A disconnect between antitumor and antiangiogenic effects of fluvastatin *in vitro* and *in vivo*[*]

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Received August 11, 2005

The possible role of statins in cancer is controversial. Indeed, among the multiplicity of biological effects ascribed to these widely used cholesterol lowering agents some could, at least in theory, inhibit tumor growth (e.g. by inhibiting Ras oncoproteins), while other actions are inert, or may even stimulate cancer aggressiveness (e.g. through promoting neovascularization). In order to address some of these controversies, we set out to compare the effects of statins on growth of cancer and endothelial cells *in vitro*, to the impact of these drugs on angiogenesis-dependent expansion of the corresponding tumors *in vivo*. Water-soluble fluvastatin was used at concentrations (0–800 ng/ml) against human umbilical vein endothelial cells (HUVEC), and several well-characterized cancer cell lines in culture, including: carcinoma (LLC), melanoma (B16F1) and fibrosarcoma driven by mutant H-*ras* (528ras1). Endpoints were based on ^{3}H-thymidine incorporation assay, cell morphology and tumorigenicity in mice. The growth inhibitory effects of fluvastatin varied among cancer cell lines (LLC>B16F1>528ras1), irrespectively of their mutant H-*ras* status. Fluvastatin also blocked the action of angiogenic factors on cultured endothelial cells, but was relatively ineffective against highly angiogenic and aggressive tumors both in young mice (6–8 weeks), and in less aggressively growing tumors in aged (80–90 weeks) mice. Thus, antitumor and antiangiogenic activity of fluvastatin *in vitro* is not recapitulated *in vivo*. Tumors may display a form of resistance to statins through a mechanism operative only *in vivo*.

Key words: statins, cancer, angiogenesis, ras, anticancer therapy, endothelium

The possible impact of statins on cancer is a subject of a considerable debate and controversy [1–3]. The importance of this debate stems from the fact that indications for statin therapy (e.g. hypercholesterolemia, atherosclerosis, coronary and vascular disease) increase with age, and thereby with prevalence of cancer in the Western population [4, 5]. In other words, a large proportion of patients receiving treatment for cardiovascular reasons may at the same time harbour dormant, cryptic or overt malignancies, the aggressiveness of which could potentially be affected by the statin therapy.

Statins exert their cholesterol lowering effects by inhibiting HMG-CoA reductase involved in converting 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate, a metabolite in the cholesterol synthesis pathway [1]. This decreases hepatic synthesis of cholesterol, leads to increase in LDL receptor turnover and enhanced cholesterol-LDL uptake, the chain of events resulting in a decrease in the plasma cholesterol levels, usually by 20–60% [1]. In so doing, statins exert a much wider, and until recently underappreciated effects, by blocking synthesis of several metabolites of mevalonate, some endowed with important biological functions in a variety of different cell types, and unrelated to regulation of blood cholesterol [1]. Thus, mevalonate is required for synthesis of cellular ubiquinone, as well as farnesyl- and geranylgeranyl diphosphates (FPP and GGPP), the latter two critical for post-translational modification and membrane localization of crucial structural and signaling proteins [1, 6]. This includes cellular lamins and a number of other proteins,

[*]This work was supported by the Canadian Cancer Society through a grant to JR from the National Cancer Institute of Canada (NCIC). HK is a recipient of a Fellowship from the International Exchange Program between McMaster University/Henderson Research Centre and the Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, while JR is a recipient of the Scientist Award from the NCIC.

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among which proto-oncogenes such as Ras, RhoA, RhoB are of particular relevance for this discussion. This is because these small GTP-ases are known to regulate growth, survival, motility and other important functions of activated (e.g. cancer or endothelial) cells, and in a manner dependent on their localization to the plasma membrane through lipid attached to their C-terminal tails by farnesyl- or geranylgeranyl transferases [6]. It is therefore not surprising that statins that deplete cellular reservoirs of quinone, FPP and GGPP residues could affect several cellular properties, such as: membrane fluidity, ubiquinone status, cellular growth and migration and several other properties, at times manifested as drug-related side effects (e.g. myalgia) [1].

Among the aforementioned 'side effects' of statins of particular interest is their possible impact on the processes of blood vessel formation and remodeling. Thus, it was argued that one of the benefits of statin therapy in cardiovascular medicine could be their apparent stimulating effect on endothelial cell replenishment, and ultimately improved perfusion of ischemic tissues [7, 8]. This was exemplified by the ability of, at least some of these drugs (e.g. simvastatin) to promote mobilization and seeding of bone marrow derived circulating endothelial cell progenitors in the lumen of denuded vascular segments [7]. In another study statins were able to promote survival of endothelial cells by activation of the Akt pathway, in a manner similar to that attributed to vascular endothelial growth factor (VEGF) and other angiogenic growth factors [9, 10].

Although the ostensibly proangiogenic effects of statins could bring considerable benefits to patients with vascular disease-dependent ischemia, they could be (and were [11]) of concern in the context of the possible accompanying, overt or cryptic (undiagnosed) malignancy. This is because vascular growth is an important constituent, and hallmark of active cancer, and a prerequisite of aggressive tumor growth, invasion and metastasis [12–14]. Indeed, overwhelming experimental evidence suggests that inhibition of tumor blood vessel formation, e.g. by blocking the activity of proangiogenic growth factors (including VEGF), or through other means, could be an effective way to treat/control cancer, a concept originally proposed by FOLKMAN [15]. This notion is supported by the recent approval of the VEGF-directed humanized monoclonal antibody known as bevacizumab (Avastin®), as the first line therapy in metastatic colorectal cancer [16]. It could be speculated that proangiogenic effects of statins could exert effects opposite to bevacizumab, and thereby promote cancer growth and metastasis [11]. These and other considerations, along with initial epidemiological analyses led to a suggestion that a linkage may exist between statin administration and cancer incidence and progression [2].

In spite of the aforementioned concerns, a more comprehensive epidemiological analysis did not provide any compelling evidence for cancer promoting effects of statins [1]. In fact, a closer examination of this class of agents suggested the opposite, i.e. anticancer properties that statins may have in various settings [1, 17, 40]. For instance, lovastatin the prototypic drug in this category, was found to induce growth inhibition and apoptosis of an number of cultured cancer cell lines, possibly due to its ability to block farnesylation and geranylgeranylation of the small proto-oncogenic GTP-ases, such as Ras and Rho [1, 17]. This is significant because Ras proteins (K-Ras, H-Ras and N-Ras) play a pivotal role in integrating growth-related signals through activation of a wide array of downstream pathways, including Raf/MEK/MAPK, PI3K/Akt, RalGDS, Rho/Rac/Cdc42 and several other modules [18, 19]. Constitutive activation of Ras is a common occurrence in human cancer, either due to frequent mutations of the respective *ras* genes (mainly K-*ras*), or through action of upstream signals emanating from activated growth factor receptors and oncogenes, such as epidermal growth factor receptor (EGFR), HER-2 and other entities [19]. In addition to promoting cellular growth, migration and metastasis activated Ras is also crucial for expression of VEGF and the related proangiogenic phenotype cancer cells, properties that synthetic farnesyltransferase inhibitors (FTIs) were shown to obliterate [14]. It is noteworthy that while these latter agents have not, as yet passed the stage of clinical exploration, they show promise as potential radiosensitizers in cancer [20]. In addition to their impact on cancer cells, Ras proteins expressed in endothelial cells play a role in their responses to angiogenic stimuli [21]. Thus, by inhibiting farnesylation or geranylgeranylation of Ras and related proteins, statins could, in fact, exert both direct anticancer as well as antiangiogenic effects [1]. Although this notion is supported by some *in vitro*, *in vivo* and preliminary clinical data [1, 3, 22], it stands in contrast to provascular or biphasic effects of statins described by other studies [7, 9, 10, 22, 23].

The seemingly contradictory conclusions, as to the possible role of statins in the context of cancer [24] and tumor angiogenesis may be, at least in part, related to inconsistencies between experimental settings employed in various reported studies, including the choice of tumor models, assays, compounds and their dosing regimens [1]. In order to address some of these potential concerns we chose to examine the effect of the same agent (fluvastatin) on the growth of the same series of cancer cells lines, both *in vitro* and *in vivo*. We show that while *in vitro* fluvastatin possesses potent growth inhibitory against some (but not all) cancer cells, or human endothelial cells, it generally fails to produce significant and consistent corresponding antitumor effects *in vivo*. Thus, as in the case of many other anticancer agents [25], also the antitumor and antiangiogenic action of statins may be obliterated by resistance mechanisms operative only *in vivo*.

Material and methods

Reagents. Fluvastatin was purchased in Henderson General Hospital Pharmacy as tablets containing 40 mg of fluvastatin yellow powder (Lescol, Novartis Pharmaceuticals).

Cells. Lewis Lung Carcinoma (LLC), B16F1 melanoma and 528ras1 fibrosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) and 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco, Invitrogen Canada). 528ras1 cell line, is a tumorigenic and angiogenic *ras* oncogene-driven, VEGF deficient fibrosarcoma, derived from murine dermal connective tissue fibroblasts, as described elsewhere [26]. LLC, B16F1 and human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HUVEC were cultured on gelatinized dishes (1% Gelatine, Sigma, St.Louis, MO, USA) in EGM-2 media (Clonetics, Cambrex BioScience, Walkersville, MD, USA) supplemented with 5% FBS, hydrocortisone (1 µg/ml), hFGF-B, VEGF, R^3-IGF-1, ascorbic acid, hEGF and GA-1000 (Gentamicin, Amphotericin B), unless otherwise indicated.

Cell proliferation assay (Thymidine incorporation assay). Cell proliferation was evaluated by ^{3}H-thymidine incorporation assay as described elsewhere in detail [27]. Briefly, the cells (LLC, B16F1 melanoma, 528ras1, HUVECs) were plated at 5,000 cells per well in 96-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for several hours and after their attachment the medium containing indicated concentrations of fluvastatin was added to the wells. In the majority of experiments the following treatments were used: 0.1; 0.2; 0.4; 0.8; 1.6; 3.1; 6.25; 12.5; 25; 50; 100; 200; 400; 800 ng/ml, the selection of which was guided by the maximum peak concentration of 150 ng/ml achieved in human plasma approximately 30 min after administration of fluvastatin. The physiological dose range of the drug, which is between 20 mg and 80 mg, could therefore be approximated by the range of *in vitro* concentrations of 9 ng/ml to 33 ng/ml. After 2–3 days of incubation with fluvastatin (different in individual experiments) 1 µCi of [methyl-^{3}H] Thymidine (Amersham Biosciences, Buckinghamshire, UK) was added to each well for 3 hours at 37 °C, at which point the plates were frozen at –70 °C, thawed, and DNA associated radioactivity harvested onto the paper filters and quantified by using the scintillation counter (Beckman, LS6000LL). Counts per minute (CPM) of drug treated and control cells were compared and changes in DNA synthesis (mitogenesis) expressed as indicated.

Tumorigenicity assays. LLC and B16F1 cells were injected subcutaneously into the flank region of C57BL/6, mice (Charles River Laboratories, Wilmington, MA, USA), while 528ras1 tumors were generated in a similar manner in mice with severe combined immunodeficiency (SCID) expressing yellow fluorescent protein (YFP) transgene [26], which were bred in house. Whenever indicated, the mice were used as tumor recipients at different ages, either at 6–8 weeks or at 80–90 weeks. The following numbers of cancer cells were injected per mouse to achieve a consistent tumor take: $1x10^6$ for LLC, $0.5x10^6$ for B16F1 and $1.2x10^6$ for 528ras1, all as single cell suspension. Daily treatment with fluvastatin 2, 4 or 16 mg/kg were administered in most experiments. These doses exceed the maximum daily dose in humans, but in preliminary experiments we found lower doses to be ineffective (data not shown). The drug was administered by oral gavage, and initiated the day after tumor cell inoculation. Fluvastatin was dissolved in sterile water which was also administered to control mice as a placebo. All *in vivo* experiments were conducted according to protocols approved by the Animal Research Ethics Board at McMaster University and in accordance with the guidelines of the Canadian Council of Animal Care (CCAC).

Data analysis. The data were presented as the mean value (±SD) derived from several independent data points (mice, tissue culture wells). Five mice were allocated to each experimental group in *in vivo* experiments. All experiments were reproduced at least twice with similar results and statistically analyzed by the Student's t-test.

Results

Diverse inhibitory effects of fluvastatin on growth of cultured cancer cell lines. One of the widely discussed effects of statins on cancer cells is their postulated impact on cellular growth, including through inhibition of post-translational processing and function of Ras oncoproteins [1, 6]. In order to test the generality of this notion further, we chose to test statin effects on DNA synthesis (growth) of three well characterized murine cancer cell lines of different origin (Fig. 1). Of those 528ras1 fibrosarcoma cells harbour a mutant H-*ras* oncogene, while B16F1 melanoma and LLC lung carcinoma are driven by unrelated and unknown transforming alterations. Fluvastatin was selected, as water-soluble properties of this statin facilitate both *in vitro* and *in vivo* analyses.

Treatment of cultured cancer cells with this agent resulted in considerable diversity of responses, in that LLC cells consistently displayed marked and dose dependent growth inhibition in the presence of fluvastatin at concentrations ranging between 50 and 800 ng/ml, and sustained over at least 48–72 hour period (Fig. 1A). In contrast, no such effect was observed in the case of B16F1, or 528ras1 cells under similar conditions (Fig. 1B and C). While B16F1 cells initially responded to low fluvastatin concentrations, this modest (40–50%) inhibition of DNA synthesis remained unchanged in spite of drug concentrations approaching 100 ng/ml to 800 ng/ml (Fig. 1B). Even less pronounced was the impact of fluvastatin treatment on 528ras1 cells. In spite of their expression of the mutant H-*ras* oncogene, a putative statin target, these cells exhibited only a slight growth inhibition at the highest drug concentrations (Fig. 1C). These experiments demonstrate that cancer cell lines differ greatly in their sensitivity to statin-mediated inhibition of cellular mitogenesis, and their responsiveness does not segregate with expression of known molecular targets of this drug, such as oncogenic Ras.

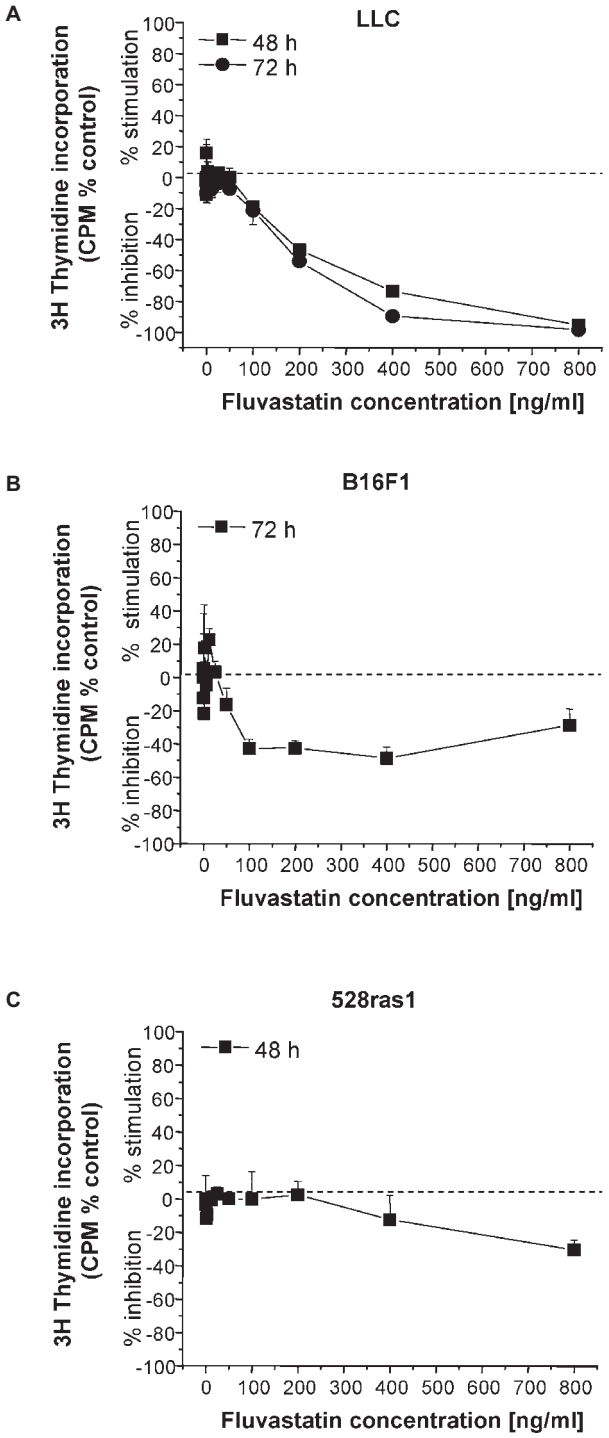

Figure 1. Inhibition of mitogenesis of cultured cancer cell lines by treatment with fluvastatin. Cells cultured in their growth media were treated with indicated concentrations of the drug for 24–72 hours and their DNA synthesis was assessed by the ^{3}H-thymidine incorporation. The effects were expressed as percentage of signal in untreated control. A. LLC carcinoma, B. B16F1 melanoma, C. 528ras1 fibrosarcoma – see text. The respective p values for 400 ng/ml were less than 0.0001, 0.0450 and 0.1541.

The impact of fluvastatin on growth and survival of cultured endothelial cells. One thought provoking activity of statins is their reported ability to activate endothelial survival pathways (e.g. Akt/PKB), and thereby mimic the effects of angiogenic growth factors, such as VEGF [10]. As this property may suggest that statins could stimulate tumor angiogenesis and growth, we decided to examine it more closely by using cultures of human umbilical vein endothelial cells (HUVEC). HUVEC cells retain some (but not all) of the properties of tumor associated endothelium and are exquisitely dependent on VEGF and bFGF for survival [27].

Contrary to some of the prior reports [10], we were unable to demonstrate the ability of fluvastatin to substitute for VEGF in cultures of growth factor starved HUVEC cells (Fig. 2A). Thus, when these cells were cultured in the presence of low (2%) serum concentrations and in the absence the essential growth factors (VEGF and bFGF), they promptly succumbed to apoptotic cell death within 48–96 hours, regardless whether fluvastatin was added to the medium, and at what concentration (Fig. 2A). Moreover, robust mitogenesis of HUVEC cells in their complete growth medium containing 5% serum, bFGF and VEGF was suppressed by fluvastatin in a time (not shown) and dose-dependent manner (Fig. 2B). A more careful examination of HUVEC cell cultures (Fig. 2C) revealed features of apoptotic cell death (rounded morphology, detachment, blebbing), the extent of which corresponded to the drug concentration (200–800 ng/kg). Collectively, these *in vitro* observations suggest that not only statins (fluvastatin) do not support survival of endothelial cells, but they also provoke their demise, growth inhibition and apoptosis, even in the presence of angiogenic factors. Hence their action in cancer would be expected to be anti- rather than proangiogenic.

In vivo resistance of tumors to anticancer and antiangiogenic effects of fluvastatin. Our *in vitro* results suggest that fluvastatin may posses at least two levels of anticancer activity. First, at least some cancer cell types (e.g. LLC) are sensitive to direct antimitogenic effects of the drug (compare Fig. 1A), and second – all tumors may be affected by its apparent antiendothelial (antiangiogenic) activity (compare Fig. 2C). In order to verify these predictions we generated subcutaneous tumors by injecting LLC or B16F1 into syngeneic mice (C57BL/6), whereas 528ras1 cells were inoculated to immunodeficient (SCID) mice and all mice were then subjected to daily administrations of fluvastatin (Fig. 3).

Unexpectedly, the inhibitory impact of fluvastatin on the growth of aggressive and highly angiogenic LLC tumors was rather modest (40–50%; Fig. 3A), and statistically insignificant (p=0.1339). We reasoned that since these initial experiments were conducted under standard conditions, i.e. involved 6–8 weeks old (relatively very young) mice, and statins are administered mainly to individuals at later stages of life, it would be of interest to examine the effects of fluvastatin also in aged (18–20 months old, i.e. 'geriatric') tumor bearing animals. Interestingly, age itself caused a mild

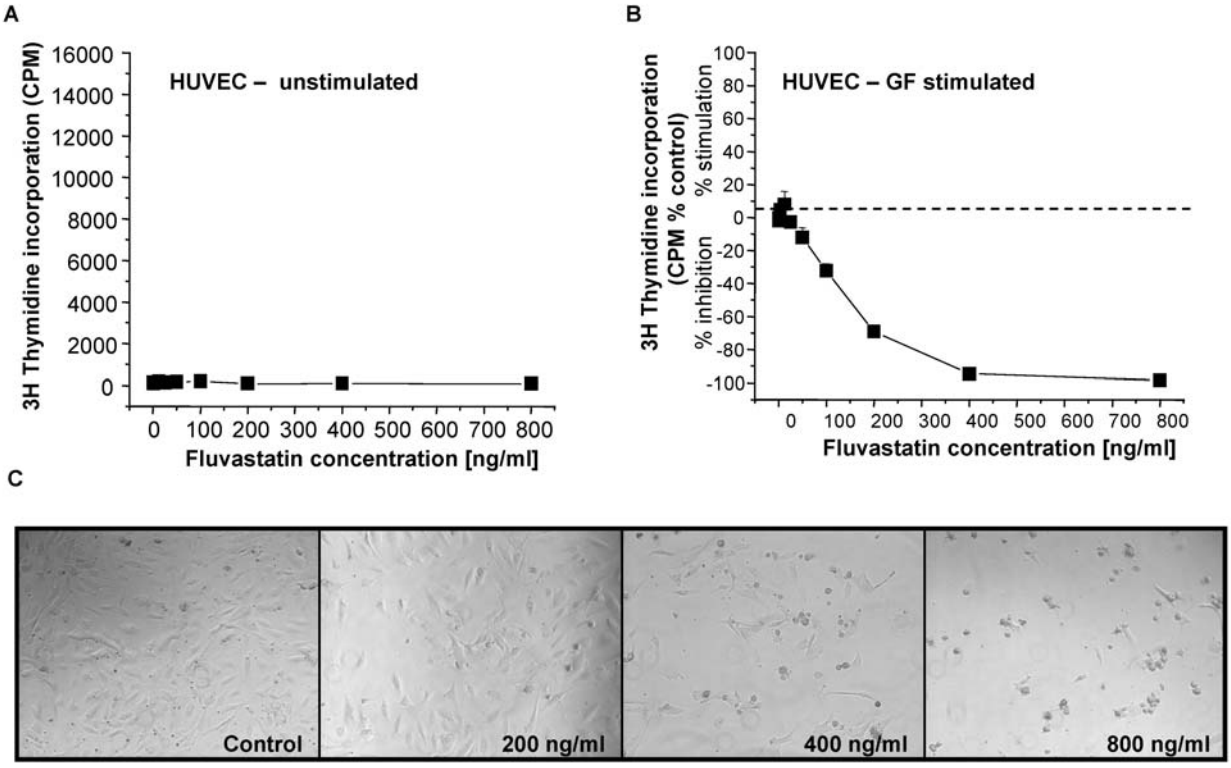

Figure 2. The impact of fluvastatin on cultured endothelial cells (HUVEC). A. ^{3}H-thymidine incorporation of fluvastatin treated HUVEC cultured in the absence of angiogenic growth factors and under low (2%) serum conditions. As the cells remain quiescent the drug does not appear to compensate for the absence of VEGF and bFGF after 48 hour period (see text). B. Dose-dependent inhibition by fluvastatin of cellular mitogenesis (DNA synthesis) in HUVEC cells cultured in the presence of essential angiogenic growth factors (VEGF and bFGF). P value for 400 ng/ml was <0.0001, however statistically significant inhibition occurred already at the dose of 50 ng/ml (p=0.0013). C. Apoptotic morphology of HUVEC in the presence of fluvastatin. As in this case (also in B) the cells were cultured in the presence of VEGF and bFGF, it appears that fluvastatin was able block the pro-survival effects of these growth factors.

tumor growth inhibition (30–40%), likely due to impairment of angiogenic processes [8, 28, 29]. However, in this setting fluvastatin exhibited a trend toward growth stimulation (rather than inhibition), though this effect was neither very pronounced nor statistically significant (Fig. 3A).

Similarly to LLC, also in the case of B16F1 and 528ras1 tumors the anticancer activity of fluvastatin was either weak or non-existent (Fig. 3B and C). This is particularly surprising in the case of 528ras1 tumors composed of *ras*-driven and VEGF-deficient cells [26], and thereby potentially more vulnerable to both, direct growth inhibitory and antiangiogenic action of statins. Thus, the effects of fluvastatin on cancer, or endothelial cells *in vitro* do not predict the anticancer activity of the drug *in vivo*.

Discussion

Our study offers several novel insights into the potential effects of statins in cancer. First, we observed that cancer cells differ in their responses to fluvastatin, in a manner that cannot be predicted by their known molecular properties (e.g. expression of mutant *ras*) [1]. This raises a question as to the true molecular targets of this, and other statins in different types of cancer cells, as exemplified by high sensitivity of LLC cells versus the relative resistance of their B16F1 and 528ras1 counterparts (compare Fig. 1).

The second important finding that emerged out of these experiments is that fluvastatin does not seem to possess endothelial cell survival-promoting activity, which was previously attributed to other statins in some, but not all published studies [10, 23]. Instead, we observed a drug induced endothelial growth arrest and apoptosis. The reasons for this discrepancy remain presently unclear [23], and could be related to unique properties of fluvastatin vis-á-vis other compounds, their inherently dual action [3] or to specific differences in experimental conditions between our study and that of KURESHI et al [10]. However, our results seem to be more consistent with the reported antiendothelial and antiangiogenic consequences of global inhibition of protein farnesylation by both statins [1, 3, 22, 30] and farnesyltransferase inhibitors [21, 27].

Third, our study suggests that *in vitro* assays predict nei-

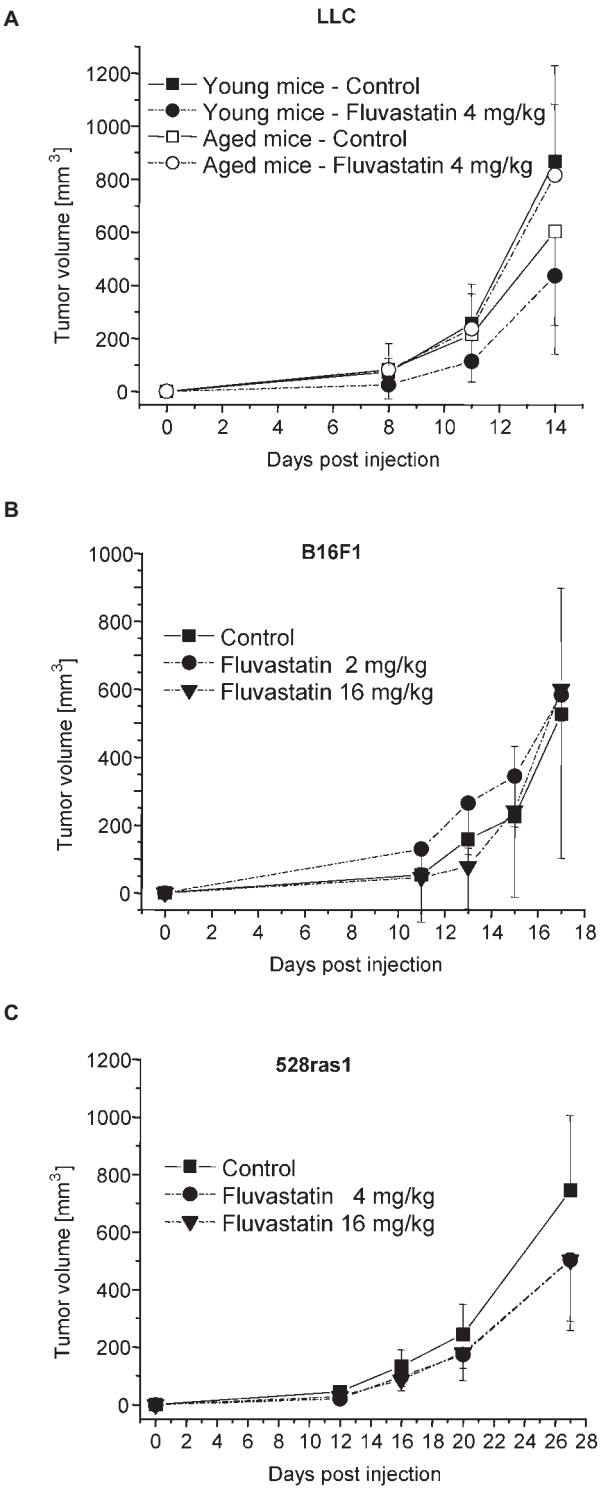

Figure 3. The impact of fluvastatin on growth of aggressive and angiogenic tumors *in vivo*. A. Moderate inhibition of LLC tumor growth by fluvastatin (dose) treatment in young (6–8 week old) C57Bl/6 mice is not recapitulated in 'geriatric' (80–90 week old) mice. B. Responsiveness of B16F1 melanoma tumors to high doses of fluvastatin. C. Moderate response of 528ras1 fibrosarcoma tumors growing in SCID/YFP mice to fluvastatin therapy.

ther the existence, nor the magnitude of anticancer activity of statins *in vivo*. Thus, even in the context of a remarkable sensitivity of both LLC and endothelial cells (HUVEC) to antimitotic and proapoptotic activity of fluvastatin *in vitro*, the corresponding (highly angiogenic/endothelial-dependent) LLC tumors in mice were affected to a much lesser degree. Interestingly, we observed a differential growth kinetics of LLC tumors implanted into young or aged mice (see Fig. 3A), a property consistent with the impact of age on the efficiency of angiogenic processes, as reported in the prior literature [8, 29, 31, 32]. This is an important consideration, as the vast majority of patients that may be receiving statins due to their cardiovascular condition, and may be at risk for cancer, are in their later decades of life. Considering that the average life expectancy of mice is between 14 and 24 months, we believe that our experiments conducted with 18–20 month old ('geriatric') animals represent a more relevant context to study the effects of statins, than studies carried out under 'standard' conditions (4–8 week old mice), presently dominating the literature [28]. Remarkably, in aged mice fluvastatin not only did not inhibit tumor growth to any significant extent, but instead actually exhibited a trend towards tumor growth stimulation, albeit without statistical significance (see Fig. 3A).

Statins were proposed to induce a number of putative antitumor effects mediated by various molecular mechanisms, related or unrelated to isoprenylation of cellular proteins. This includes one, or more of the following activities: (i) inhibition of mitogenesis and cell cycle progression due to induction of cyclin dependent kinase inhibitors $p21^{WAF/CIP1}$, $p27^{KIP1}$, (ii) proapoptotic effects related to mevalonate withdrawal, (iii) attenuation of cellular invasion, e.g. through interference with EGFR, RhoA or $NF_{\kappa}B$-dependent signaling, (iv) radiosensitization linked to inactivation of Ras, (v) antiangiogenic effect related to attenuation of the angiogenic properties of cancer cells, and/or through a direct impact on endothelium [1, 33] (vi) stimulation of the antitumor immunity through upregulation of MHC class I antigens [34], (vii) synergy with other antitumor agents [35–38]. Though, only some of these effects were confirmed by *in vivo* studies [35], several clinical phase I and phase II trials have been reported, mostly involving lovastatin, cervastatin and pravastatin [1]. Indeed, there is a growing interest in incorporating statins into therapeutic protocols in astrocytoma, gastric cancer, hepatocellular carcinoma, prostate cancer, acute myelogenous leukemia, multiple myeloma, and other malignancies in spite of the fact that the results have been mixed thus far [1, 17, 36–38].

We propose that, while statins could in principle offer a therapeutic benefit to cancer patients, particularly in combination with other anticancer agents, this effect is not universal due to existence of at least two mechanisms of *de facto* resistance to anticancer effects of these agents, i.e. cancer cell-related, host-related or both. Thus, as we documented here statin resistance may reside within intrinsic properties of

cancer cells (e.g. expressed *de novo*, or conceivably acquired during therapy). Alternatively, resistance to statins may be operative only *in vivo*, i.e. manifest a microenvironmental, multicellular and/or host/age-dependent nature [39]. While the specific mechanisms of the latter form of statin resistance remain to be established, our findings highlight the importance of testing anticancer properties of statins under realistic conditions *in vivo*.

We are grateful to our respective families and colleagues for their support, advice and encouragement. In particular we are indebted to Drs. J. DOUBEK and M. PENKA for their helpful comments.

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