

Article

HDAC Inhibition Increases HLA Class I Expression in Uveal Melanoma

Zahra Souri ¹, Aart G. Jochemsen ², Mieke Versluis ¹, Annemijn P.A. Wierenga ¹, Fariba Nemati ³, Pieter A. van der Velden ¹, Wilma G.M. Kroes ⁴, Robert M. Verdijk ^{5,6}, Gregorius P.M. Luyten ¹ and Martine J. Jager ^{1,*}

¹ Department of Ophthalmology, LUMC, Albinusdreef 2, 2333 ZA Leiden, The Netherlands; z.souri@lumc.nl (Z.S.); m.versluis@lumc.nl (M.V.); a.p.a.wierenga@lumc.nl (A.P.A.W.); p.a.van_der_velden@lumc.nl (P.A.v.d.V.); g.p.m.luyten@lumc.nl (G.P.M.L.)

² Department of Cell and Chemical Biology, LUMC, 2333 ZA Leiden, The Netherlands; a.g.jochemsen@lumc.nl

³ Laboratory of Preclinical Investigation, Department of Translational Research, Institut Curie, PSL University, 75248 Paris, France; fariba.nemati@curie.fr

⁴ Department of Clinical Genetics, LUMC, 2333 ZA Leiden, The Netherlands; w.g.m.kroes@lumc.nl

⁵ Department of Pathology, LUMC, 2333 ZA Leiden, The Netherlands; r.m.verdijk@lumc.nl

⁶ Department of Pathology, Section Ophthalmic Pathology, ErasmusMC, Dr Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

* Correspondence: m.j.jager@lumc.nl

Received: 12 November 2020; Accepted: 7 December 2020; Published: 9 December 2020



Simple Summary: Chemotherapy and immunotherapy are both used to treat malignancies. The immunotherapy of cancer often involves T cells, which recognise the antigens presented in HLA molecules. Uveal melanoma (UM) is an intraocular malignancy, which often gives rise to metastases. We determined whether high-risk tumours expressed the target of two drugs, histone deacetylase (HDAC) inhibitor Quisinostat and Tazemetostat, an inhibitor of Enhancer of zeste homologue 2 (EZH2). We observed that especially high-risk UM tumours (monosomy 3, gain of 8q, loss of BAP1) expressed several HDACs, and showed a high HLA Class I expression. We further tested whether these drugs influenced HLA Class I expression on three UM cell lines. The drug Quisinostat led to an upregulation of HLA protein and mRNA levels in three UM cell lines, while Tazemetostat had little effect. We concluded that the use of drugs that influence epigenetic regulators may impact immunotherapy approaches.

Abstract: The treatment of uveal melanoma (UM) metastases or adjuvant treatment may imply immunological approaches or chemotherapy. It is to date unknown how epigenetic modifiers affect the expression of immunologically relevant targets, such as the HLA Class I antigens, in UM. We investigated the expression of HDACs and the histone methyl transferase EZH2 in a set of 64 UMs, using an Illumina HT12V4 array, and determined whether a histone deacetylase (HDAC) inhibitor and EZH2 inhibitor modified the expression of HLA Class I on three UM cell lines. Several HDACs (HDAC1, HDAC3, HDAC4, and HDAC8) showed an increased expression in high-risk UM, and were correlated with an increased HLA expression. HDAC11 had the opposite expression pattern. While in vitro tests showed that Tazemetostat did not influence cell growth, Quisinostat decreased cell survival. In the three tested cell lines, Quisinostat increased HLA Class I expression at the protein and mRNA level, while Tazemetostat did not have an effect on the cell surface HLA Class I levels. Combination therapy mostly followed the Quisinostat results. Our findings indicate that epigenetic drugs (in this case an HDAC inhibitor) may influence the expression of immunologically relevant cell surface molecules in UM, demonstrating that these drugs potentially influence immunotherapy.

Keywords: eye diseases; uveal melanoma; oncology; HLA; HDAC; immunology; inflammation

1. Introduction

Uveal melanoma (UM) is a rare malignancy of the eye, estimated to occur in 6–7 cases per million per year in northern Europe and the United States of America [1,2]. The risk of developing metastases is around 50% [3]. The tumour arises from the uveal tract, which involves the choroid, ciliary body, and the iris [4]. In spite of the immunologically privileged nature of the eye, high-risk UM may show inflammation, and the presence of an inflammatory phenotype is related to a bad prognosis [5–7]. This inflammatory phenotype is characterised by the presence of high numbers of lymphocytes and macrophages, and a high HLA Class I and II expression [7–10]. All of these are related to the loss of one chromosome 3, a well-known risk factor for the development of metastases in this malignancy.

A high HLA Class I surface expression may protect UM cells from killing by natural killer cells (NK), and as metastases in this disease occur hematogenously, where NK cells help to remove tumour cells, a high HLA expression may help tumour cells to escape destruction [11–13]. On the other hand, the loss of HLA Class I expression has been identified as a tumour-escape mechanism in for instance cutaneous melanoma [14]. Both genetic and epigenetic events have been identified as regulators of HLA Class I and II expression [15], which is also the case in UM [16,17]. A study from our lab showed that one epigenetic regulator, EZH2, part of the Polycomb Repressive Complex 2 (PRC2), was found to influence HLA Class II expression in UM through the histone methylation of promoter IV of CIITA [18]. In other malignancies, members of the histone deacetylase (HDAC) family were identified as HLA regulators [19–22].

As HDACs are aberrantly expressed in UM [23], targeting these epigenetic regulators is considered for treatment [24]. In addition, the use of such inhibitors as adjuvant therapy in UM is being investigated [25,26]. A trial for the treatment of UM metastases has been set up [27], in which an immune checkpoint inhibitor (Pembrolizumab) is combined with the HDAC inhibitor Entinostat, under the hypothesis that the inhibition of HDACs may lead to an enhanced expression of HLA and cancer antigens, and a decreased activity of myeloid-derived suppressor cells. While this may help T cell-mediated immunotherapy, it may negatively affect NK cell-mediated lysis.

We set out to investigate the effect of two clinically relevant inhibitors of epigenetic modifiers, Quisinostat, an HDAC inhibitor, and Tazemetostat, an EZH2 inhibitor, on the expression of HLA Class I molecules on UM cell lines. We first determined the expression of HDACs, EZH2, as well as HLA expression in a set of 64 UMs.

2. Results

2.1. HDAC and EZH2 Expression in UM, Association with High Risk

Inhibitors of HDACs may be used as adjuvant treatment in combination with immune checkpoint inhibitors; as especially high-risk UM tumours with an inflammatory phenotype that express HLA Class I give rise to metastases, we wondered whether these high-risk tumours would express HDACs. We studied the mRNA expression of HDACs in a panel of 64 primary UMs, and compared their expression with the tumour's chromosome 3, chromosome 8q and BAP1 status, which are indicators of a high risk of metastases formation (Figure 1A–C).

Expression of HDAC1 ($p = 0.02$), HDAC3 ($p = 0.04$), HDAC4 ($p = 0.001$), as well as HDAC8 ($p < 0.001$) was significantly higher in M3 tumours compared to D3 tumours. In contrast, HDAC11 had a significantly lower expression in high-risk M3 tumours ($p < 0.001$). With regard to both HDAC5 as well as HDAC7 expression, we found significant differences between D3 vs. M3 tumours for only one of the two probes that had been used (HDAC5 probe 1 $p = 0.90$, HDAC5 probe 2 $p = 0.004$, HDAC7 probe 1 $p = 0.30$, HDAC7 probe 2 $p = 0.02$).

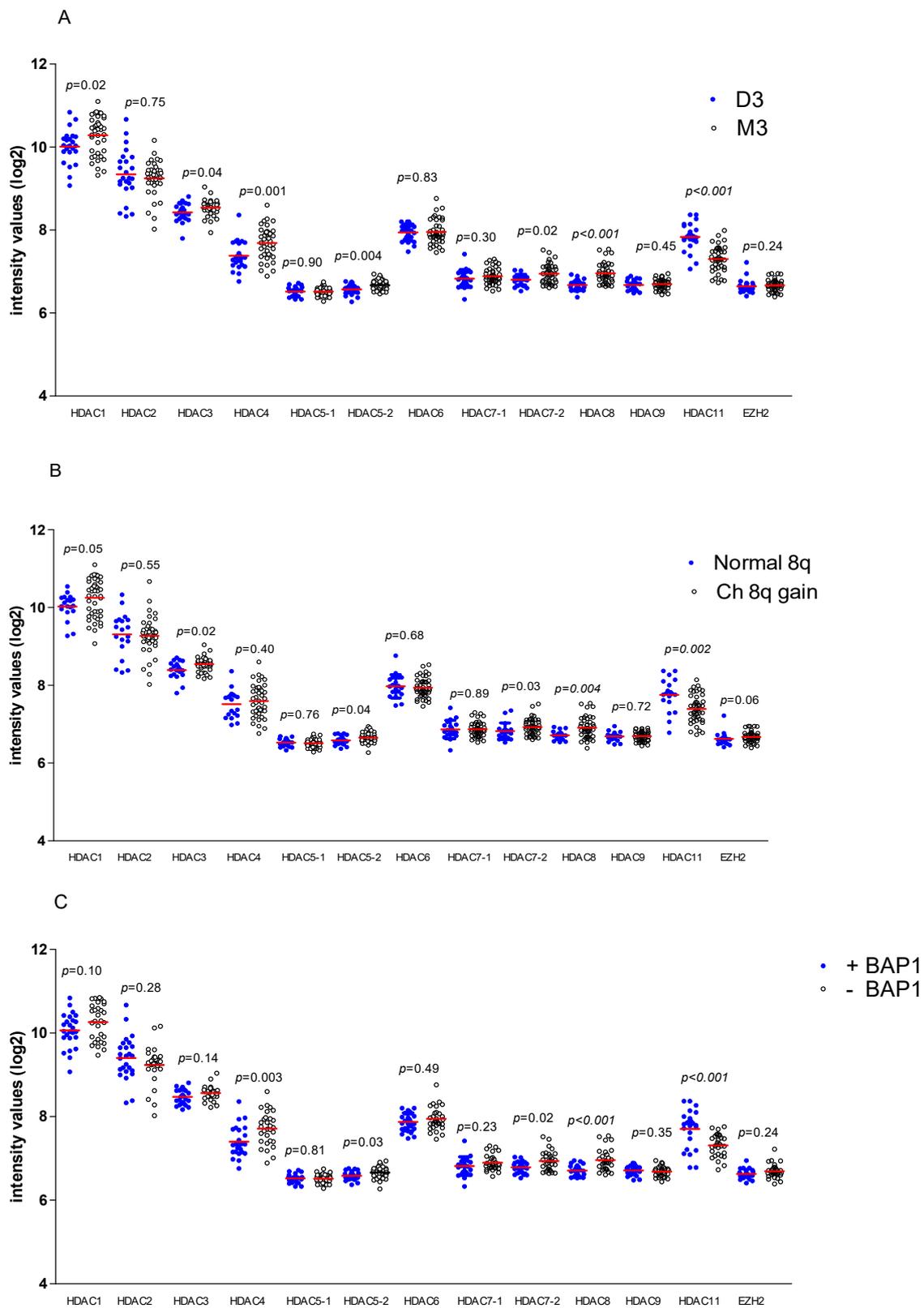


Figure 1. The expression of histone deacetylases (HDACs) in uveal melanoma in relation to the tumour’s chromosome 3, chromosome 8q and BAP1 status. The expression of several HDACs and EZH2 was compared between tumours with (A) disomy for chromosome 3 (D3, $n = 24$) and a monosomy of chromosome 3 (M3, $n = 40$); (B) normal 8q ($n = 19$) vs. 8q gain ($n = 45$); (C) positive staining for BAP1 (+BAP1, $n = 25$) vs. negative staining for BAP1 (-BAP1, $n = 30$). A Mann–Whitney U test was applied. Horizontal bars indicate mean gene expression.

The patterns for chromosome 8q gain (Figure 1B) and BAP1 expression (Figure 1C) resembled those of chromosome 3. HDAC1 ($p = 0.05$), HDAC3 ($p = 0.02$), the second probe of HDAC5 ($p = 0.04$) and of HDAC7 ($p = 0.03$), and HDAC8 ($p = 0.004$), were increased in tumours with 8q gain, while HDAC11 was decreased ($p = 0.002$). The loss of BAP1 expression as assessed by immunohistology was associated with an increase in HDAC4 ($p = 0.003$), the second probe of HDAC5 ($p = 0.03$) and HDAC7 ($p = 0.02$) and a decrease in HDAC11 ($p < 0.001$).

We also looked at EZH2, which is known to be higher in UM with a high mitotic count [28]. However, the EZH2 expression was not correlated with chromosome 3 or 8q status, or BAP1 status ($p = 0.24$, $p = 0.06$, $p = 0.24$).

2.2. Association between HDAC and EZH2 Expression and HLA Class I in UM

As high-risk tumours are known to have a higher HLA Class I expression than low risk tumours, we hypothesised that the expression of HLA Class I and M3-associated HDACs would be correlated. We tested this for all HDACs and EZH2 in our set of 64 tumours.

Expression of HDAC1, HDAC4, and HDAC8 was convincingly positively correlated with HLA-A and/or HLA-B (Table 1). The two HDAC7 probes showed diverging results, with the M3-associated probe being positively correlated with one HLA-A probe and with the HLA-B probe. The HDAC11 expression showed a significant negative correlation with HLA-A and -B expression (all P s < 0.001). These data indicate that the expression levels of three of the four HDACs, which are upregulated in M3 tumours, correlate with a high HLA-A and -B expression. HDAC11 shows the opposite pattern. No significant correlation was seen between the EZH2 and HLA Class I expression in this set of 64 tumours.

Table 1. Correlation between mRNA expression levels (determined by Illumina array) of different HDACs and EZH2 and the expression of HLA-A (three probes) and HLA-B. R = two-tailed Spearman correlation coefficient. Following Bonferroni correction, $p \leq 0.005$ is considered significant, and indicated in bold.

	HLA-A Probe 1		HLA-A Probe 2		HLA-A Probe 3		HLA-B	
	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>
HDAC1	0.367	0.003	0.392	0.001	0.380	0.002	0.370	0.003
HDAC2	-0.144	0.26	-0.167	0.19	-0.108	0.40	-0.162	0.20
HDAC3	0.262	0.04	0.241	0.05	0.293	0.02	0.318	0.01
HDAC4	0.143	0.26	0.371	0.003	0.354	0.004	0.305	0.01
HDAC5 probe 1	0.029	0.82	0.090	0.48	0.018	0.88	-0.045	0.72
HDAC5 probe 2	0.091	0.47	0.231	0.07	0.096	0.45	0.151	0.23
HDAC6	-0.179	0.16	-0.136	0.28	-0.284	0.02	-0.196	0.12
HDAC7 probe 1	-0.020	0.87	0.010	0.94	-0.014	0.91	0.108	0.39
HDAC7 probe 2	0.225	0.07	0.382	0.002	0.263	0.04	0.352	0.004
HDAC8	0.456	<0.001	0.588	<0.001	0.539	<0.001	0.550	<0.001
HDAC9	-0.066	0.60	-0.091	0.48	-0.209	0.01	-0.190	0.13
HDAC11	-0.541	<0.001	-0.548	<0.001	-0.515	<0.001	-0.607	<0.001
EZH2	0.211	0.09	0.214	0.09	0.226	0.07	0.146	0.25

2.3. In Vitro Analysis of Effect of HDAC and EZH2 Inhibition on UM Cell Lines

As mentioned earlier, high-risk M3 UMs contain more infiltrating cells, which also express HLA antigens. The correlation between the expression of certain genes and high-risk tumours is, therefore, difficult to interpret, as it cannot be excluded that the increased levels of, e.g., HLA Class I and certain

HDACs, is caused to a significant extent by the increased number of infiltrating cells and not so much by the UM tumour cells themselves.

To be able to investigate an effect of HDAC activity on HLA Class I expression, we made use of the pan-HDAC inhibitor Quisinostat and treated the UM cell lines *in vitro*.

Recent studies have shown that HDAC inhibitors are able to reduce growth in UM cell lines [29,30], but to our knowledge, no one has reported on the effect of epigenetic enzyme inhibition on the expression of immune modulators, such as HLA Class I, in UMs. We also used an EZH2 inhibitor, Tazemetostat, and studied the effect of combination therapy.

2.3.1. Morphological Analysis of UM Cell Lines after 48 h Treatment with Quisinostat and Tazemetostat

First of all, we looked at the morphological characteristics of the cell lines after 48 h treatment with Quisinostat and Tazemetostat compared with untreated cell lines (Figure 2). In the presence of Quisinostat (Q, 40 nM), at 48 h, the cell sizes of all three cell lines was reduced and more floating cells were visible. Upon treatment with Tazemetostat (T, 5 μ M), at 48 h, no large differences in the morphology or confluency were observed compared to the controls. Cells which were treated with a combination of the two drugs (40 nM Q + 5 μ M T) changed morphologically and resembled Quisinostat single treatment: plates with OMM2.5 and MP38 seemed to have fewer cells than when treated with Quisinostat alone.

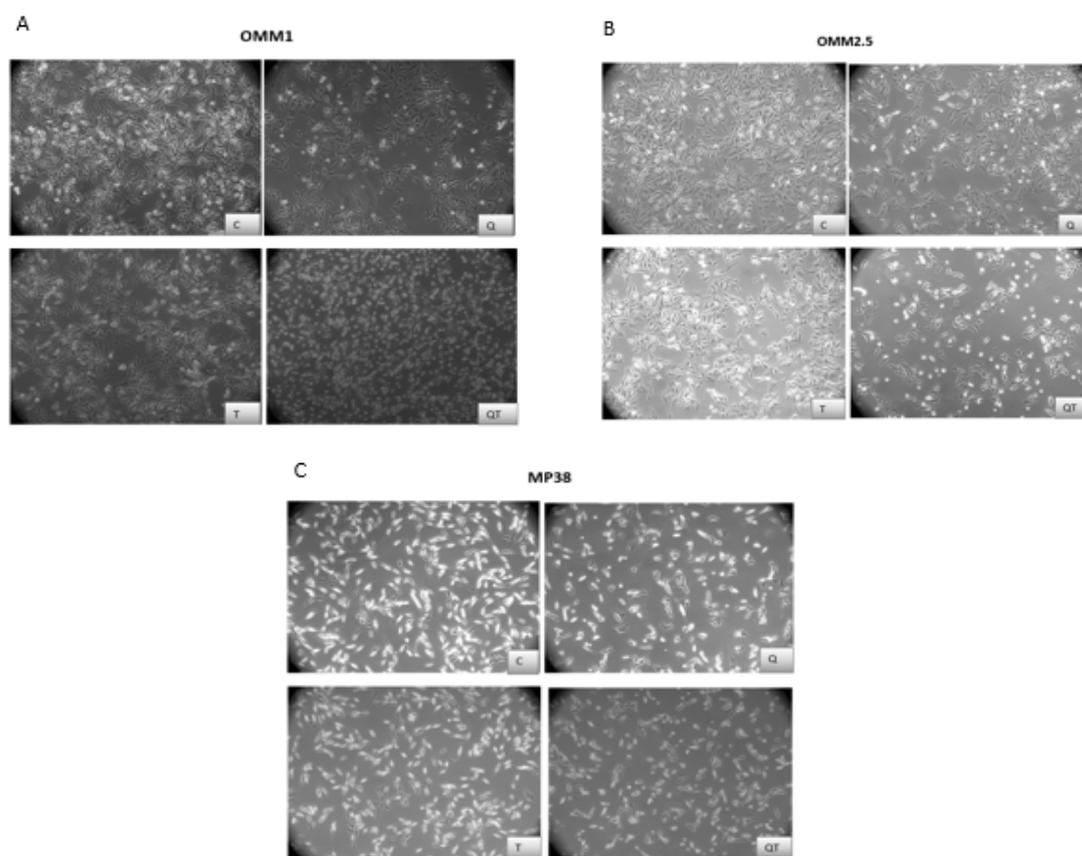


Figure 2. Photographs of uveal melanoma cell lines taken after 48 h exposure to Quisinostat (Q) and/or Tazemetostat (T): the photographs (magnification 100 \times) show fewer and more rounded cells after incubation with 40 nM Q and after combination treatment. (A) = cell line OMM1, (B) = cell line OMM2.5, (C) = cell line MP38. C: control; Q: 40 nM Quisinostat; T: 5 μ M Tazemetostat; QT: combination of 40 nM Quisinostat and 5 μ M Tazemetostat.

2.3.2. Quisinostat and Cell Growth

A quantitative analysis of cell numbers after 24 and 48 h using the Cell Titer-Blue Cell Viability Assay (Figure 3) confirmed these findings: Quisinostat reduced the cell numbers of OMM2.5 and MP38 cells after 24 h ($p = 0.01$, $p = 0.03$), and of all three cell lines after 48 h ($p < 0.001$, $p = 0.002$, $p < 0.001$). Tazemetostat by itself did not influence cell numbers. However, at 48 h, the combination of both drugs (QT) decreased cell numbers significantly in two cell lines (OMM1 and MP38) compared to the Q treatment alone ($p < 0.001$ for both).

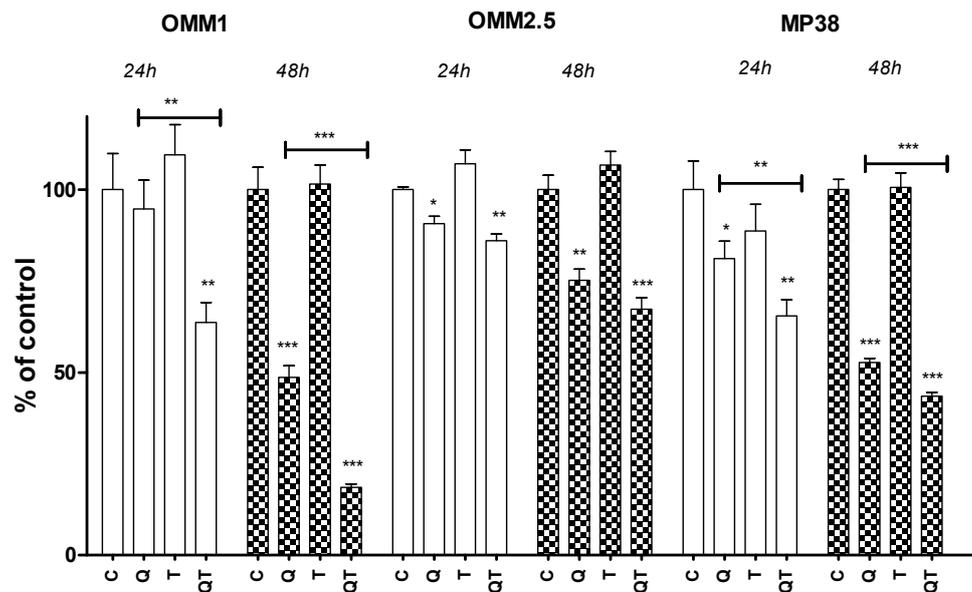


Figure 3. Cell numbers of three uveal melanoma cell lines after 48 h of treatment with Quisinostat or Tazemetostat of uveal melanoma cell lines. Cell density was determined using a cell titer blue assay. C: control; Q: 40 nM Quisinostat; T: 5 μ M Tazemetostat; QT: combination of 40 nM Quisinostat and 5 μ M Tazemetostat. * $p \leq 0.5$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate the standard error of the mean.

2.3.3. Effect of Quisinostat and Tazemetostat on HLA Class I Cell Surface Expression

We investigated the effect of Quisinostat and Tazemetostat on HLA Class I surface expression of the UM cell lines by flow cytometry after 48 h treatment. The three cell lines (OMM1, OMM2.5, and MP38) expressed HLA Class I, as determined using monoclonal antibody W6/32 (Figure 4A–C, controls). Quisinostat increased HLA Class I surface expression in all three cell lines ($p < 0.01$). Tazemetostat, on the other hand, did not affect the HLA Class I surface expression in the cell lines, while the combination treatment resulted in a significant increase compared to Quisinostat alone in OMM2.5 and MP38 ($p = 0.003$ and $p = 0.004$).

2.3.4. Effect of Quisinostat and Tazemetostat on HLA Class I mRNA Expression by qPCR

We investigated the level of HLA Class I mRNA by qPCR after 48 h of treatment with Quisinostat and/or Tazemetostat. HLA-A and HLA-B expressions were determined separately (Figure 4D–F and Figure 4G–I).

In agreement with what we found regarding broad HLA Class I cell surface expression, Quisinostat increased HLA-A in all three cell lines ($p < 0.01$). Tazemetostat gave a slight increase in cell line OMM2.5, while in all three cell lines, combined treatment led to a lower expression than with Quisinostat alone. With regard to HLA-B, Quisinostat led to a higher expression in all three cell lines, while Tazemetostat by itself gave a significant but slight increase in MP38. The combination of the two drugs led to a high expression on all three cell lines, with divergent results when compared to Quisinostat alone.

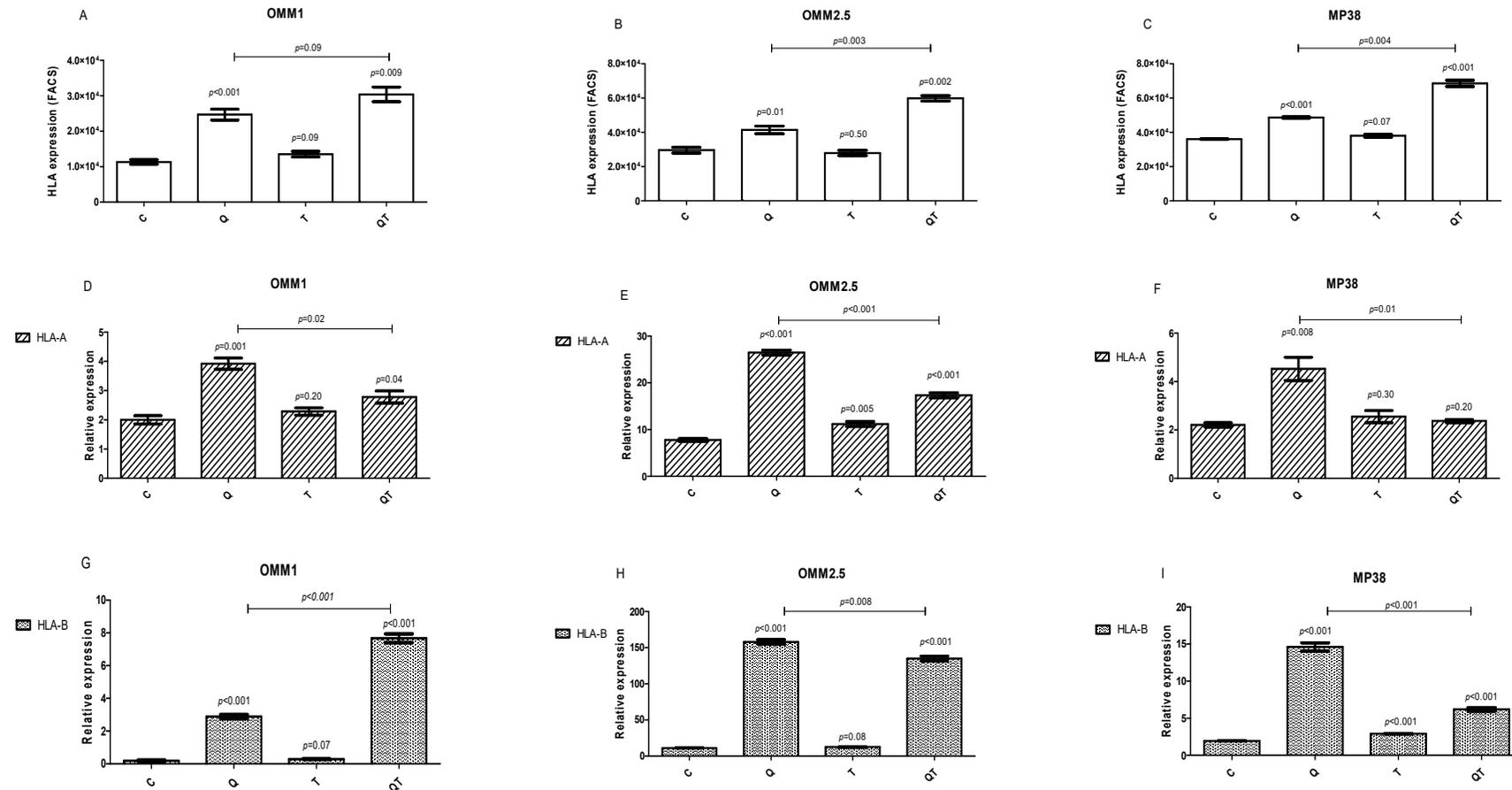


Figure 4. HLA Class I cell surface protein expression and mRNA levels after 48 h of treatment with Quisinostat and/or Tazemetostat compared to the control. Cell surface expression was determined by FACS using anti-HLA W6/32 antibody (A–C). HLA-A mRNA (D–F) and HLA-B mRNA (G–I) expression was determined by qPCR. C: control; Q: 40 nM Quisinostat; T: 5 μM Tazemetostat; QT: combination of 40 nM Quisinostat and 5 μM Tazemetostat. Using an Independent t-test, $p \leq 0.05$ was considered significant. Error bars indicate the standard error of the mean of three measurements.

3. Discussion

HDACs are a group of epigenetic regulators which have been shown to regulate inflammation in some malignancies [31,32]. Moreover, elevated HDAC expression has been associated with other inflammation-related diseases such as chronic hepatitis B [33] and rheumatoid arthritis (RA) [34]. To our knowledge, the relationship between the expression of these epigenetic regulators and the inflammatory phenotype in UM has not been investigated. As combinations of chemotherapy and immunotherapy are being used as adjuvant treatments and to treat metastases in e.g., cutaneous melanoma, we investigated the expression of several epigenetic regulators and their potential involvement in the regulation of HLA Class I, a molecule which plays an important role in immune responses, in UM.

In a set of 64 UMs, the expression of HDACs 1, 4, and 8 was positively correlated with the expression of HLA Class I. Using malignant cell lines such as from colorectal carcinoma, e.g., HDAC1 has been shown to be related to inflammation, by inducing STAT1 activation and the transcription of downstream targets of the IFN- γ signalling pathway [35,36]; such targets include HLA Class I components. In addition, we show that particularly HDAC1, 3, 4, and 8 are expressed at higher levels in high-risk UM.

We tested the effects of two drugs, Quisinostat and Tazemetostat. The pan-HDAC inhibitor Quisinostat has been shown to effectively reduce UM cell viability in vitro [29,37], and is currently being tested in clinical studies of advanced solid tumours and leukaemia [38]. Growth inhibition was accomplished by the induction of cell cycle arrest and apoptosis in other cancers such as lung cancer [39], and hepatocellular carcinoma [40]. Tazemetostat is a drug known to inhibit EZH2 and has been shown to reduce growth in medulloblastoma [41], B-cell non-Hodgkin lymphoma and epithelioid sarcoma [42]. When we applied the pan-HDAC inhibitor Quisinostat to UM cell lines, HLA Class I cell surface and mRNA expression increased, while the effect of Tazemetostat was marginal. As Quisinostat is a pan-HDAC inhibitor [30], the effect is likely due to the inhibition of deacetylation. Indeed, we have previously shown that treatment with Quisinostat strongly increased the level of acetylated Histone 3 [29]. In general, higher acetylation coincides with less chromatin compactness, higher promoter activity and increased gene transcription. We have no evidence that the effect of HDAC inhibition on HLA Class I gene transcription is caused by a direct effect on the chromatin modification around the Class I promoters. However, our results are generally in agreement with studies in other malignancies describing that HDAC inhibition will upregulate HLA Class I and some HLA regulatory components [19–22,43]. Whether increasing HLA expression in UM is helping the patient may depend on the situation: it may help to stimulate T cell-mediated cytotoxicity against metastases; however, when the primary UM is still in place, increasing HLA expression may limit the NK cell activity directed against metastatic cells which spread hematogeneously.

HDAC11 expression was significantly lower in high-risk tumours (Figure 1A–C). In 2014, Herlihy and colleagues already showed a low expression of HDAC11 in a small series of M3 UMs [23]. The gene of this HDAC is located on chromosome 3, and the loss of chromosome 3 in high-risk UMs most likely explains these findings. However, it does not necessarily mean that low HDAC11 expression has no causal relationship with an increased risk of the development of metastases. Although most studies on the function of HDAC11 suggest an oncogenic role, as in breast cancer the downregulation of HDAC11 provided the cells with an increased ability to invade from the lymph nodes to other organs [44]. It is therefore possible that the low HDAC11 expression may help the hematogenous spread to the liver.

In UMs, EZH2 plays a major role in silencing promoters and inhibiting transcription [18] and is associated with a high mitotic count [28]. Although this enzyme represses CIITA transcription by the K27m3 of histone 3, thereby silencing HLA Class II expression, we observed a high expression of EZH2 in high-risk M3 tumours, without any association to HLA Class I expression. EZH2 inhibition with Tazemetostat did not have an effect on the cell surface expression of HLA Class I, while we observed a slight increase in HLA-A mRNA in OMM2.5 and HLA-B mRNA in cell line MP38. The combination with Quisinostat led to results that varied between cell lines and HLA allele.

A Phase 1 trial is planned to use the HDAC 1, 2, and 3 inhibitor Domatinostat together with immune checkpoint inhibitors as the neo-adjuvant treatment of cutaneous melanoma patients with lymph node metastases ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04133048) NCT-4133048). This trial is set up under the assumption that the HDAC inhibitor will stimulate the interferon pathway, making the tumour “hot”. Such tumours may contribute to a good induction of cytotoxic T cells. As already mentioned earlier, high-risk UMs already have a high HLA expression, and it is unclear whether the same approach will help to treat the metastases of UMs. While HLA expression is important for tumour cell killing by T cells, a high expression blocks recognition by NK cells. Furthermore, the expression of other cell surface molecules such as checkpoint inhibitors may also be modified by blocking epigenetic regulators.

4. Materials and Methods

4.1. Study Population

Tumours were derived from 64 eyes that underwent an enucleation for UMs between 1999 and 2008 at the Leiden University Medical Center (LUMC), Leiden, The Netherlands; 51% of the patients were male and 49% female. Their mean age at the time of enucleation was 61 years.

This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The project was approved by the LUMC Biobank committee and the LUMC METC committee (19 October 2016, code G16.076/NV/gk).

4.2. Chromosome Analysis

DNA was isolated using the QIAmp DNA Mini kit (Qiagen, Venlo, The Netherlands). Single-nucleotide polymorphism (SNP) analysis was performed using an Affymetrix 250K_NSIP or Affymetrix SNP 6.0 array to detect chromosome 3 and 8 abnormalities [45].

4.3. Immunohistochemistry

Tissues were incubated with mouse monoclonal antibody (clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX, USA) for BAP1 evaluation [46,47]. Nuclear staining was evaluated by an experienced ocular pathologist.

4.4. Tumour Gene Expression

Gene expression profiling was performed with the Illumina HT12v4 array (Illumina, Inc., San Diego, CA, USA) and the data were obtained for the expression of epigenetic regulators (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, HDAC11, EZH2) and HLA Class I genes (HLA-A, HLA-B) as described previously [16]. Information regarding the Illumina probe numbers and gene expression levels is shown in Table S1.

4.5. Reagents

Quisinostat and Tazemetostat were purchased from Selleckchem (Houston, TX, USA). Drugs were dissolved in dimethyl sulfoxide (DMSO) to reach a stock concentration of 5 mM and diluted in the indicated fresh medium for in vitro studies.

4.6. Cell Lines and Cell Culture

The OMM1 cell line was established by Dr G.P.M. Luyten (LUMC, Leiden, The Netherlands) [48], while OMM2.5 was a gift from Dr B.R. Ksander (Schepens Eye Research Institute, Boston, MA, USA) [49]. Both were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI) Dutch modified media (Life technologies, Europe bv, Bleiswijk, The Netherlands) supplemented with 10% foetal bovine serum (FBS) (Greiner Bio-one, Alphen aan den Rijn, The Netherlands), 1% GlutaMAX and 1% penicillin/streptomycin (Life technologies). MP38 was kindly provided by the Curie Institute, Paris, France [50] and cultured in Iscove’s modified Dulbecco medium (IMDM) (Life Technologies) containing

20% FBS (Greiner Bio-one) and 1% penicillin/streptomycin (Gibco). Cells were incubated in 5% CO₂ at 37 °C in monolayers in tissue culture flasks in a humidified incubator.

4.7. Cell Proliferation Study

Cells were seeded in triplicate in 135 µL in 96-well plates; 24 h after seeding the cells, 15 µL of 10× concentrated drug in media was added to each well. Cell viability was determined by the Cell Titer-Blue Viability Assay: after removing the medium, 100 µL of appropriately diluted CellTiter-Blue Reagent (Promega, Fitchburg, WI, USA) was added to each well and after 60 min, fluorescence was measured in a microplate reader (Victor X3, Perkin Elmer, San Jose, CA, USA).

4.8. Flow Cytometry

Cells were incubated with an optimal dilution of mouse monoclonal anti-HLA Class I antibody (W6/32, 311414, Alexa Fluor 647; Bio Legend, Amsterdam, The Netherlands) and the data were collected (10,000–15,000 cells per live gate) using the BD LSR II system (BD Biosciences, San Diego, CA, USA); results were analysed using FACSDiva software (BD Biosciences). Three independent experiments have been performed.

4.9. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) have been described previously [51]. Briefly, total RNA was extracted from cell cultures using an RNeasy Mini Kit (Qiagen Benelux B.V., Venlo, The Netherlands). Using the iScript cDNA synthesis kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands), complementary cDNA was synthesised. Quantitative real-time polymerase chain reaction (qPCR) was performed in three independent experiments using the CFX-384 machine, with triplicates of one experiment used to make the graphs. CFX manager 3.1 (Bio-Rad) software was used to analyse the data, and the CT values of genes of interest were normalised against the geometric mean of housekeeping genes *RPS11*, *CAPNS1*, and *SRPR*. The sequences of primers used in the study are shown in Supplementary Table S2.

4.10. Statistical Analysis

Data were analysed with SPSS software version 22.0 (SPSS, nc. Chicago, IL, USA). Spearman correlation was performed in order to test correlations between non-parametric data. Bonferroni correction was applied for multiple testing, where appropriate. The Mann–Whitney U test was used to compare non-normal groups. Graphs were obtained using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). An independent t-test was used to compare the difference between the means of the in vitro studies.

5. Conclusions

To the best of our knowledge, this is the first study which reports the association between HDAC epigenetic regulators and HLA Class I expression in UMs. We showed that high-risk UMs not only show a higher expression of HLA Class I antigens but also of several HDACs. The pan-HDAC inhibitor Quisinostat increases HLA Class I expression at the protein and mRNA level while EZH2 inhibition by Tazemetostat has a slight effect on HLA expression. As epigenetic anti-cancer drugs may influence the expression of immunologically relevant molecules such as HLA in UMs, they may influence the function of T cells and NK cells, and thereby the effect of immunotherapy.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6694/12/12/3690/s1>, Table S1: Gene expression (Illumina) Probe Number in an Illumina HT12v4 array and mean expression of the genes used in this study; Table S2: Sequences of PCR primers used in this study.

Author Contributions: Conceptualisation, Z.S., M.J.J.; methodology, Z.S., A.G.J., M.V., and R.M.V.; formal analysis, Z.S.; data curation, A.P.A.W.; writing—original draft preparation, Z.S. and M.J.J.; writing—review and editing, A.G.J., M.V., A.P.A.W., F.N., P.A.v.d.V., W.G.M.K., R.M.V., G.P.M.L. and M.J.J.; supervision, A.G.J. and M.J.J.;

funding acquisition, P.A.v.d.V., A.G.J. and M.J.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant to Z.S. from The Iranian Ministry of Science Research and Technology and a Horizon 2020 grant from the European Community, CURE UM, nr. 667787.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Virgili, G.; Gatta, G.; Ciccolallo, L.; Capocaccia, R.; Biggeri, A.; Crocetti, E.; Lutz, J.-M.; Paci, E. Incidence of Uveal Melanoma in Europe. *Ophthalmology* **2007**, *114*, 2309–2315. [\[CrossRef\]](#)
2. Singh, A.D.; Topham, A. Incidence of uveal melanoma in the United States: 1973–1997. *Ophthalmology* **2003**, *110*, 956–961. [\[CrossRef\]](#)
3. Kujala, E.; Mäkitie, T.; Kivelä, T. Very Long-Term Prognosis of Patients with Malignant Uveal Melanoma. *Investig. Ophthalmol. Vis. Sci.* **2003**, *44*, 4651–4659. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Jager, M.J.; Shields, C.L.; Cebulla, C.M.; Abdel-Rahman, M.H.; Grossniklaus, H.E.; Stern, M.H.; Carvajal, R.D.; Belfort, R.N.; Jia, R.; Shields, J.A.; et al. Uveal melanoma. *Nat. Rev. Dis. Primers* **2020**, *6*, 1–25. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Jager, M.J.; Hurks, H.M.H.; Levitskaya, J.; Kiessling, R. HLA expression in uveal melanoma: There is no rule without some exception. *Hum. Immunol.* **2002**, *63*, 444–451. [\[CrossRef\]](#)
6. Bronkhorst, I.H.; Vu, T.H.K.; Jordanova, E.S.; Luyten, G.P.M.; Burg, S.H.V.D.; Jager, M.J. Different Subsets of Tumor-Infiltrating Lymphocytes Correlate with Macrophage Influx and Monosomy 3 in Uveal Melanoma. *Investig. Ophthalmol. Vis. Sci.* **2012**, *53*, 5370–5378. [\[CrossRef\]](#)
7. Robertson, A.G.; Shih, J.; Yau, C.; Gibb, E.A.; Oba, J.; Mungall, K.L.; Hess, J.M.; Uzunangelov, V.; Walter, V.; Danilova, L.; et al. Integrative Analysis Identifies Four Molecular and Clinical Subsets in Uveal Melanoma. *Cancer Cell* **2017**, *32*, 204–220.e15. [\[CrossRef\]](#)
8. Blom, D.J.; Luyten, G.P.; Mooy, C.; Kerkvliet, S.; Zwinderman, A.H.; Jager, M.J. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. *Investig. Ophthalmol. Vis. Sci.* **1997**, *38*, 1865–1872.
9. Dithmar, S.; Crowder, J.; Jager, M.J.; Vigniswaran, N.; Grossniklaus, H.E. HLA-I-Antigenexpression korreliert mit dem histologischen Zelltyp uvealer Melanome. *Der Ophthalmol.* **2002**, *99*, 625–628. [\[CrossRef\]](#)
10. Ericsson, C.; Seregard, S.; Bartolazzi, A.; Levitskaya, E.; Ferrone, S.; Kiessling, R.; Larsson, O. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. *Investig. Ophthalmol. Vis. Sci.* **2001**, *42*, 2153–2156.
11. Ma, D.; Niederkorn, J.Y. Transforming growth factor-beta down-regulates major histocompatibility complex class I antigen expression and increases the susceptibility of uveal melanoma cells to natural killer cell-mediated cytotoxicity. *Immunology* **1995**, *86*, 263–269. [\[PubMed\]](#)
12. Ma, D.; Luyten, G.P.; Luider, T.M.; Niederkorn, J.Y. Relationship between natural killer cell susceptibility and metastasis of human uveal melanoma cells in a murine model. *Investig. Ophthalmol. Vis. Sci.* **1995**, *36*, 435–441.
13. Niederkorn, J.Y. Immune escape mechanisms of intraocular tumors. *Prog. Retin. Eye Res.* **2009**, *28*, 329–347. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Garcia-Lora, A.; Algarra, I.; Garrido, F. MHC class I antigens, immune surveillance, and tumor immune escape. *J. Cell. Physiol.* **2003**, *195*, 346–355. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Campoli, M.; Ferrone, S. HLA antigen changes in malignant cells: Epigenetic mechanisms and biologic significance. *Oncogene* **2008**, *27*, 5869–5885. [\[CrossRef\]](#)
16. Van Essen, T.; Van Pelt, S.L.; Bronkhorst, I.H.G.; Versluis, M.; Némati, F.; Laurent, C.; Luyten, G.P.M.; Van Hall, T.; Elsen, P.J.V.D.; Van Der Velden, P.A.; et al. Upregulation of HLA Expression in Primary Uveal Melanoma by Infiltrating Leukocytes. *PLoS ONE* **2016**, *11*, e0164292. [\[CrossRef\]](#)
17. Souri, Z.; Wierenga, A.P.A.; Mulder, A.; Jochemsen, A.G.; Jager, M.J. HLA Expression in Uveal Melanoma: An Indicator of Malignancy and a Modifiable Immunological Target. *Cancers* **2019**, *11*, 1132. [\[CrossRef\]](#)
18. Holling, T.M.; Bergevoet, M.W.T.; Wilson, L.; Van Eggermond, M.C.J.A.; Schooten, E.; Steenbergen, R.D.; Snijders, P.J.F.; Jager, M.J.; Elsen, P.J.V.D. A Role for EZH2 in Silencing of IFN- γ Inducible MHC2TA Transcription in Uveal Melanoma. *J. Immunol.* **2007**, *179*, 5317–5325. [\[CrossRef\]](#) [\[PubMed\]](#)

19. Magner, W.J.; Kazim, A.L.; Stewart, C.; Romano, M.A.; Catalano, G.; Grande, C.; Keiser, N.; Santaniello, F.; Tomasi, T.B. Activation of MHC Class I, II, and CD40 Gene Expression by Histone Deacetylase Inhibitors. *J. Immunol.* **2000**, *165*, 7017–7024. [[CrossRef](#)]
20. Mora-García, M.D.L.; Duenas-Gonzalez, A.; Hernández-Montes, J.; De La Cruz-Hernández, E.; Perez-Cardenas, E.; Weiss-Steider, B.; Santiago-Osorio, E.; Ortiz-Navarrete, V.; Rosales, V.H.; Cantú, D.; et al. Up-regulation of HLA class-I antigen expression and antigen-specific CTL response in cervical cancer cells by the demethylating agent hydralazine and the histone deacetylase inhibitor valproic acid. *J. Transl. Med.* **2006**, *4*, 55. [[CrossRef](#)]
21. Kitamura, H.; Torigoe, T.; Asanuma, H.; Honma, I.; Sato, N.; Tsukamoto, T. Down-Regulation of HLA Class I Antigens in Prostate Cancer Tissues and Up-Regulation by Histone Deacetylase Inhibition. *J. Urol.* **2007**, *178*, 692–696. [[CrossRef](#)] [[PubMed](#)]
22. Khan, A.N.H.; Gregorie, C.J.; Tomasi, T.B. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol. Immunother.* **2008**, *57*, 647–654. [[CrossRef](#)] [[PubMed](#)]
23. Herlihy, N.; Dogrusöz, M.; Van Essen, T.H.; Harbour, J.W.; Van Der Velden, P.A.; Van Eggermond, M.C.J.A.; Haasnoot, G.W.; Elsen, P.J.V.D.; Jager, M.J. Skewed Expression of the Genes Encoding Epigenetic Modifiers in High-Risk Uveal Melanoma. *Investig. Ophthalmol. Vis. Sci.* **2015**, *56*, 1447–1458. [[CrossRef](#)] [[PubMed](#)]
24. Li, Y.; Jia, R.; Ge, S. Role of Epigenetics in Uveal Melanoma. *Int. J. Biol. Sci.* **2017**, *13*, 426–433. [[CrossRef](#)] [[PubMed](#)]
25. Landreville, S.; Agapova, O.A.; Matatall, K.A.; Kneass, Z.T.; Onken, M.D.; Lee, R.S.; Bowcock, A.M.; Harbour, J.W. Histone Deacetylase Inhibitors Induce Growth Arrest and Differentiation in Uveal Melanoma. *Clin. Cancer Res.* **2012**, *18*, 408–416. [[CrossRef](#)]
26. Triozzi, P.L.; Singh, A.D. Adjuvant Therapy of Uveal Melanoma: Current Status. *Ocul. Oncol. Pathol.* **2015**, *1*, 54–62. [[CrossRef](#)]
27. Jespersen, H.; Bagge, R.O.; Ullenhag, G.; Carneiro, A.; Helgadottir, H.; Ljuslinder, I.; Levin, M.; All-Eriksson, C.; Andersson, B.; Stierner, U.; et al. Concomitant use of pembrolizumab and entinostat in adult patients with metastatic uveal melanoma (PEMDAC study): Protocol for a multicenter phase II open label study. *BMC Cancer* **2019**, *19*, 415–421. [[CrossRef](#)]
28. Cheng, Y.; Li, Y.; Huang, X.; Wei, W.; Qu, Y. Expression of EZH2 in uveal melanomas patients and associations with prognosis. *Oncotarget* **2017**, *8*, 76423–76431. [[CrossRef](#)]
29. Heijkants, R.; Willekens, K.; Schoonderwoerd, M.; Teunisse, A.; Nieveen, M.; Radaelli, E.; Hawinkels, L.; Marine, J.-C.; Jochemsen, A.G. Combined inhibition of CDK and HDAC as a promising therapeutic strategy for both cutaneous and uveal metastatic melanoma. *Oncotarget* **2017**, *9*, 6174–6187. [[CrossRef](#)]
30. Moschos, M.M.; Dettoraki, M.; Androudi, S.; Kalogeropoulos, D.; Lavaris, A.; Mantas, D.; Damaskos, C.; Garmpi, A.; Tsatsos, M. The Role of Histone Deacetylase Inhibitors in Uveal Melanoma: Current Evidence. *Anticancer. Res.* **2018**, *38*, 3817–3824. [[CrossRef](#)]
31. Lu, J.; Sun, H.; Wang, X.; Liu, C.; Xu, X.; Li, F.; Huang, B. Interleukin-12 p40 promoter activity is regulated by the reversible acetylation mediated by HDAC1 and p300. *Cytokine* **2005**, *31*, 46–51. [[CrossRef](#)] [[PubMed](#)]
32. Shakespear, M.R.; Halili, M.A.; Irvine, K.M.; Fairlie, D.P.; Sweet, M.J. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol.* **2011**, *32*, 335–343. [[CrossRef](#)] [[PubMed](#)]
33. Zhang, H.; Li, X.; Zhang, Q.; Yang, F.; Chu, X.; Zhang, D.; Wang, L.; Gong, Z. Role of histone deacetylase expression levels and activity in the inflammatory responses of patients with chronic hepatitis B. *Mol. Med. Rep.* **2017**, *15*, 2744–2752. [[CrossRef](#)] [[PubMed](#)]
34. Angiolilli, C.; Kabala, P.A.; Grabiec, A.M.; Van Baarsen, I.M.; Ferguson, B.S.; García, S.; Fernandez, B.M.; McKinsey, T.A.; Tak, P.P.; Fossati, G.; et al. Histone deacetylase 3 regulates the inflammatory gene expression programme of rheumatoid arthritis fibroblast-like synoviocytes. *Ann. Rheum. Dis.* **2016**, *76*, 277–285. [[CrossRef](#)]
35. Nusinzon, I.; Horvath, C.M. Interferon-stimulated transcription and innate antiviral immunity require deacetylase activity and histone deacetylase 1. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14742–14747. [[CrossRef](#)]
36. Klampfer, L.; Augenlicht, L.; Huang, J.; Swaby, L.-A. Requirement of Histone Deacetylase Activity for Signaling by STAT1. *J. Biol. Chem.* **2004**, *279*, 30358–30368. [[CrossRef](#)]

37. Van Der Ent, W.; Burrello, C.; Teunisse, A.F.A.S.; Ksander, B.R.; Van Der Velden, P.A.; Jager, M.J.; Jochemsen, A.G.; Snaar-Jagalska, B.E. Modeling of Human Uveal Melanoma in Zebrafish Xenograft Embryos. *Investig. Ophthalmol. Vis. Sci.* **2014**, *55*, 6612–6622. [[CrossRef](#)]
38. Venugopal, B.; Baird, R.D.; Kristeleit, R.; Plummer, R.; Cowan, R.A.; Stewart, A.; Fourneau, N.; Hellemans, P.; Elsayed, Y.; McClue, S.; et al. A Phase I Study of Quisinostat (JNJ-26481585), an Oral Hydroxamate Histone Deacetylase Inhibitor with Evidence of Target Modulation and Antitumor Activity, in Patients with Advanced Solid Tumors. *Clin. Cancer Res.* **2013**, *19*, 4262–4272. [[CrossRef](#)]
39. Bao, L.; Diao, H.; Dong, N.; Su, X.; Wang, B.; Mo, Q.; Yu, H.; Wang, X.; Chen, C. Histone deacetylase inhibitor induces cell apoptosis and cycle arrest in lung cancer cells via mitochondrial injury and p53 up-acetylation. *Cell Biol. Toxicol.* **2016**, *32*, 469–482. [[CrossRef](#)]
40. He, B.; Dai, L.; Zhang, X.; Chen, D.; Wu, J.; Feng, X.; Zhang, Y.; Xie, H.; Zhou, L.; Wu, J.; et al. The HDAC Inhibitor Quisinostat (JNJ-26481585) Suppresses Hepatocellular Carcinoma alone and Synergistically in Combination with Sorafenib by G0/G1 phase arrest and Apoptosis induction. *Int. J. Biol. Sci.* **2018**, *14*, 1845–1858. [[CrossRef](#)]
41. Zhang, H.; Zhu, D.; Zhang, Z.; Kaluz, S.; Yu, B.; Devi, N.S.; Olson, J.J.; Van Meir, E.G. Correction: EZH2 targeting reduces medulloblastoma growth through epigenetic reactivation of the BAI1/p53 tumor suppressor pathway. *Oncogene* **2020**, *39*, 1165. [[CrossRef](#)] [[PubMed](#)]
42. Italiano, A.; Soria, J.C.; Toulmonde, M.; Michot, J.M.; Lucchesi, C.; Varga, A.; Coindre, J.M.; Blakemore, S.J.; Clawson, A.; Suttle, B.; et al. Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: A first-in-human, open-label, phase 1 study. *Lancet Oncol.* **2018**, *19*, 649–659. [[CrossRef](#)]
43. Ritter, C.; Fan, K.; Paschen, A.; Hardrup, S.R.; Ferrone, S.; Nghiem, P.; Ugurel, S.; Schrama, D.; Becker, J.C. Epigenetic priming restores the HLA class-I antigen processing machinery expression in Merkel cell carcinoma. *Sci. Rep.* **2017**, *7*, 2290–2300. [[CrossRef](#)] [[PubMed](#)]
44. Leslie, P.L.; Chao, Y.L.; Tsai, Y.-H.; Ghosh, S.K.; Porrello, A.; Van Swearingen, A.E.D.; Harrison, E.B.; Cooley, B.C.; Parker, J.S.; Carey, L.A.; et al. Histone deacetylase 11 inhibition promotes breast cancer metastasis from lymph nodes. *Nat. Commun.* **2019**, *10*, 1–12. [[CrossRef](#)]
45. Versluis, M.; De Lange, M.J.; Van Pelt, S.I.; Ruivenkamp, C.A.L.; Kroes, W.G.M.; Cao, J.; Jager, M.J.; Luyten, G.P.M.; Van Der Velden, P.A. Digital PCR Validates 8q Dosage as Prognostic Tool in Uveal Melanoma. *PLoS ONE* **2015**, *10*, e0116371. [[CrossRef](#)]
46. Koopmans, A.E.; Verdijk, R.M.; Brouwer, R.W.; van den Bosch, T.P.; van den Berg, M.M.; Vaarwater, J.; Kockx, C.E.M.; Paridaens, D.; Naus, N.C.; Nellist, M.; et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. *Mod. Pathol.* **2014**, *27*, 1321–1330. [[CrossRef](#)]
47. Van Essen, T.H.; Van Pelt, S.I.; Versluis, M.; Bronkhorst, I.H.G.; Van Duinen, S.G.; Marinkovic, M.; Kroes, W.G.M.; Ruivenkamp, C.A.L.; Shukla, S.; De Klein, A.; et al. Prognostic parameters in uveal melanoma and their association with BAP1 expression. *Br. J. Ophthalmol.* **2014**, *98*, 1738–1743. [[CrossRef](#)]
48. Luyten, G.P.M.; Naus, N.C.; Mooy, C.M.; Hagemeyer, A.; Kan-Mitchell, J.; Van Drunen, E.; Vuzevski, V.; De Jong, P.T.V.M.; Luider, T.M. Establishment and characterization of primary and metastatic uveal melanoma cell lines. *Int. J. Cancer* **1996**, *66*, 380–387. [[CrossRef](#)]
49. Chen, P.W.; Murray, T.G.; Uno, T.; Salgaller, M.L.; Reddy, R.; Ksander, B.R. Expression of MAGE genes in ocular melanoma during progression from primary to metastatic disease. *Clin. Exp. Metastasis* **1997**, *15*, 509–518. [[CrossRef](#)]

50. Amirouchene-Angelozzi, N.; Nemati, F.; Gentien, D.; Nicolas, A.; Dumont, A.; Carita, G.; Camonis, J.; Desjardins, L.; Cassoux, N.; Piperno-Neumann, S.; et al. Establishment of novel cell lines recapitulating the genetic landscape of uveal melanoma and preclinical validation of mTOR as a therapeutic target. *Mol. Oncol.* **2014**, *8*, 1508–1520. [[CrossRef](#)]
51. Bronkhorst, I.H.G.; Jehs, T.M.L.; Dijkgraaf, E.M.; Luyten, G.P.M.; Van Der Velden, P.A.; Van Der Burg, S.H.; Jager, M.J. Effect of Hypoxic Stress on Migration and Characteristics of Monocytes in Uveal Melanoma. *JAMA Ophthalmol.* **2014**, *132*, 614–621. [[CrossRef](#)] [[PubMed](#)]

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).