Histone Methylation by Temozolomide; A Classic DNA Methylating Anticancer Drug

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Abstract. *Background/Aim: The alkylating agent, temozolomide (TMZ), is considered the standard-of-care for high-grade astrocytomas –known as glioblastoma multiforme (GBM)– an aggressive type of tumor with poor prognosis. The therapeutic benefit of TMZ is attributed to formation of DNA adducts involving the methylation of purine bases in DNA. We investigated the effects of TMZ on arginine and lysine amino acids, histone H3 peptides and histone H3 proteins. Materials and Methods: Chemical modification of amino acids, histone H3 peptide and protein by TMZ was performed in phosphate buffer at physiological pH. The reaction products were examined by mass spectrometry and western blot analysis. Results: Our results showed that TMZ following conversion to a methylating cation, can methylate histone H3 peptide and histone H3 protein, suggesting that TMZ exerts its anticancer activity not only through its interaction with DNA, but also through alterations of protein post-translational modifications. Conclusion: The possibility that TMZ can methylate histones involved with epigenetic regulation of protein indicates a potentially unique mechanism of action. The study will contribute to the understanding the anticancer activity of TMZ in order to develop novel targeted molecular strategies to advance the cancer treatment.*

Malignant gliomas, or glioblastoma multiforme (GBM), are the most common primary brain malignancies found in adults (1). Despite advances in multimodal therapies of surgical extirpation, radiotherapy and chemotherapy, GBMs remain a cancer of poor prognosis that can be attributed to the aggressive nature of the tumors and the development of resistance to radiation and chemotherapy (2). Out of chemotherapeutic approaches to GBMs, temozolomide (TMZ) is the main alkylating agent used, based on its ability to increase the median survival (3-6).

TMZ possesses favorable pharmacokinetic characteristics with high oral bioavailability and blood-brain barrier (BBB) penetration (7). TMZ is a prodrug that undergoes pH-dependent degradation, first producing 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC), that is subsequently degraded into 4-amino-5-immidazole-carboxamide (AIC) – an inactive metabolite and a methyldiazonium cation, the methylating agent (Figure 1). The anticancer effects of TMZ are attributed to the methylation of DNA by TMZ with the primary cytotoxic lesion being O6-methylguanine (O6-MeG); N7-methylguanine, N3-methylguanine, and N3-methyladenine (8-10) adducts also contribute to the cytotoxicity. The DNA adducts can lead to single- and double-strand DNA breaks and eventually apoptosis; however, numerous DNA repair systems, highlighted by the methylguanine methyltransferase (MGMT) enzyme that repairs the O6-MeG lesions, can result in chemoresistance (11-13). Other DNA repair processes, such as mismatch repair (MMR) and base excision repair (BER), also influence cellular resistance to TMZ.

The field of epigenetics characterizes how chemical and enzymatic modifications to DNA and histones occur and their subsequent impact on gene expression. In fact, hypermethylation of the MGMT promoter in GBM patients (14, 15, 16) is used as a favorable prognostic biomarker and increases drug sensitivity since a lower amount of the enzyme is available to repair TMZ-induced lesions (17). Although DNA methylation has been shown to affect chromatin structure and influence histone methylation (18), it is direct enzymatic modification of histones, such as methylation and acetylation that are considered crucial to gene expression.

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Key Words: Temozolomide, malignant gliomas, chemotherapy, histone methylation.

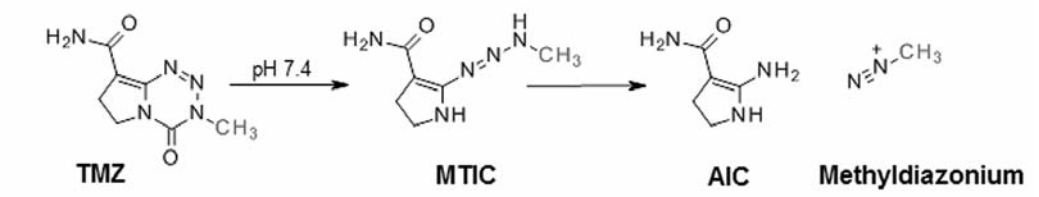


Figure 1. *TMZ metabolic cascade at physiological pH to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) and its metabolites, 4-amino-5-imidazole-carboxamide (AIC) and methyldiazonium ion.*

In the current study, we addressed whether TMZ can methylate histones directly, by examining the effects of TMZ on amino acids, peptides and histone proteins *in vitro* using mass spectrometry and protein biochemical methods. Our results demonstrated that TMZ is able to methylate histone proteins under physiological conditions. The possibility that this action could occur in GBM patients opens a new avenue of investigation to determine its significance.

Materials and Methods

Chemicals and general procedures. Arginine, lysine and TMZ (>98%) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Histone H3 (1-8) peptide H2N-ARTKQTAR-COOH (>95% by HPLC, MW=931.1 g/mol) was purchased from AnaSpec, Inc. (Fremont, CA, USA). Xenopus recombinant histone H3 (C110A) (>98% by SDS-PAGE, MW=15,239 Da), supplied as a lyophilized powder, was purchased from Active Motif (Carlsbad, CA, USA). Recombinant human histone H3.1 protein (MW=15273.2 Da by ESI-TOF) was purchased from New England BioLabs, Inc. (Ipswich, MA, USA). Human recombinant histone H3 protein (≥95% by SDS-PAGE, MW=15,500 Da by SDS-PAGE) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) as a lyophilized powder. All buffers and mobile phases were prepared using analytical grade reagents and ultrapure water from a Millipore Synergy UV water filtration system (EMD Millipore, Billerica, MA, USA) at a resistivity of 18 MΩ·cm. All reagents were used without further purification. The pH measurements were measured using a calibrated Fisher Scientific AccuMet Basic AB15 pH MV benchtop pH meter (Fisher Scientific, Pittsburgh, PA, USA).

Reactivity of amino acids and peptides with TMZ. Separate stock solutions of arginine (0.5 mM), lysine (0.5 mM), TMZ (0.2 mM) and histone H3 (1-8) peptide (10 mg/ml) were prepared in 0.01 M phosphate buffer (pH 7.4) and kept at 4˚C until use. A solution containing 10 μM amino acid or peptide and 200 μM TMZ was mixed at room temperature for 24 h. Subsequently an aliquot of the solution was diluted 1:1 with methanol and was passed through a 0.22 μm syringe filter. The filtered solution was subsequently analyzed by LC-MS/MS.

Reactivity of histone H3 Proteins with TMZ. 100 μg of histone H3 protein was resuspended in 100 μl 0.01 M phosphate buffer (pH 7.4) and 10 μM TMZ in 0.01 M (pH 7.4) phosphate buffer was mixed with the protein solution at room temperature for 24 h. The resultant reaction mixture was centrifuged in an Amicon Ultra-0.5 centrifugal filter device (EMD Millipore, Billerica, MA, USA) at 4˚C at 14,000 × *g* with a 40˚ fixed angle rotor to concentrate the protein samples and remove low molecular weight impurities and unreacted TMZ. The 100 μg concentrated protein solution was resuspended in 100 μl 0.01 M phosphate buffer (pH 7.4). Western blot analysis was then used to confirm the methylated histone H3 protein.

Mass spectrometry. Mass spectrometry experiments were performed using a QTRAP 5500 triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) coupled to a Shimadzu Prominence UFLC system (Shimadzu Corp., Torrance, CA, USA). The system was continuously supplied with ultra-high purity nitrogen, dry air, and zero air by a Source 5000 LC-MS TriGas generator (Parker Hannifin Corp., Haverhill, MA, USA). The QTRAP 5500 system was equipped with an electrospray ionization (ESI) TurboIon Spray probe utilizing zero grade air as the nebulizer (Gas 1) and heater (Gas 2) gases. Ultra-high purity nitrogen was used as the curtain (CUR) and collision (CAD) gases. Mass spectrometer control and spectral processing were carried out using Analyst 1.5.2 software (AB SCIEX). Mass spectrometer parameters were optimized for each compound analyzed (Tables I and II). A 1μl sample of unreacted amino acid, TMZ, or the reaction mixture was introduced into the electrospray source by direct injection. The mobile phase consisted of 50% methanol with 0.1% formic acid and 50% water with 0.1% formic acid and was maintained at a constant flow rate of 200 μl/min. Unreacted and reacted peptide solutions were infused directly into the electrospray source at a flow rate of 7 μl/min. All mass spectra were acquired in positive-ion mode with unit mass resolution. Mass spectra for the free amino acid studies were background-subtracted with a spectrum obtained of 100 μM TMZ in 50/50 0.01 M phosphate buffer/methanol (v/v).

Peptides were fragmented under conditions with the collision energy between 15 to 45 eV by the direct infusion method at a flow rate of 7 μl/min. The MS/MS spectra were interpreted manually, primarily by assigning the peaks corresponding to the C-terminal y-ions. The modification was mapped based on the mass difference of the y ions in the spectrum derived from the doubly charged precursor ions when compared to the y ions in the spectrum of unmethylated peptide. Mass spectrometer parameters were optimized for each methylated peptide analyzed (Table III). Mass fragmentation prediction was generated using Mascot (Matrix Science, London, UK).

Western blot analysis. Standard protein electrophoresis conditions and reagents were used for gel and sample preparation. Recombinant proteins (0.5-5 μg) and the LI-COR Chameleon Duo ladder (LI-COR Biosciences, Inc., Lincoln, NE) were loaded onto a 12% Mini-Protean-

Table I. *Optimized mass spectrometer parameters for tandem mass spectrometry of arginine and methylated arginine.*

	Q1 (Da)	CAD	CUR (psi)	DP (V)	TEM (˚C)	IS (V)	GS1 (psi)	GS2 (psi)	EP (V)	CE (V)	CXP (V)
R	174.600	MED	20	50	550	4500	60	60	10	20	12
MMR	189.100	MED	20	70	550	5500	60	60	10	20	11
DMR	203.200	MED	10	60	450	5500	60	60	10	20	11
TMR	217.100	MED	10	30	500	5500	60	60	11	25	11

R, Arginine; MMR, monomethylarginine; DMR, dimethylarginine; TMR, trimethylarginine.

Table II. *Optimized mass spectrometer parameters for tandem mass spectrometry of lysine and methylated lysine.*

	Q1 (Da)	CAD	CUR (psi)	DP (V)	TEM (˚C)	IS (V)	GS1 (psi)	GS2 (psi)	EP (V)	CE (V)	CXP (V)
K	147.000	MED	20	50	550	4500	60	50	10	18	12
MMK	161.000	LOW	20	66	600	4000	60	60	10	18	12
DMK	175.000	LOW	20	66	600	4000	60	60	10	20	12
TMK	189.000	LOW	20	66	600	4000	60	60	10	25	12

K, Lysine; MMK, monomethylysine; DMK, dimethylysine; TMK, trimethylysine.

TGX precast gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Electrophoresis was carried out in 1X Tris/Glycine/SDS gel electrophoresis buffer (Bio-Rad, Hercules, CA) for 1 h at 100 V according to the procedure recommended by the manufacturer. Proteins were then transferred overnight to a PVDF membrane pre-wet with methanol and transfer buffer (1X Tris/Glycine). The membrane was blotted with Odyssey blocking buffer (TBS) for 1 h, followed by monoclonal Anti-β-Actin antibody produced in mouse (Sigma-Aldrich Co., St. Louis, MO) and polyclonal anti-histone H3K4me1 antibody produced in rabbit (Active Motif, Carlsbad, CA) that was diluted 1:1000 in Odyssey blocking buffer plus 0.2% Tween-20 for 3 h. Following standard washing procedure, the membrane was then blotted with IRDye® 800CW Goat anti-Rabbit IgG (H+L) and IRDye® 680RD Goat anti-Mouse IgG (H+L) (LI-COR Biosciences, Inc., Lincoln, NE) diluted 1:10,000 in Odyssey blocking buffer plus 0.2% Tween-20 for 1 hour in the dark. The membrane was washed three times with TBS plus 0.1% Tween-20 and imaged by near-infrared fluorescence detection on a LI-COR Odyssey CLx scanner (LI-COR Biosciences, Inc., Lincoln, NE).

Results

Methylation of arginine and lysine by TMZ. Experimental and natural methylation sites of arginine and lysine (Figure 2A) can occur on the side-chain nitrogen atoms, both symmetrically and asymmetrically in arginine (19), and less often at the N-terminal nitrogen atoms and amide nitrogen atoms in peptide bonds (20). Methylation products of arginine reacted with formaldehyde or methyl iodide have been described as Nα-monomethylarginine (N_α -MMR), N_α,N_α-dimethylarginine (N_α,N_α -DMR), and N_α,N_α, N_α-trimethylarginine (N_α,N_α, N_α -TMR) (21), and serve as a basis to compare to methylated species produced by TMZ. The reaction of TMZ with arginine resulted in a mixture of mono-, di- and tri-methylated products that were detected at m/z 190, 204 and 218, respectively, with a 14 Da increase in the mass for each subsequent methylation (Figure 2B). The methylation positions could be either at N_α or at N_ε position. The identities of the methylated products were further investigated by the collision-activated dissociation (CID) mass fragmentation pattern to determine the potential methylation sites. The CID patterns of arginine and its methylated products displayed similar characteristic fragmentation patterns to the N_α-MMR, N_α,N_α -DMR, and N_α,N_α, N_α-TMR products as described previously,

Table III. *Optimized mass spectrometer parameters for Q1 scans of TMZ reacted with arginine (R + TMZ), lysine (K + TMZ), peptide (PEP + TMZ) and peptide alone (PEP).*

	CUR (psi)	DP (V)	TEM (˚C)	IS (V)	GS1 (psi)	GS2 (psi)	EP (V)
R + TMZ	20	66	500	5500	50	50	10
K + TMZ	20	66	500	5500	50	50	10
PEP	20	66	450	4500	40	60	11
PEP + TMZ	20	66	450	4500	40	60	11

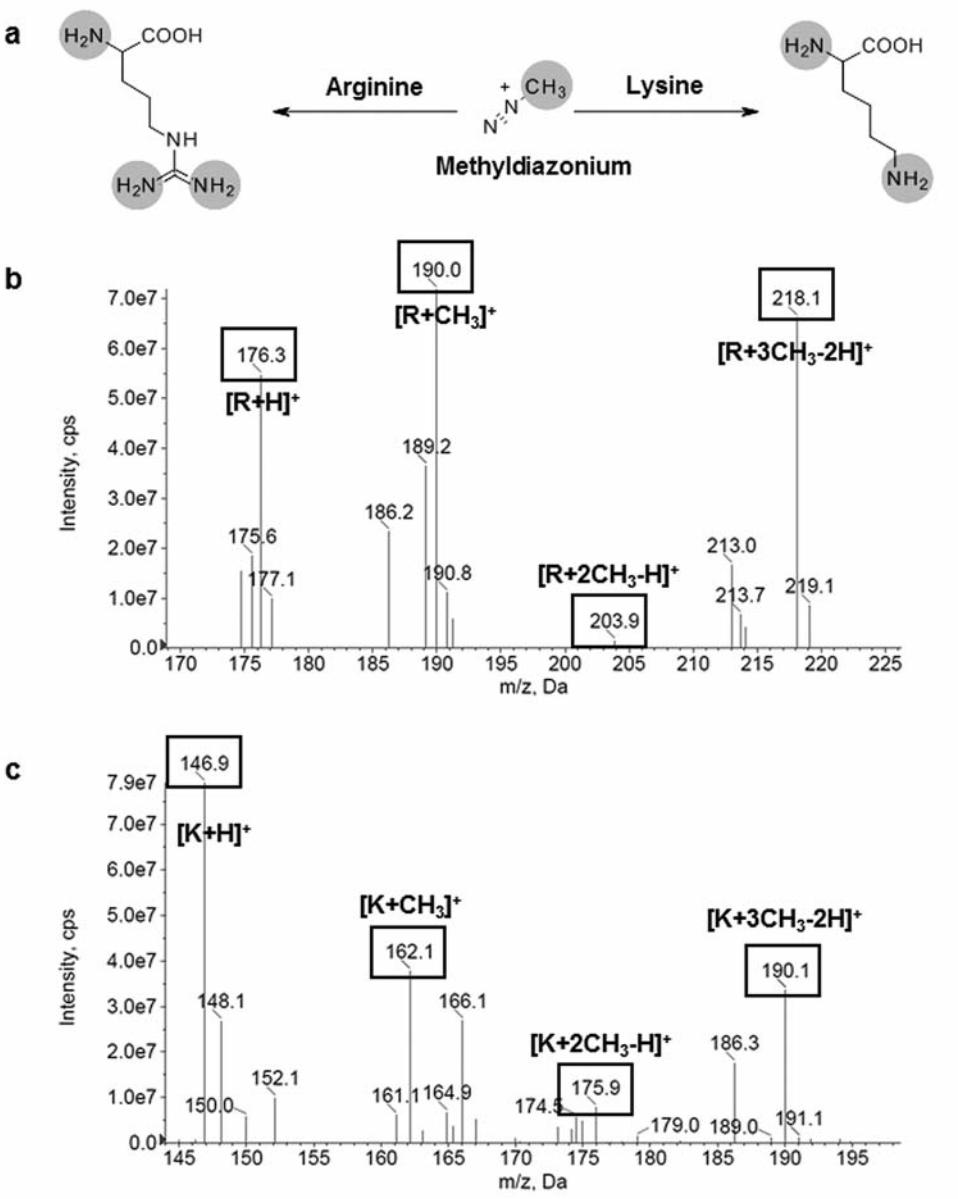


Figure 2. *(a) Potential methylation sites of arginine and lysine free amino acids. Mass spectra of the reaction between TMZ and (b) arginine or (c) lysine demonstrating multiple methylations.*

suggesting that TMZ may methylate the N-terminal nitrogen atoms of arginine or the nitrogen atoms of arginine side chains (Figure 3).

The reactions between TMZ and lysine were analogous to those with arginine and produced a mixture of mono-, di-, and tri-methylated lysine products at m/z 148, 162, and 176, respectively, with a 14-Da increase in the mass for each subsequent methylation (Figure 2C). CID fragmentation patterns of the TMZ-induced methylation products (Figure 4) suggest side chain N-methylation products previously characterized as Nε-methyllysine (N_ε-MMK), N_ε,N_ε-dimethyllysine (N_ε,N_ε- DMK), and N_ε,N_ε,N_ε-trimethyllysine (N_ε,N_ε,N_ε-TMK) (21).

Mapping the methylation site in a histone peptide following the TMZ treatment. When methyl groups (-CH_3) replace hydrogen atoms on a peptide, the isotopic mass gains are 14 Da for each methyl substitution. The substitution can occur at the amino and carboxyl termini and at the side chains of lysine and arginine (22). The b and y ions produce the

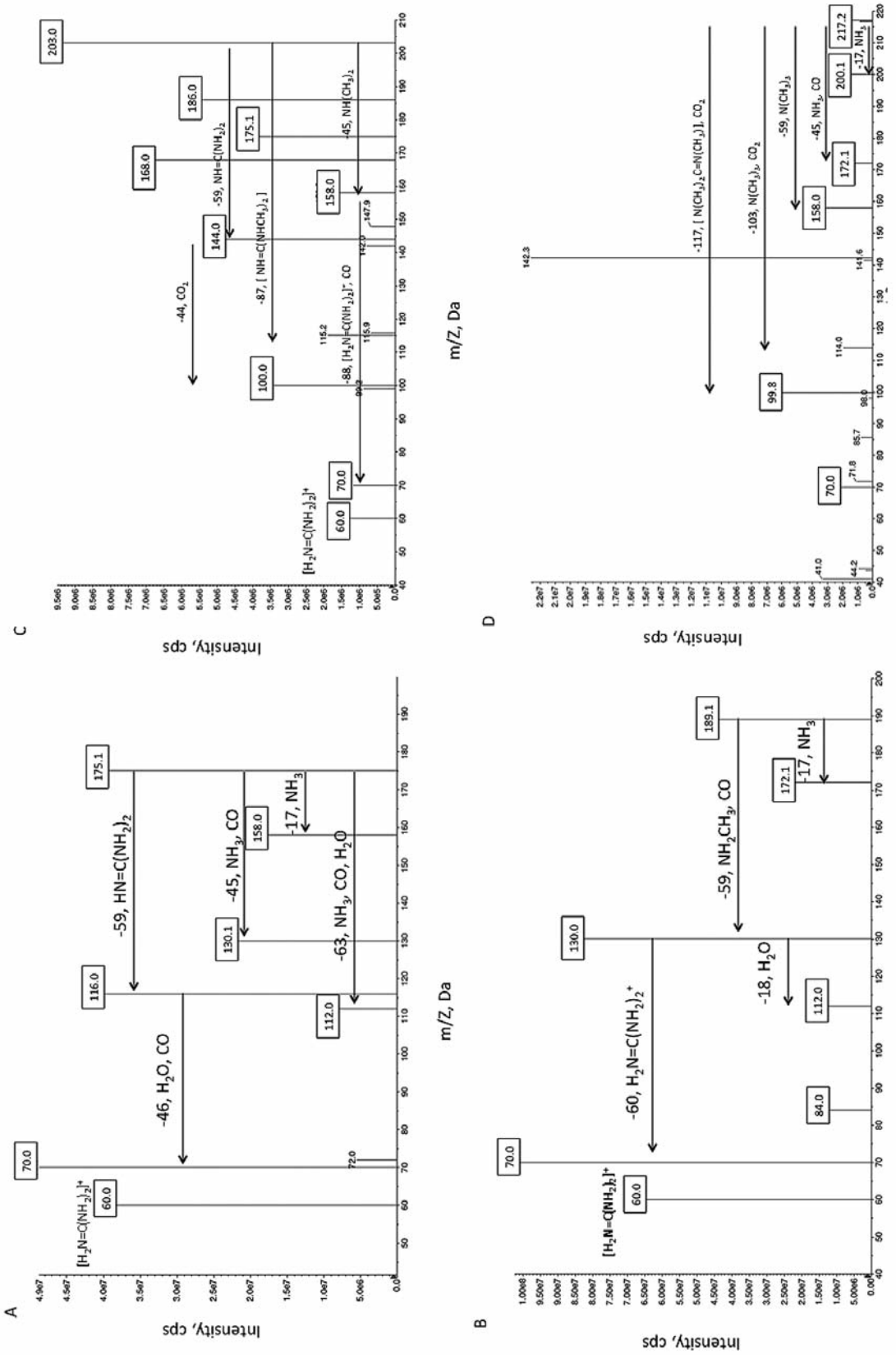


Figure 3. *ESI/CID mass spectra of (A) protonated arginine, (B) protonated monomethylated arginine (MMR), (C) protonated dimethylated arginine (DMR) and (D) protonated trimethylated arginine (TMR).*

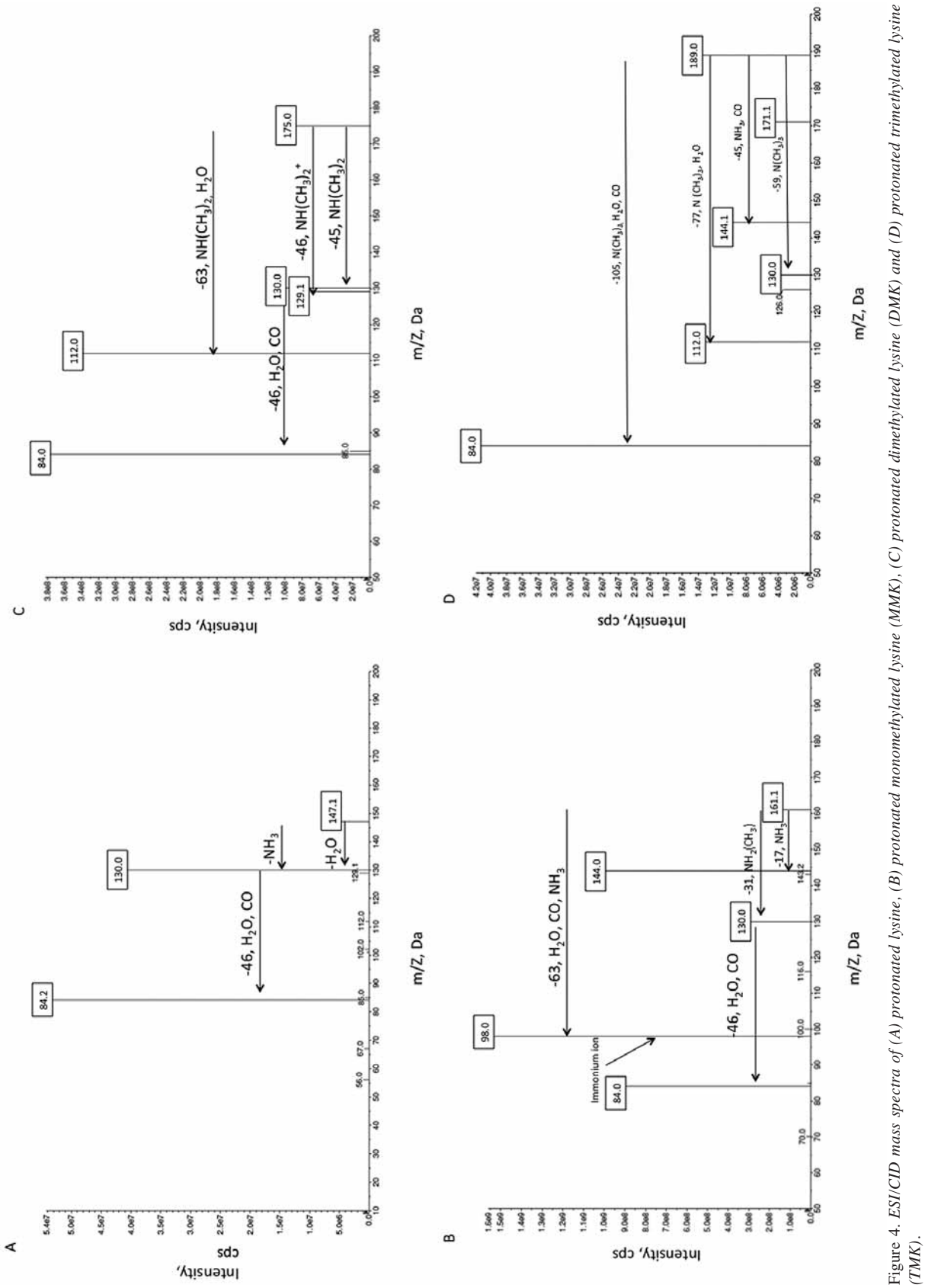


Figure 4. *ESI/CID mass spectra of (A) protonated lysine, (B) protonated monomethylated lysine (MMK), (C) protonated dimethylated lysine (DMK) and (D) protonated trimethylated lysine (TMK).*

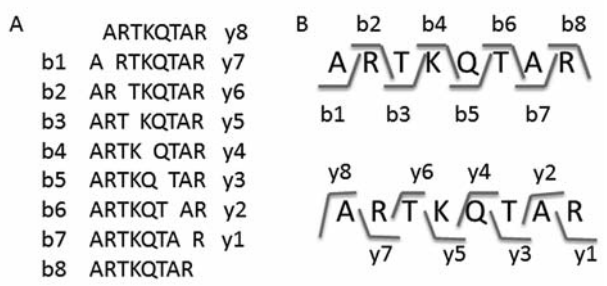


Figure 5. *Fragmentation map of human histone H3 peptide 1-8 (ARTKQTAR). A. The ladder or family of "b ions" and "y ions" generated after bond cleavage that may be observed in the fragment mass spectrum for this peptide. Y1 represents spectra for fragmentation of H3 peptide on R175, Y2 represents spectra for fragmentation of H3 peptide on R246, etc. B1 represents spectra for fragmentation of H3 peptide on A72, b2 represents spectra for fragmentation of H3 peptide on A 228, etc. B. Fully annotated fragmentation nomenclature after peptide bond ion calculator results. The blue lines represent the fragmentation of H3 peptide at the corresponding peptide bond for each amino acid. B- and y-fragment ions are denoted by blue and red, respectively.*

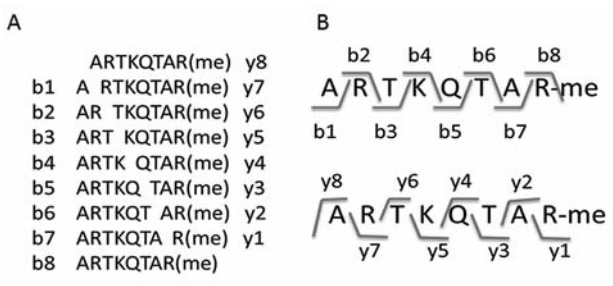
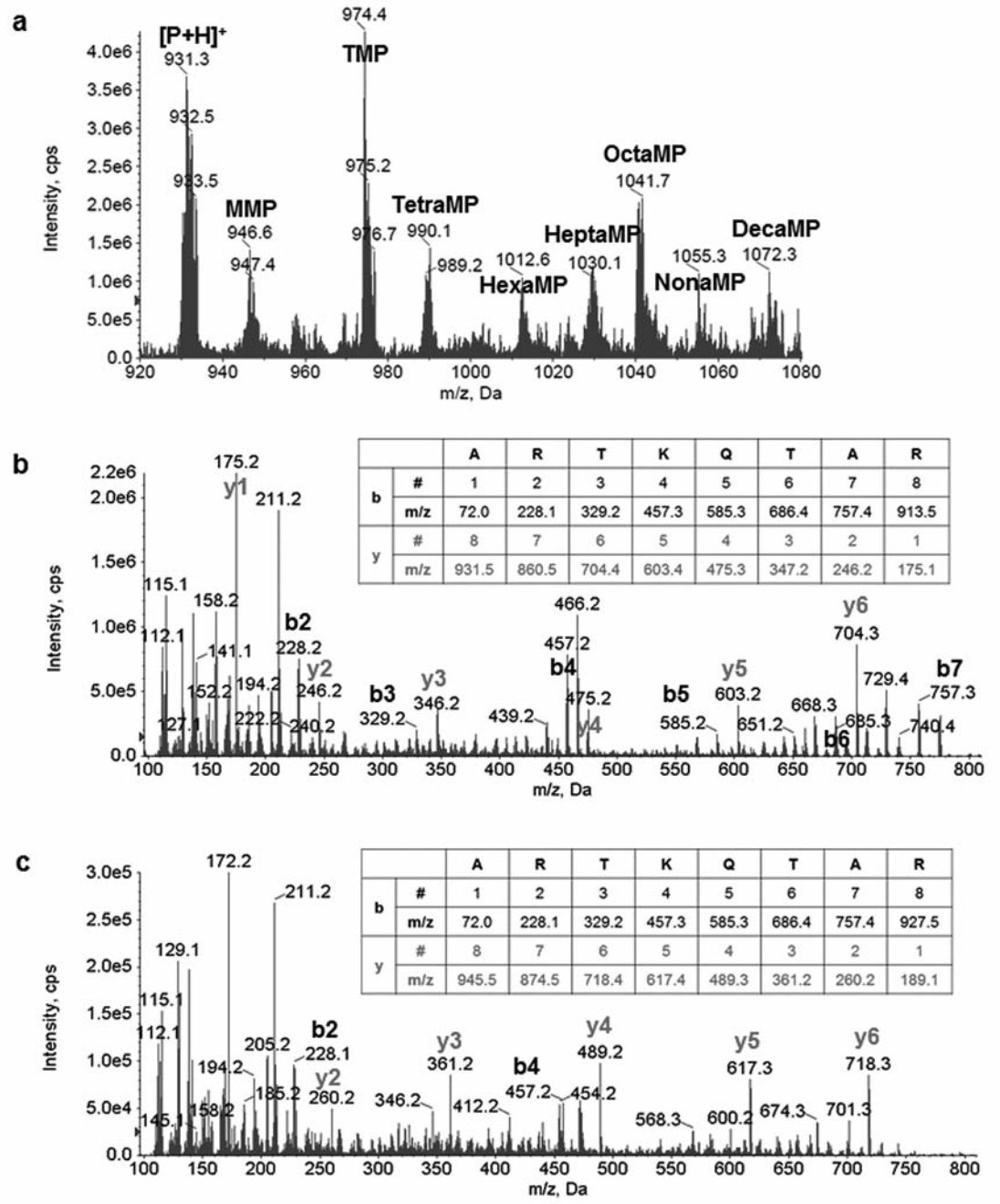


Figure 6. *Fragmentation map of methylated human histone H3 peptide 1-8 (ARTKQTAR-me). Methyl group (me) is on residue 8 (arginine). When methylation occurs on the amino acid, the m/z of b ion or y ion will display the shift based upon the position of amino acid in the peptide and molecular weight of the attachment. Y1 represents spectra for fragmentation of H3 peptide on R189, Y2 represents spectra for fragmentation of H3 peptide on R260, etc. B1 represents spectra for fragmentation of H3 peptide on A72, b2 represents spectra for fragmentation of H3 peptide on A228, etc. B. Fully annotated fragmentation nomenclature after bond cleavage between amino acids. The blue lines represent the fragmentation of H3 peptide on amino acid on each peptide bond.*

characteristic footprint necessary for the identification of possible methylation sites, which are generated by the fragmentation of the peptide bonds between two adjacent amino acid residues. Those fragment peaks that appear to extend from the amino terminus are termed "b ions", whereas groups of peptide fragment ions that appear to extend from the carboxyl terminus are termed "y ions" (Figures 5 and 6).

The reaction products of TMZ with human histone H3 peptide 1-8 (ARTKQTAR) were identified as the mono-methylated peptide (MMP), tri-methylated peptide (TMP), tetra-methylated peptide (TetraMP), hexa-methylated peptide (HexaMP), hepta-methylated peptide (HeptaMP), octa-methylated peptide (OctaMP), nona-methylated peptide (NonaMP) and deca-methylated peptide (DecaMP) at m/z 946.6, 974.4, m/z 990.1, m/z 1012.6, m/z 1030.1, m/z 1041.7, m/z 1055.3, and m/z 1072.3, respectively (Figure 7A). The methylation site for MMP was mapped using low-energy CID fragmentation (45 eV) (23). In the spectrum recorded from the unmethylated histone peptide (Figure 7B), the b ions and y ions in the spectrum derived from the doubly-charged precursor ions were displayed. The MS/MS spectrum of MMP shown in Figure 3C displayed a 14 Da shift for all y ions in comparison with the y ions in the spectrum of unmodified peptide. The methylation site was identified on number 8 arginine residue according to the mass fragmentation prediction from Mascot shown in the table above the spectra in figure 3B and 3C. The table in Figure 7B shows the m/z of the predicted b and y ions for the unmethylated peptide, while the table in Figure 7C lists the m/z for the predicted b and y ions for the methylated peptide at the Arg side chain at the C-terminus. The methylation was sufficiently stable under CID conditions that resulted in cleavage of the peptide backbone wherein it could be located in the sequence of the peptide by the observed y ions. Mapping the detailed methylation sites for multi-methylated peptides was not as conclusive as for MMP (data not shown). The result demonstrated that TMZ is able to methylate amino acid side chains in the peptide.

Methylation of histone H3.1 protein by TMZ. To demonstrate that TMZ, *via* its methyldizaonium cation, can methylate arginine and lysine within a protein, the Xenopus and human histone H3.1 recombinant proteins were reacted with TMZ. Methylation of the xenopus histone H3.1 was observed at a higher molecular weight than unmethylated protein (data not shown), as determined by gel electrophoresis. Methylated human histone H3.1 displayed a similar molecular shift (data not shown). Western blot analysis was then used to further confirm the methylation status of the proteins. As shown in Figure 8, both xenopus and human histone H3.1 proteins are positive for western blot analysis using H3K4Me antibody that is specific for the methylation of the lysine residue at the N-terminal number 4 position. Western blot analysis support that both Xenopus and human histone H3.1 proteins are methylated by TMZ under physiological conditions.



		A	R	T	K	Q	T	A	R
b	#	1	2	3	4	5	6	7	8
	m/z	72.0	228.1	329.2	457.3	585.3	686.4	757.4	913.5
y	#	8	7	6	5	4	3	2	1
	m/z	931.5	860.5	704.4	603.4	475.3	347.2	246.2	175.1

		A	R	T	K	Q	T	A	R
b	#	1	2	3	4	5	6	7	8
	m/z	72.0	228.1	329.2	457.3	585.3	686.4	757.4	927.5
y	#	8	7	6	5	4	3	2	1
	m/z	945.5	874.5	718.4	617.4	489.3	361.2	260.2	189.1

Figure 7. *(a) Mass spectrum of histone H3 peptide (1-8) after reacting with TMZ. Abbreviations: [P+H]+, native peptide; MMP, mono-methlated peptide; TMP, tri-methylated peptide; TetraMP, tetra-methylated peptide; HexaMP, hexa-methylated peptide; HeptaMP, hepta-methylated peptide; OctaMP, octa-methylated peptide; NonaMP, nona-methylated peptide; DecaMP, deca-methylated peptide. (b) Fragmentation of the doubly-charged precursor ion corresponding to the unmethylated histone H3 peptide. (c) Fragmentation of the doubly-charged precursor ion corresponding to MMP.*

Discussion

TMZ is a clinically important methylating agent used in chemotherapy against malignant gliomas whose efficacy is limited by the development of drug resistance (11). Emerging ideas in systems pharmacology beckon for a detailed molecular analysis of drug action to ensure that all drug targets are identified (24, 25). Such a comprehensive understanding of the mechanisms of drug action should provide greater insight into why drugs succeed or fail in

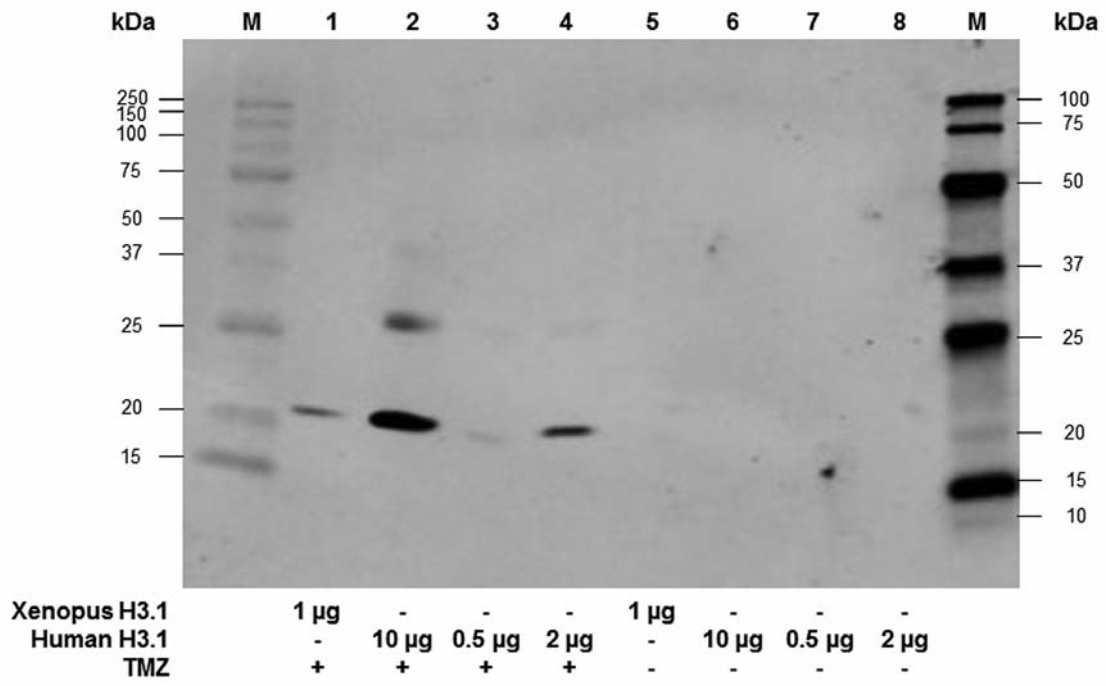


Figure 8. *Western blot analysis of histone H3.1 proteins; 1 µg methylated Xenopus histone H3.1 (Lane 1), 10 µg methylated human histone H3.1 (Lane 2), 0.5 µg methylated human histone H3.1 (Lane 3), 2 µg methylated human histone H3.1(Lane 4), 1 µg Xenopus histone H3.1 (Lane 5), 10 µg Human histone 3.1 (Lane 6), 0.5 µg human histone H3.1 (Lane 7), and 2 µg human histone H3.1 (Lane 8).*

patients. The current results indicating the ability of TMZ to methylate histone proteins suggest another target that may be involved in its mechanism of action. This is an intriguing finding since histone modifications are the basis of epigenetic modifications critical to gene transcription. Numerous anticancer drug discovery efforts are directed at histone enzymes including methylating and demethylating reactions and complexes involved in histone remodeling. The possibility that TMZ could serve as a histone methylating agent offers a new avenue for exploration.

Methylation is a well-known mechanism of epigenetic regulation. Alteration of histone methylation status influences the availability of DNA for transcriptional regulation, DNA repair, RNA processing and signal transduction (26). Histone H3 is an important component of the histone octamer core that forms nucleosomes, which are the basic repeating units of chromatin. Chromatin organization plays an important role in gene-expression regulation through conformational changes from an open to a closed configuration or *vice versa*. The core histone proteins are subjected to a variety of post-translational modifications in both their random coiled N-terminal tails and their globular central cores. One of those post-translational modifications involves methylation of lysine and arginine residues (26). Like phosphorylation, glutathionylation, myristoylation and ubiquitylation, protein methylation and demethylation play an important role in turning on and off certain cellular signaling pathways. When methyl groups replace hydrogen atoms at the side-chain of lysine and arginine residues, it will alter the structure of the protein they bind, hydrophobicity and the size of the amine group in the side chain of lysine and arginine residues. As a result, the electrostatic forces governing the histone-DNA interaction will be reduced and the gene expression of the DNA will be affected. Methylation can also change the hydrophobicity of the vicinity of protein to activate and deactivate different portions of chromatin by blocking transcription factors and other proteins from accessing the DNA. This is an important regulatory mechanism that allows different cells to express different portions of the genome. It is likely that methylation of protein can contribute to the dynamic control of biological processes.

Global levels of histone modifications differ between cell types and they have been found to be associated with the clinical outcome and progression of different cancer types. Low levels of dimethylation of histone lysine number 4 residue (H3K4me2) correlated with low survival rates in both lung and kidney cancers (27). Low levels of H3K4me2 also correlated with adverse prognosis in non-small cell lung carcinomas (NSCLC), hepatocellular carcinomas (HCC) and breast cancers

(28). On the other hand, an increase in dimethylation states of H3K4me2 was found in pancreatic cancer, adenocarcinoma, hormone factory prostate cancer (HRPC) compared to clinically localized cancers (29, 30). Histone modification can be important for potential prognostic and therapeutic targets through activation of methyltransferases and demethylases. Targeting the methylation sites of histone and elevated expression of MGMT might be able to sensitize gliomas cells associated with TMZ resistance and increase TMZ efficacy in malignant gliomas. Thus, studying the action of TMZ on histone methylation and the related consequences on gene transcription and protein signaling networks will provide new insights on its mode of action that could lead to new therapeutic strategies to enhance its efficacy.

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