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EZH2 inhibition enhances the activity of Carboplatin in aggressive-variant prostate cancer cell lines

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ABSTRACT

Background: Aggressive Variant Prostate Cancers (AVPCs) are incurable malignancies. Platinum-based chemotherapies are used for the palliative treatment of AVPC. The Polycomb Repressive Complex 2 (PRC2) promotes prostate cancer progression *via* histone H3 Lysine 27 tri-methylation (H3K27me3). *EZH2* encodes the catalytic subunit of PRC2. A recently developed nucleosome capture technology (Nu.Q®).measures H3K27me3 levels in biological fluids. EZH2 inhibitors (EZH2i) are being tested in clinical trials. We hypothesize that epigenetic reprogramming *via* EZH2i improves the efficacy of Carboplatin in AVPC and that EZH2i activity can be measured via both cellular- and cell-free nucleosomal H3K27me3 (cf-H3K27me3) levels.
Methods: We studied the expression of PRC2 genes in clinical prostate cancer cohorts (bioinformatics). We determined the effect of EZH2i on cellular- and cf-H3K27me3 levels. We measured dose-dependent effects of Carboplatin with/without EZH2i on AVPC cell viability (IC_{50}). We used RNA-Seq to study how EZH2i modulates gene expression in AVPC cells.
Results: PRC2 genes were significantly up-regulated in AVPC *vs* other prostate cancer types. EZH2i reduced both cellular and cf-H3K27me3 levels. EZH2i significantly reduced Carboplatin IC_{50}. EZH2i reduced the expression of DNA repair genes and increased the expression of p53-dependent pro-apoptotic factors.
Conclusions: EZH2i plus Carboplatin is a promising combination treatment for AVPC.

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KEYWORDS
EZH2; epigenetics; Tazemetostat; personalized epigenetic therapies; Carboplatin; aggressive variant prostate cancer; neuroendocrine prostate cancer

1. Introduction

Prolonged hormonal therapies induce phenotypic plasticity and multi-drug resistance in advanced prostate cancer [1]. Malignancies emerging from this phenomenon are androgen receptor (AR)-negative or AR-indifferent and have been classified as: (I) neuroendocrine prostate cancers (NEPCs), which express typical trans-differentiation markers (e.g., synaptophysin, FOXA2 [2]); (II) double negative prostate cancers, which are both AR-negative/-indifferent and NEPC-negative [3]; (III) aggressive-variant prostate cancers (AVPCs), which include adenocarcinomas and NEPCs that meet clinical and molecular criteria for enhanced metastatic ability and for androgen indifference [4]. Here, we will use the broader term "AVPC" to refer to all the aforementioned malignancies. Despite extensive research efforts, there is no effective treatment for AVPC. The most common palliative treatment for these patients is platinum-based therapy, which typically results in > 50% objective response rates but does not significantly improve overall survival chances (the median overall survival for these patients is shorter than 24 months) [5].

The enhancer of zeste homolog 2 (*EZH2*) *locus* encodes a methyltransferase that acts as part of the Polycomb-repressive complex 2 (PRC2) [6]. The other canonical components of PRC2 are the proteins encoded by Embryonic Ectoderm Development (*EED*) and Suppressor of zeste 12 (*SUZ12*). In the nucleus, PRC2 induces gene silencing via histone H3-Lys27 trimethylation (H3K27me3), a repressive post translational modification. EZH2 was first described as a driver of prostate cancer progression by Varambally et al. [7]. Among other results, the study by Varambally et al. showed that EZH2 silencing caused a dramatic reduction

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Article highlights

- Polycomb-mediated gene silencing promotes prostate cancer progression.
- Aggressive-variant prostate cancers (AVPCs) are characterized by increased activity of the Polycomb-Repressive Complex 2 (PRC2).
- Here we show that PRC2 inhibitors are scarcely effective as single-agent in ACPC cells.
- However, the combination of PRC2 inhibitors and Carboplatin is highly synergistic.
- RNA Seq studies revealed that PRC2 inhibitors enhance Carboplatin activity by modulating several key pathways, including DNA repair and apoptosis.

in AR-negative prostate cancer cell proliferation. We and others showed that EZH2 is up-regulated in NEPC and drives prostate cancer metastasis, phenotypic plasticity and proliferation [8–10]. Recently, several inhibitors of EZH2 have been developed and tested in clinical trials [11]. Notably, one of these inhibitors is being tested in combination with hormonal therapies in prostate adenocarcinoma (Clinical Trial: NCT03480646). However, the role of EZH2 inhibitors in AVPC therapy, particularly in combination with platinum agents, has never been explored. Here we used prostate cancer cell lines representing the main subtypes of AVPC, including DU-145 which is AR- and NEPC-negative [12] and OPT7714 which is AR-indifferent and expresses typical NEPC markers [13]. Given the heterogeneity of AVPC, it is important to test new therapies in combination with diagnostic technologies that can facilitate patient selection and treatment monitoring. We have recently demonstrated the potential clinical usefulness of a quantitative immunoassay technology that measures nucleosomal H3K27me3 levels in biological fluids (including plasma) [14]. Here we have tested this technique to measure nucleosomal H3K27me3 levels in cell supernatants and compared it with the traditional methodology of measuring H3K27me3 in cellular extracts by western blotting. This approach has allowed us to determine whether the nucleosome immunoassay technology can accurately predict the pharmacodynamic activity of EZH2 inhibitors.

Our results indicate that EZH2 inhibitors potentiate the anticancer activity of platinum agents and provide a potential mechanistic framework to explain this observation.

2. Materials and methods

2.1. Gene expression datasets

Bioinformatic analyses were conducted through the CbioPortal dataset (cBioPortal for Cancer Genomics). The correlation between PRC2 gene expression and AR-activity /NEPC features e was investigated in the "Metastatic Prostate Adenocarcinoma-SU2C-PCF" dataset (containing both adenocarcinomas and NEPC samples). The EZH2 pathway analysis and the correlation between PRC2 genes and NEPC markers was performed by analyzing the Cbioportal "Neuroendocrine Prostate Cancer-Multi Institute" dataset.

2.2. Cell culture:

The human cell line DU-145 (Cellosaurus Accession: CVCL_0105) is derived from a central nervous system metastasis and is a model of double-negative prostate cancer since it is both AR-negative and NEPC marker-negative (American Type Culture Collection (ATCC)). Being AR-negative and derived from a soft tissue metastasis, DU-145 cells are a clinically relevant model of AVPC [4]. DU-145 cells were cultured in RPMI 1640 (Gibco®, Thermo Fisher) supplemented with 10% (v/v) of heat-inactivated Foetal Bovine Serum (FBS) (Thermo Fisher) and 1% of Penicillin-Streptomycin (Pen/Strep) (Gibco®, Thermo Fisher).

AR-negative, OPT7714 (murine cell line) is a model of NEPC generated at *Istituto Nazionale dei Tumori* [13]. These cells were cultured in DMEM, high Glucose (Gibco®, Thermo Fisher) supplemented with 10% (v/v) of heat inactivated FBS (Thermo Fisher), 1% (v/v) of Pen/Strep (Gibco®, Thermo Fisher), sodium pyruvate (Gibco®, Thermo Fisher), and HBSS. No calcium, magnesium, nor phenol red (Gibco®, Thermo Fisher) was used in the culture medium.

Results obtained with the two aforementioned cell lines were further validated in human Kucap-13 cells (Cellosaurus accession CVCL_C0UV), which are a newly characterized patient-derived NEPC cell line. This cell line is the first human model of treatment-emergent NEPC. Kucap-13 cells were cultured as described in the original publication [15].

2.3. EZH2 inhibitors

Three clinically tested EZH2 inhibitors were purchased from MedChemExpress: Tazemetostat (HY-13803), GSK-126 (HY-13470), and CPI-1205 (HY-100021).

2.4. Cell quantification

Cells were trypsinized and collected for cell count by Luna automated cell counter (Logos Biosystems) in accordance with the manufacturer's instructions.

2.5. H3K27me3 measurement in cell extracts

Subconfluent cultured cells were treated with DMSO (vehicle), Tazemetostat (1, 5, and 10 µM), GSK-126 (1, 5, and 10 µM), and CPI-1205 (1, 5, and 10 µM) for 72 hours. Cells were collected by scraping, lysed in RIPA lysis buffer [20 mm Tris-HCl (pH 8.0), 150 mm NaCl, 1 mm EDTA, 0.1% SDS, 1% Igepal, 50 mm NaF, 1 mm NaVO3] containing a protease inhibitor cocktail (Merck). Protein concentration of the cell lysate was measured using the Pierce™ Gold BCA protein assay kit (Thermo Fisher).

2.6. Western blotting

The extracted proteins were resolved by gel electrophoresis on reducing SDS-polyacrylamide gels on 10–20% Tris-Glycine gels (Thermo Fisher Scientific) and transferred to nitrocellulose membranes (Fisher Scientific) following reported protocol [16]. The membranes were incubated overnight at 4°C with either anti-H3K27me3 (1:1000, Cell Signalling Technology, Tri-Methyl-

Histone H3 (Lys27) (C36B11) Rabbit mAb) diluted in 5% (w/v) BSA, or anti-GAPDH (1:10000, Sigma Aldrich) diluted in 5% dried milk.

2.7. Nucleosomal H3K27me3 measurement in cell supernatant

Cell lines were exposed to Tazemetostat, GSK-126, and CPI-1205 at 10 µM 24 hours after cell seeding. The supernatants were collected on day 3, 5, 7, and 10 after treatment, and the levels of nucleosomal H3K27me3 quantified by Nu. Q-H3K27me3 prototype colorimetric ELISA kit, (Belgium Volition SPRL). Nucleosomal vH3K27me3 levels were determined following the manufacturer's instructions.

2.8. Single-agent treatments with EZH2 inhibitors

Cell viability was determined for each cell line following exposure to different concentrations (25, 10, 1, and 0.01 µM) of EZH2 inhibitor or vehicle control (DMSO) for 5 days. The medium was replaced with fresh medium the same EZH2 inhibitors for another 5 days (the treatment lasted a total of 10 days).

2.9. Combination of platinum agents and EZH2 inhibitor

To estimate the effect of EZH2 inhibition in combination with Carboplatin (Sigma), or Cisplatin (MedChemExpress) we selected 10 µM GSK-126 because this concentration resulted in almost complete H3K27me3 ablation in at least one cell line, whilst not causing significant cell growth inhibition. Before testing the combination, we pre-treated cells with the EZH2 inhibitor for three days to ensure the occurrence of epigenetic reprogramming.

Hence, each cell line was treated with GSK-126 at 10 µM for 72 hours. Subsequently, the GSK-126 containing supernatant was replaced with a combination of GSK-126 (10 µM) and Carboplatin or Cisplatin (500, 100, 50, 10, 1, 0.1, 0.01 µM) for another 72 hours. The IC_{50} for each cell line was calculated using trypan blue exclusion cell counts. Caspase 3/7 activity was measured using the Promega Caspase-Glo assay and following Manufacturer's instructions.

2.10. Evaluation of bone marrow toxicity in vivo

C57BL/6 mice were purchased from Charles River Laboratories. 1×10^6 ST4787 NEPC cells [17] were injected on the left flank of mice. GSK-126 (30 mg/kg in saline solution) or the mock solution were administered intraperitoneally 3 days per week, for two weeks, starting when tumors reached 40–50 mm^3. Mice were sacrificed the day after the last treatment and spleens and bone marrows were collected. Cell-suspension was obtained from mechanical disgregation of spleens and from flushing of bone marrows. The antibodies used for the analysis are CD45 (Cat. 566168, BD Biosciences), CD11b (Cat. 566416, BD Biosciences) and CD3 (Cat. 100355, Biolegend). Fixable Viability Stain (FVS; BD Biosciences) was added to exclude dead cells from the analysis. Samples were acquired with BD LSRII FortessaTM and analyzed with the Flow Jo software. Animal housing and experimentation were performed following institutional guidelines and the Italian law (D.Lgs. 26/2014). *In vivo* experiments were approved by the Italian Ministry of Health (authorization number 8/2020-PR).

2.11. RNA extraction and RNA sequencing

Total RNA from DU-145 cells treated with GSK-126 or vehicle was extracted using the RNeasy Mini Kit in accordance with the manufacturer's instructions (Qiagen). NGS libraries were prepared with the Ion AmpliSeq Library Kit Plus (Thermo Fisher Scientific). For each sample, 100 ng of total RNA was reverse transcribed using the SuperScript VILO cDNA synthesis Kit (Thermo Fisher Scientific). The resulting cDNA was then amplified with the AmpliSeq Human Transcriptome Gene Expression Kit panel, targeting over 20,000 genes (>95% of the RefSeq gene database). Amplicons were digested with the proprietary FuPa enzyme (Thermo Fisher Scientific) to generate compatible ends for barcoded adapter ligation. The resulting libraries were purified using AmpureXP beads (Agencourt) at a bead to sample ratio of 1.5X and eluted in 50 µL low TE buffer. Libraries were then diluted 1:10000 and quantified by qPCR using the Ion Universal Quantitation Kit (Thermo Fisher Scientific). Individual libraries were diluted to a concentration of 50 pM, combined in batches of eight libraries, loaded on an Ion 540™ chip using the Ion ChefTM instrument and sequenced on an Ion S5™ (Thermo Fisher Scientific). Raw data was processed automatically on the Torrent ServerTM and aligned to the reference human genome reference build hg19 using the hg19 AmpliSeq Transcriptome fasta reference. Quality control was manually performed for each sample based on the following metrics; number of reads per sample > 14X106, valid reads > 95%. RNA Seq data were uploaded in the SRA database (NCBI): PRJNA1036550.

2.12. RNA-Seq analysis

Differential gene expression was performed using DESeq2 [18] and log-fold changes were shrunk using apeglm [19]. Gene Set Enrichment Analysis (GSEA) was performed using clusterProfiler [20]. Gene-pathway information was taken from the Molecular Signatures Database (MSigDB) package. Pathways with adjusted *p* value (p.adjust) values below 0.05 were retained for downstream analysis and visualization.

2.13. Statistical analyses

Experiments were conducted in three biological replicates. Data were assessed for normality using the Shapiro-Wilk test. Expression of mRNA levels from CbioPortal data and IC_{50} values were analyzed using a two-tailed Student`s t-test. H3K27me3 levels were analyzed using either a one-way ANOVA with Dunnett's post hoc test or a Kruskal-Wallis with Dunn's post hoc test. Data represent mean ± SEM and statistical analysis was performed via Graph Pad Prism 8.

2.14. Ethical approval

Animal experiments to test the myelosuppressive action of EZH2 inhibitors (Istituto Nazionale Tumori) were conducted in accordance with local regulations and upon approval by Italian Ministry of Health (authorization number 8/2020-PR).

Patient Derived Xenograft (PDX) experiments (RNA Sequencing) were conducted according to protocol #A17–0165, which was approved by University of British Columbia Animal Care Committee.

3. Results

3.1. Clinical significance of PRC2 genes in aggressive-variant prostate cancer

To investigate the clinical significance of the three main components of PRC2 (EZH2, EED, SUZ12), we queried a public dataset containing histopathological, clinical, and transcriptomic information on a cohort of metastatic prostate cancer patients [21]. Since the two main types of AVPC are characterized by reduced AR activity and/or NEPC histopathological features, we analyzed the association between each PRC2 gene and these two characteristics. All three PRC2 genes were significantly upregulated in clinical samples with histopathological features of NEPC, compared to samples with no NEPC features (Figure 1(a)). We also found a significant negative correlation between the expression of EED and EZH2 (but not SUZ12) and the "AR activity score" of clinical samples (Figure 1(b–d)). This score is a transcriptome-based estimation of AR activity, which has been calculated as previously described [22].

We then conducted a transcriptome analysis on a different dataset of clinical NEPC samples, to identify pathways that were commonly associated with EZH2 expression. This analysis showed that some key oncogenic pathways were significantly associated with EZH2 expression in NEPC samples (Figure 1(e)). Most of the enriched pathways were correlated with cell cycle (G2 and M phase), DNA repair, and chromosome maintenance. The same dataset was interrogated to study correlations between PRC2 gene expression and individual NEPC markers (SYP, ENO2). The analysis provided an independent confirmation of the strong positive correlation between PRC2 genes and almost NEPC markers (5 out of 6 significant correlations, Suppl. Tab. S1).

3.2. EZH2 inhibitors are effective in AVPC cells

In light of the bioinformatic results, we investigated whether the three clinically tested EZH2 inhibitors Tazemetostat, GSK-126, and CPI-1205 were active in NEPC cells (OPT7714) and in AR-negative cells that do not express NEPC markers (DU-145) [23]. Western blot results demonstrated that all EZH2 inhibitors reduced H3K27me3 (a marker of PRC2 activity), although to a variable degree between cell types. In DU-145 cells, GSK-126 significantly decreased H3K27me3 compared to cells exposed to vehicle (Figure 2(a)), whilst an almost complete suppression of H3K27me3 expression was observed in OPT7714 cells at

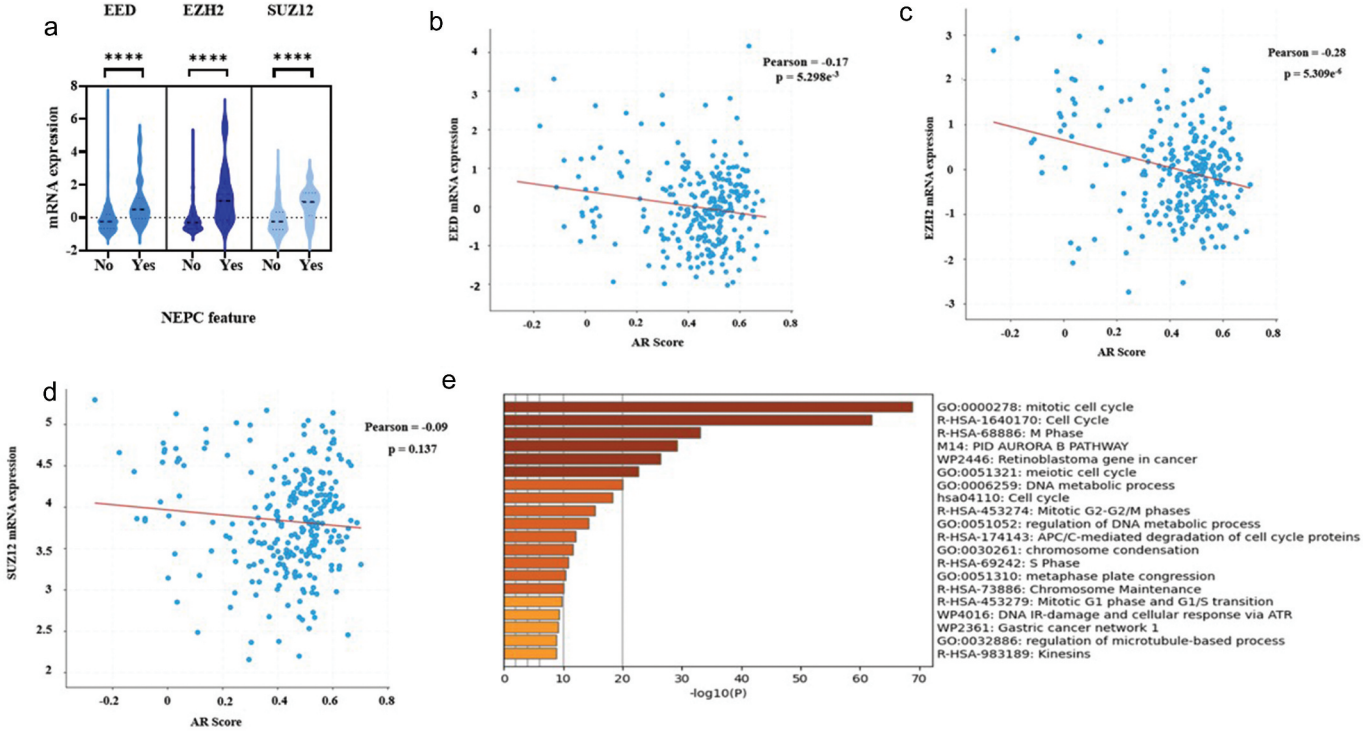

Figure 1. (a) Expression of EED, EZH2, and SUZ12 mRNA levels in $n = 264$ PCa samples (CbioPortal, accessed on 10/01/2023) with or without NEPC features. **** = $p < 0.0001$, two-tailed Student t-test Correlation between gene expression and AR activity score for genes (b) EED, (c) EZH2 and (d) SUZ12. Analises for a-d were conducted using the Metastatic Castration Resistant Prostate Cancer dataset on Cbioportal (e). Pathway analysis of genes significantly co-expressed with EZH2 in the Cbio-portal Neuroendocrine Prostate Cancer-Multi Institute dataset (cases were defined based on cell morphology and expression of NEPC makers). Genes with Pearson correlation coefficient > 0.85 (EZH2) were downloaded in Metascape for pathway analysis (https://metascape.Org/gp/index.Html#/main/step1).

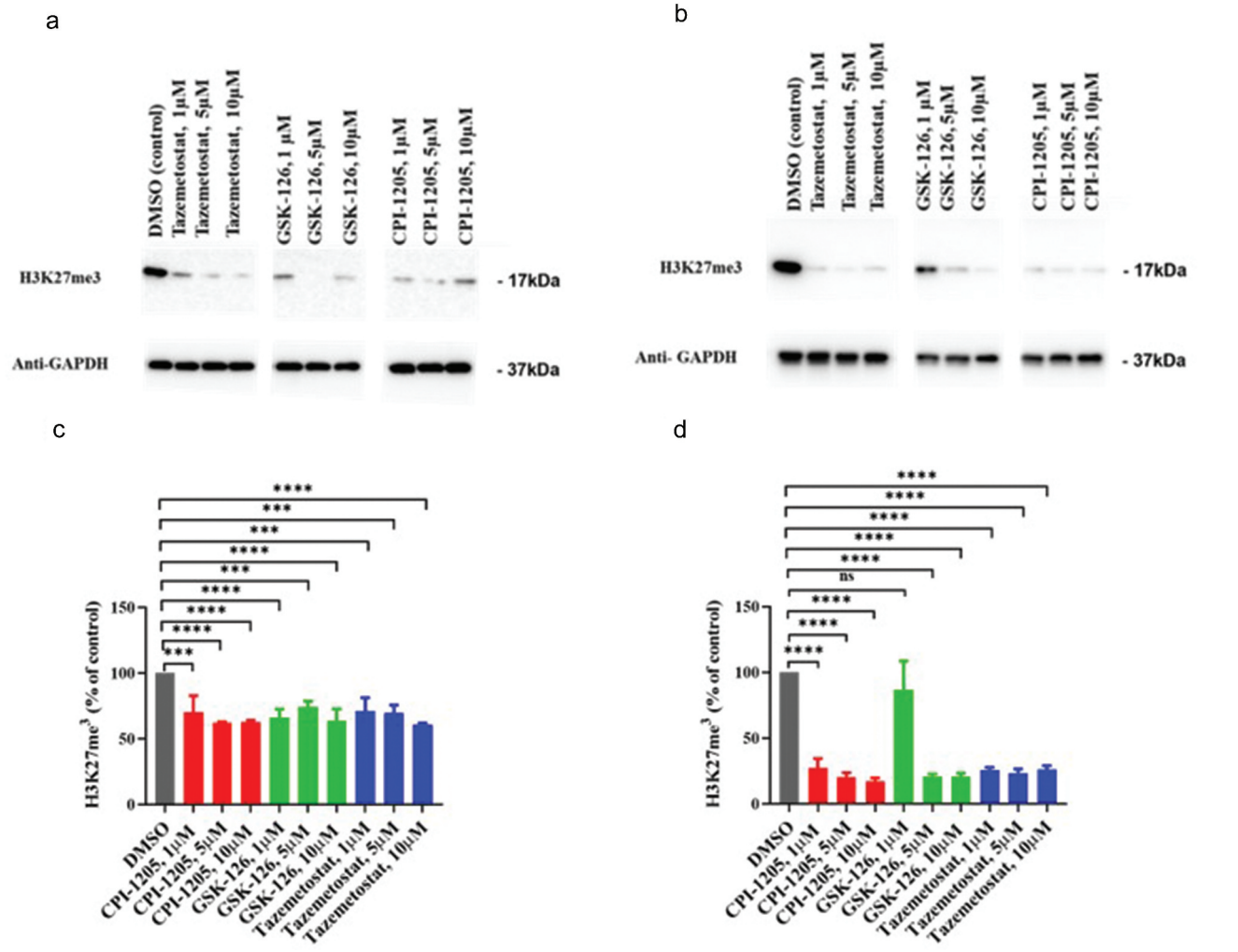

Figure 2. H3K27me3 levels in DU-145 (a and c) and OPT7714 cells (b and d) exposed to different EZH2 inhibitors (upper panel, representative image; lower panel statistical analysis of three quantified blots). Cells were treated with DMSO (vehicle), Tazemetostat 1, 5, and 10 μM, GSK-126 1, 5, and 10 μM, and CPI-1205 1, 5, and 10 μM for 72 hours; cellular protein extracts were analyzed by immunoblotting using anti-H3K27me3 and anti-GAPDH antibodies. Nu.Q Volition-measured H3K27me3 levels in the supernatant of DU-145 (c) and OPT7714 (d) cells in the same conditions described above. ***$p < 0.001$ and ****$p < 0.0001$ one-way ANOVA with Dunnett post-hoc test (c and d).

concentrations of 5 μM or higher of Tazemetostat, GSK-126 and CPI-1205 (Figure 2(b)). We also tested whether the reduction in H3K27me3 was due to a reduction in total histone H3 levels. The ratio of Nucleosomal H3K27me3/total nucleosomal H3.1 was determined in cells treated with GSK-126 or DMSO control. Our results show that the relative levels of H3K27me3/total H3.1 were significantly reduced in cells exposed to the EZH2 inhibitor (Suppl. Fig.S1).

To explore whether levels of nucleosomal H3K27me3 in biological fluids could predict the activity of the EZH2 inhibitors, we used a novel ELISA (Nu.Q) to measure the levels of this histone modifications in cell-free nucleosomes from cellular supernatants. Treatment of AVPC cells with the EZH2 inhibitors caused a significant reduction in cell-free nucleosomal H3K27me3 levels (Figure 2(c,d)). Notably, the reduction in cell-free H3K27me3 levels was more pronounced in OPT7714 than in DU-145 cells, which was consistent with the Western blot results from cellular extracts.

3.3. EZH2 inhibition increases the anticancer activity of Carboplatin in AVPC cells

Having confirmed the pharmacodynamic activity of the three EZH2 inhibitors on H3K27me3 levels, we then assessed their antiproliferative effects. As shown in Figure 3(a,b), treatment with EZH2 inhibitors alone (without Carboplatin) reduced the cell count of AVPC cells only at higher concentrations, particularly with GSK-126. We then exposed the cells to a fixed dose of GSK-126 (10 μM), followed by Carboplatin at different concentrations, or Carboplatin alone (Figure 3(c,d)). As shown in Figure 3(e,f), the combination treatment showed a significant IC_{50} reduction in both cell lines, compared to Carboplatin alone. A combination matrix analysis in DU-145 cells confirmed that the interaction between Carboplatin and GSK-126 is synergistic (Suppl. Fig. S2).

To further corroborate our hypothesis, we sought to replicate our findings in a patient-derived model of treatment emergent NEPC (Kucap-13). These cells were treated with either Carboplatin alone or Carboplatin plus GSK-126 at a fixed dose. We found that also in this model (which grows in spheroid-like

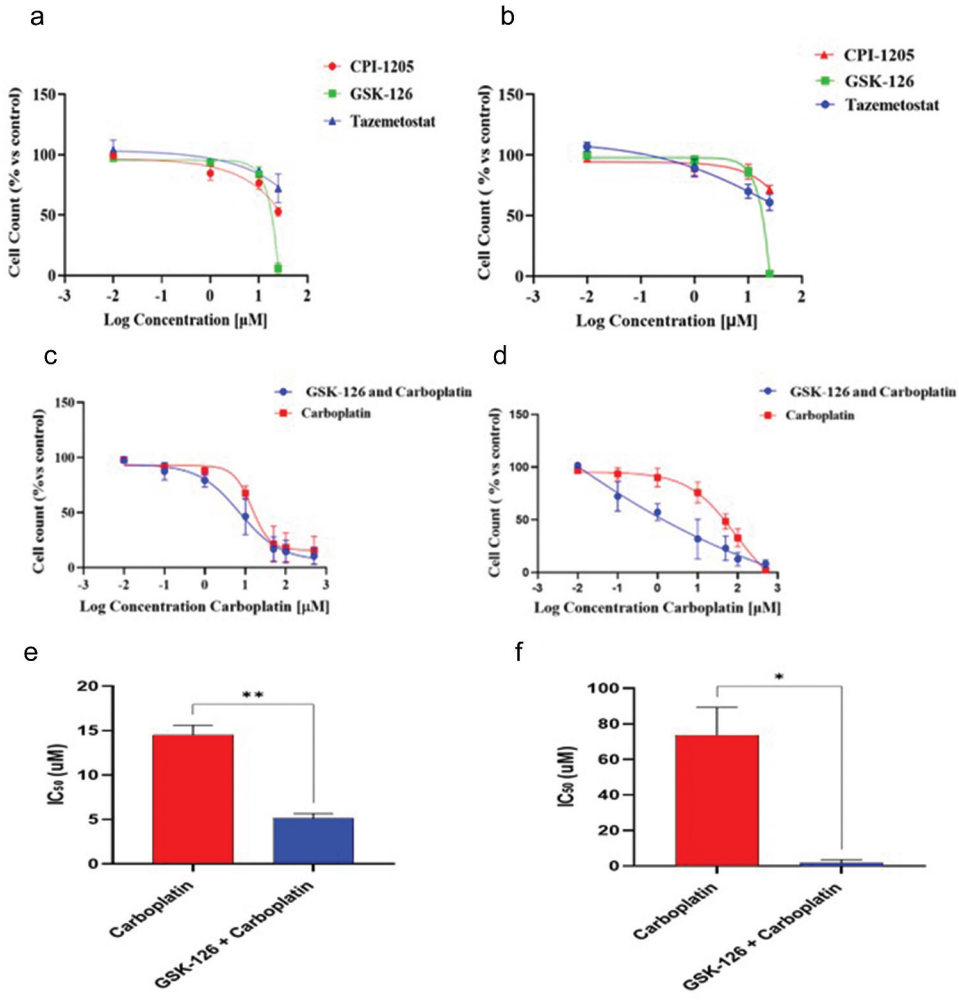

Figure 3. Growth inhibitory effects of Tazemetostat, GSK-126, and CPI-1205 on DU-145 (a) and OPT7714 (b) cells. The cells were counted after exposure to different concentrations of EZH2 inhibitor (0.01, 1, 10, and 25 µm), or DMSO for 10 days. Cell viability of DU-145 (c) and OPT7714 (d) treated with combination of GSK-126 and Carboplatin vs Carboplatin alone. Cells were exposed to 10 µm GSK-126 for 72 hours, followed by GSK-126 (10 µm) and Carboplatin (different concentrations as indicated by the X axis) for the following 72 hours. Cells were counted at the end of the experiment. IC_{50} values of DU-145 (e) and OPT7714 (f) treated with a combination of GSK-126 and Carboplatin vs Carboplatin alone. ***p<.05, ****p<.01, two-tailed Student's t-test.

aggregates) the combination induced a significant reduction in cell viability (Figure 4(a)) and an increase in apoptosis (Figure 4(b)) compared to Carboplatin only. To generalize our findings, we assessed the IC_{50} of an additional platinum agent (Cisplatin) in Kucap-13 cells which were pre-exposed (or not) to GSK-126. We found that EZH2 inhibition led to a > 5-fold reduction in Cisplatin IC_{50} (Figure 4(c)). Notably, the EZH2 inhibitor Tazemetostat does not show significant anti-proliferative effects in this cell line (single-agent treatment) [15]. Since EZH2 gene deletion is associated with myelosuppression, we assessed the frequency of myeloid ($CD11b^+$) and lymphoid ($CD3^+$) immune cell subsets in prostate cancer bearing mouse models treated with the EZH2 inhibitor GSK-126, at doses that reduce H3K27me3 [24]. As shown in Suppl. Fig. S3, we found no significant alteration of myeloid and lymphoid cell subset neither in the bone marrow nor in the periphery (spleen). Taken together, these results show that whilst EZH2 inhibitors might have limited effects as monotherapy, they significantly enhance the anti-proliferative activity of Carboplatin in AVPC cells and that EZH2 inhibitors do not cause substantial myelosuppression in vivo.

3.4. EZH2 inhibition reduces the activity of DNA repair genes

To provide a molecular explanation for the cellular response shown in Figure 3, we performed RNA sequencing on DU-145 cells exposed to GSK-126 vs control (DMSO). This analysis confirmed that EZH2 inhibition causes a profound alteration of the cell's transcriptome. Among the differentially expressed genes (DEGs), we observed more up-regulated than down-regulated transcripts upon EZH2 inhibition (Figure 5(a)). However, DEGs contained a mix of oncogenic and onco-suppressive genes. A GSEA revealed that EZH2 inhibition modulates several biological functions: in most cases this led to the activation of pathways, as shown by positive enrichment scores (Figure 5(b)). The balance between the activation of pro-apoptotic and anti-apoptotic (e.g., KRAS) pathways provides a potential explanation for the modest effects of EZH2 inhibitor as single-agents on cell growth. Notably, we found that EZH2 inhibition significantly reduced the activity of DNA repair pathways (Figure 5(b); negative enrichment score).

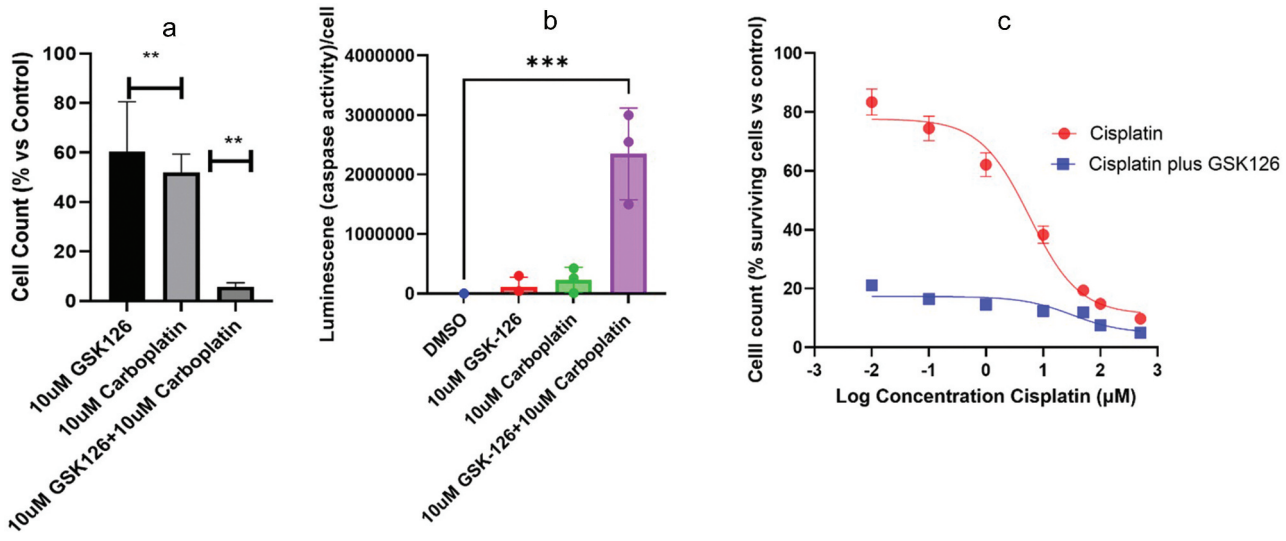

Figure 4. Cell viability in Kucap-13 NEPC spheroids exposed to GSK-126 and platinum agents. (a,b) Cells were treated at fixed doses of GSK-126 and Carboplatin as described in the Methods. At the end of the experiments, cell viability was measured with the Luna system (a) or with the Caspase-Glo 3/7 assay (Promega-b) Statistical analysis: $^{**}p < 0.01$; $^{***}p < 0.001$ (One-way ANOVA with Dunnett's multiple comparisons test). C) Cell viability of Kucap-13 cells treated with combination of GSK-126 and Cisplatin vs Cisplatin alone, as indicated in Methods. IC_{50} (Cisplatin plus GSK-126) = 5.78 µM; IC_{50} (Cisplatin) = 31.99 µm.

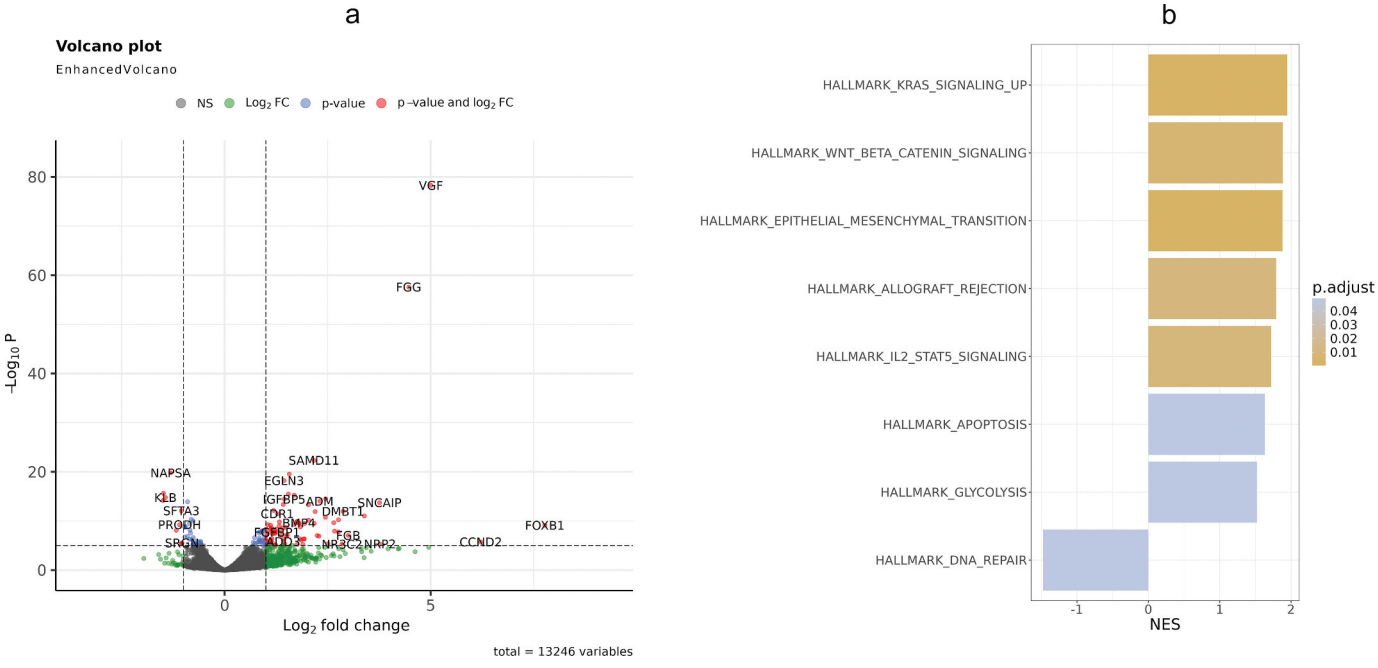

Figure 5. Transcriptome analysis of DU-145 cells exposed to EZH2 inhibitor. (a) Volcano plot of RNA Seq from cells exposed to GSK-126 vs control. Differentially expressed genes are colored in red if they are above the fold-change and -Log (p.Adjust-value) threshold; genes colored in green are only above the -Log (p.Adjust value) threshold. The names of some highly up- and down-regulated genes are shown in the plot. (b) a comparison of normalized enrichment scores (NES) that are significantly different in cells exposed to GSK-126 or control. A positive NES indicates up-regulation of the pathway in cells treated with GSK-126. A negative NES indicates down-regulation of the pathway in cells treated with GSK-126.

To gain further mechanistic insight, we plotted a heatmap of the top-20 up-regulated and down-regulated genes upon treatment with GSK-126 (Suppl. Fig. S4). GSK-126-dependent gene modulation was confirmed at protein level for one of the up-regulated (VGF) and one down-regulated (PTGES) protein (Suppl. Fig. S5). We then measured the expression of 5 of the top-20 genes down-regulated upon EZH2 inhibition in a series of high-fidelity prostate cancer patient-derived xenografts (PDXs), including castration-resistant adenocarcinomas and NEPCs. We found that most of these target genes were highly expressed in advanced prostate cancer PDXs (Suppl. Tab. S2). Notably, whilst the expression of the AR was elevated only in adenocarcinomas, PRC2 genes and most genes down-regulated upon EZH2 inhibition showed a high level of expression in all PDXs. These in vivo results corroborate the validity of our in vitro findings.

It is therefore conceivable that in addition to DNA repair inhibition, the GSK-126-dependent epigenetic reprogramming activates several drug-sensitizing pathways.

4. Discussion

In this study, we have shown that EZH2 inhibition can increase AVPC cell sensitivity to Carboplatin. To the best of our knowledge, this is the first demonstration of potential synergism between EZH2i and platinum in AVPC. Our experiments have been carried out in cell lines that represent different subtypes of AVPC (neuroendocrine and double negative) and could therefore represent a substantial fraction of clinical metastatic prostate cancers. The combination of GSK-126 and Carboplatin effectively reduced live cell count compared to the agents alone in three different preclinical models, showing at least additive effects. The translational potential of this combination should be addressed in further studies, where the pharmacokinetics of the treatments should be tested in preclinical and patient-derived models.

EZH2 inhibitors potentiate the activity of several anticancer drugs. For example, we showed that the first EZH2 inhibitor (DZNeP) increases pancreatic cancer sensitivity to gemcitabine [25]. More recently, the combination between GSK-126 and Ataxia-Telangiectasia Mutated (ATM) inhibitors was identified as synthetically lethal in breast cancer [26]. PRC2 directly silences several anticancer genes, whilst also indirectly activating numerous cancer-promoting pathways [27]. This paradigm is reflected by our RNA Seq data, showing that EZH2 inhibition resulted in both gene reactivation (most likely because of reduced H3K27me3 at specific *loci*) and gene down-regulation (most likely as an indirect or non-canonical effect). It is therefore conceivable that EZH2 inhibitors can have diverse effects on different cancer cell types. In this study, we showed that EZH2 inhibition impairs the expression of several DNA repair genes that are responsible for resistance to platinum-based chemotherapy. For example, ERCC1 is directly involved in the nucleotide excision repair pathway, which is activated by platinum-dependent DNA damage [28]. Intra-tumoral ERCC1 expression is inversely correlated with clinical outcomes in small cell lung cancer patients treated with Cisplatin, where lower expression is correlated with better response [29]. Notably, additional analysis of our RNA-Seq data indicated that other pathways could synergize with reduced DNA repair, thereby providing a further explanatory framework for the observed interaction between EZH2 inhibitors and Carboplatin. For example, GSK-126 treatment up-regulated VGF protein (Suppl. Fig. S4), which promotes prostate cancer senescence [30]. In turn, cellular senescence mediates the anticancer effect of platinum chemotherapy [31]. On the other hand, we validated the GSK-126-dependent down-regulation of PTGES (Suppl. Fig. S4), a pro-survival gene that was shown to induce chemotherapy resistance in cancer cells [32]. We acknowledge that these results were obtained in one cell line, and that other mechanisms may be at play in cells with a different genetic background. In addition, pharmacological and mechanistical results were obtained in AVPC cells, whose molecular profile is different from that of prostate adenocarcinoma cells. Hence our results cannot be extended to prostate adenocarcinomas.

Taken together, these results suggest that EZH2 inhibition, whilst not lethal *per se*, induces significant vulnerabilities in AVPC cells. It would be interesting to systematically explore other synthetically lethal combinations in this incurable cancer.

Our results also suggest a significant positive correlation between cell-free and cellular H3K27me3 post translational modification levels in nucleosomes. We described the potential prognostic value of circulating nucleosomes [14]. Here, we show that cell-free nucleosomal H3K27me3 can be detected in the supernatant from AVPC cell culture and that H3K27me3 levels decrease upon treatment with EZH2 inhibitors. Notably, the levels of cell-free H3K27me3 nucleosomes measured with the Nu.Q assays are consistent with the results obtained with standard western blot from cellular protein extracts. This suggests that the nucleosome immunoassay technology could be useful for patient selection and therapeutic monitoring. We have proposed this strategy in a previous publication [33].

Our *in vitro* evidence shows that EZH2 inhibition enhances the activity of platinum-based chemotherapies; however, further studies are needed to clarify how EZH2 inhibitors affect the local chromatin status of specific genetic *loci*, and how this translates into transcriptomic changes leading to reduced DNA repair efficiency. Our results also indicate that PRC2 genes are over-expressed in both NEPC and AR-negative cancers. However, these transcriptomic data (Figure 1) will need to be confirmed at protein level to increase the translational significance of our findings. Importantly, our synergy results were obtained *in vitro* and cannot be directly extrapolated to *in vivo* efficacy. Hence, future in vivo studies are warranted on this combination.

Another potential limitation of our study relates to the use of clinically available EZH2 inhibitors. We have decided to use these compounds because we wanted to generate clinically relevant results. In particular, we have selected three compounds that were being used in clinical trials at the beginning of our study. Notably, both the concentrations of GSK-126 and the IC_{50} concentrations of Carboplatin (combination) employed in this study are clinically achievable, as demonstrated by clinical studies [34,35]. Hence the GSK-126 plus Carboplatin schedule could be clinically tested However, currently available EZH2 inhibitors have some limitations. It is well known that EZH2 has three main functions: the canonical PRC2-dependent H3K27 methyl-transferase activity; non-canonical methyl-transferase activities (which are PRC-2-independent and target non-histone proteins); methylation-independent functions such as interacting with transcription factors and signaling molecules [36]. Whilst all these functions may be relevant to prostate cancer progression, clinically tested EZH2 inhibitors block the canonical and non-canonical methyl-transferase activity of EZH2, but not the methylation-independent functions [11]. Hence it is conceivable that future inhibitors, with a broader spectrum of action will be more effective in AVPC.

Further research could also elucidate the role of EZH2 inhibitors alone or in combination with chemotherapy *in vivo* to increase the translational clinical potential of this study.

5. Conclusions

These results suggest that clinically tested EZH2 inhibitors increase Carboplatin sensitivity in AVPC cells and that combinations of these compounds may be effective in different

subtypes of AVPC. EZH2 inhibition, using three different drugs, strongly reduces H3K27me3 levels in AVPC cells and may affect DNA repair and other cancer-relevant pathways. We also showed that the activity of EZH2 inhibitors could be monitored by a novel nucleosome immunoassay technology, via detection of secreted levels of H3K27me3-modified nucleosomes from AVPC cells.

Author contributions

Maryam Latarani: investigation, formal analysis; Perla Pucci: investigation, formal analysis, writing-first draft and editing; Mark Eccleston: conceptualization, writing-editing; Massimiliano Manzo and Ilaria Alborelli: data curation and formal analysis, writing-editing; Namra Mahmood: formal analysis, writing-editing; Priyadarsini Gangadharannambiar: validation and formal analysis; Irene Fischetti: validation and formal analysis; Mario Paolo Colombo: methodology, writing-editing; Vera Mongiardini: validation and formal analysis; Benedetto Grimaldi: validation and supervision, writing-editing; Sushila Rigas and Cheryl Hawkes: supervision, writing-editing; Shusuke Akamatsu: methodology, writing-editing; Yuzhuo Wang: conceptualization, supervision; Elena Jachetti: methodology, writing-editing; Francesco Crea: supervision, conceptualization, writing-first draft.

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Disclosure statement

Dr Mark Eccleston is a shareholder in Volition and paid consultant as well as a named inventor on several Volition patents. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Ethical declaration

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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Data availability statement

RNA Seq data were uploaded in the SRA database (NCBI): PRJNA1036550.

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