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Inhibition of Siah2 ubiquitin ligase by vitamin K3 (menadione) attenuates hypoxia and MAPK signaling and blocks melanoma tumorigenesis

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Summary

The E3 ubiquitin ligase Siah2 has been implicated in the regulation of the hypoxia response, as well as in the control of Ras, JNK/p38/NF- κ B signaling pathways. Both Ras/mitogen-activated protein kinase (MAPK) and hypoxia pathways are important for melanoma development and progression, pointing to the possible use of Siah2 as target for treatment of this tumor type. In the present study, we have established a high-throughput electro-chemiluminescent-based assay in order to screen and identify inhibitors of Siah2 ubiquitin ligase activity. Of 1840 compounds screened, we identified and characterized menadione (MEN) as a specific inhibitor of Siah2 ligase activity. MEN attenuated Siah2 self-ubiquitination, and increased expression of its substrates PHD3 and Sprouty2, with concomitant decrease in levels of HIF-1 α and pERK, the respective downstream effectors. MEN treatment no longer affected PHD3 or Sprouty2 in Siah-KO cells, pointing to its Siah-dependent effects. Further, MEN inhibition of Siah2 was not attenuated by free radical scavenger, suggesting it is ROS-independent. Significantly, growth of xenograft melanoma tumors was inhibited following the administration of MEN or its derivative. These findings reveal an efficient platform for the identification of Siah inhibitors while identifying and characterizing MEN as Siah inhibitor that attenuates hypoxia and MAPK signaling, and inhibits melanoma tumorigenesis.

Keywords

Siah2; ubiquitin ligase; Meso-scale; melanoma; hypoxia; Ras

Introduction

Protein ubiquitination is a key biological event that is involved in the control of almost every cellular function. Protein ubiquitination requires the concerted action of E1, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases (Hershko and Ciechanover, 1998; Varshavsky, 2005). E3 ligases play a central role in the recognition, and hence, targeting of substrates for ubiquitination. Accordingly, considerable efforts have been devoted in understanding mechanisms underlying E3 function, as well as their possible inhibition (Petroski, 2008).

Siah2 is a RING finger E3 ubiquitin ligase that has been implicated in the regulation of hypoxia, Ras/Raf and p38/JNK/NF- κ B signaling pathways, and thereby an important regulator of key signal transduction pathways (Habelhah et al., 2002, 2004; Nadeau et al., 2007; Nakayama et al., 2004; Zhang et al., 1998). Among the Siah2 substrates are transcription regulators (N-CoR;

Zhang et al., 1998), regulators of protein kinases involved in Ras/Raf (Sprouty 2, SPRY2; Nadeau et al., 2007), JNK, p38 (TRAF2; Habelhah et al., 2002), mitochondrial biogenesis (OGDC-E2; Habelhah et al., 2004) and hypoxia (prolyl hydroxylase 1/3, PHD; Nakayama et al., 2004). For some substrates (PHD1/3, TRAF2), Siah requires adaptor proteins such as phyllopod (PHYL) and Siah-interacting protein (SIP; Matsuzawa and Reed, 2001); whereas for others, it directly interacts with the substrate protein (OGDC-E2, SPRY2; Nadeau et al., 2007; Habelhah et al., 2004).

Most signaling pathways regulated by Siah2 are deregulated in human cancer. Consistent with this is the observation that inhibition of Siah blocks development of pancreatic, mammary, lung and melanoma tumors (Ahmed et al., 2008; Moller et al., 2008; Qi et al., 2008; Schmidt et al., 2007). These observations provide a strong rationale to develop the means for Siah2 inhibition as a new therapeutic target for cancer.

In melanoma, both the Ras-Raf-MEK-ERK (MAPK) and the hypoxia signaling pathways are activated through multiple mechanisms and play a key role in melanoma development and progression (Bedogni and Powell, 2009; Davies et al., 2002; Meier et al., 2005; Lopez-Bergami et al., 2008). Hence, the ability to inhibit both pathways by targeting one regulatory component is of great interest. The ability of ubiquitin ligase Siah to regulate both Ras/MAPK and hypoxia pathways (Nakayama et al., 2009) points to its possible use as a target for inhibition in melanoma. In agreement, inhibition of Siah2 activity using a peptide that attenuates its effect on hypoxia effectively inhibited melanoma metastasis, whereas inhibition of Siah activities that affect Ras/MAPK signaling pathways blocked melanoma tumorigenicity (Qi et al., 2008).

Here we report on an electro-chemiluminescent-based assay for screening ubiquitin ligase activity that led us to identify menadione (MEN) as a novel Siah 2 inhibitor. MEN (also known as vitamin K3) is a quinone used with cancer chemotherapeutic agents, capable of both redox cycling and arylating nucleophilic substrates (Lamson and Plaza, 2003). MEN has been extensively studied as a model stress-inducing quinone that causes cytotoxicity via generation of oxidative stress, increased peroxidase production or depleted intracellular glutathione (Lamson and Plaza, 2003). Associated with MEN's effect are changes in key signal transduction pathways, including inhibition of PTEN and activation of AKT (Yoshikawa et al., 2007), and activation of ERK (Klotz et al., 2002), AMPK (Jung et al., 2008) and p38 (Chowdhury et al., 2009). These changes were implicated in the sensitization of tumor cells to apoptosis (Criddle et al., 2006). Intriguingly, not all of the recorded effects of MEN require changes in ROS (McAmis et al., 2003). Here we demonstrate that a ROS-independent effect of MEN elicits effective and specific inhibition of Siah2.

Results

To search for putative inhibitors of Siah2 ligase activity, we used the Meso Scale Discovery electro-chemiluminescent system (Kenten et al., 2005). Polyubiquitin chains formed upon Siah2 self-ubiquitination were detected using antibodies against ubiquitin. To optimize the conditions for the Meso-scale assay we used different concentrations (in pM) of Siah2 (Figure S1). We used RNF5, another RING finger E3 ubiquitin ligase, as a control. As RNF5 and Siah2 can utilize the same E2 (UbcH5b), compounds that inhibit Siah2 ligase activity, but not that of RNF5, were considered specific as they neither affect general RING structure nor the E2 used in these reactions.

Of the 1840 compounds screened, four were identified that were able to inhibit Siah2 activity to a degree greater than 90%, without affecting RNF5 ubiquitin ligase activity (Table 1 and

Table S1). These four compounds were thus subjected to further validation using independent assays.

Using new lots of the four compounds we assessed their effect in in vitro ubiquitination assays. The strongest degree of Siah2 inhibition was seen for the compounds 1 (Acticom 160 7; MEN) and 4 (US DRUG 10), (Figure 1A), which did not reduce RNF5 self-ubiquitination activity.

As Siah2 self-ubiquitination results in its self-degradation, inhibition of the ligase activity is expected to increase the expression level of the protein. Indeed, treatment of 293T cells with MEN was sufficient to increase the steady-state levels of Siah2, but not with US DRUG 10 (Figure 1B). This observation excluded US DRUG 10 from further analysis. Consistent with this observation, the degree of Siah2, but not RNF5, auto-ubiquitination in vitro was attenuated in a dose-dependent manner (Figure 1C). As Siah1 shares structural similarities with Siah2 and augments Siah2's effect on PHD3/HIF-1 α (Nakayama et al., 2004), we also assessed the possible effects of MEN on Siah1 ligase activity. MEN also attenuated Siah1 ubiquitin ligase activity and increased its steady-state levels, as seen for Siah2 (Figures S2 and S3). In agreement, treatment of 293T cells with MEN confirmed that it effectively inhibits Siah2 ubiquitination in vivo (Figure 1D). However, the treatment with proteasome inhibitor MG132 increased the ubiquitinated and unubiquitinated forms of Siah2 (Figure 1D), confirming the role of the proteasome in the regulation of Siah turnover. These results suggest that MEN effectively inhibits Siah1/2 self-ubiquitination and increases its steady-state levels in cells, which is in agreement with the original screening and in vitro analysis data.

To further characterize the effect of MEN on Siah2 activity we monitored possible changes in the stability of Siah2 substrates. We assessed whether inhibition of Siah2 auto-ubiquitination attenuates its ability to target ubiquitination of its direct-associated (i.e. SPRY2) or adaptor-mediated substrates (i.e. PHD3).

Mouse embryonic fibroblast (MEF) cells treated with increasing concentrations of MEN revealed a corresponding increase in the steady state levels of endogenous PHD3 under hypoxia condition (Figure 1E), when Siah2 is known to exert its effect on this substrate (Nakayama et al., 2004). To confirm that this change is due to altered PHD3 stability we have monitored its half-life using cycloheximide chase assays. As shown in Figure 1(F), MEN effectively prolonged PHD3 half-life from 40 to 100 min in MEF cells.

Further, MEN also attenuated Siah2-dependent decrease in SPRY2 expression in 293T cells (Figure 2A, left panel), suggesting that MEN may affect SPRY2 availability. As in this study we have used UACC903 melanoma cells for mouse xenograft studies (see Figure 5A), we also confirmed MEN's effect on Spry2 expression in this cell line. Concomitant with the increase in SPRY2 level, ERK activity was decreased (Figure 2A, right panel), in agreement with SPRY2's role as a negative regulator of the Ras signaling pathway.

We next evaluated possible changes in the HIF-1 α expression level following MEN treatment. In agreement with the ability of MEN to inhibit Siah2 ligase activity, thereby increasing PHD3 stability, treatment of 293T cells with MEN reduced the expression of HIF-1 α in a dose-dependent manner (Figure 2B, left panel). This finding implies that MEN is expected to affect the overall hypoxia response, which has been implicated in tumorigenesis and metastasis. Treatment of Siah2 with MEN was found to inhibit its ligase activity without affecting the association with PHYL or Spry2 (Figure S4), noting that the inhibition is likely to be mediated through altered structure/conformation with consequent impacts on its ubiquitin ligase activity.

The effect of MEN was also determined in SW1 mouse melanoma cells, where Siah2 inhibition was shown to have a marked effect on HIF-1 α levels with concomitant effects on tumorigenicity and metastasis (Qi et al., 2008). Similar to the observations made in 293T cells,

MEN effectively reduced the level of HIF-1 α expression in SW1 cells (Figure 2B, right panel), albeit using a 2- to 3-fold lower concentration. MEN did not alter the level of HIF-1 α transcripts (Figure S5), confirming that the changes in HIF-1 α expression are due to its altered stability. Further, corresponding to the reduced level of HIF-1 α , MEN also inhibited the transcription of the HIF-1 α target gene, VEGF (Figure S5, right panel). In agreement, the level of VEGF secreted from SW1 melanoma cells was proportionally reduced following treatment with MEN, as determined using ELISA (Figure 2C). In vitro tube formation, an assay for the capillary formation of Human Microvascular Endothelial Cells (HMVEC) is a powerful tool for examining angiogenic potential. Consistent with reduced VEGF secretion, we observed a dose-dependent decrease in tube formation of HMVEC cells supplemented with medium from MEN-treated SW1 melanoma cells (Figure 2D), suggesting that reduced secretion of VEGF by MEN-treated SW1 cells inhibits angiogenesis in vitro. These findings establish that inhibition of Siah2 activity effectively reduces HIF-1 α expression with concomitant effects on the level of its target genes, such as VEGF, and angiogenic potential, measured in culture by monitoring tube formation.

To confirm the specificity of MEN on Siah2, we monitored changes in Spry2, PHD3 or HIF-1 α expression in Siah2 KO or Siah2/Siah1a DKO MEF cells. Significantly, MEN's ability to increase expression of PHD3 or Spry2 was attenuated in the Siah2 KO and completely abolished in the Siah2/1a DKO cells (Figure 3A, B). Correspondingly, MEN's ability to decrease expression of HIF-1 α was not apparent in the Siah2 KO (Figure 3C). Siah2/Siah1a DKO exhibits very low basal level of HIF-1 α , due to high level of PHD3 (Nakayama et al., 2004), and their treatment with MEN could not reduce HIF-1 α level further, as expected (Figure 3C). These data establish that the changes seen in the cells due to MEN treatment are Siah2-dependent.

Given that many of the cellular changes elicited by MEN have been associated with its ability to induce ROS, we have determined whether the effect on Siah2 ubiquitin ligase activity requires a change in ROS. Remarkably, the ability of MEN to stabilize PHD3 or Spry2, or affect HIF-1 α , was not affected in MEF cells that were treated with *N*-acetyl cysteine (NAC), a known ROS scavenger (Figure 3D). Fluorescence intensity analysis confirmed the generation of ROS following MEN treatment and their squelching by NAC (Figure S6). These observations suggest that changes in ROS elicited by MEN are not required for its inhibition of Siah2 ubiquitin ligase activity. Consistent with these observations, the low dose of MEN used in the cellular assays (10 μ M) and inhibition of Siah2 activity in in vitro reactions using purified bacterial proteins also point towards ROS-independent effect of MEN on Siah (Figure 1A).

To further assess the ability of MEN on Siah2 ligase activity we obtained a series of MEN derivatives. Of the 10 derivatives tested (Table S2), compound 1 (MEN-D1) was able to effectively inhibit Siah2 ligase activity at doses that are significantly lower than those used for MEN. Further assessment revealed that only MEN-D1 was able to effectively change the expression of Siah2 and Siah1a in the 293T cells (Figure 4A and data not shown). Thus we evaluated whether the MEN derivative (MEN-D1) can affect the expression of SPRY2 or HIF-1 α . Surprisingly, MEN-D1 increased Spry2 expression, with a corresponding decrease in ERK phosphorylation, but was not able to alter the levels of HIF-1 α in UACC903 cells (Figure 4B). These findings suggest that MEN-D1 may selectively affect Siah2 substrates that directly bind to this ligase (such as Spry2), and not those requiring an adaptor protein (such as PHD3).

We next sought to determine if the addition of MEN would affect tumor growth in a xenograft model. To this end we injected UACC903 melanoma cells into nude mice and monitored tumor development. Significantly, the addition of MEN or its derivative MEN-D1, in a dose as low as 2 mg/kg, efficiently blocked the growth of these melanoma tumors (Figure 5A). Analysis

of the tumors revealed lower levels of HIF-1 α expression in the MEN but not in the MEN-D1 treatment groups (Figure 5B), consistent with our data that MEN, but not MEN-D1, affects Siah2's effect on PHD3 and HIF-1 α stability. Both MEN and MEN-D1 effectively reduced the levels of pERK, in agreement with their effect on Spry2, although MEN-D1 appeared to elicit greater inhibition, as seen from immunoblot analysis (Figure 5C). The degree of apoptosis, as monitored by TUNEL assays, was found to be higher in both the MEN and MEN-D1 treated groups (Figure S7). These findings demonstrate that MEN and MEN-D1 effectively block melanoma tumor growth in a xenograft model.

Discussion

The role of MAPK and hypoxia signaling in melanoma development suggests that inhibition of these pathways could offer unique advantages for melanoma therapy. Consistent with this notion is the finding that selective inhibitors of the MAPK signaling pathways may not suffice to elicit effective inhibition of melanoma development and/or progression. Therefore, targeting regulatory proteins that control both hypoxia and MAPK signaling pathway may offer a novel modality for the treatment of this tumor type. The ubiquitin ligase Siah is among candidates that satisfy such requirement, namely – control of both Ras/MAPK and hypoxia pathways. Through the regulation of PHD3 Siah regulates the availability of HIF-1 α whereas through the regulation of Sprouty2 it effectively regulates Ras and Raf signaling (Nakayama et al., 2004, 2009; Qi et al., 2008). In agreement, inhibition of Siah2 by either peptide or dominant negative expression vector effectively blocked metastasis or tumorigenicity of melanoma, respectively (Qi et al., 2008). In this study we report on the establishment of a sensitive HTP assay that enables the screening for inhibitors of the E3 ubiquitin ligase Siah2. An initial screen of close to 2000 compounds provide a proof of concept for the sensitivity and robustness of this assay. Our screen led to the identification of MEN as a specific inhibitor of Siah2 and Siah1 ubiquitin ligase activity.

The inhibition of both Siah proteins is likely to be mediated by MEN's effect on Siah2 conformation, such that it affects its ubiquitin ligase activity without altering its association with substrates or adaptor proteins. Interestingly, in contrast to the effect of MEN, MEN derivative (D1) was found to affect Siah2 effect on Spry2 but not PHD3/HIF-1 α , suggesting that it affects the substrates that require direct interaction with Siah2, but not on those that depend on adaptor protein(s). That the effect of MEN on PHD3, HIF-1 α and Spry2 are Siah-dependent was confirmed by analysis of Siah2 KO and Siah2/Siah1a DKO cells where MEN no longer affected these substrates.

As most of MEN's biological effects are attributed to its role in redox cycling and arylating nucleophilic substrates, it is important to emphasize that the redox squelching reagent did not abolish MEN's effect on Siah2 ligase activity. Consistent with our finding that inhibition of the Siah2 pathway abolishes melanoma tumorigenesis (Qi et al., 2008), we observed that MEN as well as MEN-D1 attenuated tumorigenesis in the human melanoma UACC903 cell line. It is noteworthy that MEN-D1 was as effective as MEN in inhibition of UACC903 cells' ability to form tumors. The latter is in agreement with the inhibition of ERK activity, which is due to upregulation of Spry2, a Ras signaling inhibitor in melanoma tumors (Qi et al., 2008). Although MEN elicits a more global inhibition of Siah2 by its ability to also affect the PHD3/HIF-1 α pathway, our earlier data indicate that in vivo the inhibition of HIF signaling would occur at the level of melanoma metastasis, rather than primary tumor formation (Qi et al., 2008). This was not examined in the present study and needs to be addressed further.

The approach used in our study focuses on inhibition of Siah2 ubiquitin ligase activity, rather than protein-protein interaction, which is likely to offer another means for inhibition of this, as well as other ubiquitin ligases. Although our studies have excluded the effect of MEN on

association of Siah2 with its substrates or adaptor protein PHYL, it is expected that MEN affects the conformation of Siah2 RING domain in a selective manner, as it failed to affect another RING finger ligase, RNF5. Structural studies will allow better assessment of the precise effect of MEN on Siah, and likely offer means to improve the targeting of this ligase.

In all, the present study provides a proof of concept for the screening platform established for inhibition of Siah ubiquitin ligase activity, and for the effect of a select inhibitor, MEN, on MAPK and hypoxia signaling, and on melanoma development in a xenograft system. Siah is upregulated in melanoma (our unpublished observations) further providing the rationale for its targeting in this tumor type. Further evaluation of Siah inhibitors will be performed using a large set of melanoma cell lines bearing different genetic mutations and corresponding mouse models.

Materials and methods

Cell culture and transfections

293T cells were cultured in DMEM supplemented with 10% BS and antibiotics. SW1 cells were cultured in DMEM, supplemented with 10% FBS and antibiotics. Immortalized MEF Siah2^{+/+}, Siah2^{-/-} and Siah1a/Siah2 DKO MEF were maintained in 10% FBS with 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 0.2 mM 2-mercaptoethanol (Sigma, St Louis, MO, USA) and antibiotics. Cells were transiently transfected using the calcium phosphate transfection or the Lipofectamine 2000 plus reagent (Invitrogen) according to the manufacturer's recommendations.

Hypoxia treatment

Cells were treated under hypoxia (1% O₂) either for 5 h or for 10 h (as indicated in Results) using a hypoxia chamber (In Vivo 400; Ruskin Technologies Ltd, Bridgend, UK).

Antibodies and reagents

Antibodies against PHD3 (Abcam, Cambridge, MA, USA), β -actin, HA, Myc, GST, Flag (Sigma), Ubiquitin (Santa Cruz, CA, USA), Lamin A/C (Upstate, Temecula, CA, USA), ERK and pERK (Cell Signaling, Boston, MA, USA) were used according to the manufacturer's recommendations. HIF-1 α rabbit pAb was kindly provided by Drs. Abraham and Chiang (Burnham Institute, La Jolla, CA, USA). Spry2 rabbit pAb was a gift from Dr. Dafna Bar-Sagi (NYU, New York, NY, USA). All four selected compounds (Table 1), purchased from Timtec (Newark, DE, USA), were dissolved in DMSO and maintained as a 100 mM stock solution.

Plasmids

Flag-tagged or HA-tagged mouse Siah2, and Flag-tagged Siah1a in pcDNA3.1 vector have been described previously (Nakayama et al., 2004). GST Siah2 (Nakayama et al., 2004), GST-RNF5, GST-Siah2 RING mutant were expressed in pGEX-4T1 vector. PHYL peptide (1–130 residues) in pKH3 vector has been described earlier (Qi et al., 2008).

Library screen

Chemical libraries totaling 1840 compounds included the US FDA library (1040 compounds), Anticom160 (320 compounds), and Natural product library (480 compounds). These were tested at a compound concentration of 100 μ M in the Meso-scale ubiquitination assay (Davydov et al., 2004). Hit compounds in the primary screen were those that exhibited at least 90% inhibition of electro-chemiluminescent signal obtained in control wells. Hits were confirmed in dose-response curves; confirmed hits were repurchased in powdered form and re-tested. Selectivity was established by screening for inhibition of GST-RNF5 activity in an

assay similar to that previously described (Table S1). Further biochemical assays suggested MEN as a possible Siah inhibitor. Based on this observation, a number of commercially available compound analogs were purchased and tested (Table S2).

Screening assays

The Meso Scale Discovery (MSD, Gaithersburg, MD, USA) electro-chemiluminescent detection technology system was used for the screening. In this assay format, ubiquitinated E3 ubiquitin ligases are used to screen for small molecule inhibitors of GST-Siah2 ubiquitination. In a typical assay, 15 μ l of GST-Siah2 in complete MSD reaction buffer (5 pM final concentration of both GST-Siah2 and GST-RNF5) was adsorbed onto pre-blocked MSD MULTI-ARRAY 384-well Glutathione plates. Each compound was added in 1 μ l of 100% DMSO. Reactions were initiated by the addition of 5 μ l ubiquitin-E1-E2 mixture that had been pre-charged and allowed to proceed for 1 h at room temperature. Washing the plate with Tris-buffered solution stopped the reaction. MSD SULFO-Tag-labeled antibody against ubiquitinated proteins was then added along with Read Buffer T (MSD). Electro-chemiluminescence signals were measured with a MSD Sector Imager 2400 Reader.

Western blotting

Cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.1% SDS. The protein concentration was estimated using the Coomassie Protein assay kit (Thermo Scientific, Rockford, IL, USA). Cell lysates were subjected to SDS-PAGE and transferred onto Nitrocellulose (GE Amersham, Pittsburgh, PA, USA). Membranes were blocked for 1–2 h in either 3% BSA or 5% non-fat dry milk in Tris-buffered saline containing 0.2% Tween-20. The membranes were probed with primary antibodies (O/N at 4°C) followed by incubation with secondary antibody conjugated to fluorescent dye for 1 h at room temperature. After washing, the blots were scanned on the Odyssey Licor scanner and the image was captured using the Odyssey software. Nuclear proteins were extracted for detecting HIF-1 α using nucleo-cytoplasmic kit (Pierce, Rockford, IL, USA).

Collection of melanoma cell derived conditioned media

SW1 melanoma cells cultured in DMEM medium containing 0.2% FBS were exposed to 1% or 21% O₂ for 10 h. The supernatants were collected and centrifuged at 400 g for 5 min in a Sorvall round bucket swing-out rotor to remove debris and stored at –80°C. VEGF protein concentration in conditioned media was quantified using a VEGF ELISA kit (R&D, Minneapolis, MN, USA).

HMVEC tube formation assay

Solid gels (BD Matrigel™, BD Biosciences, San Jose, CA, USA) were prepared according to the manufacturer's instructions on a 24-well plate. HMVECs (1 $\times$ 10⁵ cells/ml) were resuspended in melanoma cell-derived conditioned medium (obtained from MEN-treated or -untreated cells under hypoxia for 10 h) and 0.5 ml/well were seeded on the surface of the solid gel. Tube formation was observed after 16 h under an inverted light microscope at 10 $\times$ magnification. The total length of the tube structures were measured using Image J software (National Institutes of Health). Each value represents the average of three samples.

Semi-quantitative RT-PCR

Total RNA was extracted using a total RNA miniprep kit (Sigma). cDNA was synthesized using 1 μ g of total RNA. The cDNA was diluted 1:10 and the PCR was carried out in the presence of α -³²p)-dCTP to amplify VEGF, actin (10, 15 and 20 cycles) or HIF-1 α (20 cycles). The signals were detected by autoradiography. Primers for PCR were as follows: VEGF:

forward, 5'-ATCTTCAAGCCGTCCT GTGT-3' and reverse, 5'-GCATTACATCTGCTGTGCT-3'. β -actin: Forward, 5'-TTCTTTGCAGCTCCTTCGTTGCCG-3' and reverse, 5'-TGGATGGCTACGTACATGGCTGGG-3'.

In vitro ubiquitination assay

GST-Siah2, GST-Siah2 RING mutant and GST-RNF5 were purified from the bacteria using Glutathione-Sepharose (Amersham Bioscience). His-UbcH5b (gift of Aaron Ciechanover, Technion, Israel) was expressed and purified from the bacteria using Ni-NTA²⁺-agarose. Purified GST-Siah2 or GST-RNF5 was subjected to an in vitro ubiquitination assay in ubiquitination buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.5 mM dithiothreitol, 2 mM NaF) supplemented with purified HA-ubiquitin (2 μ g), 2 mM ATP, E1 (50 ng) (Boston Biochem, Cambridge, MA, USA), purified E2 (UbcH5b) (250 ng) for 45 min at 37°C. Reaction mixtures were then separated on a 8% SDS-PAGE followed by Western blot analysis using an anti-ubiquitin antibody. For reactions performed on the beads, 20 μ l of GST fused proteins attached on glutathione beads, were washed twice with buffer containing (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% triton X-100, 1 mM EDTA) and once with ubiquitination buffer. After washing, reactions were carried out in 20 μ l ubiquitination buffer containing purified HA-ubiquitin (2 μ g) with shaking. The reactions were washed twice with buffer containing (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% triton X-100, 1 mM EDTA) and eluted with sample buffer for loading on to the gel.

In vivo ubiquitination

Cells were transfected with indicated plasmids and HA-tagged ubiquitin. 36 h later cells were harvested and lysed in 1 vol of 2% SDS in TBS (10 mM Tris-HCl, pH 8.0) at 95°C for 10 min. Nine volumes of 1% Triton X-100 and 2 mM in vitro EDTA in TBS were added, and lysates were incubated on ice for 30 min, followed by sonication (15 s, three times). The solution was incubated for 30 min at 4°C with protein G beads (Invitrogen) and clarified by 30 min of centrifugation (16 000 g in Eppendorf table-top centrifuge 5415R) at 4°C. The protein concentration was determined by the Bradford assay. For immunoprecipitation, 2 mg of protein was incubated with anti-flag antibody at 4°C overnight before protein G beads were added for 2 h. Beads were washed once with TBS, 1% Triton X-100, 1% SDS, twice with 0.5 M LiCl, TBS buffer and again in PBS 1% Triton X-100 containing buffer. Proteins were loaded onto 8% SDS-PAGE gels and immunoblotted with indicated antibodies.

In vitro protein-binding assays

GST-Siah2 was affinity purified by adsorption onto glutathione-Sepharose 4B beads. Spry2 and PHYLL expressing constructs were transcribed and translated using the TNT Coupled Reticulocyte Lysate System (Promega, San Luis Obispo, CA, USA) in the presence of [³⁵S]-methionine according to the manufacturer's instructions. The in vitro translated proteins were mixed with the indicated GST-fusion proteins and bound to Glutathione-Sepharose beads. The beads were washed extensively in HNTG buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, containing complete protease inhibitor cock-tail (Roche Molecular Biochemicals, Nutley, NJ, USA), and followed by separation on 12% SDS-polyacrylamide gels and autoradiography.

Melanoma xenograft

UACC 903 cells (1 $\times$ 10⁶ cells/100 μ l PBS) were injected subcutaneously to eight 4-week-old male nude mice. Different dosage (2 mg/kg, 5 mg/kg, and 10 mg/kg) of MEN or MEN-D1 (dissolved in DMSO at final concentration of 0.1%) were administered via intra-peritoneal injections twice per week. Tumor growth was monitored twice per week for 4 weeks, at which

point the mice were sacrificed and tumors were harvested for immunohistology or Western blot analyses.

Immunohistochemistry and TUNEL analysis

Melanoma xenograft tumors were fixed in Z-fix (buffered zinc formalin fixatives, Anatech, Battle Creek, MI, USA) overnight. After fixation, tumors were washed twice with PBS and processed for paraffin embedding. Tumors embedded in paraffin blocks were sliced at 5 μ m, and tumor sections were rehydrated and processed for immunohistochemistry. Antigen retrieval was performed using Dako target retrieval solution, followed by peroxidase block for 30 min with 3% hydrogen peroxide. The sections were washed with PBS and incubated with primary antibody diluted in Dako antibody diluent overnight at 4°C. After incubation, slides were washed three times with PBS/Tween-20 and incubated with Dako labeled Polymer-HRP (anti-rabbit) for at 1 h at RT. Slides were then washed four times with PBS/Tween-20, developed with DAB, and counterstained with hematoxylin. TUNEL staining was performed using an ApopTag peroxidase in situ apoptosis kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instruction.

ROS measurement

The samples were treated with H₂DCFDA (30 μ M) in warm PBS for 45 min. Cells were harvested, washed once in PBS and resuspended in PBS and analyzed by FACS.

Significance

Upregulation of mitogen-activated protein kinase (MAPK)/Ras signaling pathway is central in melanoma development and progression. Similarly, hypoxia and its master regulator HIF-1 α were shown to play an important role in melanoma progression. Hence, targeting both MAPK and hypoxia pathways is expected to effectively inhibit melanoma development and progression. The ubiquitin ligase Siah2 has been implicated in the regulation of both hypoxia response and Ras/MAPK signaling. The inhibition of Siah expression or activity was shown to effectively block mammary, pancreas, lung and melanoma cancers. Here we establish a high-throughput assay to screen for an inhibitor of Siah2 ligase activity, and demonstrate the identification and characterization of one such compound, menadione (MEN). MEN effectively attenuated hypoxia and MAPK signaling and inhibited melanoma growth in a mouse xenograft model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Inhibition of Siah2 auto-ubiquitination by positive hits from Meso Scale screen. In vitro ubiquitination reactions for GST-Siah2 and GST-RNF5 were carried out using 100 μ M of compounds 1–4 (Table S1 and Table 1). The ubiquitination was monitored by Western blot analysis using anti-ubiquitin antibodies. The position of the ubiquitinated GST-Siah2 or GST-RNF5 is indicated. The amount of GST-Siah2 or GST-RNF5 used in these reactions is shown in the lower panel using anti-GST antibodies. (B) Increase in Siah2 steady state levels by MEN. 293T cells were transfected with HA-Siah2 (1 μ g) and 24 h later cells were treated with indicated concentrations of MEN and USD 10 for 5 h. As control cells were treated with equal amounts of DMSO, Western blot analysis was performed using the indicated antibodies. β -

actin level is shown as loading control. (C) Inhibition of Siah2 auto-ubiquitination by MEN. In vitro ubiquitination reactions for GST-Siah2, GST-Siah2 RING mutant (GST-S2RM) and GST-RNF5, attached to the glutathione beads, were performed using indicated concentrations of MEN. Western blot analysis was performed using anti-ubiquitin antibodies. The position of the ubiquitinated GST-Siah2 or GST-RNF5 is indicated. Western blot with GST antibodies revealed the amount of ubiquitin ligases used in these reactions (lower panel). (D) In vivo auto-ubiquitination of Siah2 is inhibited by MEN. 293T cells were co-transfected with Flag-Siah2 with or without HA-tagged ubiquitin. After 24 h, cells were treated with MEN (25 μ M) or MG132 (40 μ M) for 6 h, or left untreated. Siah2 ubiquitination was analyzed by immunoprecipitation with an anti-Flag antibody (M2) followed by immunoblotting using monoclonal anti-HA antibodies. The same blot was reprobed with anti-Flag antibodies to monitor Siah2 levels. (E) MEN increases PHD3 protein level. MEF cells were treated with indicated concentrations of MEN under normoxia (N) or hypoxia (H; 1% O₂) for 5 h. The DMSO-treated cells were used as a control. The cells were collected, lysed and level of PHD3 determined by Western blot analysis using the indicated antibody. (F) PHD3 is stabilized by menadione treatment. The half-life of PHD3 protein was monitored by cycloheximide (CHX) pulse-chase experiment. MEF cells were treated with MEN (25 μ M) or left untreated under hypoxia for 5 h. After 5 h, CHX (20 μ g/ml) was added and cells were harvested at the indicated time points and analyzed using the indicated antibodies. Right panel: Densitometric quantification of PHD3 levels, normalized to β -actin levels.

Figure 2.

(A) Increase in Spry2 protein level by MEN. 293T cells were transfected either with Myc-Spry2 alone or in combination with HA-Siah2. 24 h later, the cells were treated with indicated concentrations of MEN for 5 h. Proteins were collected for immunoblot analysis as indicated. β -actin served as the loading control. Right panel: The UACC903 melanoma cells were treated with indicated concentrations of MEN for 5 h. Proteins were collected for immunoblot analysis as indicated. Total ERK1/2 level served as loading control. (B) MEN decreased HIF-1 α protein levels. Left panel: 293T cells were treated with indicated concentrations of MEN, under normoxia (N) or hypoxia (H; 1% O₂) for 5 h. Nuclear proteins were extracted and analyzed using the indicated antibodies. Lamin A served as the loading control. Right Panel: The experiment was performed as indicated above except using SW1 mouse melanoma cells. (C) MEN reduced VEGF secretion. SW1 melanoma cells were either treated or left untreated with indicated concentrations of MEN under normoxia or hypoxia (1% O₂) for 10 h. Culture supernatants were assayed for VEGF using a mouse VEGF ELISA kit. The values represent means $\pm$ SEM (n = 3). (D) Endothelial tube formation is attenuated by MEN treatment of

melanoma cells. SW1 melanoma cells were treated with indicated concentrations of MEN for 10 h under hypoxia (1% O₂). The conditioned media collected from these cells were used to induce tube formation in HMVEC cells (see Methods for details) cultured on reconstituted Matrigel. Capillary length was quantified by Image J and values represent means $\pm$ SEM (n = 3).

Figure 3.

(A) Analysis of the effects of MEN on PHD3 levels in Siah-KO cells. Mouse embryo fibroblasts from WT, Siah2^{-/-} and Siah1a^{-/-}/Siah2^{-/-} were treated with MEN (25 μM under hypoxia for 5 h) and proteins were prepared and analyzed by Western blots with the indicated antibodies. (B) Analysis of the effects of MEN on Spry2 proteins in Siah-KO cells. The experiment was performed as indicated in panel A, except that the analysis was performed under normoxia against Spry2. (C) Analysis of the effects of MEN on HIF-1α in Siah-KO cells. The experiment was performed as indicated in panel A, except that nuclear proteins were used for the analysis of HIF-1α. Lamin A served as the loading control. (D) The effect of MEN on Siah2 is independent of ROS generation. MEF cells were treated with MEN (25 μM) and 5 mM of *N*-

acetyl *L*-cysteine, as indicated. The level of PHD3 (under hypoxia H, 1% O₂, 5 h; upper panel) SPRY2 (under normoxia N; middle panel) and HIF-1 α , (nuclear proteins; lower panel) is shown. β -actin and Lamin A served as the loading control.

Figure 4.

(A) MEN derivative (MEN-D1) increase levels of Siah2 and Siah 1a. 293T cells were transfected with HA-Siah2 (left panel) or Flag-Siah1a (right panel; 1 μ g) and 24 h later cells were treated with indicated concentrations of MEN-D1 (Table S2). Western blot analysis was performed using the indicated antibodies. β -actin level is shown as the loading control. (B) MEN-D1 does not affect HIF-1 α protein levels. Left panel: UACC 903 Cells were treated with indicated concentrations of MEN-D1 either under normoxia or hypoxia (1% O₂) for 5 h. Nuclear proteins were analyzed using antibodies against HIF-1 α . Lamin A served as the loading control. Right panel: MEN-D1 increased the levels of Spry2 protein. UACC 903 Cells were treated with MEN D1 for 5 h, as indicated and blotted for Spry2 level. β -actin level is shown as the loading control.

Figure 5.

(A) MEN and MEN-D1 inhibit growth of UACC903 melanoma tumors. UACC903 melanoma cells were injected s.c. into the flank of nude mice. After 1 week, MEN (left graph) and MEN-D1 (right graph) were administered by intra-peritoneal injections twice per week. Tumor size was monitored at the indicated times following treatment ($n = 7$ for each group). Data are mean $\pm$ SEM. The P-values for both MEN and MEN-D1 on 29-day time point is $P < 0.01$ compared to untreated mice. (B) MEN but not MEN-D1 inhibits HIF-1 α . Tumor sections prepared from the respective groups were subjected to analysis by IHC using antibodies against HIF-1 α or pERK. (C) MEN and MEN-D1 effectively inhibit pERK. Tumors were used to prepare proteins which were analyzed by Western blots for pERK and total ERK levels. Analysis shown is for tumors obtained from three controls (C1–3), MEN-treated (M1–3) or MEN-D1-treated (MD1–3) groups.

Table 1

Select compounds identified in the Meso-Scale HTP assay and confirmed using in vitro ubiquitination assays

Compound ID	Structure
1 Acticom 160 7 ST066885
2 Acticom 160 8 AC0223
3 US Drug 5 ID01500450
4 US Drug 10 ID01500618

The name, ID, structure and IC50 for each of four compounds identified and confirmed in the in vitro based self-ubiquitination, confirming the data obtained in the initial Meso-Scale HTP screen is shown.