Article

Temozolomide Analog PMX 465 Downregulates MGMT Expression in HCT116 Colorectal Carcinoma Cells†

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Abstract

The efficacy of temozolomide (TMZ) treatment for cancers is currently limited by inherent or the development of resistance, particularly, but not exclusively, due to the expression of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) in a significant proportion of tumors. We have found that TMZ analog C8-methyl imidazole tetrazine (PMX 465) displayed good anticancer activity against the colorectal carcinoma HCT116 cells which are MGMT-overexpressing and mismatch repair (MMR)-deficient. In this study, we found that PMX 465 could downregulate the expression of MGMT in HCT116 cells at the protein and mRNA levels. We found that PMX 465 could reduce MGMT expression by increasing the binding of wild-type p53 to the *MGMT* promoter and reducing the binding of Sp1 to the *MGMT* promoter.

Keywords: PMX 465; O6-methylguanine-DNA methyltransferase; p53; Sp1; Colorectal carcinoma

Introduction

O6-methylguanine-DNA methyltransferase (MGMT), a ubiquitous DNA repair protein, reverses mutagenic and cytotoxic effects of O6-alkylguanine in DNA induced by alkylating agents. Each single alkyl group removed from O6-alkylguanine is transferred to a cysteine residue within the active site of MGMT in a stoichiometric second-order reaction, implying the inactivation of one MGMT enzyme molecule for each alkyl group removed from alkylguanine, a process termed suicide inhibition(Cabrini et al., 2015; Zhang et al., 2012). Consequently, the effciency of O6-alkylguanine repair is limited by the number of molecules of MGMT enzyme available, also considering that the dealkylating function of MGMT does not possess redundant pathways. The high expression of MGMT in tumor cells imparts resistance to TMZ chemotherapy(Cabrini et al., 2015; Ramirez et al., 2013). Therefore, combinations of MGMT inhibitors such as O6-BG with TMZ to treat cells that overexpress MGMT has been attempted(Wedge et al., 1996). However, myelosuppression limits the use of MGMT inhibitors and alkylating agent combination chemotherapy(Warren et al., 2012). In addition, resveratrol and ursolic acid (UA) have been reported to reverse TMZ resistance by downregulation of MGMT in glioblastoma cells. Moreover, experimental results in a mouse xenograft model showed that the combination treatment of resveratrol/UA and TMZ reduced tumor volumes by depleting MGMT(Lin et al., 2012; Zhu et al., 2016). Therefore, they might be candidate agents for patients with MGMT-overexpressing tumors.

MGMT expression is regulated by a variety of factors, such as promoter methylation and transcription factors(Cabrini et al., 2015). DNA methylation can repress gene transcription either by interfering with the function of transcription factors by cytosine methylation of the recognition sites or by recruiting methyl CpG binding domain (MBD) containing family proteins. Some of MBD proteins have been shown to be able to recruit histone deacetylase (HDAC) directly or through adjuvant repressors. And HDAC can cause histone to be deacetylated, resulting in chromosome compression, isolation of transcription factors and inhibition of transcription(Danam et al., 2005).

In addition, different transcription factors have been found to activate transcription of the MGMT gene, including Sp1, NF-κB, CEBP and AP-1(Cabrini et al., 2015). Among them, specificity protein (Sp) transcription factors play a critical role in embryonic and early postnatal development. The Sp family members are zinc finger proteins; with the exception of Sp2, most of Sp family members could bind GC/GT sequences that are widely distributed in the human genome(Safe et al., 2014). The study found that Sp1, Sp3 and Sp4 were highly expressed in many tumor cells, and they played a part in tumor cell proliferation, survival, inflammation, angiogenesis and infiltration by regulating cancer-causing factors. In addition, Sp1 is a negative prognostic factor for some tumor treatments (Safe et al., 2014; Vizcaino et al., 2015). The MGMT promoter contains six putative Sp1-binding sequences, and Sp1 binding could promote MGMT expression(Bocangel et al., 2009).

The transcription factor p53 has a wide range of functions in cell cycle arrest, DNA repair and apoptosis through transactivation of specific genes in response to DNA damage and other cellular stress signals(Bocangel et al., 2009). In spite of the lack of

typical p53-binding sites in the MGMT promoter, several studies have found that the tumor suppressor p53 impacts MGMT expression(Bobustuc et al., 2015; Bocangel et al., 2009; Natsume et al., 2005). In addition, it has been reported that p53 protein directly interacts with the MGMT promoter and downregulates MGMT transcription(Natsume et al., 2005).

The TMZ derivative C8-methyl imidazole tetrazine, 3-methyl-8-(1-methylimidazol-2-yl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one (PMX 465 or 465, Fig 1), is able to induce apoptosis in MGMT-overexpressing and MMR-deficient HCT116 colon cancer cells(Yang et al., 2017). In this study, we found that PMX 465 could downregulate the expression of MGMT in HCT116 cells at the mRNA and protein levels. The mechanism of MGMT downregulation by PMX 465 was likely related to modulation of MGMT promoter binding with wild-type p53 and Sp1 in HCT116 cells.

Materials and Methods

Cell lines and reagents

HCT116 (hMLH1-) colorectal carcinoma cells , H1299 ($p53^{-/-}$) non-small cell lung carcinoma cells and MCF-7 breast carcinoma cells were obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). T98G glioblastoma cells (sourced from the ATCC) were maintained in DMEM medium supplemented with 10% FBS. Cells were all incubated in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. TMZ was provided by Schering-Plough Research Institute (Kenilworth, N.J., USA). Novel analog PMX 465 was synthesized at Pharminox Ltd, BioCity, Nottingham, UK. Compounds were prepared as 100 mM stock solutions in dimethyl sulfoxide (DMSO) and stored at –20 °C. Reagents, unless specified otherwise, originated from Sigma-Aldrich Ltd.

Western Blot Analysis

Cells were lysed in RIPA lysis buffer (25 mM Tris HCl (pH 7.5), 2.5 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, 100 mM NaCl, 20 mM sodium glycerophosphate, 10 mM sodium pyrophosphate, 0.5% triton X-100) supplemented with a protease inhibitor cocktail (Roche). Cellular proteins (30 μg) were separated by SDS-PAGE, and electro-transferred onto PVDF membranes. Membranes were blocked in Tris-buffered saline (TBS) containing 5% milk and 0.1% Tween-20 at room temperature. All primary antibodies (tubulin, p53, p65 and p-p65 from Cell Signaling; Sp1 and MGMT from Abcam; GAPDH form Millipore) were incubated overnight at 4 °C; membranes were washed at room temperature before incubation with a secondary antibody (GE) conjugated with horseradish peroxidase for 1 h. Detection was performed with Super Signal Chemiluminescent reagent according to the manufacturer's protocol (Tanon ,China).

Real-time PCR

Total RNA was prepared using a total RNA kit (AXYGEN). cDNA was prepared from 1 μg of total RNA using reverse transcription kit (Promega GoScript). The reaction mixture included 1 μL of cDNA, 2 μL 10 μM forward and reverse primers mix (Songgong), 2 μL pure water and 5 μL SYBR green (SYBR Green Master Mix, RCHIO), in a total volume of 10 μL. Gene amplification was carried out using the GeneAmp 7000 Sequence Detection System (Applied Biosystems). Amplification included one stage of 10 min at 95 °C, followed by 40 cycles of a two-step loop: 15 s at 95 °C and 1 min at 60 °C. The gene expression results were normalized to the GAPDH gene.

All experiments were repeated three to five times in triplicate and data are presented as means ± SD. The primers used for real-time PCR were MGMT, 5'-ACCGTTTGCGACTTGGTACTT-3' (forward) and 5'- GGAGCTTTATTTCGTGCA GACC-3' (reverse)； GAPDH, 5'-TGCACCACCAACTGCTTAG -3' (forward) and 5'-GGCATGGACTGTGGTCAT -3' (reverse).

Chromatin immunoprecipitation assay (ChIP)

The chromatin immunoprecipitation assay was performed according to the manufacturer`s (Millipore) protocol. The specific antibodies used for immunoprecipitation were the anti-p53 antibody and accordant rabbit secondary antibody. After protein-DNA cross-links in the immunoprecipitates were reversed, the purified DNA was analyzed by PCR (35 cycles; 45 seconds at 95 °C, 45 seconds at 55 °C, 60 seconds at 72 °C) with primers that detect the MGMT promoter sequence [5'-GCTCCAGGGAAGAGTGTCCTCTGC-TCCCT-3' (forward) and 5'-GGCCTGTG GTGGGCGATGCCGTCCAG-3'(reverse)]. The PCR products were visualized on an ethidium bromide gel.

Transfection assay

HCT116 cells were cotransfected with 1.5 μg of each negative control plasmid (NC) and three p53 shRNA plasmids (678, 968 and 1043) using LipofectAMINE 2000 (Gibco Life Technologies), according to the manufacturer's instructions. These plasmids were a gift from Dr. Wei-Lin Jin (Shanghai Jiao Tong University).

Electrophoretic mobility shift analysis (EMSA)

EMSA was performed as described(Bocangel et al., 2009) with some modifications. The 5' biotin-labeled' oligo 5'-GCCCCGGCCCCGCCCCCGCGCG-3' (sense, containing a Sp1-binding site present in the MGMT promoter) was annealed with appropriate complementary strands to generate duplex oligo. Then, this experiment was performed according to the protocol of the EMSA Kit (Thermo). Specifically, the DNA (10 fmol) was incubated with extracts (10 μg) isolated from HCT116 cells for 20 min at 25 ˚C in 20 μL buffer containing 1X Binding Buffer, 5 mM $MgCl_2$, 0.05% NP-40, 2.5% glycerol and 50 ng/μL poly (dI-dC). These mixtures were separated by nondenaturing 6% polyacrylamide gels in Tris-borate buffer, and electro-transferred onto cellulose membrane. Then the membrane and mixture were crosslinked

at 120 mJ/cm^2 using 254 nm UV-light bulbs for 60 s. Finally, biotin-labeled DNA was detected by Chemiluminescence according to the EMSA Kit's protocol (Thermo).

Results

Analog PMX 465 reduced the expression of MGMT in HCT116 cells

High expression of MGMT is a major clinical reason for the resistance of tumor cells to TMZ(Cabrini et al., 2015; Ramirez et al., 2013). A previous study identified that TMZ analog C8-methyl imidazole tetrazine (PMX 465) displayed good anticancer activity against the colorectal carcinoma HCT116 cells with high MGMT expression levels and MMR deficiency(Zhikuan Yang et al., 2017). We treated HCT116 cells with TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for 48 h, and lysates from compound-treated cells were subjected to Western blot analysis for MGMT protein detection. Compared with control and TMZ treatment groups, we found that PMX 465 and O6-BG (MGMT inhibitor) could reduce the expression of MGMT (Fig 2A). Subsequently, HCT116 cells were exposed to 20, 35 and 50 μM PMX 465 for 48 h. As shown in Fig 2B, PMX 465 down-regulated MGMT in a concentration-dependent manner, and PMX 465 concentrations ≥35 μM PMX 465 could decrease the expression of MGMT. Next, HCT116 cells were treated with TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for 12, 24 and 48 h. The results showed that PMX 465 reduced MGMT expression in a time-dependent manner (Fig 2C). Next, mRNA levels of MGMT were investigated in HCT116 cells following exposure to TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for 24 and 48 h. As shown in Fig 2D, PMX 465 could decrease MGMT mRNA levels at 24 and 48 h significantly, while TMZ and O6-BG treatments had no effect on *MGMT* mRNA expression levels. These data indicate that TMZ analog PMX 465 could down-regulate the expression of MGMT in HCT116 cells at both protein and mRNA levels.

PMX 465 downregulated MGMT expression *via* protein p53

It has been found that transcription factor p53 binds directly to the MGMT promoter and inhibits the expression of MGMT(Natsume et al., 2005). Western blotting analysis was performed to monitor p53 expression after TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) treatment for 48 h. We found that only PMX 465 (50 μM) treatment increased p53 protein expression in HCT116 cells (Fig 3A). HCT116 cells were then treated with TMZ (50 μM) and PMX 465 (20, 35 and 50 μM) for 48 h. As shown in Fig 3B, PMX 465 upregulated p53 expression in a concentration-dependent manner. To further investigate whether PMX 465 disrupts p53 protein interaction with the MGMT promoter and downregulates transcription of MGMT, we carried out chromatin immunoprecipitation (ChIP) assays. Increased binding of p53 to the MGMT promoter was observed after treatment of HCT116 cells with 50 μM PMX 465 for 48 h (Fig 3C). Subsequently, p53 expression in HCT116 cells was knocked down, to further investigate whether PMX 465 regulates MGMT expression *via* p53. As shown in Fig 3D, two p53 shRNA plasmids (968 and 1043) specifically knocked down the p53 protein. It was found that knockdown of p53 by shRNA increased MGMT expression in HCT116 cells exposed to 50 μM PMX 465 for 48 h (Fig 3E). These results suggest that PMX 465 down-regulates MGMT expression in HCT116 cells *via* p53 protein.

Effect of PMX 465 on MGMT expression in cells possessing different p53 status

In order to confirm that PMX 465 caused MGMT downregulation *via* a mechanism involving p53, MCF-7 breast carcinoma cells (wt p53 and MGMT overexpression) were treated with 50 μM TMZ or PMX 465 for 48 h. Compared with control and TMZ, PMX 465 augmented p53 expression accompanied by MGMT protein reduction (Fig 4A). We further studied the influence of PMX 465 on MGMT expression in cell lines expressing mutant (mt) or no p53. H1299 ($p53^{-/-}$) NSCLC (non-small cell lung carcinoma) cells and T98G (mt p53) GBM (glioblastoma) cells were exposed to TMZ (50 μM) or PMX 465 (50 μM) for 48 h. We observed that PMX 465 had no effect on MGMT expression in these cells (Fig 4B and C). These data suggest that MGMT downregulation by PMX 465 was involved with wild type p53.

DNA damaging compounds decreased MGMT expression

P53 serves as a common transcription factor, responding to DNA damage induced by chemotherapeutic agents(Mirzayans et al., 2017). Therefore, we investigated whether other DNA damaging drugs regulate MGMT expression by inducing increased expression of p53. HCT116 cells were treated with doxorubicin (DOX), (0.25, 0.5 and 1 μg/mL) and cisplatin (DDP) (2.5, 5 and 10 μM) for 48 h; it was found that 1 μg/mL DOX, 5 and 10 μM DDP can upregulate p53 while decreasing MGMT protein expression (Fig 5A and B). These results indicate that DNA damaging chemotherapeutic compounds may down-regulate MGMT expression in HCT116 cells *via* a mechanism involving p53.

PMX 465 reduced MGMT expression by transcription factor Sp1

It has been reported that the *MGMT* promoter contains multiple Sp1 binding sites, and transcription factor Sp1 binding could promote MGMT expression(Bocangel et al., 2009). Western blotting analysis was performed to detect Sp1 expression in HCT116 cells after treatment (48 h) with TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM). We found that only PMX 465 could decrease Sp1 protein expression (Fig 6A). HCT116 cells were then exposed to TMZ (50 μM) and PMX 465 (20, 35 and 50 μM) for 48 h. As shown in Fig 6B, PMX 465 reduced Sp1 protein expression in concentration-dependently. To test whether PMX 465 interferes with Sp1 binding to its consensus site in the MGMT promoter, we performed EMSA using a consensus Sp1-binding sequence (Fig 6C) present in the *MGMT* promoter. Significant reduction in the binding of the nuclear extract was observed in HCT116 cells treated with 50 μM PMX 465 for 48 h (Fig 6D; compare lanes 3 and 4). Moreover, the signal shift observed in lane 3 could be prevented by competition from excess non-labeled DNA (Figure 6D; lane 2). These data suggest that MGMT down-regulation by PMX 465 in HCT116 cells is regulated by transcription factor Sp1.

Discussion

MGMT is critical for direct repair of O6-methylguanine DNA adducts, and its high expression confers TMZ resistance(Cabrini et al., 2015; Zhang et al., 2012). Therefore, inhibition of MGMT with several O6-methylguanine derivatives and other compounds has been explored and shown to enhance TMZ-induced cytotoxicity in tumor cells(Fan

et al., 2013). For example, O6-BG is an O6-methylguanine analog that binds to MGMT directly, inducing degradation of MGMT(Wedge et al., 1996). However, myelosuppression manifesting as neutropenia, leukopenia and thrombocytopenia was mainly responsible for the limitation of O6-BG and alkylating agent combination chemotherapy(Warren et al., 2012). In addition, other drugs such as resveratrol and ursolic acid have been reported to attenuate TMZ resistance by downregulation of MGMT in glioblastoma cells and the combination of these compounds with TMZ displayed good anticancer activity in GBM cells which over-express MGMT(Lin et al., 2012; Zhu et al., 2016).

Our earlier studies showed that TMZ analog C8-methyl imidazole tetrazine (PMX 465) (Fig 1) could induce DNA damage and apoptosis in MGMT-overexpressing, MMR-deficient HCT116 colon cancer cells (Zhikuan Yang et al., 2017). In this study, we found that PMX 465 could deplete MGMT expression in HCT116 cells at the protein and mRNA levels, while O6-BG only decreased MGMT protein (Fig 2A and D). These results indicate that PMX 465 might not act in the same way as O6-BG which depletes MGMT as its substrate. It is known that MGMT expression is regulated by a number of processes, such as MGMT promoter methylation, histone acetylation and transcription factor activity(Cabrini et al., 2015).

Studies have shown that p53 is associated with the expression of MGMT, and it has been reported that p53 can bind directly to the MGMT promoter to inhibit its expression(Natsume et al., 2005). Initially, we found that the expression of p53 was upregulated and accompanied MGMT expression reduction in HCT116 cells treated with 50 μM PMX 465 for 48 h (Fig 3A). The reduction of MGMT by PMX 465 was probably related to increasing binding of protein p53 to the MGMT promoter as evidenced by ChIP and confirmed by down-regulating p53 expression with shRNA p53 plasmid (Fig 3C and E). In order to confirm that wt p53 is essential to MGMT down-regulation, MCF-7 breast carcinoma cells (wt p53 and MGMT+) were treated with PMX 465 (50 μM) for 48 h. We found that PMX 465 could increase p53 expression and reduce MGMT protein in MCF-7 cells (Fig 4A). In addition, to further study the effect of PMX 465 on MGMT expression, cells exhibiting null and mutant p53 backgrounds, H1299 ($p53^{-/-}$) NSCLC cells and T98G (mt p53) GBM cells were treated with PMX 465 (50 μM) for 48 h; no MGMT reduction was observed in these cells (Fig 4B and C). Therefore, wt p53 appears indispensible for down-regulation of MGMT by PMX 465. P53 serves as a common transcription factor, and responds to DNA damage induced by ionizing radiation, UV light and chemotherapeutic agents(Bocangel et al., 2009; Mirzayans et al., 2017). Therefore, we speculated whether other DNA damaging agents might regulate MGMT expression *via* increased p53 expression. HCT116 cells were exposed to DOX (0.25, 0.5 and 1 μg/mL) or DDP (2.5, 5 and 10 μM) for 48 h; DOX (1 μg/mL) and DDP (5 and 10 μM) were able to reduce MGMT protein expression by up-regulating p53 expression (Fig 5A and B). These results may imply that some DNA damaging compounds may down-regulate MGMT expression *via* protein p53.

Sp family members sp1, sp3 and sp4 are highly expressed in many tumors and play a critical role in embryonic and early postnatal development. There is evidence that Sp1 expression decreases with age in humans and is a negative prognostic factor

for survival of gastric, pancreatic cancer and glioma patients(Safe et al., 2014; Vizcaino et al., 2015). In addition, previous studies have shown that several pro-oncogenic Sp-regulated genes are important for cell growth, survival, angiogenesis and inflammation. The MGMT promoter contains six putative Sp1-binding sites, and Sp1 can bind directly to the promoter to promote MGMT expression(Bocangel et al., 2009). We found that PMX 465 reduces MGMT expression *via* interference with Sp1. Firstly, PMX 465 is able to reduce Sp1 protein levels in HCT116 cells (Fig 6A). Moreover, as evidenced EMSA experiments, binding between Sp1 and the MGMT promoter was reduced following treatment of cells with 50 μM PMX 465 for 48 h (Fig 6D). Sp1 protein could be downregulated by caspases, activation of proteasomes or drug-induced ROS generation(Safe et al., 2014; Vizcaino et al., 2015). However, the mechanism by which PMX 465 decreases Sp1 expression remains to be elucidated.

Notably, nuclear factor-κB (NF-κB) is also involved in transcriptional regulation of MGMT through direct interaction with two putative NF-κB binding sequences within the MGMT promoter region(Lavon et al., 2007). It has been reported that resveratrol, fluoxetine and bortezomib attenuated MGMT expression by disrupting NF-κB signaling(Lin et al., 2012; Song et al., 2015; Vlachostergios et al., 2013). Our results showed that PMX 465 treatment did not change p65（the subunit of NF-κB）and active P-p65 protein levels (Fig S1A). We performed EMSA using a consensus NF-κB binding sequence present in the MGMT promoter and found that the binding did not change in HCT116 cells treated with 50 μM PMX 465 for 48 h (Fig S1B).

In addition to transcription factor activation, promoter methylation levels and chromatin remodeling can also affect MGMT expression(Nakagawachi et al., 2003). We also investigated the effect of PMX 465 on MGMT promoter methylation in HCT116 cells using bisulfate sequencing PCR (BSP). The promoter sequence contains the usual MSP detection sites. The results showed that PMX 465 did not alter methylation in HCT116 MGMT promoter regions (Fig S2B). In addition, adenovirus E1A protein has been shown to bind with p300 protein to inhibit the transcription of MGMT, leading to silencing of the MGMT promoter(Alonso et al., 2007). Retinoblastoma binding protein 4 (RBBP4) is a component of several chromatin modifying protein complexes evoking varying effects on gene expression, RBBP4 interacts with CBP/ p300 to form a complex that drives the expression of MGMT. Moreover, it has been reported that RBBP4 is a negative modulator of TMZ sensitivity and that disruption of this protein enhances TMZ sensitivity through down-regulation of MGMT(Kitange et al., 2016). We explored whether RBBP4 expression was altered by PMX 465 (50 μM) treatment (48 h) in HCT116 cells (Fig S3). However, the expression of RBBP4 was not changed after treatment with PMX 465.

In summary, we found that TMZ analog PMX 465 could down-regulate the expression of MGMT in HCT116 cells at the protein and mRNA levels. As shown in Fig.7, PMX 465 could reduce MGMT expression by increasing the binding of wild-type p53 to the *MGMT* promoter and reducing the binding of Sp1 to the *MGMT* promoter. Experiments performed in p53 null cells, or cells possessing mutant p53 demonstrated that the effects of PMX 465 on MGMT expression were dependent upon wt p53. Indeed, other DNA damaging chemotherapeutic agents may also down-regulate

MGMT expression in HCT116 cells *via* a wt p53-dependent manner.

In conclusion, the mechanism of action of MGMT down-regulation by PMX 465 in human colorectal carcinoma HCT116 cells was associated with increased binding of wt p53 with the *MGMT* promoter and disruption of Sp1 binding to the *MGMT* promoter.

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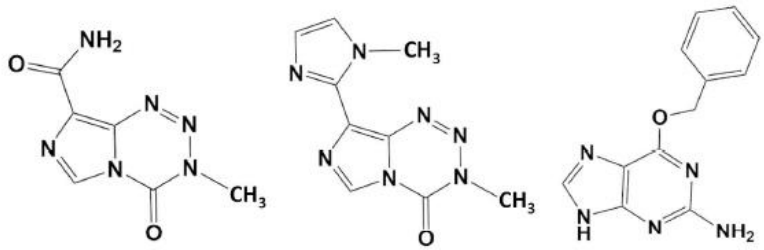

Cell line	IC_{50}（μM）	
	TMZ	PMX465
HCT116	592.88± 16.10	25.37± 2.21

Figure. 1. Chemical structures of TMZ, PMX 465 and O6-BG. And IC50 values in table show the response of HCT116 cells to TMZ and PMX 465.

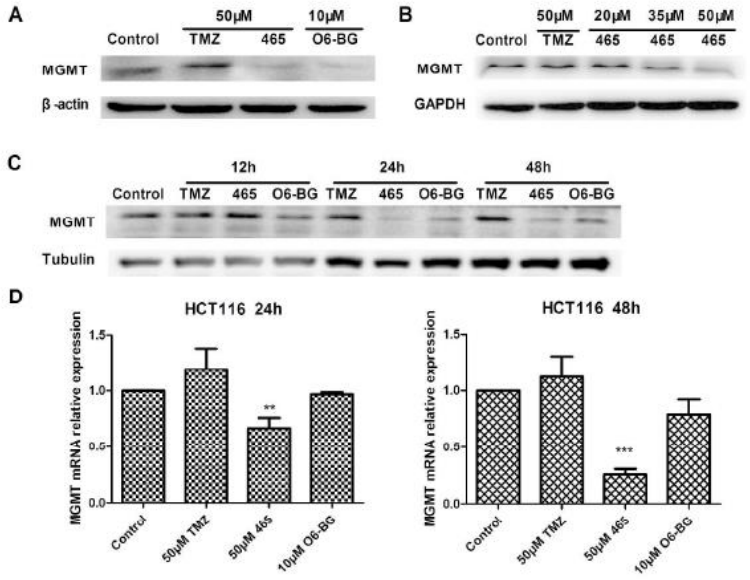

Figure. 2. PMX 465 down-regulates MGMT expression in HCT116 cells. (A) Detection of MGMT expression by Western blot. HCT116 cells were treated with TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for 48 h. (B) and (C) Detection of MGMT expression in HCT116 cells following treatment of cells with different concentrations PMX 465 for 48 h or TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for different time 12, 24 and 48h. (D) mRNA level of MGMT in HCT116 cells. Cells were treated with TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for 24 and 48 h, then analyzed by real time PCR (**P < 0.01, ***P < 0.005).

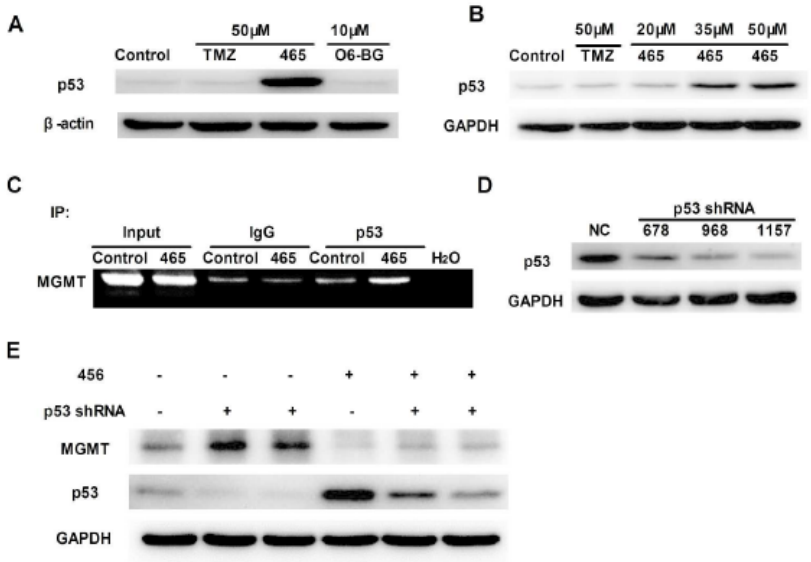

Figure. 3. PMX 465 down-regulates MGMT expression in HCT116 cells via protein p53. (A) Detection of p53 expression by Western blot. HCT116 cells were treated with TMZ (50 μM), PMX 465 (50 μM) and O6-BG (10 μM) for 48 h. (B) Detection of p53 expression in HCT116 cells following treatment of cells with different concentrations PMX 465 for 48 h. (C) Chromatin immunoprecipitation assay detected protein p53 interacting with the MGMT promoter in HCT116 cells which were treated with PMX 465 (50 μM) for 48 h. (D) shRNA interference experiments for p53. At 48 h after NC plasmids (negative control plasmids) and three p53 shRNA plasmids (678, 968 and 1043) transfection, the p53 level in HCT116 cells was analyzed by Western blot. (E) Knocking down of p53 by shRNA in HCT116 cells and treated with 50 μM PMX 465 for 48 h in the same time, MGMT level in HCT116 cells was detectd by Western blot.

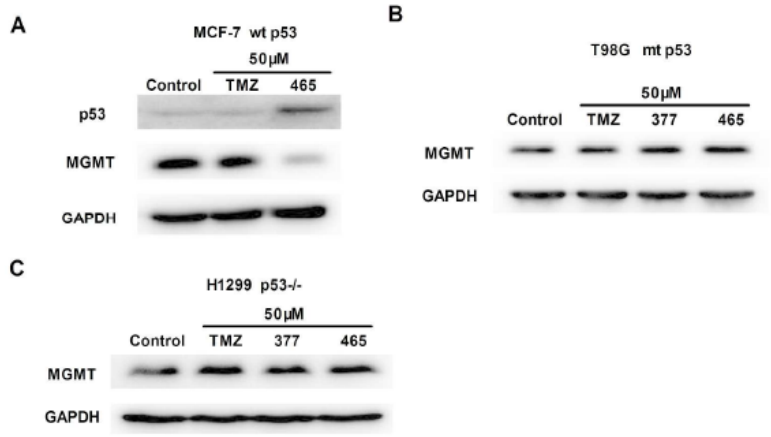

Figure. 4. Effect of PMX 465 on MGMT expression in different p53 background cells. (A) Detection of wt p53 and MGMT expression by Western blot in HCT116 cells treated with TMZ (50 μM) and PMX 465 (50 μM) for 48 h. (B) and (C) Analysis of MGMT expression in H1299 (p53-/-) NSCLC cells and T98G (mt p53) GBM cells following treatment of cells with TMZ (50 μM) and PMX 465 (50 μM) for 48 h.

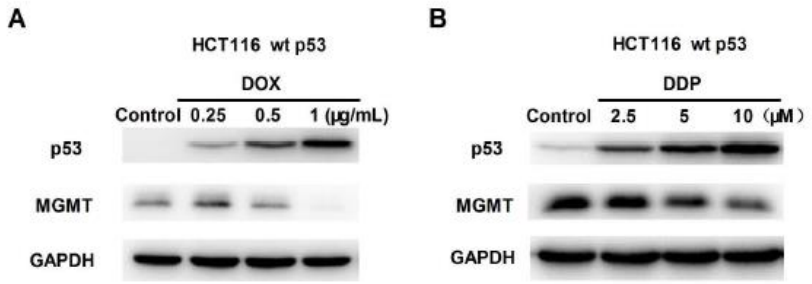

Figure. 5. Effect of DNA damage agents on MGMT expression in HCT116 cells. (A) P53 and MGMT expression after doxorubicin treatments. Cells were treated with DOX (0.25, 0.5 and 1 μg/mL) for 48 h. (B) P53 and MGMT expression after cisplatin treatments. HCT116 cells were treated with DDP (2.5, 5 and 10 μM) for 48 h.

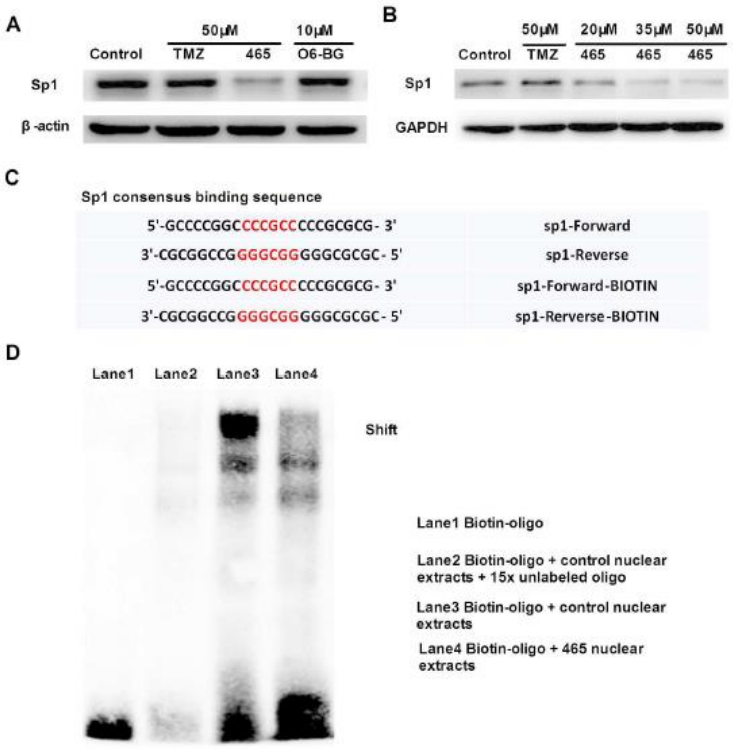

Figure. 6. PMX 465 reduces MGMT expression in HCT116 cells by protein Sp1. (A) Detection of p53 expression by Western blot in HCT116 cells treated with TMZ (50 μM), and PMX 465 (50 μM) for 48 h. (B) Analysis of MGMT expression in HCT116 cells following treatment of cells with different concentrations PMX 465 for 48 h. (C) The sequence of probe containing Sp1 binding site. (D) Electrophoretic shift mobility analysis detected protein Sp1 in the nuclear extract of HCT116 cells treated with 50 μM PMX 465 for 48 h interacting with probe. Lane 1 biotin-oligo; lane 2 biotin-oligo, control nuclear extracts and 15 times unlabeled probe; lane 3 biotin-oligo and control nuclear extracts; lane 4 biotin-oligo and the nuclear extracts of HCT116 cells treated with 50 μM PMX 465 for 48 h.

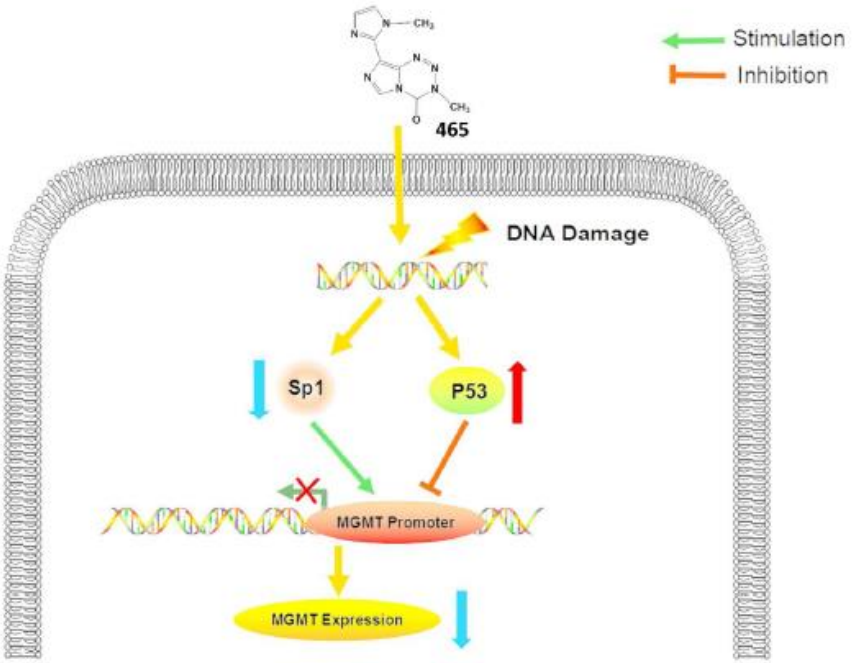

Figure. 7. The depiction of MGMT reduction by PMX 465.