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# The Role of Adhesion Receptors in Melanoma Metastasis and Therapeutic Intervention Thereof

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## 1. Introduction

The metastatic spread of solid tumors is the most fatal complication in malignant diseases and the major cause of tumor-related mortality. This is of high relevance for malignant melanoma which are highly proliferating tumors with aggressive metastatic tendency. The majority of melanoma cells tend to metastasize primary via the lymph system and secondary into different organs, most likely distant skin regions, liver, lungs, brain, and heart via hematogenous distribution, which is associated with bad prognosis. The molecular mechanisms of the metastatic processes are complex and not fully elucidated. Although cytostatic agents or anti-angiogenic drugs (e.g. Avastin/bevacizumab) affect, beside their effects on the primary tumors, also the growth and development of metastases, there is at present no antimetastatic pharmacological strategy in the clinics to interfere with tumor cell dissemination or spread.

Adhesion receptors are strongly involved in the process of tumor cell metastasis, either by deregulation of adhesive functions and subsequently the detachment of tumor cells from the primary tumor and the overcoming of tissue borders, or by mediating manifold cell contacts with blood components in the phase of hematogenous distribution. An intensive and ongoing preclinical research provided essential insight and several postulated factors and mechanisms for the hematogenous dissemination, i.e. the interaction of tumor cells with the different blood elements, soluble factors and cells. P- and L-selectin, members of a family of carbohydrate binding adhesion receptors are regarded as functional key players in the contact formation of tumor cells with platelets and leukocytes, thus facilitating microemboli formation and accelerating metastatic dissemination. The antimetastatic activities of heparin, confirmed by a number of prospective clinical trials, are to a large extent ascribed to the inhibition of these two selectins.

Besides the important role of selectins, integrins as ubiquitous cell adhesion receptors are also involved in the mediation of manifold adhesive interactions in the metastasis of tumor cells. Integrins bind ligands of the extracellular matrix (ECM), such as fibronectin, collagen, laminin and vitronectin to stabilize cell attachment with the surrounding tissues or to

mediate migration in the metastatic cascade. Furthermore, integrins mediate cellular contacts of the tumor cells to platelets, leukocytes, and endothelium. However, in contrast to the selectins, the integrin function has hardly been considered as target for antimetastatic approaches.

Integrins possess important tasks in cell signalling that can be summarized as defining the cellular shape, mobility, invasion and cell cycle regulation. The regulation of these processes via contribution of the actin and microtubule cytoskeletons is well known to be controlled by Rho-GTPases (Ras homologue; guanosin triphosphatases), which belong to the superfamily of Ras-GTPases (Rat adeno sarcoma). GTPases of the Ras-superfamily are important for cell proliferation, metastasis, migration, apoptosis, gene expression and multiple other functions in the cell. Since the integrin signalling is also cross-linked to the function of GTPases, a therapeutic influence on the GTPase activity could be a novel and attractive approach to control integrin bindings. Recent data on the treatment of melanoma cells with lysophosphatidylcholine (LysoPC) referred to reduced integrin functions, which might be related to reduced GTPase signalling.

This book chapter will deal with the molecular function of adhesion receptors in the process of hematogenous metastasis of melanoma cells and the therapeutic potential and prospects to interfere with adhesion receptor activity as an antimetastatic approach. The focus was put on the integrins, which will be explained with their functions, abilities and connections to other indispensable proteins, such as cytoskeletal components in the context of cancer and metastasis. Finally the hypothesis to influence the integrin functions in metastatic cascade at a signalling level will be introduced and discussed as an interesting novel target for antimetastatic approaches.

## **2. The metastasis of melanoma cells**

### **2.1 The course of the metastatic cascade**

Tumor cell metastasis is a complex cascade which consists of various molecular events. Metastatic cells have to exit the primary tumor by a deregulation of the cellular contacts, have to migrate the basement membrane of the tumor, degrade the extracellular matrix and intravasate lymphatic vessels or local post capillary veins. Several factors are known which contribute to the malignant transformation of melanocytes and melanoma development and progression, including microenvironmental influences, or UV radiation for triggering a cascade of proinflammatory factors and mediators (Lee & Herlyn 2006; Schwarz & Luger 1989).

Once in the blood system, the tumor cells have to escape the immune surveillance and physical stress of the blood stream to finally seed at the vascular bed at distant organs. This is the rate-limiting step of the metastatic cascade before they can extravasate and form metastases (**Figure 1**).

An insight into the hematogenous phase of tumor cell distribution is given by numerous animal models of experimental metastasis, which dominantly used melanoma cell lines due to their rapid metastatic colonization tendency (Ludwig et al., 2004; Bereczky et al., 2005; Ludwig et al., 2006; Mousa et al., 2006; Niers et al. 2009). Although these models do not completely recapitulate the natural processes of metastatic spread, the timely defined presence of tumor cells in the blood systems allows for characterization and evaluation of cellular contacts within the phase of hematogenous distribution.

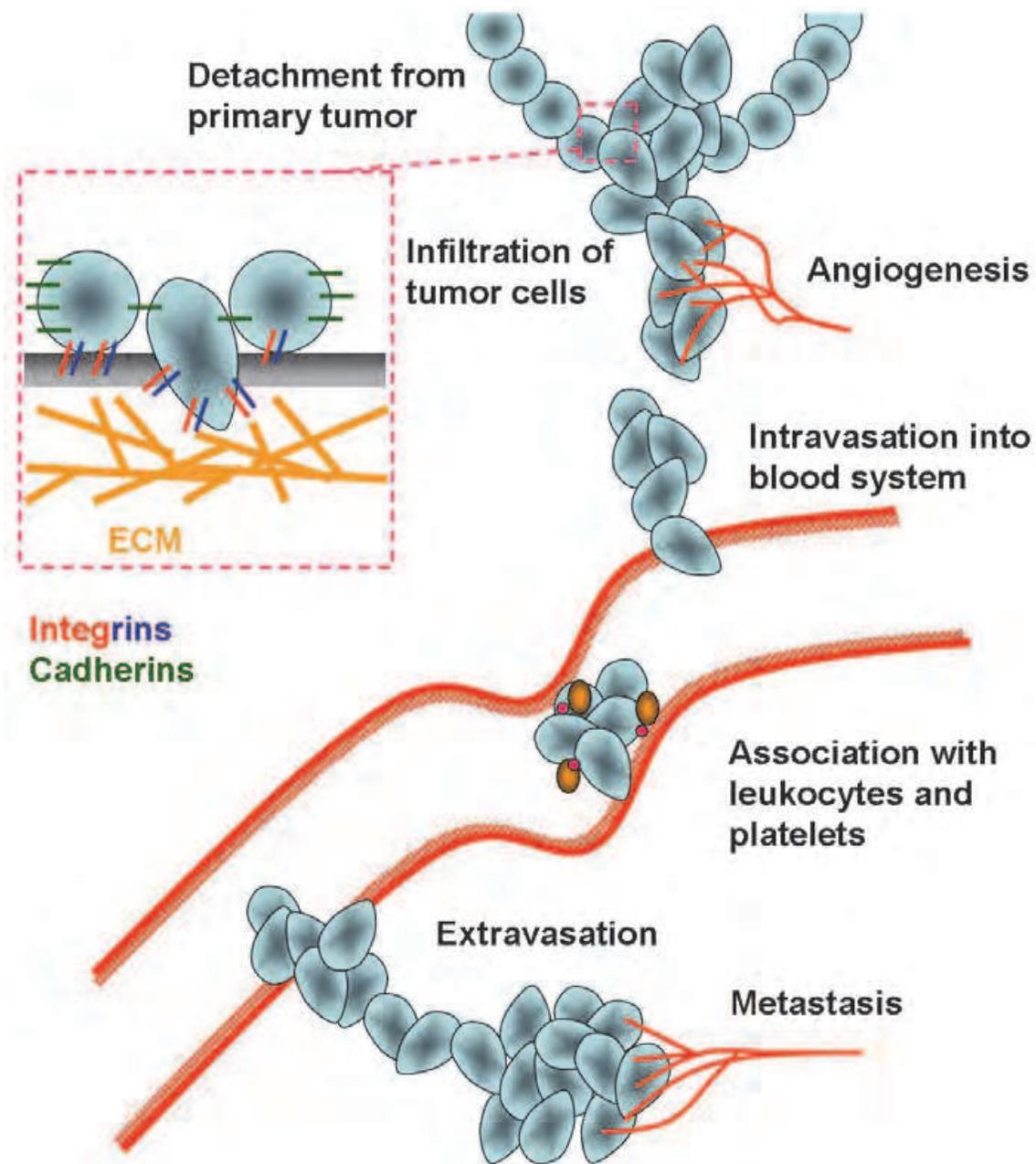


Fig. 1. Schematic representation of the hematogenous metastasis.

The illustration give insights into the step-by-step process of the hematogenous metastasis: Detachment of tumor cells from primary tumor; overcoming of ECM, intravasation into the blood system, association with platelets and leukocytes to prevent shear forces and immune defense, transport through the organism, physical arrest or arrest via interaction with the vascular endothel, extravasation, generation of micrometastases and angiogenesis to the blood supply.

Modified from (Guo W. & Giancotti F. G., 2004)

The massive interaction of tumor cells forming a platelet cloak is a vital strategy to evade the immune defense and was shown to correlate with metastatic progression. Furthermore, the recruitment of leukocytes to form microemboli is thought to support microvascular arrest at distant sites and contributes to activation of endothelial cells. In general, tumor cell and/or

leukocyte interaction with the microenvironment at site of colonization induces an inflammatory-like activation of endothelial cells during the initial steps of metastasis progression, including the chemokine network and different procoagulative factors (Lazennec & Richmond, 2010). However, cellular adhesion receptors play a pivotal role for this complex cellular communication.

## **2.2 The impact of selectins on the hematogenous tumor cell dissemination**

The selectins, a family of carbohydrate binding proteins, represent the crucial importance linked with hematogenous metastasis (Läubli & Borsig, 2010). P-selectin, expressed by platelets is dominant for the platelet contact of tumor cells. Its role for protecting the tumor cells and facilitating metastatic progression is confirmed by experiments using P-selectin knock-out mice, which displayed strongly attenuated experimental metastasis (Ludwig et al., 2004; Kim et al., 1998). It became evident that P-selectin, expressed by activated endothelial cells also contributes to the metastatic progression (Ludwig et al., 2004). L-selectin, which is constitutively expressed by all types of leukocytes, supports the comprehension of leukocyte into the microemboli and vascular contact formation and activation (Läubli et al., 2006). Endothelial activation is associated with the upregulation of other adhesion receptors, such as E-selectin or the integrin ligand vascular cell adhesion molecule-1 (VCAM-1), which again advises to the inflammatory-like reaction (Auguste et al., 2007).

Consequently, the competitive blocking of the selectin function appears as a promising therapeutic approach to interfere with the metastatic cascade.

## **2.3 The role of heparin for antimetastatic approaches**

The evidence for those selectin-blocking approaches came indirectly from clinical efforts to treat cancer-associated thromboembolic events, which are frequent complications in malignant diseases. Heparin, or low molecular weight heparin (LMWH) are the anticoagulant drug of choice in the clinical treatment or prophylactic treatment of cancer-associated thrombosis. Based on the retrospective evaluation of clinical data, which referred to a survival benefit of heparin treated cancer patients, a number of prospective clinical trials have been launched (Zacharski & Lee, 2008). Animal experiments supported the assumption that heparin hardly affects the primary tumor but interferes with the process of metastasis. Several molecular mechanisms exist to explain the antimetastatic efficiency of heparin. The reader is referred to excellent reviews in this field (Borsig, 2010; Casu et al. 2008) from which the inhibitory capacity of heparin towards P- and L-selectin binding should be highlighted here.

Heparin as a highly sulfated, acidic polysaccharide has the ability to compete with the natural mucin-like selectin ligands. The capacity of heparin to interfere with P- and L-selectin binding has already been described in the early 90<sup>th</sup> (Skinner et al., 1991), but in the context of tumor cell metastasis the heparin effects as competitors of P- and L-selectins were accumulated during the last decade. Further insight was obtained into the structural requirements of heparin for selectin binding (Hostettler et al., 2007), and binding affinities of heparin to both selectins were shown to be in the low micromolar range (Simonis et al., 2007).

## **2.4 Integrins as targets for heparin**

The platelet integrin IIb IIIa also contributes to the bond formation between platelets and melanoma cells via fibronectin or vWF to melanoma integrins. A recent study reported that

heparin has also an inhibitory capacity to this binding (Zhang et al., 2009). Thrombin, interacting with its receptor PAR-1 on melanoma cells (protease-activated receptors-1) has a strong impact for regulating this interaction. PAR-1, which is predominantly overexpressed in malignant melanoma cells induces diverse procoagulant and metastatic events, such as matrix degradation, secretion of angiogenetic factors or integrin activation (Melnikova et al., 2008).

Melanoma integrins can also directly contribute to the firm adhesion to the vasculature. The integrin  $\alpha 4\beta 1$  (very late activation antigen-4, VLA-4), which interacts with the VCAM-1 as counter receptor, is described as another pathway for endothelial arrest of malignant melanoma cells (beside osteosarcoma and rhabdomyosarcoma cells), thereby promoting the transmigration (Okahara et al., 1994; Liang & Dong, 2008) and metastasis (Garofalo et al., 1995; Schadendorf et al., 1995). Although these findings suggest that the inhibition of VLA-4 could be promising in the treatment of melanoma metastasis, and despite VLA-4 inhibition by antibodies or small molecules is a vital strategy to interfere with pathological inflammations, such approaches have not been described so far in the cancer field. We could recently show that the VLA-4/VCAM-1 interaction of murine or human melanoma cell lines can be efficiently blocked by heparin (Schlesinger et al., 2009). Nevertheless, beside those approaches for a competitive blockade of integrin receptor function, the manifold signalling functions of integrins open a new way for a therapeutic interference, which will be discussed below.

### **3. The biology of integrins in the context of cell adhesion processes and metastasis**

In addition to the selectin-mediated cell adhesion processes in metastasis, the protein families of immunoglobulins (IGs), cadherins and integrins play important roles - in the case of malfunction - in the development and progression of melanoma metastasis.

Several members of the IG superfamily such as ICAM-1, L1CAM and MCAM/MUC18 are significantly associated with progression of melanoma metastasis (Meier et al., 2006; Yamada et al., 2006; Johnson et al., 1997). The MUC18/MCAM expression confers metastatic potential and increased tumorigenicity to human melanoma cells (Johnson et al., 1999). A switch of the cadherin molecules from E-cadherin to N-cadherin is responsible for the disassociation of melanoma cells from keratinocytes. The loss of E-cadherin function is connected with the upregulation/induction of MUC18/MCAM and  $\alpha v\beta 3$  integrin in melanocytic cells in vitro and with changes in the levels and the cellular distribution of the transcriptional regulator  $\beta$ -catenin in melanomas in vivo (Johnson et al., 1999). Thus, melanoma cell invasion through the dermis is mediated by this change (Hsu et al., 1996; Hsu et al., 2000).

One of the most prominent examples that is frequently associated with the progression of melanoma is the integrin  $\alpha 4\beta 1$  (VLA-4) (Braeuer et al., 2011). It was observed that VLA-4 overexpression and interaction with VCAM-1 is clearly correlated with experimental lung metastasis and the tumor stage (Schadendorf et al., 1995; Okahara et al., 1994). Due to the fact that the interaction of VLA-4 and VCAM-1 and other integrins are of great interest as possible targets for melanoma cancer therapy, the following chapter will handle with details on the integrin family and their structural and functional connections in the context of cancer metastasis.

### 3.1 The general structure of integrins

The integrins are large and complex transmembrane glycoproteins which act as adhesion receptor molecules that are responsible for the mediation of attachment and anchorage between cells or to the underlying extracellular matrix (ECM) (Morgan et al., 2007). In detail they span the plasma membrane and work alongside other proteins such as cadherins and selectins to mediate cell-cell and cell-matrix interaction and communication. One important task of integrins is the binding of the cell surface and ECM components such as fibronectin, vitronectin, collagen, and laminin.

Many cells have multiple types of integrins on their surface. That means the use of integrin-targeted reagents is not specific for all but for a few or one specific integrin. This provides additional mechanistic insights into the functions of integrin adhesion receptors.

The structure of integrins can be divided in two distinct chains, the  $\alpha$ - and  $\beta$ -subunit (**Figure 2**) which form a non-covalent heterodimer (Morgan et al., 2007; Lau et al., 2009; Shattil et al., 2010). In mammals, 18  $\alpha$ - and 8  $\beta$ -integrin genes have been characterized that encode polypeptides that combine to form 24 unique, canonical  $\alpha/\beta$  receptors out of 144 possible combinations. The *Drosophila* genome encodes only five  $\alpha$ - and two  $\beta$ -subunits, and the *Caenorhabditis nematodes* possess two  $\alpha$  and one  $\beta$  genes (Shattil et al., 2010; Morgan et al., 2007; Humphries, 2000). In addition, variants of some integrin subunits are formed by differential splicing, for example four variants of the beta-1 subunit exist (Hynes, 2002).

Both the  $\alpha$ - and  $\beta$ -subunits form separate tails, which penetrate the plasma membrane and possess small cytoplasmic domains (Nermut et al., 1988; Lau et al., 2009). The extracellular  $\alpha/\beta$ -domains are relatively huge compared to the short cytoplasmic domain and the structure of each subunit is conserved between isoforms, excluding a subset of  $\alpha$ -subunits, which include an inserted 'A-domain' in their ligand-binding pocket (Shattil et al., 2010; Morgan et al., 2007). The pathway of extracellular binding of integrins to intracellular transformation of the binding is most often mediated by the cytoplasmic tail of the  $\beta$ -subunit (Morgan et al., 2007).

### 3.2 The cellular functions of integrins

Concerning the function, integrins are involved in a wide range of biological activities, including immune patrolling, cell migration, defining cellular shape, cell cycle regulation, growth, invasion, proliferation, differentiation, survival/apoptosis and binding to cells by certain viruses (Desgrosellier & Cheresh, 2010; Assoian & Klein, 2008).

The integrin proteins act as receptors which are able to communicate between the ECM and the cell. They also transport information from the status inside the cell to the extracellular space. This allows rapid and flexible responses in both directions. Thus integrin tasks can be divided into two main functions: Attachment of the cell to the ECM and signal transduction from the ECM to the cell. Within the past decade it has become apparent that adhesion molecules such as integrins play an important role for the mediation of critical cytosolic signalling events in the cell (Stupack, 2007).

Integrins act to regulate complex processes in cancer disease such as angiogenesis, tumor growth and metastasis (Hynes, 2002), and the signalling has a dramatic impact on cell proliferation, survival and motility. For this reason they have become attractive therapeutic targets for the development of pharmaceutical compounds. Several effective integrin antagonists are now under clinical evaluation (Stupack, 2007).

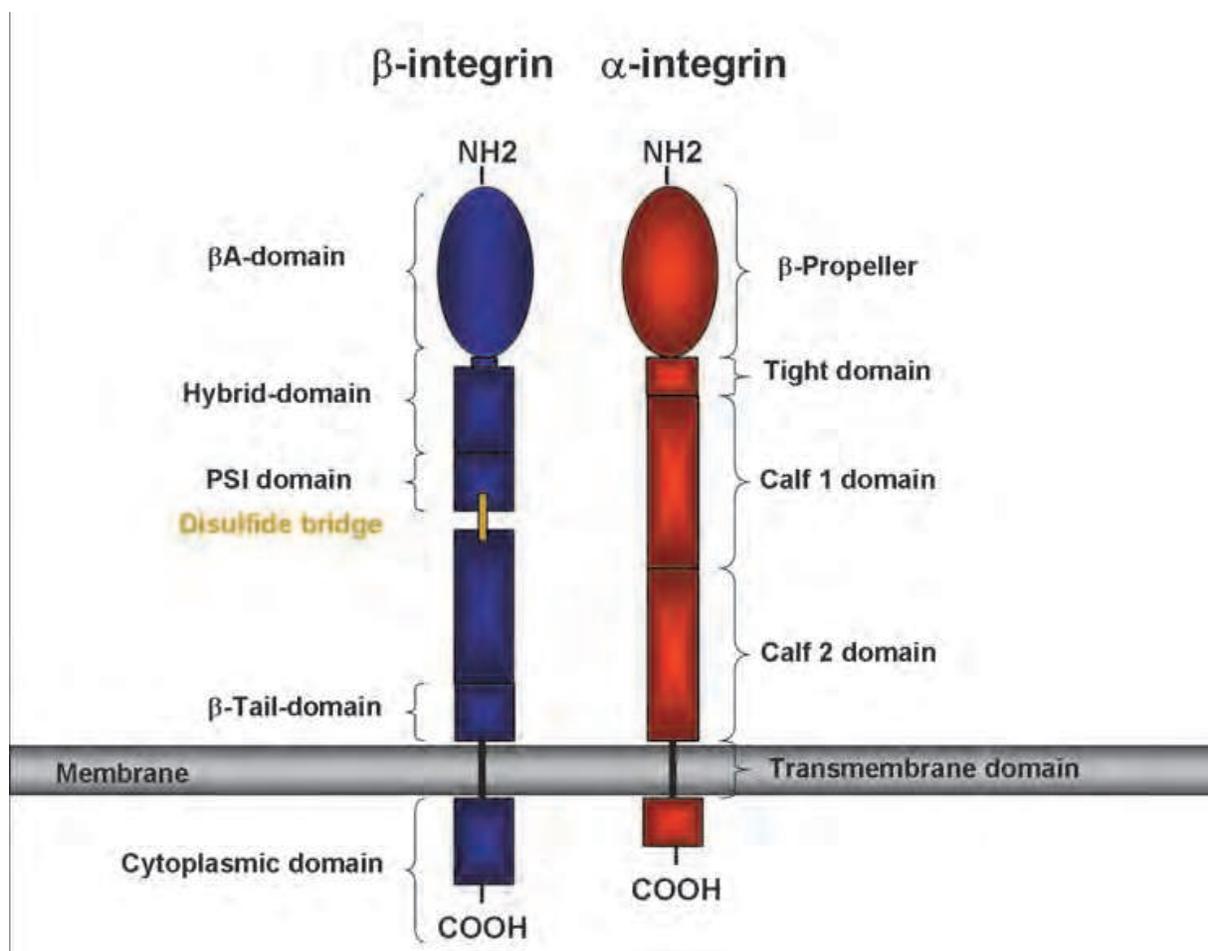


Fig. 2. Domain architecture of the heterodimeric transmembrane domains which show how integrins are designed to act as bidirectional signalling machines. Integrins are heterodimeric adhesive receptors consisting of an  $\alpha$ - and a  $\beta$ -subunit. The ligand binding site is provided by the N-terminal domain of the  $\alpha$ - and  $\beta$ -integrin subunits (the  $\beta$ -propeller and the  $\beta$ A domain, respectively) which are assembled in most integrins by non-covalent interactions to form a “head”. It is known that in 8  $\alpha$  integrin subunits ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 10$ ,  $\alpha 11$ ,  $\alpha L$ ,  $\alpha M$ ,  $\alpha X$  and  $\alpha D$ ), the  $\alpha A$  domain, which is homologous to the  $\beta A$  domain of the  $\beta$ -integrin subunit, is inserted into the  $\beta$ -propeller domain. This is the main ligand-binding site in these integrins. Integrins that lack an A domain (e.g. the depicted schematic architecture of  $\alpha IIIb\beta 3$  integrin), the  $\beta A$  domain forms the main ligand-binding site. The PSI-domain (plexin, semaphorin and integrin) is at the N terminus of the  $\beta$ -integrin subunit, but is joined by disulfide bonds to more C-terminal residues. The remaining C-terminal extracellular domains of the  $\alpha$ - and  $\beta$ -subunit comprise two long ‘legs’ which are anchored in the PM. The low affinity state of the integrin for its ligands is maintained by non-covalent interactions between the  $\alpha$ - and  $\beta$ -integrin transmembrane and cytoplasmic domains. Figure is modified from (Shattil et al., 2010);  $\alpha$ -subunit and  $\beta$ -subunit

In particular integrins operate as mechanistic biosensors in a context-dependent manner. On one hand, integrins that ligate substrate-immobilized ligands typically transduce positive signals into the cell. On the other hand, antagonized or unligated integrins promote negative signalling into the cell, which leads to cell cycle arrest or apoptosis. Thus, integrins constantly interrogate the local ECM and modulate cell behaviour accordingly. Typically, receptors

inform a cell of the molecules in its environment and the cell evokes a response. Integrin receptors have two ways of signalling. They are able to perform outside-in signalling - response to molecules in its environment - and they also operate in an inside-out mode (Huveneers & Danen, 2009; Shattil et al., 2010; **Figure 3**). Therefore they are known as bidirectional, allosteric molecular signalling machines, although the relationship between specific conformations and activation remains controversial (Hynes, 2002; Shattil et al., 2010).

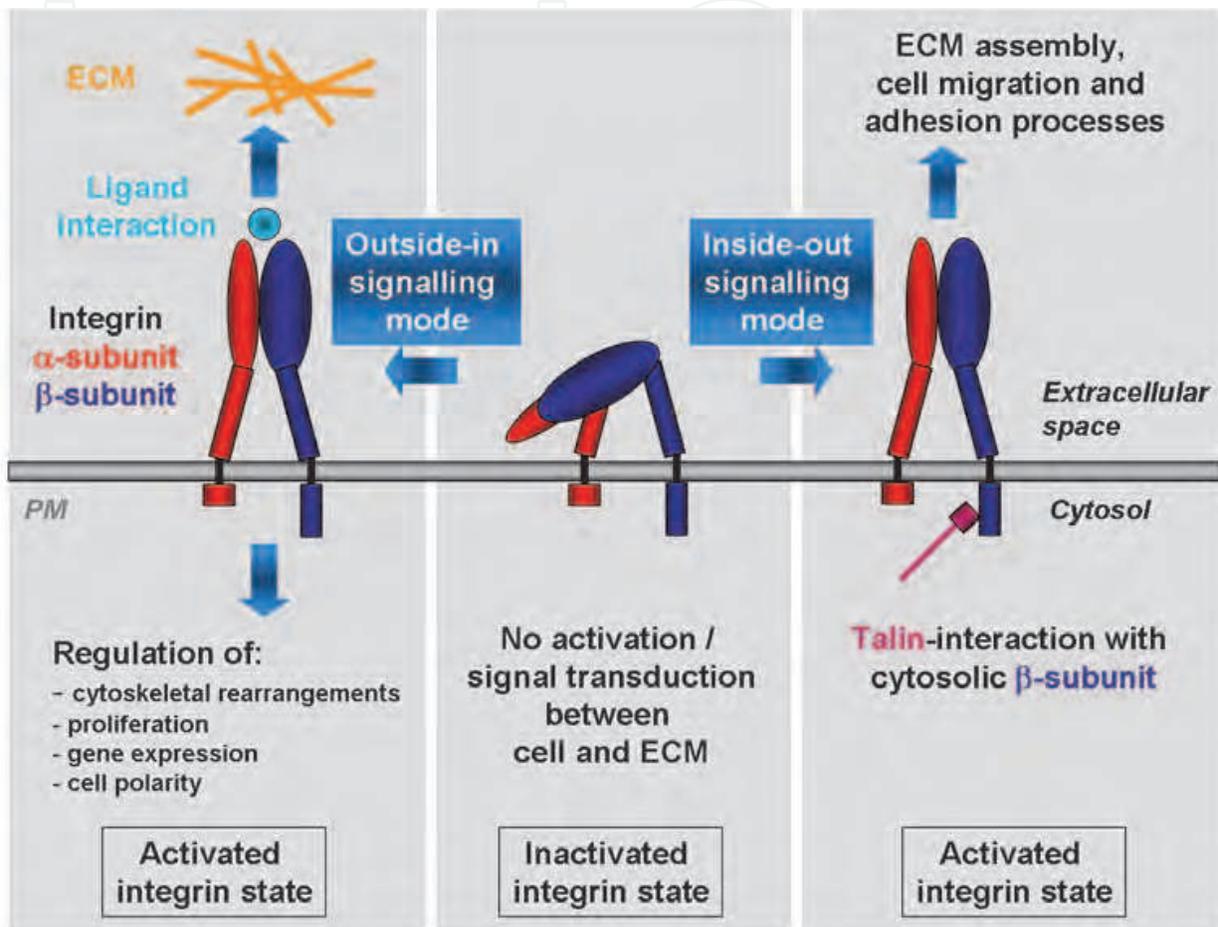


Fig. 3. Cellular signalling modes of integrins.

The two ways of integrin signalling can be divided in “outside-in” (left) and “inside-out” (right) modes. Both directions have different biological and biochemical consequences. In the case of “outside-in” signalling, binding of integrins to their extracellular ligands changes the conformation of the integrin and - because many of the ligands are multivalent - contributes to integrin clustering. The combination of these two events leads to intracellular signals that control cell polarity, cytoskeletal structure, gene expression, cell survival and proliferation. During ‘inside-out’ signalling, an intracellular activator, such as talin or kindlins, binds to the  $\beta$ -integrin tail, leading to conformational changes that result in increased affinity for extracellular ligands (integrin ‘activation’). Inside-out signalling controls adhesion strength and enables sufficiently strong interactions between integrins and ECM proteins to allow integrins to transmit the forces required for cell migration and ECM remodelling and assembly. Both modes of signalling are often closely linked; for example, integrin activation can increase ligand binding, resulting in outside-in signalling. Conversely, ligand binding can generate signals that cause inside-out signalling. Modified from (Shattil et al., 2010)

As mentioned before, the cytoplasmic tail of the  $\beta$ -subunit is known to be the main region of mediating protein interaction (e.g. talin, a cytoskeletal protein), signal transduction and direct integrin activation (Shattil et al., 2010; Morgan et al., 2007).

#### 4. The integrins in the context of signal transduction processes

The mechanisms of integrin interaction at the cytoplasmic side are strongly connected to the regulation of GTPases. For example, activated Rac1 and RhoA transduce signals to integrin activation via phospholipase D (PLD) and phosphatidylinositol-4-phosphate 5-kinase 1 $\gamma$  (PIP5K1 $\gamma$ ) (Tybulewicz & Henderson, 2009). Furthermore it is known that integrin signalling is inhibited by RhoH through an unknown mechanism. In this case, integrin signalling leads to the activation of the GEF  $\alpha$ PIX (PAK-interacting exchange factor- $\alpha$ ) and the following activation of Rac1 and PAK (p21-activated kinase) (Tybulewicz & Henderson, 2009). PAKs are well known to serve as targets for the small GTPases Cdc42 and Rac and they have been implicated in a wide range of biological activities, such as regulating the cell motility and morphology (PAK1), involvement in apoptotic processes (PAK2), or in the rapid cytoskeletal reorganization in dendritic spines (PAK3) or mediation of filopodia formation (PAK4).

Integrin signalling works predominantly through the recruitment and activation of Src-family kinases (SFKs). Most integrins recruit focal adhesion kinase (FAK) through their cytoplasmic domain of the  $\beta$ -subunit (Guo & Giancotti, 2004). FAK works as a phosphorylation-regulated scaffold to recruit Src to focal adhesions. From this important point of origin, several signal pathways influence the cell proliferation, survival and migration (Figure 4). Activating signalling via FAK, phosphatidylinositol 3-kinase (PI3K) over phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>) to AKT and protein kinase B (PKB) can induce the mentioned effects (e.g. adhesion, cell proliferation, etc.). In addition, there is a direct crosstalk via GEF to activate Rac (another possibility for activation is from SFK via CAS, Crk and DOCK180 to Rac) which in turn can activate PAK, Jun amino-terminal kinase (JNK) and nuclear factor kappa-B (NF $\kappa$ B) (Parsons & Parsons, 1997; Schlaepfer & Hunter, 1998; Cary et al., 1999). Furthermore FAK can activate extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) by recruiting the Rap1 GEF C3G through Crk. Rap1 is then able to activate ERK/MAPK through B-Raf. An alternative way for ERK/MAPK activation is via the direct recruitment of growth-factor-receptor-bound-2 (GRB2) and son-of-sevenless (SOS) complex. Here, certain integrins, including  $\alpha$ 5 $\beta$ 1,  $\alpha$ 1 $\beta$ 1 and  $\alpha$ v $\beta$ 3, are coupled to palmitoylated SFKs through their  $\alpha$ -subunits. The palmitoylated SFKs recruit and phosphorylate the adaptor Shc, which combines with GRB2-SOS to activate ERK/MAPK signalling from Ras (Wary et al., 1996; 1998).

Beside the interaction of integrins with SFKs mentioned before, some integrins are able to interact directly with SFKs via the cytoplasmic tail of their  $\beta$ -subunits (Arias-Salgado et al., 2003). As an example, the  $\alpha$ 6 $\beta$ 4 integrin is palmitoylated at its  $\beta$ -subunit. This palmitoylation is required for the incorporation of the complete  $\alpha$ 6 $\beta$ 4 integrin in lipid rafts where the receptor is able to interact with SFKs that are similarly palmitoylated (Gagnoux-Palacios et al., 2003). Here, the SFKs phosphorylate several tyrosine residues in the cytoplasmic domain of  $\beta$ 4, which causes the recruitment of SHC and activation of Ras-ERK/MAPK and PI3K signalling (Mainiero et al., 1995; 1997; Shaw et al., 1997; 2001). The known pathways that integrins can activate through SFKs are sufficient for the induction of cell migration, invasion and proliferation or to confer some protection from apoptosis on cells.

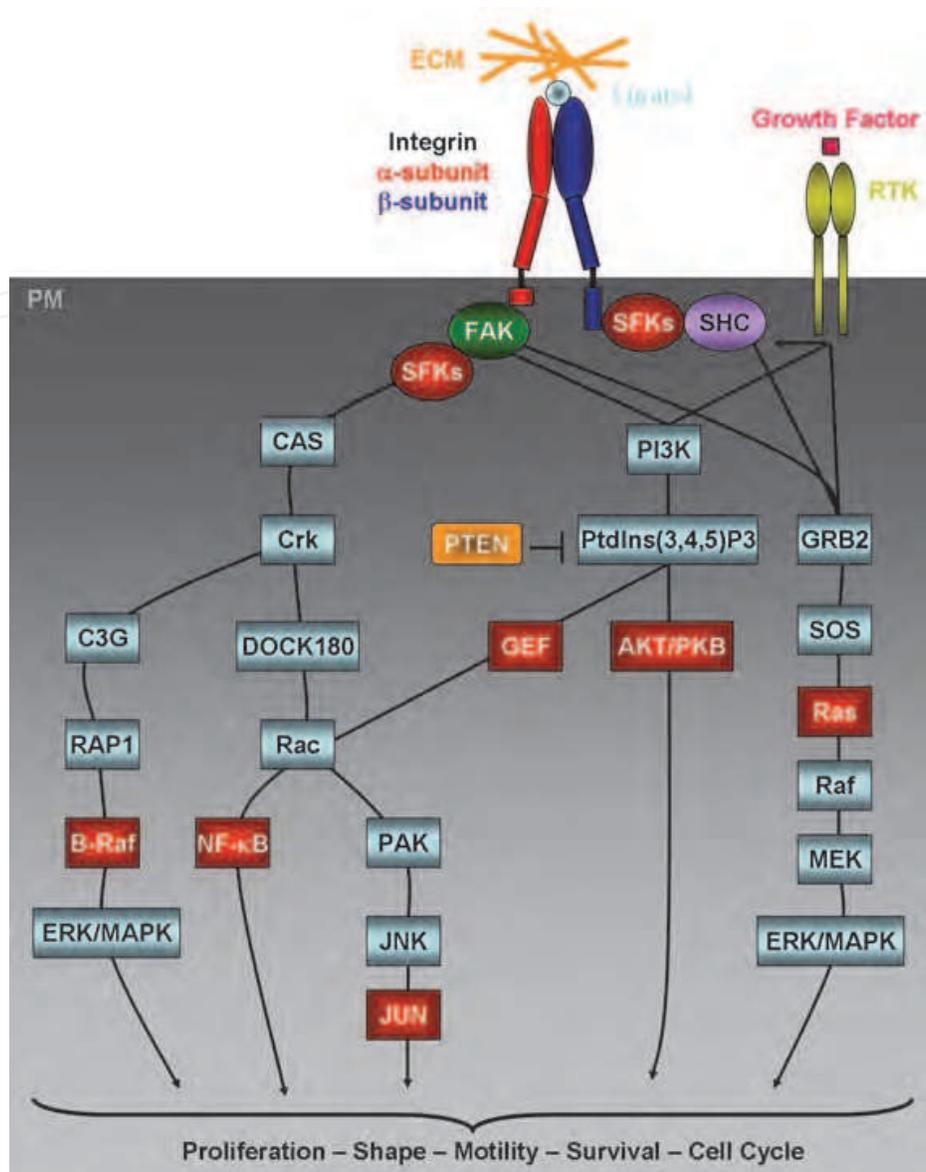


Fig. 4. General overview of alternative integrin signalling pathways.

The figure shows a section of the possible signalling cascades which lead to the activation of cellular survival proliferation and migration. Further details to the interactions are explained in the main text.

Modified from (Guo & Giancotti, 2004).

In addition to the  $\alpha6\beta4$  integrin,  $\alpha4\beta1$  (VLA-4) and  $\alphaL\beta2$  have been identified to colocalize with the lipid raft marker GM-1 in T-cells. Disruption of raft integrity through depletion of membrane cholesterol with methyl- $\beta$ -cyclodextrin (MbCD) completely disrupted  $\alpha4\beta1$  cluster formation, implying that the lipid rafts are required for  $\alpha4$  integrin clusters (Leitinger & Hogg, 2002). The integrins  $\alpha\nu\beta3$ ,  $\alpha11\beta3$  and  $\alpha2\beta1$  are associated with specific integrin interacting proteins in cholesterol-dependent microdomains distinct from classical rafts (Green et al., 1999). These results hypothesize that the mechanism of membrane compartmentalization - as identified for the mentioned integrins - also operates in other integrin-signalling systems, which might be an explanation for several specific aspects of diverse integrins.

## 5. Possible ways for pharmacological antimetastatic approaches by intervention with integrins on the plasma membrane and signalling level

The integrins, especially the integrin VLA-4 has been for longer time in the interest as target for the design of small molecule competitive inhibitors as potential antiinflammatory drugs (Singh et al, 2004).

Concerning the “non-competitive” influence on integrins, there basically exist two different ways for integrin modulation: An interference with the signal transduction at the cytoplasmatic site or an influence on the integrin compartmentalization at the plasma membrane. As mentioned before the influence on integrins is mainly mediated via the cytoplasmatic tail of their  $\beta$ -subunits which allows the inside-out signalling (Arias-Salgado et al., 2003).

In general, integrins essentially need the specific membrane positioning and membrane anchorage of signalling proteins like GTPases of the Ras-superfamily for their signalling processes. The distribution of Ras proteins is determined by different C-terminal lipid modifications. Extensive experimental studies on Ras-GTPases have revealed that the proteins only operate at the plasma membrane (Meder & Simons, 2005; Pechlivanis & Kuhlmann, 2006). Several publications have pointed out the importance of Ras compartmentalization for signal transduction (Roy et al. 2002; Chiu et al., 2002). In particular, the palmitoylation allows the anchorage for H-Ras / N-Ras and the spatial and temporal organization plus segregation of the GTPases signal transfer duration. The time of PM-Golgi apparatus cycling, for N-Ras the transfer time from the PM to the Golgi apparatus during cycling, was remarkably shortened compared to H-Ras (Rocks et al., 2010; Rocks et al., 2005). This is in line with their palmitoylation status - two palmitoyl anchors for H-Ras and one palmitoyl anchor for N-Ras. Therefore, impact on the GTPase localization by affecting the membrane characteristics might have strong consequences for the integrin activity too.

In addition to the  $\alpha 6\beta 4$  integrin mentioned before, which possesses as laminin-5 receptor unique functions in epithelial growth and carcinoma invasion (Mainiero et al., 1997; Shaw et al., 1997) a palmitoylation of the  $\beta 4$ -subunit as a prerequisite for the incorporation of  $\alpha 6\beta 4$  in rafts and the compartmentalization with SFKs (Gagnoux-Palacios et al., 2003), other integrins were also reported to depend critically on their membrane localization. The  $\alpha 4\beta 1$  integrin (VLA-4) was also identified to be localized in lipid rafts, which revealed that lipid rafts also play a key role in regulating integrin activity, function and its further downstream signalling (Leitinger & Hogg, 2002; Schadendorf et al., 1995; Okahara et al., 1994). Non-raft integrins are excluded from the rafts by cytoskeletal constraints and are no more able to perform signalling from raft microdomains (Leitinger & Hogg, 2002). Thus, the positioning of integrins inside or outside of rafts for their physical interaction with important signalling switches, such as GTPases or FAKs depends critically on the surrounding lipid composition. Consequently, an influence on the membrane characteristics and lipid composition of tumor cells appears as attractive way to influence integrin activities.

In a recent study, such an aspect of attenuated melanoma metastasis has been reported (Jantscheff et al., 2011), which refers to reduced receptor mediated binding by non-competitively interfering with integrin function. This study is based on earlier findings that empty liposomes consisting of saturated phosphatidylcholine (PC) displayed strong antimetastatic effects in a murine pancreatic mice model (Graeser et al., 2009). Jantscheff et al. (2011) postulated that liposomes, passively accumulated in the tumor, release LysoPC as

a degradation product of the saturated PC which affects the capability of tumor cells for metastases. In order to follow this hypothesis, the authors incubated murine melanoma B16.F10 cells with physiological and increased concentrations of saturated LysoPC. The melanoma cells fastly removed the LysoPC from the medium which was accompanied by a radical shift in tumor cell membrane fatty acid composition towards saturated fatty acids. This had strong morphological and functional consequences for the tumor cells. Electron microscopic images suggested that the changed membrane composition leads to a strong increase in number and size of filopodial-like membrane protrusions. It became evident that these morphological changes are based on cytoskeletal contractions. However, these concentrations of LysoPC used did not possess direct cytotoxicity. An induction of apoptosis could also be excluded.

The functional basis for antimetastatic effects of LysoPC became exposed by investigating the adhesion receptor binding. As mentioned before, melanoma cells make use of their integrin VLA-4 binding the endothelial ligand VCAM-1 for vascular arrest in the metastatic cascade. LysoPC incubation affected crucially the VLA-4 activity in a concentration dependent manner, although the expression levels of this integrin were not changed. Exceeding the physiological LysoPC concentration, the melanoma cells lost their ability for VCAM-1 binding. Furthermore, even though the treated cells exhibited a remarkably augmented number of protrusions, the cell motility on fibronectin as essential requirement for distinct steps of metastasis, e.g. tissue transmigration was significantly attenuated. In addition, the interaction with platelets via P-selectin was also strongly diminished, which is a further factor for reduced metastasis. These *in vitro* findings were totally reflected in a syngenic intravenous lung-invasion model. Using *ex vivo*-treated B16.F10 cells, LysoPC concentrations above the threshold (450  $\mu\text{M}$ ) resulted in significantly reduced metastasis-like lesions in lung tissue.

The search for the molecular basis of these promising data is ongoing yet, but might lead to some preliminary postulations. On the one hand, biophysical aspects of membrane properties and their change by the saturated lipids can be discussed to affect integrin function. On the other hand, an interference of LysoPC with the integrin signalling at the level of GTPases can be assumed.

Referring to the first assumption, a balance between saturated and unsaturated fatty acids is a fundamental biophysical determinant of membrane fluidity (Mansilla et al., 2008). The so-called homeoviscous adaptation is highly regulated and influences important membrane properties as flexibility, and lipid raft composition (Mansilla et al., 2008; Stulnig et al., 2001; Callaghan et al., 1993; Hac-Wydro et al., 2007). This strongly indicates that the addition of exogenous fully hydrogenated LysoPC and the subsequent change of membrane composition might have a clear impact on the deregulation of these factors. Since the integrin function is dependent on several aspects of membrane compartmentation (as described before), a reduced VLA-4 function could result from changes in e.g. raft domains.

Concerning the expression of important signalling proteins, we observed slightly reduced gene expression of the GTPases RhoA, RhoB and others after LysoPC exposition (300  $\mu\text{M}$  and 450  $\mu\text{M}$ ) indicating an altered signal transduction in the context of membrane shape and adhesion (Alexander M. et al., 2010; 2011). RhoA and RhoB are known to participate in the regulation of cytoskeleton, proliferation, formation of filopodia, lamellipodia, stress fibers and adhesion complexes (Hall A., 1998; Bishop & Hall, 2000; Etienne-Manneville & Hall, 2002; Etienne-Manneville & Hall, 2001; Hall A., 2009). Thus, activation of Rho GTPases is necessary for signalling between cells and ECM and for maintenance of cell shape and associated focal adhesion complexes (Burridge & Wennerberg, 2004).

Furthermore, one main part of cellular crosstalk and adhesion is the signal transduction via the integrins and SFKs (Mainiero et al., 1995; 1997; Shaw et al., 1997; 2001). In addition, to the reduced gene expression of GTPases, we also identified that several integrin subunits (ITGA4, ITGA2 and ITGB1) are reduced in transcription by LysoPC exposition in a concentration-dependent manner (Alexander M. et al., 2010). Thus, one can assume interplay between the LysoPC incubation and the altered adhesion, membrane morphology and gene expression of the examined melanoma cells.

The exact signal pathway that is affected by LysoPC exposition has to be investigated in future analyses due to the fact that not only one or a few defined signal pathways are triggering the adhesion process via integrins (**Figure 4**). Nevertheless it is suggested that - due to the kind of deregulated genes - the ERK/MAPK pathway may be one key part of the promising antimetastatic effects initiated by LysoPC.

Genes which are regulated in expression levels by the ERK/MAPK pathway could influence the processes of invasion and adhesion. It is not completely understood if ERK/MAPK is activated or inhibited by LysoPC but specific kinases such as the dual-specificity phosphatase 1 (DUSP1) - a member of the threonine-tyrosine dual-specificity phosphatases - are deregulated by LPC treatment. Several members of the dual-specificity phosphatase (DUSPs) family are able to dephosphorylate MAPK isoforms with different specificity, cellular and tissue localization (Bermudez et al., 2010; Calvisi et al., 2008; Liu et al., 2007). The MAP kinases phosphatase (MKP) DUSP1/MKP-1 was shown to dephosphorylate ERKs (extracellular-regulated kinases), JNK and p38MAPKs (Liu et al., 2007). DUSP1 displays a rather broad specificity for inactivation of the ERK, p38 and JNK MAP kinases (Keyse, 2008). In addition, DUSP1 has detectable binding to ERK in vivo and is suggested to act as a positive activator of ERK in EGFR-mutant lung cancer cell lines independent of the ability to bind to ERK (Britson et al., 2009). Recent findings support the involvement of DUSPs in cancer progression and resistance (Bermudez et al., 2010) due to the fact that abnormalities in MAPK signalling have important consequences for processes critical to the development and progression of human cancer.

LysoPC exhibit a comparable chemical structure to the experimental anticancer drugs miltefosine and edelfosine (**Figure 5**). The treatment of cancer cells with those alkylphosphocholines (APCs), which are able to incorporate into specific membrane compartments can disturb the well balanced and organized lipid network and thus influence the signalling of associated proteins. Such effects were reported for edelfosine and miltefosine, which are known to induce changes in receptor interaction and consequently signalling processes (Ausili et al., 2008; Gajate et al., 2009; Mollinedo & Gajate, 2010).

Since LysoPC possess a remarkably rapid uptake in the membranes of cancer cells, a comparable cellular mechanism by the critical alteration of the integrity and functionality of specific membrane microdomains could be hypothesized for LysoPC with regard to the global gene expression data. Although LysoPC possesses an evident similarity in chemical structure compared to the APCs, LysoPC seems to activate different signalling pathways. One difference is that exposition with edelfosine leads to selective promotion of apoptosis in leukemic cells (Mollinedo et al., 1997; Gajate et al., 2000; 2004).

Edelfosine was tested as a pharmaceutical compound against prostate cancer (Berdel et al., 1981), human brain tumors, lung tumors and other cancer types (Berdel et al. 1984; Denizot et al., 2001; Haugland et al., 1999; Houlihan et al., 1987; Kosano & Takatani, 1988; Ausili et al., 2008). In detail, edelfosine exposition leads to accumulation in lipid rafts (van der Luit et al., 2002; Gajate et al., 2004) and to a reorganization of the lipid and protein composition of

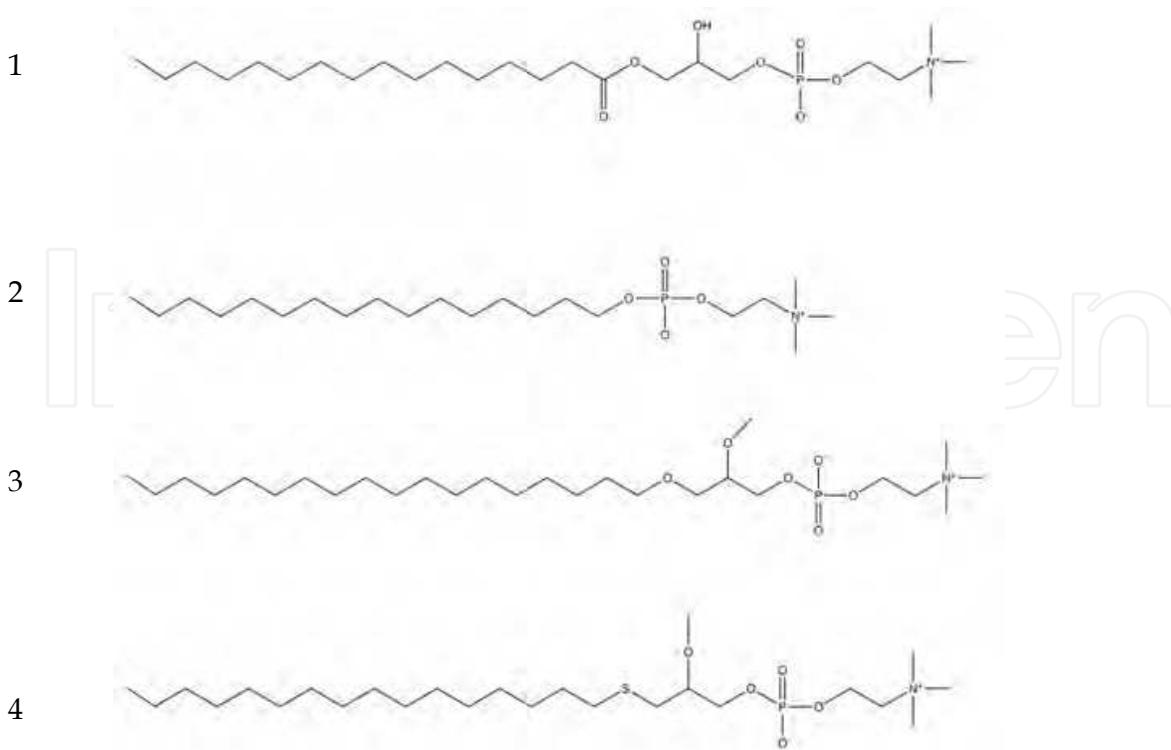


Fig. 5. Chemical structures of: Lysophosphatidylcholine (1), Miltefosine (2), Edelfosine (3) and Ilmofosine (4).

membrane caveolae (Gajate et al., 2004; Zaremborg et al., 2005; Gajate & Mollinedo, 2007). In a multiple myeloma animal model, oral administration of edelfosine showed a potent *in vivo* anti-myeloma activity and the drug accumulated preferentially in the tumor (Mollinedo et al., 2010). These data suggest that edelfosine incorporation in lipid rafts leads to a redistribution of sterols from the plasma membrane (Zaremborg et al., 2005). The redistribution of a major lipid raft component - they consist of cholesterol, glycolipids and sphingolipids - is likely to alter the biophysical properties of the lipid raft microdomain with putative important consequences for cell fate, due to the fact that the association of raft-targeted proteins is strongly assumed to be altered. However, there are no data available how edelfosine affects the integrin status of tumor cells.

In addition, the APC analog miltefosine (hexadecylphosphocholine) acts as another membrane-directed anti-tumoral and also anti-leishmanial drug (Santa-Rita et al., 2004). It activates anti-tumor effects against a broad spectrum of established tumor cell lines and solid tumors (Boggs et al., 1998; Wieder et al., 1998; Rybczynska et al., 2001; Jendrossek et al., 2002). Initial clinical studies have shown promising results: for example, miltefosine may be used for the treatment of cutaneous metastases of mammary carcinomas (Clive et al., 1999; Jimenez-Lopez et al., 2010).

Presently it remains open whether the antimetastatic effects and the serious affect on integrin activity by LysoPC are in line or on a comparable mechanistic level with the APC membrane effects. Future studies will provide insight into the hypotheses on membrane effects and the consequences for integrin localization and signalling. However, the non-competitive influence on the integrin activity by changing the lipid microenvironment appears as an interesting approach to interfere with integrin function in tumor cell metastasis.

## 6. Conclusion

The mortality rate of melanoma diseases is to a great extent related to the high tendency to form metastases via the lymph and blood system. An increasing insight into the molecular mechanisms of hematogenous metastasis offers new therapeutic options to interfere with the metastatic spread. Cellular adhesion receptors appear as attractive targets in that context, since adhesion molecules mediate several key events to allow the tumor cells the survival in the blood system and the settlement in the vascular bed of distant organs.

For a competitive blockade of the adhesion receptor function, heparin or non-anticoagulative heparin products possess most promises, since heparin is clinically accepted as anticoagulant and numerous preclinical data confirm the capacity of heparin to interfere with the selectins and selected integrins.

A novel strategy refers to a non-competitive influence on the adhesion receptors. A recent example is given by studies using LysoPC to melanoma cells, which drastically reduced the binding capacity of the integrin VLA-4 and thus, metastatic rate in mice. Although the exact molecular mechanisms are not fully elucidated, this might open new potential therapeutic options to reduce metastasis by interfering with adhesion molecules at the signalling level.

## 7. Acknowledgment

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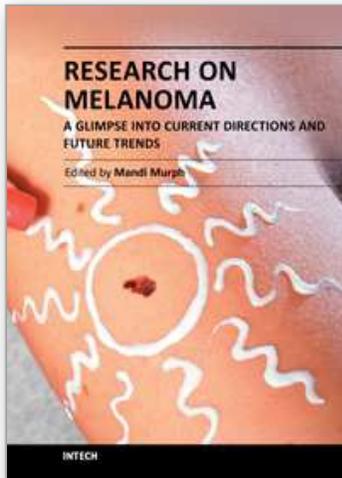
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## **Research on Melanoma - A Glimpse into Current Directions and Future Trends**

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The book *Research on Melanoma: A Glimpse into Current Directions and Future Trends*, is divided into sections to represent the most cutting-edge topics in melanoma from around the world. The emerging epigenetics of disease, novel therapeutics under development and the molecular signaling aberrations are explained in detail. Since there are a number of areas in which unknowns exist surrounding the complex development of melanoma and its response to therapy, this book illuminates and comprehensively discusses such aspects. It is relevant for teaching the novice researcher who wants to initiate projects in melanoma and the more senior researcher seeking to polish their existing knowledge in this area. Many chapters include visuals and illustrations designed to easily guide the reader through the ideas presented.

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