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Original Article

The proteasome inhibitor ixazomib targets epigenetic chromatin modification enzymes upregulated by m2c macrophage polarisation in lung cancer*

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ABSTRACT

Background and Aims: Poor prognosis in lung cancer is associated with tumor-associated macrophages (TAMs) that exhibit M2c-like behaviours that support multiple pathways in the tumour microenvironment. The interplay between epigenetic modifications and the ubiquitin-proteasome pathway involves three key mechanisms: regulation of epigenetic enzymes by ubiquitin, interaction between ubiquitin and epigenetic modifiers, and epigenetic silencing of critical genes involved in cellular processes. Therefore, we investigated the effects of ixazomib, a proteasome inhibitor, on gene expression changes in epigenetic chromatin modification enzymes in a co-culture of M2c macrophages and A549 lung cancer cells.

Methods: The IC_{50} concentration of ixazomib was determined to be 2.19 µM using a real-time cell analyser. THP-1 monocytes were polarised into M0 macrophages with 100 ng/mL phorbol 12-myristate 13-acetate (PMA), rested, and then exposed to 1 mM hydrocortisone to become M2c macrophages. A549 cells were seeded in the lower chamber of a co-culture plate. M2c macrophages were then co-cultured with A549 cells for 24 h with or without 2.19 µM ixazomib. After being isolated, mRNA was converted to cDNA and analysed using a gene panel with RT-PCR.

Results: The findings showed that 56 genes had exceptionally high expression levels (up to 1848-fold. Ixazomib downregulated these overexpressed genes.

Conclusion: Ixazomib effectively modulates the expression of genes involved in epigenetic chromatin modification in the lung cancer microenvironment, indicating its utility in lung cancer therapy. Further studies are needed to explore the combined use of epigenetic drugs and proteasome inhibitors.

Keywords: Macrophage, polarisation, epigenetics, ixazomib, lung cancer.

INTRODUCTION

Macrophages are functionally complex players in adaptive and innate immune responses to pathogens, tissue regeneration and development. Macrophages adopt a variety of polarisation states, which have been characterised as M1- and M2-polarized subtypes, to facilitate this. Understanding phenotypic heterogeneity and transcriptional regulation is important because in many disorders, macrophages have emerged as essential because of their essential phagocytic function in defence and homeostasis (Guerriero, 2018).

Tumour-associated macrophages (TAMs) can contribute up to half of the tumour mass and exert potent effects on metastatic processes and tumour growth. Poor clinical outcomes have been linked to high numbers of cells with macrophage-associated markers in several solid tumours (Hu et al., 2016). Among other types of cancer, M2-like macrophages outnumber M1-like macrophages in lung cancer (Zhang et al., 2011). Based on the features that stimulated their polarisation, M2 macrophages are further classified as M2a, M2b, and M2c. Poor prognosis is caused by macrophage acquisition of M2a- and M2c-like phenotypes in the microenvironment of lung cancer, which promote epithelial–mesenchymal transition, cell infiltration, and tumour growth. M1-like macrophages, on the other hand, cause less lung cancer cell proliferation, lower angiogenesis, and apoptosis (Atri, Guerfali, & Laouini, 2018).

Nearly all cellular processes, including cell cycle, gene transcription and translation, survival and apoptosis, cell

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metabolism, and protein quality control, are dependent on the ubiquitin-proteasome pathway or partially modified transcription factors. Growing evidence indicates that the ubiquitin-proteasome system controls epigenetic changes. Recent studies have demonstrated that, in comparison to non-epigenetic regulatory mechanisms like genetic modifications or conventional signal transduction pathways, epigenetic regulation is equally important for practically both for pathological conditions like cancer and all biological processes (Yerlikaya, Kanbur, Stanley, & Tümer, 2021). Epigenetic regulation modifies gene function without modifying the DNA sequence. Chromatin conformation regulates the accessibility of DNA and thus the binding elements of transcription, gene expression, and phenotype. Closely packed chromatin, also known as heterochromatin, has poor DNA accessibility and is linked to gene silencing. On the other hand, open-chromatin conformation of DNA is easily accessible, facilitating transcription factor binding and gene expression (Baardman, Licht, De Winther, & Van den Bossche, 2015). A potential connection between the environment, metabolism, gene regulation, and the consequent macrophage polarisation state is made feasible by epigenetic enzymes using the metabolites created by cell metabolism (Van den Bossche, Neele, Hoeksema, & De Winther, 2014).

Ixazomib, a medication that is orally bioavailable and has a lower toxicity profile, has been designed to reversibly, selectively, and potently inhibit proteasomes (Xie, Wan, Liang, Zhang, & Jiang, 2019). Ixazomib is widely used for the treatment of multiple myeloma, a type of cancer that affects plasma cells in bone marrow. It is used in combination with lenalidomide and dexamethasone to treat relapsed or refractory multiple myeloma (Lee et al., 2024). Considering these findings, the effects of M2c macrophage polarisation on genes associated with epigenetic chromatin regulation in lung cancer and the therapeutic effect of ixazomib on this pathway were investigated.

MATERIAL AND METHODS

Cell Culture Studies

Human lung cancer A549 (ATCC® CCL-185TM) and human monocyte THP-1 (ATCC® TIB-202) cells were grown in RPMI-1640 growth media containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humid incubator containing 5% CO_2.

Proliferation assay using a real-time cell analysis system

By monitoring the impedance changes of linked cells and generating a cell index (CI) value, the real-time cell analysis system (RTCA DP) system continuously analyzes cell behaviour. Every hour during the experiment, the well impedance for each group and concentration was measured and converted into a CI value. Cell proliferation in A549 lung cancer cells was investigated, and the IC_{50} doses of ixazomib were calculated using this system.

The cells were seeded at a density of 1x104 cells per well on 16-well E-plates in 100 µL media for this purpose. After 24 h of incubation, the instrument was stopped, and the current medium was replaced with fresh medium containing ixazomib at several concentrations (100 nM, 1 µM, 10 µM and 20 µM). The xCELLigence RTCA DP Instrument (ACEA Biosciences, USA) was used to evaluate the impedance of each well every hour for 24 h. The assays were performed in 8 wells, and the average CI was calculated. The cell proliferation levels and IC_{50} of ixazomib were calculated according to the CI values using RTCA DP Software 1.2.1. (Dikmen, Canturk, Kaya-Tilki, & Engur, 2017).

Macrophage Polarisation and Co-Culture Studies

THP-1 human monocyte cells were polarised into M2c macrophages and co-cultured with A549 cells for 24 h before mRNA isolation for gene expression arrays. The steps followed during the polarisation from monocyte to M2c macrophages are described as follows:

THP-1 monocytes were incubated with 100 ng/mL PMA in 1% serum for 48 h to differentiate into M0 macrophages. After removing the PMA-containing medium, the cells were washed with phosphate-buffered saline (PBS) three times. The cells were then incubated in normal medium for 48 h to eliminate the effects of PMA. During PMA application, medium serum concentration was maintained at 1% to support differentiation (Chanput, Mes, & Wichers, 2014).

For M2c macrophage polarisation, firstly M0 macrophage cells were stained with Trypan blue solution and $2.5x10^5$ cells counted using a cell counter (Cedex XS®, Innovatis, USA) and plated into the 0.4 µm pore-sized upper chamber of the co-culture plate in serum-free medium. After 48 h, the rested cells were treated with 1 µM hydrocortisone containing serum-free medium and incubated for 72 h (Martinez, Sica, Mantovani, & Locati, 2008). Polarised macrophages were washed with PBS to eliminate remaining debris, and the cells were incubated in growth medium for another 24 h. Using BD Accury C6 flow cytometry, the expression of the cell surface markers CD-206 (M2) and CD163 (M2c) (BioLegend) was used to determine the macrophage subtypes. Macrophages M0 and M2c were imaged morphologically using a Leica DM inverted light microscope.

In accordance with this co-culture, 5x105 A549 cells were seeded into the lower chamber of a second 6-well plate and allowed to adhere the day before the co-culture. The upper chambers containing the M2c macrophages were placed into six-well plates containing A549 cells, and the two cell populations were co-cultured for 24 h with or without the addition of 2 µM ixazomib (24h IC_{50} concentration for A549 cells) (Engür-Öztürk & Dikmen, 2022). Untreated A549 cells were used as the control.

Isolation of mRNA and Real-Time Polymerase Chain Reaction (RT-PCR)

mRNA was isolated from cells co-cultured for 24 hours to investigate the interaction between the gene expression levels of epigenetic chromatin modification enzymes in M2c macrophages and A549 lung cancer cells. In this co-culture, 5×105 A549 cells were seeded into the lower chamber of a 6-well plate and allowed to adhere the day before co-culturing. The upper chambers containing M2c macrophages were then placed into the wells with A549 cells, and the two cell populations were co-cultured for 24 hours with or without the addition of 2 µM ixazomib, which is the 24-hour IC_{50} concentration for A549 cells. Untreated A549 cells served as the control.

mRNA isolation were carried out using the MagNA Pure Compact RNA Isolation Kit® (Catalogue No: 04802993001, Roche, Germany) in the MagNA Pure Compact System. The total mRNA concentrations of the samples were assessed using a NanoDrop 2000® (Thermo Fisher, USA) spectrophotometer at 260 and 280 nm. Using the Transcriptor High Fidelity cDNA Synthesis Kit® (Catalogue no. 05091284001, Roche, Germany), according to the manufacturer's instructions, 500 ng/µL of mRNA from each sample was converted to cDNA. Realtime Ready Custom Gene Panel 96 (Catalogue no. 100141919, Roche, Germany), which contains the enzymes responsible for epigenetic chromatin modification, was used to measure gene expression levels after cDNA samples were replicated using the PCR method. The internal housekeeping gene actin-beta (ACTB) was employed. Using the instrument's analytic software, the results were examined in relation to changes in amplification levels compared with the control group.

Statistical analysis

In Graphpad Prism 8.4.2, one-way ANOVA and Tukey's post hoc tests were used to examine the findings. The mean standard deviation (±) represents the average of three separate studies. *P* values ($P>0.05$ n.s, $P<0.05$*, $P<0.01$**, $P<0.001$***, and $P<0.0001$****) are used to indicate how significant the results are in relation to the control and M2c groups.

RESULTS

M2c Macrophage Polarisation

Macrophage polarisation, which is crucial for tissue repair and homeostasis preservation, is the process by which macrophages change into different functional phenotypes in response to particular microenvironmental stimuli and signals. According to cell surface markers, released cytokines, and biological roles, M2 macrophages are classified into subcategories (Yao, Xu, & Jin, 2019). As macrophages probe and move through their surrounding matrix, their morphology shifts, and changes in cell shape are linked to changes in cell function. As shown in Figure 1, in the presence of M2c-inducing cytokines, they transform morphologically from a round and flattened shape to one that is significantly more elongated (McWhorter, Wang, Nguyen, Chung, & Liu, 2013).

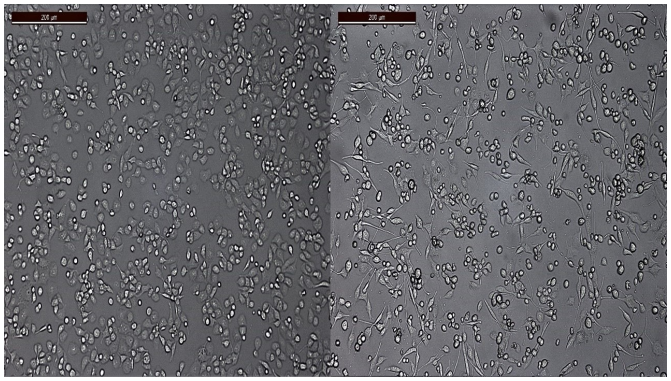

Figure 1. THP-1-origin macrophages (M0) (left) and M2c polarised macrophage (right) observed under a light microscope (10X).

Determination of Antiproliferative Effects of Ixazomib Using RTCA DP

The RTCA DP generates real-time data by gathering measurements at specified intervals over a period of days, indirectly detecting the cell number by measuring the electrical impedance (Dikmen et al., 2020). Real-time and time-dependent analyses of the IC_{50} concentrations of A549 lung cancer cells were performed using the RTCA DP system. The correct ixazomib concentrations for the xCELLigence system evaluation were selected using the WST-1 approach (data not shown). Ixazomib dose-dependently reduced the viability of A549 cells (Figure 2. In A549 lung cancer cells, the ixazomib IC_{50} value was calculated to be 2.45 µM at 24 h (Figure 3).

Determination of Gene Expression Levels of Epigenetic Chromatin Modification Enzymes

mRNA was isolated from cells co-cultured for 24 hours to examine the gene expression of epigenetic chromatin modification enzymes in M2c macrophages and A549 lung cancer cells. After co-culturing with or without 2 µM ixazomib, RT-PCR experiments were conducted to analyze the gene expression levels. Untreated A549 cells were used as the control.

The RT-PCR results revealed that 56 genes involved in epigenetic chromatin changes had incredibly high expression levels up to 1848-fold, including DNA methyl transferase (DNMT), histone deacetylase (HDAC), protein arginine specific methyl transferase (PRMT), ubiquitin conjugating (UBE), lysine demethylase (KDM), aurora kinase (AURK), mixed lineage leukaemia (MLL), and SET domain-containing (SETD) enzyme families (Table 1). Overexpressed genes were downregulated in the presence of the proteasome inhibitor ixazomib, except SETD5 and SETD6, which were increased by 3,84 and 870,69-fold, respectively.

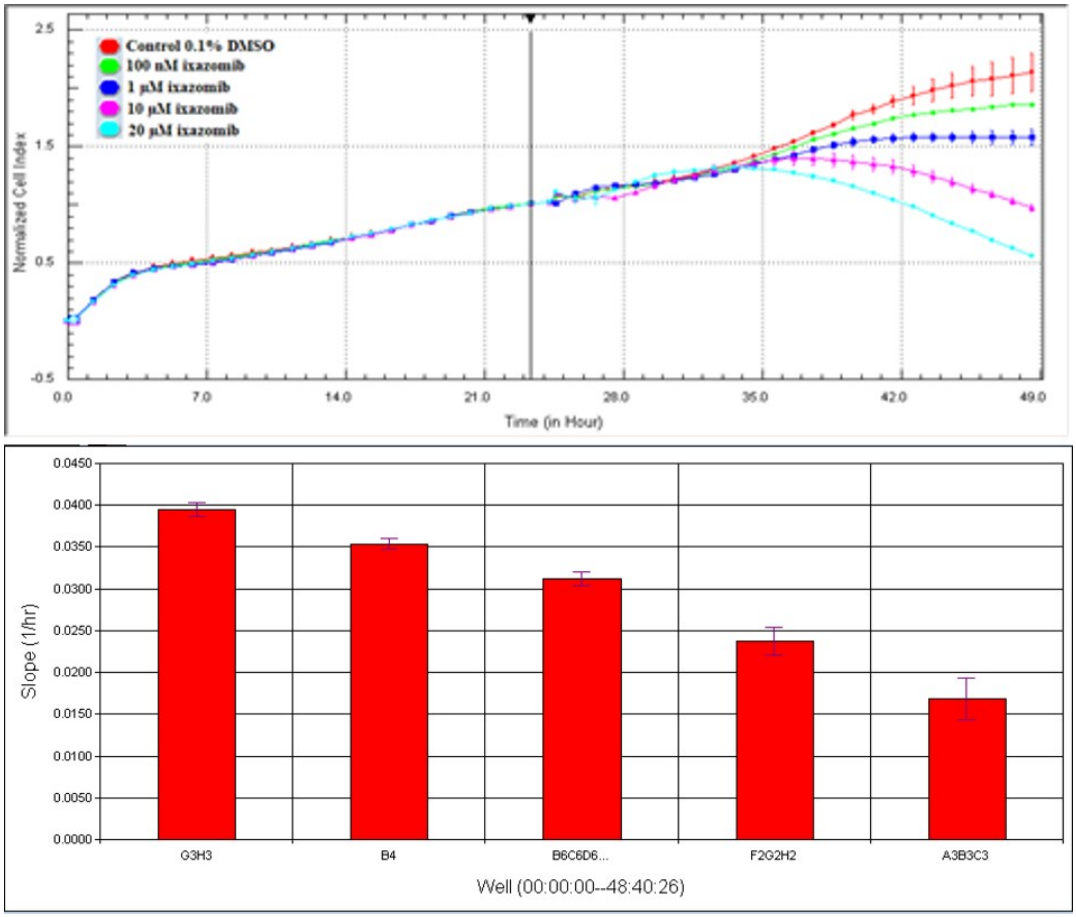

Figure 2. Real-time monitoring of the antiproliferative effects of ixazomib in A549 cells using RTCA DP Software 1.2.1 (n=8). (A) After 24 hours of incubation with ixazomib at various concentrations, real-time CI values were monitored to determine the IC_{50} value. (B) The slope graph is plotted according to the CI data from the 24th hour.

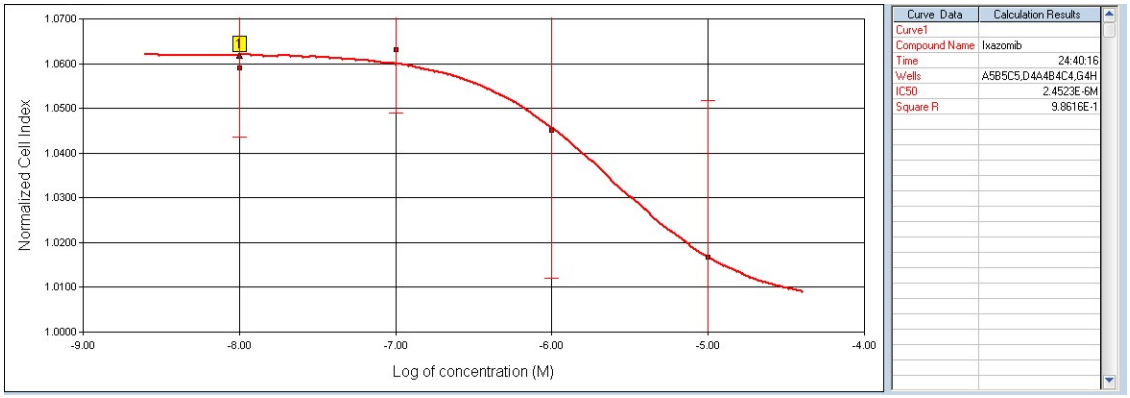

Figure 3. The IC_{50} concentration of ixazomib in A549 cells was determined using the dose-response curves of the cell index and RTCA DP Software 1.2.1 at 24 h (IC_{50}: 2.45 µM).

In the presence of M2c macrophages, HDAC enzymes, particularly HDAC4, were significantly upregulated, showing a remarkable 1848-fold increase in expression. Treatment with ixazomib resulted in a significant reduction (1112-fold) in HDAC4 expression. Additionally, UBE2A and UBE2B expression increased significantly in the presence of ixazomib but decreased substantially when ixazomib was present. The M2c-induced increase in the expression of DNMT1, DNMT3a, and DNMT3b was reversed in the presence of ixazomib. Furthermore, SETD6, which was activated in the presence of ixazomib, showed potential in suppressing inflammatory responses and influencing tumour suppression and anti-inflammatory responses through its effect on NF-κB signalling.

Table 1. Fold changes in the mRNA expression levels of epigenetic chromatin modification enzymes in M2c co-cultured A549 cells treated with or without 2 µM ixazomib.

Gene Name	Gene Symbol	Fold Change (Mean±Std. Deviation)		*P* value					
		M2c	**M2c+Ixazomib**	**Control vs. M2c**		**Control vs. M2c + ixazomib treatment**		**M2c vs. M2c + ixazomib**	
Aurora Kinase A	AURKA	6,24±0,1	0,52±0,2	<0,0001	****	0,052	ns	<0,0001	****
Aurora Kinase B	AURKB	2,91±0,2	0,16±0,06	0,0001	***	0,0112	*	<0,0001	****
ASH1 Like Histone Lysine Methyltransferase	ASH1L	2,17±0,4	0,45±0,05	0,0053	**	0,1153	ns	0,0007	***
Activating Transcription Factor 2	ATF2	20,19±0,06	2,96±0,3	<0,0001	****	0,0002	***	<0,0001	****
Coactivator-associated Arg1 methyltransferase 1	CARM1	5,55±0,3	0,62±0,3	<0,0001	****	0,4658	ns	<0,0001	****
CSRP2-binding protein	CSRP2BP	9,29±0,6	0,33±0,2	<0,0001	****	0,1674	ns	<0,0001	****
Disruptor of Telomeric Silencing 1-like	DOT1L	3,67±0,3	0,41±0,3	<0,0001	****	0,0824	ns	<0,0001	****
Dipeptidase 2	DPEP2	3,58±0,4	0,16±0,2	<0,0001	****	0,0156	*	<0,0001	****
DNA Methyltransferase 1	DNMT1	2,26±0,14	0,11±0,02	0,002	**	0,0114	*	0,0001	***
DNA Methyltransferase 3A	DNMT3A	64,71±1,3	0,1±0,03	<0,0001	****	0,381	ns	<0,0001	****
DNA Methyltransferase 3B	DNMT3B	15,27±1,1	0,41±0,37	<0,0001	****	0,6089	ns	<0,0001	****
Histone Deacetylase 1	HDAC1	40,02±1,59	8,5±0,27	<0,0001	****	0,0002	***	<0,0001	****
Histone Deacetylase 2	HDAC2	8,91±0,13	0,73±0,44	<0,0001	****	0,5397	ns	<0,0001	****
Histone Deacetylase 3	HDAC3	4,94±1,23	0,64±0,26	0,0014	**	0,8231	ns	0,0009	***
Histone Deacetylase 4	HDAC4	1848,02±1	1112,46±0,57	<0,0001	****	<0,0001	****	<0,0001	****
Histone Deacetylase 7	HDAC7	3,69±1,2	0,2±0,03	0,0125	*	0,2516	ns	0,0022	**
Histone Deacetylase 8	HDAC8	32,35±1,24	4,64±0,69	<0,0001	****	0,0069	**	<0,0001	****
Histone Deacetylase 10	HDAC10	7,13±0,96	1,27±0,17	<0,0001	****	0,9514	ns	<0,0001	****
Histone Deacetylase 11	HDAC11	3,09±0,17	0,2±0,09	<0,0001	****	0,0004	***	<0,0001	****
Histone Acetyltransferase 1	HAT1	10±0,87	1,02±0,45	<0,0001	****	0,9991	ns	<0,0001	****
Lysine Acetyltransferase 2B	KAT2B	9,97±1,13	0,38±0,14	<0,0001	****	0,5241	ns	<0,0001	****
Lysine Demethylase 1A	KDM1A	2,68±0,47	0,24±0,13	0,001	***	0,0428	*	0,0001	***
Lysine Demethylase 4A	KDM4A	13,01±1,17	2,75±0,47	<0,0001	****	0,0613	ns	<0,0001	****
Lysine Demethylase 4C	KDM4C	3,85±0,79	0,29±0,07	0,0007	***	0,2194	ns	0,0002	***
Lysine Demethylase 5C	KDM5C	2,77±0,45	1,09±0,22	0,0009	***	0,9286	ns	0,0011	**
Lysine Methyltransferase 2A	MLL	2,88±0,24	0,49±0,12	<0,0001	****	0,0166	*	<0,0001	****
Lysine Methyltransferase 2C	MLL3	12,99±0,18	0,1±0,08	<0,0001	****	0,0002	***	<0,0001	****
Lysine Methyltransferase 2E	MLL5	9,18±0,78	0,74±0,31	<0,0001	****	0,8059	ns	<0,0001	****
Myb Like, SWIRM, and MPN Domains 1	MYSM1	3,2±0,45	0,34±0,11	0,0002	***	0,0608	ns	<0,0001	****
Lysine Acetyltransferase 8	MYST1	11,72±1,1	1,85±0,23	<0,0001	****	0,3267	ns	<0,0001	****
Lysine Acetyltransferase 7	MYST2	5,88±0,47	2,26±0,58	<0,0001	****	0,0271	*	0,0001	***
Lysine Acetyltransferase 6A	MYST3	7,74±0,25	2,06±0,48	<0,0001	****	0,023	*	<0,0001	****
Lysine Acetyltransferase 6B	MYST4	2,4±0,2	0,27±0,04	0,0006	***	0,0153	*	<0,0001	****
Nuclear Receptor Co-activator 1	NCOA1	7,47±0,04	1,02±0,26	<0,0001	****	0,9859	ns	<0,0001	****
Nuclear Receptor Co-activator 3	NCOA3	5,08±1,01	0,53±0,14	0,0004	***	0,6318	ns	0,0002	***
Nuclear Receptor Co-activator 6	NCOA6	5,12±0,36	0,49±0,12	<0,0001	****	0,0668	ns	<0,0001	****
Nuclear Receptor-binding SET domain protein 1	NSD1	2,79±0,24	0,23±0,03	<0,0001	****	0,0018	**	<0,0001	****
P21 (RAC1) Activated Kinase 1	PAK1	2,15±0,42	0,38±0,16	0,0039	**	0,0601	ns	0,0004	***
Protein Arginine Methyltransferase 1	PRMT1	10,96±0,74	1,52±0,48	<0,0001	****	0,53	ns	<0,0001	****
Protein Arginine Methyltransferase 2	PRMT2	3,31±0,6	0,17±0,07	0,0005	***	0,0641	ns	<0,0001	****
Protein Arginine Methyltransferase 3	PRMT3	2,9±0,2	0,39±0,09	<0,0001	****	0,0041	**	<0,0001	****
Protein Arginine Methyltransferase 5	PRMT5	2,05±0,05	0,66±0,24	0,0003	***	0,0613	ns	<0,0001	****
Protein Arginine Methyltransferase 7	PRMT7	3,64±1,05	0,36±0,19	0,0048	**	0,4628	ns	0,0016	**
Ring Finger Protein 2	RNF2	2,3±0,3	0,17±0,03	0,0007	***	0,0072	**	<0,0001	****

Table 1. Continued

Ribosomal Protein S6 Kinase A3	RPS6KA3	4,79±0,78	0,42±0,22	0,0002	***	0,3536	ns	<0,0001	****
SET Domain Containing 1A	SETD1A	1,71±0,56	0,17±0,03	0,0892	ns	0,0514	ns	0,0032	**
SET Domain Containing 1B	SETD1B	6,2±0,4	0,65±0,11	<0,0001	****	0,2588	ns	<0,0001	****
SET Domain Containing 2	SETD2	2,22±0,22	0,39±0,22	0,0024	**	0,0548	ns	0,0003	***
SET Domain Containing B2	SETDB2	5,87±0,23	1,44±0,13	<0,0001	****	0,0684	ns	<0,0001	****
SET Domain Containing 3	SETD3	9,07±0,66	3,56±0,48	<0,0001	****	0,0015	**	<0,0001	****
SET Domain Containing 4	SETD4	5,6±0,3	0,91±0,27	<0,0001	****	0,9072	ns	<0,0001	****
SET Domain Containing 5	SETD5	0,94±0,04	3,84±0,13	0,7714	ns	<0,0001	****	<0,0001	****
SET Domain Containing 6	SETD6	0,06±0,03	870,69±0,76	0,0877	ns	<0,0001	****	<0,0001	****
SET Domain Containing 7	SETD7	0,69±0,05	0,1±0,03	<0,0001	****	<0,0001	****	<0,0001	****
SET Domain Containing 8	SETD8	10,41±0,62	6,51±0,28	<0,0001	****	<0,0001	****	<0,0001	****
SET and MYND Domains Containing 3	SMYD3	3,85±0,82	0,09±0,08	0,0008	***	0,1254	ns	0,0002	***
Suppressor Of Variegation 3-9 Homologue 1	SUV39H1	1,85±0,14	0,13±0,06	0,0001	***	0,0001	***	<0,0001	****
Lysine Methyltransferase 5B	SUV420H1	2,61±0,25	0,05±0,02	<0,0001	****	0,0005	***	<0,0001	****
Ubiquitin-Conjugating Enzyme E2 A	UBE2A	65,69±0,73	43,46±0,24	<0,0001	****	<0,0001	****	<0,0001	****
Ubiquitin-Conjugating Enzyme E2 B	UBE2B	5,02±0,49	0,59±0,16	<0,0001	****	0,3892	ns	<0,0001	****
Wolf-Hirschhorn Syndrome Candidate 1 (2019)	WHSC1	3,16±0,25	0,16±0,03	<0,0001	****	0,0043	**	<0,0001	****

DISCUSSION

Inflammatory processes and macrophage polarisation are necessary for cells to respond to microenvironmental changes and stimuli. Epigenetic pathways improve macrophage diversity and plasticity in order to defend the host from various threats. However, disorders are persistent because of maladaptive epigenetic modifications. As a result, the goal of pharmacological action is to convert a damaged epigenetic landscape into a healthy one. To identify harmful epigenetic signatures and predict disease vulnerability, the epigenetic landscapes of major immune cells should be screened.

Many HDAC isoenzymes are upregulated in various cancers. However, because of the complexity of the linked pathways, the complete mechanisms of action of HDACs have yet to be fully explored (Damaskos et al., 2018). According to our results, HDAC enzymes were dramatically upregulated in the presence of M2c macrophages. HDAC4, whose expression was increased 1848-fold between these enzymes, was the most striking result. Due to its participation in various biological processes, HDAC4 (a class IIa HDAC) has been identified as a significant enzyme in cancer pathogenesis (Jin et al., 2018). HDAC4 expression is not only related with tumour size in malignant thyroid lesions, but it also promotes tumour development in ovarian, colon, glioblastoma, and gastric cancer cells by inhibiting p21 expression (Zeng et al., 2016). In human A549 lung cancer cells, HDAC4 overexpression determines etoposide resistance via P-glycoprotein expression (Kaewpiboon et al., 2015). According to our findings, ixazomib treatment reduced HDAC4 expression by 1112-fold. This is an intriguing finding concerning the importance of combining epigenetic medications with cancer drugs for treating lung cancer, particularly in terms of preventing resistance.

One of the significant results was the upregulation of ubiquitin conjugating enzyme E2A (UBE2A) and UBE2B to 65.7- and 5-fold levels, respectively. In DNA damage-induced mutagenesis, mismatch repair, and gene silencing, UBE2A and UBE2B play crucial roles (Bruinsma et al., 2016). Both genes are members of the E2 family of ubiquitin-conjugating enzymes, which are essential for protein degradation via the ubiquitin proteasome pathway (UPP). The expression of these genes decreased to 45.5 and 0.6-fold, respectively, when ixazomib, a proteasome inhibitor, was present. In addition, abnormal DNMT activity plays a key role in the onset and development of malignancies, leading to the methylation of tumour suppressor genes (Robertson, 2001). DNMT1 is a maintenance enzyme that maintains the semi-methylation of the CpG dinucleotide in newly cloned DNA (Bestor, 1992). As a result, its role in the maintenance of methylation in the genomes of growing cells is critical. DNMT3a and DNMT3b are critical for *de novo* methylation and the establishment of a novel methylation state *in vivo* (Okano, Bell, Haber, & Li, 1999). In the presence of ixazomib, the increase in M2c-induced expression of these genes was eliminated.

Unlike M2c-activated genes, SETD6, which was activated in the presence of ixazomib in the gene panel, yielded some interesting findings. Numerous biological and pathological processes have been linked to the methylation of lysine residues in proteins, although many human protein lysine methyltransferase have unclear catalytic activity or substrate specificity. SETD6 was shown to be a methyltransferase that monomethylated the Lys310 position of the transcription factor linked with

the chromatin NF-kB subunit RelA. Through SETD6-mediated methylation, RelA was rendered inactive, and RelA-driven transcriptional pathways involved in the inflammatory response in primary immune cells were diminished (Levy et al., 2011). Because NF-κB signalling is connected to pathological inflammatory processes and cancer, SETD6 upregulation can be effective for a number of mechanisms through which protein methylation and chromatin regulation influence tumour suppression and anti-inflammatory responses.

CONCLUSION

In conclusion, our study highlights the significant impact of the interplay between M2c macrophages and lung cancer cells on the induction of a notable increase in gene expression within epigenetic pathways in the tumour microenvironment. Proteasome inhibitors are effective in alleviating these dysfunctions. Although proteasome inhibitors have primarily excelled in treating haematological malignancies, their potential in lung cancer treatment is noteworthy, particularly when combined with epigenetic therapies. However, it is essential to acknowledge that our study did not directly assess the effectiveness of combined epigenetic drugs and proteasome inhibitors. Further investigations are essential to explore the potential synergistic effects of combined therapy against lung cancer.

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