Rho-GTPases and Statins: A Potential Target and a Potential Therapeutic Tool Against Tumors?

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ABSTRACT

Rho GTPases, which control processes such as cell proliferation and cytoskeleton remodeling, are often hyperexpressed in tumors. Several members, such as RhoA/B/C, must be isoprenylated to interact with their effectors. Statins, by inhibiting the synthesis of prenyl groups, may affect RhoA/B/C activity and represent a promising tool in anticancer therapy.

Key words: Rho GTPases, cancer, isoprenylation, statins, chemotherapy

INTRODUCTION

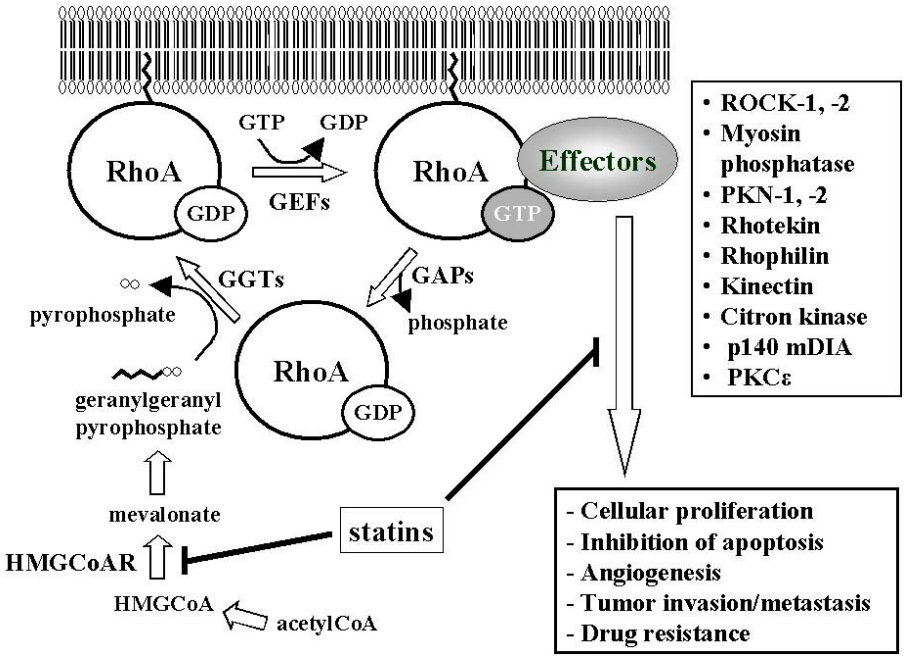
Rho GTPases belong to the Ras superfamily of low molecular weight (MW 20-30 kDa) monomeric GTP-binding proteins and are found in all eukaryotic cells [1; 2; 3; 4]. Until now, twenty mammalian genes encoding Rho GTPases have been described [4; 5; 6]. The most investigated members are Rho (Ras homologous), Rac (Ras-related C3 botulinum toxin substrate) and Cdc42 (cell division cycle 42). In this chapter we have focused our attention on the RhoA, RhoB and RhoC isoforms and on the effects of statins on Rho activity in human tumors. Similar to other regulatory GTPases, Rho proteins act as molecular switches cycling between an inactive GDP-bound state and an active GTP-bound state: in their GTP-bound form the Rho GTPases are localized at membranes and are able to interact with effector molecules initiating downstream responses. Their intrinsic GTPase activity turns the proteins back into the GDP-bound state thereby terminating signal delivery [2]. The activation of growth factor receptors and integrins can promote the exchange of GDP for GTP on Rho proteins: among the upstream activating agonists, we can mention epidermal growth factor (EGF), hepatocyte growth factor (HGF), lysophosphatidic acid (LPA), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), int-1/wingless (WNT1) [7]. The cycling between the GTP- and GDP-bound states is regulated by three types of regulatory proteins: (a) guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP to activate the switch [8]; (b) GTPase-activating proteins (GAPs), which stimulate the intrinsic GTPase activity to inactivate the switch [9]; and (c) guanine nucleotide dissociation inhibitors (GDIs), which, by binding many (but not all) Rho proteins, prevent their spontaneous activation in the cytosol [10] and favor their removal from the membranes at the end of the signaling process [11]. Besides activating Rho GTPases, GEFs participate also in the selection of downstream effectors [12]. To perform their biological functions, most Rho proteins have to dock onto cell membranes, by means of a lipid moiety, either a geranylgeranyl or farnesyl residue, attached to the cystein of the C-terminal CAAX box (C = Cys, A = aliphatic amino acid, X = any amino acid) [13; 2], a process catalyzed in the cytoplasm by either geranylgeranyltransferases or

farnesyltransferases, respectively [14]. The majority of Rho family proteins (i.e. RhoA, RhoC, Rac1, Cdc42, Rab, Rap1A) are geranylgeranylated, while only few members, such as RhoB, RhoD, Rnd, are farnesylated. Rho B has a unique behavior amongst Rho family members, since it may be geranylgeranylated as well as farnesylated; moreover it has an additional tail of palmitic acid [5]. The attachment of the isoprenyl group to the CAAX box promotes the translocation of the GTPases to the endoplasmic reticulum, where the AAX tripeptide tail is cleaved and the new C terminus is methylated. Following full processing, GTPases are directed to their cellular location, which is often the cytoplasmic surface of cell membranes, through mechanisms that are still poorly understood [15]. The Rho-specific GDI (RhoGDI) plays an important role in this regulatory context, because it masks the isoprenyl group, thereby promoting the cytosolic sequestration of Rho [10; 16]. Finally, Rho GTPases can be regulated through direct serine phosphorylation or ubiquitination, but the meaning of these covalent modifications in normal physiology is still unclear [4].

Activated Rho GTPases interact with a large number of effector molecules that, in turn, lead to the stimulation of signaling cascades promoting general cellular responses, such as cell migration, cell adhesion, cell polarity, gene expression, cell cycle progression and transformation, cell survival, secretion, phagocytosis, endocytosis and NADPH oxidase activation [3; 4]. RhoA is ubiquitous and seems to be strongly involved in all these cellular processes (**Fig. 1**). Also RhoB and RhoC proteins, which show a 85% homology with RhoA and are expressed in a great number of human tissues [5], regulate cell proliferation, polarity and migration [7; 17]. It is widely thought that Rho proteins may contribute to cancer due to their effects on cell migration (influencing invasion and metastasis) and proliferation (favoring the cell survival and growth), but, in contrast to the oncogenic Ras proteins (N-Ras, H-Ras, K-Ras), which are frequently mutated in human cancers, until now there are no reports of mutated, constitutively active forms of Rho proteins in tumors [7]. Only in haematopoietic cells of patients affected by non-Hodgkin's lymphoma it has been shown that RhoH gene is often mutated and rearranged, but it is not clear if this gene translocation may contribute to

the onset and progression of the disease [17; 18]. However, recent works have shown that several Rho proteins are overexpressed in human tumors and in some cases such increased expression is associated with a poor clinical outcome [7; 18].

Figure 1

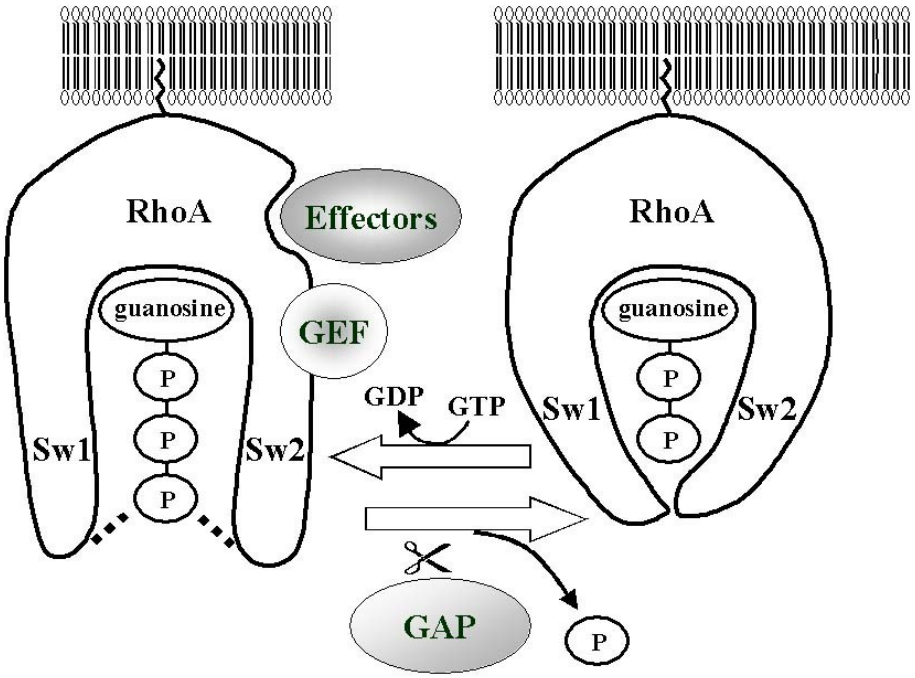


ROLE OF RHOA IN NORMAL AND TUMOR CELLS.

RhoA is a 21-kDa protein containing 193 amino acids. Crystal structure-based comparative analysis of GDP- versus GTP-bound Rho revealed conformational differences in two surface regions of the N-terminal half: *Switch region 1* and *Switch region 2*. These two domains interact with GDP or GTP, as well as with Rho-specific GEF [19]: in the GDP-bound protein, the Switch 2 region is close onto Switch 1 and has a disordered conformation. The binding of Rho-GEF to Switch 2 domain causes extensive conformational changes, facilitating the loss of GDP and unmasking the binding site for GTP. Aminoacidic residues involved in GTP binding lay on both Switch 1 and Switch 2 regions [19] (**Fig. 2**). The N-terminal half of RhoA contains the majority of the amino acids involved in GTP binding and hydrolysis, together with the Switch 1 and 2 regions [2]. The C-terminus of RhoA is essential for the correct localization of the protein, which is subsequent to the post-translational geranylgeranylation or farnesylation of the C-terminal cysteine [14; 15]. In

addition, the C-terminal peptide of RhoA has been recently indicated as an allosteric activator of AGAP proteins, a class of GAPs that recognize Arf proteins as substrates [20]. The activation of Arf proteins by AGAP controls membrane trafficking and actin organization.

Figure 2



RhoA usually shuttles between cytosol and plasma membrane, RhoB may localize on plasma membrane and endosomal vesicles, RhoC may be cytosolic or associated to perinuclear structure [5]. RhoA is a target for several bacterial toxins, which modify key conserved amino acids involved in its regulation [21]. *Clostridium botulinum* exoenzyme C3 transferase specifically ADP-ribosylates RhoA at asparagine-41, inhibiting its biological activity, probably by stabilizing the Rho/GDI complex and inhibiting the GEF-mediated nucleotide exchange of RhoA [22]. The large toxins A and B from *Clostridium difficile* block the RhoA interaction with downstream effectors by glucosylating the protein at threonine-37 [21].

RhoA and RhoC mRNA and protein are constitutively expressed during the cell cycle; on the opposite, the amount of RhoB protein is usually low, increasing during the G1/S phase transition, and is upregulated by growth factors [5]. Activated RhoA interacts with several effector molecules

including Rho-kinases (ROCK or ROK) 1 and 2, the myosin-binding subunit (MBS) of myosin phosphatase, protein kinase N (PKN) 1 and 2, rhotekin, rhophilin, kinectin, citron kinase, Lim kinase, p76RBE, protein kinase C (PKC)ε, p140 mDIA and DB1 transcription factor [23; 2; 4]. Similarly to GEFs and GAPs, effectors bind to RhoA through the Switch 1 and 2 regions, but the amino acids involved in the interaction with each target are different [2]. Although the downstream effectors of Rho proteins are often similar, slight differences exist among RhoA, RhoB and RhoC concerning their binding to specific GEF [24] or GAP proteins [25]. Furthermore, it has been reported that RhoC interacts with ROCK more efficiently than RhoA [26]. p120 β-catenin, a cytosolic effector of E-cadherin, can recruit and control the activation of ROCK1, which increases the actin polymerization, or of RhoAGAP, which turns off RhoA activity and ROCK1 effects [27]. This cycling represents the first known feed-back mechanism that controls the activity of RhoA and overcomes the schematic division into RhoA downstream and upstream effectors, since p120 β-catenin belongs to both classes. The progression of our knowledge on RhoA activation and deactivation will likely uncover other feed-back loops.

RhoA's functions in the cell are primarily related to cytoskeletal regulation. RhoA plays a central role in regulating cell shape, polarity and locomotion through its effects on actin polymerization, actomyosin contractility, cell adhesion and microtubule dynamics [2; 3; 4]. Amongst the ascertained effects of RhoA, it is known that RhoA is required for the generation of contractile force leading to rounding of the cell body [12] and that the proper localization of RhoA in the nucleus is essential during cytokinesis [28]. In particular the activity of the RhoA effectors Citron Kinase [28] and GEF-H1 [29] is necessary for the correct control of cytokinesis in non-transformed cells. RhoA is important for cell cycle progression through G1, since it regulates the expression of cyclin D1 and cyclin-dependent kinase inhibitors [4] and it is required for processes involving cell migration [30]. RhoA regulates the activity of a variety of biochemical pathways, including the activation of MAP kinases (MAPK), in particular c-Jun-N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and p38 kinase [31], as well as numerous transcription factors, such as serum

response factor (SRF) [32], activator protein 1 (AP-1) [33], nuclear factor kB (NF-kB) [34], c/EBPb, FHL-2, PAX6, GATA-4, E2F, ER-α, ER-β, CREB [35; 36] and STAT proteins [37; 38]. Rho GTPases show transforming activity by their own [7; 38; 39]: indeed, the overexpression of constitutively activated Rho proteins, such as RhoA, RhoG, Rac, Cdc42 and TC10, induces tumoral transformation in non-transformed fibroblasts [7; 40; 41]. Active Rho proteins are necessary for Ras-mediated oncogenic transformation [40; 42], whereas dominant negative mutants of Rac1 and RhoA inhibit the Ras transforming activity [40]. Although at a lesser extent, also the overexpression of RhoC seems to be related to the oncogenic transformation [5; 7]. On the opposite, RhoB has been described as an oncosuppressor gene [43; 44], and the loss of RhoB expression has been shown to be involved in lung carcinogenesis [45]. Curiously, the anti-tumoral action of RhoB in murine fibroblasts is evident only when RhoB is geranylgeranylated, while it is lost if the protein is farnesylated [46].

RhoA overexpression confers to cancer cells a highly invasive phenotype. LPA, a strong activator of RhoA, promoted matrix invasion and metalloproteinase activity in ovarian cancer [47]. A highly active RhoA is necessary for the cellular motility in prostate cancer [48], where the GTPase is negatively controlled by the endocannabinoid receptors-dependent signaling [49]. RhoA favors cell motility also in tumors with aberrant activity of ephrin-B receptor [50] or E-cadherin/epidermal growth factor receptor [51]. The hyperactivity of RhoA-related proteins, such as ROCK [52] or Dia1 [53], enhanced the invasive attitude in tumors, while the overexpression of the tumor suppressor gene Deleted in Liver Cancer (DLC1) greatly reduced the cell motility in hepatocellular carcinoma because of the RhoGAP activity of DLC1 [54]. In mice injected with human pancreatic cancer cells, liver metastatic nodules were reduced when cells were transfected with the p190 RhoGAP, which slackens the RhoA signaling [55]. In normal and transformed breast epithelium, EGFR and β3-integrin control p190 RhoGAP and RhoA, which increases filopodia formation and cell migration [56]. This mechanism is important for the shape change that occurs during epithelial-mesenchymal transition and matrix invasion by breast cancer cells. RhoA GEF-H1 is another factor

favoring cell invasion: in breast cancers it is under the transcriptional control of the "human pituitary tumor-transforming gene" oncogene, which up-regulates RhoA GEF-H1 and RhoA activity, increasing cell migration [57].

RhoC has a minor effect than RhoA on cell proliferation [58], but confers to cancer cells a highly invasive attitude [58; 59] and is directly related to an increased number of lung metastasis [60].

Several types of human cancers have been analyzed for Rho proteins mutations or overexpression [61]. RhoA levels are significantly increased in breast cancer, correlating with the tumor grade [62; 63; 64]. RhoA mRNA is higher in ovarian carcinoma: such an increase is particularly significant in metastatic lesions of peritoneal dissemination than in the respective primary tumors [65]. Protein expression of RhoA and its two downstream effectors ROCK1 and ROCK2 is significantly higher in testicular germ cell tumors [66]. The overexpression of RhoA GEF-H1 has been also described in aggressive cancers, where it is associated to high aneuploidy due to aberrant mitosis [29] and high invasion [57].

RhoA may control several autocrine loops in tumor cells: for instance, in transformed lung epithelium, active RhoA increases the synthesis and secretion of prostaglandin E2 [67], which is critical for epithelial tumor growth. Another attractive autocrine mechanism is the tumoral secretion of exosomes, small vesicles produced by tumor cells and carrying growth factors, cytokines, receptors, miRNA, which support or repress cancer cell proliferation. Recently, the RhoA/ROCK1/Lim Kinase pathway has been identified as a controller of secretion of exosomes with transforming activity on mitotically arrested cells [68]. RhoA also mediates the effects of endocrine messengers, as suggested by the higher responsiveness to androgens in prostate cancers overexpressing RhoA [69]. This effect is due to the RhoA/ROCK1-operated nuclear localization of transcription factors, like the so-called "serum response factor megakaryocytic acute leukemia cofactor", which cooperate with androgen receptor in androgen-dependent prostate tumors [69].

Furthermore, RhoA has been suggested as an useful prognostic factor of the invasion and metastasis of upper urinary tract cancer: RhoA and ROCK protein levels are elevated in bladder cancer,

showing higher expression in less differentiated tumors and metastatic lymph nodes [70]. The expression and activation of RhoA is greater in small cell lung carcinoma than non-small cell lung carcinoma cell lines [71]. Patients with esophageal squamous cell carcinoma overexpressing RhoA tended to have poor prognosis compared with patients with RhoA under-expression [72]. RhoA was found frequently overexpressed in gastric cancer compared with normal tissue [73]. Invasiveness of hepatocellular carcinoma is facilitated by the RhoA/ROCK pathway and is likely to be relevant to tumor progression [74]. A high proportion of colon cancers overexpresses RhoA [75] and the inhibition of RhoA activity through the introduction of dominant negative mutants completely abolishes the invasive capacity of colonic epithelial cells [76]. Plasminogen Activator Inhibitor type-1 is important for matrix invasion by colon cancer cells: its localization in the connective tissue surrounding transformed cells creates selective "hot spots" in the plasma membrane of tumor cells where RhoA and ROCK1 are activated and promote cells blebbing and epithelial-mesenchymal transition [77], one of the first steps of metastasis. The epithelial-mesenchymal transition in colon cancer is also supported by the increased activity of the mammalian Target of Rapamycin Complexes mTORC1 and mTORC2, which have RhoA and ROCK as downstream effectors [78]. These results suggest that the RhoA/ROCK axis may act as a collector of multiple signals, all promoting matrix invasion and cell migration. Furthermore, the RhoA/ROCK pathway has been implicated in the vascular endothelial growth factor (VEGF)-mediated angiogenesis [79], which is also increased in highly proliferating tumors. These evidences suggest that RhoA activation should be considered a strong marker of aggressive tumors.

As far as RhoC is concerned, its expression has been related to a more aggressive phenotype in ovarian [65], head and neck cancer [80] and in melanoma [81]. In contrast, only one contradictory study reports that RhoC enhances the tissue invasion, without affecting the directional motility of prostate cancer cells [82]. Recently, RhoC has been also proposed as a novel biomarker of tumor invasiveness, metastasis [83] and poor prognosis [84]. The selective silencing of RhoC increased the expression of oncosuppressor genes and reduced cell migration and anoikis in breast and

prostate cancer cells [85]. Both RhoA and RhoC activities are necessary to explain the highly metastatic behavior of Erb2-overexpressing breast cancers, where ErB-2 oncogene recruits and phosphorylates the semaphorin receptor Plexin-1, which activates both GTPases [86].

On the other hand, RhoA and RhoC have sometimes mutually exclusive signals, due to the competition for RhoA-GDI: for instance, only when RhoC is removed by gene silencing, RhoGDIα can stabilize RhoA and promote its activation by RhoGEF, resulting in an increased activity of RhoA-operated pathways [85]. In the case of cell migration, RhoC preferentially promotes a directed and polarized migration, through the downstream effector FMNL3, which reduces the spreading of lamellipodia; the effects of RhoA are more variable, depending on which type of kinases is predominantly activated: ROCK2 promotes a polarized cell movement, whereas ROCK1 is specifically involved in the tail retraction events on the opposite side of the migratory front [87].

These and other *in vitro* and *in vivo* studies provided good evidence that RhoA and RhoC activation is highly relevant for tumor progression and invasiveness [88;89], and have suggested that abrogation of RhoA and RhoC functions could be a promising strategy to attenuate tumor metastasis [90; 91; 92; 93].

Synthetic compounds affecting the geranylgeranylation [94] or the post-translational modifications of RhoA [95], bacterial toxins [96] and specific anti-RhoA small interfering RNA (siRNA) [97] have shown anti-tumor activity. However, many of these strategies have dose-limiting toxicity [94] and have only been tested *in vitro* [93]. Other therapeutic tools have been addressed to inhibit the downstream RhoA effectors. Y-27632, which specifically inhibits the ROCKs [98], largely reduced metastasis in animal models [90] and the newly developed ROCK inhibitor Wf-536 reduced angiogenesis, tumor growth and metastasis *in vivo* [99; 100]. Fasudil [1-(5-isoquinolinesulfonyl)-homopiperazine, also known as HA-1077 and AT877], another ROCK inhibitor currently used in the treatment of cardiovascular [101] and neurological disorders [102], blocked the tumor progression in animal models [103] and exhibited anti-angiogenic properties [104]. A further strategy is to reduce the amount of active geranylgeranylated RhoA by statins.

STATINS INHIBIT RHOA ACTIVITY

By inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), statins decrease the synthesis of cholesterol and isoprenoids molecules, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) [105]. By this way, statins may impair the isoprenylation and the activity of Ras and Rho family G-proteins [92]. Nowadays, many natural and synthetic statins (**Table 1**) are used in clinical practice as anti-cholesterolemic agents [105], in the prevention therapy of coronary artery disease (to view the structures of main statins, see [106]). Statins inhibit HMGCoAR by binding to the HMGCoA pocket with a common hydrophobic bulk, whereas the other substitute groups are positioned in a non polar groove [105]. In consequence of the high number of van derWaals interactions formed with the enzyme, statins tightly bind at nanomolar concentrations, displacing the physiological substrate HMGCoA, which binds at micromolar concentrations [107]. Small differences in the chemical structure account for the different kinetic properties of each drug [108].

Factors other than the reduction of cholesterol synthesis have been invoked to justify such a variety of therapeutic properties [109]. Many statins' effects appear more related to the inhibition of RhoA activity than to the decrease of cholesterol synthesis. For instance fluvastatin prevents heart dysfunction and interstitial myocardial fibrosis in diabetic rats by inhibiting RhoA activity [110]. Using the same mechanism, statins inhibit the smooth muscle cells proliferation [111] and the cardiac remodeling [112] in hypertensive rats, and decrease the secretion of lipoprotein-associated phospholipase A2 by macrophages in atherosclerotic lesions [113]. Recently, pitavastatin has been employed as inhibitor of the accumulation of Tau protein in neurons, an effect due to the decrease in RhoA/ROCK1 pathway [114] and that opens new perspectives for the therapeutic use of statins in Alzheimer disease.

Table 1. Chemical, pharmacodynamic and pharmacokinetic properties of the most employed statins.

Compound	Chemical properties	Ki (nM) HMGCoAR *	IC_{50} ** (nM)	Biovailability *** (%)	Plasma $t_{1/2}$ *** (h)
Cerivastatin	- Hydrophobic drug - Entry in cells by passive diffusion	1.3	5	60	2-3
Simvastatin	- Hydrophobic drug -Administered as a lactone prodrug, which needs to be activated in liver -Entry in cells by passive diffusion - Substrate of ABC-transporters	0.1	345-1500	< 5	1.9
Atorvastatin	- Hydrophobic drug - Entry in cells by passive diffusion	0.5-1	40-100	41	12-58
Lovastatin	- Hydrophobic drug - Administered as a lactone prodrug, which needs to be activated in liver - Substrate of ABC-transporters	0.6	24-50	< 5	1.5
Pravastatin	- Hydrophilic drug - Substrate of ABC-transporters	2.3	700-2650	10-26	1.8
Fluvastatin	- Hydrophilic drug - Substrate of ABC-transporters	0.3	30-43	25	0.5

Adapted from Moghadasian [107].

* HMGCoAR: 3-hydroxy-3-methylglutaryl coenzyme A reductase.
** Concentrations resulting in the 50% inhibition of cholesterol synthesis in HepG2 human hepatoma cells.
*** After oral administration.

STATINS AND TUMOR GROWTH/APOPTOSIS

Since the overexpression of the enzymes of mevalonate pathway cooperates with Ras to promote malignant transformation [115], drugs inhibiting this pathway have been regarded in the last years

as attractive anti-cancer tools. It is conceivable that statins slacken the rate of cell proliferation by lowering the synthesis of cholesterol, a major component of cellular membranes. However, an increasing number of experimental evidences suggest that the inhibition of RhoA isoprenylation is a crucial mechanism in reducing tumor growth and eliciting apoptosis [116;92]. Statins exert *in vitro* and *in vivo* anti-proliferative effects in solid [117; 118] and hematopoietic malignancies [119; 120]. The statin-mediated mitotic arrest was related to the reduced RhoA isoprenylation: for instance, the addition of GGPP or mevalonate, but not FPP or cholesterol, and the expression of constitutively active RhoA prevented the lovastatin-induced G1 phase cell cycle arrest and cell senescence in human prostate cancer cells [121]. The pro-apoptotic effect of statins has been related to the lowering of protein geranylgeranylation also in glioblastoma [122], melanoma [123] and acute myeloid leukemia [124]. By gene microarray approach, RhoA has been shown to be one of the genes modulated by lovastatin in cervix and head and neck squamous carcinomas cells [125]. The statin-induced apoptosis in these tumors was prevented by supplying GGPP and restoring RhoA isoprenylation [125]. The mechanism by which the reduced RhoA isoprenylation leads to growth arrest and apoptosis of tumor cells still remains to be elucidated. The lovastatin-mediated mitotic arrest in human prostate cancer cells was associated with a rapid alteration of phosphorylation state of Rb protein, a decrease in E2F-1, cyclin A and cdc2, and an accumulation of p27 protein level, leading to a significant reduction in the proportion of S phase cells [121]. Similarly, lovastatin decreased cell proliferation of anaplastic thyroid cancer cells by reducing RhoA/ROCK1 activity, which lowered cyclin A2 and cyclin D3 and increased the amounts of p27 and cyclin-dependent kinase 4, producing a G0/G1-arrest [126]. In prostate PC3 cancer cells, the cell cycle arrest induced by atorvastatin was accompanied by an increased expression of LC3-II, indicative of enhanced autophagy; this event was prevented by the addition of geranylgeraniol, suggesting that the statin inhibited a geranlygeranylated protein [127]. Such GTPase has not yet been identified.

In human breast cancer cells the simvastatin-induced apoptosis was mediated by the JNK pathway [128], while in human osteosarcoma lipophilic statins promoted apoptosis by inhibiting RhoA

activity and decreasing phospho-p42/p44 levels [129]. In contrast with all these evidences, we did not find any anti-tumor activity by atorvastatin in Her2/neu-overexpressing mammary cancer: although atorvastatin decreased Ras and extracellular-regulated kinase (ERK) 1/2 activity, thus slowing down pathways that are critical for cell proliferation, it simultaneously decreased RhoA/ROCK signaling, which resulted in an increased activity of the pro-survival factor NF-kB [130]. Interestingly, Ras and RhoA displayed a differential sensitivity to atorvastatin and the latter was the most inhibited by the drug: it means that at doses compatible with those used in hypercholesterolemic patients, the proliferative signals derived from the inhibition of RhoA balanced the anti-proliferative signals derived from the inhibition of Ras [130]. To our knowledge this is the first evidence proving an antagonistic effect between Ras and RhoA in terms of tumor growth and suggests that the anti-tumor effect of statins can be highly variable and tumor-dependent.

It cannot be excluded that the anti-proliferative and pro-apoptotic effects of statins may be mediated by Rho proteins other than RhoA: for instance, the downregulation of the RhoC protein by antisense oligonucleotides [131] or siRNA [132] induced the arrest of proliferation as well as the apopotic death of cancer cells. However, no reports link the statin action to a selective inhibition of RhoC proteins.

In addition statins may also increase cellular differentiation: for instance, lovastatin was able to promote differentiation in neuroblastoma cells and in acute myeloid leukemia cells [133]. The effect of lovastatin on immature leukemia cells was similar to that evoked by retinoic acid: both drugs increased the expression of the integrins CD11b and CD18 and decreased the expression of bcl-2 protein. These changes were associated with late stage differentiation of the myeloid cells and were considered as an index of myeloid blasts maturation [133]. Lovastatin also promoted the neurite growth and immature pheochromocytoma cells, transforming them into more differentiated neuronal cells [134]. Again, such an effect was reverted by mevalonate and geranylgeraniol [134].

Not all statins exert a pro-apoptotic effect at the same extent, because of the different

pharmacokinetic and pharmacodynamic properties [124]. Besides being direct pro-apoptotic agents, statins also enhanced the apoptosis induced by other chemotherapeutic drugs [118; 135]. Such effect was prevented by GGPP [118]. In several cases, statins have been also observed to exert anticancer effects independently of the mevalonate pathway [136; 137].

Recently, the overexpression of mevalonate pathway genes has been reported in breast cancers with mutated p53, where it is predictive of poor prognosis [138]: mutated p53 enhanced the transcription of mevalonate pathway genes and increased the invasive growth of cancer cells. Since simvastatin and the inhibitor of geranylgeranyltransferase fully prevented the latter event [138], it is likely that a geranylgeranylated protein of Rho family is involved.

STATINS AND ANGIOGENESIS

Both pro- and anti-angiogenic effects of statins have been widely described [139; 140; 141]. On one hand, statins increased the differentiation of endothelial progenitor cells in mice and humans [142] and stimulated the capillary formation through a hsp90- and nitric oxide (NO)-dependent mechanism [139]. On the other hand, statins blocked the proliferation and promoted the tumor necrosis factor (TNF)-α-mediated apoptosis of endothelial cells [141], inhibited the formation of vascular tubes [140], and prevented the matrix remodeling [143]. Recently it has been reported that simvastatin, fluvastatin and cerivastatin reduce the endothelial cell growth also under hypoxia [144], an environmental condition resembling that occurring in the inner core of solid tumors. The sensitivity to the anti-angiogenic effect of statins is strictly dose- and cell type-dependent [145; 146]. In human vascular smooth muscle cells and microvascular endothelial cells, which constitutively produce large amounts of VEGF, statins reduced the VEGF secretion; on the opposite, in primary macrovascular endothelial cells, which do not basally secrete VEGF, statins were pro-angiogenic at less than 1 μM and anti-angiogenic at higher concentrations [146].

In a recent screening aimed to discover new anti-angiogenic drugs for prostate cancer, four statins (mevastatin, lovastatin, simvastatin, rosuvastatin) have been identified amongst the leading anti-angiogenic compounds; rosuvastatin was the most potent in vitro and efficiently decreased the

tumor growth in mice xenografts, thanks to a dual action, i.e. the reduction of microvessel density within tumor and the induction of apoptosis in tumor cells [147].

There is general agreement that most statins' anti-angiogenic effects are mediated by RhoA and RhoC inhibition. The active RhoA/ROCK pathway stimulates angiogenesis by increasing the secretion of VEGF, interleukin (IL)-6 [148] and IL-8 [149], by modulating the activity of metalloproteinase-9 [150] and by regulating the cytoskeletal remodeling and the cellular migration [143]. The ROCK inhibitor Fasudil indeed has demonstrated to possess anti-angiogenic properties in human endothelial cells [151]. The overexpression of RhoC in breast cancer cells led to increased secretion of pro-angiogenic factors, such as VEGF, basic fibroblast growth factor, IL-6 and IL-8 [152], in a MAP-kinase dependent way [153]. Both the cerivastatin-induced decrease of endothelial cell locomotion *in vitro* and the simvastatin-elicited decrease of capillary growth *in vivo* were reversed by GGPP [143; 154]. The available experimental evidences suggest that RhoA and RhoC are mainly involved in favoring angiogenesis and may be considered promising targets in the anti-angiogenic therapy. Recently RhoB expression has been shown to be crucial to regulate the endothelial survival and proliferation during the physiological vascular development [155]; however the role of RhoB in the tumor angiogenesis and the effects of statins on RhoB activity still remain to be elucidated.

STATINS AND METASTASIS

Statins inhibited the invasiveness of human colon carcinoma cells [156], human pancreatic cancer cells [157] and human anaplastic thyroid cancer cells [158]. It has been reported above that RhoA overexpression is highly relevant for tumor progression and invasiveness. In the aggressive breast cancer MDA-MB-231 cells the anti-invasive properties of statins were related to the inhibition of the RhoA/ROCK/NF-kB pathway [159]. NF-kB, whose nuclear translocation may depend on RhoA activity [48; 160; 161], in turn up-regulates the expression of genes involved in cellular invasiveness, such as urokinase-type plasminogen, tissue factor and metalloproteinase 9 [159]. Statins inhibited cell motility also by disrupting the RhoA/Focal-Adhesion-Kinase (FAK)/Akt

signaling [162]: it has been reported that RhoA activity is necessary for the tyrosine phosphorylation and activation of FAK [162; 163], which is then responsible for the activation of the Akt kinase [164]. Akt may further enhance the nuclear translocation of NF-kB [162]. Interestingly, the effects of lovastatin were nearly absent in the less invasive breast cancer MCF-7 cells [159], but a differential activity of RhoA was not further investigated. Moreover, lovastatin impaired the TNF-α- and RhoA-dependent increase of E-selectin in human endothelial cells, reducing a potential mechanism of cancer cell adhesion and transendothelial migration [165]. RhoB seems responsible for the increase of E-selectin caused by TNF- α as well [165]. Statins showed good efficacy in reducing metastasis also in vivo: fluvastatin and lovastatin decreased the metastatic ability of renal cancer cells [166] and mammary carcinoma cells [167]. In the latter model lovastatin impaired the secretion of urokinase, a key proteolytic enzyme during tumor invasion [167]. In a murine model of melanoma, simvastatin and fluvastatin reduced the number of lung metastasis by decreasing the expression of metalloproteinases and α2-, α4-, α5-integrins, other molecules important for tumor cell invasion; these effects have been attributed to the lower amount of RhoA localized at the plasma membrane and to the lower phosphorylation of Lim kinase and myosin light chain in animals exposed to statins [168]. A similar decrease of integrins, due to the low activity of RhoA/ROCK pathway, was reported in invasive hepatocellular carcinoma cells treated with simvastatin [169].

Due to the central role of RhoC in tumor invasion and metastasis [60], several studies pointed out a relationship between the anti-metastatic effect of statins and the specific inhibition of RhoC in human cancers: for instance atorvastatin lowered the metastatic attitude of melanoma cells by decreasing the RhoC isoprenylation [170]. By preventing the activation of both RhoA and RhoC, fluvastatin impaired the transendothelial migration of MDA-MB-231 cells [171]. Furthermore, the inhibition of both RhoA and RhoC, by specific siRNA [132; 172], prevented the matrix invasion by human breast cancer cells.

STATINS, CHEMOTHERAPY EFFICACY AND MULTIDRUG RESISTANCE

In vitro studies reported that statins synergized with γ rays [173], doxorubicin, paclitaxel and 5-fluorouracile [174] in reducing cancer growth. Besides increasing the sensitivity to doxorubicin, lovastatin also reduced the drug cardiotoxicity in mice, via an hypothetical lipid-lowering effect [175]. On the other hand, in a limited group of experimental works, statins and chemotherapeutic agents had no synergistic effects [176; 177]. It has been hypothesized that the p53 level may influence the efficacy of statins: indeed pravastatin and atorvastatin sensitized p53-deficient tumor cells to etoposide, doxorubicin and 5-fluorouracil, but failed in p53 wild-type cells [178]. Several evidences pointed out that the inhibition of RhoA isoprenylation is involved in modulating the response to chemotherapy. For instance, lovastatin increased the apoptotic effect of 5-fluorouracil or cisplatin in human colon cancer cells, whereas the addition of GGPP prevented the cell death [118]. Fluvastatin enhanced the pro-apoptotic effect of gemcitabine in pancreatic cancer *in vitro* and *in vivo* and such an effect was prevented by the administration of mevalonic acid [135]. Interestingly, fluvastatin increased the expression of deoxycytidine kinase, the enzyme required for the activation of gemcitabine, and simultaneously reduced the level of 5α-nucleotidase, responsible for its catabolism [135].

Multidrug resistance (MDR), an acquired or constitutive cross-resistance towards many unrelated anti-cancer drugs, is the major obstacle to a successful pharmacological therapy of tumors [179]. Many statins are substrates of ATP-binding cassette (ABC) transporters, like P-glycoprotein (Pgp) and MDR-related proteins (MRPs) [180; 181], whose overexpression mediates the enhanced efflux of chemotherapeutic agents [179]. ABC transporters are membrane pumps which bind and hydrolyze ATP, thus mediating the active efflux of endogenous metabolites and drugs [179]. Lovastatin, simvastatin, fluvastatin and pravastatin are transported out of the cells by Pgp [182], which is also responsible for the efflux of anthracyclines, Vinca alkaloids, epipodophyllotoxins, taxanes, actinomycin-D, mitoxantrone [179]. Therefore, statins might affect the accumulation of chemotherapeutics in cancer cells by competing with them for the same ABC pump-mediated

transport [181]. Statins induced a selective apoptosis in drug-resistant cancer cells [183; 184]: the molecular mechanism was not fully clarified, but it has been reported that drug-resistant cells were partially protected from statins-induced apoptosis by the addition of FPP and GGPP [185]. Furthermore, a recent study implicates RhoA in MDR: hepatocellular carcinoma cells overexpressing the Rho-specific GEF Lymphoid blast crisis (Lbc) were resistant to doxorubicin, but this resistance was reverted by the C3 exotoxin from *C. Botulinum* [186]. These evidences suggest that statins could revert MDR by impairing the RhoA operation. Indeed, atorvastatin increased the doxorubicin's cytotoxic efficacy and accumulation in both sensitive and drug-resistant human colon cancer cells [187]. Interestingly, such effect of atorvastatin was mediated by its ability to induce the cellular synthesis of NO, which in turn may nitrate the ABC transporter MRP3, leading to a reduced efflux of doxorubicin [187]. The increased synthesis of NO followed by the nitration on ABC transporters was not statin-specific: indeed simvastatin produced the same sequence of events in colon cancer [188]. The molecular basis of the statins' effect was clarified in the human malignant mesothelioma, which is highly resistant to a large number of chemotherapeutic agents: both mevastatin and simvastatin corrected the doxorubicin resistance of mesothelioma cells by inhibiting the RhoA/ROCK pathway [189]. The statins' effects, reverted by mevalonic acid and mimicked by Y-27632, were NO-dependent [189]. These results led to hypothesize that the inhibition of RhoA/ROCK causes the activation of the NF-kB transcription factor and the subsequent induction of NO synthase: in mesothelioma cells the increased synthesis of NO was accompanied by the nitration of another ABC transporter, the Pgp [189]. The central role of RhoA GTPase in modulating NO synthesis and MDR was confirmed in RhoA-silenced doxorubicin-resistant colon cancer cells, where the only depletion of RhoA was sufficient to turn the drug-resistant phenotype into a drug-sensitive one [190].

A cell adhesion-mediated drug resistance (CAM-DR), dependent on Wnt3 overexpression and RhoA/Rho kinase activity [191], is often observed in myeloma cells. Also CAM-DR was totally overcome by statins and specific inhibitors of geranylgeranyltransferases and ROCKs [192].

The inhibition of RhoA does not always produce a chemosensitization: for instance, lovastatin conferred cross-resistance to doxorubicin and etoposide in human endothelial cells [177] and the expression of constitutive active RhoA induced a significant resistance to etoposide, 5-fluorouracil and taxol, but increased the sensitivity to vincristine in human prostate carcinoma cells [193]. In the light of these findings, we cannot exclude that the inhibition of RhoA by statins may modulate both chemotherapy efficacy and MDR, with different effects depending on the anti-cancer agent and on the type of tumor.

STATINS AND CHEMOPREVENTION OF TUMORS

In a small number of studies, statins exhibited a carcinogenic and genotoxic effect, but HMGCoAR inhibitors were used at concentrations higher than the common therapeutic doses [194; 195]. By inhibiting cellular proliferation and invasion, statins are likely to exert rather a cancer-preventing effect. Indeed the chemopreventive action of statins was confirmed in *in vivo* models of chemical carcinogenesis [196; 197] or pre-cancerous diseases, such as ulcerative colitis [198] and familial adenomatous polyposis [199]. The oral administration of statins, at a dose very close to that used in the treatment of cardiovascular diseases, efficiently reduced the growth of breast cancer in mice, through a MAP-kinase- and NF-kB-dependent mechanism [200].

In mice with pancreatic intraepithelial neoplasms, atorvastatin prevented the transition into invasive adenocarcinoma, reducing the activity of RhoA and of other molecules favoring survival and/or proliferation, such as PI3K, Akt, PCNA, p27, cyclin D, survivin, β-catenin [201].

Yet, when considering the cancer prevention in patients regularly taking statins, conflicting data exist: some case-control studies and randomized controlled trials found no association between the use of statins and reduced frequency of solid tumors [202; 203]. Only a long-term therapy with statins partially lowered the incidence of tumors [204]. On the opposite, other studies showed that statins efficiently reduced the incidence of pancreatic cancers [205], as well as metastasis and mortality in advanced stages of prostate cancer [206]. Randomized controlled trials for preventing

cardiovascular disease indicated that statins reduced the incidence of colorectal cancer and melanoma [207].

Experimental evidences are not yet available in support of the hypothesis that the *in vivo* chemopreventive action of statins is due to the inhibition of Rho proteins. Interestingly, statins in combination with non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to prevent colorectal cancer. In mice affected by adenomatous polyposis, atorvastatin and the cyclooxygenase 2 (COX2) inhibitor celecoxib synergistically prevented the development of colon adenocarcinoma [199]. Similarly, in a population-based case-control study, the association of aspirin and statins was more chemopreventive than the single drugs [208]. It has been reported that COX2 induces the activation of the RhoA/ROCK pathway, leading to the disruption of cellular adherens junctions and increased motility of colon cancer cells [209]. Since Rho and COX2 activities appear to be strictly related in colon cancer cells [210], the synergistic effect of statins and NSAIDs could be exerted by inhibiting a COX2/Rho/ROCK pathway, but this hypothesis needs to be still confirmed.

STATINS IN CANCER TREATMENT

The anti-cancer effect of statins was analyzed in different human clinical trials: the therapy with statins was well tolerated and did not enhance the adverse effects of anti-cancer drugs [211; 212] or radiotherapy [213], but conflicting results were reported about its efficacy [211]. The limited number of patients taking statins [202], the advanced stage of the disease and the too small median survival of patients [211] may affect the statistical potency of these studies. Some variability of response in hepatocellular cancer has been described: fluvastatin exerted a different anti-proliferative effect in mice, depending on the tumor stage [214], and the addition of pravastatin to the 5-fluorouracil therapy significantly prolonged the patients survival [215]. However this result was not confirmed by subsequent studies [216].

Better results have been obtained in hematological malignancies: simvastatin stabilized the disease progression in patients both sensitive and resistant to chemotherapy [217] and reversed the resistance to bortezomib and bendamustine in patients with relapsed myeloma [218]. Similarly,

lovastatin improved the clinical response and the overall survival of patients with relapsed myeloma, if added to the standard therapeutic regimen (thalidomide and dexamethasone) [219]. The statins' effect in myeloma was attributed to the reduced prenylation of small G-proteins, including the Rho homologue Rap1 [217]. In a phase 1 study, pravastatin, added to idarubicin and cytarabine, obtained encouraging response rates in patients with acute myeloid leukemia [212]. In this type of tumor the exposure to cytotoxic drugs evoked an increase of cholesterol synthesis and chemoresistance, whereas statins restored the chemosensitivity by lowering the cholesterol levels [220]. Most of these experimental works provided only preliminary results and did not investigate the molecular mechanisms of the action of statins.

Other drugs targeting the mevalonate pathway, like the anti-osteoporotic drugs aminobisphosphonates, which inhibit isopentenyl diphosphate (IPP) isomerase and FPP synthase [221], showed anti-tumor activity and slackened the progression of metastasis in cancer patients [222]. Interestingly aminobisphosphonates exhibited anti-angiogenic properties by suppressing RhoA activity [223]. The association of statins and bisphosphonates was more effective than the single drugs in reducing the geranylgeranylation of proteins [224], and clinically achievable concentrations of fluvastatin and zoledronic acid synergistically induced apoptosis in cancers [225]. Another noteworthy recent study reported that simvastatin decreases the invasive attitude of p53-mutated breast cancer cells by impairing the activity of an unknown geranylgeraylated protein [138]: this result looks particularly appealing because it is the first evidence that statins treatment corrects the phenotypical consequences of a genetic mutation. RhoA, RhoB and RhoC are under intensive investigations as antitumor targets, with promising results: the C3-transferase homologue CT04, a cell-permeant inhibitor of the three Rho GTPases, but not of other geranylgeranylated or farnesylated proteins, efficiently reduced cell migration in ovarian cancer [226], one of most invasive and chemoresistant tumors. Narciclasine, a novel selective RhoA inhibitor extracted from Amaryllidaceae plants, has been tested *in vivo* against primary and metastatic brain tumors, showing good anti-tumor efficacy and few side-effects [227]. Furthermore the experiments in mice

also suggest that this new inhibitor has an excellent delivery across the brain-blood barrier, which is hardly crossed by many other anti-cancer drugs.

Taken as a whole, present evidences suggest that the inhibition of RhoA might be an important anti-cancer tool *in vitro* and *in vivo*. Moreover, also the reduction of RhoC activity may decrease the tumor invasiveness and metastasis. The relative importance of the inhibition of these two isoforms in the efficacy of anti-tumor therapy with statins has to be still clarified. As to RhoB, which may have differential (enhancing or suppressive) effects on carcinogenesis, depending on the nature of its prenylation [46], the prevailing effect of statins is not known. Specific siRNA have been constructed to knock-down Rho proteins separately, but they have been only applied in mice models or in *in vitro* studies [93; 94; 97]. Presently it can be only affirmed that, by inhibiting the isoprenylation, statins lower the activity of RhoA and RhoC, and subsequently may impair the promoting effects of these GTPases in the development of many tumors. This is a stimulus to keep on investigating statins (and other inhibitors of Rho and Rho-associated regulators and effectors) as potential tools in the future anti-tumor therapy.

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Figure Legends

Fig.1. Schematic representation of the activation/inactivation cycle of the small GTPase RhoA, of the ultimate effects of RhoA activation and of the site of action of statins. The mechanism by which Rho GTPases lose the prenyl chain during the cycle is still poorly known. Abbreviations: GAPs: GTPase-activating proteins; GDIs: guanine nucleotide dissociation inhibitors; GEFs: guanine nucleotide exchange factors; GGT: geranylgeranyl transferase; HMGCoA: 3-hydroxy-3-methylglutaryl coenzyme A; HMGCoAR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; PKC: protein kinase C; PKN: protein kinase N; ROCK: Rho-kinase.

Fig. 2. Role of Switch 1 and Switch 2 regions in the RhoA cycle. When bound to GDP, RhoA is in a "closed" conformation, with the Switch 2 region laying down on Switch 1 and avoiding any interaction with GTP or effectors. The binding of Rho-specific GEF to the Switch 2 domain modifies the shape of RhoA into an "open" conformation, which favors the loss of GDP and unmasks the binding site for GTP and downstream effectors. Following the action of Rho-specific GAP, GTP is hydrolysed into GDP and the protein returns in the "closed" conformation. Abbreviations: GAP: GTPase-activating protein; GEF: guanine nucleotide exchange factor; Sw1 and Sw2: Switch domains 1 and 2, respectively.