Statins-Mediated Inhibition of Rho GTPases as a Potential Tool in Anti-Tumor Therapy

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Abstract: Rho GTPases, which control processes such as cell proliferation and cytoskeleton remodeling, are often hyper-expressed 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 [1-4]. Until now, twenty mammalian genes encoding Rho GTPases have been described [4-6]. The most investigated members are Rho (Ras homologous), Rac (Ras-related C3 botulinum toxin substrate) and Cdc42 (cell division cycle 42). In this review we have focused our attention on the RhoA, RhoB and RhoC isoforms, with particular interest to the A isoform, since it is the most investigated Rho GTPase known to be modulated by statins. 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), that catalyze the exchange of GDP for GTP to activate the switch [8]; (b) GTPase-activating proteins (GAPs), that 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 [16, 10]. 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), that are frequently mutated in

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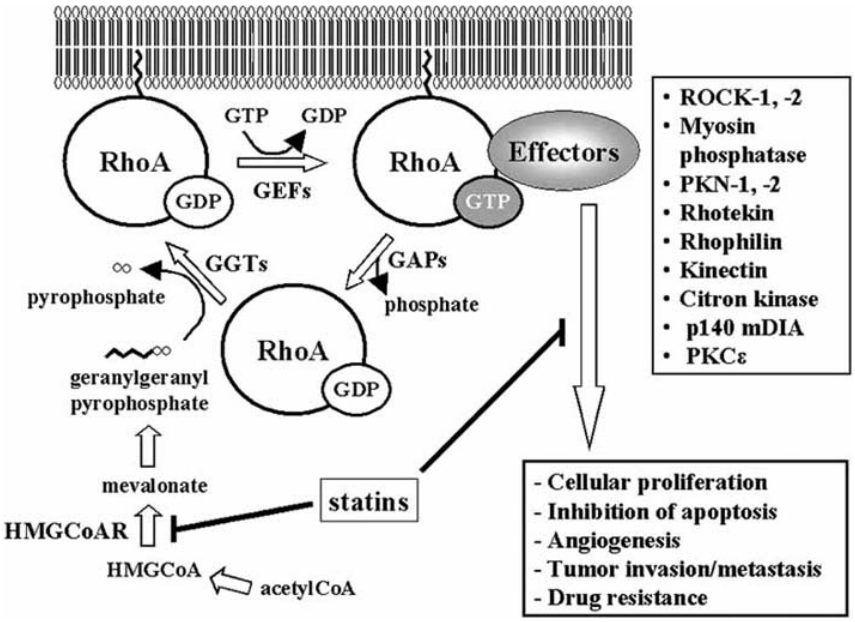

Fig. (1). Schematic representation of the activation/inactivation cycle of the small GTPase RhoA, of the ultimate effects of RhoA activation and of the site of action of statins. The mechanism by which Rho GTPases lose the prenyl chain during the cycle is still poorly known. Abbreviations: GAPs: GTPase-activating 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.

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 [18, 17]. 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].

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]. RhoA usually shuttles between cytosol and plasma membrane, RhoB may localize on plasma membrane and endosomal vescicles, RhoC may be cytosolic or associated to perinuclear structure [5]. RhoA is a target for several bacterial toxins, that modify key conserved amino acids involved in its regulation [20]. *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 [21]. The large toxins A and B from *Clostridium difficile* block the RhoA interaction with downstream effectors by glucosylating the protein at threonine-37 [20].

RhoA and RhoC mRNA and protein are constitutively expressed during the cell cycle; on the opposite, the amount of RhoB protein is usually lower, 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, p76RBE, protein kinase C (PKC) ε, p140 mDIA and DB1 transcription factor [2, 4, 22]. 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 [23] or GAP proteins [24]. Furthermore, it has been reported that RhoC interacts with Rho kinase more efficiently than RhoA [25].

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

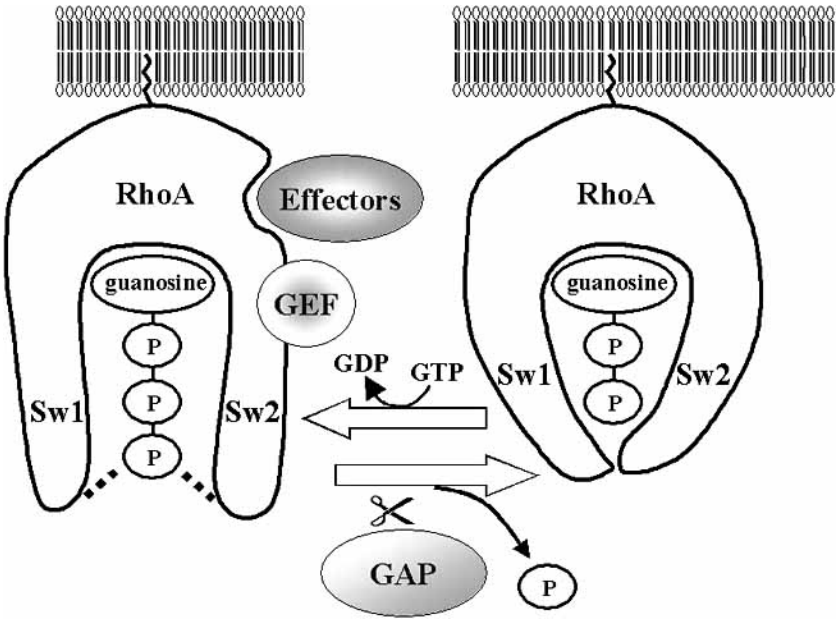

Fig. (2). Role of Switch 1 and Switch 2 regions in the RhoA cycle. When bound to GDP, RhoA is in a "closed" conformation, with the Switch 2 region laying down on Switch 1 and avoiding any interaction with GTP or effectors. The binding of Rho-specific GEF to the Switch 2 domain modifies the shape of RhoA 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.

actin polymerization, actomyosin contractility, cell adhesion and microtubule dynamics [2-4]. RhoA is required for the generation of contractile force leading to rounding of the cell body [12]. But RhoA is also 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 [26]. 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) [27] and p38 kinase [27], as well as numerous transcription factors, such as serum response factor (SRF) [28], activator protein 1 (AP-1) [29], nuclear factor kB (NF-kB) [30], c/EBPb, FHL-2, PAX6, GATA-4, E2F, ER-α, ER-β, CREB [31, 32] and STAT proteins [33, 34].

Rho GTPases show transforming activity by their own [7, 34, 35]: indeed, the overexpression of constitutively activated Rho proteins, such as RhoA, RhoG, Rac, Cdc42 and TC10, induces tumoral transformation in non-transformed fibroblasts [7, 36, 37]. Active Rho proteins are necessary for Ras-mediated oncogenic transformation [36, 38], whereas dominant negative mutants of Rac1 and RhoA inhibit the Ras transforming activity [36]. Although at a lesser extent, also the overexpression of RhoC seems to be related to the oncogenic transformation [5, 7, 39]. On the opposite, RhoB has been described as an oncosuppressor gene [40, 41], and the loss of RhoB expression has been shown to be involved in lung carcinogenesis [42]. 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 [43].

RhoA overexpression confers to cancer cells a highly invasive phenotype. Lysophosphatidic acid, a strong activator of RhoA, promoted matrix invasion and metalloproteinase activity in ovarian cancer [44]. A highly active RhoA was necessary for the cellular motility in prostate cancer [45] and in transformed cells with aberrant activity of ephrin-B receptor [46] or E-cadherin/epidermal growth factor receptor [47]. The hyperactivity of RhoA-related proteins, such as ROCK [48] or Dia1 [49], 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 [50]. 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 [51]. Also RhoC activation gives to cancer cells a highly invasive attitude [39, 52, 53] and is directly related to an increased number of lung metastasis in several *in vivo* models [39, 54].

Several types of human cancers have been analyzed for Rho proteins mutations [55]. RhoA levels are significantly increased in breast cancer, correlating with the tumor grade [56-58]. 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 [59]. Protein expression of RhoA and its two downstream effectors ROCK1 and ROCK2 is significantly higher in testicular germ cell tumors [60]. 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 [61]. The expression and activation of RhoA is greater in small cell lung carcinoma than non-small cell lung carcinoma cell lines [62]. Patients with esophageal squamous cell carcinoma overexpressing RhoA tended to have poor prognosis compared with patients with RhoA underexpression [63]. RhoA was found frequently overexpressed in gastric cancer compared with normal tissue [64]. Invasiveness of hepatocellular carcinoma is facilitated by the RhoA/ROCK pathway and is likely to be relevant to tumor progression [65]. A high proportion of colon cancers overexpresses RhoA [66] and the inhibition of RhoA activity through the introduction of dominant negative mutants completely abolishes the invasive capacity of colonic epithelial cancer cells [67]. Furthermore, the RhoA/ROCK pathway has been implicated in the vascular endothelial growth factor (VEGF)-mediated angiogenesis [68]. As far as RhoC is concerned, its expression has been related to a more aggressive phenotype in ovarian [59], head and neck [69] and gastric cancer [52], and also in melanoma [70]. In contrast, only one contradictory study reports that RhoC enhances the tissue invasion, without affecting the directional motility of prostate cancer cells [71]. Recently, RhoC has been also proposed as a novel biomarker of tumor invasiveness, metastasis [52, 72] and poor prognosis [73]. 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 [74, 75], and have suggested that abrogation of RhoA and RhoC functions could be a promising strategy to attenuate tumor metastasis [76-79].

Synthetic compounds affecting the geranylgeranylation [80] or the post-translational modifications of RhoA [81], bacterial toxins [82] and specific anti-RhoA small interfering RNA (siRNA) [83] have shown anti-tumor activity. However, many of these strategies have dose-limiting toxicity [80] and have only been tested *in vitro* [79]. Other therapeutic tools have been addressed to inhibit the downstream RhoA effectors. Y-27632, which specifically inhibits the ROCKs [84], largely reduced metastasis in animal models [76] and the newly developed ROCK inhibitor Wf-536 reduced angiogenesis, tumor growth and metastasis *in vivo* [85, 86]. Fasudil [1-(5-isoquinolinesulfonyl)-homopiperazine, also known as HA-1077 and AT877], another ROCK inhibitor currently used in the treatment of cardiovascular [87] and neurological disorders [88], blocked the tumor progression in animal models [89] and exhibited anti-angiogenic properties [90]. 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) [91]. By this way, statins may impair the isoprenylation and the activity of Ras and Rho family G-proteins [78]. Nowadays, many natural and synthetic statins (Table **1**) are used in clinical practice as anti-cholesterolemic agents [91], in the prevention therapy of coronary artery disease (to view the structures of main statins, see [92]). 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 [91]. 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 [93]. Small differences in the chemical structure account for the different kinetic properties of each drug [94].

Factors other than the reduction of cholesterol synthesis have been invoked to justify such a variety of therapeutic properties [95]. For instance, the statins effects on Ca^{2+} mobilization in endothelial cells [96], smooth muscle cells proliferation [97], leukocyte activation [98] and bone remodeling [99] appear more related to the inhibition of RhoA activity than to the reduction of cholesterol synthesis.

STATINS AND TUMOR GROWTH/APOPTOSIS

It is conceivable that statins slacken the rate of cellular proliferation by lowering the synthesis of cholesterol, a major component of cellular membranes. However, an increasing number of experimental evidences suggests that the inhibition of RhoA isoprenylation is a crucial mechanism in reducing tumor growth and eliciting apoptosis [78, 100]. Statins exert *in vitro* and *in vivo* anti-proliferative effects in solid [101, 102] and hematopoietic malignancies [103]. 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 [104]. The pro-apoptotic effect of statins has been related to the lowering of protein geranylgeranylation also in glioblastoma [105], melanoma [106] and acute myeloid leukemia [107]. 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 [108]. The statin-induced apoptosis in these tumors was prevented by supplying GGPP and restoring RhoA isoprenylation [108]. 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 [104]. In human breast cancer cells the simvastatin-induced apoptosis was mediated by the JNK pathway [109], while in human osteosarcoma lipophilic statins promoted apoptosis by inhibiting RhoA activity and decreasing phospho-p42/p44 levels [110].

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 [111] or siRNA [112] induced the arrest of proliferation as well as the apoptic death of cancer cells. However, no reports link the statins action to a selective inhibition of RhoC proteins.

In addition statins may also increase cellular differentiation: for instance, lovastatin was able to promote differentia-

Table 1. Chemical, Pharmacodynamic and Pharmacokinetic Properties of the Most Employed Statins. Abbreviations: HMGCoAR: 3-hydroxy-3-methylglutaryl Coenzyme A Reductase. Adapted from Moghadasian [93]

Compound	Chemical properties	Ki (nM) HMGCoAR	IC_{50} * (nM)	Bioavailability ** (%)	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

* Concentrations resulting in the 50% inhibition of cholesterol synthesis in HepG2 human hepatoma cells.

** After oral administration.

tion in neuroblastoma cells and in acute myeloid leukemia cells [113]. 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 [113]. Lovastatin also promoted the neurite growth in immature pheochromocytoma cells, transforming them into more differentiated neuronal cells [114]. Again, such an effect was reverted by mevalonate and geranylgeraniol [114]. Not all statins exert a pro-apoptotic effect at the same extent, because of the different pharmacokinetic and pharmacodynamic properties [107]. Besides being direct pro-apoptotic agents, statins also potentiated the apoptosis induced by other chemotherapeutic drugs [102, 115]. Such potentiating effect was prevented by GGPP [102]. In several cases, statins have been also observed to exert anticancer effects independently of the mevalonate pathway [116, 117].

STATINS AND ANGIOGENESIS

Both pro- and anti-angiogenic effects of statins have been widely described [118-120]. Statins augmented the differentiation of endothelial progenitor cells in mice and humans [121] and stimulated the capillary formation through a hsp90- and nitric oxide (NO)-dependent mechanism [118]. On the other hand, statins blocked the proliferation and promoted the tumor necrosis factor (TNF)-α-mediated apoptosis of endothelial cells [120], inhibited the formation of vascular tubes [119], and prevented the matrix remodeling [122]. Recently it has been reported that simvastatin, fluvastatin and cerivastatin reduce the endothelial cells growth also under hypoxia [123], 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 [124, 125]. 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 [125].

There is general agreement that most statins' anti-angiogenic effects are mediated by RhoA and RhoC inhibition. The active RhoA/Rho kinase pathway stimulates angiogenesis by increasing the secretion of VEGF [68], interleukin (IL)-6 [126] and IL-8 [127], by modulating the activity of metalloproteinase-9 [128] and by regulating the cytoskeletal remodeling and the cellular migration [122]. 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 [129], in a MAP-kinase

dependent way [130]. 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 [122, 131]. 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 [132]; 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 [133], human pancreatic cancer cells [134] and human anaplastic thyroid cancer ARO cells [135]. 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/Rho kinase/NF-kB pathway [136]. NF-kB, whose nuclear translocation may depend on RhoA activity [45, 137, 138], in turn up-regulates the expression of genes involved in cellular invasiveness, such as urokinase-type plasminogen, tissue factor and metalloproteinase 9 [136]. Statins inhibited cellular motility also by disrupting the RhoA/Focal-Adhesion-Kinase (FAK)/Akt signaling [139]: it has been reported that RhoA activity is necessary for the tyrosine phosphorylation and activation of FAK [139, 140], which is then responsible for the activation of the Akt kinase [141]. Akt may further enhance the nuclear translocation of NF-kB [139]. Interestingly, the effects of lovastatin were nearly absent in the less invasive breast cancer MCF-7 cells [136], 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 [142]. Also RhoB seems responsible for the increase of E-selectin caused by TNF-α [142]. Statins showed a great efficacy also in syngeneic BALB/c mice models: fluvastatin and lovastatin reduced the metastatic ability of renal cancer cells [143] and mammary carcinoma cells [144]. In the latter model lovastatin impaired the secretion of urokinase, a key proteolytic enzyme during tumor invasion [144]. Due to the central role of RhoC in tumor invasion and metastasis [39, 52-54], 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 [145]. By preventing the activation of both RhoA and RhoC, fluvastatin impaired the transendothelial migration of MDA-MB-231 cells [146]. Furthermore, the inhibition of both RhoA and RhoC, by specific siRNA [112, 147], 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 [148], doxorubicin and cisplatin [149] in reducing cancer growth. Besides increasing the sensitivity to doxorubicin, lovastatin also reduced the drug cardiotoxicity in mice, *via* an hypothetical lipid-lowering effect [150]. On the other hand, in a limited group of experimental works, statins and chemotherapeutic agents had no synergistic effects [151, 152]. 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 [153]. 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 [102]. 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 [115]. 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 [115].

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 [154]. Many statins are substrates of ATP-binding cassette (ABC) transporters, like P-glycoprotein (Pgp) and MDR-related proteins (MRPs) [155, 156], whose overexpression mediates the enhanced efflux of chemotherapeutic agents [154]. ABC transporters are membrane pumps which bind and hydrolyze ATP, thus mediating the active efflux of endogenous metabolites and drugs [154]. Lovastatin, simvastatin, fluvastatin and pravastatin are transported out of the cells by Pgp [157], which is also responsible for the efflux of anthracyclines, Vinca alkaloids, epipodophyllotoxins, taxanes, actinomycin-D, mitoxantrone [154]. Therefore, statins might affect the accumulation of chemotherapeutics in cancer cells by competing with them for the same ABC pump-mediated transport [156]. Statins induced a selective apoptosis in drug-resistant cancer cells [158, 159]: 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 [160]. 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* [161]. 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 [162]. 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 [162]. 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 [163]. The statins' effect, reverted by mevalonic acid and

mimicked by Y-27632, was NO-dependent [163]. 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 [163]. A cell adhesion-mediated drug resistance (CAM-DR), dependent on Wnt3 overexpression and RhoA/Rho kinase activity [164], is often observed in myeloma cells. Also CAM-DR was totally overcome by statins and specific inhibitors of geranylgeranyltransferases and ROCKs [165].

The inhibition of RhoA does not always produce a chemosensitization: for instance, lovastatin conferred cross-resistance to doxorubicin and etoposide in human endothelial cells [152] 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 [166]. It is thus conceivable that the inhibition of RhoA by statins differentially modulates both chemotherapy efficacy and MDR, 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 [167, 168]. 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 several *in vivo* models of chemical carcinogenesis [169, 170] or pre-cancerous diseases, such as ulcerative colitis [171] and familial adenomatous polyposis [172]. 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 [173]. 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 [174]. Only a long-term therapy with statins partially lowered the incidence of tumors [175]. On the opposite, other studies showed that statins efficiently reduced the incidence of pancreatic cancers [176], as well as metastasis and mortality in advanced stages of prostate cancer [177]. Randomized controlled trials for preventing cardiovascular disease indicated that statins reduced the incidence of colorectal cancer and melanoma [178].

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 [172]. Similarly, in a population-based case-control study, the association of aspirin and statins was more chemopreventive than the single drugs [179]. 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 [180]. Since Rho and COX2 activities appear to be strictly related in colon cancer cells, 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 [181, 182] or radiotherapy [183], but conflicting results were reported about its efficacy [181, 184]. The limited number of patients taking statins [184], the advanced stage of the disease and the too small median survival of patients [181] 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 [185], and the addition of pravastatin to the 5-fluorouracil therapy significantly prolonged the patients survival [186]. However this result was not confirmed by subsequent studies [187].

Better results have been obtained in hematological malignancies: simvastatin stabilized the disease progression in patients both sensitive and resistant to chemotherapy [188] and reversed the resistance to bortezomib and bendamustine in patients with relapsed myeloma [189]. The statins' effect was attributed to the reduced prenylation of small G-proteins, including the Rho homologue Rap1 [188]. In a phase 1 study, pravastatin, added to idarubicin and cytarabine, obtained encouraging response rates in patients with acute myeloid leukemia [182]. 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 [190]. Most of these experimental works provided only preliminary results and did not investigate the molecular mechanisms of the action of statins and the role of RhoA, RhoB or RhoC.

Recently, the anti-osteoporotic drugs aminobisphosphonates, which inhibit isopentenyl diphosphate (IPP) isomerase and FPP synthase [191], showed anti-tumor activity and slackened the progression of metastasis in cancer patients [192]. Interestingly aminobisphosphonates exhibited anti-angiogenic properties by suppressing RhoA activity [193]. The association of statins and bisphosphonates was more effective than the single drugs in reducing the geranylgeranylation of proteins [194], and clinically achievable concentrations of fluvastatin and zoledronic acid synergistically induced apoptosis in cancers [195].

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 [43], 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 [79, 80, 83]. 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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