

S100A4-induced cell motility and metastasis is restricted by the Wnt/ β -catenin pathway inhibitor calcimycin in colon cancer cells

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ABSTRACT The calcium-binding protein S100A4 is a central mediator of metastasis formation in colon cancer. S100A4 is a target gene of the Wnt/ β -catenin pathway, which is constitutively active in the majority of colon cancers. In this study a high-throughput screen was performed to identify small-molecule compounds targeting the S100A4-promoter activity. In this screen calcimycin was identified as a transcriptional inhibitor of S100A4. In colon cancer cells calcimycin treatment reduced S100A4 mRNA and protein expression in a dose- and time-dependent manner. S100A4-induced cellular processes associated with metastasis formation, such as cell migration and invasion, were inhibited by calcimycin in an S100A4-specific manner. Calcimycin reduced β -catenin mRNA and protein levels despite the expression of $\Delta 45$ -mutated β -catenin. Consequently, calcimycin inhibited Wnt/ β -catenin pathway activity and the expression of prominent β -catenin target genes such as S100A4, cyclin D1, c-myc, and dickkopf-1. Finally, calcimycin treatment of human colon cancer cells inhibited metastasis formation in xenografted immunodeficient mice. Our results demonstrate that targeting the expression of S100A4 with calcimycin provides a functional strategy to restrict cell motility in colon cancer cells. Therefore calcimycin may be useful for studying S100A4 biology, and these studies may serve as a lead for the development of treatments for colon cancer metastasis.

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INTRODUCTION

S100A4 is a ubiquitous small, calcium-binding protein that enables cell migration and invasion to increase cell motility (Garrett *et al.*, 2006). Consequently, S100A4 expression is up-regulated during wound healing, neurite outgrowth, fibrosis, or neovascularization—

all physiological processes that rely on increased cell motility. However, overexpression of S100A4 is often correlated with pathological conditions such as epithelial–mesenchymal transition, tumor outgrowth, and metastasis formation (Schneider *et al.*, 2008; Boye and Maelandsmo, 2010).

In colon cancer distant metastases are the major cause for cancer death, rendering this disease the second-most-frequent cause of cancer-related death in the Western world (Jemal *et al.*, 2008). S100A4 expression is significantly associated with metastasis formation in colorectal cancer patients, and the expression of S100A4 represents a significant prognostic marker in colorectal carcinoma (Gongoll *et al.*, 2002; Cho *et al.*, 2005; Stein *et al.*, 2006). Despite S100A4 increasing the metastatic potential of the cell, S100A4 transgenic mice are not phenotypically changed (Ambartsumian *et al.*, 1996). However, when these are crossed with spontaneous tumor-forming mice, S100A4 overexpression leads to highly

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Abbreviations used: ANOVA, one-way analysis of variance; DKK-1, dickkopf-1; GAPDH, glycerin-aldehyde-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; LOPAC, library of pharmacologically active compounds; MMP, matrix metalloproteinase; NOD/SCID, nonobese diabetic/severe immune deficient; TCF, T cell factor.

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aggressive primary tumors and formation of metastasis (Davies *et al.*, 1996). Highly metastatic mouse mammary carcinoma cells injected into S100A4 null mice are unable to form metastases (Grum-Schwensen *et al.*, 2005). These observations suggest that S100A4 is not simply a marker for metastatic disease but rather has a causal role in mediating this process.

Tumor growth and metastasis depend on increased cell migration, invasion, and angiogenesis. S100A4 is involved in all of these processes by interaction with multiple proteins in the cytoplasm and extracellular space. For instance, intracellular S100A4 increases cell migration by rearranging proteins of the cytoskeleton. S100A4 binds tropomyosin and nonmuscle myosin II in a Ca²⁺-dependent manner and inhibits the actin-regulated ATPase activity of myosin II (Kriajevska *et al.*, 1994; Ford *et al.*, 1997). It thus promotes the disassembly of myosin filaments and inhibits their reassembly (Ford *et al.*, 1997; Li *et al.*, 2003). S100A4 is located at the leading edge of migrating cells (Kim and Helfman, 2003), where it induces the formation of flexible protrusions. Moreover, in the presence of a chemoattractant, S100A4 enhances directed migration (Li and Bresnick, 2006). Thus the S100A4–myosin II interaction not only increases cell motility, but it also enhances cell polarization and directed migration.

S100A4 expression is associated with reduced expression levels of E-cadherin, thus loosening epithelial cell adhesion (Keirsebilck *et al.*, 1998). In addition, S100A4 reduces cell adhesion by interacting with liprin-β1 (Kriajevska *et al.*, 2002). S100A4 can access the extracellular space by a yet-unknown mechanism, by which it interacts with endothelial-bound annexin II and thus enhances angiogenesis (Ambartsumian *et al.*, 2001; Kriajevska *et al.*, 2002). Extracellular S100A4 can also bind to the receptor for glycation end products and thereby activate matrix metalloproteinase-13 (MMP-13) expression (Yammani *et al.*, 2006). Furthermore, up-regulation of MMP-2 and MMP-9 expression by extracellular S100A4 allows cell invasion and thus promotes metastasis formation (Mathisen *et al.*, 2003; Saleem *et al.*, 2006).

S100A4 gene transcription in colon cancer cells is regulated by the canonical Wnt/β-catenin pathway, one of the most frequently deregulated pathways in colon cancer (Stein *et al.*, 2006). The pathway strictly controls the cytoplasmic level of β-catenin via a destruction complex. In this complex β-catenin is phosphorylated and thus marked for proteasomal degradation (Barker and Clevers, 2006). Mutations within this pathway lead to intracellular accumulation of β-catenin, which then translocates to the nucleus and activates β-catenin/T cell factor (TCF) target gene transcription (Giles *et al.*, 2003; Stein *et al.*, 2006).

Because S100A4 plays such a central role in the process of metastasis formation, the inhibition of S100A4 expression provides a promising strategy for novel antimetastatic treatments (Sack and Stein, 2009). To identify potential S100A4 transcription inhibitors, we performed a high-throughput screen for compounds that inhibited S100A4-promoter activity. Thus we identified calcimycin as a novel inhibitor of S100A4 transcription, which overcomes a constitutively active Wnt/β-catenin pathway and significantly restricts S100A4-induced cell motility in colon cancer cells.

RESULTS

Identification of S100A4 transcription inhibitor calcimycin

Calcimycin was identified as an inhibitor for S100A4 expression in a high-throughput screen of the Library of Pharmacologically Active Compounds (LOPAC) 1280, a collection of 1280 pharmacologically active compounds that have shown effects on major drug target classes such as kinases, G proteins, and ion channels. HCT116/S100A4pLUC cells expressing S100A4-promoter-driven firefly lu-

ciferase were exposed to 20 twofold dilutions of calcimycin. After 24 h, luciferase activity was determined as a read-out for S100A4-promoter activity (Figure 1A). From the high-throughput data it was found that calcimycin inhibited luciferase activity in a concentration-dependent manner, with an EC₅₀ of 2.7 μM (95% confidence interval [CI], 1.0–4.0 μM). At the same time growth inhibition was assessed from the high-throughput data, yielding an IC₅₀ of ~80 μM (95% CI, 15.2–403.9 μM).

To define cytotoxic side effects potentially affecting further experiments, the effect of calcimycin on cell viability of HCT116 cells was analyzed more precisely by manual pipetting. Exposure of HCT116 cells to calcimycin concentrations ranging from 2 nM to 300 μM for 24 h resulted in a reduction of cell viability in a concentration-dependent manner with an IC₅₀ of 2.1 μM (95% CI, 1.5–2.2 μM; Figure 1B).

We next analyzed the ability of calcimycin to reduce endogenous S100A4 expression in HCT116 cells. Exposure of HCT116 cells to increasing concentrations of calcimycin for 24 h resulted in a concentration-dependent reduction of S100A4 mRNA and protein. Treatment with 1 μM calcimycin reduced S100A4 expression to 30% of that for the solvent-treated control (Figure 1C). Cell viability was not affected by this concentration of calcimycin after 24 h. Therefore we chose the calcimycin concentration of 1 μM for use in the following studies.

S100A4 expression is inhibited in a time-dependent manner by calcimycin

To assess the kinetics of the calcimycin effect, HCT116 cells were exposed to 1 μM calcimycin, and S100A4 expression was analyzed every 6 h. S100A4 mRNA was reduced in a time-dependent manner. After 12 h of treatment a basal reduction level of ~30% of the solvent-treated control was observed (Figure 1D). After 18 h of treatment the S100A4 protein levels were also reduced. S100A4 expression inhibition continued for 5 consecutive days after a single dose of 1 μM calcimycin (Figure 1E). Similar to HCT116 cells, HCT116/vector cells showed reduced S100A4 mRNA to ~30% of solvent-treated control cells after 1 μM calcimycin treatment for 24 h (Figure 1F). In contrast, HCT116/S100A4 cells showed no significant reduction in S100A4 mRNA or protein upon calcimycin treatment. These cells overexpressed S100A4 cDNA under the control of a cytomegalovirus (CMV) promoter, which was not targeted by calcimycin treatment.

S100A4-induced cell migration and invasion is inhibited by calcimycin

We previously showed that S100A4 expression in colon tumors significantly correlates with metastasis formation (Stein *et al.*, 2006). Moreover, S100A4 drives metastasis formation by increasing cell migration and invasion (Garrett *et al.*, 2006). Therefore we next examined S100A4-induced cell motility in the presence of calcimycin. Exposure of HCT116 cells to calcimycin significantly reduced the number of migrated cells to 20% of that for solvent-treated HCT116 cells (Figure 2A). Similarly, calcimycin treatment inhibited cell migration of HCT116/vector cells to 16% of that for the control cells. In contrast, HCT116/S100A4 cells showed no significant reduction in cell migration upon calcimycin treatment. Cell invasion of calcimycin-treated HCT116 and HCT116/vector cells was inhibited to <30% of that for the solvent-treated control cells (Figure 2B). In contrast, cell invasion of HCT116/S100A4 cells was not significantly changed upon calcimycin treatment. Directed migration analyzed by wound-healing assay revealed that calcimycin clearly inhibited wound closure in HCT116 and HCT116/vector cells but not in HCT116/S100A4 cells (Figure 2C). In summary, calcimycin displayed an antimigratory

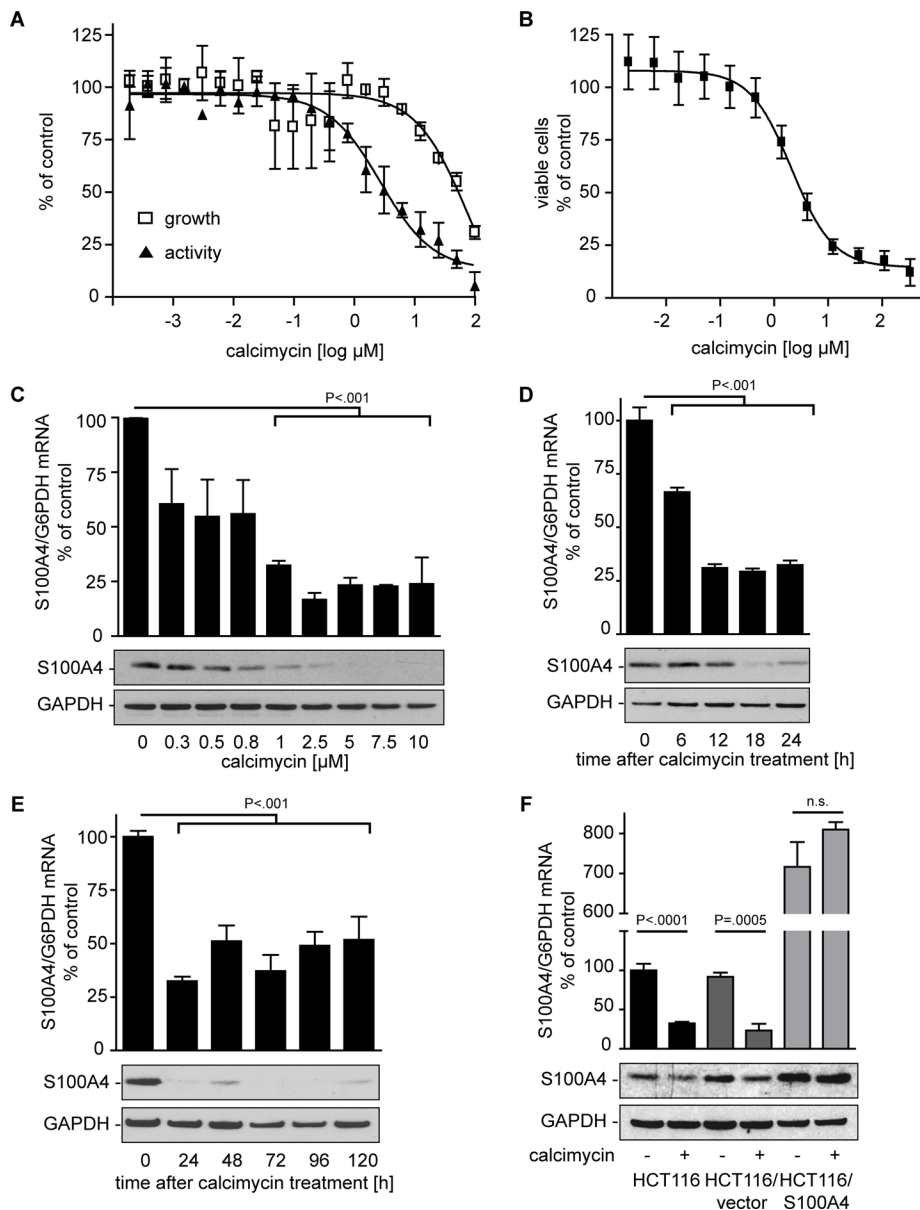


FIGURE 1: S100A4-promoter activity is inhibited by calcimycin in a concentration- and time-dependent manner. Luciferase activity and cell viability of calcimycin-treated HCT116/S100A4pLUC cells were determined after 24 h. S100A4/G6PDH mRNA ratios were determined by qRT-PCR. S100A4 and GAPDH protein levels were measured by Western blot. (A) High-throughput screening data of calcimycin inhibiting S100A4 promoter-driven reporter activity and cell viability. (B) Cell viability of HCT116 cells treated with increasing concentrations of calcimycin for 24 h is reduced in a concentration-dependent manner. (C) S100A4 mRNA and protein levels are reduced in HCT116 cells treated with increasing concentrations of calcimycin. (C, D) S100A4 expression is reduced in HCT116 cells treated with a single dose of 1 μM calcimycin for the time indicated. Data represent mean ± SE (n = 4). Statistical significance was analyzed by two-sided ANOVA and Bonferroni post hoc multiple comparison test. (E) CMV promoter-driven S100A4 expression is not affected by calcimycin treatment. Data represent mean ± SE (n = 3). Statistical significance was analyzed by Student's t test.

and anti-invasive effect in HCT116 cells, and this effect was overcome by the exogenous overexpression of S100A4.

Calcimycin inhibits anchorage-dependent and anchorage-independent cell proliferation

We next analyzed cell proliferation under calcimycin treatment. Exposure of HCT116, HCT116/vector, and HCT116/S100A4 cells to a single dose of 1 μM calcimycin resulted in a clear inhibition of an-

chorage-dependent proliferation after day 3 (Figure 3A). Calcimycin further inhibited anchorage-independent growth as analyzed by colony formation. On exposure to calcimycin the number of colonies formed by HCT116, HCT116/vector, and HCT116/S100A4 cells was significantly reduced to <5% of that for solvent-treated control cells (Figure 3B). Furthermore, as shown in the insets of the microphotographs, the size of colonies was clearly reduced by calcimycin treatment. Proliferation of cells that exogenously overexpress S100A4 was also affected by calcimycin treatment. Thus the antiproliferative effect of calcimycin on anchorage-dependent and anchorage-independent growth was not overcome by an increased S100A4 expression level.

Calcimycin inhibits S100A4 expression, cell motility, and proliferation in other human colon cancer cell lines

We next analyzed calcimycin effects in other human colon cancer cell lines. Exposure of SW620, LS174T, and SW480 cells to 1 μM calcimycin for 24 h reduced the S100A4 mRNA level to <40% of that for the respective solvent-treated controls (Figure 4A). S100A4 protein in these cells was clearly reduced upon calcimycin treatment. In DLD-1 cells no S100A4 mRNA and protein expression was detected, and therefore no further effect by calcimycin was possible.

Exposure of SW620, LS174T, and SW480 cells to 1 μM calcimycin for 24 h inhibited cell migration to <25% of that for the respective solvent-treated controls (Figure 4B). DLD-1 cells presented the lowest migration rate, which was not further affected by calcimycin treatment. Cell invasion of SW620, LS174T, and SW480 cells was reduced to <30% of that for the respective solvent-treated controls by calcimycin treatment (Figure 4C). The low cell invasion rate of DLD-1 cells was not significantly changed upon calcimycin treatment. In the wound-healing assay solvent-treated SW620, LS174T, and SW480 cells were able to close the wound by day 4 after wound insertion (Figure 4D). In contrast, in solvent-treated DLD-1 cells wound closure was not completed by day 4, which was consistent with their decreased migration and invasion rates. Following a single dose of 1 μM calcimycin, wound closure, as measurement for directed migration, was impaired in all four colon cancer cell lines for 4 d post-wound insertion.

and anti-invasive effect in HCT116 cells, and this effect was overcome by the exogenous overexpression of S100A4.

Anchorage-dependent cell proliferation was inhibited upon calcimycin treatment in all four colon cancer cell lines as measured on day 4 (Figure 4E). In a colony formation assay solvent-treated SW620, LS174T, and SW480 cells formed large colonies by day 7 (Figure 4F). Colony formation was inhibited in all four colon cancer

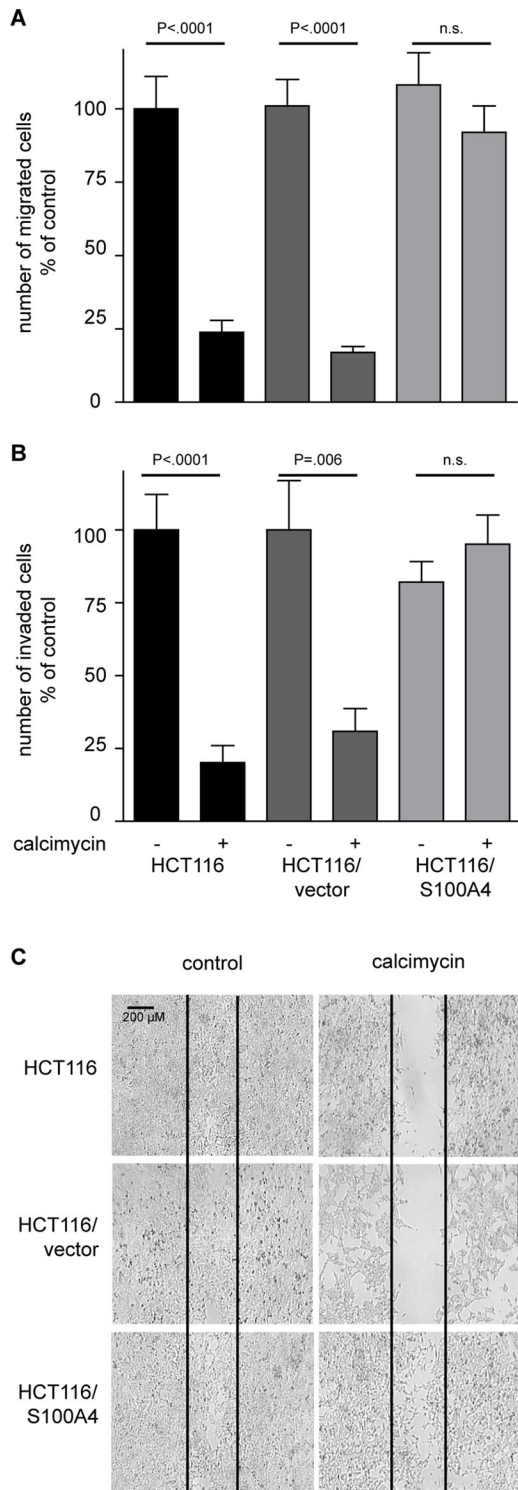


FIGURE 2: S100A4 induced cell motility is inhibited by calcimycin. Cell migration was determined with Boyden chamber and wound-healing assay. Cell invasion was measured with a Matrigel-covered Boyden chamber assay (A) Cell migration is inhibited in HCT116 and HCT116/vector cells but not in HCT116/S100A4 cells when treated with calcimycin. (B) Cell invasion is inhibited in calcimycin-treated HCT116 and HCT116/vector cells but not in HCT116/S100A4 cells. Data represent mean \pm SE (n = 5). Statistical significance was analyzed with Student's t test. (C) Directed migration is inhibited in calcimycin-treated HCT116 and HCT116/vector cells but not in HCT116/S100A4 cells. Microphotographs were taken with 10 \times magnification 4 d after wound was entered.

cell lines following calcimycin treatment. Colonies of solvent-treated DLD-1 cells were smaller than colonies of other solvent-treated colon cancer cell lines. However, the size of DLD-1 colonies was still reduced upon calcimycin treatment. Quantification of formed colonies revealed that calcimycin treatment significantly reduced the number of colonies in all four colon cancer cell lines to <10% of the respective-solvent treated controls (Figure 4G). In summary, calcimycin inhibited cell migration and invasion of various colon cancer cell lines in relation to their S100A4 expression level. Cell proliferation was inhibited independent of the S100A4 expression level of S100A4 in these cells.

Calcimycin inhibits constitutively active Wnt/ β -catenin pathway signaling

We previously reported that S100A4 is a β -catenin target gene (Stein *et al.*, 2006). Therefore we investigated Wnt/ β -catenin pathway activity using the TOP/FOPflash reporter assay to assess the mechanism underlying calcimycin inhibition of S100A4 expression. We used HCT116 cells that are heterozygous for mutated β -catenin. The mutated β -catenin lacks serine 45, the initial phosphorylation site needed for proteasomal degradation (Amit *et al.*, 2002). Consistent with this mutation, in these cells expression of the TOPflash reporter is increased. Treatment of HCT116 cells with calcimycin reduced the TOPflash reporter activity to <30% upon calcimycin treatment (Figure 5A). In HAB68^{mut} cells, which are homozygous for this β -catenin mutation, calcimycin treatment reduced TOPflash reporter activity to <10% of that for solvent-treated HCT116 cells. TOPflash reporter activity of solvent-treated HAB92^{wt} cells, which are homozygous for wild-type β -catenin, was <30% of the activity found in cells that bear a constitutively active Wnt/ β -catenin pathway due to mutated β -catenin. Moreover, calcimycin treatment of HAB92^{wt} cells further reduced this TOPflash reporter activity to 12%.

We next analyzed the expression level of β -catenin, which represents the central player of this pathway. In calcimycin-treated HCT116, HAB68^{mut}, and HAB92^{wt} cells the β -catenin mRNA levels were reduced to <50% of that for solvent-treated HCT116 cells (Figure 5B). Consistent with this result, the β -catenin protein level was diminished upon calcimycin treatment of these cells.

Reduced β -catenin levels should result in reduced target gene transcription. Therefore we analyzed the expression levels of prominent β -catenin/TCF target genes such as cyclin D1 (Shtutman *et al.*, 1999), c-myc (He *et al.*, 1998), and dickkopf-1 (DKK-1; Gonzalez-Sancho *et al.*, 2005). In calcimycin-treated HCT116 cells the expression of cyclin D1, c-myc, and DKK-1 was inhibited to 35, 25, and 2% of the solvent-treated controls, respectively (Figure 5C).

We analyzed the effect of calcimycin on S100A4 expression in the presence of mutated or wild-type β -catenin and found that S100A4 mRNA was reduced to 35% in HAB68^{mut} and HCT116 cells and was hardly detectable in HAB92^{wt} cells (Figure 5D). In parallel, calcimycin treatment reduced S100A4 protein levels in HCT116 and HAB68^{mut} cells, whereas no S100A4 protein was detectable in HAB92^{wt} cells. Cell migration in HCT116 and HAB68^{mut} cells was inhibited to the extent of HAB92^{wt} cells, which was <25% of that for solvent-treated HCT116 cells (Figure 5E). These results provide evidence that calcimycin restricts the Wnt/ β -catenin pathway-driven S100A4 expression and thus S100A4-induced cell motility.

Calcimycin inhibits the metastatic potential of human colon cancer cells in xenografted mice

To test the effect of calcimycin on the ability of cells to form metastasis *in vivo*, we used HCT116/LUC cells, which stably expressed the firefly luciferase protein and therefore allowed the

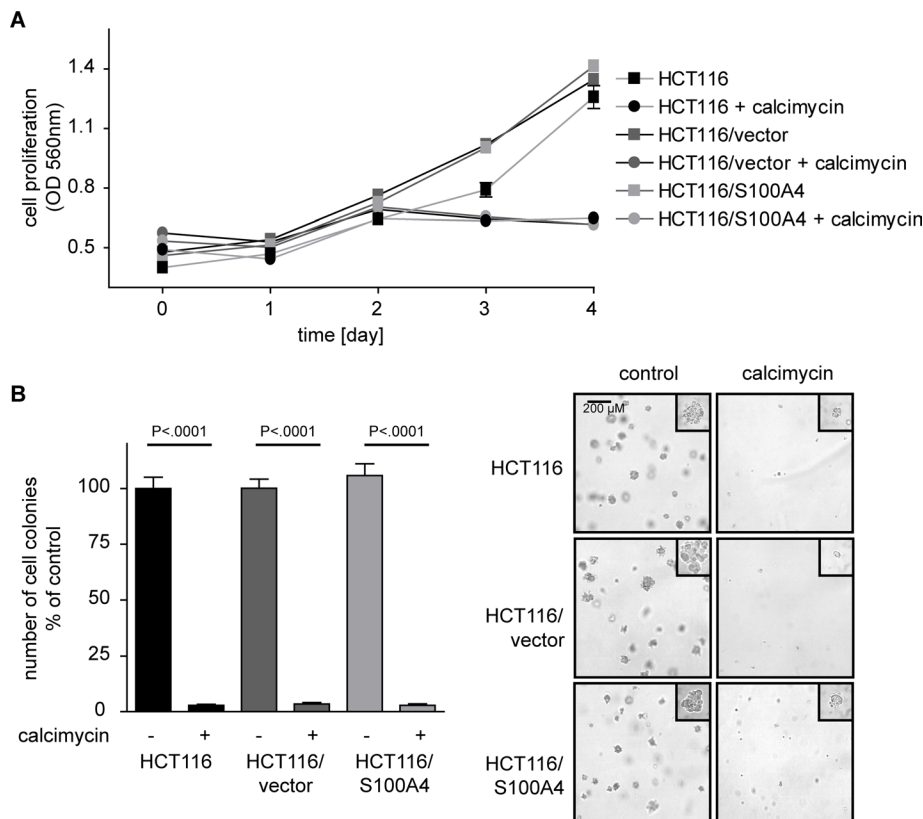


FIGURE 3: Calcimycin inhibits anchorage-dependent and anchorage-independent cell growth. Anchorage-dependent growth was determined by MTT assay; anchorage-independent growth was measured in a soft-agar colony formation assay. (A) Anchorage-dependent cell proliferation of HCT116, HCT116/vector, and HCT116/S100A4 cells was reduced upon calcimycin treatment (■, solvent-treated HCT116; ●, calcimycin-treated HCT116; ▣, solvent-treated HCT116/vector; ●, calcimycin-treated HCT116/vector; ▤, solvent-treated HCT116/S100A4; ●, calcimycin-treated HCT116/S100A4 cells). (B) Anchorage-independent cell proliferation of HCT116, HCT116/vector, and HCT116/S100A4 cells was inhibited by calcimycin. Number of colonies was counted on day 7, when microphotographs were taken with 10× and 40× (insets) magnification. Data represent mean ± SE (n = 3). Statistical significance was determined by Student's *t* test.

detection of metastases via noninvasive *in vivo* luminescence imaging. Cells were treated with 1 μM calcimycin or solvent for 24 h and subsequently inoculated intrasplenically into nonobese diabetic/severe immune-deficient (NOD/SCID)-IL2R⁻ mice. Six days posttransplantation, luminescence signals were detected in the lateral and ventral abdominal cavity, where the spleen and liver are situated, respectively (Figure 6A). In control mice, the ventral luminescence signal was stronger than in mice from the calcimycin group.

To investigate the origin of luminescence, the spleen and the liver were dissected. All animals developed tumors in the spleen, which were clearly visible in the bright-field analysis (Figure 6B). A strong luminescence signal originated from the spleen tumor, indicating that the tumor was formed by HCT116/LUC cells. Bright-field analysis of the resected livers revealed no clear differences between mice of the control and calcimycin groups. However, the luminescence signal from livers of control mice was clearly stronger than that from livers of calcimycin mice.

Quantification of the luminescence signal revealed no significant differences in the signal intensity of spleen tumors from mice of the control and calcimycin groups (Figure 6C). In contrast, the luminescence signal of livers was reduced to 30% in mice of the calcimycin group compared with control mice.

From the reduced liver luminescence signal in mice of the calcimycin group, we hypothesized that the calcimycin treatment hampered HCT116/LUC cells from forming metastases in the liver tissue. We therefore performed immunohistological staining for human cytokeratin-19 in liver cryosections of mice injected with control and calcimycin-treated cells. In the livers of control mice the incidence of micrometastases was clearly higher than in those of mice of the calcimycin group (Figure 6D). Moreover, the size of the micrometastases was reduced in livers of mice injected with calcimycin-treated cells.

We next quantified the amount of human DNA in the liver sections of control and calcimycin mice by quantitative PCR amplification of human satellite DNA as previously described (Becker *et al.*, 2002). In livers from mice of the calcimycin group the amount of human DNA was reduced to ~25% of that of control mice, indicating that the calcimycin treatment indeed inhibited human colon cancer cells from forming liver metastases (Figure 6E). Analysis of the human-specific S100A4 expression in liver metastases of the xenografted mice revealed that in livers from mice of the calcimycin group the S100A4 mRNA was hardly detectable, in contrast to the control mice (Figure 6F). From these data we conclude that the treatment of colon cancer cells with calcimycin restricted their potential to form liver metastases *in vivo*.

DISCUSSION

Intensive research has demonstrated the central role of S100A4 in the process of cancer metastasis, which qualifies S100A4 as a potentially promising target for therapeutic intervention against metastasis (Sherbet, 2009; Boye and Maelandsmo, 2010). Most of the work concerning S100A4 has concentrated on the elucidation of the mechanism by which this molecule drives metastasis. Less work has focused on the inhibition of S100A4 to reduce S100A4-induced cell motility. Here we report the calcium ionophore calcimycin as an inhibitor of S100A4 transcription in colon cancer cells. We show that calcimycin treatment inhibits a constitutively active Wnt/β-catenin pathway, thereby inhibiting S100A4 expression and leading to restricted S100A4-induced cell migration and invasion *in vitro* and *in vivo*.

In a high-throughput screen we identified calcimycin as one of the most effective inhibitors of S100A4-promoter activity. In this screen we used HCT116 cells in which the S100A4-promoter was highly active. Inhibition of S100A4-promoter activity by calcimycin indicated that calcimycin targets S100A4 at the transcription level. Calcimycin reduced S100A4 mRNA levels in a concentration- and time-dependent manner in colon cancer cell lines with increased basal S100A4 expression levels. In line with our observation in human colon cancer cells, calcimycin has been reported to reduce the S100A4 mRNA level in mouse mammary adenocarcinoma cells, as well as in human monocytes and lymphocytes

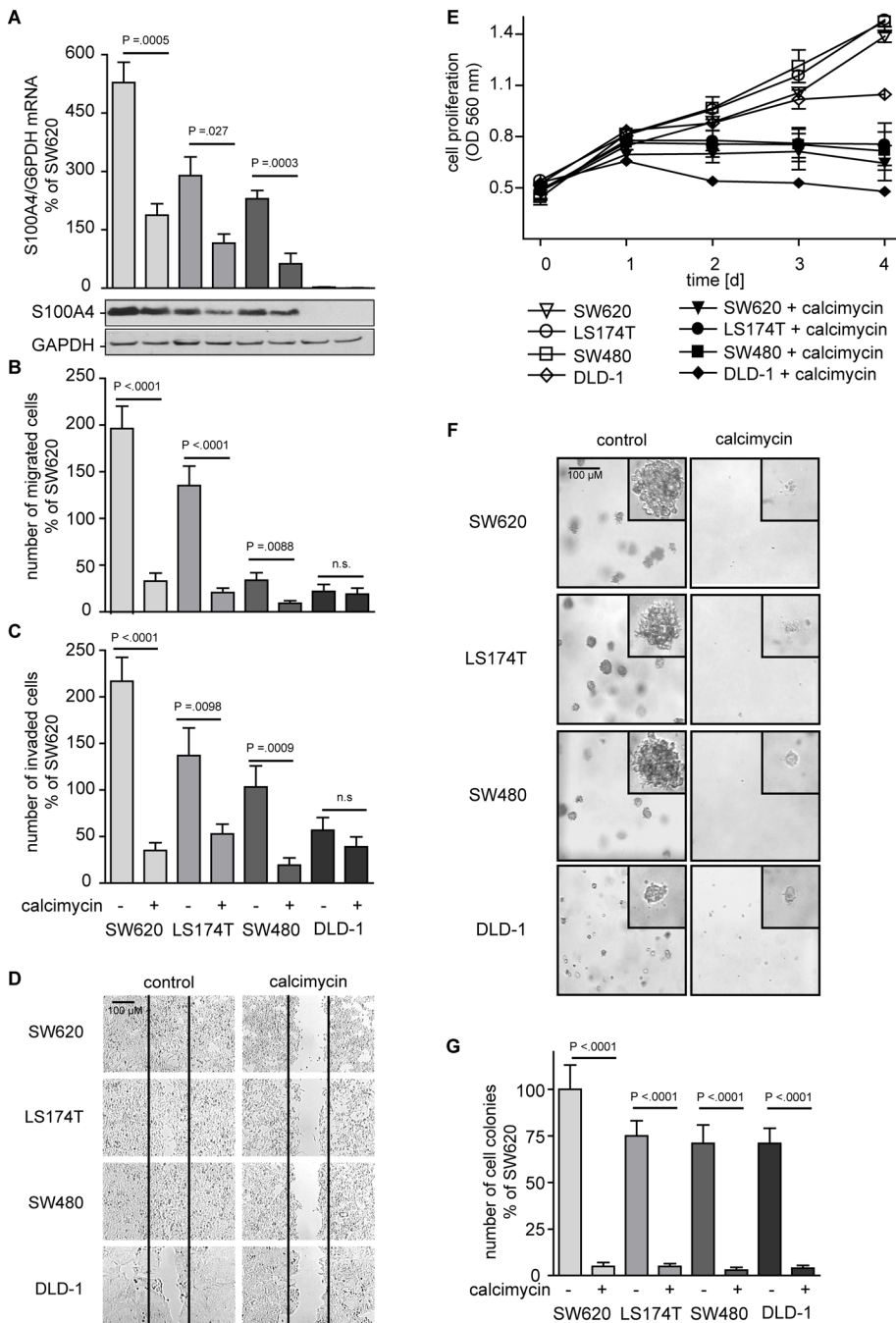


FIGURE 4: Calcimycin inhibits S100A4 expression, cell motility, and proliferation in human colon cancer cell lines. S100A4/G6PDH mRNA ratios were determined by qRT-PCR. S100A4 and GAPDH protein levels were measured by Western blot. Cell migration was determined with Boyden chamber and wound-healing assay. Cell invasion was measured with a Matrigel-covered Boyden chamber assay. Anchorage-dependent growth was determined by MTT assay; anchorage-independent growth was measured in a soft-agar colony formation assay. (A) S100A4 expression was reduced upon calcimycin treatment. (B) Calcimycin treatment inhibited cell migration. (C) Cell invasion was inhibited upon calcimycin treatment. (D) Calcimycin inhibited directed migration. (E) Calcimycin inhibited cell proliferation. (F, G) Calcimycin reduced the size and number of colonies formed, respectively. Number of colonies was counted on day 7, when microphotographs were taken with 10 \times and 40 \times (insets) magnification. Data represent mean \pm SE (n = 3). Statistical significance was determined by Student's *t* test.

(Grigorian *et al.*, 1994). We further showed that S100A4 protein levels were also suppressed in a concentration- and time-dependent manner following calcimycin treatment in several colon cancer cell lines.

Calcimycin is an ionophorous, polyether antibiotic isolated from *Streptomyces chartreusensis*. Calcimycin facilitates the transport of divalent cations across the membrane, which makes it a useful tool to study calcium signaling (Pressman, 1976). Because calcimycin elevates intracellular calcium levels (Gwak *et al.*, 2006), one would expect that calcimycin treatment would increase S100A4 protein activity, which depends on calcium ions (Santamaria-Kisiel *et al.*, 2006). However, in our study, we did not see an increased migratory or invasive phenotype in exogenously S100A4-overexpressing cells that were treated with calcimycin. Although we cannot completely exclude that calcimycin caused increased S100A4 protein activity, we definitely show here that reducing the overall expression level of S100A4 significantly inhibits S100A4-induced cell motility.

S100A4 protein drives metastasis by interaction with a multitude of partner proteins, leading to increased cell migration and invasion (Belot *et al.*, 2002; Stein *et al.*, 2006). Consequently, down-regulation of S100A4 expression by calcimycin restricted cell migration in colon cancer cells. This is in line with observations from RNA interference experiments in which knockdown of S100A4 mRNA was shown to reduce cell migration (Gao *et al.*, 2005; Tabata *et al.*, 2009). Of interest, calcimycin was not able to suppress cell migration or invasion in cells that exogenously overexpressed S100A4. From these observations we conclude that the antimigratory and anti-invasive effect of calcimycin was caused by the down-regulation of S100A4 expression from its native promoter. Knockdown of S100A4 mRNA levels with short hairpin RNA (shRNA) was shown to inhibit cell proliferation in vitro and to reduce tumor growth and metastasis in vivo (Shi *et al.*, 2006). Moreover, in gastric cancer cells, shRNA knockdown of S100A4 increased the occurrence of apoptosis (Hua *et al.*, 2009). In our study, knockdown of S100A4 expression by treatment with calcimycin was accompanied by reduced cell proliferation. Exogenous overexpression of S100A4 was ineffective in overcoming the antiproliferative effect of calcimycin, which suggests that this effect is independent of the S100A4 expression level.

S100A4 is a target gene of the Wnt/ β -catenin pathway (Stein *et al.*, 2006). We found that calcimycin treatment inhibits β -catenin transcription, which leads to the inhibition of the Wnt/ β -catenin pathway activity in colon cancer cells and explains its inhibitory effect on S100A4 expression. Of interest, calcimycin was able to overcome a constitutively active Wnt/ β -catenin pathway, since it reduced β -catenin also in cells that are heterozygous or even homozygous for $\Delta 45$ -mutated

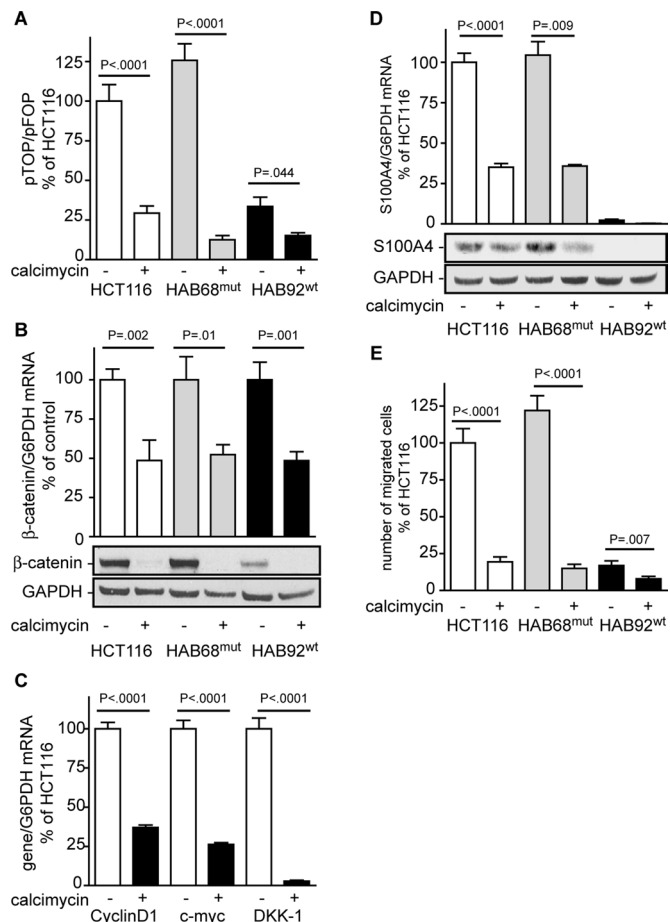


FIGURE 5: Calcimycin inhibits constitutively active Wnt/β-catenin pathway. Activity of the Wnt/β-catenin pathway was analyzed by TOP/FOPFlash reporter assay. Quantification of mRNA was performed with qRT-PCR; protein expression was determined by Western blot. (A) Wnt/β-catenin pathway was reduced in calcimycin-treated HCT116 cells and in derivative cell lines HAB68^{mut} and HAB92^{wt}. (B) The expression of β-catenin mRNA and protein was reduced in calcimycin-treated in HCT116, HAB68^{mut}, and HAB92^{wt} cells. (C) mRNA levels of β-catenin/TCF transcription target genes cyclin D1, c-myc, and DKK-1 were reduced upon calcimycin treatment. (D) S100A4 expression was inhibited in HCT116, HAB68^{mut}, and HAB92^{wt} cells treated with calcimycin. (E) Cell migration of HCT116, HAB68^{mut}, and HAB92^{wt} cells was decreased upon calcimycin treatment. Data represent mean ± SE (n = 3). Statistical significance was analyzed by Student's t test.

β-catenin. In line with our findings, calcimycin treatment also abolished Wnt/β-catenin pathway activity in HEK293 cells that stably expressed the TOPflash reporter plasmid (Gwak *et al.*, 2006).

In line with reduced Wnt/β-catenin pathway activity, we found that S100A4 and other β-catenin/TCF target genes such as cyclin D1, c-myc, and DKK-1 were down-regulated following calcimycin treatment. Cyclin D1 and c-myc are known oncogenes, and their overexpression causes increased cell proliferation (He *et al.*, 1998; Shtutman *et al.*, 1999). Knockdown of β-catenin expression levels by shRNA was shown to result in reduced levels of cyclin D1 and c-myc, and this reduction was associated with reduced cell proliferation (Huang *et al.*, 2007). Consistent with this observation, in our study calcimycin reduced the cyclin D1 and c-myc mRNA levels, which might explain the observed inhibition of anchorage-dependent and anchorage-independent cell proliferation following calcimycin treatment.

Intrasplenic inoculation of S100A4 overexpressing cells was shown to induce liver metastases in xenografted mice (Stein *et al.*, 2006). Because S100A4 is a major regulator of colon cancer metastasis, its inhibition should result in restricted metastasis formation. Indeed, we found in this study that calcimycin treatment of human colon cancer cells restricted their potential to form liver metastases *in vivo*. The number and size of liver metastases formed by calcimycin treated cells were significantly reduced. Bioluminescence imaging, as well as immunohistochemistry, visualized larger and more frequent micrometastases in livers of control animals than in livers of mice from the calcimycin group. The amount of human satellite DNA was reduced in livers of calcimycin mice, supporting the observation that fewer human colon cancer cells invaded the liver tissue. Thus calcimycin treatment restricted the metastatic potential of colon cancer cells *in vivo*.

We are aware that by interfering with the Wnt/β-catenin pathway, calcimycin will have several different functions and actions within the cell. Among these functions we show that calcimycin inhibits S100A4 expression and that this inhibition leads to reduced cell motility. We showed that HCT116/S100A4 cells migrated and invaded despite the calcimycin treatment. The inhibitory effect of calcimycin on cell migration and invasion was overcome by exogenous overexpression of S100A4. Thus we provide evidence that the antimigratory and anti-invasive effect of calcimycin was specific to the calcimycin-mediated S100A4 expression inhibition.

In conclusion, our study reports calcimycin as a novel inhibitor of S100A4-promoter activity, which leads to reduced S100A4 expression and thus impairs S100A4-induced cell motility and metastasis. Because metastasis is the major cause of colon cancer death, there is an urgent need for antimetastatic treatment. S100A4, as a mediator of this disease progression, provides a promising therapeutic target. We provide evidence that targeting S100A4 expression by application of calcimycin restricts S100A4-induced cell migration and invasion *in vitro* and *in vivo*. Therefore we show that calcimycin is a useful compound not only to study S100A4 biology, but also to form the basis for the development of treatments against colon cancer metastasis.

MATERIALS AND METHODS

Cell lines and treatments

The human colon cancer cell line HCT116 and its derivatives HAB-68^{mut} and HAB-92^{wt} were kindly provided by Todd Waldman (Georgetown University, Washington, DC). HCT116 cells are heterozygous for gain-of-function mutated Δ45-β-catenin. In HAB-68^{mut} and HAB-92^{wt} the wild-type or mutated allele of β-catenin was deleted, respectively, by homologous recombination (Kim *et al.*, 2002). These cell lines were expanded briefly in culture and cryopreserved in multiple replicate vials. The cell banks were tested by PCR and culture methods and found to be free of mycoplasma. To authenticate the HAB-68^{mut} and HAB-92^{wt} cell lines as HCT116 derivatives, short tandem repeat (STR) genotyping was performed in August 2010 using the ABI Identifier Kit (Life Technologies, Darmstadt, Germany). The STR genotypes were consistent with published genotypes for HCT116. All cells were tested for the correct β-catenin genotype by restriction fragment length polymorphism as previously described (Stein *et al.*, 2006). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) in a humidified incubator at 37°C and 5% CO₂. HCT116 cells were transfected to express S100A4-promoter controlled firefly luciferase (HCT116/S100A4pLUC cells), CMV promoter-controlled firefly luciferase (HCT116/LUC), CMV promoter-controlled S100A4 cDNA (HCT116/S100A4 cells), or the empty vector as control (HCT116/vector cells). The S100A4 promoter was a kind gift from

Gene	Primer/probe	Sequence 5'–3'
β-Catenin	Forward primer	gtg cta tct gtc tgc tct agt a
	Reverse primer	ctt cct gtt tag ttg cag cat c
	FITC probe	agg act tca cct gac aga tcc aag tca-FITC
	Lcred640-probe	LCRed640-cgt ctt gtt cag aac tgt ctt tgg act ctc-phosphate
Cyclin D1	Forward primer	ctg ttt ggc gtt tcc cag agt cat c
	Reverse primer	agc ctc ctc ctc aca cct cct c
c-myc	Forward primer	acc ctt gcc gca tcc acg aaa c
	Reverse primer	cgt agt cga ggt cat agt tcc tgt tgg
DKK-1	Forward primer	tag cac ctt gga tgg gta ttc
	Reverse primer	ata ttt cta gtc cat gag agc c
	FITC probe	gtc tcc ggt cat cag act gtg cc-FITC
	Lcred640 probe	LCRed640-agg att gtg ttg tgc tag aca ctt ctg g-phosphate
S100A4	Forward primer	ctc agc gct tct tct ttc
	Reverse primer	ggg tca gca gct cct tta
	FITC probe	tgt gat ggt gtc cac ctt cca caa gt-FITC
	Lcred640 probe	LCRed640-tcg ggc aaa gag ggt gac aag t-phosphate
Human satellite DNA	Forward primer	ggg ata att tca gct gac taa aca g
	Reverse primer	aaa cgt cca ctt gca gat tct ag
	FITC probe	ctt cac ata aaa act aca cag atg cat tct cag g-FITC
	LCRed640 probe	LCRed640-ctt ttt ggt gat gtt tgt att caa ctc cca g-phosphate

FITC, fluorescein isothiocyanate.

TABLE 1: Primers and probes used for qRT-PCR.

David Allard (University of Exeter and Plymouth, Exeter, United Kingdom) and comprised the sequence from –1487 to +33 base pairs around the S100A4 transcription start site (Hernan *et al.*, 2003). The S100A4 cDNA was kindly provided by Claus Heizmann (University of Zurich, Zurich, Switzerland; Engelkamp *et al.*, 1992).

Substances from the LOPAC 1280 and additional samples of calcimycin (both Sigma-Aldrich, St. Louis, MO) were dissolved in dimethylsulfoxide. Dilutions were performed with RPMI-1640 medium.

High-throughput screening and cell cytotoxicity assay

A BIOMEK2000 automatic pipetting system (Beckman Coulter, Brea, CA) was used to seed 2.5×10^3 HCT116/S100A4pLUC cells/well into 384-well plates. Cells were exposed to dilutions of each compound of the LOPAC 1280 for 24 h. Luciferase expression was determined using Britelite reagent in a Wallac Victor reader (both PerkinElmer, Waltham, MA). In parallel, cell cytotoxicity of the compounds was measured by Alamar blue cytotoxicity assay. Briefly, after 24 h of incubation with cells, plates were treated with Alamar blue (Sigma-Aldrich) solution for 4 h and then read on a fluorescence plate reader at excitation and emission wavelengths of 530 and 590 nm, respectively. Compounds showing inhibition in the S100A4 promoter screen were counterscreened using an HIF-1 promoter screen to establish selective activity (Rapisarda *et al.*, 2002).

Quantitative real-time PCR, Western blot analysis, and immunohistochemistry

Quantitative real-time (qRT) PCR was carried out using the LightCycler480 (Roche, Mannheim, Germany) as described previously (Stein *et al.*, 2006) with the primers and probes summarized in Table 1. For β-catenin, S100A4, and DKK-1 cDNA quantification HybProbe Fast-

start master mix (Roche) was used. Cyclin D1 and c-myc cDNAs were quantified with SYBR green (Roche). For quantification of the housekeeping gene glucose-6-phosphate dehydrogenase (G6PDH) cDNA the hG6PDH Roche Kit (Roche) was used.

Western blot was performed with the following antibodies: a polyclonal rabbit anti-human S100A4 antibody (Dako, Glostrup, Denmark), a polyclonal goat anti-human glycerin-aldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal mouse anti-human β-catenin antibody. Immunoblotting for GAPDH served as loading control.

Immunohistochemistry was performed as previously described (Stein *et al.*, 2011). Briefly, cryosections were stained with rabbit anti-human-specific cytokeratin-19 antibody (dilution 1:50; Acris Antibodies, Herford, Germany) and horseradish peroxidase-coupled anti-rabbit antibody (dilution 1:1000; Promega, Madison, WI).

Cell migration, cell invasion, and wound-healing assay

For the Boyden chamber assay (Boyden, 1962), 2.5×10^5 cells were seeded into transwell filter membrane chambers (pore size, 12.0 μm; Millipore, Billerica, MA) and allowed to accommodate for 15 h. Cells were treated with 1 μM calcimycin for 18 h. The number of cells that migrated to the lower chamber was counted in a Neubauer chamber. For the invasion assays, transwell membranes were coated with 1:3 diluted Matrigel (BD Biosciences, Heidelberg, Germany). For the wound-healing assay, 2.5×10^5 cells were seeded at 60% confluence 24 h before a wound of ~300 μm width was made with a pipette tip. Medium was exchanged daily, and microphotographs were taken on day 4.

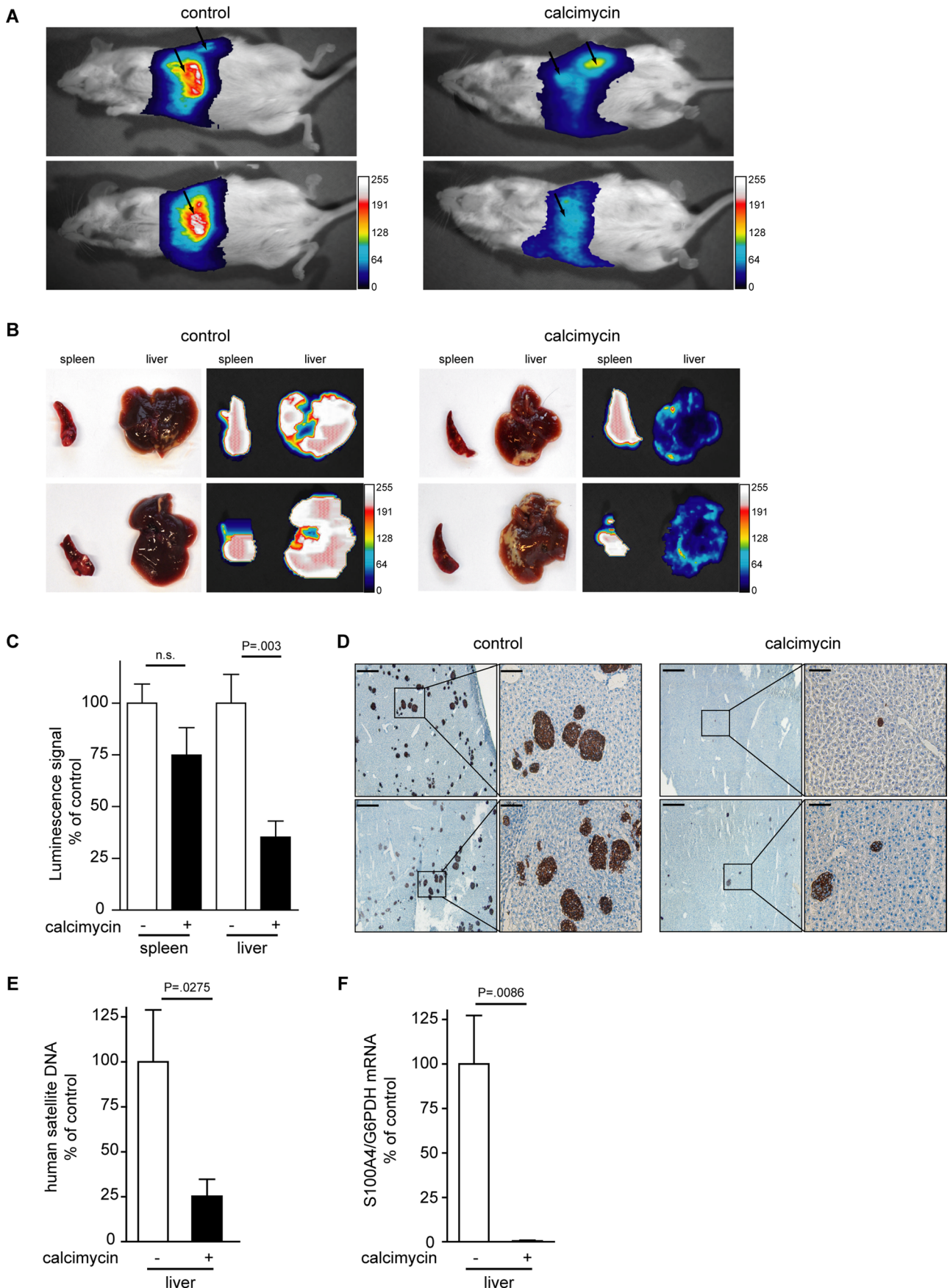


FIGURE 6: Calcimycin reduces the metastatic potential of human colon cancer cells in xenografted mice. Mice were intrasplenically injected with calcimycin- or solvent-treated HCT116/LUC cells. Two representative mice per group are shown. (A) Bioluminescence was measured 6 d posttransplantation in the region of spleen and liver (metastases target

Cell proliferation and colony formation assay

For determination of cell proliferation, 8×10^3 cells were treated daily for 4 d with $1 \mu\text{M}$ calcimycin. For determination of viable cells 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) was added to a final concentration of 0.5 mg/ml, incubated for 3 h, and dissolved by 10% SDS in 10 mM HCl. The optical density was measured at 560 nm. For colony formation assays 1×10^3 cells were resuspended in $1 \mu\text{M}$ calcimycin- or solvent-containing medium supplemented with 0.33% (wt/vol) agarose and seeded as single cells. After incubation in a humidified incubator at 37°C and 5% CO_2 for 7 d, colonies were analyzed by light microscopy. Colonies were counted if they consisted of more than three cells.

TOP/FOPflash reporter assay

Transfection of 8×10^5 cells with TOPflash or FOPflash plasmids (Promega) occurred 24 h before the cells were treated with $1 \mu\text{M}$ calcimycin. After 18 h of treatment luciferase activity was measured by the Steady Glow Luciferase Assay System (Promega). TOPflash reporter gene expression (representing the Wnt/ β -catenin pathway activity) was normalized to FOPflash reporter gene expression (representing basal reporter gene expression).

In vivo bioluminescence imaging of xenograft mice

All experiments were performed in accordance with the United Kingdom Coordinating Committee for Cancer Research guidelines and approved by the State Office of Health and Social Affairs, Berlin, Germany. HCT116/LUC cells were treated with $1 \mu\text{M}$ calcimycin or solvent 24 h before 2×10^6 cells were intrasplenically injected into eight NOD/SCID-IL2R⁻ mice per group. Mice were anesthetized by intraperitoneal injection of 35 mg/kg Hypnomidate (Jassen-Cilag, Neuss, Germany) and received 150 mg/kg D-luciferin (Biosynth, Staad, Switzerland) intraperitoneally for bioluminescence imaging. Imaging was performed with the NightOWL LB 981 system (Berthold Technologies, Bad Wildbad, Germany). ImageJ, version 2.3, was used for color coding of signal intensity (presenting a 256 grayscale) and for quantification of the luminescence signal. Mice were killed 6 d posttransplantation, when a clear liver signal was detected. The spleen (the tumor implantation site) and the liver (the metastasis target organ) were shock frozen in liquid nitrogen, and cryosections were performed for isolation of genomic DNA (Qiagen, Hilden, Germany) and mRNA (Roboklon, Berlin, Germany) and for immunohistochemistry.

Statistical analysis

Statistical analyses were performed with GraphPad Prism, version 4.01. Comparison of two groups was done by Student's *t* test. Comparison of a control versus several treated groups was performed by one-way analysis of variance (ANOVA) and Bonferroni post hoc multiple comparison. The inhibiting concentration 50 (IC₅₀) was defined as the concentration that reduced cell viability to 50% of solvent-treated control cells. The effective concentration 50 (EC₅₀) was defined to be the concentration at which reporter activity was reduced to 50% of solvent-treated control cells. The IC₅₀ and EC₅₀ were cal-

culated by sigmoidal dose-response curve fit of $x = \log(x)$ transformed data. IC₅₀ and EC₅₀ values were given as geometric means with 95% confidence interval. All significance tests were two-sided. $p < 0.05$ was defined as statistically significant.

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organ). (B) Spleen tumor and liver metastases are visualized by bioluminescence imaging. (C) No significant difference in the luminescence signal of spleen tumors was found between mice injected with control or calcimycin-treated cells. The liver luminescence signal was significantly reduced in the calcimycin group. Data represent mean \pm SE ($n = 6$). Statistical significance was analyzed by Student's *t* test. (D) Immunohistochemistry for human cytokeratin-19 identified smaller and fewer micrometastases in the calcimycin group. (E) The amount of human satellite DNA was reduced in livers from the calcimycin group. (F) The S100A4 mRNA expression normalized to hG6PDH was absent in livers of mice of the calcimycin group. Data represent mean \pm SE ($n > 3$). Statistical significance was analyzed by Student's *t* test.

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