Death receptor pathway activation and increase of ROS production by the triple epigenetic inhibitor, UVI5008

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Running title: Mechanisms of UVI5008 epi-drug action

Keywords: epigenetic treatments, cancer, apoptosis, SIRT, HDAC

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ABSTRACT

Deregulation of the epigenome is recognized as cause of cancer and epigenetic factors are receiving major attention as therapeutic targets; yet the molecular mode of action of existing epi-drugs is largely elusive. Here, we report on the decryption of the mechanism of action of UVI5008, a novel epigenetic modifier, that inhibits histone deacetylases, sirtuins and DNA methyltransferases. UVI5008 highly efficiently induces cancer cell-selective death in a variety of models and exerts its activities in several human tumor xenografts and genetic mouse models of human breast cancer in vivo. Its anticancer activity involves independent activation of death receptors and ROS production. Importantly, UVI5008 action is not critically dependent on p53, Bcl-2 modifying factor (BMF), and/or TNF-related apoptosis-inducing ligand (TRAIL) as cell death is efficiently induced in cells mutated or deficient for these factors limiting the risk of drug resistance development and maximizing its application spectrum. The simultaneous modulation of multiple (epigenetic) targets promises to open new avenues with unanticipated potential against cancer.
INTRODUCTION

Cancer is a multistep process involving acquisition of unlimited replicative potential, self-sustained growth with loss of sensitivity towards apoptogenic and check-point controls (1). These aberrations are caused by a series of genetic events that involve proto-oncogene activation, tumor suppressor gene (TSG) inactivation and senescence escape. Studies on leukemogenesis have revealed the intimate relationship between initial somatic mutations, often a chromosomal translocation, and epigenetic deregulation (2, 3). In the case of acute promyelocytic leukemia (APL) the formation of a PML-RARα oncofusion protein leads to multiple deregulations, that include HDAC recruitment (4) to retinoic acid (RA) target genes resulting in obstruction of differentiation programs (2, 5). While aberrant HDAC recruitment is a hallmark of APL, recent data suggest that HDAC mis-targeting is necessary, but not sufficient for leukemogenesis (6), supporting the novel concept that multiple targeting by combination therapy may achieve maximal anti-cancer effects. This concept is also supported by the multiple genetic and epigenetic aberrations present in cancer that might need a multi-target therapy approach. It has become clear that cancer originates from, and is supported by epigenetic deregulation (7-9). Deposition of epigenetic ‘marks’ on chromatin is accomplished by enzymes residing in multi-subunit complexes (10). The control of the enzymatic machinery including histone acetylation (HAT)/deacetylation (HDAC) and methylation/demethylation (11) or DNA methylation (DNMT) (12) is central to transcription regulation. Chromatin modifying enzymes, in particular HDACs and DNMTs, have emerged as new anticancer targets. The fact that epigenetic modifications can be reversed makes epi-drugs promising for anti-tumor therapy (13). Proof-of-principle comes from studies with HDAC inhibitors (HDACi) that
are in clinical practice (14).

HDACs are considered leading targets for therapy to reverse epi-aberrations (15). The eighteen mammalian HDACs are divided in two families, the Zn\(^{2+}\)-dependent HDACs (Class I: HDACs 1-3, 8; Class II: HDACs 4-7, 9, 10; Class IV: HDAC11) and the NAD\(^+\)-dependent sirtuins (SIRTs1-7; Class III) (16). SIRT1 has only recently been implicated in malignancies (17). DNMTs are responsible for CpG methylation within the genome. Three active enzymes have been identified in mammals: DNMT1 responsible for maintenance of pre-existing methylation and DNMT3a/b for \textit{de novo} methylation (18).

Notwithstanding the clinical use of HDAC and other inhibitors, little is known about the molecular mode of action. We -and others- have demonstrated that HDACis induce TRAIL-apoptosis (TNF-related apoptosis inducing ligand/TNSF10/Apo2L) in acute myeloid leukemia (AML) cells, \textit{in vivo} AML models and \textit{ex vivo} blasts (19, 20). Here we report on UVI5008 molecular characterization and anti-cancer activities. UVI5008 inhibits three epi-enzymes and independently affects the death receptor (DR) pathway and ROS production. UVI5008 targets p53, the Bcl-2 modifying factor BMF and/or TRAIL in cancer cells harboring the wild-type factors as well as tumors, which are deficient for one of them.

**MATERIALS AND METHODS**

**Cell lines**

Cell lines and primary cell cultures are deeply described as supplementary methods. Briefly, U937, K562, K812-F, HL60 (leukemia), DU-145 (prostate), HCT116 (colon), MDA-MB231 and MCF7 (breast) cancer cells were obtained from ATCC. HCT116 p53\(^{-/-}\) and HCT116-DK0 colon cancer cells were a gift from B. Vogelstein, John Hopkins
Kimmel Cancer Center, Baltimore, US. BJ, normal foreskin fibroblast and Ras transformed BJELR cells were a gift from W. C. Hahn (Dana-Farber Cancer Institute, Boston, US). For AML primary samples, bone marrow containing 80% to 90% leukemic blasts was purified over Ficoll and processed as previously described (20). Primary CD34+ normal progenitors (obtained from donors) have been cultured in ex vivo medium as recommended by the supplier. This study was approved by the Ethical Committee of the Second University of Naples. KD clones used in this study have been published previously (20) or have been created by standard procedures using the Mission technology (Sigma).

**Inhibitors**

SAHA (Merck), AZA (Sigma), EX527 (Alexis) and MS-275 (Bayer Schering Pharma) were dissolved in DMSO and used at 5x10^{-6} M or as indicated. All other compounds described were dissolved in DMSO and used at 1 or 5 μM.

**Cell cycle, differentiation and cell death assays** are described as supplementary methods and carried out as in (20, 21).

**Western blot analyses**

Western blots were performed following supplier’s suggestions. For the determination of p21^{WAF1/CIP1} and p16^{INK4} 100 µg of extracts were separated on 15% polyacrylamide gels and blotted. Western blots are shown for p21 (Transduction Laboratories), p16 and total ERKs (Santa Cruz). For α-tubulin acetylation 25 µg of extracts were separated on 10% polyacrylamide gels and blotted. Western blots are shown for acetylated α-tubulin, total tubulin (Sigma) and total ERKs (Santa Cruz). For the detection of histone H3 acetylation we used Upstate antibodies, whereas for histone H3K9 trimethylation, and H3K9, H3K14 and H3K18 acetylations we used Abcam antibodies.
Histone extraction protocol and MS analysis, P53K382 acetylation, p21 expression and histone extraction in xenograft tumors, Xenograft and MMTV-MYC mouse models, Isolation and digestion of genomic DNA are described as supplementary methods.

In vitro methylation of genomic DNA

Digested DNA was methylated with 8U of Sss1 methylase (New England Biolabs) in the presence of 3.2 mM SAM for 2 h at 37 °C. After phenol-chloroform extraction, DNA was precipitated and resuspended with 20 µL water.

Bisulfite treatment and PCR for RARβ and p16 methylation

Bisulfite treatment and PCR for RARβ and p16 methylation are described in the Supplementary Methods.

Fluorimetric human cell based HDAC1 and 4 assays and in vitro HDAC assays

HDAC assays were performed as previously described (21, 22)

In vitro assays of Sirt1 and Sirt2, DNMTs assay, ChIP assay, Immunofluorescence assays are described in the Supplementary Methods. ChIP assays have been performed as in (23).

RNA Preparation for RNA-Seq, Illumina High-Throughput Sequencing and MethylCap-seq are described in the Supplementary methods and in (2, 24, 25).

RESULTS

UVI5008 efficiently Induces Apoptosis

We tested the activity of UVI5008 (Fig. 1A) activity on a series of cancer cells.
UVI5008 induced cell death (Fig. 1B) and apoptosis by activation of both initiator, executor caspases (Fig. 1C) and loss of mitochondrial membrane potential in cancer cells derived from of leukemias (U937, Ku-812F and K562) or solid tumors, such as breast (MCF7), osteosarcoma (U2OS), prostate (DU145), colon carcinoma (HCT116) and melanoma (A375) (Supplementary Fig. S1A and data not shown). Several cell lines derived from colon, breast and prostate carcinomas, exhibited anti-proliferative responses with IC_{50}s ranging from about 200 nM to 3.1 µM (Supplementary Methods and data not shown).

UVI5008 Anti-Cancer Action in vivo and ex vivo

To test UVI5008 anti-tumor activity in vivo, xenograft assays were performed with luciferase-expressing human cancer cells. At 40 mg/kg UVI5008 (MW 710.46 g/mol) efficiently blocked HCT116 colon carcinoma growth as visualized by in vivo imaging (Fig. 2A) and quantified by direct photon counting (Fig. 2B). No adverse side effects were noticed, while the same dose of SAHA (Vorinostat) (MW 264.33 g/mol) was lethal (data not shown). Similarly, UVI5008 strongly inhibited MCF7 breast cancer growth in vivo in presence of estradiol; tamoxifen co-treatment attenuated the growth without affecting the median overall tumor size (Fig. 2C left panel). Importantly, UVI5008 strongly reduced tumor growth in a genetic mouse model (MMTV-myc) of human breast cancer, when treatment was initiated as soon as the tumors became palpable (Fig. 2C, right panel). The weight profiles of the treated vs. control groups supported the absence of toxicity. Similar tumor growth inhibition by 40 mg/kg UVI5008 was observed in the MMTV-cerbB2 breast cancer model (data not shown). Thus, UVI5008 displays a strong anti-cancer effect not only in xenografts, but also in two genetic mouse models that
mimic human breast cancer. Pharmacokinetics analyses revealed half-lives of up to ~7 h (p.o. application) for the in vivo formed glutathione conjugate of UVI5008 (see Supplementary Methods).

UVI5008 mediated Tumor-Selective Apoptosis

To assess the cancer-selective potential of UVI5008 we first used the isogenic BJ stepwise cellular tumorigenesis model (26). While primary fibroblasts (BJ) were largely insensitive to UVI5008, 80% of the transformed BJELR cells were killed (Fig. 2D, left panel).

The efficient induction of tumor selective cell death by UVI5008 prompted us to test its efficacy in ex vivo assays in primary AML blasts. Within 24h, UVI5008 induced caspase 3 activation and apoptosis in 14/14 independent ex vivo blasts (Fig. 2E and Supplementary Fig. S1B). No significant toxicity was seen in normal CD34+ progenitors, whereas all AML blasts were sensitive (Fig. 2D, right panel).

UVI5008 is a triple HDAC, DNMT and SIRT inhibitor

To decipher the basis of UVI5008 anticancer activity we assessed inhibition of DNA methylation. Using methylation-specific PCR (MSP), we corroborated that UVI5008, like its parental compound psammaplin A (27), induced significant demethylation of the RARβ and p16INK4a promoters (Supplementary Fig. S2A). In keeping with the p16 promoter reactivation, UVI5008 induced an increase of p16 protein levels in U937 cells that was higher than the one seen upon combined treatment with 5-aza-cytidine (5-aza-C) and SAHA (Supplementary Fig. S2B, top panel). In vitro assays demonstrated that
UVI5008 acted as a direct inhibitor of DNMT3a (Supplementary Fig. S2B) and only marginally inhibited DNMT1 (Supplementary Fig. S2B, lower panel), when compared to the DNMT1 inhibitors RG108 (28) or SGI-1027 (29). To the best of our knowledge UVI5008 is the first DNMT3a-selective inhibitor.

Next, we assessed whether UVI5008, like psammaplin A (30, 31), acts as HDAC inhibitor. UVI5008 blocked the activity of HDAC1-4 both in vitro and in cell-based assays (Fig. 3A and Supplementary Fig. S3). Moreover, UVI5008 treatment of U937 cells induced the well-known HDACi target p21, inhibited deacetylation of the HDAC6/SIRT2 substrate α-tubulin and affected histone acetylation (H3K9, H3K14 and H3K18) (Fig. 3B). UVI5008 also induced global histone H3 hyperacetylation in ex vivo blasts as exemplified for AML #116 (Fig. 3C and data not shown), thus acting both in vitro and ex vivo. Immunoblotting of tumors derived from both HCT116-xenografted mice and ‘MMTV-myc’ model treated with UVI5008 revealed global (pan-H3) and specific (H3K9, H3K14) hyperacetylation, thereby confirming UVI5008 HDACi action in tumori. Immunohistochemical analysis showed a stronger induction of H3K9 acetylation in HCT116 xenografts of mice treated with UVI5008 than in animals treated with SAHA (Fig. 3D bottom panel).

To investigate whether UVI5008 induced other histone modifications, we defined the spectrum of modifications in bulk histones from U937 cells treated with UVI5008 (4h) using mass spectrometric analyses. In addition to the increase of histone H3 acetylation expected for an HDACi, we identified a histone H3-derived peptide that is acetylated at K56 (Supplementary Fig. S2C and Supplementary Table S1). In yeast, deacetylation of H3K56ac is mediated by the sirtuin homologues Hst3 and Hst4 (32), and in humans
H3K56ac acts as SIRT1 substrate (33). Hence, we investigated whether UVI5008 possesses sirtuin inhibitory (SIRTi) activity. In vitro assays with recombinant human sirtuin-1 and -2 revealed that UVI5008 is a powerful inhibitor of SIRT1 and SIRT2 (Fig. 4A). Analyses with anti-H3K56ac antibodies (34) revealed H3K56 acetylation in UVI5008-treated U937 cells, U2OS osteosarcoma cells (Fig. 4A-B) and other cell lines (Fig. 4B and data not shown). The observation that tumors from HCT116-xenografted mice and from the ‘MMTV-myc’ breast cancer genetic mouse model revealed increased H3K56ac when treated with UVI5008 but not SAHA, established the in vivo SIRT inhibitory activity of UVI5008 (Fig. 4B-C).

Considering that acetylation activates p53 (35) and that SIRT1 deacetylates p53K382 (36), we hypothesized that UVI5008 may sustain p53 activity. Indeed, treatment of MCF7 and HCT116 cells with etoposide and UVI5008 increased p53K382 acetylation similar to the SIRT1i EX527 (37), thus extending our observations that UVI5008 inhibits sirtuins (Fig. 4C). Immunohistochemical analysis in tumors from HCT116-xenografted mice treated with UVI5008 confirmed efficient SIRT inhibition in vivo (37) (Fig. 4D). Our in vivo studies confirm that UVI5008, which displays a favorable PK (see Supplementary methods for PK and PD study), reaches tumors in vivo to exert HDACi and SIRTi activity and to display anticancer action at doses that may be tolerable and efficacious in the context of a clinical trial. These results demonstrate that UVI5008 is a potent HDAC and sirtuin inhibitor in vitro and in vivo.

**UVI5008 re-Activates Tumor Suppressor Gene Programs**

A measure of importance for the activity of epigenetic inhibitors and their application as anti-cancer drugs is the ability to induce growth arrest by altering the expression of tumor...
suppressor genes (TSG) such as the cyclin-dependent kinase inhibitor p21\(^{\text{Waf1/Cip1}}\) CDKN1A (38). UVI5008 efficiently induced transcription of p21\(^{\text{Waf1/Cip1}}\) in HCT116 cells and in the corresponding xenografts \textit{in vivo} (Fig. 5A). UVI5008 also induced p21\(^{\text{Waf1/Cip1}}\) expression in p53\(^{-/-}\) HCT116 cells and xenografts, indicating that p21 targeting is p53-independent (Fig. 5A). Expression of SFRP1, SFRP2 and MLH1 TSGs was strongly induced in UVI5008-treated HCT116 cells (Fig. 5A, right panel), HCT116-xenografts, MDA-MB231 and MCF7 cells, supporting the SIRTi activity of UVI5008 (Fig. 5B). Note that UVI5008 mediated higher activation of SFRP1 expression when compared with exposure to SIRTi or DNMTi alone (Fig. 5B).

We also tested the ability of UVI5008 to activate transcription from genes with DNA methylated CpG-island in HCT116 cells and a double knockout of DNMT1 and DNMT3b (“HCT116-DKO” cells) by RNA-seq; in parallel, the DNA methylation status was determined by MethylCap (25). UVI5008 was able to partially reactivate loci such as UCHL1 gene whose CpG-island is DNA methylated in line with its albeit weak DNMTi action (Fig. 5C). Upon complete loss of DNA methylation at this CpG-island as in HCT116-DKO, the gene is strongly reactivated. UVI5008 treatment of HCT116-DKO did not further enhance transcription as compared to the parental HCT116 suggesting that its DNMT but not HDAC and/or sirtuin inhibition caused the low but significant activation of the UCHL1 locus in WT cells. TIMP3, a TSG known both to be repressed by DNA methylation (39, 40) and to be SIRT1 target (41) was reactivated by UVI5008 only in HCT116-DKO cells and not in the untreated or wt cells (Fig. 5C). Possibly due to its low DNMT-inhibitory activity UVI5008 could not overcome the repressive action of DNA methylation. Induction of p21\(^{\text{Waf1/Cip1}}\) (CDKN1A) expression by UVI5008 in wt
and DKO cells (Fig. 5D) is most likely due to the non-DNMTi activities of UVI5008, as the promoter-proximal CpG-island is not DNA methylated in wt or DKO HCT116 cells.

**Mechanisms of Pro-Apoptotic UVI5008 action**

The induction of apoptosis in cells, *ex vivo* and *in vivo* by UVI5008 and its *bona fide* tumor-specific action prompted us to decipher the underlying molecular pathway(s). RT-PCR and ELISA assays showed a strong increase of TNFSF10 mRNA and protein levels upon UVI5008 exposure (Supplementary Fig. S4A) extending our previous findings that the TRAIL is a target of HDACi (19, 20). In line with the transcription activation of the TNFSF10 promoter by UVI5008, chromatin immunoprecipitation (ChIP) assays (23) revealed a rapid increase in histone H3 acetylation at the TNFSF10 promoter as exemplified by H3K9ac spreading to the TNFSF10 intron within 2h of treatment (Supplementary Fig. S4B). Similar activation was seen at the promoter of the death receptor-5 (DR5/TRAIL-R2; data not shown). These results show that UVI5008 activates the tumor-selective TRAIL pathway, which accounts for, or contributes to its tumor-selectivity.

To assess the contribution of the TRAIL pathway to tumor-selective activity of UVI5008, we expressed a dominant-negative FADD (dnFADD) and the non-functional FADD mutant (dnFADDmut); the dnFADD reduced UVI5008-induced death only to ~50%, while completely blocking TNFSF10-induced apoptosis (Supplementary Fig. S4C). Similarly, only a moderate reduction of apoptosis was detectable in UVI5008-treated U937 cells expressing stably integrated shTNFSF10 (KD) (20), whereas a complete
reduction was obtained upon MS275, a bona fide ‘pure’ HDACi (Supplementary Fig. S4C). Moreover, neither the simultaneous knockdown of TNFSF10 and p21 nor the blocking of DR-mediated death pathways by neutralizing antibodies affected UVI5008 death (Supplementary Fig. S5A and B). These observations strongly suggest that while TRAIL induction is a key factor in HDACi apoptosis, this is not the only mechanism by which UVI5008 induces apoptotic pathways distinct from or additional to TRAIL.

Concomitant Death receptors and ROS activation cause UVI5008-mediated apoptosis

To gain further insight into the UVI5008 death activities, we assessed the implication of p53 and Bcl-2 modifying factor (BMF). The p53 gene is a SIRT target, whereas BMF silencing was reported to rescue cells from HDACi-induced apoptosis (42) in some cancers. Despite efficient knockdown of BMF and p53 no significant rescue from UVI5008-induced apoptosis was detected (Fig. 6A) suggesting that neither p53 nor BMF are singly essential. In agreement with these results, strong anti-tumor action of UVI5008 was observed in HCT116 p53−/− xenografts in vivo (Fig. 6A, right panel), re-emphasizing its therapeutic potential for the therapy of p53 mutant or deficient cancers. On the other hand, caspase 8 and 9 inhibition, or caspase 8 silencing resulted in a major inhibition (Fig. 6B), implicating both the extrinsic and intrinsic pathways in UVI5008-induced death. Interestingly, while necroptosis inhibition by Nec-1 did not affect UVI5008-mediated death, N-acetyl-cysteine (NAC), a scavenger of reactive oxygen species (ROS), significantly decreased it (Fig. 6C; compare lanes 3 and 7 with lanes 2 or 6). Most
importantly, NAC fully blocked UVI5008-induced apoptosis in cells stably expressing shTNFSF10 (compare lanes 14 and 15). Strikingly, experiments carried out ex vivo in primary AML blasts, corroborated the dual involvement of the TRAIL-induced death signaling and ROS production as the main apoptotic mechanism of action of UVI5008 (Fig. 6D, lane 6). Thus, the anticancer activity of UVI5008 originates from the cumulative activation of multiple pathways due to the concomitant activation of death receptors and ROS production. More specifically, the triple action inhibitor UVI5008 displays i) strong anticancer action in vitro, ex vivo and in vivo, ii) reactivation of TSGs in vitro and in vivo and iii) antitumor efficacy independent of p53 and BMF in vitro and in vivo.

DISCUSSION

The fact that epigenetic modifications can be reversed by epi-drugs makes epi-compounds highly promising for anti-tumor therapy. However, the results have been lagging behind expectation. Investigation of the spectrum of epi-mutations in cancer has only recently started and FDA (Food and Drug Administration)-approved epi-drugs are currently limited by a number of factors. Due to their moderate toxicities, HDACis and DNMTis entered therapy without a detailed molecular characterization. Beside this, no real markers for their apoptotic action or patient’s response have been reported so far, thus currently hampering clinical stratification. This lack of knowledge about pathways and targets might have led to unfocused therapeutic applications of epi-drugs.

We have identified and characterized the mechanism resulting in the apoptogenic action of UVI5008 in detail. We have previously reported that induction of TRAIL is part of the
action spectrum of HDACis (19, 20). Hence, the contribution of TRAIL tumor-selective death by UVI5008 is likely to be linked to its HDACi action. Our observation that both TNFSF10-silenced and TNFSF10-deficient cells (cell lines and blasts) undergo apoptosis upon UVI5008 treatment strongly supports the contribution of SIRT and DNMT inhibition to UVI5008 anti-cancer action. Our data suggests that UVI5008 may be effective even in HDACi-resistant and hyper-mutated tumors. The same applies to tumors in which p53 is silenced or mutated (Figures 5 and 6). Importantly, p53, BMF and TRAIL are often altered in cancers, thus suggesting the beneficial application of UVI5008 in multiple settings. Considering that SIRT1 mediates resistance to intrinsic apoptosis in cancer (43), SIRT1i may enforce the higher UVI5008 apoptotic activity possibly enhanced by its other inhibitory activities. We show that UVI5008 anticancer activity is causally linked to DR pathway activation and ROS production (Fig. 6).

Although the involvement of oxidative stress in TRAIL-mediated apoptosis has been reported (44), our data shows that only the concomitant impairment of both pathways fully blocks UVI5008 action. Thus the targeting of these two molecular pathways in both in vitro and ex vivo settings by UVI5008 is independent (Fig. 6). Interestingly, SIRT1 inhibits apoptosis induced by oxidative stress. SIRT1 deacetylates and activates FOXO1 (45), FOXO3a (46), and FOXO4 (47), promoting expression of the DNA repair factor GADD45, and of the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD). These proteins may contribute to the induction of ROS tolerance by SIRT1 in tumors. Perturbation of the mechanisms that tightly couple ROS production, oxidative stress signaling, and FOXO activity to the subsequent cellular response is a pivotal step in cancer. Consequently, the ROS-FOXO pathway is a promising therapeutic target in
cancer, not only as it mediates chemotherapy response, but also because it underpins drug resistance. As the intimate, reciprocal relation between FOXO and ROS is being unraveled (48), new applications arise to apply SIRTis to circumvent resistance to conventional drugs. The tight link between SIRTis and ROS production (48) may explain UVI5008 ROS-dependent action and its higher anticancer action when compared to single HDACi and SIRTi.

Analysing DNMT3a inhibition, UVI5008 effect on DNA methylation on the p16 promoter is comparable to that of 5-azacytidine, whereas the induction of p16 protein expression by UVI5008 is higher (Supplementary Fig. S3), which likely results from the simultaneous inhibition of DNMT3a and SIRT1/2. Indeed, the indication that upon induction of DNA double strand breaks, SIRT1 recruitment to the E-cadherin promoter is a pre-requisite for DNMT3 recruitment and subsequent heritable CpG methylation at the promoter strongly favors the use of an anti-cancer drug that targets both SIRT and DNMT. The reported de-regulation/over-expression of HDACs, DNMTs and SIRTs in cancer (17, 33), also rationalizes UVI5008 use.

As suggested for HDACi and DNMTi combinations (49), also SIRT1i reactivates TSGs without altering DNA hypermethylation (8). The fact that TSGs, such as TIMP3 (Fig. 5), become re-expressed upon UVI5008 exposure is relevant. The mechanism of TIMP3 silencing in colon cancer and its role in tumorigenesis remain elusive. That DNA demethylation fails to reactivate the gene indicates that multiple activities potentiated by UVI5008 are needed for its re-expression. A further illustration that UVI5008 fulfills the criteria of a 'multiple targeting' drug is the activation of SFRP1 (Fig. 5) which is far higher with UVI5008 than with canonical combo-treatments, supporting the winning
strategy of applying a single drug exerting multiple-interventions against cancer.

The use of a single drug with multiple epi-activities, such as UVI5008, has great promise for therapy, as the synergies between its activities may reduce both the effective dose and development of resistance (50). The pharmaceutical development of a single compound is less complex than that of combinatorial therapies, complicated by divergent pharmacokinetics, pharmacodynamics and complex ADMET profiles. UVI5008 belongs to this novel class of compounds, as it inhibits three distinct classes of epigenetic enzymes, namely DNMT3a, HDACs, and sirtuins. The rationale at the basis of targeting drugs could radically shift with the introduction of molecules interfering simultaneously with multiple targets as this might be more effective than single target agents thus representing a new generation of anti-cancer drugs. In this view the inhibition of at least three epi-enzymes offers a promising alternative to combo-treatments of cancer using ‘poly-pharmacology’ approaches. Given the ability of cancer to bypass signaling routes via complex networks, the simultaneous modulation of multiple epi-targets promises to open new avenues against cancer.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

HGS, ARdL, HG and LA are inventors for the patent n° WO2008125988 entitled: ‘Novel Derivatives of Psammaplin A, a method for their synthesis and their use for the prevention or treatment of cancer’.

ACKNOWLEDGEMENTS
We are grateful to W. Hahn and R. Weinberg for stepwise tumorigenesis model. We thank P. Jansen, C. Erb, and G. Lemaire for technical help; E. Carrillo de Santa Pau and H. Marks, for bioinformatics analysis; V. Carafa and F. Rodriguez-Barrios for technical help and discussions.

GRANT SUPPORT

This work was supported by: Associazione Italiana ricerca contro il cancro (AIRC to LA), MIUR to LA, Association for International Cancer Research (AICR 00-108 to HG), Ligue National Contre le Cancer (HG), ANR-07-EMPB-012-01 ‘EPI_DRUG’, MEC (SAF2010-17935 FEDER to AdL), Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale (PRIN2009PX2T2E to LA), EU LSHC-CT-2005-518417 (EPITRON), HEALTH-F4-2007-200767 (APO-SYS), HEALTH-F2-2007-200620 (CANCERDIP), HEALTH-F4-2009-221952 (ATLAS).

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FIGURE LEGENDS

Figure 1. Structure and apoptogenic action of UVI5008 in U937 cells. A. Structure of UVI5008; B. Apoptosis of UVI5008 at 40h revealed by pan-caspase activation assays; C. Selective assays for activation of caspases 8, 9 and 3-7. In (B) and (C) UVI5008 was used at μM UVI5008. Evaluation of the loss of mitochondrial potential after UVI5008 treatment (5 μM).

Figure 2. UVI5008 displays anticancer action in vivo and tumor-selective apoptosis. A. False-color images revealing tumor growth in vivo of luciferase-tagged HCT116 cells, xenografted into nude mice and treated with vehicle or 40mg/kg of UVI5008; the increase of luminescence determined by direct photon counting reveals the growth inhibitory effects; B. Kinetics of tumor mass development as in A; C. left panel. Tumor mass development as in A of MCF7 xenografted mice treated with 40mg/kg of UVI5008;
right panel. Tumor mass development in MMTV-Myc transgenic mice treated with 40mg/kg of UVI5008; treatment was initiated when tumors became palpable; normalized mouse weights are shown on the right side. D. Left panel. Primary BJ fibroblasts and their tumorigenic derivatives were exposed to UVI5008 and survival was measured by MTT assays; right panel. Survival rate of CD34+ cells and AML blasts upon UVI5008 (5 µM) treatment after 48 h. E. Example of the apoptotic UVI5008 action (20 h, 5 µM) in the AML blast #116, both in bone marrow (BM) and periphery (P).

Figure 3. HDAC inhibitory activities of UVI5008 in vitro, ex vivo and in vivo. A. Cell-based HDAC1 and HDAC4 assay carried out in MCF7 cells with UVI5008 at 5 µM; B. Western blot analyses of p21, α-tubulin and histone H3 acetylation, K9H3me3x and H3K9, H3K14, H3K18 acetylations in U937 cells after 24 h with UVI5008 at 5 µM; C. Immunofluorescence of histone H3 acetylation levels in ex vivo AML 116 blasts; D. Stimulation of histone H3 specific acetylations both in the tumors of MMTV-Myc transgenic mice (data with HCT116 cells treated in vitro are shown for comparison) and in xenografted HCT116 p53−/− tumors treated with 40 mg/kg dose of UVI5008 and 20 mg/kg dose of SAHA analysed at day 22. Immunohistochemical staining for H3K9 acetylation in tumor sections from UVI5008 or SAHA-treated HCT116-xenografted mice.

Figure 4. SIRT inhibitory activities of UVI5008 in vitro, ex vivo and in vivo. A. Human recombinant SIRT1 and 2 assays in vitro with the indicated compounds; histone H3K56 acetylation in U937 and U2OS cells treated for 24 h with UVI5008 (5 µM); B.
Western blot analyses of H3K56 acetylation in histone extracts of U937 cells treated with UVI5008 (5 µM); C. Stimulation with UVI5008 (40 mg/kg dose) induced H3K56 specific acetylation in both tumors of MMTV-Myc transgenic mice (data with HCT116 cells treated in vitro are shown for comparison) and in xenografted HCT116 p53<sup>+/+</sup> tumors analysed at day 22; H3K56ac immunohistochemical staining of tumor sections from UVI5008-treated HCT116-xenografted mice. D. Top panel. Analysis of p53K382 acetylation in MCF7 cells after 6 h treatment with the indicated compounds; middle panel. p53K382 acetylation levels in HCT116 cells treated in vitro; bottom panel. p53K382 acetylation immunohistochemical staining of tumor sections from HCT116-xenografted mice treated with 40mg/kg of UVI5008. Both UVI5008 and EX243, but not MS275, stimulate p53K382 acetylation in HCT116 cells in vitro (middle panel) and in tumori in treated xenografted mice (bottom panel).

**Figure 5. UVI5008 displays Tumor Suppressor Gene reactivation in vitro and in vivo**

A. Left panel. Western blot revealing p21 expression in HCT116 cells and in mice bearing HCT116 p53<sup>+/+</sup> and HCT116 p53<sup>−/−</sup> tumors; right panel. 48h treatment of UVI5008 re-activates TSGs in HCT116 cells; B. Left panel. UVI5008 re-activates TSGs in HCT116-based xenografts; 36B4 is an inert internal control RNA used for normalization; (right panel, top) UVI5008 reactivates TSG in breast cancer cells treated for 30h; (right panel, bottom) combined treatment with UVI5008 and AZA or EX527 and AZA (36 h) synergizes in gene-re-expression in MDA-MB231 cells; C. Reactivation of UCHL1 gene: browser screen shot of RNA-seq and DNA methylation (MethylCap-seq) profiling of HCT116 (WT) and HCT116-DKO cells after treatment with UVI5008 at 5
µM for 24 h. RNA-seq was performed using poly(A)+ RNA. In MethylCap, fragmented genomic DNA (average 400-500bp) is separated into three fractions (low, medium and highly methylated DNA). The degree of methylation is graded from black (high) to white (unmethylation). Green boxes indicate CpG Islands. The organization of the gene is schematically indicated.; D. DNA methylation-dependent and -independent reactivation of TIMP3 and CDKNIA (p21) genes. Browser screens shots as in C.

Figure 6. Only multiple pathway interventions can block UVI5008 action in vitro and in vivo. A. (Left) Caspase 3-7 assay in WT and BMF KD U937 cells upon UVI5008 at 5 µM; annexin V assay carried out in MCF7 cells WT or p53 KD upon treatment with UVI5008 at 5 µM; (Right) tumor growth kinetics of luciferase-tagged HCT116 p53<sup>−/−</sup> mouse xenografts exposed to vehicle or 40 mg/kg UVI5008. B. Caspase 8 and 9 inhibitors (50 µM) and siRNA targeting Casp8 do not fully impair UVI5008 apoptosis in U937 cells. As control, TRAIL-mediated apoptosis was fully blocked by si-Casp8; C. NAC, but not NEC1, blocks apoptosis in TRAIL KD, but not in WT U937 cells treated with UVI5008. D. Only the combined action of NAC and TRAIL blockage impairs apoptosis in primary ex vivo AML blasts treated with UVI5008 (24 h).
Figure 4

A

B

C

D
Molecular Cancer Therapeutics

Death receptor pathway activation and increase of ROS production by the triple epigenetic inhibitor, UVI5008

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Mol Cancer Ther Published OnlineFirst October 6, 2011.

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