMA84, obtained as described above, together with thePCR master mix using respective primers. Primer sequences for CXCR1 were 5'TCCTGGGAAATGACACAGCA'3 and 5'AAGCCAAAGGTGTGAGGCAG'3 and for CXCR2 were 5'GGGCAACAATACAGCAAACT'3 and 5'GCACTTAGGCAGGAGGTCTT'3. The reaction conditions were: 95°C 5 min, 94°C for 30 s, 58°C for30 s, 72°C for 30 s for 35 cycles and a final extension phase at 72°C for5min. The PCR products were separated on a 3% agarose gel and stained with 5μl ethidium bromide prior to examination under UV light and photographs taken.

Western blot

LAMA84 cells were treated or not for 2, 10 and 30 minutes with 10 ng/ml of r-IL8 in presence or not of 1000 nM of SB225002. Total protein cell lysate were obtained and analyzed by SDS-PAGE follone by Western blotting as previously described [25]. Antibodies used in the experiments were: Akt, phospho Akt, ErK 1/2, phospho Erk 1/2, β-actin (all from Cell Signalling Technology, MA, USA); CXCR1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Colony formation assay

In order to test the effect of IL8 on the growth of CML cells in methylcellulose, cells were cultured at low cell density using semisolid medium. LAMA84 were plated in 6-well at low density(2000 cell/ml/well) in Iscove's methylcellulose medium (Methocult H4230, Stem Cell Technologies, Vancouver, Canada) containing or not r-

IL8 (2, 5, 10 ng/ml) and in presence or not of 300- 600 nM of SB225002.After 14 days of culture, LAMA84 colonies were evaluate by phase-contrast microscopy and photographed. The area of twenty LAMA84 colonies per condition was measured with the IMAGE-J software (http://rsbweb.nih.gov/ij/).

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. Mice were randomly assigned to four groups of seven.Each mouse was inoculated subcutaneously (sc) in the right flank with viable single cells (2×10^7) suspended in 0.2 ml of PBS additioned with r-IL8 (200ng/mouse), SB225002 (1000nm), PBS (vehicle) or r-IL8 plus SB225002. Treatments were repeated twice a week, in the tumour site.The day of injection and beginning of treatment was considered as day 0. Tumour xenografts were measured and mice were weighed twice a week.Tumour volume was determined by calliper by using the following formula: L X W2/2 = mm3 where L and W are the longest and shortest perpendicular measurements in millimeters, respectively. The same formula was used to calculate tumour weights assuming that 1 mm3 = 1 mg. Animals were euthanized at day 50 after inoculation and tumours were prelevated.

Statistics

Data were expressed as means +/- SEMs of three independent experiments. Statistical analyses were performed by using a paired samples t test. Differences were considered to be significant when p values were smaller than 0.05.

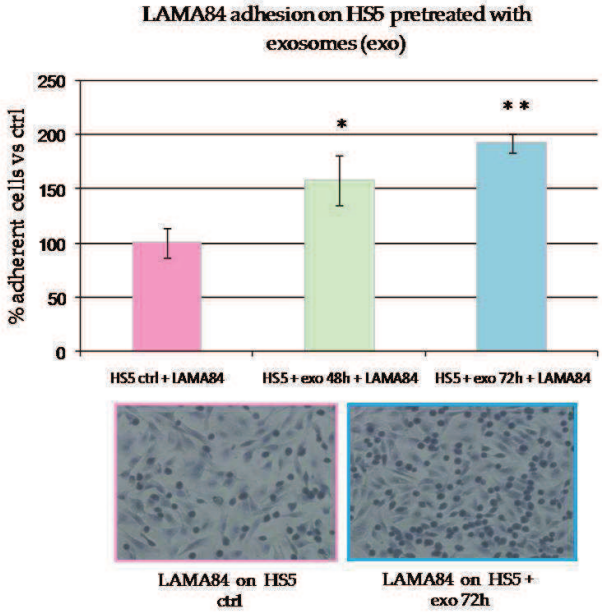
Results

LAMA84-exosomes promote the adhesion and the migration of CML cells

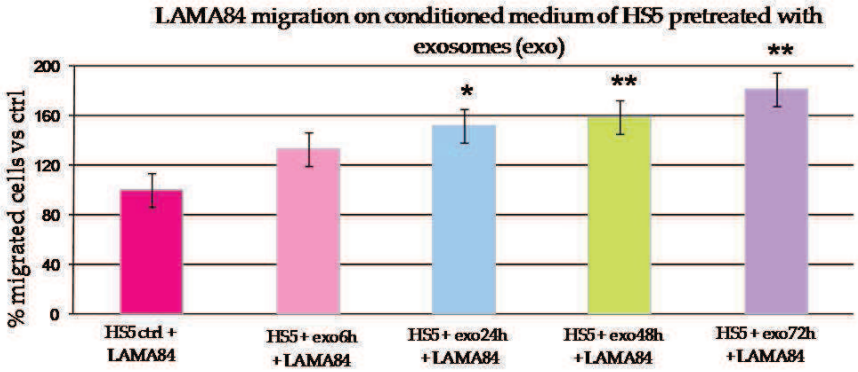
Normal haematopoietic cell regulation involves interaction between marrow stromal cells and haematopoietic progenitor cells which may be facilitated by both soluble factors and direct cell-cell interactions. It is well described that an altered interaction between CML cells and bone marrow stromal cells occurs in the neoplastic disease. To evaluate the potential to which CML exosomes are able to modify the surrounding microenvironments, we first investigated whether the release of CML-derived exosomes could affect the adhesion of leukaemic cells to stromal cells and their migratory capability towards the conditioned medium of HS5, a human stromal cell line. Figure 1a shows that after 48h or 72h of pre-treatment of HS5 with 50ug/ml of LAMA84-derived exosomes, there is an increase of adhesion of LAMA84 to stromal monolayer. Moreover, we collected the conditioned medium of HS5 stimulated for 6-24-48-72h with LAMA84-exosomes and we performed a motility assay using transwell inserts. We used as chemoattractant the conditioned medium collected after the different time points. As shown in figure 1b, the conditioned medium of HS5 pre-treated with exosomes favours the migration of LAMA84. These results demonstrate that LAMA84-derived exosomes promote the adhesion and migration of leukaemic cells towards stromal cells, suggesting that exosomes may favour the retention of CML cells within the bone marrow, thus providing a protective niche for leukaemic cells. Moreover, our data suggest that the ability of LAMA84 exosomes to induce adhesion and migration of CML cells to stromal cells depends on both soluble factors and direct cell-cell interactions.

Figure 1

a



b



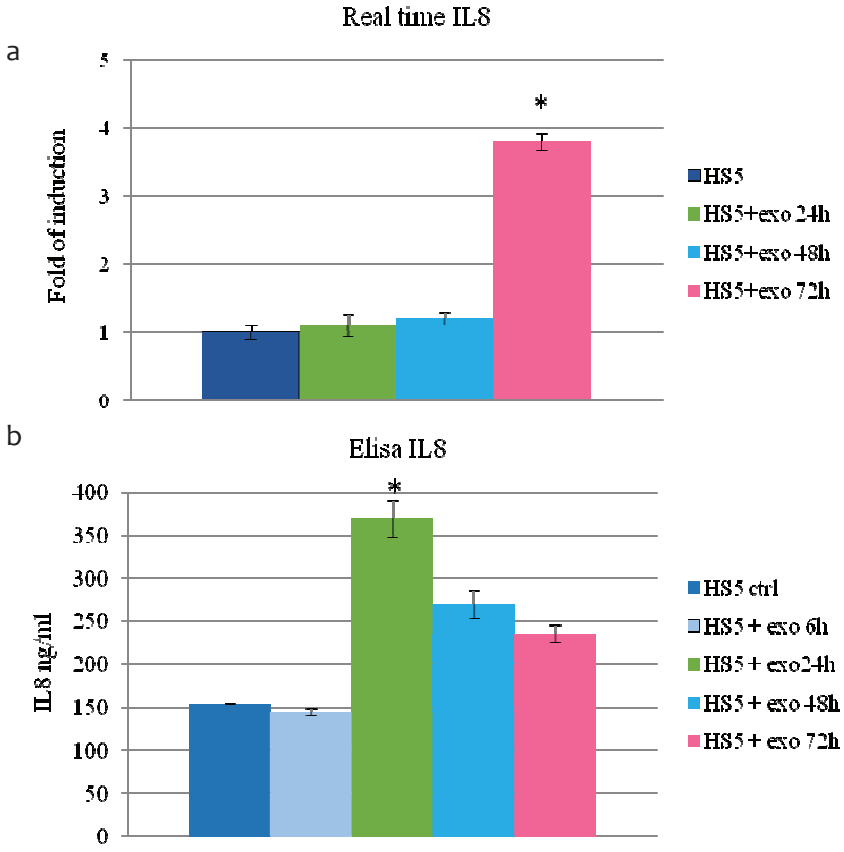
LAMA84-exosomes promote LAMA84 adhesion and migration: (a) 48 and 72h of pre-treatment of HS5 monolayer with 50 µg/ml of LAMA84-exosomes induces an increase of CML cell adhesion to stromal cells (* $p < 0.001$; ** $p < 0.0001$). (b) Migration of LAMA84 was evaluated on transwell using, as chemoattractant, the conditioned medium of HS5 pre-treated for 6,24,48,72h with 50 µg/ml of LAMA84-exosomes (*$p < 0.05$;**$p < 0.01$).

LAMA84-exosomes induce the expression of IL8 in HS5

We postulate, and previous data suggest us that soluble factor secreted by HS5 stromal cells after pre-treatment with exosomes could influence the migration and attachment of LAMA84 within the bone marrow microenvironment. We then investigate which components of the conditioned medium of HS5 stimulated with LAMA84-exosomes could cause these effects on LAMA84. Recent data from our laboratory suggest that the pro-angiogenic chemokine, IL-8, may have a role within

the bone marrow microenvironment. The role of IL-8 in the progression of solid tumours is well described, but its role in the biology of CML is yet to be elucidated. Figure 2a shows that after 72h of pre-treatment of HS5 with LAMA84-exosomes, there is an increase of the expression of the mRNA of IL8; moreover in figure 2b is shown the increase of the protein release in the conditioned medium of HS5 pre-treated with exosomes.

Figure 2



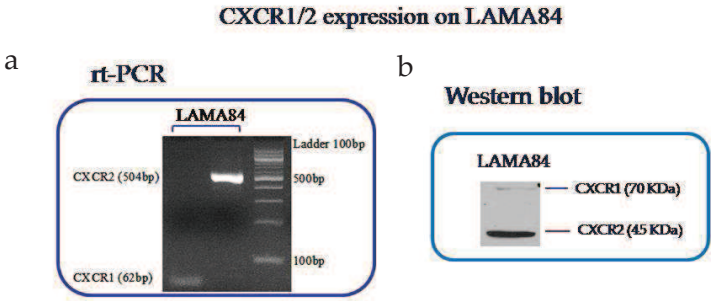
IL8 expression: (a) treatment of HS5 monolayer with 50 μg/ml of LAMA84-derived exosomes for 24, 48, 72h increases the mRNA expression of IL-8.(b) ELISA assay shows that treatment of HS5 monolayer with 50 μg/ml of LAMA84-derived exosomes for 6, 24, 48, 72h induces the release of IL-8 in the conditioned medium.(*$p<0.05$).

IL-8 promotes CML cells adhesion and migration

Interleukin 8 is a well known promigratory and proangiogenic stimulus in many cancers; little is known on its role in CML. To confirm and to better understand the role of IL-8 in mediating the crosstalk between CML and stromal cells, we blocked both IL8 receptors, CXCR1 and CXCR2, using a commercially available inhibitor (SB

225002). We first evaluated and confirmed the expression of both receptors in LAMA84 (figure 3).

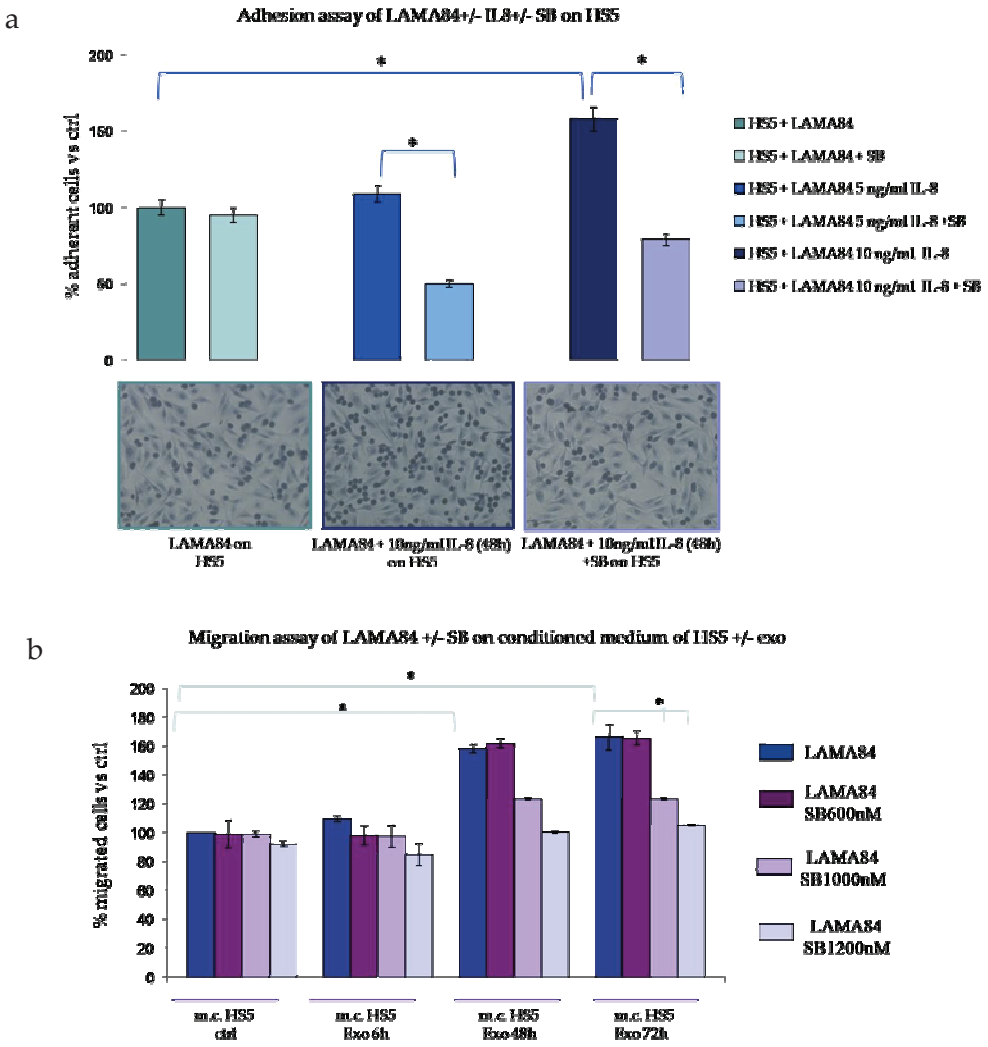
Figure 3



CXCR1 and CXCR2 expression in LAMA84 has been validate through rt-PCR (a) and western blot (b).

Adhesion of leukaemia cells to BM stroma is known to enhance cell survival. We performed an adhesion assay of LAMA84, pre-treated or not with IL-8 and CXCR1-2 inhibitors, on stromal monolayer. As we have observed after pre-treatment of HS5 with exosomes, the treatment of LAMA84 with 5 or 10 ng/ml of recombinant IL8 (r-IL8) for 48h, promotes the adhesion of LAMA84 on stromal cells in a dose dependent manner; moreover the co-treatment of LAMA84 with r-IL8 and CXCR1-2 inhibitors (SB), reverts the effects previously observed (figure 4a). Furthermore, the treatment of LAMA84 with SB decreases the ability of LAMA84 to migrate towards the conditioned medium of HS5 pre-treated with CML-derived exosomes as we show in figure 1b. These data suggest that IL8 released in the conditioned medium of HS5 pre-treated with CML-exosomes is able to modulate LAMA84 behaviour.

Figure 4



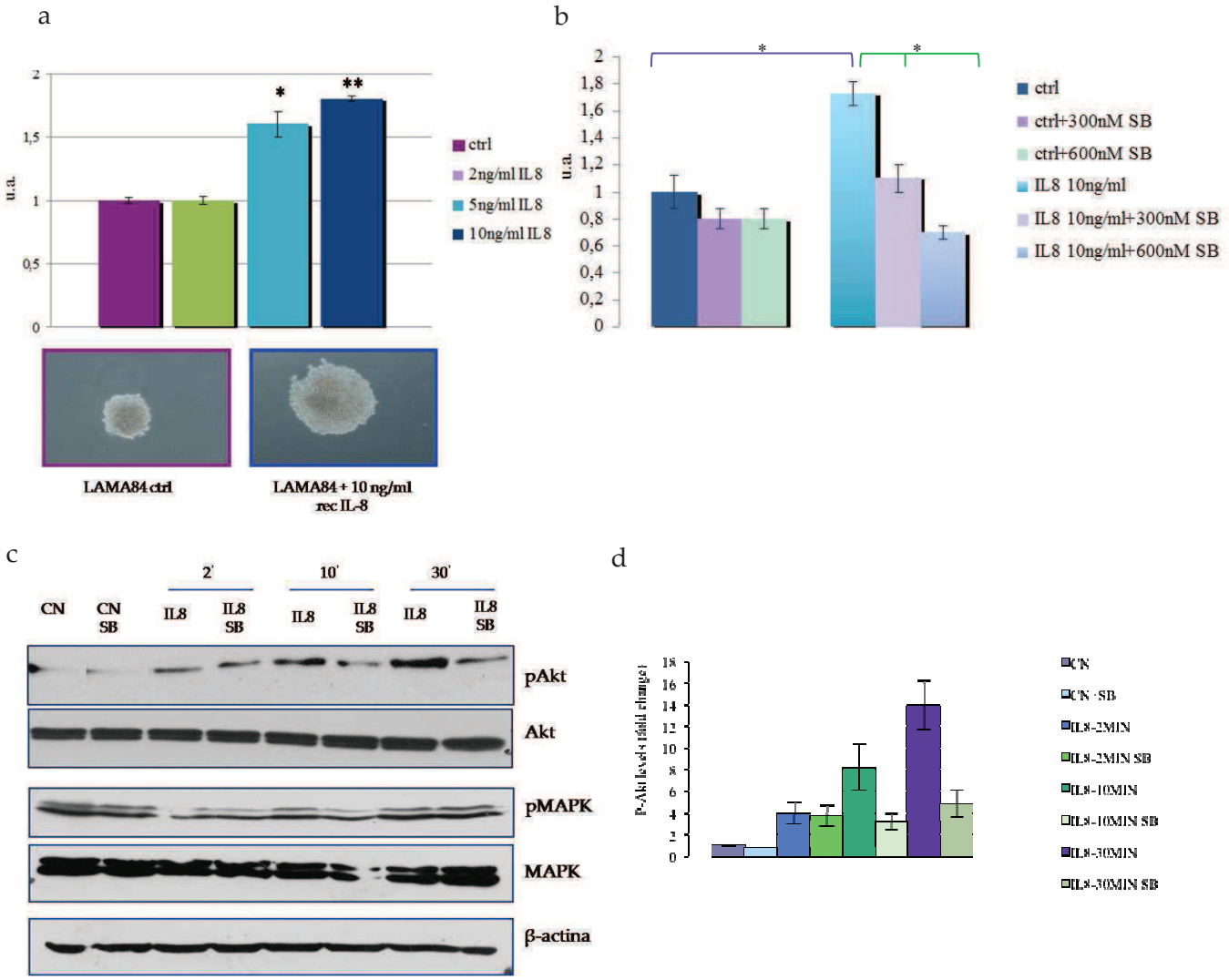
Treatment of LAMA84 with CXCR1-2 inhibitors blocked LAMA84 adhesion and migration: (a)48h of pre-treatment of LAMA84 with 5 and 10 ng/ml of rIL-8 induces an increase of CML cell adhesion to stromal cells. 48h of co-treatment of LAMA84 with 5 and 10 ng/ml of r-IL8 and 1000 nM of IL8 receptors inhibitor (SB 225002), reverts these effects (* $p < 0.001$). (b) migration of LAMA84 was evaluated on transwell inserts using as chemoattractant the conditioned medium of HS5 pre-treated for 6, 48, 72 h with 50 ug/ml of LAMA84-exos0mes. The addition of 600, 1000, 1200 nM of SB, decreases LAMA84 migration. (*$p< 0.01$)

IL8 promotes CML cells survival *in vitro*

We further evaluated the role of IL8 in the survival of leukaemic cells. Colony formation assay in methylcellulose shows that LAMA84, treated with escalating doses of r-IL8 (2, 5, 10 ng/ml), are able to form colonies in methylcellulose with a greater area than control cells (figure 5a). The addition of 300 or 600 nM of SB significantly reduced the size of the IL-stimulated colonies areas without affecting the area of control colonies (figure 5b). We then investigated the signal transduction pathways activated in LAMA84 after IL8 treatment. As shown in figure 3c, western

blot analysis show that after 2, 10 and 30 minutes of r-IL8 treatment there is a time-dependent increase of the Akt phosphorylation reaching a 15 fold increase after 30 minutes of 10 ng/ml of r-IL8 treatment (figure 5d), thus suggesting a role of IL8 in promoting the survival of leukaemic cells. In our model IL8 doesn't trigger the phosphorylation of MAPK, suggesting that this chemokine doesn't affect CML proliferation; these data have been supported by proliferation assays (data not shown).

Figure 5



IL8 promotes the survival of LAM84:(a) the addition of 2, 5 and 10 ng/ml of rIL-8 increases the area of LAMA84 colony in methylcellulose with respect to control (*p<0.05; **p<0.01). (b) addition of 300 or 600 nM of SB reverts these effects (*p<0.01). (c) wb analysis show that 2, 10, 30 minutes treatment of CML cells with 10 ng/ml of IL8 triggers phosphorylation of Akt but not phosphorylation of MAPK; treatment of LAMA84 with 1000 nM SB reduces these effects. Blots were then stripped and subsequently reprobed with antibody against b-actin to ensure equal loading. (d) histogram representing densitometric analysis of P-Akt levels.

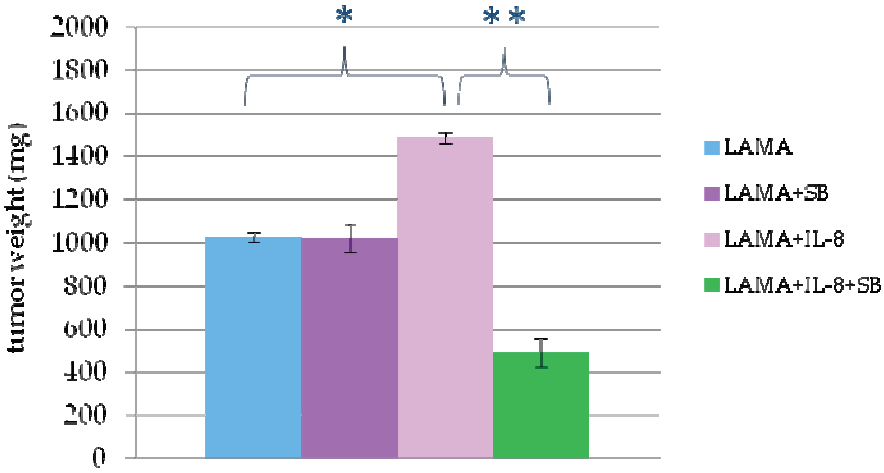
In vivo effect of IL8 on tumour growth

On the basis of the *in vitro* effects of IL8 on LAMA84 malignant behaviour, we examined the *in vivo* role of the cytokine using a xenograft CML tumour model. For this purpose, LAMA84 cells were inoculated subcutaneously in NOD/SCID mice and subsequently treated locally twice a week with IL8, vehicle (PBS), SB or IL8 plus SB (figure 6a). The histogram in figure 6b represents the mean of tumour weight for each group. Mice treated with IL8 develop largers tumours than control groups (PBS or SB); co-treatment with SB results in a slower tumour growth compared with mice treated with IL8 alone.

Figure 6

a

b



IL8 promotes tumour growth *in vivo*:(a) schematic representation of mice treatment: NOD/SCID mice was inoculated subcutaneously with viable LAMA84 cells ($2x10^7$) suspended in 200ul of PBS. Mice were randomly assigned to 4 groups of 7 mice and were treated with IL8 (200ng/mouse), PBS (vehicle), SB (1000 nM) and co-treated with IL8 and SB. All treatments were administrated in the tumour site, twice a week. Tumour xenografts were measured twice a week and tumour weight was calculated. All mice were euthanized 50 days after cells injection. (b) Histogram represents the mean of tumour weight for each group +/- SD. *p<0.05; **p<0.01.

Conclusions

The tumour microenvironment plays a pivotal role in the survival and persistence of tumour cells *in vivo*. It is believed the microenvironment contributes to numerous cellular mechanisms conducive to the survival of malignancies, including CML. Recently, emphasis has been placed on identifying and understanding those mechanisms.

Adhesion of leukaemic cells to BM stroma represents a mechanism for retention of progenitor cells in contact with anti-apoptotic signals derived from the marrow microenvironment. A better understanding of the molecular mechanisms and of molecules that drive such processes could lead to the identification of new targets.

Exosomes have been recently considered as new vehicles of these molecules into the tumour microenvironment and, for this reason, data is beginning to accumulate on their role as new actors in the crosstalk between cancer and normal cells in the tumour microenvironment. Exosomes are not simply vehicles of molecules targeted into recipient cells, but also induce phenotypic changes in neighboring cells by activating specific cell signaling pathways, leading to cancer progression.

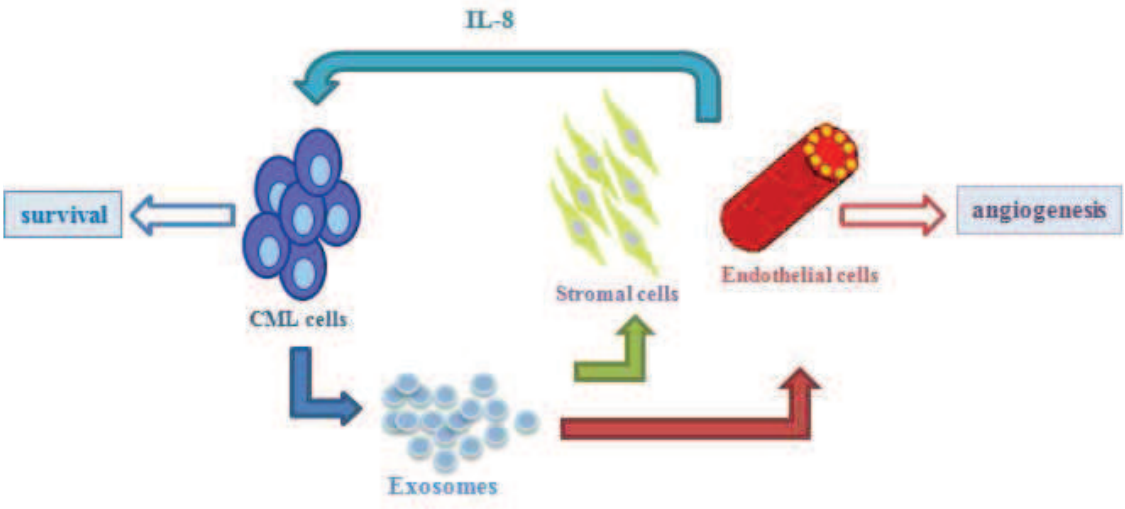
In this work we show that CML-derived exosomes stimulate bone marrow stromal cells to secrete IL8, which is significantly increased in the leukaemic bone marrow. Furthermore we show that IL8 enhances CML cells adhesion stromal cells and migratory abilities; on the other side, IL8, produced after exosome stimulation of bone marrow stromal cells, is able to sustain the survival of Chronic Myelogenous Leukaemia cells.

The release of exosomes by CML cells and the consequent increase of IL8 in the microenvironment, may represent a molecular mechanism by which leukaemia cells contributes to the establishment of a malignant BM microenvironment.

Concluding remarks

Concluding remarks

It is now recognized that tumour microenvironment undergoes extensive changes during tumour development and influences the evolution and progression of cancer. While current anti-CML therapies have been largely ''leukaemia cell-centered,'' emerging evidence highlights the importance of the BM stroma for the growth, survival and resistance to tyrosine kinase inhibitor of leukaemia cells. A greater understanding of molecules involved in the modulation of the bidirectional crosstalk within the tumour microenvironment may provide alternative opportunities to improve the efficacy of anti-CML treatment. We showed that chronic myelogenous leukaemia cells, both sensitive and resistant to imatinib, release exosomes that are able to modulate the crosstalk between CML cells and the cell component of bone marrow microenvironment, promoting the release of IL8. Moreover, IL8 produced after exosome stimulation cause, in an autocrine way, the activation of an angiogenic phenotype and by paracrine mechanisms is able to sustain the survival of leukaemic cells. We also reported that CTO, is able to target both cancer cells and the tumour microenvironment, by inhibiting exosome-stimulated angiogenesis, thus suggesting a potential therapeutic utility in leukaemia patients.



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Publications and acts in congresses

Publications discussed in this thesis

- C. Corrado*, **S**. **Raimondo*,** L. Saieva*, Flugy A, De Leo G. and Alessandro R. Exosomes mediate a paracrine interplay between chronic myelogenous leukaemia and stromal cells: a role for interleukin 8. *Manuscript in preparation*. **(*co-first authorship)**
- C. Corrado*, **S. Raimondo***, A. Chiesi, F. Ciccia, G. De Leo, R. Alessandro. Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications. Review. *International Journal of Molecular Sciences. 2013* **(*co-first authorship)**(Impact Factor: 2.59)
- C. Corrado, A.M. Flugy, S. Taverna, **S. Raimondo**, G. Guggino,R. Karmali, G. De Leo, and R. Alessandro. Carboxyamidotriazole-orotate inhibits the growth of Imatinib-1 resistant chronic myelogenous leukaemia cells and modulates exosomes-stimulated angiogenesis. *Plos One* 2012;7(8):e42310(Impact Factor: 4.4)

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- C. Corrado, **S. Raimondo**, A.M. Flugy, S. Fontana, A. Santoro, A. Marfia, E.C. Kohn, R. Arlinghaus, G. De Leo and R. Alessandro. Carboxyamidotriazole inhibits cell growth of Imatinib-resistant chronic myelogenous leukaemia cells including T315I Bcr-Abl mutant by a redox-mediated mechanism. Cancer Letters, 2011; 300: 205-214 (Impact Factor: 4.86)
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- CicciaF, Accardo-PalumboA, Rizzo A, Guggino G, **Raimondo S**, Giardina A, Cannizzaro A, Colbert R, Alessandro R and Triolo G. Evidence that autophagy, but not the unfolded protein response, regulates the expression of IL-23 in the gut of patients with ankylosing spondylitis and subclinical gut inflammation. *Annals of Rheumatic Disease*, 2013 (Impact Factor: 8.72)
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- F. Ciccia, G. Guggino, A. Rizzo, A.R. Giardina, **S. Raimondo**, A. Ferrante, P. Cipriani, F. Carrubbi, A. Cannizzaro, G. Sireci, F. Dieli, G. Campisi, G. De Leo, R. Giacomelli, R. Alessandro, G. Triolo. Aberrant expression of IL-22RA1 on hematopoietic cells as immunologically signature of primary Sjogren's Syndrome and Sjogren-associated non-Hodgkin lymphomas. Submitted to *Annals of the Rheumatic Diseases* (Impact Factor: 8.72)
- Allegra A*, **Raimondo S***, Volpes A, Fanale D, Marino A, Cicero G, De Leo G, Sammartano F, Allegra G, Alessandro R. Gene expression profiling of cumulus oophorus cells reveals altered pathways in patients with

endometriosis. Submitted to Reproductive biomedicine online. **(*first co-authorship**) (Impact Factor: 2.04)

Acts in congresses

-***Raimondo S**, Corrado C, Saieva L,Flugy A., De Leo G. and Alessandro R. Exosomes mediate a paracrine interplay between Chronic Myelogenous Leukaemia and stromal cells: a role for interleukin 8. XXVI congresso AICC (Associazione Italiana Colture Cellulari) Brescia, 20-22 Novembre 2013. ***Presenting author**.

- Corrado C, Saieva L, ***Raimondo S**, Flugy A., De Leo G. and Alessandro R crosstalk between chronic myelogenous leukaemia and bone marrow stromal cells: role for IL-8 and CML-derived exosomes. XV AIBG, Cosenza 27-29 Settembre 2013. ***Selected for oral communication**.

- Corrado C., **Raimondo S**., Saieva L., Flugy A., De Leo G. and Alessandro R. Role of exosomes and interleukin 8 in the crosstalk between chronic myelogenous leukaemia and bone marrow stromal cells. 55rd Annual Meeting of the Italian Cancer Society (SIC), Catanzaro, 23-26 settembre 2013.

- G. Guggino, F. Ciccia, A. Rizzo, **S. Raimondo**, A. Giardina, F. Carubbi, P. Cipriani, G. Sireci, R. Giacomelli, R. Alessandro, G. Triolo. Aberrant expression of IL-22RA1 on hematopoietic cells as immunologically signature of primary Sjrogen's syndrome and Sjrogen-associated non-Hodgkin lymphomas.EULAR (The European League Against Rheumatism). Madrid, 12-15 June 2013.

- F. Ciccia, A. Accardo-Palumbo, A. Rizzo, G. Guggino, **S. Raimondo**, A. Giardina, M. Peralta, R. Colbert , R. Alessandro, G. Triolo. Autophagy, but not the unfolded protein response, regulates the expression of IL-23 in the gut of patients with ankylosing spondylis and subclinical gut inflammation. EULAR (The European League Against Rheumatism). Madrid, 12-15 June 2013.

- C. Corrado, ***S. Raimondo**, A. Flugy, G. De Leo, R. Alessandro. Role of interleukin 8 in exosome-mediated crosstalk between Chronic Myelogenous Leukaemia

cells and bone marrow stromal cells. Second International Meeting of International Society for Extracellular Vesicles (ISEV), Boston, April 17-20 2013. ***Presenting author**.

- ***S. Raimondo**, C. Corrado, S. Taverna, A. Flugy, G. De Leo, R. Alessandro. CarboxyAmido-Triazole Orotate inhibits the growth of Imatinib-resistant chronic myelogenous leukaemia cells and modulates exosomes-stimulated angiogenesis. XXV congresso AICC (Associazione Italiana Colture Cellulari, Palermo 21-23 Novembre 2012 (abstract book pag. 64). ***Selected for oral communication and best poster award**.
- **S. Raimondo**, F. Ciccia, V. Rodolico, G. Campisi, G. Guggino, G. De Leo, R. Alessandro, G. Triolo. IL-34 contributes to the development of a pro-inflammatory microenvironment in patients with Sjrogen's syndrome. XIV AIBG, Assisi-Perugia 28-29 Settembre 2012.
- C. Corrado, A.M. Flugy, S. Taverna, **S. Raimondo**, G. Guggino, R. Karmali, G. De Leo, R. Alessandro. CarboxyAmido-Triazole Orotate inhibits the growth of Imatinib-resistant chronic myelogenous leukaemia cells and modulates exosomes-stimulated angiogenesis. XIV AIBG, Assisi-Perugia 28-29 Settembre 2012.
- S. Taverna, C. Corrado, **S. Raimondo**, L. Saieva, F. Favaloro, A. Russo, D. Fanale, G. De Leo, R. Alessandro. Role of CML exosomes in the crosstalk between chronic myelogenous leukeaemia and bone marrow-derived cells. XIV AIBG, Assisi-Perugia 28-29 Settembre 2012.
- **S. Raimondo**, C. Corrado, S. Taverna, A. Flugy, G. De Leo, R. Alessandro. *In vitro* and *in vivo* effect of CarboxyAmido-Triazole Orotate (CTO) on Imatinib resistant Chronic Myelogenous Leukaemia cells. RIKEN RCAI International Summer Program, Yokohama, Japan, June 22-27, 2012.
- G. Guggino, F. Ciccia, **S. Raimondo**, A. Giardina, G. Sireci, F. Dieli, R. Alessandro, G. Triolo. NKP44+NK cells are expanded and produce high amounts of IL-22 in the salivary glands of Sjrogen syndrome patients. 8th International Congress on Autoimmunity. Granada, Spain, May 9-13, 2012

- R. Alessandro, C. Corrado, S. Taverna, **S. Raimondo**, A.M. Flugy, R. Karmali and G. De Leo. Carboxyamidotriazole-orotate inhibits the growth of Imatinib resistantchronic myelogenous leukaemia cells and modulates exosomes stimulated Angiogenesis. First International Meeting of International Society for Extracellular Vesicles (ISEV), Gothenburg Sweden April 18-21. Abstract book page 53; abstract number 49.
- Taverna S., Flugy A., Saieva L., Corrado C., **Raimondo S**., Fontana S., Karmali R., De Leo G. and Alessandro R (2011) "Tumour microenvironment modulation by exosomes in chronic myelogenous leukaemia". 53rd Annual Meeting of the Italian Cancer Society (SIC) Turin, 19 – 22 October 2011.
- S. Taverna, A. Flugy, L. Saieva, S. Fontana, C. Corrado, **S. Raimondo**, E.C. Kohn, A. Santoro, S. Meraviglia, G. De Leo, R. Alessandro (2011) "Role of exosomes released by chronic myelogenous leukaemia cells in angiogenesis". XXIII Congresso Nazionale AIBG (Associazione Italiana di Biologia e Genetica generale e molecolare). Padova 30 settembre-1 ottobre 2011.