Antitumor effect of SIRT1 inhibition in human HCC tumor models in vitro and in vivo

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Abstract

Sirtuins (SIRT1-7) are a highly conserved family of NAD+ dependent enzymes that control the activity of histone and non-histone regulatory proteins. SIRT1 is known to promote longevity and to suppress the initiation of some cancers. Nevertheless, SIRT1 is reported to function as a tumor suppressor as well as an oncogenic protein. Our data show that compared to normal liver or surrounding tumor tissue, SIRT1 is strongly over-expressed in human hepatocellular carcinoma (HCC). Additionally, human HCC cell lines (Hep3B, HepG2, HuH7, HLE, HLF, HepKK1, skHep1) were screened for the expression of the sirtuin family members and only SIRT1 was consistently overexpressed compared to normal hepatocytes. To determine its effect on HCC growth, SIRT1 activity was inhibited either with lentiviruses expressing shRNAs or with the small molecule inhibitor, cambinol. Knockdown or inhibition of SIRT1 activity had a cytostatic effect, characterized by an altered morphology, impaired proliferation and an increased expression of differentiation markers and cellular senescence. In an orthotopic xenograft model, knockdown of SIRT1 resulted in 50% fewer animals developing tumors and cambinol treatment resulted in an overall lower tumor burden. Taken together, our data demonstrate that inhibition of SIRT1 in HCC cells impairs their proliferation in vitro and tumor formation in vivo. These data suggest that SIRT1 expression positively influences the growth of HCC and support further studies aimed to block its activity alone or in combination as a novel treatment strategy.
Introduction

Sirtuins are a highly conserved family of enzymes that are homologues to the silent information regulator (Sir) 2 gene family found in yeast. The sirtuin family consists of 7 members (SIRT1-7) that share a highly conserved catalytic core domain and rely on NAD⁺ for their enzymatic function. The family members differ in their N- and C- terminal extensions and in their subcellular localization, which together are responsible for their various binding partners and diverse metabolic functions (1). (Additional information on sirtuin family members (SIRT2-7) is provided in the Supplementary Material).

SIRT1 is the best-studied family member due to its purported ability to prolong the lifespan in Caenorhabditis elegans, Drosophila melanogaster and mammals (2,3,4,5). SIRT1 deacetylates histone proteins and other key transcriptional regulators such as p53 (6,7,8), NF-κB (9), Foxo (10), Ku70 (11), E2F1 (12), PPARγ co-activator 1α (PGC-1α) (13) and hypoxia inducible transcription factors (HIF) (14,15,16). Its broad effect on gene transcription has centralized its role in cellular events involved in aging, metabolism, inflammation and stress responses (17). In the past decade, a controversial view of SIRT1 has emerged regarding its role in tumorigenesis. SIRT1 is found to be overexpressed in many cancers such as prostate, colon and acute myeloid leukemia (18,19,20). Nevertheless, it is reported to function as a tumor promoter and as a tumor suppressor protein. The indeterminate role of SIRT1 suggests that its involvement in tumorigenesis may differ between tumor types and depend on the temporal or spatial distribution of upstream and downstream factors that regulate its function (21).

In the study we investigated the expression SIRT1 in the context of hepatocellular carcinoma (HCC). HCC is the third most common cause of cancer-related deaths
worldwide (22). It is a highly aggressive cancer with a complex and multimodal pathogenesis (23). Only early stages of HCC are curable with today’s treatment protocols therefore new therapeutic strategies are currently needed. Whole-genome sequencing of HCCs has identified recurrent mutations in chromatin regulators that can directly influence chromatin’s structure and activity. (24). Chromatin regulators exert post-translational modifications of histone proteins by altering their methylation, ubiquitinylation and acetylation status and have a direct influence on gene expression. Histone deacetylases (HDACs) in particular, class I & II HDACs are being targeted with compounds such as panobinostat as a novel treatment strategy for HCC (25,26). Sirtuins make up the class III HDACs and therefore may represent another class of chromatin regulators for potential targeting.

In this study we demonstrate that SIRT1 is frequently overexpressed in human HCC and HCC cell lines. Inhibition of SIRT1 activity results in the impairment of tumor cell growth and increased expression of differentiation markers in in vitro and in vivo models. These observations suggest that SIRT1 expression promotes the growth of HCC and new treatment strategies inhibiting its activity may be a novel means for the treatment of HCC.
Materials and Methods

Immunohistochemistry

Human liver and HCC samples were obtained from consented patients in our institution by standard surgical oncology procedures. Tissue samples from patients diagnosed with HCC were compared to samples taken from tumour-free resection margins or non-tumor liver. SIRT1 was detected using a rabbit polyclonal anti-SIRT1 antibody (Santa Cruz Biotechnology). Additional details can be found in Supplementary Materials and Methods.

Cell culture and human tissue

All HCC cell lines were from human origin. Hep3B and HepG2 cells were purchased from ATCC (LCG Promochem). HLE, HLF, HepKK1 and skHep1 cell lines were kindly provided by L. Wilkens (University of Bern) and HuH7 cells by J-F. Dufour (University of Bern). All lines were tested for mycoplasma contamination and only lines testing negative were used in the study. The HCC cell lines were not authenticated further. Cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin and 100µg/ml streptomycin (Life Technology) at 37°C in a humidified incubator with 5% CO₂. For primary human hepatocytes, liver specimens were obtained from patients undergoing liver resection. Informed consent was obtained prior to surgery in compliance with the local ethical committee. Details of the isolation method can be found in Supplementary Materials and Methods. Hypoxic culture conditions were performed in a microaerophilic system (Ruskinn, Biotrace International) at 1% O₂, 5% CO₂ and 94% N₂.

Chemicals and reagents
Cambinol and nocodazole (Sigma-Aldrich) were solved in DMSO as stock solutions (cambinol 100mmol/L; nocodazole 5mg/mL). D-luciferin (Synchem OHG) was dissolved in saline (250mM) at the time of use.

**RNA extraction and quantitative RT-qPCR**

RNA was isolated by Trizol Reagent and followed the manufacturer’s protocol (Life Technologies). cDNA was synthesised by using Omniscript® RT kit 200 (Cat. No. 205113, Qiagen). mRNA was analysed by RT-qPCR (ABI 7900, SDS 2.3 software). A detailed description of probes and primers can be found in the Supplementary Materials and Methods.

**Proliferation assays**

Proliferation assays (alamarBlue®, mitotic index, colony formation and EdU incorporation) are described in detail in the Supplementary Materials and Methods.

**Senescence-associated β-galactosidase (SA-β-gal) staining**

Cells fixed with 2% formaldehyde and 0.2% glutaraldehyde were incubated for 14h with staining solution (40mM citric acid sodium phosphate, pH 6.0, 1mg/ml 5-bromo-4-chloro-3-isyl-β-D galactoside [X-gal], 5mM potassium ferricyanide, 150mM NaCl, 2mM MgCl₂). Photographs were taken with a Nikon camera.

**Cell cycle analysis by FACS**

Cells fixed with 70% EtOH were incubated for 2h with RNase A (40ug) (Promega) / propidium iodide solution (50ug) (Sigma-Aldrich). Cell cycle distribution was determined by FACS using the LSR II and FACSDiva software (Becton Dickinson).
Caspase-3 assay

Caspase-3 assay was determined using fluorogenic caspase-3 substrate II (Millipore: Cat. No. 235425). Fluorescence was measured with a TECAN Infinite 200 plate reader at 380nm.

HCC xenograft mouse model

Six to 8 week old female Rag2/common gamma-null mice (Rag2-/-/γc-/-) (Taconic) were used for an orthotopic xenograft HCC model. Five x 10^5 HepG2 cells (with or without shRNAs) were injected under the capsule of the left liver lobe in a 1:1 ratio with Matrigel® (BD Biosciences). Cambinol (Sigma) was prepared in a vehicle of 10% ethanol, 10% cremophore (Sigma). Animals were treated with 100uL of 100 mg/kg cambinol or vehicle by intra-peritoneal injection daily, five times per week. For bioluminescent tumor detection, anesthetised animals were injected with 30μl i.p. with D-luciferin [250mM]. Luminescence was measured exactly 10min after injection using the NightOWL in vivo Imaging System (Berthold Technologies). All experiments were performed in strict accordance with Swiss Federal Veterinary Office article 18 Animal Welfare Act, article 141 Animal Welfare Ordinance and article 30 Animal Experimentation Ordinance. All protocols were approved by Bernese cantonal authorities, LANAT Amt für Landwirtschaft und Natur Veterinärdienst (VeD), Permit Nr: 87/09. Further details of the model can be found in the Supplementary Materials and Methods.

Lentiviral vectors expressing firefly luciferase or shRNA targeting SIRT1

Lentivirus production, titer determination and transduction were carried out as previously described [35]. A detailed description can be found in the Supplementary Materials and Methods.
**Statistical Analysis**

All graphs represent mean value of triplicates ± standard deviation (SD). Statistics were performed using unpaired Students t-test and graphs made using GraphPad Prism software, with definitions for p values as follows: *p = 0.01-0.05 (significant), **p = 0.001-0.01 (very significant), ***p < 0.0001 (extremely significant).
Results

*SIRT1 is overexpressed in HCC and liver cancer cell lines*

SIRT1 protein expression was examined in a panel of twenty human HCC tumors and adjacent non-tumor tissue by immunohistochemistry. We observed a strong distinct staining for SIRT1 with predominant nuclear localization in tumor cells, whereas SIRT1 protein expression in normal or adjacent non-malignant liver was minimal or absent (Fig. 1A). Samples were scored for intensity of SIRT1 staining and average tumor score (2.00 ± 0.58, p<0.001) and maximum tumor score (2.5 ± 0.7, p<0.001) were both significantly higher than adjacent non-malignant liver (1.0 ± 0.07) (Fig. 1B). Nevertheless, the levels of SIRT1 mRNA were not significantly different in eight samples of HCC and paired non-tumour liver (Fig. 1C). We next determined if other sirtuin family members were overexpressed in HCC, by comparing mRNA expression of SIRT1-7 in seven human liver cancer cell lines to non-malignant primary human hepatocytes. Of the sirtuin family members, only SIRT1 mRNA was significantly increased in six out of the seven cell lines (Fig. 1D). The other family members, SIRT2 (Fig. 1E) and SIRT3-7 (Supplementary Fig. 1) were expressed at equal or lower levels (exceptions: SIRT5 and SIRT7 in Hep3B and HepG2; SIRT6 in Hep3B). Similar results were obtained by analyzing the protein expression of SIRT1 and SIRT2 by Western blot. SIRT1 protein was consistently expressed at higher levels in HCC cells lines, whereas, equal or lower levels were observed for SIRT2 (Fig. 1F).

*Knocking down SIRT1 has a cytostatic effect in HCC cell lines*

To determine whether SIRT1 is essential for HCC cell growth, SIRT1 was knocked down with shRNA expressing lentiviruses (shSIRT1_3206 and shSIRT1_1958) in HepG2 (Fig. 2) and Hep3B and HuH7 cell lines (Supplementary Fig. 2). SIRT1 knockdowns were
compared to a scrambled control (shScr). SIRT1 mRNA expression was significantly reduced in shSIRT1 infected cells (Fig. 2A and Supplementary Fig. 2A) with a coinciding loss of protein (Fig. 2B and Supplementary Fig. 2B). Clone shSIRT1_1958, targeting SIRT1 on the CDS was more efficient than clone shSIRT1_3206, which targets SIRT1 mRNA in the 3′-UTR. SIRT2 protein (Fig. 2B and Supplementary Fig. 2B) or mRNA (Supplementary Fig. 2C) was not decreased when SIRT1 mRNA was targeted, however, an increase of its mRNA, was observed in cells transduced with clone shSIRT1_1958. The morphology of cells infected with shSIRT1 viruses was transformed into a larger, flattened shape compared to shScr and non-infected controls (Fig. 2C Supplementary Fig. 2D). Morphological changes in HepG2 cells were associated with a decrease in proliferation, measured by colony formation (Fig. 2D) and alamarBlue® assays (Fig. 2E). Phosphorylation of histone 3, a marker of cells exiting mitoses was lower in SIRT1 knockdown cells (Fig. 2F) and this was associated with more cells arrested in G1 and less in S phase of the cell cycle (Fig. 2G). There were no visual signs of cell death or a measureable increase in activity of the apoptotic protein caspase-3 (data not shown). A characteristic of HCC cells is the expression of de-differentiation markers glypican (GPC3) and α-fetoprotein (AFP) and a loss of the epithelial marker E-cadherin (CDH). Loss of SIRT1 led to a significant decrease in AFP and GPC3 (Fig. 2H and Supplementary Fig. 2E) and an increase of CDH mRNA expression (Fig. 2H). Interestingly, a stable SIRT1 knockdown line was unable to be generated in Hep3B and HuH7 cells. Their complete lack of growth was accompanied with an induction of cellular senescence as shown by enlarged cell size and expression of pH-dependent beta-galactosidase activity (Supplementary Fig. 2F). Taken together, these data suggest that loss of SIRT1 has a cytostatic effect on HCC cells by arresting cells in G1 thus leading to a lower mitotic index and impairment of cell proliferation. The change in their morphology
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was accompanied by changes of differentiation markers and in Hep3B and HuH7 cells an induction of cellular senescence could be observed.

**Knocking down SIRT1 reduces tumor formation in an orthotopic xenograft model**

We next tested the effect of SIRT1 knockdown in vivo using an orthotopic xenograft mouse model. To monitor tumor growth non-invasively, a tagged HepG2 cell line was generated with a luciferase containing lentivirus. The resulting HepG2_luc cells were further infected with shScr, shSIRT_3206 or shSIRT1_1958 shRNA and selected cells were injected subcapsularly in the liver of immune deficient mice. Tumor growth was measured by bioluminescent imaging (Fig. 3A). While 60% of the mice developed tumors in the control group after day 4 and 80% after day 11, the animals harboring cell lines with knocked down SIRT1 expression formed tumors in 50% of the animals for the shSIRT1_3206 and only 33% for the shSIRT1_1958 for up to 30 days (Fig. 3B). In HepG2_luc +shSIRT1 cells, we observed either a complete lack of tumor formation or tumor growth was delayed, as they never reached an exponential growth phase compared to the shScr control group (Fig. 3C). Histological analysis of tumors with SIRT1 knockdown revealed a reduced number of intratumoral blood vessels (Fig. 3D). This impairment of tumor angiogenesis is supported by our observation that hypoxia-induced VEGF expression is inhibited in SIRT1 knockdown cells (Fig. 3E).

**SIRT1 is a potential drug target**

We next questioned whether SIRT1 could be a target for pharmacological intervention. SIRT1 was inhibited with cambinol, a cell permeable β-naphthol pharmacophore that blocks its NAD+ dependent deacetylase activity (Fig. 4). Cambinol altered the morphology of two HCC cell lines, with a more striking change observed in HepG2 cells (Fig. 5A) than in Hep3B cells (Supplementary Fig. 3A). The morphological changes were
not associated with visible signs of cell death or with activity of the apoptotic protein caspase-3 (Fig. 5B). Inhibition of SIRT1 reduced colony formation in a dose-dependent manner (Fig. 5C & Supplementary Fig. 3B) and fewer colonies were reflected by a significant decrease in the percentage of proliferating, EdU positive cells (Fig. 5D). Interestingly, cambinol lead to a G1 arrest only in p53wt-HepG2 cells, not p53del-Hep3B (Fig. 5E & Supplementary Fig. 3C). As we observed in SIRT1 knockdown cells, cambinol reduced the expression of de-differentiation markers AFP and GPC3 (Fig. 5F & Supplementary Fig. 3D). And finally, cambinol impaired cell migration in a dose-dependent manner (Supplementary Fig. 3E).

Using an orthotopic xenograft model, we next tested if cambinol could impair HCC growth in vivo. First, to determine if cambinol had a hepatotoxic effect itself, mice were treated daily with 50 and 100 mgkg⁻¹ cambinol for two weeks. At the end of treatment, we performed a 70% partial hepatectomy to assess liver damage as an impairment of regeneration. Serum alanine aminotransferase (ALT) levels were not increased in cambinol treated animals (Supplementary Fig. 3F). Moreover, cambinol did not impair the regenerative capacity of normal liver compared to vehicle only controls (Supplementary Fig. 3G). Nonetheless, in animals harboring HCC xenografts, tumor growth was impaired in animals treated with cambinol starting on day 8-post tumor cell injection (Fig 5G). Tumors in vehicle treated animals reached an exponential growth rate starting on day 16, whereas cambinol suppressed tumor growth in 3 animals and reverted tumor growth in 1 animal after day 23. Taken together, our data demonstrate that inhibiting SIRT1 activity in HCC tumor cells leads to a decrease in cell proliferation, an increase of differentiation and impaired tumor growth in vivo, whereas, it does not impair the proliferative potential of normal liver parenchyma.
Discussion

Current literature describes how activation of SIRT1 is able to prevent the development of age or carcinogen-induced cancers. For example, Herranz et al., showed that transgenic animals overexpressing SIRT1 are protected against DEN/high fat diet-induced liver carcinogenesis compared to non-transgenic controls (27). The protective effect was explained, in part, by SIRT1-mediated regulation of NF-κB activity; SIRT1 overexpression reduced NF-κB-mediated inflammation and hepatic cell malignant transformation. Although SIRT1 expression is purposed to prevent HCC in this model, we observed a strong overexpression of SIRT1 in human HCC tumors and HCC cell lines. This observation confirms other studies that have documented SIRT1 overexpression in panels of human HCC samples (28,29,30). In cancer research, there are more examples emerging of how malignant cells have the ability to hijack survival mechanisms used by non-malignant cells for their own endurance (31,32). These observations prompted our lab to hypothesize that SIRT1 activity in healthy liver tissue may provide protection against malignancy, however after transformation, SIRT1 expression or its over expression may be providing a protective or survival advantage for the tumor cells. Our data supports this by demonstrating that inhibition of SIRT1 in HCC cells by knockdown with shRNA or with the inhibitor cambinol can impair tumor cell growth in vitro and in vivo. This is the first preclinical study in HCC to demonstrate that inhibition of SIRT1 impairs tumor cell growth in animal models and that it is possible to use small molecule inhibitors to achieve an anti-tumor effect in vitro and in vivo and collectively suggest that SIRT1 may be a novel target for cancer therapy.

In our study, we inhibited SIRT1 with the small molecule inhibitor cambinol that previously was shown to impair the growth of Burkitt lymphoma xenografts (33). Interestingly, cambinol did not impair a normal physiological growth response in non-malignant liver
Inhibition of SIRT1 impairs growth of HCC cells

cells, as mice treated with cambinol displayed no signs of hepatic damage or impairment of liver cell proliferation in response to partial hepatectomy. In agreement, it was previously shown that knockdown of SIRT1 with siRNA led to growth arrest in human epithelial cancer cell lines but not primary epithelial cells (34) and that cambinol is well tolerated by mice in the absence of other noxious stimuli (33). SIRT1 is not a specific target of cambinol, it also inhibits SIRT2 NAD-dependent deacetylase activity in vitro with IC₅₀ values of 56 and 59 μmol/L, respectively (33). Although it has been previously shown that combined SIRT1 and SIRT2 inhibition induces cell death, we observed a cytostatic as opposed to a cytotoxic effect in cells treated with cambinol (35).

SIRT1 has a direct effect on cell survival and proliferation by targeting key transcription factors such as FOXO, E2F1 and p53 (referenced in introduction) and can promote cancer cell growth by blocking cellular senescence and differentiation (36). Therefore, and as demonstrated here, inhibition of SIRT1 removes this growth-promoting signal and forces cells in a non-proliferating, senescent or more differentiated phenotype.

Regardless of their p53, wild-type (HepG2), null (Hep3B) or mutated (HuH7) status all lines overexpressed SIRT1 and all responded to SIRT1 inhibition with an inhibition of cell proliferation. Therefore, the cytostatic effect achieved was not dependent on functioning p53. As p53 activity is impaired by SIRT1 we expected a stronger effect with SIRT1 inhibition in p53wt cells. However, in the absence of other stresses or DNA-damaging agents, it was the p53-mutated and null cells that showed a profound inhibition of cell proliferation and induction of cellular senescence by SIRT1 inhibition. As we observed cellular senescence by SIRT1 inhibition this further supports a cytostatic effect, as senescence is an arrested state in which cells still remain viable. When examining the differentiation state of cells with altered SIRT1 expression or activity, we observed a loss of expression of the malignancy markers AFP and GPC3. GPC3 promotes growth of
HCC cells through stimulation of the canonical Wnt signaling pathway that regulates the expression of many downstream oncogenic proteins. A recent study has shown that molecular targeting of GPC3 enhanced TGF-β2 expression and signaling, which in turn inhibited HCC cell proliferation and cell cycle progression, and induced replicative senescence (37). Loss of expression of AFP and GPC3 together with an increase of E-cadherin suggests that cells are gaining a more differentiated phenotype. This observation might be beneficial as poorly differentiated cancer cells are more resistant to conventional chemotherapies (38). Further studies will determine whether SIRT1 has a direct effect on AFP and GPC3 gene expression, as it has since been shown that SIRT1 localizes to the promoter element of E-cadherin to repress its expression directly (39).

SIRT1 was strongly expressed in human samples of HCC and it appears that its overexpression is unique as the other family members displayed equal or lower protein levels. mRNA analysis of HCC cell lines suggested that SIRT1 was transcriptionally increased in liver cancer cell lines. The transcription of SIRT1 is negatively regulated by the tumor suppressor proteins p53 and HIC1, both of which are frequently mutated in cancers. Nonetheless, this observation did not correspond with our patient samples; an increase of SIRT1 protein was not associated with an increase of its mRNA, suggesting a posttranslational stabilization of its protein. It is still unclear at which point and by which mechanism HCC cells gain and become reliant on SIRT1 expression. It has been suggested that SIRT1 is critical in all stages of tumor formation (reviewed in (40)). However, it still needs to be determined if SIRT1 overexpression is indeed an initiating event, as no tumors were reported to be induced in SIRT1 overexpressing transgenic mice, in fact, these mice had fewer spontaneous carcinomas and sarcomas (41) (27). Further investigations may reveal that as important as SIRT1 overexpression is; perhaps
equally important is loss of expression of other family members, for example SIRT3 and SIRT6 in promoting tumor growth in HCC.

Our study supports SIRT1 as a novel therapeutic target for the treatment of HCC. Although our results suggest that targeting SIRT1 may be effective as a single modality, the cytostatic rather than cytotoxic of SIRT1 inhibition suggests that targeting SIRT1 inhibition in combination with other cancer therapies to enhance sensitivity and to impair tumor cell escape mechanisms may be a future approach.

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References


Figure Legends

**Figure 1.** Overexpression of SIRT1 in HCC patient samples and human HCC cell lines.
A) Immunohistochemistry was performed on normal liver (n=8) and HCC tissue (n=20) for SIRT1 protein expression. (NT)=non-tumor adjacent tissue and (T)= tumor. Magnification 100x. B) Intensity of SIRT1 staining was scored on a scale of 1-3. C) SIRT1 mRNA was quantified by RT-qPCR in 8 HCCs compared to adjacent non-tumor tissue. D) SIRT1 and E) SIRT2 mRNA were measured by RT-qPCR in 7 liver cancer cell lines and fold increase was calculated in reference to primary human hepatocytes. F) Western blot analysis of SIRT1 and SIRT2 protein in HCC cell lines compared to normal hepatocytes. To account for the decrease levels of α-tubulin in cultured hepatocytes, two times more protein was loaded for hepatocytes than HCC cells to show equal levels of control proteins. ImageJ software was used for quantitative measurements, setting normal hepatocytes as one. Experiment was repeated four times with independently isolated primary hepatocytes.

**Figure 2.** Effect of SIRT1 knockdown in HepG2 cells, in vitro. A) RT-qPCR of SIRT1 mRNA. B) Western blot for SIRT1 and SIRT2. β-actin was used as a loading control. C) Effect on cellular morphology was detected by microscopy, 200x. D) Colony formation assay, stained with crystal violet. Proliferation was measured by performing E) alamarBlue® assay, detected as relative light units (RLU) and F) a mitotic index assay, expressed as relative phosphorylated H3 levels. G) Cell cycle analysis was performed by FACS and expressed as % Watson pragmatic. H) RT-qPCR for AFP, GPC3 and CDH expression.
Figure 3. Influence of SIRT1 knockdown in HepG2 cells, in vivo using an intrahepatic xenograft mouse model in Rag2-/-γc-/- mice. A) Intrahepatic tumors were detected by bioluminescent imaging. B) Tumor formation expressed as tumor free animals over time. C) Tumor growth over time was measured as relative light units (RLU). D) H & E staining of xenograft, 100x magnification. E) RT-qPCR of VEGF mRNA of HepG2 cells cultured under hypoxic conditions.

Figure 4. Structure of cambinol (33).

Figure 5. Inhibition of SIRT1 activity in HepG2 cells using cambinol in vitro and in vivo. A) Cell morphology of DMSO (control) or cambinol treated cells observed by microscopy, 200x. B) Caspase 3, a marker for apoptosis, expressed as pMol/min/mg protein in cambinol or staurosporin (positive control) treated cells. Cell proliferation was measured by C) colony formation assay and D) FACS analysis of EdU incorporation. E) Cell cycle was determined by FACS analysis after cambinol treatment. F) AFP and GPC3 mRNA measured by RT-qPCR. G) Tumor growth curve over time measured as relative light units (RLU).
Figure 3

A

B

C

D

E

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Figure 4
**Figure 5**

**A**

DMSO | 100 μM Cambinol
---|---

**B**

Caspase-3 protein

**C**

Surviving fraction

**D**

%EdU positive cells

**E**

% Watson Pregnatic

**F**

mRNA fold change

**G**

**vehicle (n=4)**

**cambinol (n=1)**

**cambinol (n=3)**

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