

RESEARCH PAPER



Vitamin C in combination with inhibition of mutant IDH1 synergistically activates TET enzymes and epigenetically modulates gene silencing in colon cancer cells

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ABSTRACT

Mutations in the enzyme isocitrate dehydrogenase 1 (IDH1) lead to metabolic alterations and a sustained formation of 2-hydroxyglutarate (2-HG). 2-HG is an oncometabolite as it inhibits the activity of α -ketoglutarate-dependent dioxygenases such as ten-eleven translocation (TET) enzymes. Inhibitors of mutant IDH enzymes, like ML309, are currently tested in order to lower the levels of 2-HG. Vitamin C (VC) is an inducer of TET enzymes. To test a new therapeutic avenue of synergistic effects, the anti-neoplastic activity of inhibition of mutant IDH1 via ML309 in the presence of VC was investigated in the colon cancer cell line HCT116 *IDH1*^{R132H/+} (harbouring a mutated *IDH1* allele) and the parental cells HCT116 *IDH1*^{+/+} (wild type *IDH1*). Measurement of the oncometabolite indicated a 56-fold higher content of 2-HG in mutated cells compared to wild type cells. A significant reduction of 2-HG was observed in mutated cells after treatment with ML309, whereas VC produced only minimally changes of the oncometabolite. However, combinatorial treatment with both, ML309 and VC, in mutated cells induced pronounced reduction of 2-HG leading to levels comparable to those in wild type cells. The decreased level of 2-HG in mutated cells after combinatorial treatment was accompanied by an enhanced global DNA hydroxymethylation and an increased gene expression of certain tumour suppressors. Moreover, mutated cells showed an increased percentage of apoptotic cells after treatment with non-cytotoxic concentrations of ML309 and VC. These results suggest that combinatorial therapy is of interest for further investigation to rescue TET activity and treatment of *IDH1/2* mutated cancers.

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Introduction

Initiation and progression of cancerous lesions arise not only from genetic mutations but also from aberrant epigenetic mechanisms such as DNA methylation. Here, failure to maintain a balance between DNA hypomethylation and hypermethylation gives rise to subsequent pathological processes in the cell. While genome-wide DNA hypomethylation is associated with chromosomal instability, aberrant DNA hypermethylation in CpG islands of gene promoter regions can lead to gene silencing [1–3]. This, in turn, can facilitate tumorigenesis and cancer progression due to decreased expression of, e.g., tumour suppressor genes and disruption of cell cycle regulation, apoptosis, and/or DNA repair.

Aberrant DNA hypermethylation has been associated with several types of cancer, including gliomas, acute myeloid leukaemia (AML) and cancers of the lung, breast, ovaries, and colon [4–6]. Members of the ten-eleven translocation (TET) dioxygenase family are capable of oxidizing methylated 2'-deoxycytidines to hydroxymethylated 2'-deoxycytidines – e.g., 5-methyl-2'-deoxycytidine (5-mdC) to 5-hydroxymethyl-2'-deoxycytidine (5-hmdC) [7] – a known mechanism of active DNA demethylation. TETs can further oxidize 5-hmdC to 5-formyl-2'-deoxycytidine (5-fdC) and 5-carboxy-2'-deoxycytidine (5-cadC), which are eventually replaced by unmodified 2'-deoxycytidines as a result of thymine-DNA glycosylase (TDG)-mediated base excision repair [8–10]. This cascade of 5-mdC oxidation by TETs might represent an active gene demethylation mechanism leading to

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 Supplementary data for this article can be accessed [here](#).

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transcriptional reactivation. In addition to its role as a demethylation intermediate, 5-hmdC has unique epigenetic properties that result in specific gene expression profiles [11,12]. Indeed, a genome-wide decrease in 5-hmdC levels is considered an epigenetic hallmark in many cancers [13,14] and several studies have highlighted the diagnostic and prognostic value of this mechanism.

From a therapeutic point of view, a limited number of known TET enzyme activators can significantly increase genome-wide 5-hmdC levels. Vitamin C (VC) is one of the best-known substrates of the TET enzymes as well as a potent antioxidant and reducing agent [15]. It has been proposed that VC is responsible for the restoration of TET enzyme catalytic activity through the reduction of Fe(III) to Fe(II) [16,17]. This hypothesis has, however, been contradicted by other studies in which other strong reducing agents were unable to enhance the TET-mediated hydroxylation of 5-mdC [18–20]. The mechanism by which VC contributes to increased levels of TET-mediated hydroxylation of 5-mdC is, therefore, presently unclear.

Aberrant DNA hypermethylation has been identified in AML and gliomas, as well as in colorectal cancer and was linked to decreased TET enzyme activity due to mutations in the isocitrate dehydrogenase (IDH) family, specifically *IDH1* or *IDH2* [4,21,22]. Mutations at the codon 132 of *IDH1* result in replacement of an arginine by a histidine (*IDH1*^{R132H}) and are present in >70% of adult WHO grade II/III gliomas and in >80% of secondary glioblastoma multiforme (GBM), a highly aggressive brain tumour [23–25]. In gliomas, the *IDH1*^{R132H} mutation is the most frequent, though other types of cancer causing base substitutions in *IDH1* and *IDH2* have also been reported [25,26]. Colorectal cancers harbour an *IDH1* mutation exclusively accompanied by the CpG island methylator phenotype (CIMP), which is characterized by aberrantly coordinated DNA hypermethylation of CpG dinucleotides in a subset of promoters of genes silenced during tumorigenesis [27].

The switch of amino acids at the active site of IDH enzymes is crucial for the enzyme's function. The *IDH1*^{R132H} substitution not only disrupts physiological oxidative decarboxylation of isocitrate to α -ketoglutarate as performed by the wild type enzyme, it can also irreversibly convert α -

ketoglutarate to the structurally similar oncometabolite 2-hydroxyglutarate (2-HG) in large amounts and as a consequence inhibit α -ketoglutarate-dependent dioxygenases such as the TET enzymes [28–31]. Indeed, a specific CpG island methylator phenotype (CIMP) in gliomas and colorectal cancers, that is characterized by DNA hypermethylation, has been associated with *IDH* mutations, which has led to several speculations that *IDH* mutations contribute to tumorigenesis via altered epigenetic regulation [27,32].

Additionally, a previous study demonstrated 'rewiring' of metabolic profiles in *IDH1* mutant (mutated *IDH1*) gliomas: the production of pyruvate from lactate, along with glutamate anaplerosis, was used to replenish the tricarboxylic acid (TCA) cycle and produce isocitrate and α -ketoglutarate, the latter of which serves as a substrate for mutant IDH enzymes to further generate 2-HG [33]. Concordantly, other work has shown >80% of 2-HG produced by mutant IDH enzymes in colorectal carcinoma cells derives from glutamine, which is converted to glutamate [34,35]. This is in contrast to *IDH1* wild type gliomas, which predominantly use pyruvate for lactate production and predominantly depend on glycolysis and acetate anaplerosis. The clear metabolic 'rewiring' exhibited by *IDH1* mutated gliomas has several other implications such as enzyme inhibition and epigenetically mediated silencing of genes encoding for metabolic enzymes [33].

For these tumours, *IDH* mutations have become a target for novel therapeutic approaches including the use of hypomethylating agents – i.e., DNMT inhibitors or enhancers of TET enzyme activity – and inhibitors of mutant IDH enzymes [36,37]. Although the hypomethylating agents like decitabine or 5-azacytidine were originally developed as cytostatic agents for leukaemia chemotherapy, their epigenetic properties have since been revealed to play an important role in their anti-cancer activity [38–42]. A multitude of new compounds for the treatment of tumours bearing mutated *IDH1* and *IDH2* are presently in clinical trials [25]. Innovative combinations of epigenetically active substances and chemotherapeutics have been shown to improve actions against solid tumours and leukaemia. However, combinations of mutant IDH inhibitors and compounds capable of reactivating DNA hydroxymethylation, such as VC (ascorbate), have

yet to be investigated as a novel therapeutic avenue in *IDH* mutated cancers. This newly discovered function of VC prompted us to determine whether additive or synergistic effects can be achieved when sub-toxic concentrations of the mutant *IDH1* inhibitor ML309 are used in combination with high-doses of VC. Investigations were conducted in mutated *IDH1* (HCT116 *IDH1*^{R132H/+}) and wild type colon cancer cells (HCT116 *IDH1*^{+/+}) and to assess whether such an epigenetic therapy has therapeutic application in cancers.

Due to the excessive generation of the oncometabolite 2-HG, mutant *IDH1* drives tumorigenesis and cancer progression by impairing the efficacy of epigenetically active TET enzymes, promoting aberrant DNA methylation patterns. Targeting one of the most frequent cancer-associated mutant alleles, *IDH1*^{R132H}, may be a promising therapeutic approach. The aim of this work was, therefore, to investigate TET-mediated DNA hydroxymethylation and how it is affected by the repercussions of mutated *IDH1*. As such, this work aims to contribute to the identification of mechanisms capable of mitigating the carcinogenic effects of the *IDH* mutations frequently found in a variety of cancer types, including colorectal cancer and brain tumours.

Results

2-HG levels in *IDH1*^{R132H/+} cells decrease upon treatment with ML309 and VC

IDH1 mutations are associated with altered *IDH1* enzyme function which induces the overproduction

of the neomorphic metabolite 2-HG. In order to validate the increased abundance of 2-HG in HCT116 *IDH1*^{R132H/+} cells compared to HCT116 *IDH1*^{+/+} cells, 2-HG content was analysed by LC-MS/MS analysis (Figure 1(a)). Indeed, the intracellular concentration of 2-HG was 56-fold higher in *IDH1*^{R132H/+} cells (8.5 nmol/mg protein) compared to that in parental wild type cells (0.15 nmol/mg protein). Mutant *IDH1* inhibition by 10 μ M ML309 significantly reduced 2-HG levels in HCT116 *IDH1*^{R132H/+} cells. However, the level of 2-HG in wild type cells was not achieved (Figure 1(b)). VC (1 mM) on its own reduced 2-HG levels only marginally in *IDH1* mutated cells, but combinational treatment with ML309 and VC decreased 2-HG levels to those found in *IDH1*^{+/+} cells.

Genome-wide DNA hydroxymethylation is decreased in *IDH1*^{R132H/+} cells

As 2-HG inhibits TET activity, we aimed to assess whether elevated 2-HG levels in mutant cells are accompanied by a lower DNA hydroxymethylation status. Indeed, the 5-hmdC/dC levels were more than 50% lower in *IDH1*^{R132H/+} cells as compared to *IDH1*^{+/+} cells (0.004% hmdC/dC and 0.008% hmdC/dC, respectively) (Figure 2(a)). It should be mentioned that the genome wide DNA methylation was not affected in *IDH1*^{R132H/+} cells. As shown in Figure 2(b), 5 -mdC/dC levels were similar in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells, both cell types contained approximately 5% mdC/dC.

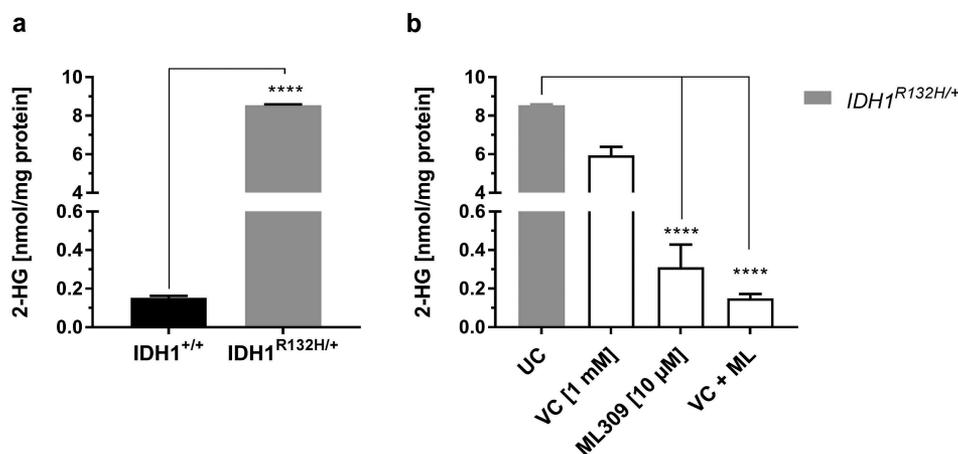


Figure 1. Measurement of 2-HG levels in HCT116 wild type *IDH1* (*IDH1*^{+/+}) and mutant *IDH1* (*IDH1*^{R132H/+}) cells. (a) Comparison of 2-HG levels in untreated *IDH1*^{+/+} and *IDH1*^{R132H/+} (mean \pm SD; **** $p < 0.0001$; $n = 3$), (b) Influence of VC (1 mM) and ML309 (10 μ M) treatment on 2-HG levels in *IDH1*^{R132H/+} cells after 48 h (mean \pm SD; **** $p < 0.0001$; $n = 3$)

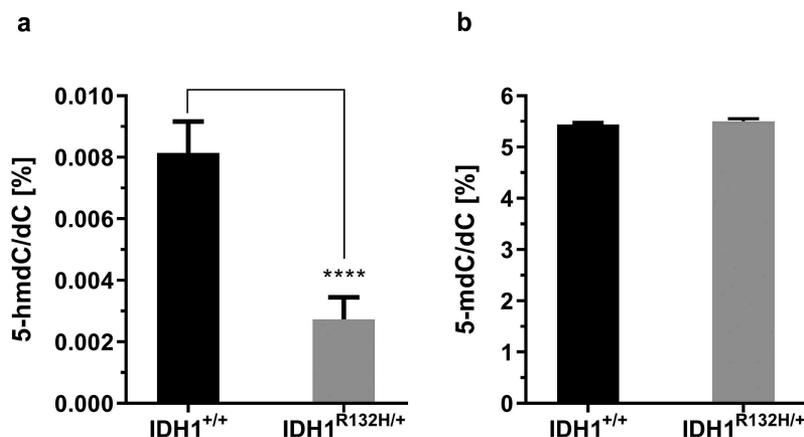


Figure 2. Genomic 5-hydroxymethyl-2'-deoxycytidine (5-hmdC) and 5-methyl-2'-deoxycytidine (5-mdC) levels in HCT116 wild type *IDH1* (*IDH1*^{+/+}) and mutant *IDH1* (*IDH1*^{R132H/+}) cells.

(a) 5-hmdC/dC levels in untreated *IDH1*^{+/+} and *IDH1*^{R132H/+} (mean \pm SD; *****p* < 0.0001; *n* = 3), (b) The 5-mdC/dC levels in *IDH1*^{+/+} and *IDH1*^{R132H/+} (mean \pm SD; *n* = 3)

VC increases genome-wide DNA hydroxymethylation in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells

VC is known to activate TET dioxygenases and increase global DNA hydroxymethylation. Therefore, 5-hmdC/dC levels were measured in HCT116 *IDH1*^{+/+} and *IDH1*^{R132H/+} cells in response to VC via isotope-dilution LC-MS/MS. The *IDH1*^{+/+} cells exhibited increased 5-hmdC/dC levels after 48 h treatment with VC in a concentration-dependent manner. Differences were significant at 10 μ M VC and reached maximal

at 1 mM VC (Figure 3(a)), which is consistent with a previous report [36]. However, in *IDH1*^{R132H/+} cells VC incubation only slightly increased 5-hmdC/dC levels, but not in a significant manner. To evaluate whether VC has an impact on the DNA methylation status of HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} cells, 5-mdC/dC ratios were plotted against increasing concentrations of VC. As it is evident from supplementary Figure S2, VC did not contribute to an altered DNA methylation status neither in mutated nor in wild type cells.

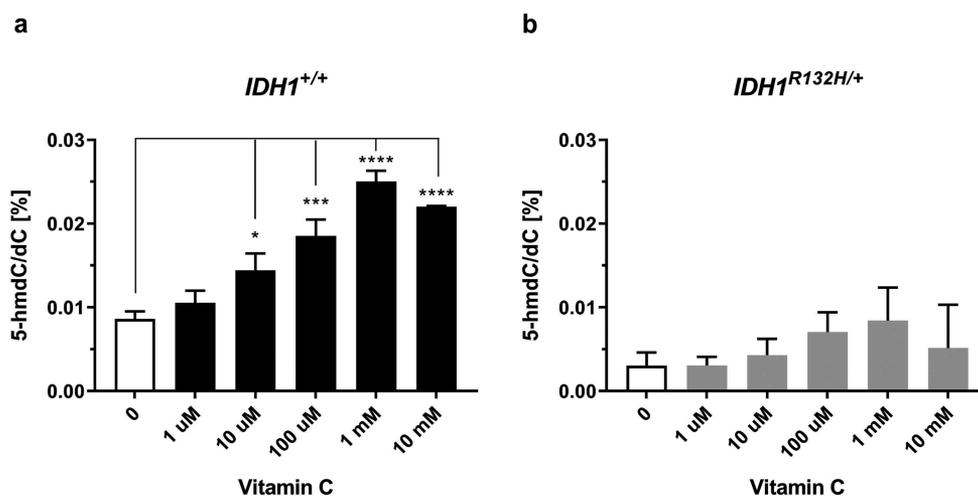


Figure 3. Impact of VC on genomic 5-hmdC/dC levels in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells.

(a,b) Quantification of 5-hmdC/dC levels in *IDH1*^{+/+} and *IDH1*^{R132H/+} treated for 48 h with increasing concentrations of VC as indicated (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 – significant results; error bars = SD; *n* = 3).

Combinatorial treatment of ML309 and VC increases the genome-wide DNA hydroxymethylation

As 2-HG is highly overproduced in *IDH1* mutated cells, which is accompanied by an inhibition of TET enzymes and reduced 5-hmdC/dC levels, it can be assumed that inhibition of mutant *IDH1* will restore TET dioxygenase activity and increase 5-hmdC/dC levels. Surprisingly however, treatment of *IDH1*^{R132H/+} cells with the mutant *IDH1* inhibitor ML309, although reducing 2-HG contents, but not to the level of wild type cells, did not significantly alter 5-hmdC/dC levels (Figure 4). Therefore, it was of major interest to determine whether co-incubation of both compounds, VC and ML309, improved the epigenetic outcome with regards to genomic 5-hmdC/dC levels. Remarkably, the combination of both compounds synergistically increased the DNA hydroxymethylation in *IDH1*^{R132H/+} cells. After co-incubation of 1 mM VC with 1 or 10 μ M ML309, we found significantly enhanced 5-hmdC/dC levels. In the presence of 1 mM VC a significant increase relative to matched ML309 only treated cells was seen ($p < 0.001$ for 1 μ M ML309 and $p < 0.01$ for 10 μ M ML309) (Figure 4(b)).

In contrast, VC and ML309 combinations had no impact on the DNA methylation status of HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} cells. As shown in Figure S2, 5-mdC/dC ratios were plotted against increasing concentrations of ML309, but did not contribute to an altered DNA methylation status in wild type or mutant *IDH1* harbouring cells.

VC and ML309 induce significant changes in mRNA profile of *IDH1*^{+/+} and *IDH1*^{R132H/+} cells

To examine whether the increased genome-wide 5-hmdC levels in response to VC and ML309 led to a direct impact on the mRNA expression of epigenetically regulated genes, differential gene expression of 25 specific target genes was analysed. Indeed, the treatment with VC and ML309 alone or in combination had a significant impact on the expression of the analysed genes in both, mutated and wild type cells. Apparently, the treatment modulated the expression of genes involved in glucose uptake, apoptotic pathways and vitamin C uptake. Though we found certain similar expression changes, there were significant differences between HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} cells (Figure 5).

Both cell lines showed a significant decrease of gene expression in *ALKBH5*, *DKK1*, *DNMT1*, *DNMT3B*, *GLUT1*, *GLUT3*, *NOXA* and *TET2* after combined treatment with VC and ML309. Interestingly, in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells, the treatment with VC and ML309 led to an increased expression of *BAD*, which is part of the p53 signalling pathway. This gene can be activated in part by the 14-3-3 protein, whose gene expression was also up-regulated in both cell types. Accordingly, we found an increase in transcripts of down-stream gene products like *p16*, as well. This leads to the assumption that in *IDH1*^{R132H/+} cells, at least in part, the induction of apoptosis and cell cycle arrest may be associated to the combinatorial treatment of ML309 and VC via

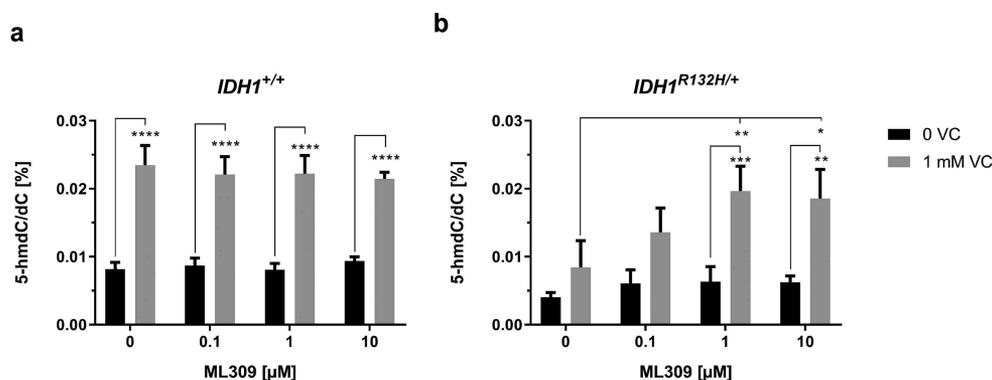


Figure 4. Impact of ML309 and VC on genomic 5-hmdC/dC levels in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells. (a,b) Quantification of 5-hmdC/dC levels in *IDH1*^{+/+} and *IDH1*^{R132H/+} treated for 48 h with increasing concentrations of ML309 alone or in combination with vitamin C (1 mM) as indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ – significant results; error bars = SD; $n = 3$).

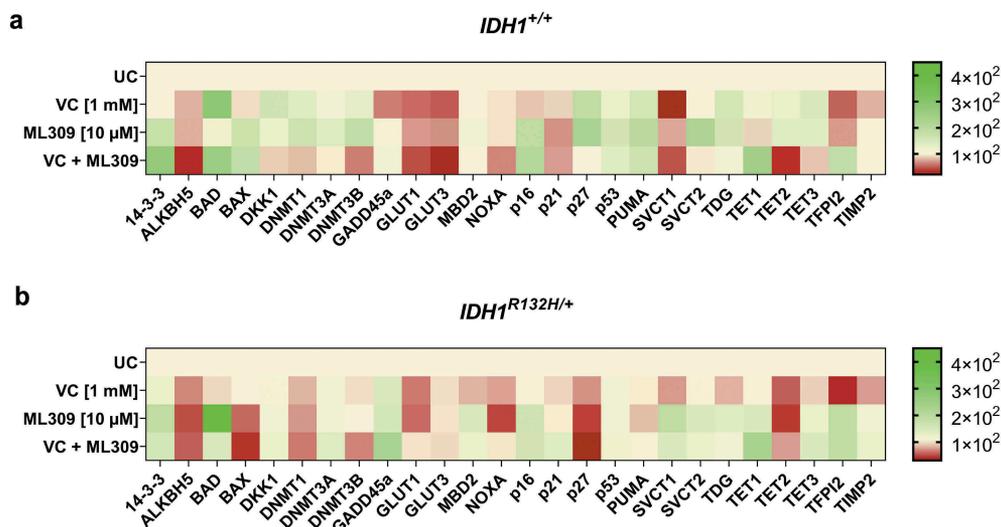


Figure 5. Gene expression profile of 25 genes after single or combinatorial treatment with VC and ML309 for 48 h in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells.

Heat map of relative mRNA-levels of the differential expressed genes in (a) *IDH1*^{+/+} and (b) *IDH1*^{R132H/+} cells after treatment with VC (1 mM), ML309 (10 μM) and combinatorial treatment. (relative to the HMBS housekeeper gene, using the $2^{\Delta\Delta CT}$ method)

the *GADD45a* pathway. Indeed, mutated cells showed an increased *GADD45a* mRNA expression. Frequently, cancer cells show a high expression of glucose transporters *GLUT1* and *GLUT3* due to the metabolic changes and high demands of energy through neoplastic transformation [43–45]. In *IDH1*^{+/+} cells, *GLUT1* and *GLUT3* were significantly reduced upon treatment with VC, whereas in *IDH1*^{R132H/+} cells also a reduction, though to a smaller extent, was detected. As overexpression of these transporters is associated with poor survival in several cancers [46], we suggest a positive outcome of high-dose VC treatment as therapeutically avenue in *IDH1*^{+/+} and *IDH1*^{R132H/+} cancer cells. Further, several genes involved in epigenetic regulations showed a different mRNA profile after treatment with VC and ML309. The combinatorial treatment led to a *DNMT1* and *DNMT3B* down-regulation in both cell types. As one could speculate, that the increase of 5-hmdC/dC levels after treatment with VC was due to an increase of *TET* gene expression, we measured the differential expression, as well. The combinatorial treatment led to an increase of *TET1* mRNA expression in mutated and wild type cells. In contrast, *TET2* mRNA expression was down-regulated after treatment with VC and ML309. The controversial regulation does not explain the increase of 5-hmdC/dC levels in the cells. As the expression analysis of

the selected genes indicated a differential regulation in response to VC and ML309, it was of interest to examine whether this gene regulation influences cell growth and apoptosis.

Enhanced cell death by VC in pharmacological concentrations in combination with ML309

The cytotoxic potential of increasing concentrations of mutant *IDH1* inhibitor ML309 with or without VC (1 mM) on HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} was evaluated by means of MTT reduction assay (Figure 6). Upon the 24 h treatment with increasing concentrations of ML309, mutated and wild type *IDH1* cells became less viable. Mutant cells exhibited a higher response rate with a calculated IC₅₀ of approximately 45 μM and wild type cells did not have a relative viability lower than 55% after treatment with the highest tested concentration of 50 μM ML309. The co-incubation with 1 mM VC and ML309 led to a significant decrease of cell viability in mutant cells (IC₅₀: 19 μM) as well as in wild type cells (IC₅₀: 29 μM). These data indicate that co-administration of 1 mM VC was detrimental to cell viability at all ML309 concentrations for both tested cell lines. Overall, cell toxicity was more pronounced for *IDH1*^{R132H/+} compared to *IDH1*^{+/+} cells with all tested treatment strategies.

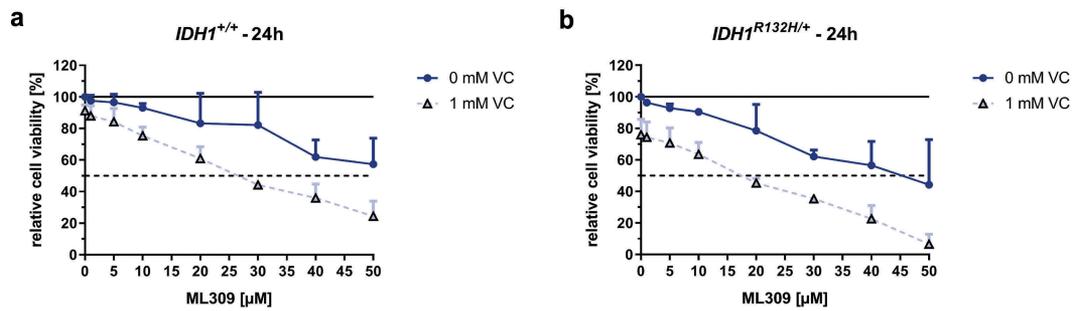


Figure 6. Effect of mutant IDH1 inhibitor ML309 and VC on cell viability of HCT116 $IDH1^{+/+}$ or $IDH1^{R132H/+}$.

HCT116 cells were exposed to increasing concentrations of ML309, as indicated. The incubation was carried out for 24 h in HCT116 $IDH1^{+/+}$ (a) or $IDH1^{R132H/+}$ (b) with ML309 alone or in combination with VC (1 mM), and cell viabilities assessed by MTT assay. Data are expressed as a percentage of the untreated control (100% are indicated as black line), viable cell levels <75% were taken to indicate cytotoxic induction (error bars = SD; n = 3), dotted line shows 50% viability for determination of IC50 values.

Apoptosis induction in $IDH1^{R132H/+}$ colon cancer cells upon treatment with ML309 and VC

For further elucidation, whether the single or combinatorial treatment with ML309 and VC was able to induce apoptosis in HCT116 $IDH1^{+/+}$ or $IDH1^{R132H/+}$ cells, selected treatments were analysed by flow cytometry after staining with Annexin V and propidium iodide (Figure 7). A significant induction of

total apoptosis relative to untreated cells was only evident in HCT116 $IDH1^{R132H/+}$ cells. Stimulation with ML309 (10 μM) led to a significant increase of the apoptotic rate, which was further enhanced after combinatorial treatment with 1 mM VC (Figure 7). Interestingly, the increased rate of apoptosis after combinatorial treatment was due to an enhanced amount of early apoptotic cells.

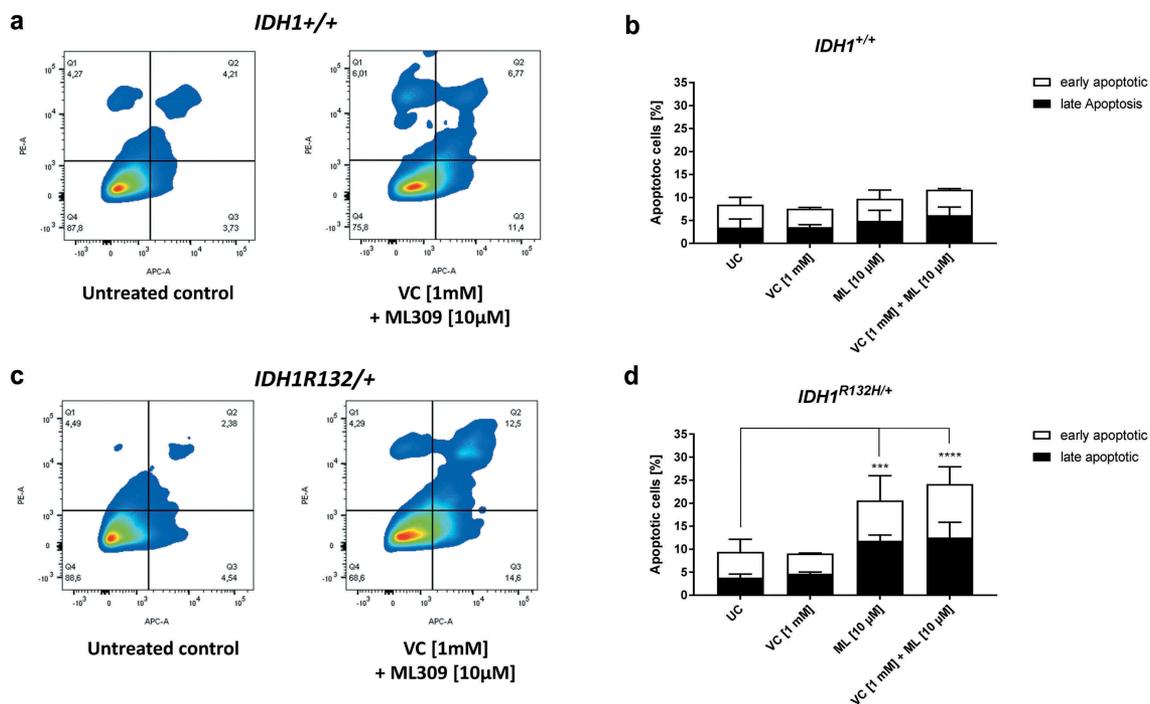


Figure 7. Measurement of apoptotic cells upon VC and ML309 incubation.

(a, c) Representative contour plots of Annexin V/propidium iodide staining in cells treated for 48 h with combination of VC and ML309, as indicated. Q1 – gates for necrotic cells, Q2 – for late apoptosis, Q3 – for early apoptosis and Q4 – for healthy cells. (b, d) Quantitative representation of early and late apoptotic cells after treatment with VC, ML309 or combined with indicated concentrations. (error bars = SD; n = 3; Statistical significance of the treated groups to the untreated control was calculated using 2-way ANOVA and Tukey post-test: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).

Discussion

In this study, we present the first attempt to investigate a synergistic effect of VC and mutant IDH1 inhibitors in *IDH1* mutated cancer cells. By combining the compounds, we aimed to reverse the epigenetic aberrations of mutated *IDH1* cancer cells by activating the TET enzymes with VC and simultaneously inhibit the neomorphic action of mutated IDH1 enzymes via the mutant IDH1 inhibitor ML309.

Aberrant epigenetic modifications are a hallmark of cancer progression, and restoring a balance between hyper- and hypomethylating events has emerged as a promising counteraction against neoplasia for therapeutic use. Originally, VC has attracted attention as a potential cancer therapy through potential induction of oxidative stress that selectively kills cancer cells [47,48]. VC has also been shown to act as a potent activator and possible co-factor of the TET dioxygenases, which mediate the oxidation of 5-mdC to 5-hmdC in genomic DNA, a possible therapeutic avenue for active demethylation in aberrantly hypermethylated cancer loci [18,49]. Notably, there is an underestimated prevalence of VC deficiencies in cancer patients [50–52], and pharmacologically active concentrations of up to 1 g/kg bw VC have been shown to significantly improve the outcomes of the epigenetic cancer therapies [50].

Cells harbouring *IDH1* mutations show a significant overproduction of the oncometabolite 2-HG, which is able to promote cellular transformation towards cancer. As this effect can be reversed by inhibition of the production of 2-HG, the development of specific compounds able to repress IDH activity is of great interest. Here, we investigated whether VC can enhance the anti-carcinogenic actions of the mutant IDH1 inhibitor ML309 in *IDH1* mutated cells, and whether this innovative combination of these compounds can be used as a model therapy option for cancers harbouring *IDH1* mutations. Although our studies were only performed with the human colorectal carcinoma cell line HCT116 with a heterozygous knock-in of IDH1 dominant-negative (R132H) point mutation, a similar outcome can be speculated in other *IDH1* mutated cancer cells.

Mutations in *IDH1* and *IDH2* are frequently observed in various cancers ranging from solid cancers like glioblastomas, chondrosarcoma or colorectal cancers and in different forms of leukaemia like AML [25]. Unusually to other cancer-related aberrations, the most common mutation in *IDH1* and the less common mutation in *IDH2* confer a neomorphic gain-of-function. One prevalent *IDH1* mutation involves a histidine substitution for an arginine (*IDH1*^{R132H/+}), which leads to an aberrantly working IDH1 enzyme and to the overproduction of the oncometabolite 2-HG. Especially high-grade gliomas show a high prevalence of mutated *IDH1* or *IDH2* genes of up to 90%, whereas *IDH1* is most often mutated. Even in up to 30% of AML leukaemia *IDH1/2* mutations are found [29]. Certain subclasses of colorectal cancers with a CpG island show *IDH1* mutations, whereas 2-HG can effectively inhibit the function of Fe(II)-dioxygenases like TET enzymes. One aim of the present study was to determine whether 2-HG production in *IDH1*^{R132H/+} HCT116 cells could be reduced by application of the mutant IDH1 inhibitor ML309. In addition, it was of interest whether TET reactivation mediated by VC could effectively rescue 5-hmdC/dC levels in *IDH1*^{R132H/+} HCT116 cells.

Firstly, the excessive production of 2-HG in *IDH1*^{R132H/+} cells was confirmed by a highly sensitive LC-ESI-MS/MS approach, revealing up 56-fold 2-HG content relative to HCT116 *IDH1*^{+/+} cells. As expected, this was also accompanied by a significant reduction of the 5-hmdC/dC levels in line with the binding of 2-HG to numerous class of Fe(II)-dependent dioxygenases, including the TET enzymes, and their subsequent inhibition. However, genome-wide DNA hypermethylation in *IDH1*^{R132H/+} cells was not observed. Similarly, treatment with ML309 or VC failed to evoke marked changes 5-mdC/dC levels. By contrast, the treatment with ML309 significantly reduced the 2-HG levels in the *IDH1*^{R132H/+} cells, where treatment with VC alone did not. VC is a potent activator of TET activity, as seen by the increased 5-hmdC/dC levels in *IDH1*^{+/+} cells. Although in *IDH1* mutant cells, the VC mediated increase of 5-hmdC/dC was not significant, we found a synergistical activation of TET activity after co-incubation with VC and ML309. In detail, the combinatorial treatment significantly put

the 5-hmdC/dC levels up to 0.02%, around 4 times higher than in untreated *IDH1*^{R132H/+} cells. This was surprising with regard to the currently presumed model of VC action on TET enzymes. Until now, it was not clear that VC can reactivate TET-enzymes in *IDH1*^{R132H/+} gain-of-function cells. Due to its structure, VC itself should be unsuitable to efficiently rescue 2-HG-mediated inhibition of TET activity. In contrast, 2-HG exhibits structural similarities to the TET cofactor α -ketoglutarate and thereby performs competitive inhibition of α -ketoglutarate-dependent oxygenases [53,54], including TET enzymes. Yet, it is unlikely that competitive displacement of 2-HG can be exerted by VC itself, the detailed mode of action needs to be further explored. The previously proposed idea by Monfort [17] and others was that VC exerts its positive effects on DNA hydroxymethylation by recycling Fe(II) for TET enzyme activity. In that case, VC might not be able to rescue DNA hydroxymethylation in cells that produce excessive amounts of 2-HG, which is a competitive antagonist of the TET cofactor α -ketoglutarate. Based on our findings, we suggest that in some way VC and ML309 act synergistically in order to mediate a displacement of 2-HG at the catalytically active site of TET and to reactivate its full function. This disparity of proposed scientific approaches punctuates the requirement for further investigation. In contrast to the combinatorial treatment, we found that incubation with increasing concentrations of mutant IDH1 inhibitor ML309 alone led to no significant increase of 5-hmdC/dC levels neither in *IDH1*^{R132H/+} mutant cells nor *IDH1*^{+/+} wild type cells. Here, the reduction of 2-HG levels was successful; however without increase of 5-hmdC/dC levels. This discrepancy can be explained by the fact that the mutant IDH1 inhibitor ML309 was not able to diminish 2-HG to those contents found in wild type cells. Further studies to investigate the mode of action of the mutant IDH1 inhibitor on the reactivation of TETs will be needed here.

In addition, we show that VC and ML309 alone, but especially the combination, induced a significant increase of apoptotic cells in *IDH1*^{R132H/+} cells. However, in *IDH1*^{+/+} cells, the treatment did not lead to any detectable apoptosis induction. This finding underlines the *IDH1* mutant-specific mode of action of the combinatorial treatment of these two

compounds, which was evident for the TET activity and 5-hmdC/dC increase, as well. Most interestingly, apoptosis induction was accompanied by significant gene activation of a subset of analysed genes. Here, we found that *GADD45a* was one of the highest re-expressed genes after combinatorial treatment. The *GADD45a* gene product is increased following stressful growth arrest conditions. The DNA damage-induced transcription of this gene is mediated by both p53-dependent and -independent mechanisms. Interestingly, in *IDH1*^{+/+} and *IDH1*^{R132H/+} cells, the treatment with VC and ML309 led to increases in the expression of *BAD*, which is part of the p53 signalling pathway. This gene can be activated in part by the 14-3-3 protein, whose gene expression was up-regulated in both cell types, as well. Accordingly, we found an increase in transcripts of down-stream gene products like *p16*, as well. This leads to the assumption that in *IDH1*^{R132H/+} cells, at least partly, the induction of apoptosis and cell cycle arrest may be associated to the combinatorial treatment of ML309 and VC via the *GADD45a* pathway.

Frequently, cancer cells show a high expression of glucose transporters *GLUT1* and *GLUT3* due to the metabolic changes and high demands of energy through neoplastic transformation [43,55,56]. In *IDH1*^{+/+} and *IDH1*^{R132H/+} cells, *GLUT1* and *GLUT3* levels were reduced upon treatment with VC. As overexpression of these transporters is associated with poor survival in several cancers [44], we purpose a positive outcome of high-dose VC treatment as therapeutical avenue in *IDH1*^{+/+} and *IDH1*^{R132H/+} cancer cells.

Further, genes involved in epigenetic regulations show a different mRNA profile between *IDH1*^{+/+} and *IDH1*^{R132H/+} cells. The combinatorial treatment led to a *DNMT1* and *DNMT3B* down-regulation in both cell types. As one could speculate that the increase of 5-hmdC/dC levels after treatment with VC was due to increase of *TET* gene expression, we measured the differential expression, as well. Surprisingly, *TET1* and *TET2* gene expressions were regulated in an opposite manner after treatment with VC and ML309. Therefore, the synergistic effect on the 5-hmdC/dC levels after combinatorial treatment in *IDH1*^{R132H/+} cells cannot be explained solely by *TET* expression. Further studies for the exact

mode of action by VC and ML309 on TET activity are needed.

It has been shown that *IDH1* mutated cancer cells confer certain liabilities of other enzymes, which may open different avenues of treatment [25,29,45]. This can lead to a decrease of resistance to specific therapies during the cancer treatment. As shown in this study, we found that the combinatorial treatment with VC and ML309, as one mutant *IDH1* inhibitor, can effectively induce apoptosis and gene expression of certain tumour suppressors. Therefore, it would be of great interest to further investigate the beneficial outcome of this treatment routine in mutated *IDH1* cancers *in vivo*.

Several studies have underlined the clinical potential of high-dose ascorbate in anticancer therapies [46]. However, due to further insights into the physiological mechanisms of VC, a combinatorial treatment with other epigenetic [36,57] or immunomodulatory [58,59] compounds can be recommended [60,61]. The benefit of co-treatment with VC and decitabine in cancer cells or with checkpoint inhibitors, such as CTLA4 or PD-L1 has been proved and even, radiotherapy [62] and chemotherapy [63,64] can be supported by high-dose VC co-treatment.

Conclusion

This study is the first to investigate the combination of the mutant *IDH1* inhibitor ML309 and high VC doses as a potential anti-cancer therapy. In summary, combinatorial treatment showed an important impact on the epigenetic landscape of *IDH1* mutant cancer cells that corresponded to significant increases of apoptotic cell population within these. Furthermore, the combination of both compounds led to significant increases in genome-wide 5-hmdC/dC levels and expression of previously down-regulated tumour suppressor genes, as well as a shift towards a more apoptotic state without significant cytotoxic induction. To the best of our knowledge, this is the first time a direct link between VC treatment and a decrease in genome-wide 2-HG levels has been observed. Although this study mainly concentrated on the HCT116 cell line with an introduced *IDH1* mutation, we can conclude that our results indicate anticancer therapies in *IDH1*^{R132H/+} mediated by non-toxic concentrations of ML309 are

capable of enhancing anti-cancer activities when administered in combination with high-dose VC. As such, our data suggest that therapeutic responses to low, non-toxic doses of ML309 can be improved by additional VC supplementation.

Materials and methods

Cell culture and treatment

For cell culture experiments the human colorectal carcinoma cell line HCT116 with a heterozygous knock-in of *IDH1* dominant-negative (R132H) point mutation was used. HCT116 *IDH1*^{+/+} (parental) and HCT116 *IDH1*^{R132H/+} knock-in human colorectal cell lines were generated by rAAV targeting technology GENESIS [65] and obtained from Horizon Discovery (Cambridge, United Kingdom) as frozen cultures.

For the cultivation of cells pre-warmed RPMI 1640 medium with GlutaMAX supplement (Thermo Fisher, Darmstadt, Germany), containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (growth medium) was used. The cells were maintained in 5% CO₂ at 37°C. The cells were allowed to reach 90% confluency before subcultivation. The cell lines were confirmed as negative for mycoplasma infection within 6 months prior to use.

VC and ML309 [66] were purchased from Sigma (Schnelldorf, Germany). HCT116 *IDH1*^{+/+} and HCT116 *IDH1*^{R132H/+} cells were treated, if not other stated, with 1 mM VC and increasing concentrations of ML309 (1–50 µM) for 24 h within the scope of a cell viability assay. The maximal tolerable doses of VC was determined in a previous study [36]. Based on the results of the cell viability assay the concentrations of ML309 for further experiments were chosen. For the apoptosis assay, the cells were treated with 10 µM ML309 and 1 mM VC. The gene expression analysis was conducted after treatment of the cells with selected concentrations of 1 mM VC and 10 µM ML309. In order to analyse the genome-wide 5-mdC/dC and 5-hmdC/dC levels, the cells were treated with increasing concentrations of VC (1 µM – 10 mM) and 0.1, 1 and 10 µM ML309 alone or in combination with 1 mM VC.

For all cell-based assessments, test compounds were dissolved and diluted in RPMI 1640 medium supplemented with 1% FBS and 1% penicillin/

streptomycin (test medium). Cells were treated with the respective test compounds in test medium 24 h after seeding. To ensure continuous stability of the test compounds throughout the experiments, the test medium was changed every 24 h with freshly dissolved or diluted test compounds.

Cytotoxicity assessment via MTT assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used to indirectly determine the cytotoxic potential of VC (1 mM) with increasing concentrations of ML309 (1 μ M to 50 μ M) in HCT116 *IDH1*^{+/+} and HCT116 *IDH1*^{R132H/+} cells. The reduction of yellow MTT to blue formazan via mitochondrial reductase is an effective indicator for metabolic activity. As this reaction can only proceed in metabolically active cells, the amount of generated blue dye correlates with the amount of viable cells. By exploiting this process, one can indirectly measure cell viability of proliferating cells via colorimetric measurement [67].

For the assessment of the cytotoxic potential of the test compounds, HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} cells were seeded in 96-well plates and incubated in test medium for 24 h at 37°C. Cells in unamended medium were used as vehicle control, and cells treated with 0.01% or 0.005% SDS were used as positive control. After the respective incubation times, test media were substituted for medium containing 0.5 mg/ml MTT for 4 h at 37°C. Following the substitution of MTT medium for DMSO, the plates were shaken at 350 rpm in darkness at room temperature for 20 min. Measurement of the samples' respective optical densities was performed at $\lambda = 540$ nm by means of a FLUOstar OPTIMA plate reader. The biologically relevant threshold of 80% cell viability was defined as the onset of cytotoxicity upon administration of test compounds.

Quantification of 2-hydroxyglutarate using liquid chromatography tandem-mass spectrometry (LC-MS/MS)

The quantification of 2-HG levels in HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} cells was conducted according to a previously described method [68]. Cell pellets were lysed in deionized water using

a Bead Ruptor 12 (Omni International, Kennesaw, USA). Protein content (by Bradford assay) and 2-HG were determined in the cell lysates. For 2-HG quantification, 5 nmol of 3-amino-4-hydroxybenzoic acid (AHBA) were added to lysate aliquots (50 μ l) as internal standard. Analyses were conducted with an Agilent 1260 Infinity LC system coupled to an Agilent 6490 triple quadrupole-mass spectrometer (both from Waldbronn, Germany) interfaced with an Agilent Jet Stream (AJS) electrospray ion source operating in the negative ion mode (ESI⁻). Chromatographic separation was carried out using an YMC-Triart C18 plus column (3 μ m, 3 \times 100 mm) equipped with a guard column of the same material. Water and methanol, both supplemented with 10 mM ammonium formate and 0.02% formic acid, were used as eluents. Cell lysate supernatants were injected into a mobile phase consisting of 100% water. 2-HG and AHBA were eluted from the column with an 8-min linear gradient to 70% methanol at a flow rate of 0.25 ml/min. 2-HG and AHBA eluted from the separation column at 3.8 and 6.5 min, respectively. The total run time for one analysis was 14 min, including re-equilibration of the LC system. The following ion source parameters were used: drying gas temperature = 225°C, drying gas flow = 15 l/min of nitrogen, sheath gas temperature = 350°C, sheath gas flow = 12 l/min of nitrogen, nebulizer pressure = 40 psi, capillary voltage = 4000 V, nozzle voltage = 500 V. The optimized ion funnel parameters were: high pressure RF voltage = 90 V and low pressure RF voltage = 60 V. 2-HG was quantified by external calibration in relation to the internal standard AHBA (2-HG range: 0–500 μ M, fixed AHBA: 50 μ M) using the multiple reaction monitoring (MRM) approach. The following mass transitions were recorded (optimized collision energies in parentheses): 2-HG: m/z 147.0 \rightarrow 129.1 (8 eV), m/z 147.0 \rightarrow 85.1 (12 eV), m/z 147.0 \rightarrow 56.9 (20 eV); AHBA: m/z 152.0 \rightarrow 107.9 (12 eV). The water loss, represented by m/z 147.0 \rightarrow 129.1 for 2-HG, was used for quantification. The dwell time for each of the four mass transitions recorded was 175 ms. The determined 2-HG amounts were normalized to the actual protein content of the cell pellet used for lysis.

Determination of genome-wide DNA methylation and hydroxymethylation using isotope-dilution liquid chromatography tandem-mass spectrometry (LC-MS/MS)

HCT116 *IDH1*^{+/+} and HCT116 *IDH1*^{R132H/+} cells were treated with VC and ML309 in different concentrations as indicated for 24 h. The genomic DNA was then isolated with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Samples of genomic DNA (ranging between 10 and 20 µg) were hydrolysed to 2'-deoxynucleosides following a protocol previously described [69]. [¹⁵N₂,¹³C₁]dC, 5-mdC-d₃ and 5-hmdC-d₃ (all from Toronto Research Chemicals, Toronto, Canada) were used as internal standards for quantification of dC, 5-mdC and 5-hmdC in DNA hydrolyzates by isotope-dilution LC-MS/MS as reported recently [36]. Briefly, analytes were chromatographically separated with an Agilent 1260 Infinity LC system coupled to an Agilent 6490 triple quadrupole mass spectrometer (both from Agilent Technologies, Waldbronn, Germany) that was operated in multiple reaction monitoring (MRM) mode for detection of 2'-deoxynucleoside analytes. Precursor ions were generated by positive mode electrospray ionization (ESI+). The following mass transitions were used for quantification (accordant internal standard in parentheses): dC: *m/z* 228.1 (231.1) → 112.0 (114.9); 5-mdC: *m/z* 242.1 (245.1) → 126.0 (129.0) and 5-hmdC: *m/z* 258.1 (261.1) → 142.0 (145.0).

Apoptosis assessment via flow cytometric analysis

Levels of apoptotic and dead cells were determined by flow cytometry using eBioscience™ Annexin V Apoptosis Detection Kit APC (Thermo Fisher, Darmstadt, Germany). The differentiation between late-apoptotic or necrotic and early-apoptotic cells was facilitated by staining the cells with the fluorescent dye propidium iodide and allophycocyanin (APC)-coupled Annexin V prior to flow cytometric analysis [70]. For this purpose, HCT116 *IDH1*^{+/+} or *IDH1*^{R132H/+} cells were seeded and treated with VC (1 mM) or ML309 (10 µM) alone and with combination of both for 48 h. Cells grown in unamended medium were used as vehicle control, and cells treated with TNFα and cycloheximide (20

ng/ml, 25 µg/ml, respectively) 24 h prior to cell harvest were used as positive control for the induction of apoptosis. The protein synthesis inhibitor cycloheximide was included in the positive control to induce pro-apoptotic effects of TNFα. Per run 10,000 events were counted and analysed on a FACSCanto II (BD Biosciences, Heidelberg, Germany). FlowJo software (Treestar, Ashland, USA) was used for data analysis.

RNA extraction and quantitative real-time PCR

Altered gene expression in treated or untreated HCT116 *IDH1*^{+/+} and HCT116 *IDH1*^{R132H/+} cells was analysed by means of quantitative real-time polymerase chain reaction (qPCR). The procedure included the treatment of cells, isolation of RNA, cDNA synthesis and qPCR, as previously described [36]. RNA extraction was performed with the RNA High Pure RNA Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The cDNA synthesis was then conducted using the RevertAid reverse transcriptase (Thermo Fisher, Darmstadt, Germany). Therefore, the synthesis of cDNA, 3 µg of isolated total RNA from treated and untreated HCT116 *IDH1*^{+/+} and HCT116 *IDH1*^{R132H/+} cells was brought to a volume of 11 µl with nuclease-free water, mixed with 1 µl oligo (dT)₁₈ primers and incubated at 65°C for 5 min. Nuclease-free water (1 µl), 5X Reaction Buffer (4 µl), 10 mM dNTP mix (2 µl) and RevertAid M-MuLV Reverse Transcriptase (1 µl) were added to each sample and gently mixed. Samples were incubated at 45°C for 1 h, followed by a 5-min heating period at 70°C and cooling off at 4°C. The qRT-PCR was performed using the Maxima SYBR Green qPCR Mix (ThermoFisher, Darmstadt, Germany) on a LightCycler 480 II Real-Time PCR system (Roche, Mannheim, Germany). Quantification was performed using the ΔΔ Ct method with hHMBS expression as an internal reference. Melt curve analysis confirmed that all the qRT-PCR products were generated in the form of double-stranded DNA. An overview of the analysed target genes is given in Table 1. Forward and reverse primers were optimized or verified for optimization and obtained from Eurofins Genomics (Hamburg, Germany).

Table 1. Primer sequences for the RT-qPCR.

Target gene	Primer sequence (5' – 3')
<i>14-3-3s</i>	forward: TAG GCG CTG TTC TTG CTC CAA reverse: ACC AGT GGT TAG GTG CGC TCA
<i>BAD</i>	forward: GAT CGG GCT TGG GGT GAG AC reverse: TCA TCT GTC TGC CGG GTC TG
<i>BAX</i>	forward: GAC ATT GGA CTT CCT CCG GG reverse: ACA GGG ACA TCA GTC GCT TC
<i>DKK1</i>	forward: GGG TCT TTG TCG CGA TGG TA reverse: CGG GTA CGG CTG GTA GTT G
<i>DNMT1</i>	forward: ACC TGG CTA AAG TCA AAT CC reverse: ATT CAC TTC CCG GTT GTA AG
<i>DNMT3A</i>	forward: ACT ACA TCA GCA AGC GCA AG reverse: CAT CCA CCA AGA CAC AAT GC
<i>DNMT3B</i>	forward: CCA GCT CTT ACC TTA CCA TC reverse: CAG ACA TAG CCT GTC GCT TG
<i>GADD45a</i>	forward: ACG ATC ACT GTC GGG GTG TA reverse: AAT GTG GAT TCG TCA CCA GCA
<i>GLUT1</i>	forward: CTG CTC ATC AAC CGC AAC reverse: CTT CTT CTC CCG CAT CAT CT
<i>GLUT3</i>	forward: CAG CGA GAC CCA GAG ATG reverse: TTG GAA AGA GCC GAT TGT AG
<i>HMBS</i>	forward: ACC AAG GAG CTT GAA CAT GC reverse: GAA AGA CAA CAG CAT CAT GAG
<i>ITGA4</i>	forward: GTT TTC CAG AGC CAA ATC CA reverse: GCC AGC CTT CCA CAT AAC AT
<i>NOXA</i>	forward: GCT GGA AGT CGA GTG TGC TA reverse: GGA GTC CCC TCA TGC AAG TT
<i>p16</i>	forward: GAG CAG CAT GGA GCC TTC reverse: CCT CCG ACC GTA ACT ATT CG
<i>p21</i>	forward: AGT GGA CAG CGA GCA GCT GA reverse: TAG AAA TCT GTC ATG CTG GTC TG
<i>p27</i>	forward: AAA CGT GCG AGT GTC TAA CGG GA reverse: CGC TTC CTT ATT CCT GCG CAT TG
<i>p53</i>	forward: TGT GGG ATG GGG TGA GAT TTC Reverse: CTG TTG GTC GGT GGG TTG
<i>PUMA</i>	forward: GAC CTC AAC GCA CAG TAC GA Reverse: TAA GGG CAG GAG TCC CAT GA
<i>SVCT1</i>	forward: TCA TCC TCC TCT CCC AGT ACC T reverse: AGA GCA GCC ACA CGG TCA
<i>SVCT2</i>	forward: TCT TTG TGC TTG GAT TTT CGA T reverse: ACG TTC AAC ACT TGA TCG ATT C
<i>TET1</i>	forward: GCT GCT GTC AGG GAA ATC AT reverse: ACC ATC ACA GCA GTT GGA CA
<i>TET2</i>	forward: CCA ATA GGA CAT GAT CCA GG reverse: TCT GGA TGA GCT CTC TCA GG
<i>TET3</i>	forward: TCG GAG ACA CCC TCT ACC AG reverse: CTT GCA GCC GTT GAA GTA CA
<i>TFPI2</i>	Forward: GCA CAT GCA CGT TTG CAA TC Reverse: CCA GAT GAA GCT ACT TGT ATG
<i>TIMP2</i>	forward: CCA GAA GAA GAG CCT GAA CCA reverse: GTC CAT CCA GAG GCA CTC ATC
<i>TRAIL</i>	forward: TGC GTG CTG ATC GTG ATC TT reverse: TCT TGG AGT CTT TCT AAC GAG C

Statistical analysis

Statistical differences were determined by multiple t-tests between untreated and treated groups, where p-values ≤ 0.05 were considered as significant. Differences in apoptosis induction were calculated by 2 way ANOVA analysis, where p-values ≤ 0.05 were considered as significant. All statistical analyses

were conducted using the software GraphPad Prism (GraphPad Software, Inc., La Jolla, USA).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author Contributions

CG and BK designed experiments; CG, FS and AB performed experiments; CG, FS, TH and AB analysed data; CG, FS, AB, TH and BK wrote the manuscript, CG and BK advised on experimental design and provided critical feedback, all authors reviewed the manuscript.

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