

The differential effects of statins on the metastatic behaviour of prostate cancer

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BACKGROUND: Although statins do not affect the incidence of prostate cancer (CaP), usage reduces the risk of clinical progression and mortality. Although statins are known to downregulate the mevalonate pathway, the mechanism by which statins reduce CaP progression is unknown.

METHODS: Bone marrow stroma (BMS) was isolated with ethical approval from consenting patients undergoing surgery for non-malignant disease. PC-3 binding, invasion and colony formation within BMS was assessed by standardised *in vitro* co-culture assays in the presence of different statins.

RESULTS: Statins act directly on PC-3 cells with atorvastatin, mevastatin, simvastatin (1 μM) and rosuvastatin (5 μM), but not pravastatin, significantly reducing invasion towards BMS by an average of 66.68% (range 53.93–77.04%; $P < 0.05$) and significantly reducing both number (76.2 ± 8.29 vs 122.9 ± 2.48 ; $P = 0.0055$) and size ($0.2 \pm 0.0058 \text{ mm}^2$ vs $0.27 \pm 0.012 \text{ mm}^2$; $P = 0.0019$) of colonies formed within BMS. Statin-treated colonies displayed a more compact morphology containing cells of a more epithelial phenotype, indicative of a reduction in the migrational ability of PC-3 cells. Normal PC-3 phenotype and invasive ability was recovered by the addition of geranylgeranyl pyrophosphate (GGPP).

CONCLUSION: Lipophilic statins reduce the migration and colony formation of PC-3 cells in human BMS by inhibiting GGPP production, reducing the formation and the spread of metastatic prostate colonies.

British Journal of Cancer (2012) **106**, 1689–1696. doi:10.1038/bjc.2012.138 www.bjcancer.com

Published online 24 April 2012

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Keywords: prostate cancer; metastasis; statins; bone marrow stroma

Prostate cancer (CaP) is the second most common malignancy in men worldwide, with 910 000 new cases diagnosed in 2008 (Ferlay *et al*, 2010). CaP has a predilection to metastasise to the bone marrow stroma (BMS), and development of CaP bone metastases almost invariably result in CaP-related mortality (George, 1988). The metastatic process is a complex, multistep process, which can be modelled with modified *in vitro* invasion chambers utilising human primary BMS (Lang *et al*, 1998; Scott *et al*, 2001; Hart *et al*, 2005). These *in vitro* models not only allow the determination of the mechanism of CaP metastasis to the BMS but enable the elucidation of how therapeutic agents may interfere with the metastatic process.

Recent evidence links lipid metabolism and statin use with the behaviour of CaP. Large scale epidemiological data showing lower rates of CaP progression in patients taking statins (Platz *et al*, 2006) have been consolidated by reports showing that CaP incidence in screened (Murtola *et al*, 2010) and non-screened (Breau *et al*, 2010) populations is reduced in men taking these drugs and that individuals with lower cholesterol levels had lower rates of high risk disease (Platz *et al*, 2008). Further clinical data from histological analysis of large case after prostatectomy series

showed less aggressive features in men taking statins (Loeb *et al*, 2010) and in patients undergoing radiotherapy for CaP indices of CaP treatment failure were reduced in men taking statins, especially in those with high risk features (Gutt *et al*, 2010).

The basis for these observations is poorly understood but the effects probably relate to the pleiotropic actions of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) inhibitors on cellular behaviour, cell-cell interaction and cellular motility in relation to lipid metabolism (Stancu and Sima, 2001). To better understand this process, we have studied the effects of different statins in our in-house, well-categorised models of CaP behaviour, evaluating the differential effects of specific statins on cellular binding, migration and early cellular survival in human BMS.

MATERIALS AND METHODS

Cell culture

PC-3 (ATCC-LGC, Teddington, UK) cells were cultured in HAM'S-F12 media supplemented with 7% foetal calf serum (FCS) and 2 mM L-glutamine at 37 °C, 5% CO₂ in air. PC3-GFP were cultured as for PC-3 except for addition of 0.15 mg ml⁻¹ hygromycin B. Human BMS was obtained from volunteers undergoing surgery for benign disease and cultured according to Coutinho *et al* (1993). Briefly, 2×10^6 cells ml⁻¹ in long-term bone marrow culture medium

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Received 23 January 2012; revised 13 March 2012; accepted 21 March 2012; published online 24 April 2012

(LTBCM) (Iscove's modified Dulbecco's medium at 350 mOsm, 10% foetal calf serum, 10% horse serum, 5×10^{-7} M hydrocortisone) were grown at 33 °C in 5% CO₂ in air for 4–5 weeks until haematopoietically active areas were observed. Bone marrow endothelial cells (BMEC) were cultured in LTBCM conditioned by BMS on fibronectin-(50 mg ml⁻¹ in PBS) treated flasks. All cell lines were verified by the Paterson Institute for Cancer Research tissue typing service.

Statins and metabolites

Atorvastatin, rosuvastatin (Discovery Fine Chemicals Ltd, Dorset, UK), mevastatin, and simvastatin (Sigma-Aldrich, Poole, UK) were dissolved in dimethyl sulphoxide (DMSO) and pravastatin (Sigma-Aldrich) was dissolved in water at concentrations of 100 mM. Activated simvastatin was also assessed. Briefly, 4 mg of simvastatin was dissolved in 100 µl of ethanol to which 150 µl of 0.1 N NaOH was added and incubated at 50 °C for 2 h. The pH was brought to 7.0 by HCl and diluted to a final concentration of 100 mM before storing at 4 °C. All statins were used at final non-toxic concentrations as defined by trypan blue exclusion. All metabolites were purchased from Sigma-Aldrich. Geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) were dissolved in methanol at a final concentration of 1 mg ml⁻¹. Cholesterol was dissolved in chloroform at 100 mM. Mevalonate was made up in water at a stock concentration of 10 mg ml⁻¹ (40 mM).

Binding assay

Prostate epithelial cell (PEC) binding to BMS was determined as previously described (Scott *et al*, 2001); 5×10^4 PC3-GFP cells, treated with statin or DMSO vehicle control, were added to confluent BMS in a 96-well plate. Wells were washed and fluorescence was read using a FLUOstar OPTIMA spectrometer (BMG Labtech, Aylesbury, UK). Binding was expressed as percent of pre-washed total fluorescence.

Invasion assay

Prostate epithelial cell invasion towards BMS was measured as previously described (Hart *et al*, 2005); invasion towards statin-pretreated BMS or in the presence of statins was assessed. The FluoroBlok (BD Biosciences, Oxford, UK) tumour cell invasion system used phenol red-free RPMI 1640 media and FluoroBlok cell culture inserts coated with 100 µl of Matrigel (BD Biosciences) (1:25 dilution with RPMI 1640) above chemoattractant (BMS or tissue culture plastic (TCP)). 2×10^5 PC3-GFP in RPMI 1640/0.1% BSA were added to each FluoroBlok insert and the co-cultures incubated at 37 °C, 5% CO₂ for 18 h. Bone marrow stroma was pretreated daily with the different statins. PC-3 cells were treated with statins 30 min before addition. Final readings utilised the FLUOstar OPTIMA spectrometer. Recovery assays were conducted as above or in the presence of a confluent BMEC cell layer barrier (Hart *et al*, 2005). PC3-GFP cells were treated with 10 µM GGPP, 100 µM mevalonate, 16 µM cholesterol or 10 µM FPP immediately before the additions of statins and adding to the invasion chamber.

Co-culture colony assay

Prostate epithelial cell growth in BMS co-culture was measured as previously described (Lang *et al*, 1998); confluent primary human BMS, pretreated with statins for 60 min, was seeded with 500 PC-3 cells. Statins were refreshed daily for 8 days before 4% paraformaldehyde fixation. Co-cultures were permeabilised using ice-cold methanol and blocked with 10% rabbit serum, followed by 0.3% hydrogen peroxide. Co-cultures were incubated with mouse anti-human pan-cytokeratin at 1:200 (Sigma-Aldrich) followed by

biotinylated rabbit anti-mouse 1:400 (DAKO Ltd., Cambridge, UK). A complex of avidin DH and biotinylated horseradish peroxidase H (Vector Laboratories, Peterborough, UK) was then added and developed with DAB substrate.

Clonogenic assay

Five hundred PC-3 cells were treated at day 1 and day 7 with statins, before methanol acetone fixation and staining with crystal violet on day 14; colonies (>32 cells) were then counted using a standard microscope graticule.

Statistics

Values are presented as mean ± s.e. Assays were compared using the two-tailed Student's *t*-test with significance set at $P < 0.05$.

RESULTS

Reduction of BMS invasion by lipophilic statins

We have previously shown that BMS is the most potent chemoattractant for metastatic PECs and that the metastatic process can be modelled using primary human BMS co-cultures (Hart *et al*, 2005; Brown *et al*, 2006). We therefore addressed three different questions: do statins affect the BMS microenvironment (Figure 1A), the PECs directly (Figure 1B) or the interaction between BMS and PECs (Figure 1C). Statins did not affect the ability of BMS to induce PEC invasion; although atorvastatin and simvastatin treatment of BMS reduced invasion as compared with the controls ($84 \pm 8.8\%$; $P = 0.18806$ and $80 \pm 10.57\%$; $P = 0.14755$), this did not reach significant levels (Figure 1A). All statins, except pravastatin, induced a similar and marked reduction in PEC invasion towards BMS ($P > 0.05$), averaging 66.68% of control (range 54–77%; $P < 0.05$). Bone marrow stroma pretreatment (Figure 1C) did not provide additional benefit to treating PECs alone (58.86%; $P > 0.05$ of PC-3's invading compared with controls), suggesting a direct effect on the PC-3 cells and not on the BMS 'soil'.

The effect of statins was more significant in the presence of a confluent BMEC/Matrigel barrier, which models the blood BMS barrier. Both 5 µM rosuvastatin and 1 µM simvastatin (Figure 1D) completely inhibited direct invasion towards BMS (9.37% vs 100%, $P = 0.0201$ and 25.6% vs 100%, $P = 0.0496$ for rosuvastatin and simvastatin vs BMS + vehicle control, respectively), with 5 µM rosuvastatin reducing invasion to levels similar to TCP ($P = 0.21212$). Activation of simvastatin according to manufacturer's instructions had no effect on the ability of simvastatin to inhibit transendothelial invasion towards BMS ($13.5\% \pm 7.1\%$ and $14.8\% \pm 12.8\%$, respectively; $P = 0.142$) (Supplementary Figure 1).

Effect of statins on PEC binding to BMS

When malignant PECs enter the BMS, they migrate towards and bind to niches within the BMS before proliferating and forming viable colonies (Brown *et al*, 2010). We sought to assess the inhibitory effect of statins on this process. Incubation of PECs with statins for up to 120 min did not alter the ability of PECs to bind to BMS (Figure 2).

Reduction of PEC colony formation in BMS by lipophilic statins

Previous studies have documented the antiproliferative effect of high-dose statin treatment of PECs. However, the antiproliferative effect of statins on PEC within the lipid-rich environment of the BMS is unknown. We therefore compared the effect of statins on

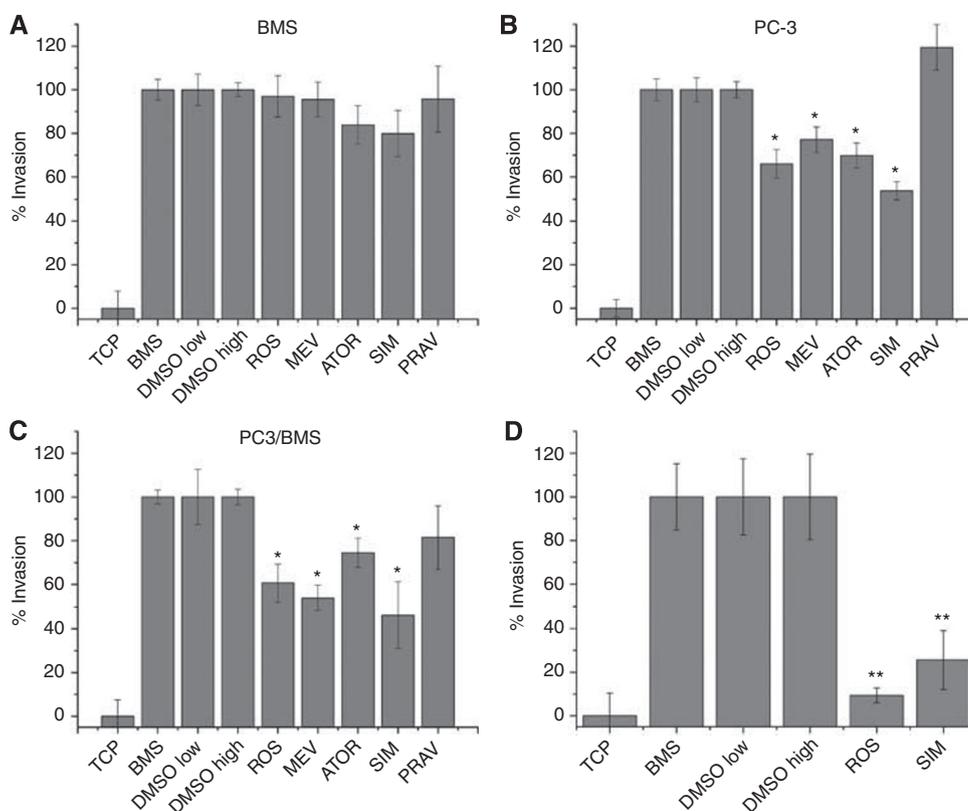


Figure 1 Statins directly affect the ability of CaP to invade towards BMS. **(A)** Bone marrow stroma treated with statins (rosuvastatin (ROS), mevastatin (MEV), atorvastatin (ATOR), simvastatin (SIM) or pravastatin (PRAV)) for 7 days before washing and using as an invasion stimulus in a Boyden chamber assay in the absence of statins. **(B)** PC-3 treated with statins 30 min before adding to the top of the Boyden chamber invasion assay. **(C)** PC-3 cells were pretreated for 30 min before invasion assay in the presence of statins towards BMS previously treated with statins for 7 days. Invasion normalised to TCP and to either BMS (PRAV) or DMSO vehicle controls (atorvastatin, MEV, ROS, SIM); $n = 3$. **(D)** PC-3 treated with either $1 \mu\text{M}$ SIM or $5 \mu\text{M}$ ROS 30 min before adding to the top of the Boyden chamber invasion assay, which contained a confluent BMEC layer on top of a Matrigel layer. * denotes $P < 0.05$ from vehicle control. ** denotes $P > 0.05$ between statins.

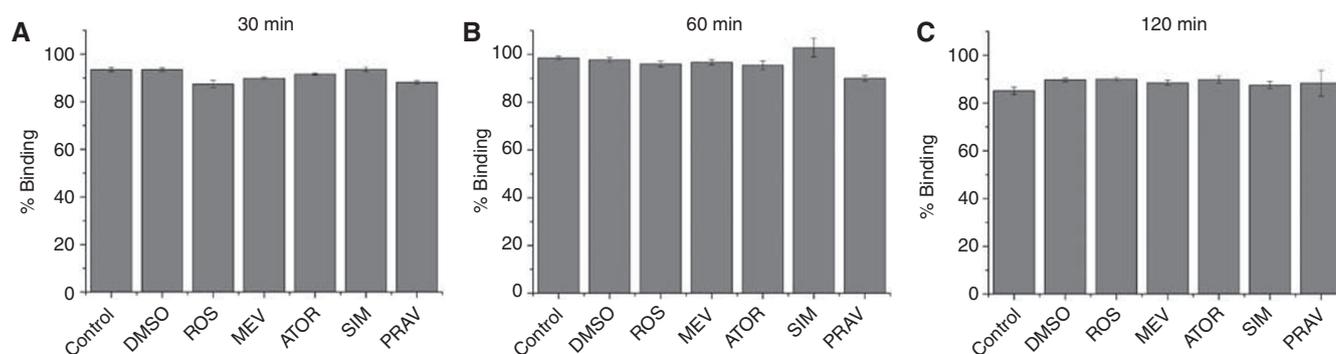


Figure 2 Statins do not inhibit PEC binding to human BMS. PC3-GFP cells were incubated with $1 \mu\text{M}$ atorvastatin, SIM, MEV, $5 \mu\text{M}$ ROS or $10 \mu\text{M}$ PRAV for **(A)** 30, **(B)** 60 or **(C)** 120 min before binding to confluent cultures of BMS. After 60 min, unbound cells were washed off and the mean well fluorescence was determined using a FLUOstar OPTIMA spectrometer. Data expressed as a percentage of the maximum well fluorescence pre-washing; $n = 3$.

PEC colony formation and proliferation in isolation with clonogenic assay or in BMS co-culture.

Pravastatin, at doses up to $100 \mu\text{M}$, had no effect on PC-3 colony formation or cellular proliferation in either clonogenic or BMS co-culture assays (Figure 3A). However, the lipophilic statins and rosuvastatin all had a significant effect on colony formation in both assays and cellular proliferation. Lipophilic statins and rosuvastatin significantly reduced the number of colonies to a similar degree in clonogenic assays and BMS co-culture (Table 1), with simvastatin being the most potent (reduction of 75.44% and

49.44%, respectively). Bone marrow stroma colonies were smaller than controls ($0.19 \pm 0.025 \text{ mm}^2$, $0.21 \pm 0.03 \text{ mm}^2$, $0.21 \pm 0.03 \text{ mm}^2$, $0.19 \pm 0.03 \text{ mm}^2$; $P < 0.05$) resulting in a reduction of the total PEC area ($12.8 \pm 3.34 \text{ mm}^2$, $20.7 \pm 4.5 \text{ mm}^2$, $18.68 \pm 4.1 \text{ mm}^2$, $14 \pm 3.8 \text{ mm}^2$) in co-cultures treated with rosuvastatin, atorvastatin, mevastatin and simvastatin, respectively (Figure 3B). There was no difference ($P > 0.05$) in the colony size, number or overall PEC load between the four statins.

Typically, PC-3 colonies in BMS co-culture formed large diffuse colonies of migrating PECs within the BMS. Cells displayed a

mesenchymal morphology, being teardrop shaped, with ruffled leading edges of lamellipodia in the absence of therapeutic agents or in the presence of DMSO vehicle control (Figure 3C, control). The lipophilic statins induced significant morphological changes

in the BMS colonies. Prostate epithelial cell colonies were more tightly packed, containing fewer cells than controls. At a higher magnification they displayed differing morphology compared with the mesenchymal morphology seen in control co-cultures, with

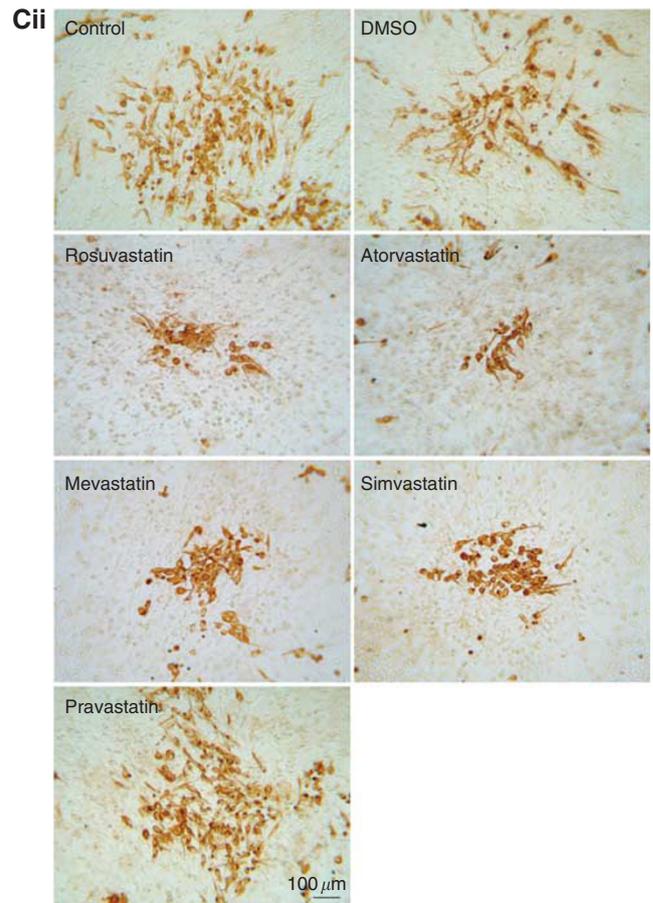
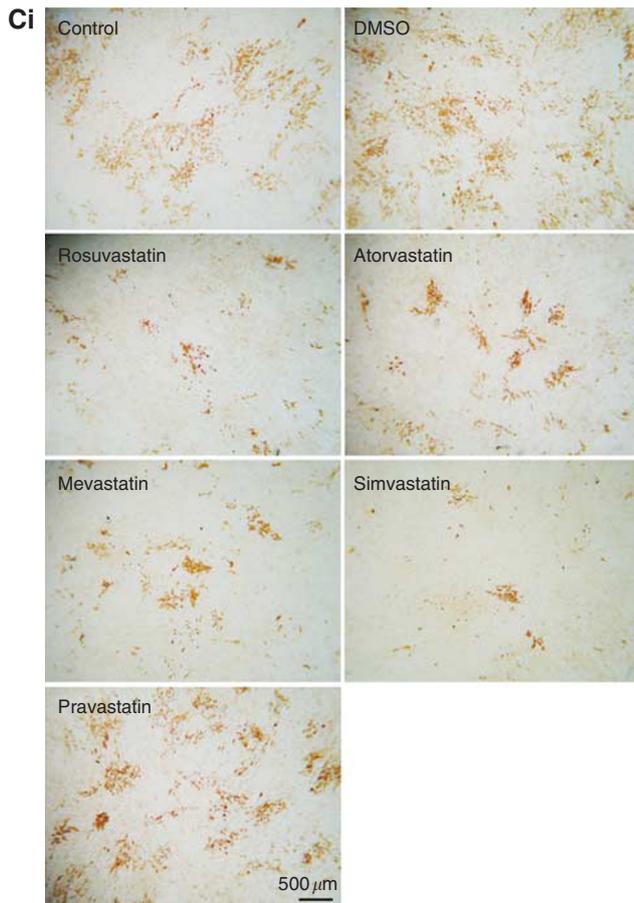
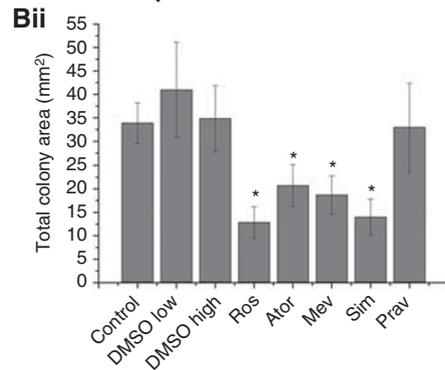
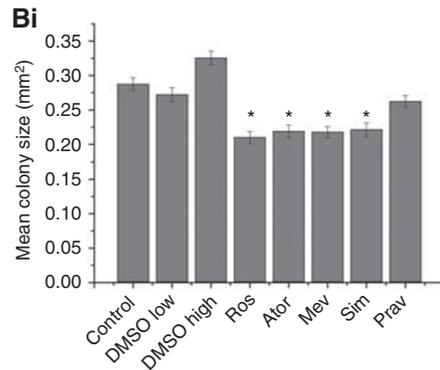
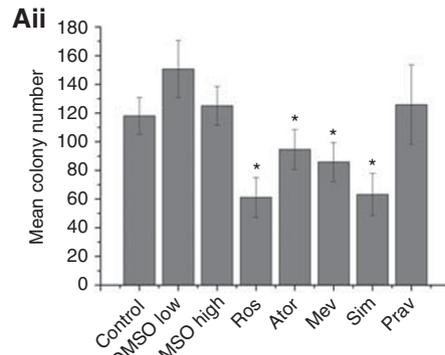
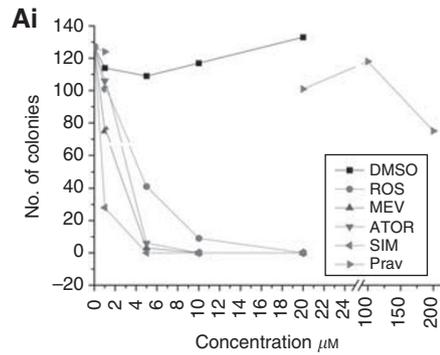


Table 1 Effect of statins on prostate epithelial colony formation in BMS co-culture

	Mean colony number	s.e.	Fold reduction	P-value
No drug control	118	12.84		
DMSO low control	150.7	19.97		
DMSO high control	125	13.54		
Rosuvastatin	61.2	13.76	2.04	0.0095
Atorvastatin	94.6	13.91	1.59	0.055
Mevastatin	85.8	13.56	1.76	0.0315
Simvastatin	63.2	4.72	2.38	0.0117
Pravastatin	125.8	27.79	0.94	0.7928

Abbreviations: BMS = Bone marrow stroma; DMSO = dimethyl sulphoxide; PC-3 colony formation after statin treatment in BMS co-culture assay. PC-3 cells were treated with 1 μ M atorvastatin, mevastatin, simvastatin, 5 μ M rosuvastatin or 10 μ M pravastatin daily for 8 days in BMS co-culture. Colonies >32 cells were counted. Table 1 shows the mean number of colonies, the fold reduction in numbers as compared with the relevant vehicle controls and the significant difference between treatments and their respective vehicle controls.

PECs being more rounded, without obvious lamellipodia at the cell's leading edge. Although some cells retained their teardrop appearance, the leading edge did not possess lamellipodia and the tails were distorted, appearing longer, wider and curved (Figure 3C). These morphological effects were not seen with pravastatin.

Mechanism of statin inhibition of invasion

Statins act by blocking the conversion of HMG-CoA to mevalonic acid through inhibition of HMG-CoA reductase (Figure 4A). We therefore sort to recover the invasion by supplying the downstream metabolites mevalonate, cholesterol or the prenylating agents GGPP and FPP. Treating PC-3-GFP cells with either GGPP or mevalonate had no significant effect on their ability to invade towards BMS ($P=0.259$ and 0.619 , respectively). Both cholesterol and FPP reduced PC3-GFP invasion towards BMS, but this reduction was not significant ($75.51\% \pm 2.25\%$; $P=0.158$ and $54.9\% \pm 8.24\%$; $P=0.056$, respectively). PC3-GFP cells were treated with mevalonate, GGPP, FPP or cholesterol before treatment with

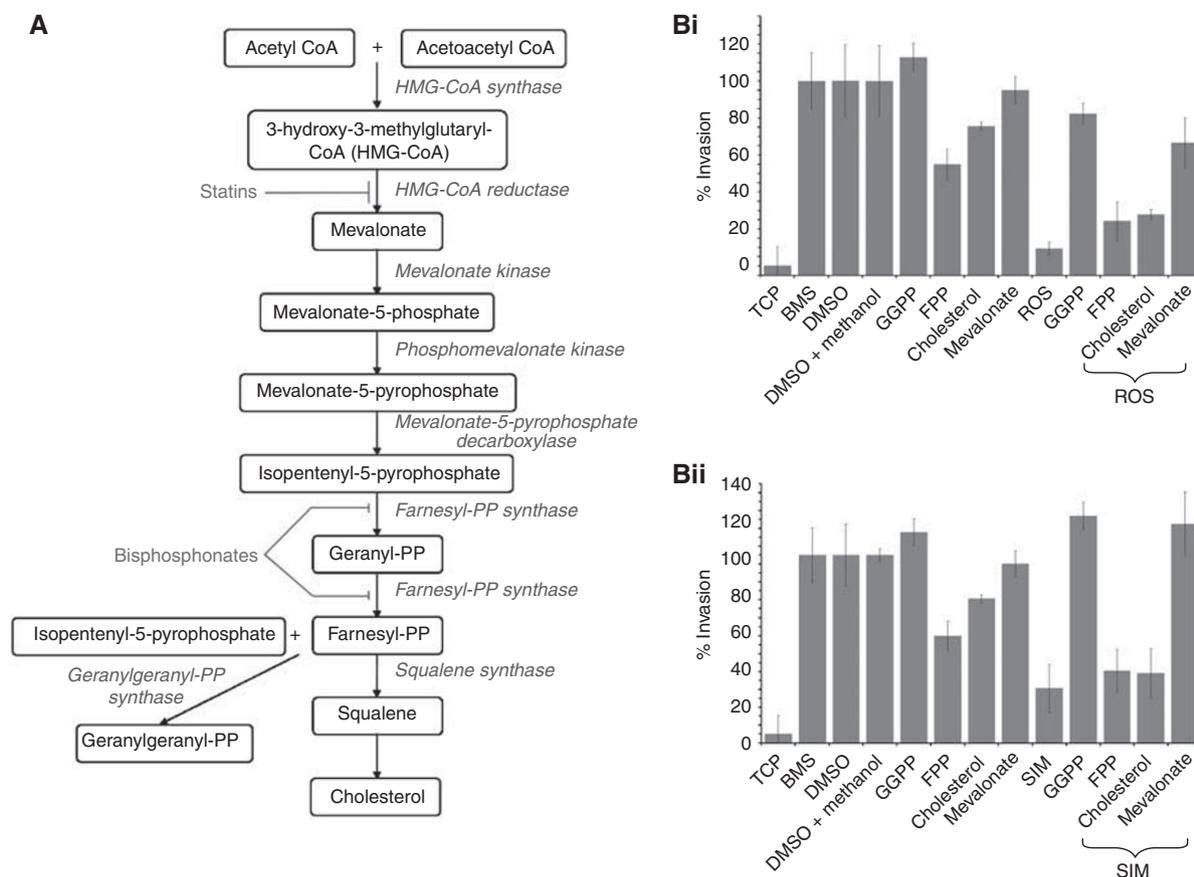


Figure 4 Geranylgeranyl pyrophosphate recovers statin-inhibited invasion towards BMS. **(A)** Diagrammatic representation of the mevalonate pathway highlighting the metabolic blocks of statins and bisphosphonates. **(B)** Histogram depicting the effect of the addition of the mevalonate pathway metabolites (mevalonate, FPP, GGPP or cholesterol) downstream of the statin block in a BMS co-culture assay utilising a BMEC barrier layer. **(Bi)** Invasion recovery assay in the presence of 5 μ M ROS. **(Bii)** Invasion recovery assay in the presence of 1 μ M SIM.

Figure 3 Statins inhibit both colony formation in mono and BMS co-culture and spread in BMS co-culture. **(Ai)** Statin toxicity on PC-3 cells was determined by 14-day clonogenic assays with statin refreshed at day 7. Colonies of >32 cells were counted. **(Aii)** Confluent BMS was pretreated for 60 min with statins before the addition of 500 PC-3 cells. Statins were refreshed daily for 8 days before co-cultures were fixed and PECs stained with pan-cytokeratin DAB. Histogram shows the number of prostate colonies formed, **(Bi)** histogram showing the mean size of colonies formed in BMS co-culture and **(Bii)** histogram showing the total prostate colony area formed in BMS co-culture in the presence of statins; * denotes $P < 0.05$ from vehicle control. **(Ci)** Photomicrographs of typical prostate colonies formed in BMS co-culture in the presence of statins. **(Cii)** High magnification photomicrographs of a single colony formed in bone marrow stromal co-culture in the presence of media alone, DMSO, ROS, ATOR, MEV, SIM, PRAV; $n = 3$.

either 1 μM simvastatin or 5 μM rosuvastatin, and invasion through a BMEC/ Matrigel barrier towards untreated BMS was assessed. The addition of mevalonate to the invasion assay completely restored/enhanced the invasion towards BMS ($66.67\% \pm 13.26\%$; $P=0.1126$ and $117.42\% \pm 17.79\%$; $P=0.0033$ for rosuvastatin and simvastatin, respectively). However, of the addition of downstream metabolites, only GGPP was able to completely restore rosuvastatin-treated invasion towards BMS ($82.42 \pm 5.52\%$; $P=0.229$). Addition of GGPP to simvastatin-treated cells resulted in an enhanced invasion as compared with the statin-free controls ($122 \pm 7.83\%$; $P=0.0185$). Addition of either FPP or cholesterol did not affect the ability of rosuvastatin or simvastatin to inhibit invasion towards BMS. Activation of simvastatin had no effect on the recovery profile, with both GGPP ($111.5 \pm 8.59\%$; $P=0.9079$) and mevalonate ($67.04 \pm 8.03\%$; $P=0.0547$) restoring the invasive ability of PC3-GFP cells towards BMS ($P=0.1391$ and $P=0.2267$ comparing activated to not activated simvastatin in the presence of GGPP or mevalonate, respectively) (Supplementary Figure 2).

DISCUSSION

This is the first study to report the comparative effects of different statins on CaP cellular migration towards and within human BMS. The results demonstrate a clear effect on CaP migration towards and through BMS and on malignant PEC's ability to grow clonally in that location. However, this effect was limited to the lipophilic statins and was not seen with the hydrophilic statin, pravastatin. The differential effect of the two basic subtypes of this class of drug has been well described; lipophilic statins diffuse across cellular membranes and exert their metabolic effects in the liver and other tissues; hydrophilic statins require active transport across the cell membrane in order to exert their actions intracellularly. This action of hydrophilic agents is therefore predominantly hepatic and not peripheral (Stancu and Sima, 2001; Garwood, 2010) and this fact has significant consequences for the effects observed in neoplasms such as breast and other cancers (Campbell *et al*, 2006; Kotamraju *et al*, 2007; Koyuturk *et al*, 2007). This differential action was clearly evident in the results presented herein and may be an important consideration when interpreting data from population studies of the effect of statins or when planning statin-based prevention trials in CaP.

Epidemiological studies have shown that although statin use does not affect CaP incidence, it does reduce the risk of clinical progression and CaP-related mortality; the mechanisms of this are unknown. Statins can induce apoptosis in a range of malignant epithelia *in vitro* but the concentrations of 10–20 μM used far exceed those achievable physiologically in humans. We utilised concentrations of statins with reduced PEC toxicity, allowing assessment of their potential role in invasion and early metastasis. The statin concentrations we used were higher than the serum levels detected with current statin dosing regimes, for example, peak plasma concentration of simvastatin in patients receiving 40 mg per day is 7.2 nM (3 ng ml^{-1}) (Najib *et al*, 2003). However, in trials with lymphoma or myeloma patients (van der Spek *et al*, 2006), the maximum tolerated dose of simvastatin was 15 mg per kg per day, which would increase the circulating serum levels reaching peripheral organs significantly. A regime of 45 mg per kg per day lovastatin in patients with solid tumours induced peak serum levels 3.9 μM (Thibault *et al*, 1996).

Using validated human BMS co-culture models (Hart *et al*, 2005), we have shown that lipophilic statins all affect the ability of PECs to invade towards and through BMS (Figures 1 and 3) to initiate and develop tumour colonies. BMS pretreatment (Figure 1A) had no effect on statins' ability to promote or inhibit this process. Figure 1B (PC-3 treatment alone) and Figure 1C (pretreatment of BMS and PC-3) showed similar reductions in invasion. Utilising the more complex model of metastasis using

BMECs and basement membrane, which recapitulates the blood BMS barrier, both rosuvastatin and simvastatin completely inhibited directed invasion towards BMS. These data suggest that statins affect PECs directly but have no effect on their microenvironment. The exact mechanism underlying statins' ability to block CaP migration is unknown but there are several possible aetiological factors.

A recent large cohort study of cancer-free males over the age of 35, showed that men with desirable/borderline cholesterol levels ($<200 \text{ mg dl}^{-1}$ – $<240 \text{ mg dl}^{-1}$) had a lower risk of developing high grade CaP but no risk reduction in their overall incidence of CaP (Mondul *et al*, 2010). This supports other studies where low cholesterol was not associated with organ confined or low grade CaP but was associated with reduced risk of high grade cancer (Platz *et al*, 2008). A further epidemiological study also showed that low cholesterol was inversely associated with advanced disease (OR = 0.42, 95% CI = 0.13–1.36). The numbers in this study were small but the senior author (Platz *et al*, 2008) and others (Solomon and Freeman, 2008) have gone on to propose that statins affect CaP's intracellular cholesterol metabolism, which is known to be dysregulated. The uptake of statins by the liver and the requirement for liver-specific esterases, for example, in the case of the pro-drug simvastatin, limits statin availability in the peripheral circulation (Merck, 2005). It is therefore hypothesised that statins act by blocking HMG-CoA reductase in the liver and not in tumours, and that the reduction in cholesterol affects tumours at extrahepatic sites. Here we have shown that simvastatin can act directly on the PC3-GFP cells in the absence of activation, (Supplementary Figure 1) suggesting that simvastatin may act within the periphery. Cholesterol is an essential component of lipid rafts, cellular membrane microdomains that regulate signalling cascades originating from membrane-bound receptors such as tyrosine kinases. Treatment of CaP overnight or glioma cells for 48 h with simvastatin induced a 70% reduction in lipid raft cholesterol, an effect reversed by addition of exogenous cholesterol (Zhuang *et al*, 2005; Wu *et al*, 2009). This reduction inhibited the Akt1 serine-threonine kinase/ protein kinase-B signalling pathway and induced apoptosis, an effect that could be reversed by cholesterol replenishment. Corroborating *in vivo* studies showed that elevated cholesterol levels increased pAkt1 signalling and promoted tumour growth, with a coincident reduction in apoptosis.

The studies above demonstrate a role of cholesterol in CaP progression by promoting tumour growth and preventing apoptosis. However, in the study presented here we are specifically modelling the effects of statins on the invasive and migratory abilities of CaP cell towards and within human BMS. Within these validated invasion co-culture models, cholesterol was unable to recover the invasive phenotype after statin treatment. We have shown previously that invasion towards BMS occurs predominantly within the first 4 h (Hart *et al*, 2006). In this study, PC-3 cells were pretreated for 30 min before assay, suggesting that a reduction in cholesterol synthesis by CaP cells is not the factor inhibiting invasion. This observation is supported by co-culture assay data; the co-cultures were grown in the presence of FCS and horse serum, both of which contain cholesterol. It is also noteworthy that invasion was towards haematopoietically active adipocyte-rich human BMS, which contains high cholesterol levels. All the co-cultures treated with lipophilic statins showed proliferation of PC-3 cells but formed smaller colonies of tightly packed epithelial cells, differing in their morphology to the controls, losing the ruffled leading edges of their lamellipodia and displaying distorted trailing edges, indicating motility impairment. Taken together these models suggest that the inhibition of invasion towards/through BMS, but not proliferation, is not related directly to circulating and local tissue cholesterol levels.

A critical factor may be the impairment of the transmigration mechanisms of cancer cells by the effects of statins, in particular, their effect on the mevalonate and isoprenoid axis. Tumour cell exposure to statins downregulates mevalonate expression through

HMG-CoA reductase inhibition. This affects membrane integrity, cell signalling, protein synthesis and cell cycle progression. Disruption of these processes by statins may result in inhibition of tumour initiation, growth and metastasis (Boudreau *et al*, 2007). Mevalonate is the precursor of the isoprenoids FPP and GGPP, essential for the translocation of signalling proteins, such as the G-proteins Ras and Rho to the plasma membrane, where they mediate signal transduction by controlling cell differentiation, proliferation and migration (Hager *et al*, 2006). Addition of GGPP, unlike cholesterol or FPP, to our invasion assays completely restores the ability of PC-3 cells to invade towards the BMS, suggesting that the lipophilic statins act by preventing the translocation of nascent signalling proteins, which require geranylgeranylation, to the cell surface. This would lead to a reduction of signalling proteins at the cell surface and thereby preventing the cell from detecting the invasive stimulus from the BMS or BMS adipocytes (Brown *et al*, 2010).

Further studies support the notion that the mevalonate/G-protein axis is important. Zhao *et al* (2010) demonstrated that 10 μ M lovastatin inhibited EGFR dimerisation and signalling via AKT and its downstream targets, 4E-binding protein 1 and S6 kinase 1, in a GGPP-dependent manner. Lovastatin also induced cytoskeletal disorganisation associated with increased inactive RhoA expression, which lacked the GGPP tag. Co-incubation with mevalonate or GGPP restored RhoA activity and EGF signalling. This suggests that inhibition of invasion towards and through BMS is more likely to arise from isoprenoid loss rather than loss of endogenous cholesterol production by the CaP cells. This latter factor may be critical; the ability of the cancer cell to move is

fundamental to metastasis and its inhibition prohibits metastatic CaP survival in BMS (Lang *et al*, 1998). *In vitro* studies have demonstrated the critical importance of the Rho/Rac axis in mesenchymal to amoeboid transition and invasion/migration (Sanz-Moreno *et al*, 2008); these steps are fundamental to progression in prostate and other cancers. Thus, important elements of metastatic behaviour are potentially inhibited by lipophilic statins and these anti-migrational effects alone may be responsible for the CaP-related observations presented herein and reported clinically (Platz *et al*, 2006; Gutt *et al*, 2010).

Here we have used validated models of malignant prostate epithelial invasion to examine the effects of statins on the migratory pathway in CaP metastasis. We have shown for the first time in this laboratory setting that statins act directly on malignant PEC and block the formation of GGPP from HMG-CoA. Loss of GGPP leads to a significant reduction in the ability of malignant PEC to invade towards and through BMS and so reduce their ability to form colonies within the BMS. This statin effect is not universal across all classes of statins as the hydrophilic statin pravastatin had no effect on the PC-3 cell line in our models. In summary, statins appear to act on two key components on CaP metastasis; reduce tumour growth, possibly through the reduction of cholesterol but also, as described here, through inhibiting geranylgeranyl prenylated pathways required for transendothelial migration and BMS invasion.

Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

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