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REPORT

A PTEN inhibitor displays preclinical activity against hepatocarcinoma cells

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ABSTRACT

Phosphatase and tensin homolog (*PTEN*) gene is considered a tumor suppressor gene. However, PTEN mutations rarely occur in hepatocellular carcinoma (HCC), whereas heterozygosity of PTEN, resulting in reduced PTEN expression, has been observed in 32–44% of HCC patients. In the present study, we investigated the effects of the small molecule PTEN inhibitor VO-OHpic in HCC cells. VO-OHpic inhibited cell viability, cell proliferation and colony formation, and induced senescence-associated β-galactosidase activity in Hep3B (low PTEN expression) and to a lesser extent in PLC/PRF/5 (high PTEN expression) cells, but not in PTEN-negative SNU475 cells. VO-OHpic synergistically inhibited cell viability when combined with PI3K/mTOR and RAF/MEK/ERK pathway inhibitors, but only in Hep3B cells, and significantly inhibited tumor growth in nude mice bearing xenografts of Hep3B cells. Therefore, we demonstrated for the first time that VO-OHpic inhibited cell growth and induced senescence in HCC cells with low PTEN expression, and that the combination of VO-OHpic with PI3K/mTOR and RAF/MEK/ERK inhibitors resulted in a more effective tumor cell kill. Our findings, hence, provide proof-of-principle evidence that pharmacological inhibition of PTEN may represent a promising approach for HCC therapy in a subclass of patients with a low PTEN expression.

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AKT; HCC; PTEN; senescence; Sorafenib; VO-OHpic

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer-related mortality worldwide.[1] In the last years, the clinical diagnosis and management of early-stage HCC has improved significantly, however, the prognosis in patients with advanced HCC is still extremely poor. Sorafenib, a multikinase inhibitor which targets the RAF/MEK/ERK signaling pathway, as well as multiple tyrosine kinase receptors (i.e. VEGFR-2/-3, PDGFR-β, Flt-3 and c-Kit), is the only approved systemic therapies that improve survival in HCC patients.[2,3] However, although sorafenib improves prognosis in advanced HCC, response to sorafenib remains low and median overall survival is only extended by a few months.[2,3] Molecular therapies targeting signaling cascades involved in hepatocarcinogenesis have been explored in several phase III clinical trials.[4,5] However, none of the drugs tested have shown positive results in first or second treatment lines after progression on sorafenib.[6]

Due to the current limitations of therapeutic options, there is an urgent need to identify novel targets for systemic therapy and to develop new specific treatments.[4,5] Recently, pro-senescence therapy has emerged as a promising anticancer therapy in several types of cancer,[7,8] including HCC.[9]

The phosphatidylinositol-3-kinase (PI3K)/phosphatase and tensin homolog deleted on chromosome ten (PTEN)/AKT/mammalian target of rapamycin (mTOR) pathway has a key role in HCC as its activation induces cell proliferation and increases survival.[10] This pathway is activated after the binding of various growth factors to specific cell surface receptors, such as EGFR and IGF-1R. The PI3K/AKT oncogenic signal is frequently hyperactivated in HCC.[10]

However, multiple lines of evidence indicate that increased PI3K/AKT signaling may also induces cell senescence,[11-15] although the mechanisms mediating PI3K/AKT-induced senescence are poorly understood.

Negative regulation of the PI3K/AKT pathway is primarily accomplished through the action of PTEN, a tumor suppressor protein. PTEN is inactivated in a wide range of tumors, including 40–60% of human liver cancers.[16,17] However, frequent loss of heterozygosity of PTEN has been observed in 32–44% of HCC patients, resulting in reduced PTEN expression.[18,19] Although PTEN loss in human cancers has been documented, the exact role of PTEN in HCC has not been fully elucidated.

Notably, in contrast to its function as a tumor suppressor, pharmacological inhibition of PTEN with the water-soluble vanadium-based complex (VO-OHpic), a potential anti-diabetic drug,[20] has recently been discovered to trigger some cancer cells to enter into an irreversible non-dividing state.[21] This condition, referred to as PTEN-Induced Cellular Senescence (PICS) was observed only in cells with partial PTEN activity

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(heterozygous).[21] Interestingly, VO-OHpic did not cause an evident effect on cells with wild type PTEN. This finding indicated that VO-OHpic could be developed as a potential "pro-senescence" anti-cancer drug for the treatment of cancers with reduced PTEN expression.[22]

On this basis, we aimed to test whether PTEN inhibition would represent an effective therapeutic modality for hepatocellular carcinoma.

Results

PTEN expression and effects of VO-OHpic on cell viability, cell proliferation and colony formation in human HCC cell lines

We first analyzed the basal expression of PTEN on the human HCC cell lines Hep3B, PLC/PRF/5 and SNU475. As documented by Western blot analysis, the HCC cell lines showed different expression levels of PTEN (Fig. 1A).

PLC/PRF/5 cells expressed the highest levels (arbitrarily fixed at 1) while Hep3B cells showed the lowest expression of the protein. SNU475 cells were PTEN negative (Fig. 1A).

To address the question of whether pharmacological inhibition of PTEN could affect the growth of HCC cells, we used the new PTEN inhibitor VO-OHpic (Fig. 1B).[20] As PTEN activity is critically involved in the regulation of downstream AKT activity, we initially verified the effects of VO-OHpic on AKT phosphorylation levels. HCC cells were treated with varying concentrations of VO-OHpic, and phosphorylation status on Ser473 was analyzed by Western blot in three representative cell lines (Fig. 1C). As expected, inhibiting PTEN activity resulted in a dose-dependent increase in expression levels of activated AKT (phospho-AKT) in Hep3B cells, but no detectable phospho-AKT was observed in PLC/PRF/5 cells. Furthermore, expression of the mammalian target of rapamycin (mTOR), a downstream target of activated AKT, was analyzed. Treatment with VO-OHpic increased the expression levels of the active form of mTOR, phospho-mTOR, in Hep3B cells, but not in the other cell lines (Fig. 1C).

Emerging studies have suggested that PTEN may also negatively regulate the extracellular signal-regulated kinase (ERK)1/2 pathway.[23,24] We therefore analyzed the expression of ERK1/2 levels after VO-OHpic treatment by Western blot. As reported in Figure 1C, in the Hep3B cells treatment with VO-OHpic increased expression levels of the active form of ERK1/2, phospho-ERK1/2, in a dose-dependent manner, while it had no effect on the total amount of ERK1/2 proteins. Similar results were obtained in the PLC/PRF/5 cells. Not surprisingly, these effects on p-AKT, p-mTOR and p-ERK1/2 were absent in the PTEN-negative SNU745 (Fig. 1C).

HCC cell lines were therefore treated with increasing concentrations of VO-OHpic, after which cell viability (Fig. 2A)

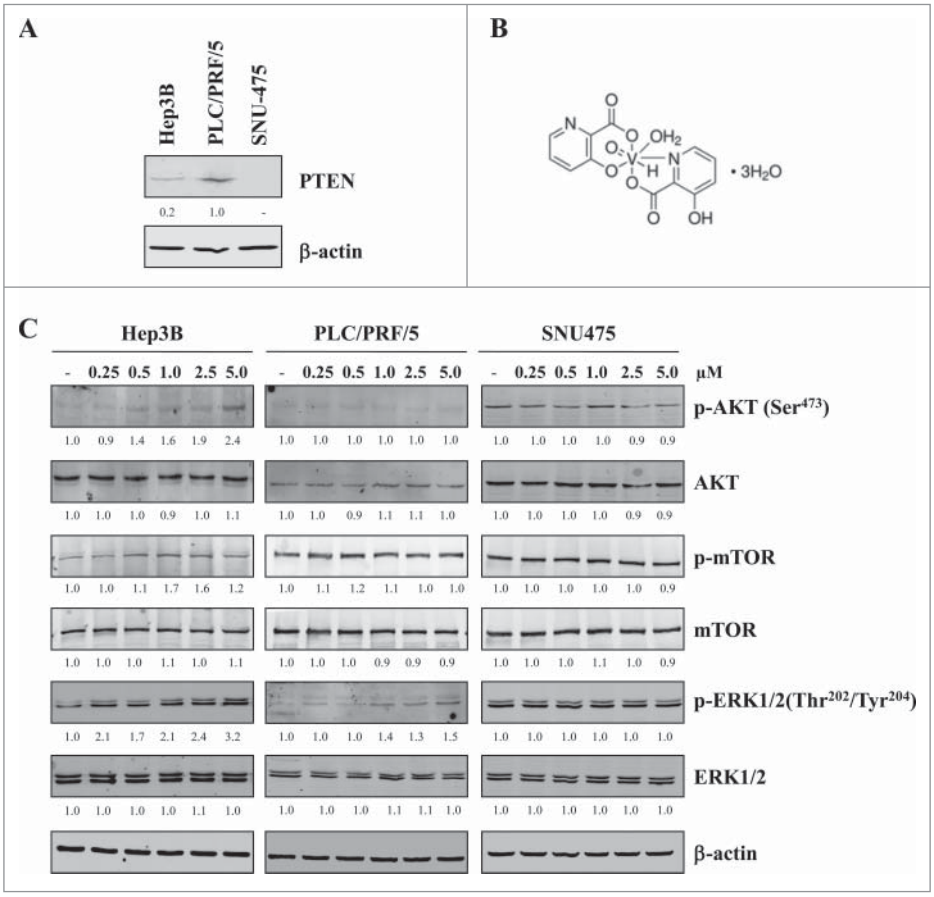

Figure 1. (A) Western blot analysis of PTEN basal expression on human HCC cell lines. (B) Chemical structure of PTEN inhibitor hydroxy(oxo)vanadium 3-hydroxypyridine-2-carboxylic acid trihydrate (VO-OHpic). (C) Western blot analysis of PTEN-regulated phospho proteins AKT, mTOR and ERK1/2 in Hep3B, PLC/PRF/5 and SNU475 cells. The numbers represent the ratio of the relevant protein normalized with β-actin, with vehicle-treated control samples (–) arbitrarily set at 1.0. The data shown represent two independent experiments with comparable outcomes.

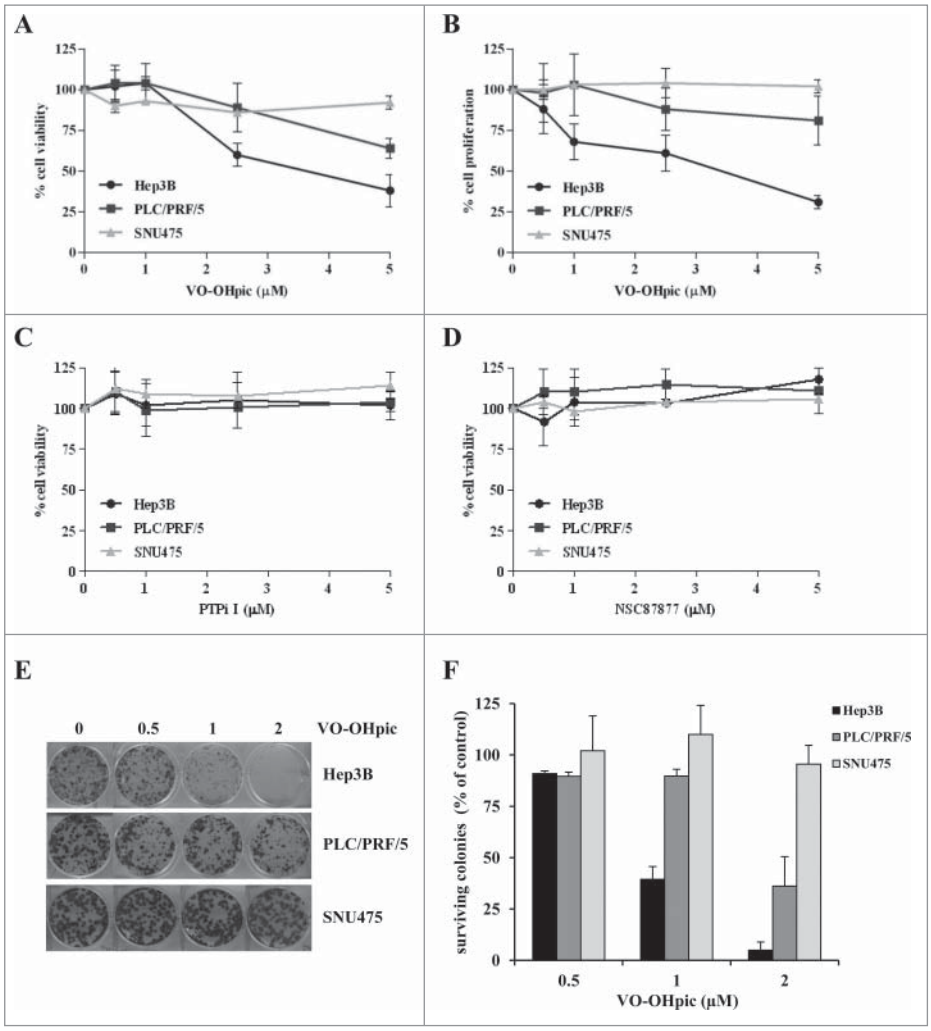

Figure 2. PTEN inhibition reduces cell viability, cell proliferation and colony forming ability in Hep3B and PLC/PRF/5 cells. (A) Cell viability in each HCC cell line was assessed by MTS assays. Cells were treated with increasing concentrations of VO-OHpic for 120 hours. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. (B) DNA synthesis was measured by BrdU incorporation into DNA. Cells were treated with the indicated concentrations of VO-OHpic. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. (C-D) Cell viability in each HCC cell line was assessed by MTS assays. Cells were treated with increasing concentrations of PTPi I (C) or NSC87877 (D) for 120 hours. Data are expressed as the percentage of control cells and are the means ± SD of three separate experiments, each of which was performed in triplicate. (E) Representative images of clonogenic assay after treatment with VO-OHpic. Hep3B, PLC/PRF/5 and SNU475 cells were plated overnight and exposed to VO-OHpic at the indicated concentrations every 48 hours. These experiments continued for 14 days in Hep3B and PLC/PRF/5 cells and for 10 days in SNU475 cells. Surviving colonies were stained and counted. (F) Data are expressed as the number of colonies and are the means ± SD of two separate experiments, each of which was performed in duplicate.

and cell proliferation (Fig. 2B) were analyzed by MTS and BrdU assays, respectively. Figure 2A shows that SNU475 cells were resistant to VO-OHpic, even at the highest concentrations tested, whereas although treatment with VO-OHpic increased the pro-survival pathway AKT (Fig. 1C), a dose-dependent decrease in cell viability and cell proliferation was surprisingly seen in the other two cell lines tested, *i.e.*, Hep3B and PLC/PRF/5 (Fig. 2A-B). However, Hep3B cells were more sensitive to the drug than PLC/PRF/5 cells, displaying IC_{50} values at 120 hours of 3.4 and > 5 μM in Hep3B cells and PLC/PRF/5 cells, respectively (Fig. 2A-B). Although some reports have reported that VO-OHpic is a specific and potent inhibitor of PTEN,[21,25-29] others have raised concerns about its specificity.[30] Therefore, to ascertain whether the VO-OHpic effects were due to inhibition to other phosphatases, such as Src homology region 2 domain-containing phosphatase (SHP) or protein-tyrosine phosphatase 1B (PTP1B), we analyzed the effects of PTP inhibitor I, which inhibits SHP-1 and PTP1B, and of NSC87877, which inhibits SHP-1 and SHP-2, on cell viability. HCC cell lines were therefore treated with increasing concentrations of the two inhibitors, after which cell viability was analyzed by MTS assays. Cell viabilities of all HCC cell lines were not affected by treatment with either PTP1B or NSC87877, even at the highest concentrations tested (Fig. 2C-D).

The effects of VO-OHpic on cell growth were further confirmed using a clonogenic assay (Fig. 2E-F). There was a dose-dependent decrease in colony-forming ability due to VO-OHpic treatment in Hep3B and PLC/PRF/5 cells, but not in SNU475 cells.

All together these data demonstrate that PTEN-negative SNU475 cells were insensitive to the drug in all the experimental conditions. In addition, these data demonstrate that cells with low PTEN expression (Hep3B cells) are more responsive than cells with high PTEN expression (PLC/PRF/5 cells).

Since the anti-growth effects of the treatments could have been due to increased cell death, we examined the effects of the drug on the induction of apoptosis. Treatment of all cell lines with up to 5 μM VO-OHpic had negligible effects on apoptosis induction as evaluated by DNA staining with Hoechst 33258 (Fig. S1A). In addition, the expression the apoptosis-related proteins PARP (a well-known caspase-3/7 substrate) was also analyzed by Western blot. In all cell lines, treatment with VO-OHpic failed to induce PARP cleavage and expression of PARP maintained the baseline levels observed in untreated cells (Fig. S1B). Therefore, none of these analyses revealed activation of apoptotic response after VO-OHpic treatment.

Pharmacological inhibition of PTEN with VO-OHpic drives senescence in Hep3B cells

Several lines of evidence indicate that increased AKT/mTOR signaling induces cellular senescence response[11,14] and, as mentioned before in the prostate cancer model, inhibition of the residual activity of PTEN in low PTEN expressing cells forces prostate cancer cells to PICS.[21] Therefore, to investigate the mechanisms of the growth inhibition effects of VO-OHpic in HCC cells, cells were treated with the drug for five days and the hallmark feature of senescent cells, β-galactosidase activity, was quantified. As demonstrated in Figure 3 prolonged treatment drove cells into a senescent state.

A considerable, dose-dependent fraction of cells of up to 56% showed senescence-associated β-galactosidase (SA-β-GAL) activity in Hep3B cells, while in PLC/PRF/5 and SNU475 cells SA-β-GAL activity was unaffected by VO-OHpic (Fig. 3A-B). These results demonstrated that the inhibitor drives senescence only in cells with low basal levels of PTEN.

Cellular senescence is frequently accompanied by the production of secreted proteins, referred as senescence-associated secretory phenotype (SASP), which mediate the different effects of senescence on the tissue microenvironment. To better characterize the observed senescence after treatment with VO-OHpic, senescence-associated changes in expression of genes involved in SASP were analyzed by quantitative real-time PCR. As reported in Figure 3C, treatment with VO-OHpic increased the expression levels of *interleukin 8* (IL8) and *matrix metalloproteinase 9* (MMP9) genes, two factors

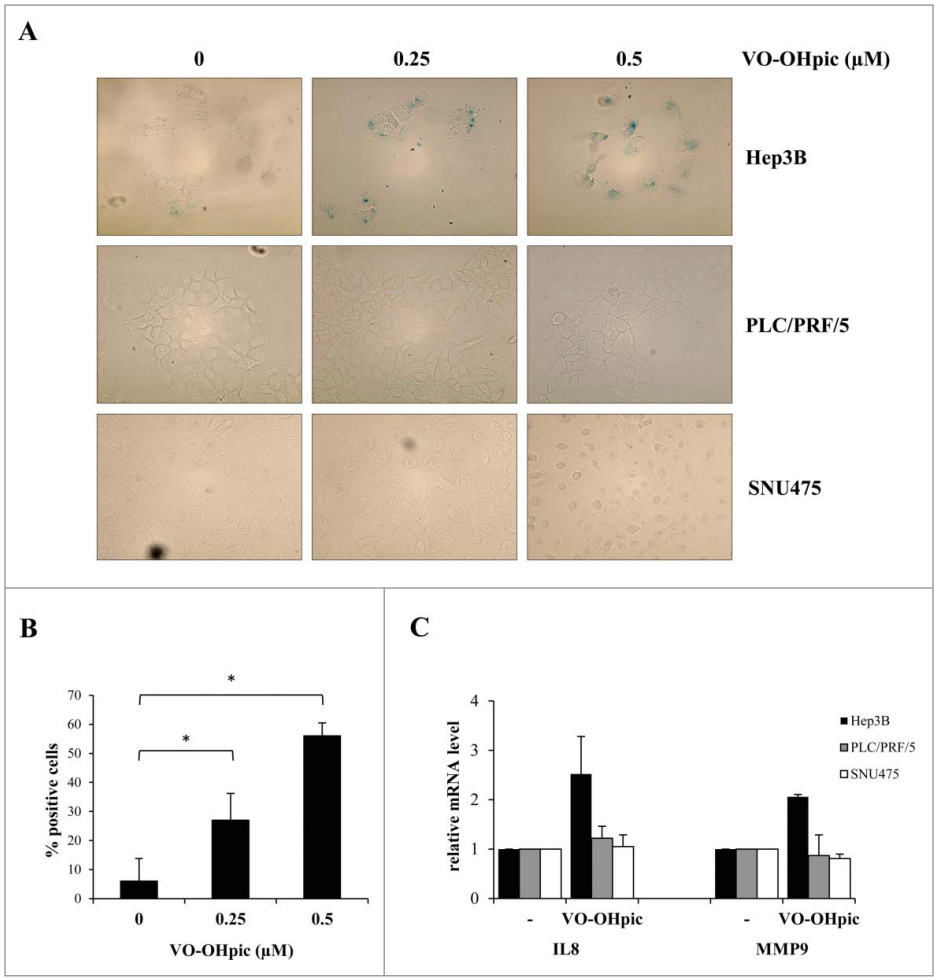

Figure 3. VO-OHpic treatment induced senescence-associated β-galactosidase (SA-β-Gal) and senescence-associated secretory phenotype (SASP) in Hep3B cells. (A) Representative images of Hep3B, PLC/PRF/5 and SNU475 cells treated with the indicated concentrations of VO-OHpic. VO-OHpic was added every 72 hours, and after five days senescent cells were identified by SA-β-Gal assay. (B) Data are expressed as the number of SA-β-Gal positive cells and are the means ± SD of two separate experiments, each of which was performed in duplicate. (C) Expression of IL8 and MMP9 mRNAs were analyzed by quantitative RT-PCR in HCC cells. Hep3B, PLC/PRF/5 and SNU475 cells were treated with the 500 nM of VO-OHpic for 72 hours. Relative expression was calculated as ratio of drug-treated samples versus control (DMSO) and corrected by the quantified expression level of β-actin. The results shown are the means ± SD of three experiments, each performed in triplicate.

known to be secreted by senescent cells, in Hep3B cells, while in PLC/PRF/5 and SNU475 cells gene expression was unchanged upon VO-OHpic treatment.

VO-OHpic induces a G2/M arrest and increases the expression of cell-cycle inhibitor p21

To investigate the mechanism of the senescence-associated growth inhibition, we analyzed the cell cycle profile, by flow cytometric analysis, of HCC cells after VO-OHpic treatment. After 72 hours of treatment with VO-OHpic, an accumulation of cells in G2/M phase of the cell cycle was observed in Hep3B cells, when compared with untreated cells, while in PLC/PRF/5 and SNU475 cells no changes in the distribution of different cell cycle phases were observed (Fig. 4A).

Cell cycle phase progression is regulated by a number of the cyclin-dependent kinases (CDKs) and cyclins which can be negatively regulated by kinase inhibitor proteins, such as p21 and p16, two well known CDK inhibitors involved in the control of cellular senescence. To further elucidate the mechanism of VO-OHpic induced cell cycle arrest in HCC cells, we determined the levels p16 and p21 mRNAs in all cell lines exposed to different concentrations of VO-OHpic (Fig. 4B). The levels of p16 mRNA were only slightly increased in Hep3B and SNU475 cells, whereas p21 mRNA was increased only in Hpe3B cells, but not in PLC/PRF/5 and SNU475 cells, suggesting that it may play a role in VO-OHpic-induced senescence.

VO-OHpic synergizes with PI3K/mTOR and Raf/MEK/ERK inhibitors

The observation that treatment with VO-OHpic altered AKT and ERK1/2 signaling prompted us to investigate the functional roles of the activation of these signaling pathways. Therefore, we next analyzed the effect on cell viability in Hep3B cells of various treatment combinations: VO-OHpic with the multi-kinase inhibitor sorafenib, with the MEK inhibitor U0126, with the dual PI3K/mTOR inhibitor

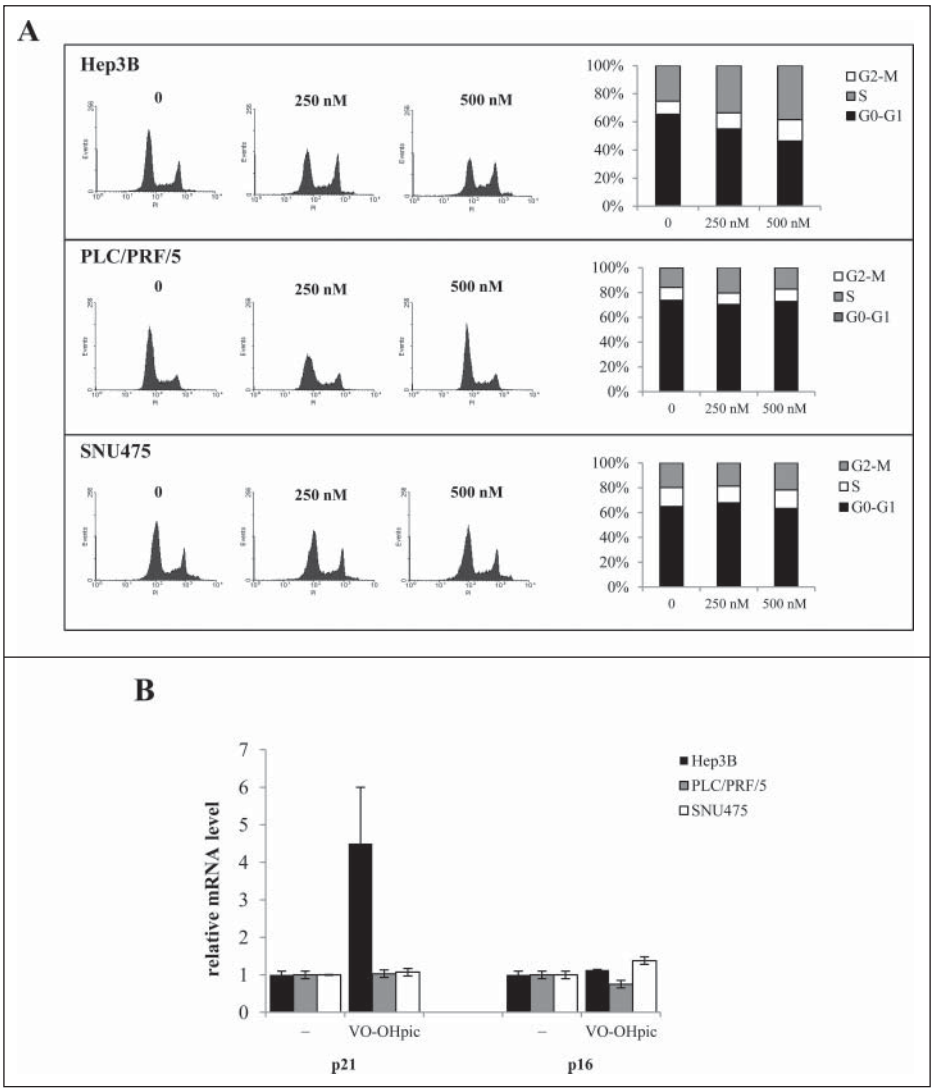

Figure 4. VO-OHpic induced cell cycle arrest and increased the expression of p21 mRNA in Hep3B cells. (A) Representative images of cell cycle analysis in Hep3B, PLC/PRF/5 and SNU475 cells treated with 500 nM of VO-OHpic for 72 hours. Cells were stained with propidium iodide and DNA content of cells was analyzed by flow cytometry. (B) Expression of p21 and p16 mRNAs were analyzed by quantitative RT-PCR in HCC cells. Hep3B, PLC/PRF/5 and SNU475 cells were treated with the 500 nM of VO-OHpic for 72 hours. Relative expression was calculated as ratio of drug-treated samples versus control (DMSO) and corrected by the quantified expression level of β-actin. The results shown are the means $\pm$ SD of three experiments, each performed in triplicate.

Table 1. VO-OHpic in combination with sorafenib, U0126, and BEZ235 elicited synergistic inhibition of cell viability in Hep3B cells. The combination index (CI) values are indicated.

VO-OHpic (μM)	Sorafenib (5 μM)	U0126 (10 μM)	BEZ235 (50 nM)
0.5	0.599	0.982	1.094
1	0.619	0.570	0.764
2.5	0.538	0.403	0.868
5	0.541	0.360	0.418

CalcuSyn software was used to calculate the combination index (CI), where a CI $<$ 1 indicated synergy, 1 indicated an additive effect and $>$ 1 indicated antagonism. In experiments with U0126, cells were pre-treated with U0126 for 2 h and then treated with VO-OHpic in combination for a 72 additional hours in the presence of U0126.

BEZ235. According to the combination index (CI), the combination of varying concentrations of VO-OHpic with all these inhibitors resulted in a synergistic inhibition of cell viability in Hep3B cells, as evaluated by MTS assay after 72 hours of treatment (Table 1).

Pharmacological inhibition of PTEN with VO-OHpic inhibited tumor growth in vivo

To demonstrate the *in vivo* effectiveness of VO-OHpic on HCC, a mouse xenograft tumor model of Hep3B cells was used. Treatment with VO-OHpic significantly reduced tumor volume when compared with tumors of the untreated group (Fig. 5A).

As a control for drug-associated cytotoxicity, changes in animal body weight were also monitored. Mice treated with VO-OHpic did not show a significant loss of body weight when compared with mice treated with vehicle alone, suggesting a satisfactory level of drug cytotoxicity (Fig. 5B).

Western blot analysis performed on homogenates from tumor tissues of mice treated with VO-OHpic showed higher p-AKT and p-ERK1/2 levels (Fig. 5C) than those of untreated mice, confirming data observed in *in vitro* experiments (Fig. 1C). Immunohistochemical analysis showed a lower expression of cell proliferation marker Ki-67 in tumor tissues from animals treated with VO-OHpic, than in the tissues of the untreated animals (Fig. 5D-E), confirming data obtained using an *in vitro* proliferation assay (BrdU assays) (Fig. 2B).

Discussion

In the present study using human HCC cells expressing different levels of PTEN, we present a new insight into the antitumor effects of the PTEN inhibitor VO-OHpic, as well as the putative mechanisms involved. First, we demonstrated the effect of VO-OHpic by analyzing expression of PTEN-regulated phosphoproteins (*i.e.* p-AKT, p-ERK1/2). We then determined that VO-OHpic inhibited the cell viability, cell proliferation and colony-forming ability of HCC cells in relation to PTEN levels.

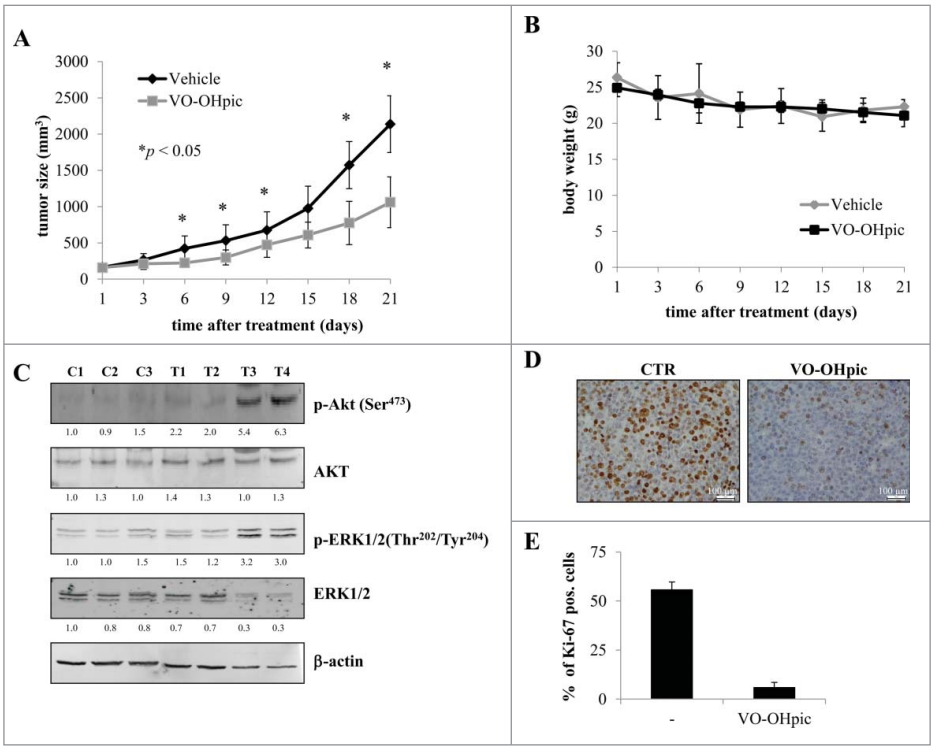

Figure 5. The effect of VO-OHpic on xenograft models of Hep3B cells. (A) Effect of VO-OHpic on tumor growth. Once tumors were engrafted and palpable, mice (n=6) were treated daily (6 days/week) with VO-OHpic at 10 mg/kg, as described in Materials and Methods. The curve of tumor growth was compared with that of control mice treated with vehicle alone, $^{*}p < 0.05$. (B) Body weight alteration analysis. Mice were weighed twice a week and the weights presented in the graphs. (C) Representative Western blotting showing phospho-AKT, AKT, phospho-ERK1/2 and ERK1/2 levels of three mice treated with vehicle alone (control; C1, C2 and C3) and four mice treated with VO-OHpic (T1, T2, T3 and T4). The numbers represent the ratio of the relevant protein normalized with β-actin, with vehicle-treated control sample C1 arbitrarily set at 1.0. (D) Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tumor tissues. Tissues from the control mice and mice treated with VO-OHpic were stained for Ki-67 proliferation index (20 x magnification). (E) Data are expressed as the number of positive cells and are the means $\pm$ SD of five fields in three tumor sections from mice treated with vehicle alone and in four tumor sections from mice treated with VO-OHpic.

Although some reports have reported that VO-OHpic is a specific and potent inhibitor of PTEN,[21,25-29] others have raised concerns about its specificity.[30] In particular, Spinelli *et al.*, have shown that besides PTEN other phosphatases, such as Src homology region 2 domain-containing phosphatase-1 (SHP1), encoded by *protein tyrosine phosphatase non-receptor type 6* (*PTPN6*) gene, may be inhibited by VO-OHpic at least as potently as PTEN.[30] Our results demonstrated that inhibition of other phosphatases, such as SHP1, SHP2 and PTP1B, by NSC87877 and PTPi I inhibitors, has no effect on HCC cell viability, even at the highest drug concentrations tested for each inhibitor.

Treatment with VO-OHpic has recently been demonstrated to specifically and differentially induce senescence in PTEN$^{+/-}$ prostate tumors, with no deleterious effect on PTEN wild-type cells.[21] This senescence response is referred to as PTEN-loss-induced senescence (PICS). Although it is clear that PICS has a central role in blocking tumor progression in prostate tumorigenesis,[21] the extent to which PICS might restrict the development of other tumors is not completely clear. Cellular senescence is a complex process that can be triggered in a number of ways. We demonstrated that VO-OHpic treatment induced cellular senescence, but only in HCC cells with low endogenous PTEN levels. Furthermore, treatment with VO-OHpic induced cell cycle arrest and induced expression of senescence-associated secretory phenotype (SASP) pro-inflammatory cytokine and protease mRNAs, IL8 and MMP9 respectively.

Interestingly, PTEN-negative SNU475 cells, which in basal conditions already show a moderate number of SA-β-gal positive cells, failed to show further induction of senescence after VO-OHpic treatment. These results are in agreement with those obtained by Alimonti *et al.* in which treatment with VO-OHpic at 500 nM did not induce further senescence in PTEN negative MEF cells, suggesting that the effect of VO-OHpic is dependent on PTEN expression levels.[21]

However, targeting PTEN might seems a contradiction owing its role as tumor suppressor, acting as a negative regulator of the PI3K/AKT/mTOR pro-survival pathway. However, Chen *et al.* demonstrated that complete acute loss of *PTEN* did not give a proliferative advantage as would be expected, but instead promoted a strong senescence response that opposes tumor progression.[12] In addition, Alimonti *et al.* provide evidence in support of the idea that, at least in the context of low PTEN expression, further inactivation of PTEN can suppress, rather than promote, tumorigenesis.[21] On the other hand, others have shown that overexpression of PTEN or inhibition of PI3K promotes senescence response.[31] On the bases of these observations Pandolfi's group postulated the so called "continuum model of tumor suppression," in which both complete loss ("no dose") or overexpression ("high dose") of the tumor suppressor PTEN promote senescence, which can be also induced upon pharmacological inhibition (such as inhibition with VO-OHpic) in cells expressing "low dose" (30–50% of the normal dose present in WT cells) of PTEN.[32,33]

It is well established that in various tumor types the tumor suppressor p53 is essential in inducing cell cycle arrest, apoptosis and senescence in response to various stress signals.[34] However, emerging evidence suggests that cellular senescence can also be triggered in a p53-independent manner.[35,36] In our model, the Hep3B cell line had mutated *p53* genes, therefore VO-OHpic treatment-induced senescence was independent of p53 gene function. It is plausible that other molecular alterations might influence the effect of PTEN inhibition in HCC cells.

PTEN activity is already known to be critically involved in the regulation of AKT activity, but recent data suggest that PTEN may also regulate the ERK1/2 pathway.[11,23,24,37,38] We demonstrated that VO-OHpic treatment induced a significant increase in activation of both pathways. Paradoxically, several lines of evidence indicate that over-activation of AKT and ERK signaling pathways, which are considered oncogenic signaling pathways, might induces growth arrest and cellular senescence response.[11,13,14,39-43] Therefore, we hypothesize that activation of AKT and ERK as observed during VO-OHpic treatment might, if protracted, ultimately activate cellular senescence. Importantly, we also confirmed the effects of activation of these pathways by VO-OHpic in *in vivo* experiments. In the Hep3B mouse xenograft model, PTEN inhibition by VO-OHpic strongly suppressed tumor growth, and this was associated with an increase in p-AKT and p-ERK1/2 levels, together with reduced levels of the cell proliferation.

Activation of AKT and ERK1/2 pathways is also important in drug resistance. HCC is a cancer type which is difficult to treat and a complex disease which requires interacting approaches for therapy to be effective. To date, the multikinase inhibitor, sorafenib, is the only drug approved to treat patients with advanced HCC.[3] Several promising novel anticancer agents are currently under investigation in phase II and III clinical trials for the treatment of HCC.[4,5] For example, AKT and MEK inhibitors have shown promising effects for HCC treatment both in *in vitro* and *in vivo* HCC models.[44-48] However, most of these drugs have failed as single agents in clinical trials conducted on HCC patients.[4-6] Hence, a multi-targeting-based approach using a rational combination of different drugs is of particular relevance in HCC treatment.

The observation that treatment with VO-OHpic activates the AKT and ERK signaling pathways prompted us to investigate whether inhibition of PTEN activity may control the sensitivity of Hep3B cells to various targeted therapies. We demonstrated synergistic antitumor effects when VO-OHpic was combined with PI3K/mTOR pathway inhibitor (*i.e.*, NVP-BEZ235) and a MEK pathway inhibitor (*i.e.* U0126). Given that the Raf/MEK/ERK inhibitor sorafenib is the standard of care in the first-line setting for advanced HCC patients, the new agents and new drug combinations must be compared head-to-head with sorafenib. Therefore, we also combined VO-OHpic with sorafenib, and a synergistic growth inhibitor effect was again observed. These data demonstrated that a combined targeted approach of PTEN inhibitor with PI3K/mTOR and RAF/MEK/ERK inhibitors may kill tumor cells more effectively and may allow the use of this type of therapy in HCC subclasses with a low PTEN expression.

In conclusion, both *in vitro* and *in vivo* experiments demonstrated the efficacy of the pro-senescence therapy based on the inhibition of PTEN phosphatase activity via VO-OHpic

treatment of HCC cells expressing low levels of PTEN. Our results lead us to propose the use of PTEN inhibitor VO-OHpic for the treatment of certain HCC subclasses with low PTEN expression levels.

Materials and methods

Cell lines, cell culture and reagents

The human hepatocellular carcinoma cell lines Hep3B, PLC/PRF/5, and SNU475 used in this study had a low passage number and were maintained in RPMI medium (SIGMA, Milan, Italy), containing 10% (v/v) Fetal Bovine Serum (FBS) (GIBCO, Life Technologies, Monza MB, Italy). Hep3B and SNU475 cell lines were obtained from the American Type Culture Collection (ATCC). PLC/PRF/5 cells used in this study were a gift from Prof. O. Bussolati (Unit of General and Clinical Pathology, Department of Experimental Medicine, University of Parma, Parma, Italy). All cell lines were authenticated by short tandem repeat (STR) profiling (BMR Genomics, Padova, Italy), and used within 6 months of receipt. All cultures were routinely tested and found to be free of mycoplasma contamination. VO-OHpic and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorafenib and U0126 were purchased from Alexis Biochemicals (San Diego, CA, USA). BEZ235 was purchased from Cayman Chemical (Ann Arbor, MI, USA). PTPi I and NSC87877 were purchased from Santa Cruz Biotechonogy (Dallas, TX, USA).

Cell viability assays

Cells (3×10^3/well) were distributed into each well of 96-well plates and then incubated overnight. At time 0, the medium was replaced with fresh complete medium and various doses of compounds were added. For combined treatment, cells were treated with VO-OHpic and with target specific inhibitors for the indicated time. At the end of treatments, MTS assays were performed as previously described.[48] For the synergistic activity, the data were analyzed using CalcuSyn software version 2.0 (Biosoft, Cambridge, UK) to determine if the combination of VO-OHpic and PI3K/Akt/mTOR and Raf/MEK/ERK inhibitors was additive or synergistic. When CI = 1, effects were additive. When CI < 1.0, effects were synergistic. CI < 0.1 indicates very strong synergism as defined by the CalcuSyn manual.

BrdU incorporation assays

Cell proliferation was determined by estimating the amount of bromodeoxyuridine (BrdU) incorporation into DNA by a colorimetric immunoassay (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. In brief, 3×10^3 cells were cultured in 96-well plates with varying concentrations of VO-OHpic for 72 hours. BrdU was added 24 hours before the end of the treatments. Results were expressed as the percentage inhibition of BrdU incorporation over the control. Values were expressed as means ± SD of three separate experiments, each performed in triplicate.

Colony formation assays

The effect of different inhibitor concentrations on cell growth was also assessed using a clonogenic assay. For this analysis, 500–750 cells were plated in 6-well plates in growth medium, and after overnight attachment cells were exposed to VO-OHpic for 48 hours. Medium with or without VO-OHpic was replaced every 48 hours. The Hep3B and PLC/PRF/5 cells were grown for 14 days and SNU475 cells for 10 days. At the end of treatments, colonies were stained and counted as previously described.[48] Experiments were performed in duplicate and repeated twice.

Western blot analysis

Whole cell lysates were obtained using RIPA buffer (Cell Signaling Technologies Inc., Beverly, MA, USA) and Western blots were performed using the methodology for the Odyssey® infrared imaging system (LI-COR Biosciences, NE, USA) as previously described.[48] Antibody signals were analyzed as integrated intensities of regions defined around the bands of interest in either channel, with primary antibodies raised against β-actin (SIGMA), phospho-AKT, AKT, phospho-ERK1/2, ERK1/2, phospho-mTOR, mTOR, PARP and PTEN (Cell Signaling Technologies).

Senescence detection assay

Cells (1×10^3) were grown on 8-well chamber slides. At time 0, the medium was replaced with fresh complete medium and compounds were added. Five days after the treatment, senescent cells were identified by an SA-β-gal assay performed as previously described.[49]

Extraction of cellular RNA and real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Monza, MB, Italy) according to the manufacturer's instructions.1.5 μg of total RNA were subjected to reverse transcription to generate cDNA. Expression of selected genes was quantified by quantitative SYBR Green fluorescence Real-Time PCR (Qiagen, Milan, Italy) using the StepOnePlus (Applied Biosystems, Carlsbad, CA, USA). QuantiTect Primer assays for *IL8* (QT00000322), *MMP9* (QT00040040), *p21* (QT00062090) and *p16* (QT00089964) were purchased from Qiagen and amplified as recommended. Relative expression was calculated using the comparative Ct method. Relative quantity of the gene of interest was calculated as ratio of drug-treated samples versus control (DMSO) and corrected by the quantified expression level of β-actin (QT00095431). The results shown were the mean ± SD of three experiments, each performed in triplicate.

Cell cycle analysis

Cells (10×10^3) were grown on 100 mm tissue culture dishes. After 3 days of treatment, cells were washed twice with ice-cold PBS and then resuspended at 1×10^6/ml in a hypotonic fluorochrome solution containing propidium iodide 50 μg/ml in 0.1% sodium citrate plus 0.03% Nonidet P-40. After 1 hour of

incubation in this solution, the samples were filtered through nylon cloth, 40 μm mesh, and their fluorescence was analyzed as single-parameter frequency histograms using a FACSort instrument (Becton Dickinson, Montain View, CA, USA). The data were analyzed with CellQuest software (Becton Dickinson).

In vivo studies

Male nude athymic mice (Fox1 nu/nu) aged 4 weeks were obtained from Harlan (Udine, Italy) and allowed to acclimatize for 1 week. Suspensions of 10×10^6 Hep3B cells in 0.2 ml of PBS were inoculated into the right flank of the animal. When tumors became palpable (around 150 mm^3), the mice were randomly divided into two groups, with the various tumor volumes equally distributed between the two groups. One group was treated daily (6 days/week) with 10 mg/kg VO-OHpic suspended in DMSO, further diluted in a solution of 25% ethanol and administered via IP injection. The control group received the vehicle alone. Tumor volumes and body weight were recorded as previously described.[48] Tumors were then harvested: half of each tumor was frozen in liquid nitrogen and stored at −80°C for Western blot analyses, while the other half was fixed in formalin and used for immunohistochemistry analyses. All procedures were carried out according to institutional guidelines, which are in compliance with national (D.L., 116 G.U., Suppl.40; 18 February 1992) and international laws and policies (ECC Council Directive 86/609, OJ L358.1, 12 December 1987). This study was authorized by the Italian Ministry of Health (D.M. n. 39/2014-B).

Immunohistochemistry analyses

Immunohistochemical studies were performed as previously described.[48] To study Ki-67 expression ImmunoRatio® software was used (http://jvsmicroscope.uta.fi/immunoratio/). This software calculates the percentage of positively-stained area (DAB-stained area) divided by total nuclear area, using a color deconvolution algorithm for separating the staining components (diaminobenzidine and hematoxylin) and adaptive thresholding for nuclear area segmentation. Statistical analysis was performed using Student's two-tailed t test. The criterion for statistical significance was $p < 0.05$.

Abbreviations

ERK	extracellular signal-regulated kinase
HCC	Hepatocellular Carcinoma
mTOR	mammalian target of rapamycin
PI3K	phosphatidylinositol-3-kinase
PICS	PTEN-Induced Cellular Senescence
PTEN	Phosphatase and tensin homolog
SA-β-GAL	senescence-associated β-galactosidase
SHP1	Src homology region 2 domain-containing phosphatase-1

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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