

COMPOSITION AND METHOD FOR TREATING A DRUG-RESISTANT CANCER

CROSS REFERENCE

【0001】 This non-provisional application claims the priority under 35 U.S.C. §119(a) on US Patent Provisional Application No. 63/194,585 filed on May 28, 2021, the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

【0002】 The present invention pertains to a new composition and method for treating a drug-resistant cancer, particularly a TMZ-resistant cancer.

BACKGROUND OF THE INVENTION

【0003】 Glioblastoma multiforme (GBM) is one of the most malignant tumors, and it has an aggressive pattern and a high recurrence rate; it is a World Health Organization grade IV astrocytoma [1]. Despite multimodalities treatment with surgery and concomitant radiation and chemotherapy, patients with GBM still have a poor prognosis, with a mean survival of less than 15 months, which indicates therapeutic resistance [2-4].

【0004】 Colon cancer or colorectal cancer (CRC) is one of the most prevalent kinds of malignancy tumors and the third leading cause of cancer mortality globally. Although colon cancer or CRC standard treatment was already well studied and established, it still remains high mortality and clinical challenge issues. The disease is less symptomatic since patients are frequently diagnosed with advanced cancer at an initial evaluation, and the consequent five-year survival rate is around 10% [5-6]. The standard treatments of CRC are surgery, radiation and/or chemotherapy, in which Oxaliplatin (Oxp) and its prodrug capecitabine are widely used in clinical practice [7-8]. Unfortunately, the recurrence under this kind of DNA crosslink agent treatment is still common within the first few years even after completing the whole cycle [9].

【0005】 Temozolomide (TMZ) is an imidazotetrazine lipophilic prodrug of the alkylating agent dacarbazine with good penetration of the blood-brain barrier. TMZ is

though spontaneous nonenzymatic conversion at physiologic pH to the reactive compound 5-(3-methyltriazene-1-yl)-imidazole-4-carboxamide (MTIC)[10]. The cytotoxicity of MTIC is thought to be primarily due to alkylation (methylation) of DNA, which occurs mainly at the O6 and N7 positions of guanine. TMZ has been widely used as the standard chemotherapy for newly-diagnosed glioblastoma multiforme (GBM) since its initial FDA approval in 2005. Besides GBM, TMZ has been proven to have equal efficacy to dacarbazine. Therefore, it is also used “off-label” in patients with malignant melanoma after standard treatment. In addition, many clinical studies are ongoing to demonstrate the effectiveness of TMZ in other indications, such as brain metastases, lymphomas, neuroendocrine tumors, pituitary tumors, Ewing’s sarcoma, primitive neuroectodermal tumors, refractory leukemia, lung cancer, and other tumors [11]. TMZ is a well-tolerated treatment for elderly, pediatric, or palliative patients in clinical, which can be used as a single agent or as an adjuvant (radiotherapy or chemotherapy) first-line or x-line treatment. However, the drug resistance of TMZ-treated patients will happen due to the over-expression of O6-methylguanine methyltransferase (MGMT). Therefore, it is a clinically meaningful and substantial obstacle that must be overcome to treat GBM successfully.

【0006】 However, less than 50% of patients respond to TMZ due to the overexpression of O6-methylguanine methyltransferase (MGMT), which reverses the methylation of the O6 position of guanine, thereby repairing DNA in GBM cells and resisting the chemotherapeutic effect [12-14]. Comparing the MGMT protein level between newly diagnostic and recurrent CRC patients who had received TMZ treatment supported MGMT reduction may promote efficacy of TMZ treatment[15-17]. In addition to promoter methylation, MGMT is regulated by various transcription factors, such as p53, Sp1, NF-κB, CEBP, and AP-1. Among these, p53 downregulates MGMT transcription by directly interacting with the MGMT promoter [18,19]. Thus, in addition to MGMT promoter methylation, p53 could regulate MGMT expression and cause TMZ resistance. Therefore, additional mechanisms regulating MGMT must be identified to overcome TMZ resistance.

【0007】 Accordingly, it is desirable to develop a new and better therapy or treatment for a drug-resistant cancer, particularly TMB-resistant GBM or CRC.

SUMMARY OF THE INVENTION

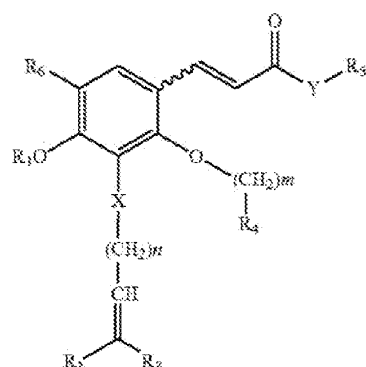
【0008】 Accordingly, the present invention provides a new method for treatment of a drug-resistant cancer, such as TMZ-resistant GBM or CRC.

【0009】 It is unexpectedly found in the present invention that a compound X, such as BMX, can enhance the TMZ-mediated cytotoxic effect on GBM-R cell lines and CRC cell lines HT29, HCT116 and RKO. Accordingly, the present invention provides a new method for treatment of a drug-resistant cancer in a patient, particularly TMZ-resistant GBM or CRC, comprising administering said patient a combination of BMX and TMZ.

【0010】 In the invention, BMX (NBM-T-L-BMX-OS01) which is a histone deacetylase 8 inhibitor (HDAC8i), shows significantly anti-cell proliferation effects in colorectal cancer cell, human umbilical endothelial cells, lung cancer cells and glioblastoma cell, and it also presents tumor suppression ability in animal xenograph model [20, 21]. However, it is unexpectedly found in the present invention that BMX could overcome drug- resistance in cancer cells. In one example of the invention BMX could overcome GBM-R cells by enhancing the TMZ-mediated cytotoxic effect by downregulating the β -catenin/c-Myc/SOX2 signaling pathway and upregulating p53-mediated MGMT inhibition. It was confirmed that high HDAC8 expression in human GBM tissues and GBM-R cell lines correlated with MGMT levels, and the combination of BMX and TMZ induced WT(Wild Type)-p53 mediated apoptosis through WT-p53 mediated MGMT inhibition in GBM-R cell lines. Moreover, the combination of BMX and TMZ suppressed cell proliferation and GSC phenotype activity via the β -catenin/c-Myc/cyclin D1/SOX2 signaling pathway in GBM-R cell lines.

【0011】 In one example of the invention, it was evidenced that a combination of BMX and TMZ triggered on cell cycle arrest, senescence, autophagy and apoptosis in CRC cells via upregulating p53/p21/Puma/Bax is compromised by the crosstalk of the downregulating Wnt / β -catenin/cyclin D1/c-Myc /p62 pathways. Accordingly, BMX could be a promising strategy for the precision personal treatment of TMZ-resistant GBM patients or CRC patients with WT-p53.

【0012】 Accordingly, in one aspect, the present invention provides a combination for treating a TMZ-resistant cancer, which comprises TMZ and a compound A having the structure of Formula A, or a pharmaceutically acceptable salt, stereoisomer, enantiomer, prodrug or solvate thereof.



Formula A

wherein

R^1 is hydrogen, alkyl, alkenyl, C_5 - C_6 cycloalkyl, 5-membered or 6-membered unsaturated carbocycle or 5-membered or 6-membered heterocycle, or $(CH_2)_m R^4$;

X is C, -O-, -N- or -S-;

Y is -O-, -NH or -O- C_1 - C_4 alkyl;

n is an integer of 0 to 10;

m is an integer of 0 to 5;

R^2 and R^3 is independently C_1 - C_6 alkyl;

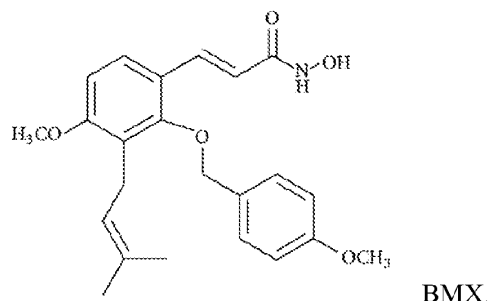
R^4 is C_5 - C_6 cycloalkyl or 5-membered or 6-membered unsaturated carbocycle or heterocycle which may be substituted with halogen, $-CF_3$, $-OR^7$ or $-NR^7R^8$, wherein R^7 and R^8 are independently hydrogen or C_1 - C_6 alkyl;

R^5 is OH, NH_2 or C_5 - C_6 cycloalkyl, 5-membered or 6-membered unsaturated carbocycle or heterocycle wherein the cycloalkyl, carbocycle and heterocycle may be optionally substituted with halogen, NH_2 , NO_2 , C_1 - C_6 alkoxy, C_1 - C_6 alkylthio, $OR^{7'}$, NR^7R^8 or CF_3 ; and

R^6 is H, C_1 - C_{10} alkyl which may be substituted by hydroxy or C_2 - C_{10} alkenyl, or together with R_1 being $-C_2H_2-$; and

wherein TMZ and the compound A are combined at a relative ratio to effectively overcome TMZ resistance.

【0013】 In one embodiment of the invention, the compound A is a compound BMX having the structure of



【0014】 According to the present invention, the TMZ resistance is overcome by enhancement of TMZ-mediated cytotoxic effect by downregulating the β -catenin/c-Myc/SOX2 signaling pathway and upregulating WT-p53 mediated MGMT inhibition.

【0015】 In the invention, TMZ and the compound A, e.g., BMX, are separately or sequentially administered.

【0016】 In one example of the invention, the cancer is glioblastoma multiforme (GBM) or colorectal cancer (CRC).

【0017】 In another aspect, the present invention provides a method for treating a TMZ-resistant cancer in a patient, which administering the patient a therapeutically effective amount of the combination according to the invention.

【0018】 In one example of the invention, the cancer is GBM or CRC.

【0019】 In a further aspect, the present invention provides a method for treating precision personal treatment for a drug-resistant cancer in a patient, comprising determining the expression of WT-p53 in the patient, and administering the patient a therapeutically effective amount of BMX or its combination with said drug if the expression of WT-p53 is present in the patient.

【0020】 In one embodiment of the invention, the drug is TMZ.

【0021】 In one particular embodiment of the invention, the drug-resistant cancer is TMZ-resistant cancer, particularly GBM or CRC.

【0022】 In the invention, it is confirmed that BMX is effective in enhancing inhibition of WT-p53 cancer cells.

【0023】 In a further aspect, the present invention provides a use of the combination of BMX and TMZ for manufacturing a medicament or a kit for treating a TMZ-resistant cancer.

【0024】 The present invention will be further described by way of the following examples. However, it should be understood that the following examples are solely intended for the purpose of illustration and should not be construed as limiting the present invention in practice.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

【0025】 The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred.

【0026】 In the drawings:

【0027】 Figure 1 provides the pathway analysis for genes potentially associated with HDAC8 by bioinformatics tools; wherein shRNA HDAC8 was entered into the CLUE database, and CP and PCL with a score of >90 were selected (A). The target genes were entered into the CPDB pathway analysis database (B) for further experiments. (C) Top 10 pathways for selecting CP and PCL (score > 90) for shRNA HDAC8. 10 pathways as below: VEGF; PI3K-Akt Signaling Pathway; JAK STAT pathway and regulation; Signaling Pathway; MAPK signaling pathway - Homo sapiens (human); Apoptosis; Autophagy; HIF-1 signaling pathway; TNF related weak inducer of apoptosis (TWEAK) Signaling Pathway; Wnt Signaling Pathway. VEGF; PI3K-Akt Signaling Pathway; JAK STAT pathway and regulation; Signaling Pathway; MAPK signaling pathway - Homo sapiens (human); Apoptosis; Autophagy; HIF-1 signaling pathway; TNF related weak inducer of apoptosis (TWEAK) Signaling Pathway; Wnt Signaling Pathway.

【0028】 Figure 2 shows that BMX inhibited the growth and proliferation of GBM cells (U87MG and A172) and the BMX and TMZ combination inhibits the growth and proliferation in GBM-R cells (U87MG-R and A172-R). (A) Chemical structure of BMX. (B) Cell viability of GBM and GBM-R cell lines after treatment with 0.5, 10, 15, 30, or 50 μ M BMX. (C) Cell viability of GBM and GBM-R cell lines after

treatment with 0.25, 50, 100, 200, 400, or 800 μ M TMZ. (D) GBM and GBM-R cell viability after treatment with 10 μ M BMX with or without TMZ at various concentrations (0.25, 50, 100, 200, 400, or 800 μ M) for 24 hrs. (E) GBM and GBM-R cell viability after treatment with 50 μ M TMZ with or without BMX at various concentrations (0.5, 10, 15, 30, or 50 μ M) for 24 hrs. (F) GBM-R cell viability after treatment with 50 μ M TMZ with or without 10 μ M BMX for 24, 48, and 72 hrs. (G) Colony formation assay of GBM and GBM-R cell lines with BMX (0, 5, or 10 μ M) with or without TMZ (50 μ M) for 14 days. Data are represented as means $\pm$ SEM from three experiments. * $p < 0.05$ vs. control (A172 and U87MG); # $p < 0.05$ vs. (A172-R and U87MG-R).

【0029】 Figure 3 shows that BMX enhanced the TMZ-mediated cytotoxic effect by targeting the Wnt/ β -catenin/GSK3 β pathway to suppress cell proliferation in GBM-R cells. (A) GSK-3 β and β -catenin activation status of GBM-R cells after treatment with 5 or 10 μ M BMX with or without 50 μ M TMZ for 48 hrs. (B) c-Myc and cyclin D1 protein levels of GBM-R cells after treatment with 5 or 10 μ M BMX with or without 50 μ M TMZ for 48 hrs. (C) β -catenin (Ser33/37/41) phosphorylation status and changes in c-Myc and cyclin D1 protein expression in GBM-R cells after treatment with 10 μ M BMX and 50 μ M TMZ with or without 10 μ M MG132.

【0030】 Figure 4 shows that BMX and TMZ combination enhanced the TMZ-mediated cytotoxic effect by promoting TMZ-mediated apoptosis in GBM-R cells. (A) Cell cycle distribution of GBM (U87MG and A172) and GBM-R (U87MG-R and A172-R) cells treated with BMX for 48 hrs with or without a TMZ. (B) Percentages of cells in G0/G1, S and G2/M phases are presented in the histograms. (C) Bar graph of the percentage of sub-G 1. (D) Annexin V/PI apoptosis assay of GBM (U87MG and A172) and GBM-R (U87MG-R and A172-R) cells treated with BMX for 48 hrs with or without TMZ. (E) Histograms illustrating the percentages of apoptotic cells.

【0031】 Figure 5 shows that the combination of BMX and TMZ enhanced the TMZ-mediated cytotoxic effect by WT-p53 mediated MGMT inhibition in GBM-R cells. (A) The expression pattern of WT-p53 and MGMT on the GBM (U87MG and A172) and GBM-R (U87MG-R and A172-R) cell lines. (B) Protein alterations of WT-p53, MGMT, P21, Bax, Bcl-2, Puma, and cleaved caspase-3 after treatment with 5 or 10 μ M BMX with or without 50 μ M TMZ for 48 hrs on U87MG-R and A172-R cells. (C) Treatment of GBM (U87MG and A172) and GBM-R (U87MG-R and

A172-R) cells with 5 or 10 μ M BMX with or without 50 μ M TMZ for 48 hrs reduced MGMT levels and increased WT-p53 and Phospho-WT-p53 levels (ser 15). β -actin was used as an internal control.

【0032】 Figure 6 shows that the combination of BMX and TMZ reduced GSC formation in GBM-R cells. (A) Status of CSC-related genes (CD133, CD44, and SOX2) expression between parental and resistant daughter cell lines. (B) Changes in CD133, CD44, and SOX2 protein levels after receiving 5 and 10 μ M BMX with or without 50 μ M TMZ for 48 hrs on U87MG-R and A172-R cells. (C) Immunohistochemical staining for HDAC8 and CSC-related genes (CD133 and CD44) in human primary GBM (the same patient before concomitant radiation and chemotherapy) and recurrent GBM tumor tissues (after concomitant radiation and chemotherapy) obtained through surgical biopsies.

【0033】 Figure 7 provides a working model of the mechanism of combination of BMX with TMZ to overcome TMZ resistance in GBM-R cells.

【0034】 Figure 8 shows the genetic features of GBM cell lines (A)The expression of HDAC8 from Abcam company in GBM cells by western blot.

【0035】 Figure 9 shows that BMX was a potent semi synthesized HDAC8 inhibitor. (B) The examination of MGMT methylation pairs GBM through big data analysis. The wide-type and mutant are labeled with blue and red, respectively (one-sided t test, *: $p < 0.05$). (A) HDAC8 expression levels stimulated with different doses of BMX (0-10 μ M) in the presence or absence of TMZ (50 μ M) were determined using qRT-PCR assays. (B) HDAC8 expression levels stimulated with BMX (0-10 μ M) with or without TMZ (50 μ M) were determined using Western blotting.

【0036】 Figure 10 shows that BMX and BMX with TMZ inhibited the growth and proliferation of U87MG, U87MG-R, A172 and A172-R. (A) Cell viability of GBM and GBM-R cell lines after treating with indicated concentrations of BMX (0.5, 10, 15, 30 or 50 μ M) for 24, 48 and 72 hrs. (B) Cell viability of GBM and GBM-R cell lines after treating with the indicated concentrations of TMZ (0.25, 50, 100, 200, 400 or 800 μ M) 24, 48 and 72 hrs. (C) Cell viability of GBM and GBM-R cells lines after treating with 10 μ M BMX with or without TMZ at different concentrations (0.25, 50, 100, 200, 400 or 800 μ M for 24, 48 and 72 hrs. (D) Cell viability of GBM and GBM-R cells lines after treating with 50 μ M TMZ with or without BMX at different concentrations (0.5, 10, 15, 30 or 50 μ M) for 24, 48 and 72 hrs. (E) Cell viability of

GBM-R cells lines after treating with 50 μ M TMZ with or without 10 μ M BMX for 24, 48 and 72 hrs.

【0037】 Figure 11 shows that in vitro cytotoxicity of BMX in GBM cells. (A) U87 and U87R cells and (B) A172 and A172R cells were treated with TMZ (50 and 100 μ M). This inhibitory effect of TMZ on GBM cells was determined using a clonogenic assay. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

【0038】 Figure 12 shows the drug and gene information in Glioblastoma, including the HDAC8 expression levels of two parent GBM cell lines (A172 and U87MG), which are wild-type p53 (WT-p53), and two TMZ-resistant GBM cell lines.

【0039】 Figure 13 shows BMX, TMZ, oxaliplatin and doxorubicin combination inhibited cell proliferation in CRC cells. (A) The proliferation of BMX, TMZ, Oxp, Dox, BMX plus TMZ, BMX plus Oxp or BMX plus Dox in HT29, HCT116 and RKO cells with various drug concentrations and treatment durations were assayed using the CCK-8 method. (B) Colony formation capability assay with different treatments of BMX, TMZ, Oxp, BMX plus TMZ, and BMX plus Oxp in HT29, HCT116 and RKO cells, the clones were quantified and presented as a statistical figure. (C) Cell cycle analysis after 48 hrs treatment with different concentrations of BMX or BMX combined with TMZ in HT29, HCT116 and RKO cells and the proportion of cells in each cell cycle phase. (D) Apoptosis analysis after 48 hrs treatment with different concentrations of BMX or BMX combined with TMZ and the apoptotic rate of cells in HT29, HCT116 and RKO cells. All results are shown as mean $\pm$ s.d. from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control (HT29 cells); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. control (HCT116 cells); † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. control (RKO cells).

【0040】 Figure 14 shows effects of BMX, BMX and TMZ combination-induced apoptosis and autophagy were mediated by p53 mediated MGMT inhibition. (A) Western blot analysis of P53, p53 Lys382 acetylation, p53 Ser15 phosphorylation, p21, p16, MGMT, γ H2AX, E2F1 and E2F3 expression in HT29, HCT116 and RKO cells treated with various concentrations BMX (5 and 10 μ M) and BMX combined with TMZ for 48 hrs. (B) Expressions of cleaved caspase-3, -7, -8, -9 and cleaved PARP proteins in HT29, HCT116 and RKO cells treated with various concentrations BMX (5 and 10 μ M) and BMX combined with TMZ for 48 hrs. (C) Expressions of Bax, Bcl-2, BID, Bim, Bak and Puna proteins in HT29, HCT116 and RKO cells

treated with various concentrations BMX (5 and 10 μ M) and BMX combined with TMZ for 48 hrs. GAPDH was used as the loading control.

【0041】 Figure 15 shows BMX, BMX and TMZ combination induces cellular senescence in HT29, HCT116 and RKO cells. Senescence-associated β -galactosidase (SA β -gal) staining of BMX and TMZ combination. Cells were treated with 10 μ M of BMX plus TMZ (50 μ M) for 48 hrs, the cells were stained with SA β -gal (blue cytoplasmic stain). Scale bar, 50 μ m. Quantification of SA β -gal activity. All results are shown as mean $\pm$ s.d. from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control (HT29 cells); # p < 0.05, ## p < 0.01, ### p < 0.001 vs. control (HCT116 cells); † p < 0.05, †† p < 0.01, ††† p < 0.001 vs. control (RKO cells).

【0042】 Figure 16 shows BMX and TMZ combination reduced CSC formation in HT29, HCT116 and RKO cells. Changes in CD133, CD44, and SOX2 protein levels after receiving 5 and 10 μ M BMX with or without 50 μ M TMZ for 48 hrs on HT29, HCT116 and RKO cells. GAPDH was used as the loading control.

【0043】 Figure 17 shows BMX enhanced the TMZ-mediated cytotoxic effect by targeting the Wnt/ β -catenin/GSK3 β pathway in CRC cells. (A) GSK-3 β , β -catenin activation status, c-Myc and Cyclin D1 of HT29, HCT116 and RKO cells after treatment with 5 or 10 μ M BMX with or without 50 μ M TMZ for 48 hrs. (B) GSK-3 β , β -catenin activation status, c-Myc and Cyclin D1 expression is upregulated by BMX with or without 50 μ M TMZ and MG132 in T29, HCT116 and RKO cells. (C) P53 and MGMT expression is upregulated by BMX with or without 50 μ M TMZ and MG132 in T29, HCT116 and RKO cells. GAPDH was used as the loading control.

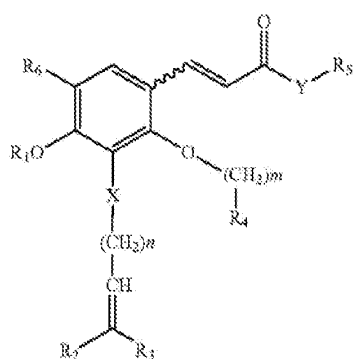
【0044】 Figure 18 shows autophagy was responsible for BMX along, BMX and TMZ combination induced cell death. (A) LC3 and P62/SQSTM1 expression in HT29, HCT116 and RKO cells treated with BMX (5 and 10 μ M) with or without 50 μ M TMZ evaluated by western blot. (B) p62/SQSTM1 expression is downregulated by BMX with or without 50 μ M TMZ and MG132 in T29, HCT116 and RKO cells. (C) Pre-treatment with BAF and VAD reduced the cell apoptosis in HT29, HCT116 and RKO cells exposed to BMX (5 and 10 μ M) with or without 50 μ M TMZ for 48 hrs. (D) Effects of VAD and BAF on BMX (5 and 10 μ M) with or without 50 μ M TMZ induced cleaved caspase-3, cleaved PARP, P62 and LC3 expression. GAPDH was used as the loading control.

DETAILED DESCRIPTION OF THE INVENTION

【0045】 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person skilled in the art to which this invention belongs.

【0046】 The invention provides a new method for treating a TMZ-resistant cancer (e.g., GBM and CRC) patient is provided using a combination of TMZ and a Compound A.

【0047】 Compound A is a novel small-molecule isoform-selective HDAC8 inhibitor. The compound A is disclosed in US Patent No. 7,994,357, the content of which is hereby incorporated by reference in its entirety. The compound A has a structure of Formula A, or a pharmaceutically acceptable salt, stereoisomer, enantiomer, prodrug or solvate thereof:



Formula A

wherein

R^1 is hydrogen, alkyl, alkenyl, C_5 - C_6 cycloalkyl, 5-membered or 6-membered unsaturated carbocycle or 5-membered or 6-membered heterocycle, or $(CH_2)_mR^4$;

X is C, -O-, -N- or -S-;

Y is -O-, -NH or -O- C_1 - C_4 alkyl;

n is an integer of 0 to 10;

m is an integer of 0 to 5;

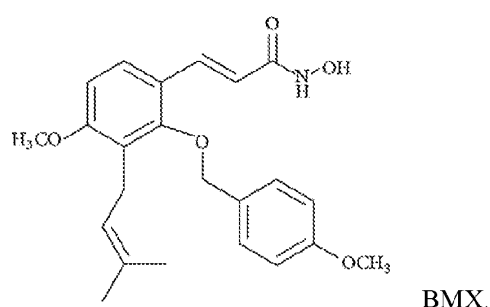
R^2 and R^3 is independently C_1 - C_6 alkyl;

R^4 is C_3 - C_6 cycloalkyl or 5-membered or 6-membered unsaturated carbocycle or heterocycle which may be substituted with halogen, $-CF_3$, $-OR^7$ or $-NR^7R^8$, wherein R^7 and R^8 are independently hydrogen or C_1 - C_6 alkyl;

R^5 is OH, NH_2 or C_5 - C_6 cycloalkyl, 5-membered or 6-membered unsaturated carbocycle or heterocycle wherein the cycloalkyl, carbocycle and heterocycle may be optionally substituted with halogen, NH_2 , NO_2 , C_1 - C_6 alkoxy, C_{1-6} alkylthio, $OR^{7'}$, NR^7R^8 or CF_3 ; and

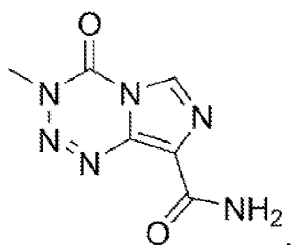
R^6 is H, C_1 - C_{10} alkyl which may be substituted by hydroxy or C_2 - C_{10} alkenyl, or together with R_1 being $-C_2H_2-$.

【0048】 In one particular embodiment of the present invention, the Compound A is BMX, which was derived from the semi-synthesis of osthole and play a novel role in learning and memory as reported in Yang YC et al. [22]:



【0049】 It is known that BMX is an isoform-selective HDAC8 inhibitor, having the lowest toxicity and the ability to cross the blood-brain barrier[22].

【0050】 As used herein, the term “Temozolomide” or “TMZ”, which is sold under the brand name “Temodar” among others, refers to a medication used to treat some brain tumors such as glioblastoma multiforme or anaplastic astrocytoma. TMZ has the structure below:



【0051】 Temozolomide (TMZ) is an alkylating agent used for treatment of some cancers, such as a second-line treatment for astrocytoma and a first-line treatment for glioblastoma multiforme. It is also found that Olaparib in combination with temozolomide demonstrated substantial clinical activity in relapsed small cell lung cancer.

【0052】 As used herein, the term “glioblastoma multiforme,” “glioblastoma” or “GBM” refers to a brain cancer, which can either start from normal brain cells or develop from an existing low-grade astrocytoma. There is no known method of preventing GBM. Treatment usually involves surgery, after which chemotherapy and radiation therapy are used. The medication temozolomide (TMZ) is frequently used as part of chemotherapy.

As used herein, the term “colon cancer,” “colorectal cancer” or “CRC” also known as bowel cancer, or rectal cancer, refers to a cancer developed from the colon or rectum (parts of the large intestine). Its signs and symptoms may include blood in the stool, a change in bowel movements, weight loss, and fatigue. The standard treatments of CRC are surgery, radiation and/or chemotherapy, in which Oxaliplatin (Oxp) and its prodrug capecitabine are widely used in clinical practice. Unfortunately, the recurrence under this kind of DNA crosslink agent treatment is still common within the first few years even after completing the whole cycle.

【0053】 In the present invention, BMX is found to overcome TMZ resistance by enhancing TMZ-mediated cytotoxic effect by downregulating the β -catenin/c-Myc/SOX2 signaling pathway and upregulating WT-p53 mediated MGMT inhibition. The results in the present invention indicated that BMX or its combination with TMZ is promising for precision personal treatment of TMZ-resistant WT-p53 GBM or CRC cells.

【0054】 The present invention is further illustrated by the following examples, which are provided for the purpose of demonstration rather than limitation.

【0055】 Example 1

【0056】 1.1 Materials and Methods

【0057】 1.1.1 Cell culture and reagents

【0058】 Four GBM cell lines U87, U87R, A172, and A172R were used in this study. The American Type Culture Collection (ATCC; Manassas, VA, USA)

provided human GBM cell lines U87-MG (ATCC HTB-14; GBM of unknown origin) and A172 (ATCC CRL-1620; ATCC). U87R and A172R cells were obtained from Dr. Tsung-I Hsu and Dr. Jian-Ying Chung (The Ph.D. Program for Neural Regenerative Medicine, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 50 μ M TMZ for at least 60 days. TMZ resistance in U87R and A172R cells was confirmed using the colony formation assay (Figure 11). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin (all from Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and maintained in a humidified incubator at 37 °C and 5% CO₂. NBM-BMX (BMX), (E)-2-(4-Methoxybenzyloxy)-3-prenyl-4-methoxy-N-hydroxycynamide, was provided by NatureWise Biotech & Medicals Corporation (Taipei, Taiwan).

【0059】 1.1.2 Cell proliferation and colony formation assays

【0060】 We plated 3000 GBM cells per well in 96-well plates and allowed them to adhere overnight. To validate cell line responsiveness to BMX and TMZ monotherapy, the cells were treated with different doses of BMX or TMZ for 24, 48, and 72 hrs. To confirm cell responsiveness to the BMX-TMZ combination, cells were either treated with different doses of TMZ (0-800 μ g/mL) with or without BMX (10 μ M) for 24, 48, and 72 hrs or with different doses of BMX (0-50 μ M) with or without TMZ (50 μ M) for 24, 48, and 72 hrs. Following treatment, the absorption value was measured using a CCK8 kit (Targetmol, Shanghai, China) at the indicated time points. The results are reported as the mean $\pm$ standard deviation of at least three replicates.

【0061】 A172, A172-R, U87MG, and U87MG-R cells were seeded (1000 cells/dish) into 6-cm culture dishes and incubated for 14 days. The cells were washed three times with phosphate-buffered saline, fixed in 4% paraformaldehyde for 30 min, and stained with 0.1% crystal violet for 20 min at 25 °C. The colonies were carefully washed with tap water, and then the number of colonies, defined as at least 50 cells, were counted and analyzed. The results are expressed as the average colony count $\pm$ SE from three independent experiments.

【0062】 1.1.3 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

【0063】 The ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for quantitative analysis of mRNA expression. The cells (2×10^5) were seeded in 6-well plates, and total RNA was extracted using Tissue Total RNA Mini Kit (Geneaid, Taipei, Taiwan). A 10-ng sample of total RNA was transcribed into cDNA by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was quantified using Fast SYBR Green Master Mix (Applied Biosystems) following the procedures provided by the manufacturer, with 18s as the inner reference. All procedures were performed according to the manufacturer's protocols. The thermocycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 s. Each sample was analyzed in triplicate. The threshold cycle (Ct) values were calculated using the StepOnePlus (Applied Biosystems) software. The relative expression of each mRNA was calculated using the $2^{-\Delta Ct}$ method. The primer sequences for HDAC8 were as follows:

HDAC8 forward 5'-GCGTGATTTCAGCACATAA-3' (SEQ ID NO: 1);

HDAC8 reverse 5'-ATACTTGACCGGGGTCATCC-3' (SEQ ID NO: 2).

18s forward 5'-TCAAGTGCAGTGCAACAACCTC-3' (SEQ ID NO: 3);

18s reverse 5'-AGAGGACAGGGTGGAGTAATCA-3' (SEQ ID NO: 4).

【0064】 1.1.4 Flow cytometric analysis of the DNA cell cycle

【0065】 For the DNA cell cycle, following treatment with different doses of BMX (0–10 μ M) in the presence or absence of TMZ (50 μ M) for 48 hrs, the cells were harvested through trypsinization, washed twice with phosphate-buffered saline, and fixed in methanol. The cells were then washed again, subjected to RNase A at a final concentration of 0.05 mg/mL (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), and incubated with 10 μ g/mL propidium iodide (PI; Sigma-Aldrich; Merck Millipore) at 4 °C for 15 min in the dark. Cell cycle analysis was conducted using a fluorescence-activated cell sorting (FACS) flow cytometer (Attune NxT flow cytometer, Thermo Fisher Scientific).

【0066】 1.1.5 Flow cytometric analysis of apoptosis

【0067】 To analyze cell apoptosis in different doses of BMX (0–10 μ M) in the presence or absence of TMZ (50 μ M), FITC-labeled annexin V/PI staining was performed using the CF®488A Annexin V and PI Apoptosis Kit (Fremont, CA, USA), according to the manufacturer's instructions. The analysis by flow cytometry of PI

and annexin was performed 48 hrs post-treatment. A total of 10,000 nuclei were measured using an FACS flow cytometer (Attune NxT flow cytometer, Thermo Fisher Scientific).

【0068】 1.1.6 Immunohistochemical staining

【0069】 Immunohistochemical staining was performed on 4- μ m-thick paraffin sections. The sections were dewaxed hydrated and placed at 4 °C overnight. For antibodies against CD133 (AP1802a, Abgent, San Diego, CA, USA), P62 (ab56416, Abcam, Cambridge, MA, USA), and LC3II (AP1802a, Abgent), the standard avidin-biotin complex procedures were employed. After the sections were returned to room temperature, biotinylated secondary antibodies and horseradish-labeled streptavidin were added. The samples were then incubated in an oven at 37 °C. Subsequently, DAB color development, hematoxylin counterstaining, gradient alcohol dehydration, and xylene transparent were conducted. All samples were sealed with neutral gum afterwards. Human brain tissues: The ethics statements in this study were approved by the Institutional Review Board of Kaohsiung Medical University Hospital (No. KMHIRB-F(I)-20200024). Informed consent was obtained from all subjects involved in the study.

【0070】 1.1.7 Western blot analysis

【0071】 The cells were collected and lysed in RIPA lysis buffer (EMD Millipore Billerica, MA, USA, 10 $\times$ RIPA buffer) containing a protease inhibitor. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The SDS loading buffer was mixed with the protein samples. Proteins (20 μ g/lane) were separated using 8%–12% SDS-PAGE and transferred to a PVDF membrane, which was blocked with 5% bovine serum albumin at room temperature for 1 hr in tris-buffered saline (TBS)-Tween 20 (0.5%; TBS-T), incubated with primary antibodies overnight at 44°C, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hr. After washing thoroughly with TBS-T, the HRP signals were detected with a chemical HRP substrate. The antibodies we used are listed in Table 3. Signal of each target protein was visualized by incubation with an enhanced chemiluminescent reagent and exposure to X-ray film.

【0072】 1.1.8 Statistical analysis

【0073】 Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using one-way analysis of variance. Data were compared using the Student's t test. The level of statistical significance was set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3. Experimental antibodies.

Designation	Source or Reference	Identifiers	Additional Information
GAPDH	Arigo, Hsinchu, Taiwan	ARG10112	1:5000
β -actin	Santa Cruz, CA, USA	sc-47778	1:2000
HDAC8	ABclonal, Woburn, MA, USA	A8865	1:1000
c-Myc	Cell Signaling Technology, Inc	#5605s	1:1000
cyclin D1	Cell Signaling Technology, Inc	#2978s	1:1000
cyclin B	Cell Signaling Technology, Inc	#12231s	1:1000
Caspase-3	p15;	NB100-56708	1:1000
Bax	Cell Signaling Technology, Inc	#2772s	1:1000
p21	Cell Signaling Technology, Inc	#2947s	1:1000
Bcl-xL	Cell Signaling Technology, Inc	#2764s	1:1000
phospho-p53	p15;	#9284s	1:1000
puma	Cell Signaling Technology, Inc	#12450s	1:1000
MGMT	Cell Signaling Technology, Inc	#2739s	1:1000
p53	Santa Cruz, CA, USA	sc-126	1:1000

CD133	Cell Signaling Technology, Inc	#5860s	1:1000
CD44	Abcam, Cambridge, MA, USA	ab157107	1:1000
SOX-2	Cell Signaling Technology, Inc	ab97959	1:1000

【0074】 1.1.9 Predict the potential mechanism of a HDAC8 inhibitor through multi-database platform

Indirect process: CLUE calculated the connectivity score of shRNA HDAC8 among their one million profiles and ordered the similarity to opposite compounds as well as gene perturbations. The criteria were filtered above 90 positive scores and collected each instance targeted-genes as shRNA HDAC8 regulators in biological function. Input this genes list in CPDB platform for enrichment analysis to obtain clear pathways information. Direct process: Based on BMX-L1000 gene expression data, both up-regulation and down-regulation gene list which respond to the drug biological function in HepG2 cells. BMX (1 μ M) compared to DMSO control to define the significant differential expression genes (DEGs) according to ± 1.5 fold change, p-value < 0.05 . Hence, the DEGs were used as inputs to query CPDB for pathway analysis. To narrow down the prioritized pathways, we interested two results and selected the common element.

【0075】 1.2 Results:

【0076】 1.2.1. Pathway analysis for potential expression profile of HDAC8 inhibitor through bioinformatics tools

【0077】 To explore possible mechanisms of HDAC8 inhibitor and gene involvement, we used Connectivity map (C-Map) and the Library of Integrated Network-Based Cellular Signatures Unified Environment (CLUE) systemic database (<https://clue.io/>) and ConsensusPathDB (CPDB) platform (<http://cpdb.molgen.mpg.de/>) for the comprehensive mechanism analysis. We utilized two bioinformatics processes, direct and indirect analyses, respectively (Figure 1 A). For the direct analysis, HepG2 cells were treated with BMX in L1000 plate, which responded to the biological function of BMX (Figure 1 A, right). The significant

differentially expressed genes (1583 up-regulation and 900 down-regulation) with 1.5-fold change were used to query the CPDB platform to reveal the potential pathways ($p\text{-value} < 0.05$). Next, we analyzed the HDAC8 inhibition function through an indirect approach, pattern matching algorithm of the CLUE platform. Using shRNA *HDAC8* signature as the simulation of BMX treatment (HDAC8 inhibitor), we then accessed CLUE, which computed over 1 million profiles to match the similar signature-pattern from 19,811 small molecule compounds or gene perturbations (e.g., 18,493 shRNAs, 3,462 over-expression constructs), and then obtained the connectivity score. The positive score denoted a similar mechanism between query and instance signatures; while the negative meant the opposite function. Our criteria were selected above 90 connectivity scores of compounds (CPs), knockdown genes (KDs), overexpression genes (OE), and perturbation class (PCLs). CLUE clustered the similar function compounds or same family genes into a particular group which could postulate as the mechanism of action. However, this big data system did not offer detailed pathway information. Thus, we combined the CPDB platform for complementary analysis from shHDAC8 and BMX-treated cells (Figure 1 A, left). These different bioinformatics pipelines would obtain several mechanisms/pathways and we intersected these two datasets to filter the possible potential pathways. Wnt signaling pathway is one of the top-ranking mechanisms uncovered via our multi-databases platform (Figure 1 B).

【0078】 1.2.2 BMX enhanced the TMZ-mediated cytotoxic effect to inhibit the growth and proliferation in GBM-R cells

【0079】 To investigate whether HDAC8 is correlated with therapy-resistant GBM, we examined the HDAC8 expression level of two parent GBM cell lines (A172 and U87MG, A172 and U87MG are wild-type p53 (WT-p53), Figure 12) and two TMZ-resistant GBM cell lines (A172-R and U87MG-R, variants of WT-p53). HDAC8 overexpression was detected in both GBM-R cell lines (Figure 8A and 8B).

【0080】 In the example, NBM-BMX (provided by Nature Wise Biotech & Medicals Corporation; BMX was used in this manuscript) was used as a HDAC8 inhibitor to mimic the effect of shRNA HDAC8 for further experiment. The structure of BMX (397.46 Da) is shown in Figure 2A. It was confirmed that BMX is a HDAC8 inhibitor by treating the four cell lines with BMX and detecting BMX-induced inhibition of HDAC8 mRNA and protein expression (Figure 9A and 9B).

【0081】 It is believed that BMX might enhance the sensitivity of TMZ-mediated cytotoxic effect in both GBM and GBM-R cells. In this example, it was found that a combination effect exists between BMX and TMZ in treating GBM and GBM-R, in A172/A172-R and U87MG/U87MG-R cells. An MTT assay was performed to assess cell proliferation and cell viability for the BMX-alone, TMZ-alone, and combination groups under different concentrations at 24, 48 and 72 hrs. In each treatment alone group, the results suggested that the cytotoxic effect in each group increased in a time-dependent manner (Figure 10A). The results revealed that the IC_{50} values of BMX alone were $21.00 \pm 2.34 \mu M / > 52.64 \pm 3.62 \mu M$ in A172/A172-R cells and $29.84 \pm 2.32 \mu M / > 68.13 \pm 4.69 \mu M$ in U87MG/U87MG-R cells (Figure 2B), suggesting that BMX alone could inhibit GBM cell proliferation, but not inhibit GBM-R cell proliferation. In addition, the IC_{50} values of TMZ alone were $73.48 \pm 3.65 \mu M / 80.99 \pm 1.68 \mu M$ in A172/U87MG cells and $595.07 \pm 23.42 \mu M / 302.51 \pm 15.24 \mu M$ in A172-R/U87MG-R cells, confirming the reliability of GBM-R cells (Figure 2C). In the combination treatment group, BMX $10 \mu M$ was used to combine with different dose of TMZ (Figure 2D) and TMZ $50 \mu M$ (the same as the maintenance concentration in GBM-R cell lines) combined with different concentrations of BMX (Figure 2E) to determine which dose of BMX and TMZ that can most enhance TMZ-mediated cytotoxic effect in GBM-R cells. The data revealed that $50 \mu M$ TMZ with $10 \mu M$ BMX exerted the highest cytotoxic effect in both GBM-R cell lines. We used this combination in a time-dependent manner and noted a cytotoxic effect in 48 hrs (Figure 2F, in 48hrs BMX $10 \mu M$: $0.88x$, $0.77x$, $0.63x$; BMX and TMZ: $0.74x$, $0.56x$, $0.47x$). The clonogenic assay also revealed that $10 \mu M$ BMX with $50 \mu M$ TMZ, rather than BMX alone, suppressed GBM-R cells (Figure 2G). In view of the above, the data suggest that the combination treatment inhibits the growth and proliferation of GBM cells (U87MG and A172) and GBM-R cells (U87MG-R and A172-R), and the combination of $10 \mu M$ BMX and $50 \mu M$ TMZ exerts the highest cytotoxic effect (suppression of cell proliferation and cell viability) on GBM-R cells. Nevertheless, the cell viability of BMX alone was still moderately decreased to show the partial ability of pharmacological cytotoxic effect in A172R/U87R, comparing with TMZ alone with no suppression effect. Thus, the combined BMX and TMZ treatment was compared with BMX alone in the further experiments.

【0082】 1.2.3 BMX enhanced the TMZ-mediated cytotoxic effect by targeting the Wnt/ β -catenin/GSK3 β pathway in GBM-R cells

【0083】 The mechanism was investigated by the enhancement of in the TMZ-mediated cytotoxic effect in GBM-R cells. On the basis of the pathway analysis, it was postulated that the canonical Wnt signaling (also known as Wnt/ β -catenin) pathway was involved in the proliferation of GBM-R cells. The genetic background for each cell indicated no mutation in Wnt genes, such as adenomatous polyposis coli and β -catenin (CTNNB1). The phospho- β -catenin (Ser33/Ser37/Thr41) as a β -catenin active form for detecting the β -catenin status. GSK3 β (S9) was used for β -catenin phosphorylation to degrade β -catenin. The results indicated that 10 μ M BMX with 50 μ M TMZ reduced the protein levels of β -catenin directly and reduced the protein levels of phospho- β -catenin (Ser33/Ser37/Thr41) through phosphorylation by GSK3 β in U87R and A172R cells, whereas BMX alone only slightly reduced these levels. Moreover, the phosphorylation level of GSK3 β (S9) also decreased, indicating that GSK3 β activity increased and β -catenin was phosphorylated (Figure 3A). To examine the effects of BMX on the proliferative markers c-Myc and cyclin D1, it was noted that BMX both with and without TMZ could decrease their levels (Figure 3B).

【0084】 The GBM-R cells were treated with the proteasome inhibitor MG132 to verify that the β -catenin protein level decreased due to protein degradation. The results revealed that MG132 application reversed β -catenin degradation and increased c-Myc and cyclin D1 expression under 10 μ M BMX and 50 μ M TMZ (Figure 3C). These results demonstrated that BMX enhanced GSK3 β activity through Ser9 phosphorylation downregulation, which in turn enhanced β -catenin phosphorylation at Ser33/Ser37/Thr41, triggering protein degradation. Taken together, these data revealed that 10 μ M BMX and 50 μ M TMZ enhanced TMZ-mediated cytotoxic effects, partly via the Wnt/ β -catenin/GSK3 β pathway, thus reducing GBM-R cell proliferation.

【0085】 1.2.4 BMX enhanced the TMZ-mediated cytotoxic effect by promoting TMZ-mediated apoptosis in GBM-R cells

【0086】 To investigate whether BMX can induce cell cycle arrest, it was analyzed the effects of BMX (5 μ M and 10 μ M) alone and combined with 50 μ M TMZ on the cell cycle in the A172-R and U87MG-R cell lines. The results revealed that 10 μ M BMX alone induced cell cycle arrest in the G0/G1 phase in A172-R cells (70.34%)

and U87MG-R cells (77.95%). Next, 5 and 10 μ M BMX with 50 μ M TMZ not only increased the amount of cell cycle arrest in G0/G1 but also caused arrest in the sub-G1 phase (apoptosis) in both GBM-R cell lines (Figure 4A-C).

【0087】 Flow cytometry revealed that BMX combined with TMZ yielded a high percentage of apoptotic cells in A172-R/U87MG-R cell lines (21.7%/25.95%) in a dose-dependent manner (Figure 4D). Moreover, late apoptosis was also predominant after treatment with 10 μ M BMX and 50 μ M TMZ (Figure 4E). Thus, BMX alone could only induce cell cycle arrest and suppress cell proliferation but not induce apoptosis, whereas the BMX and TMZ combination could also promote TMZ-mediated apoptosis, leading to enhanced cytotoxicity, in GBM-R cells.

【0088】 1.2.5 BMX enhanced the TMZ-mediated cytotoxic effects by WT-p53 mediated MGMT inhibition in GBM-R cells

【0089】 Because the BMX and TMZ combination could promote TMZ-mediated apoptosis, we speculated that BMX might enhance TMZ-mediated apoptosis through WT-p53 mediated MGMT inhibition. First, we examined WT-p53 and MGMT levels in A172/A172-R and U87MG/U87MG-R cells, confirming that TMZ resistance is related to WT-p53 and MGMT (Figure 5A). The bioinformatics analysis also suggested that only 33 % patients have p53 mutation, others are p53 WT (Table 2). Next, the TCGA and driverDB databases were assessed to check the overall survival rate between p53 mutation and p53 WT. By colony formation assay (Figure 11), it was clearly revealed that p53 WT cases showed poor prognosis in GBM patients as compared to the mutated cases.

【0090】 We examined the proapoptotic signaling system in WT-p53 mediated apoptosis. MGMT was also examined for TMZ repair ability. The results revealed that the levels of proapoptotic makers, such as P21, Bax/Bcl2, and Puma, increased and that those of MGMT decreased after treatment with BMX both without and with 50 μ M TMZ. However, cleaved caspase-3 was only noted with the combination of 10 μ M BMX and 50 μ M TMZ (Figure 5B). To clarify whether apoptosis is induced by BMX alone, TMZ alone, or their combination in WT-p53 mediated MGMT inhibition, A172-R and U87MG-R cells were treated with 50 μ M TMZ without or with 5 and 10 μ M BMX. It was found that TMZ alone could only moderately suppress MGMT expression without increasing WT-p53 and DNA damage marker (WT-p53-ser15). However, MGMT expression obviously decreased with the combination of 10 μ M

BMX and 50 μ M TMZ. Moreover, WT-p53 and DNA damage markers (WT-p53-ser15) expression levels also increased, meaning that MGMT was negatively regulated by WT-p53 mediated apoptosis (Figure 5C).

【0091】 Moreover, by assessing scatter plot for p53 WT and mutant cells (Figure 8B), it was found that GBM p53 WT cells were MGMT hypermethylated and lower the MGMT mRNA and protein expression as well. In addition, TMZ alone could not induce WT-p53 mediated apoptosis in GBM-R cells. However, these data suggested that BMX and TMZ combination could enhance the TMZ-mediated cytotoxic effect through WT-p53 mediated MGMT inhibition in GBM-R cells. BMX alone could moderately decrease MGMT levels but not induce WT-p53 mediated apoptosis in GBM-R cells.

Table 2. CCLE p53 genetic features

Tp53-WT		Tp53-mut	
SF126	KS1	LN229	YKG1
BECKER	MOGGUVW	SW1783	X8MGBA
GOS3	U87MG	TM31	MOGGCCM
KNS81	LN443	U138MG	
KG1C	LN235	SF295	
AM38	SF172	M059K	
H4	U343	G11	
D283MED	U178	KNS60	
NMCG1	F5	KALS1	
YH13	SF767	CAS1	
DKMG	A1207	KNS42	
U118MG	LN319	SNU201	
GB1	LN382	SNU1105	
SNU489	LNZ308	SNU738	
SNU466	LN340	SNU626	
CCFSTTG1	CH157MN	DAOY	
D341MED	SF268	SW1088	
ONS76	SF539	HS683	
DBTRG05MG	SNB75	LN18	

A172		T98G	
X132N1		GMS10	
SNB19		X42MGBA	
U251MG		GAMG	

【0092】 1.2.6 The combination of BMX and TMZ reduced GSC formation in GBM-R cells.

【0093】 Because GSC markers are the core of GBM resistance, we examined the levels of GSC markers in all cell lines; high expression levels of CD133, CD44, and SOX2 were detected in A172-R and U87MG-R cells, implying that TMZ resistance is partly related to GSC markers (Figure 6A). Furthermore, treatment with 10 μ M BMX and 50 μ M TMZ clearly reduced the expression levels of CD133, CD44, and SOX2 in both GBM-R cell lines (Figure 6B). Thus, the BMX and TMZ combination could enhance the TMZ-mediated cytotoxic effect by attenuating GSC markers to convert the stemness phenotype in GBM-R cells.

【0094】 We also examined HDAC8 and GSC markers in TMZ-resistant GBM human tissues through immunohistochemistry (Figure 6C). The results revealed that HDAC8 and GSC are closely related to TMZ resistance in GBM.

【0095】 1.3. Conclusion

【0096】 Although preclinical studies have indicated that HDACis have antitumor effects in glioma, none of the previous studies mentioned or expected the treatment of chemotherapy-resistant GBM. It was first found in the present invention that BMX, a novel iso-selective HDAC8 inhibitor, can enhance TMZ-mediated cytotoxic effect not only by downregulating the β -catenin/c-Myc/SOX2 pathway to inhibit stemness, but also by upregulating WT-p53 mediated MGMT inhibition to induce apoptosis in TMZ-resistant GBM cells. Moreover, it was also revealed that the inverse correlation of WT-p53/MGMT reversion and the Wnt/ β -catenin/GSK β signaling pathway may be involved in the oncogenic role in GBM and GBM with TMZ resistance.

【0097】 Based on the above-mentioned results, the following working model is proposed (see Figure 7):

【0098】 First, β -catenin/c-Myc/cyclin D1/SOX2 signaling pathway in TMZ-resistant GBM (Pathway in right side). According to our previous studies and the

bioinformatics analysis in this study, the Wnt/ β -catenin/GSK3 β pathway can influence therapy selection for GBM [17]. It was demonstrated in the present invention that both of BMX without and with TMZ (thin and thick lines) could enhance GSK3 β activity by downregulating Ser9 phosphorylation, which in turn enhanced β -catenin phosphorylation at Ser33/Ser37/Thr41, triggering β -catenin protein degradation. β -catenin degradation was confirmed with MG132 as a proteasome inhibitor. Undegraded β -catenin translocates into the nucleus to bind to TCL4 and activate downstream target genes, such as c-Myc and cyclin D1, to induce cell proliferation and continue the cell cycle. Both BMX alone (thin lines) and BMX with TMZ (thick lines) suppressed c-Myc and cyclin D1 expression and induced cell cycle arrest. However, BMX alone could not induce cell cycle arrest in the sub-G1 phase. Only the combination of BMX and TMZ induced profound cell cycle arrest and proceeded to the sub-G1 phase, meaning that it induced late-apoptosis in GBM-R cells (dotted line in the right lower part in Figure 7).

【0099】 In addition, GSCs play a vital role in therapeutic resistance in GBM. They are characterized by their self-renewal ability, both in vitro and in vivo, through high expressions of neuronal stem cell markers, such as CD133 and CD44, as well as transcription factors, such as SOX2 [23]. It was revealed in the invention that BMX both without and with TMZ attenuated not only CD133 and CD44 but also SOX2 by downregulating the GSC phenotype to suppress stemness. As previously reported, c-Myc is also required to maintain glioma CSCs [24]. It could be concluded that BMX alone and BMX with TMZ suppressed cell proliferation by enhancing the TMZ-mediated cytotoxic effect via the β -catenin/c-Myc/cyclin D1/SOX2 signaling pathway in GBM-R cells.

【0100】 Furthermore, WT-p53 mediated MGMT inhibition in TMZ-resistant GBM (Pathway in left side in Figure 7). The mechanism of action of TMZ in GBM is methylation of the O6 position to guanine to damage DNA. MGMT reverses methylation to repair DNA in GBM cells and to exert GBM resistance. Although an MGMT-independent pathway also plays a critical role in TMZ resistance[25-27], the MGMT-dependent pathway is still considered the major pathway for TMZ resistance. In the present invention, GBM-R cell lines (A172-R and U87MG-R) expressed high levels of the MGMT protein, thus verifying that the MGMT-dependent pathway is indeed a major mechanism for TMZ resistance in these cell lines. It may be

speculated that BMX suppressed MGMT expression in MGMT-dependent GBM-R cell lines to weaken the ability of MGMT for DNA damage repair. In the GBM-R cell lines, BMX alone moderately decreased MGMT expression and TMZ alone did not (Figure 5B and Figure 5C), but their combination obviously reduced the MGMT protein levels and enhanced TMZ-mediated apoptosis in an MGMT-dependent manner.

【0101】 As shown in Figure 7, the model includes two main signaling pathways. The pathway on the right side: β -catenin/c-Myc/cyclinD1/Sox2 signaling pathway. When GBM-R cell lines were treated with BMX alone (thin lines) or BMX with TMZ (thick lines), GSK3 β (S9) and active β -catenin decreased. The following c-Myc and cyclin D1 also decreased to induce cell cycle arrest and attenuate stemness activity, However, only BMX with TMZ (dashed lines) possibly induce apoptosis. The pathway on the left side: WT-p53 mediated MGMT inhibition. When GBM-R cell lines were treated with BMX alone or BMX with TMZ, WT-p53 increased and downregulated MGMT levels, cell cycle arrest and stemness in both BMX alone (thin lines) and BMX with TMZ (thick lines). However, WT-p53 and DNA damage marker (WT-p53-ser15, not shown) increased the following the activation of cell cycle arrest marker (P21) and proapoptotic markers (BAX/Bcl2, and Puma) to induce apoptosis and cell death only in BMX with TMZ (thick lines), indicating as profoundly DNA damage. Color red indicated up-regulation. Color green indicated down-regulation.

【0102】 It was found in the example that the BMX provided an enhanced effect in inhibition of HDAC to to decrease MGMT via WT-p53 restoring (all P53 lanes in Figure 5A-C). It was demonstrated in the present invention that BMX alone (thin lines in the left part) moderately increased WT-p53 level to moderately downregulate MGMT expression, leading to still maintain the ability of DNA repair (Figure 5B). It was also speculated that HDAC inhibition may reduce MGMT expression through WT-p53 reactivation. It was demonstrated that BMX alone moderately increased the WT-p53 level and moderately downregulated MGMT expression, leading to the maintenance of DNA repair. Moreover, BMX alone also induced cell cycle arrest marker (P21). In the present invention, the combination of BMX and TMZ (thick lines in left part) induced extensive DNA damage through WT-p53 (and ser15) overexpression and downregulated MGMT expression, eventually leading to WT-p53 mediated apoptosis (Figure 5C). This combination (compared with BMX alone) could

also increase the expression of cell cycle arrest marker (P21), proapoptotic proteins (Bax/Bcl2 and Puma) and induce cleaved caspase-3 expression for WT-p53 mediated apoptosis. Taken together, these results implied that BMX (thin lines in left part in Figure 7) alone only partially induced WT-p53 mediated MGMT inhibition but that the BMX and TMZ combination (thick lines in left part in Figure 7) enhanced TMZ cytotoxic effect in GBM-R cell lines to overcome TMZ resistance.

【0103】 In conclusion, it was unexpectedly found in the present invention that BMX overcomes TMZ resistance by enhancing TMZ-mediated cytotoxic effect by downregulating the β -catenin/c-Myc/SOX2 signaling pathway and upregulating WT-p53 mediated MGMT inhibition. These findings indicate that the combination of BMX and TMZ is promising for precision personal treating of TMZ-resistant WT-p53 GBM cells.

【0104】 Example 2

【0105】 2.1. Materials and Methods

【0106】 2.1.1 Cell lines and cell culture

【0107】 Three CRC cell lines HT29, HCT116 and RKO were used in this study. The American Type Culture Collection (ATCC; Manassas, VA, USA) provided human CRC cell lines HT29 (ATCC HTB-38; mutant TP53, p.R273H; APC frame shift, p. E1554fs; wild type β -catenin), HCT116 (ATCC CCL-247; wild type TP53; wild type APC; deletion β -catenin, p. S45del) and RKO (ATCC CRL-2577; wild type TP53; wild type APC; wild type β -catenin). Three CRC cells lines as listed above were cultured in adherent culture condition, maintained at 37°C inside the cell incubation containing 5% CO₂. The two cell lines of HCT-116 and HT-29 cells were cultured in McCoy's 5A medium with supplemented 10% fetal bovine serum (Gibco; Thermo Fischer Scientific, Grand Island, NY, USA), 1% penicillin and 1% streptomycin. RKO cell was cultured in MEM medium with supplemented 10% FBS, 1% penicillin, 1% streptomycin and 1% sodium pyruvate. The cell cultures were passaged by trypsinization every three days. BM-BMX (BMX), (E)-2-(4-Methoxybenzyloxy)-3-prenyl-4-methoxy-N-hydroxycinamide, was provided by Nature Wise Biotech & Medicals Corporation (Taipei, Taiwan).

【0108】 2.1.2 Cell proliferation assays

【0109】 We plated 4000 CRC cells per well in 96-well plates and allowed them to adhere overnight. To validate cell line responsiveness to BMX and TMZ monotherapy, the cells were treated with different doses of BMX or TMZ for 24, 48, and 72 hrs. To confirm cell responsiveness to the BMX-TMZ combination, cells were either treated with different doses of TMZ (0-1000 $\mu\text{g/mL}$) with or without BMX (5 μM) for 24, 48, and 72 hrs or with different doses of BMX (0-10 μM) with or without TMZ (50 μM) for 24, 48, and 72 hrs. Following treatment, the absorption value was measured using a CCK8 kit (Targetmol, Shanghai, China) at the indicated time points. The results are reported as the mean $\pm$ standard deviation of at least three replicates.

【0110】 2.1.3 Flow cytometric analysis of DNA cell cycle

【0111】 Cells were treated with different doses of BMX (0-10 μM) in the presence or absence of TMZ (50 μM) for 48 hrs. Untreated cells were used as a negative control. All samples were run in triplicate in at least three independent experiments. Flow cytometric analysis for propidium iodide (PI) was performed. For the DNA cell cycle, the cells were trypsinized, centrifuged, washed with phosphate-buffered saline (PBS), and fixed in methanol. The cells were then washed again, and incubated with PI-working solution (10 $\mu\text{g/mL}$ PI and 20 mg/mL RNase A) for 15 min at 37°C in the dark. Using a flow cytometer (Attune NxT flow cytometer, Thermo Fisher Scientific) the PI fluorescence of 10,000 individual nuclei was calculated. The fractions of the cells in G0/G1, S, G2/M, and sub-G0/G1 phase were analyzed using Attune NxT flow cytometry software and were determined for each histogram as the mean peak fluorescence intensity.

【0112】 2.1.4 Flow cytometric analysis of apoptosis

【0113】 Apoptosis induction in different doses of BMX (0-10 μM) in the presence or absence of TMZ (50 μM) was assayed by the detection of membrane externalization of phosphatidylserine with the with the CF®488A Annexin V and PI Apoptosis Kit (Fremont, CA, USA) following the manufacturer's instructions [16]. All samples were then immediately analyzed by flow cytometry.

【0114】 2.1.5 Quantitative real time RT-PCR

【0115】 RNA was extracted from the cells (2×10^5) using Tissue Total RNA Mini Kit (Geneaid, Taipei, Taiwan) following the manufacturer's instructions. RNA concentration and purity was examined using a NanoDrop® spectrophotometer

(Thermo Scientific, Waltham, MA, USA) at 260–280 nm. Then, cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), also following the manufacturer's instructions. qPCR reactions were performed in the 7500 Real-time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems), according to manufacturer's recommendations, with 18s as the inner reference. The threshold cycle (Ct) values were calculated using the StepOnePlus (Applied Biosystems) software. The relative expression of each mRNA was calculated using the $2^{-\Delta Ct}$ method. The primer sequences for HDAC8 were as follows:

HDAC8 forward 5'-GCGTGATTTCAGCACATAA-3' (SEQ ID NO: 1);

HDAC8 reverse 5'-ATACTTGACCGGGGTCATCC-3' (SEQ ID NO: 2).

18s forward 5'-TCAAGTGCAGTGCAACAACCTC-3' (SEQ ID NO: 3);

18s reverse 5'-AGAGGACAGGGTGGAGTAATCA-3' (SEQ ID NO: 4).

【0116】 2.1.6 Colony formation assay

【0117】 For the anchorage-dependent growth assay, 1000 cells were resuspended in the medium and seeded in 6-well plates. The culture media only added in various concentrations of BMX (0-10 μ M) in the presence or absence of TMZ (50 μ M) were changed every 2–3 days. After 14 days, the media were removed and the cells were washed and fixed with 4% paraformaldehyde for 30 min, and stained with 0.1% crystal violet for 20 min at 25 °C. The intensity of crystal violet was quantified by the absorbance of 570 nm after dissolving the stained cells with dimethyl sulfoxide (DMSO). The results are expressed as the average colony $\pm$ SE from three independent experiments.

【0118】 2.1.7 Senescence-associated (SA) β -galactosidase (SA- β -gal) analysis

【0119】 SA expression of β -gal activity was done with a Senescence Detection kit (CS0030-1KT; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Briefly, cells were treated with different doses of BMX (0-10 μ M) in the presence or absence of TMZ (50 μ M) for 48 hrs, were washed with PBS and fixed using the fixative solution for half an hour at room temperature and then incubated at 37°C overnight with the SA- β -gal staining solution. SA- β -gal activity was examined by X-gal (5-bromo-4-chloro-3-indolyl β -D-galactoside) staining at pH 6.0. The senescent cells stained with blue were photographed. Randomly selected fields (n=3) were analyzed by light microscope to quantify the percentage of senescent cells.

【0120】 2.1.8 Western blot analysis

【0121】 Western blot analysis was used to examine the indicated protein expression levels under various concentrations of BMX (0-10 μ M), and SAHA, VPA or PCI-34051 in the presence or absence of TMZ (50 μ M) or OXP (5 μ M) of the tested cell lines. SDS-PAGE and Western blot analysis was performed on lysates prepared as outlined previously [16]. The specific primary antibodies against acetyl-histone H3 (Lys9/Lys14), acetyl-histone H4 (Lys8), P53, acetyl-p53 (Lys382), phospho-p53 (Ser15), P21, P16, MGMT, phospho-H2AX (S139), E2F1, E2F3, Cleaved Caspase-3, Cleaved Caspase-8, Cleaved Caspase-7, Cleaved Caspase-9, PARP, Bax, Bcl-2, Bid, Bim, Bak, Puma, β -catenin, phospho- β -catenin(Ser/33/37/41), GSK3 β , phospho-GSK3 β (Ser 9), c-Myc, Cyclin D1, P62, LC3B, CD133, CD44, SOX-2 and HDAC8 were used for detection, and GAPDH, α -tubulin or β -actin was used as the internal control. After incubation with the primary antibodies, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, the HRP signals were detected with a chemical HRP substrate. The antibodies we used are listed in Table 4. Signal of each target protein was visualized by incubation with an enhanced chemiluminescent reagent and exposure to X-ray film.

Table 4. Key resources

Reagent Type (Species) or Resource	Designation	Source or Reference	Identifiers	Additional Information
Antibody	Rabbit anti-Acetyl-Histone H3(Lys9/Lys14)	Cell signaling (Beverly, MA, USA)	s9677	1:1000
Antibody	Rabbit anti-Acetyl-Histone H4(Lys8)	Cell signaling (Beverly, MA, USA)	s2594	1:1000
Antibody	Mouse anti-GAPDH	Santa Cruz (Santa Cruz, CA, USA)	sc-32233	1:1000
Antibody	Mouse anti-P53	Santa Cruz (Santa Cruz, CA, USA)	sc-126	1:1000
Antibody	Rabbit anti-Acetyl-p53(Lys382)	Cell signaling (Beverly, MA, USA)	s2525	1:1000
Antibody	Rabbit anti- Phospho-p53 (Ser15)	Cell signaling (Beverly, MA, USA)	9284	1:1000
Antibody	Rabbit anti-P21	Cell signaling (Beverly, MA, USA)	s2947	1:1000

Antibody	Mouse anti-P16	Santa Cruz (Santa Cruz, CA, USA)	sc-56330	1:1000
Antibody	Rabbit anti-MGMT	Cell signaling (Beverly, MA, USA)	s2739	1:1000
Antibody	Rabbit anti-phosphor-H2AX (S139)	Elabscience	E-AB-68087	1:1000
Antibody	Mouse anti-E2F1	Santa Cruz (Santa Cruz, CA, USA)	SC-251	1:1000
Antibody	Mouse anti-E2F3	Santa Cruz (Santa Cruz, CA, USA)	SC-56665	1:1000
Antibody	Rabbit anti-Cleaved Caspase-3	Cell signaling (Beverly, MA, USA)	s9661	1:1000
Antibody	Rabbit anti-Cleaved Caspase-9	Cell signaling (Beverly, MA, USA)	s9501	1:1000
Antibody	Rabbit anti-Cleaved Caspase-7	Cell signaling (Beverly, MA, USA)	s9491	1:1000
Antibody	Rabbit anti-Cleaved Caspase-8	Cell signaling (Beverly, MA, USA)	s9496	1:1000
Antibody	Rabbit anti-PARP	Cell signaling (Beverly, MA, USA)	s9542	1:1000
Antibody	Rabbit anti-Bax	Cell signaling (Beverly, MA, USA)	s2772	1:1000
Antibody	Rabbit anti-Bcl-2	Cell signaling (Beverly, MA, USA)	s2870	1:1000
Antibody	Rabbit anti-Bid	Cell signaling (Beverly, MA, USA)	s2002	1:1000
Antibody	Rabbit anti-Bim	Cell signaling (Beverly, MA, USA)	s2819	1:1000
Antibody	Rabbit anti-Bak	Cell signaling (Beverly, MA, USA)	s3814	1:1000
Antibody	Rabbit anti-Puma	Cell signaling (Beverly, MA, USA)	s4976	1:1000
Antibody	Rabbit anti-catenin	Cell signaling (Beverly, MA, USA)	s9562	1:1000
Antibody	Rabbit anti-Phospho-	Cell signaling (Beverly, MA, USA)	s9561	1:1000

	catenin (Ser/33/37/41)	USA)		
Antibody	Rabbit anti-Phospho-GSK3 (Ser 9)	Cell signaling (Beverly, MA, USA)	s9323s	1:1000
Antibody	Mouse anti-GSK3	BD Biosciences	610202	1:1000
Antibody	Rabbit anti-c-Myc	Abcam (Cambridge, MA, USA)	ab32072	1:1000
Antibody	Mouse anti-Cyclin D1	Santa Cruz (Santa Cruz, CA, USA)	sc-8396	1:1000
Antibody	Mouse anti- α -tubulin	Sigma-Aldrich (St. Louis, MO, USA)	T5168	1:5000
Antibody	Mouse anti-P62	Abcam (Cambridge, MA, USA)	ab56416	1:2000
Antibody	Rabbit anti-LC3B	Cell signaling (Beverly, MA, USA)	s3868	1:1000
Antibody	Rabbit anti-CD133	Cell signaling (Beverly, MA, USA)	s64326	1:1000
Antibody	Rabbit anti-CD44	Proteintech (, USA)	15675-1-AP	1:2000
Antibody	Rabbit anti-SOX-2	Abcam (Cambridge, MA, USA)	ab97959	1:1000
Antibody	Rabbit anti-HDAC8	ABclonal	a8865	1:1000

【0122】 2.1.9 Statistical analysis

【0123】 Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using one-way analysis of variance. Data were compared using the student's t test. The level of statistical significance was set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

【0124】 2.2. Result

【0125】 2.2.1 Optimize the combination of BMX and TMZ in three CRC cell lines

【0126】 To investigate the influence of BMX or TMZ on CRC cell growth, three human colorectal cancer cell lines, HT29 (p53 mutation), HCT116 (p53 wild type) and RKO (p53 wild type), were utilized. They were separately treated with BMX (0.313, 0.625, 1.25, 2.5, 5 and 10 μ M) or TMZ (25, 50, 100, 200, 400, 800 and 1000 μ M) for 24, 48 and 72 hrs. The results showed that CRC cell viability was inhibited

significantly in a dose-dependent manner. The half maximal inhibitory concentration (IC₅₀) values of BMX or TMZ alone in the HT-29, HCT-116 and RKO cells were derived (Table 5). With clonogenic assay, which represented *in vivo* tumorigenicity, TMZ was effective against tumor sphere formation in the clonogenic assay of the HT29, HCT116 and RKO cells, the IC₅₀ values of TMZ were 359.45±50.43, 137.66±22.73, and 244.01±29.42 μ M, respectively. The results showed that the basic cell proliferation inhibition rate of BMX and TMZ with three incubation time on three colorectal cancer cells including HT-29, HCT-116 and RKO.

Table 5. BMX and TMZ combination inhibited cell proliferation in CRC cells

BMX alone				TMZ alone		
Time(hrs) Cell lines	IC ₅₀ of BMX (μ M)			IC ₅₀ of TMZ (μ M)		
	24	48	72	24	48	72
HT29	42.6±2.4	9.9±0.5	2.9±0.2	>1000	930.8±47.7	257.6±20.53
HCT116	24.8±2.5	7.7±0.3	1.5±0.3	>1000	515.2±21.6	192.0±14.7
RKO	38.5±3.5	7.2±0.6	1.5±0.2	>1000	991.6±52.4	380.5±40.3
BMX 5 μ M was used to combine with different doses of TMZ				TMZ 50 μ M combined with different concentrations of BMX		
BMZ (5 μ M)				TMZ (50 μ M)		
Time(hrs) Cell lines	IC ₅₀ of TMZ (μ M)			IC ₅₀ of BMZ (μ M)		
	24	48	72	24	48	72
HT29	>400	128.3±18.3	--	>10	9.1±0.2	2.2±0.1
HCT116	>400	41.56±2.4	--	>10	3.2±0.3	0.9±0.1
RKO	>400	21.9±2.7	--	>10	3.6±0.4	0.9±0.1

【0127】 To evaluate whether BMX improved the chemosensitivity of TMZ, BMX and TMZ were administered together to HT-29, HCT-116 and RKO cells. The combination of BMX (5 μ M) and TMZ (25, 50, 100, 200 and 400 μ M) exhibited a greater inhibitory effect on cell growth than BMX and TMZ did individually. The combination of BMX (5 μ M) and TMZ (25, 50, 100, 200 and 400 μ M) exhibited a greater inhibitory effect on cell growth than BMX and TMZ did individually. Subsequently, TMZ 50 μ M combined with different concentrations of BMX (0.313, 0.625, 1.25, 2.5, 5 and 10 μ M) were selected to verify that TMZ and BMX suppressed

cell proliferation in a time-dependent manner. Notably, BMX decreased the IC₅₀ of TMZ in HT-29, HCT-116 and RKO cells (Table 5). These findings suggested that BMX inhibited CRC cell proliferation and improved the chemosensitivity of TMZ. 50 μ M TMZ with 5 μ M BMX exerted the highest cytotoxic effect in HT-29, HCT-116 and RKO cells. We used this combination in a time dependent manner and noted a cytotoxic effect in 48 hrs. This finding suggested that BMX improved the chemosensitivity of TMZ. BMX in combination with TMZ suppressed cell proliferation in a time-dependent manner. Thus, all subsequent experiments were performed using TMZ 50 μ M combined with different concentrations of BMX (2.5, 5 and 10 μ M) for 48 hrs.

【0128】 We next examined colony formation in the presence of BMX alone or combined with TMZ. In regular continuous fashion, we found that this inhibitory effect increased when combined BMX with 50 μ M TMZ. If increase to TMZ (150 μ M), BMX could be lower down to 1-2 μ M instead of 5-10 μ M. Taken together, these results have demonstrated the combined use of BMX and TMZ synergistically inhibits proliferation and colony formation of CRC cancer cells. Thus, all subsequent experiments were performed using TMZ 50 μ M combined with different concentrations of BMX (2.5, 5 and 10 μ M) for 48 hrs.

【0129】 2.2.2 The effects of the combination of BMX and TMZ compared with conventional drugs on CRC

【0130】 Cell cycle arrest is one of the main causes of the inhibition of cell proliferation. To evaluate the possible mechanisms through which BMX or combined treatment inhibited cell growth, cell cycle profiles were assayed using flow cytometry. As shown in Figure 13, the combined treatment significantly induced G2/M phase arrest and exhibited much stronger effects on G2/M phase arrest than any other single drug did in HT29 and HCT116 cells. 2.5, 5 and 10 μ M BMX with 50 μ M TMZ not only increased the amount of cell cycle arrest in G0/G1 but also caused arrest in the sub G1 phase (apoptosis) in RKO cell lines.

【0131】 The synergistic effect of BMX with TMZ after a 48 hrs treatment was measured by Annexin V binding in three CRC cells. Treatment with BMX and TMZ induced a prominent increase in apoptotic cell percentage compared with each agent individually. BMX increased early apoptotic cells up to 23.78%, 49.34% and 59.18% in HT29, HCT116 and RKO cells, so did the late apoptosis. While in combination

treatment population of late apoptosis increased from 1.08% to 10.36%, 3.67% to 19.37% and 0.32% to 16.48% in HT29, HCT116 and RKO cells after 48 hrs incubation.

【0132】 2.2.3 BMX, and the combination of BMX and TMZ induced apoptosis were mediated by p53 mediated MGMT inhibition

【0133】 The p53 pathway has been reported to be involved in apoptosis of various cancer cells induced by chemotherapy drugs [28]BMX has been shown to activate p53, leading to cell death, mediated by β -catenin pathway [16]. In order to elucidate the anticancer effect of BMX and TMZ resulted from DNA damage or not, we examined the DNA damage and corresponded p53 pathway markers in three CRC cell lines that had different p53 phenotypes. In view of the basic protein expression status of the markers including p53, Acetyl-p53 (Lys382), p53(Ser 15), p21, p16, MGMT, γ -H2AX, E2F1, E2F3, GAPDH in HT29, HCT116 and RKO cells, BMX alone not only enhanced p53 expression, but possibly also regulated other important genes interfering with cell growth. Treatment with BMX alone or BMX with TMZ combination dose-dependently increased the levels of p53 phosphorylation (Ser15) and γ -H2AX phosphorylation (Ser139) in HT29, HCT116 and RKO cells. In the HT29, HCT116 and RKO cells, acetylation of p53 at Lys382 increased in a time-dependent manner, enhanced expression of p53 downstream target p21 and p16. As shown in the results of the Western blotting for p53 wildtype and mutant cells, CRC p53 wildtype cells were MGMT hypermethylated and lower the MGMT protein expression as well. In addition, BMX with TMZ combination significantly decreased E2F3 expressions (Figure 14A). Interestingly, acetylation of histone H3 was also increased by BMX or BMX with TMZ. This suggests that BMX affects the activity of histone acetyltransferases and/or HDACs in cells, which leads to acetylation of proteins, including p53. Combine BMX with TMZ can increase P21, P16 expression and H2AX phosphorylation through enhancing p53 expression and activating p53 functions mediated MGMT inhibition (Figure14A).

【0134】 The balance between pro-apoptotic (stress or death) signals and anti-apoptotic molecules, including Bcl-2 and Bid, Bax or poma, is a main reason, causing apoptotic response through caspases-dependent pathway [29]. The cleavage of Caspases, exhibited in Figure 14B, suggested that Caspase-7, Caspase-8, Caspase-9 and Caspase-3 activities showed no significant change in BMX under lower

concentrations, while combined with TMZ in HT29 cells, they were highly up-regulated in a dose-dependent manner, contributing to PARP cleavage and apoptosis eventually. Apoptosis protein expression levels of cleaved caspase 3, caspase 7, caspase 9 and caspase PARP were found to significantly increase in a concentration dependent manner following BMX 10 μ M treatment in the HCT116 and RKO cell lines. Moreover, we examined the proapoptotic signaling system in p53 wildtype cell mediated apoptosis. The results revealed that the BMX treatment decreased the level of the antiapoptotic proteins Bcl-2 and increase proapoptotic proteins Bax, Bim and Puma. However, BMX treatment did not lead to the upregulation of the proapoptotic Bcl-2 family proteins Bak and Bid. In addition, the synergistic effect of BMX and TMZ was better than BMX alone (Figure 14C). The combination of TMZ plus BMX resulted more senescent cell than each treatment alone, especially in p53 wildtype cell, such as HCT116 and RKO (Figure 15). Because CD133, CD44, and SOX2 is highly related to the drug resistance of CSCs and is used as a phenotypic marker for CSC including CRC, treatment with BMX and TMZ clearly reduced the expression levels of CD133, CD44 and SOX2 in a dose dependent manner in HT29, HCT116 and RKO (Figure 16). Thus, the BMX and TMZ combination could enhance the TMZ-mediated cytotoxic effect by attenuating CSC markers to convert the stemness phenotype in CRC cells. Therefore, the results above indicated that caspase-dependent signaling pathway was activated by BMX and TMZ combinational treatment in CRC cells to induce cell apoptosis.

【0135】 2.4 BMX enhanced the TMZ mediated cytotoxic effect by targeting the Wnt / β catenin/GSK3 β pathway in CRC cells

【0136】 Next, investigated the mechanism by which BMX enhances the TMZ-mediated cytotoxic effect on Wnt/ β -catenin activity was investigated in three CRC cells. As shown in Figure 3A, β -catenin, phospho- β -catenin (Ser33/Ser37/Thr41) and phospho-GSK-3 β (Ser9) protein expression levels were increased, while phospho- β -catenin (Ser33/Ser37/Thr41) and phospho-GSK-3 β (Ser9) levels were decreased by BMX treatment in three CRC cells. Combined treatment with 5 μ M BMX and TMZ reduced the protein levels of β -catenin directly and reduced the protein levels of phospho β -catenin (S33/S37/T41) through phosphorylation by GSK3 β in three cell lines. Furthermore, we further examined the effects of BMX on the proliferative markers c-Myc and cyclin D1 and noted that BMX both with and without TMZ could

decrease proliferative markers c-Myc and cyclin D1 (Figure 17A). These results demonstrated that combined treatment with 5 μ M BMX and TMZ enhanced GSK3 β activity through Ser9 phosphorylation downregulation, which in turn enhanced β -catenin phosphorylation at Ser33/Ser37/Thr41, triggering protein degradation (Figure 17B). In addition, MG132 application reversed β -catenin degradation and increased MGMT expression under 5 μ M BMX and 50 μ M TMZ (Figure 17C). Taken together, these data revealed that BMX and TMZ enhanced TMZ-mediated cytotoxic effects, partly via the Wnt/ β -catenin/GSK3 β pathway, thus reducing CRC cell proliferation.

【0137】 2.5 Autophagy served as a key regulator in BMX, BMX and TMZ combination induced cell death

【0138】 Lipidated LC3 and autophagy substrate p62 are frequently used as markers to assess autophagosomes and autophagy[17]. Treatment with BMX or combined treatment BMX and TMZ also yielded a concentration-dependent increase in the expression of P62 and LC3-II, which is the processed form of LC3 (Figure 18A). β -catenin negatively regulates P62 expression [17,30]. To verify that the decreased protein level of P62 was caused by β -catenin protein degradation, the proteasome inhibitor MG132 was applied to the BMX or BMX and TMZ combination treated cells. As expected, BMX induced β catenin degradation was reversed and P62 expression was also suppressed when MG132 was applied (Figure 18B). Due to β -catenin protein degradation with combination treatment, the P62 was no longer inhibited and then triggered downstream autophagy pathway (Figure 18B). To determine the role of autophagy in BMX or BMX and TMZ combination-induced cell death, we used BAF, a protein biosynthesis inhibitor that inhibits the late stages of autophagy and Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone), a cell-permeant pan caspase inhibitor, to treat cells before BMX or BMX and TMZ combination addition, and we found that Z-VAD-FMK suppressed early apoptosis that induced by BMX plus TMZ treatment in three cell lines. In addition, pre-treatment with BAF A1 reduced BMX or BMX and TMZ combination induced cell death obtained via flow cytometry, which was consistent with the results of the reduced expression of cleaved caspase-3, caspase-7, caspase-8 and caspase-9 induced by BMX or BMX and TMZ combination. Interactions between different autophagy/apoptosis related proteins and the corresponding signaling pathways have been identified, implying crosstalk exists between two pathways. To test the role of

apoptosis in 6c-induced autophagy, we treated cells with BAF or Z-VAD-FMK before adding BMX or BMX and TMZ combination. As shown in Figure 18D, although Z-VAD-FMK and BAF showed early apoptosis suppression, BAF inhibited BMX and TMZ combination induced caspase 3 activation without interfering LC3I/II in all cells. However, Z-VAD-FMK inhibited BMX and TMZ combination induced caspase 3 activation with interfering LC3 I/II in p53 mutant type cell line. Taken together, these results emphasized the importance of stimulating autophagy during cell death.

【0139】 2.3. Conclusion

【0140】 CRC treatments using traditional radio-chemotherapy are sometimes inefficient, partly because of CRC patients does not respond to this therapy regimen and/or suffer from severe drug toxicities. It is demonstrated in the present invention that the combination of BMX and TMZ exhibited the specific and efficient, synergistic anti-proliferative and apoptotic effect in HCC cells, especially in HCT116 and RKO. In conclusion, BMX and TMZ provided the best synergistic efficacy and its mechanism is the most important link at present. It is also concluded that BMX, a specific HDAC8i, in combination with temozolomide (TMZ) inhibited cell proliferation, induced cell cycle arrest, cell senescence, autophagy and apoptosis, resulting in cell death.

【0141】 It is also found that the combination of BMX and TMZ induced the synergistic apoptotic cell death through caspase-3 cleavage and PARP activation. It was demonstrated in the present invention that the combination of BMX and TMZ induced augmentation of phospho-p53 (ser15) as well as DNA damage such as increased γ -H2AX foci. The increased expression of phospho-p53 (ser15) might result from the increase in total p53 expression, which was reported in our previous study [16, 17]. In addition, our study revealed that BMX might have an HDAC-dependent synergistic effect with TMZ on the viability of CRC cells.

【0142】 Given the above, the high HDAC8 expression in human GBM tissues and GBM-R cell lines are correlated with MGMT levels. The combination of BMX and TMZ induced WT-p53 mediated apoptosis through WT-p53 mediated MGMT inhibition in GBM-R cell lines. Moreover, the combination of BMX and TMZ also suppressed cell proliferation and GSC phenotype activity via the β -catenin/c-Myc/cyclin D1/SOX2 signaling pathway in GBM-R cell lines. Therefore, BMX could

be a promising strategy for the precision personal treatment of WT-p53 and TMZ-resistant GBM patients.

【0143】 Overall, as an indication of the synergistic mechanism, it was demonstrated in the present invention that the combination of BMX and TMZ was effective in the induction of cell death of CRC through upregulating p53/p21/E2F3/Bax and downregulating Wnt / β -catenin/cyclin D1/c-Myc /p62 pathways. Therefore, it was found that the combination of BMX and TMZ provides the potential effect on cell death, including induction of apoptosis and autophagy. The results in the Examples indicate the potential role of the combination of BMX and TMZ by helping us understand their HDAC8 dependent synergistic effect on CRC cell death. These findings suggest a highly clinically relevant new mechanism of developing resistance against combined chemo-regimens.

【0144】 While this specification contains many specifics, these should not be construed as limitations on the scope of the invention or of what may be claimed, but rather as descriptions of features specific to particular embodiments or examples of the invention. Certain features that are described in this specification in the context of separate embodiments or examples can also be implemented in combination in a single embodiment.

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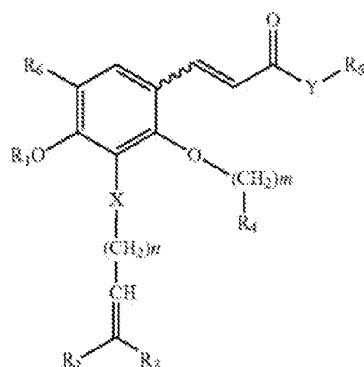
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What is claimed is:

1. A combination for treating a TMZ-resistant cancer in a patient, which comprises Temozolomide (TMZ) and a Compound A having the structure of Formula A, or a pharmaceutically acceptable salt, stereoisomer, enantiomer, prodrug or solvate thereof:



Formula A

wherein

R^1 is hydrogen, alkyl, alkenyl, C_5 - C_6 cycloalkyl, 5-membered or 6-membered unsaturated carbocycle or 5-membered or 6-membered heterocycle, or $(CH_2)_mR^4$;

X is C, -O-, -N- or -S-;

Y is -O-, -NH or -O- C_1 - C_4 alkyl;

n is an integer of 0 to 10;

m is an integer of 0 to 5;

R^2 and R^3 is independently C_1 - C_6 alkyl;

R^4 is C_5 - C_6 cycloalkyl or 5-membered or 6-membered unsaturated carbocycle or heterocycle which may be substituted with halogen, $-CF_3$, $-OR^7$ or $-NR^7R^8$, wherein R^7 and R^8 are independently hydrogen or C_1 - C_6 alkyl;

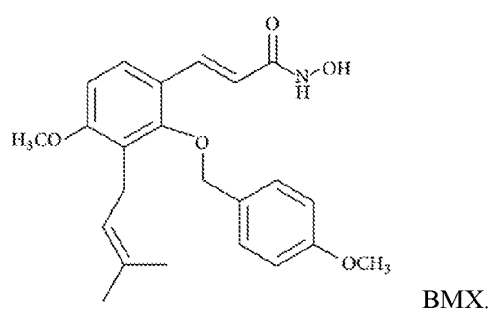
R^5 is OH, NH_2 or C_5 - C_6 cycloalkyl, 5-membered or 6-membered unsaturated carbocycle or heterocycle wherein the cycloalkyl, carbocycle and heterocycle

may be optionally substituted with halogen, NH₂, NO₂, C₁-C₆ alkoxy, C₁₋₆ alkylthio, OR^{7'}, NR⁷R⁸ or CF₃; and

R⁶ is H, C₁-C₁₀ alkyl which may be substituted by hydroxy or C₂-C₁₀ alkenyl, or together with R₁ being -C₂H₂-; and

wherein TMZ and the compound A are combined at a relative ratio to effectively overcome TMZ resistance.

2. The combinatin of claim 1, wherein the compound A is a compound BMX having the structure of



3. The combination of claim 1 or 2, wherein the TMZ resistance is overcome by enhancement of TMZ-mediated cytotoxic effect.

4. The combination of claim 3, wherein the enhancement of TMZ-mediated cytotoxic effect is by downregulating the β -catenin/c- Myc/SOX2 signaling pathway and upregulating WT-p53 mediated MGMT inhibition the resistant.

5. The combination of in any of claims 1-4, wherein TMZ and the compound A are separately or sequentially administrated.

6. A combination of in any of claims 1-5, wherein the combination comprises TMZ and BMX.

7. The combination of in any of claims 1-6, wherein the cancer is glioblastoma multiforme (GBM) or colorectal cancer (CRC).

8. A method for treating a TMZ-resistant cancer in a patient, which administering the patient a therapeutically effective amount of the combination as defined in any of claims 1-6.

9. The method of claim 8, wherein the cancer is GBM or CRC.

10. A method for a precision personal treatment for a drug-resistant cancer in a patient, which comprises determining the expression of WT-p53 in the patient, and administering the patient a therapeutically effective amount of the compound A as defined in claim 1 or its combination with said drug if the expression of WT-p53 is present in the patient.

11. The method of claim 10, wherein the drug is TMZ.

12. The method of claim 10 or 11, wherein the drug-resistant cancer is TMZ-resistant GBM or CRC.

13. A use of the combination as defined in any of claims 1-6 for manufacturing a medicament or a kit for a TMZ-resistant cancer patient.

14. The use of claim 11, wherein the cancer is GBM or CRC.

15. A use of the combination as defined in any of claims 1-6 for manufacturing a medicament or a kit for precision personal treatment for a TMZ-resistant WT-p53 GBM patient or CRC patient.