Synthesis and Antitumor Activity Evaluation of a Novel Combi-nitrosourea Prodrug: BGCNU

Yameng Wang, Ting Ren, Xinxin Lai, Guohui Sun, Lijiao Zhao,*^{ORCID} Na Zhang, and Rugang Zhong

Beijing Key Laboratory of Environmental and Viral Oncology, College of Life Science and Bioengineering, Beijing University of Technology, Beijing 100124, P. R. China

Supporting Information

ABSTRACT: Chloroethylnitrosoureas (CENUs) are an important type of alkylating agent employed in the clinical treatment of cancer. However, the anticancer efficacy of CENUs is greatly decreased by a DNA repairing enzyme, O⁶-alkylguanine-DNA alkyltransferase (AGT), by preventing the formation of interstrand cross-links (ICLs). In this study, a combi-nitrosourea prodrug, namely, *N*-(2-chloroethyl)-*N'*-2-(O⁶-benzyl-9-guanine)ethyl-*N*-nitrosourea (BGCNU), which possesses an O⁶-benzylguanine (O⁶-BG) derivative and CENU pharmacophores simultaneously, was synthesized and evaluated for its ability to induce ICLs. The target compound is markedly more cytotoxic in human glioma cells than the clinically used CENU chemotherapies ACNU, BCNU, and their respective combinations with O⁶-BG. In the AGT-proficient cells, significantly higher levels of DNA ICLs were observed in the groups treated by BGCNU than those by ACNU and BCNU, which indicated that the activity of AGT was effectively inhibited by the O⁶-BG derivatives released from BGCNU.

KEYWORDS: Combi-nitrosourea, chloroethylnitrosourea, anticancer activity, DNA interstrand cross-links, AGT inhibition

Chloroethylnitrosoureas (CENUs) are an important family of alkylating agents and widely used in the clinical treatment of cancers, including brain tumor, malignant lymphomas, neuroglioma, and various solid tumors.¹ The anticancer activity of CENUs is believed to be closely related to the formation of DNA interstrand cross-links (ICLs). As depicted in Figure 1, CENUs spontaneously undergo decomposition to produce a chloroethylating intermediate, which attacks guanine at the O⁶-position to form O⁶-chloroethylguanine (O⁶-ClEtG); then O⁶-ClEtG cyclizes to N1,O⁶-ethanoguanine (N1,O⁶-EthanG) followed by a second attack on the N3 site of the complementary cytosine, resulting in the formation of dG-dC cross-links. The yielded DNA ICLs inhibit the separation of double-strands during DNA replication and transcription, leading to the apoptosis of cancer cells. However, there is a DNA repairing enzyme, namely, O⁶-alkylguanine-DNA alkyltransferase (AGT), which restores the O⁶-alkylated guanine by transferring the alkyl groups from the O⁶-position of guanine to the cysteine 145 (Cys145) in its active site followed by a ubiquitination-dependent proteolysis of the inactivated protein.^{2–4} As a result, the formation of dG-dC cross-links will be blocked once the CENU-induced O⁶-ClEtG or N1,O⁶-EthanG is repaired by AGT. This process plays an important role in the formation of DNA ICLs, and thus, high levels of AGT can cause a strong resistance of tumor cells to CENU chemotherapies. In order to improve the therapeutic efficacy of CENUs and other O⁶-guanine alkylating agents, several strategies have been employed to inhibit the

drug resistance induced by AGT. O⁶-Benzylguanine (O⁶-BG) is the first AGT inhibitor reaching clinical trials, which reacts with Cys145 at the active site of AGT to form S-benzylcysteine to inactivate the enzyme.^{5–7} A number of evidence demonstrated that O⁶-BG can effectively increase the sensitivity of cancer cells to chemotherapies by dissipating the activity of AGT.^{8–11} Another AGT inhibitor, O⁶-(4-bromophenyl)guanine (O⁶-4-BTG), also entered clinical trials and exhibited even higher potency than O⁶-BG.^{12,13}

Except for the application of AGT inhibitors as adjuvants to improve the chemotherapeutic effects of CENUs, a series of studies have moved toward hybrid anticancer drugs by combining two functional groups into one molecule, known as a “combi-molecule”.^{14–20} These combi-nitrosourea prodrugs have two pharmacophores, in which the nitrosourea moiety releases a DNA damaging agent and the other pharmacophore exerts bioactivity via another mechanism. Since hybrid anticancer drugs are promising chemotherapeutic strategies for their ability to overcome the drawbacks of conventional anticancer drugs, we tried to develop a novel combi-nitrosourea prodrug with the potency of inducing DNA ICLs and simultaneously sensitizing cancer cells to chemotherapies by inhibiting AGT activity. In this study, we present the synthesis, antitumor activity evaluation, and mechanism of action of a novel combi-

Received: September 11, 2016

Accepted: January 13, 2017

Published: January 13, 2017

Figure 1. General mechanisms for the formation and repair of dG-dC cross-links.

nitrosourea prodrug, *N'*-2-(*O*⁶-benzyl-9-guanine)ethyl-*N*-(2-chloroethyl)-*N*-nitroso-urea (BGCNU), which possesses an *O*⁶-BG and a CENU pharmacophore. The combi-nitrosourea prodrug was designed to release the chloroethylating intermediate to induce dG-dC cross-links, along with the *O*⁶-BG derivative as an AGT inhibitor. Bearing the AGT inhibitor and the cross-linking pharmacophores simultaneously, the combi-nitrosourea prodrug is expected to exhibit higher efficiency and eliminate drug resistance compared with conventional CENU chemotherapies. According to our previous study on the three-dimensional quantitative structure–activity relationships (3D-QSAR) of *O*⁶-BG derivatives as AGT inhibitors, there is a wide entrance in the active pocket of AGT near the N9 position of the *O*⁶-BG derivatives. This indicates that a bulky substituent group on the N9 site is tolerated.²¹ Therefore, in this study, the CENU pharmacophore was conjugated to *O*⁶-BG on the N9 site by an ethylene bridge.

The preparation of BGCNU is depicted in Scheme 1 using *O*⁶-BG (1) as a starting material. Briefly, 1 was treated with 1,2-

Scheme 1. Synthesis of the Combi-nitrosourea Prodrug BGCNU

dibromoethane and K_2CO_3 , resulting in the formation of *N*9-bromomethyl-*O*⁶-benzyl-guanine (2) by a simple bromine substitution at the N9 position. Then, the bromine atom was reduced by phthalimide potassium in anhydrous *N,N*-dimethylformamide (DMF) followed by a substitution reaction with hydrazine hydrate to obtain the resulting intermediate *N*9-(2-amino)ethyl-*O*⁶-benzyl-guanine (4). Compound 4 was then treated with 2-chloroethyl isocyanate in anhydrous DMF yielding *N*-(2-chloroethyl)-*N'*-2-(*O*⁶-benzyl-9-guanine)ethyl-urea (5). Finally, 5 was converted to the target compound 6 via a nitrosation reaction with nitrosonium tetrafluoroborate in acetone containing a moderate amount of glacial acetic acid at 0 °C. The anticipated structures of all intermediate products and the final compound were in agreement with the spectral (IR, ¹H NMR, and ¹³C NMR) data and were further substantiated by HRMS. All characterization data are summarized in the Supporting Information. As classical CENUs, BGCNU undergoes spontaneous hydrolysis to yield *O*⁶-BG analogues 7 and 4 (see Scheme 2).^{22,23} The stability of BGCNU was investigated, and the results were described in the Supporting Information.

Scheme 2. Pathway of BGCNU Decomposition

To evaluate the anticancer activity of the synthesized BGCNU, a cell survival assay against human glioma cells was performed using the Cell Counting Kit-8 (CCK8) method. Three human glioma cell lines with different levels of AGT expression were employed for the activity investigation, including SF126 (AGT[−], little or no AGT activity), SF767 (AGT⁺, 61 fmol/10⁶ cells), and SF763 (AGT⁺, 119 fmol/10⁶ cells).^{24–27} The cytotoxicity of BGCNU was compared with those of 1-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-3-(2-chloroethyl)-3-nitrosourea (ACNU), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), and their respective combinations with *O*⁶-BG. The details of the experimental protocol and all measurements can be found in the Supporting Information. As shown in Figure 2A,B, in SF763 cells with high levels of AGT expression, the synthesized BGCNU (IC₅₀ = 60 μM) exhibit 27- and 17-fold stronger inhibitory activity than the clinically used ACNU (IC₅₀ = 1600 μM) and its combination with *O*⁶-BG (IC₅₀ = 1000 μM), respectively; BGCNU also was significantly more cytotoxic than BCNU (IC₅₀ = 350 μM) and its combination with *O*⁶-BG (IC₅₀ = 200 μM) by 5.8 and 3.3 times, respectively. Similarly, BGCNU also exhibited higher cytotoxicity against SF767 (IC₅₀ = 58 μM), which express moderate levels of AGT, than ACNU (IC₅₀ = 570 μM), BCNU (IC₅₀ = 130 μM), and their respective combinations with *O*⁶-BG (400 and 120 μM, respectively) (see Figure 2C,D). For the AGT-deficient SF126 cells (see Figure 2E,F), BGCNU (IC₅₀ = 52 μM) was still 7 to 9 times more cytotoxic than ACNU (IC₅₀ = 450 μM) and its combination with *O*⁶-BG (IC₅₀ = 360 μM); however, it exhibited slightly higher toxicity than BCNU (IC₅₀ = 85 μM) alone and similar potency to the combination of BCNU with *O*⁶-BG (IC₅₀ = 55 μM). The result of the cell survival assays indicated that the sensitivity of the AGT⁺ cells (SF763 and SF767) toward BGCNU was increased compared

Figure 2. Cell survival curves for human glioma SF763, SF767, and SF126 cells treated with the indicated drugs. (A) SF763, (C) SF767, and (E) SF126 cells treated with ACNU, ACNU combined with 20 μ M O⁶-BG, and BGCNU; (B) SF763, (D) SF767, and (F) SF126 cells treated with BCNU, BCNU combined with 20 μ M O⁶-BG, and BGCNU.

to conventional CENUs (ACNU and BCNU). This provided direct evidence that the drug resistance of the AGT⁺ cells was decided by the high levels of AGT activity, which could be suppressed by the O⁶-BG derivatives generated from the combi-nitrosourea.

Previous studies reported that dG-dC cross-links may be employed as an indicator for predicting the anticancer efficiency of CENUs.²⁸ Therefore, in this study, we determined the levels of dG-dC cross-link in human glioma cells exposed to ACNU, BCNU, and BGCNU using the previously reported high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) method.^{29,30} The details of the experimental protocol, the HPLC-ESI-MS/MS method validation, and all the measurements are described in the [Supporting Information](#). The levels of dG-dC cross-link were determined in DNA extracted from three human glioma cells (SF763, SF767, and SF126) and in calf thymus DNA (CT-DNA) treated with ACNU, BCNU, and BGCNU at different concentrations (0.1, 0.2, 0.4, and 0.6 mM). In the samples of both cellular DNA and CT-DNA, the levels of dG-dC cross-link displayed a dose-dependence with increasing drug concentrations from 0.1 to 0.6 mM. In the samples of CT-DNA, the levels of dG-dC cross-link induced by BGCNU were lower than those induced by ACNU or BCNU for each group with the same drug concentration (see Figure S1 in the [Supporting Information](#)). This result may be due to the decreased water solubility of BGCNU and the absence of AGT in the reaction mixture.

In contrast to the results of CT-DNA, in all DNA samples from human glioma cells, significantly higher ($p < 0.01$) levels of dG-dC cross-links were observed in the groups treated by BGCNU than those treated by ACNU or BCNU at all drug

concentrations (see Figure 3, data are listed in Table S1 in the [Supporting Information](#)). As shown in Figure 3, in SF763 cells,

Figure 3. DG-dC crossing levels in SF763, SF767, and SF126 cells treated by ACNU, BCNU, and BGCNU with the drug concentrations at (A) 0.1, (B) 0.2, (C) 0.4, and (D) 0.6 mM. Symbol designations are (stripes) for BCNU, ■ for ACNU, and □ for BGCNU treatment ($n = 3$).

the levels of dG-dC cross-link induced by BGCNU are 43, 38, 28, and 19 times those induced by BCNU at the drug concentrations of 0.1, 0.2, 0.4, and 0.6 mM, respectively; and 23, 20, 20, and 13 times those induced by ACNU at the drug concentrations of 0.1, 0.2, 0.4, and 0.6 mM, respectively. In SF767 cells, the cross-linking levels induced by BGCNU were 7 to 14 and 5 to 11 times higher than those induced by BCNU and ACNU in all drug concentrations, respectively. These results indicated that the AGT activity in SF763 and SF767 cells was greatly inhibited by the O⁶-BG derivatives yielded from BGCNU, which resulted in the significant enhancement of the dG-dC cross-linking levels. Interestingly, increased levels of dG-dC cross-link were also observed in the AGT-deficient SF126 cells. The levels of BGCNU-induced cross-links in SF126 cells were 3 to 9 and 2 to 6 times higher than those induced by BCNU and ACNU, respectively. This finding may be attributable to the increased lipophilicity of BGCNU (LogP = 1.81) by introducing an O⁶-BG moiety into the molecule, which led to the higher ability of BGCNU than BCNU and ACNU to penetrate the cell membrane resulting in the formation of DNA ICLs.

Comparing the cross-linking levels in the three glioma cell lines exposed to ACNU and BCNU, it can be observed that the levels of dG-dC cross-link generally follow the order of SF126 > SF767 > SF763. However, in the groups treated by BGCNU, this order changed because of the considerable enhancement of the cross-linking levels in the AGT-proficient SF763 and SF767 cells. In the groups treated at high drug concentrations (0.4 and 0.6 mM), the highest cross-linking levels were observed in SF763 cells followed by SF767 and SF126 cells. Moreover, the promotion of dG-dC cross-linking levels by BGCNU was more prominent at lower drug concentrations. In our previous work, another combi-nitrosourea prodrug, 3-(3-(((2-amino-9H-purin-6-yl)-oxy)methyl)benzyl)-1-(2-chloroethyl)-1-nitrosourea, was synthesized and was demonstrated to induce significantly higher levels of dG-dC cross-link in human glioma cells than ACNU and BCNU.³¹ However, the levels of dG-dC cross-link in SF763 cells treated by the previously reported combi-nitrosourea is 90% lower than those induced by BGCNU at the

same drug concentrations. This may be attributed to the substitution of the nitrosoourea moiety on the N9 site of O⁶-BG, where it was demonstrated by a 3D-QSAR study to be highly tolerated for O⁶-BG derivatives as AGT substrates.²¹ For an in-depth insight into the mechanism of AGT inhibition, a molecular docking study was performed to explore the interaction between AGT protein and the O⁶-BG derivatives released from BGCNU.

The details for the procedure of molecular docking is described in the [Supporting Information](#). Figure 4 displays the

Figure 4. Key hydrogen bonding interactions between 7 (A) or 4 (B) and the amino acid residues in the active pocket of AGT (PDB code: 1T39).

ligand-binding surface of the docked molecular complexes and the key hydrogen bonding interactions of O⁶-BG derivatives released from BGCNU with the active pocket of the AGT protein. In contrast to the four hydrogen bonds formed between O⁶-BG and Tyr114, Cys145, and Ser159 (see Figure S2 in the [Supporting Information](#)), six specific hydrogen bonds were observed between compound 7 or 4 and the four amino acid residues in the active site of AGT. In the complex of AGT binding to compound 7 (see [Figure 4A](#)), the isocyanate group, a “tail” on the N9 site of guanine, was observed to have interactions with AGT by forming two more hydrogen bonds with Asn137 and Tyr114 residues compared to the O⁶-BG...AGT complex. Similarly, in the complex of compound 4 with AGT (see [Figure 4B](#)), two new hydrogen-bonding interactions were observed between Gly131 and the aminoethyl group on the N9 site and between Tyr114 and the amino group on the N2 site of guanine. Moreover, it can be observed from the ligand-binding surface of the complexes that the substituent “tails” on the N9 site of 7 and 4 were precisely embedded in the groove adjacent to the active site pocket of AGT protein.

In this study, we synthesized a new combi-nitrosoourea prodrug, BGCNU, and evaluated its anticancer activity using human glioma cells. By introducing an O⁶-BG pharmacophore, the novel combi-nitrosoourea exhibits significantly higher cytotoxicity and DNA interstrand cross-linking potency than the clinically used CENU chemotherapies due to the efficient inhibition of the AGT activity in AGT⁺ cells. This study has

paved the way for the development of a novel CENU chemotherapy with high efficiency and eliminated drug resistance. However, BGCNU exhibits nontarget inhibition to tumor cells, which is likely to be a potential disadvantage hampering the further investigations of BGCNU in animal experiments and clinical studies. To solve this problem, we are working on the design and synthesis of BGCNU derivatives containing a hypoxia activated moiety, which will enable the prodrug to be selectively activated to release AGT inhibitor in the oxygen-deficient region of solid tumor.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](#) at DOI: [10.1021/acsmmedchemlett.6b00358](https://doi.org/10.1021/acsmmedchemlett.6b00358).

Experimental details for synthetic procedures and biological assays, full compound characterization, and molecular docking ([PDF](#))

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (+86)10-67396139. Fax: (+86)10-67392001. E-mail: zhaolijiao@bjut.edu.cn.

ORCID

Lijiao Zhao: [0000-0002-4876-323X](https://orcid.org/0000-0002-4876-323X)

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. 21277001), Natural Science Foundation of Beijing Municipality (No. 7162015), Beijing Municipal Education Commission Science and Technology Project (No. PXM2015_014204_500175), and Jinghua Talent Project of Beijing University of Technology (No. 015000514115001).

Notes

The authors declare no competing financial interest.

■ REFERENCES

- (1) Gnewuch, C. T.; Sosnovsky, G. A critical appraisal of the evolution of N-nitrosooureas as anticancer drugs. *Chem. Rev.* **1997**, *97*, 829–1013.
- (2) Daniels, D. S.; Woo, T. T.; Luu, K. X.; Noll, D. M.; Clarke, N. D.; Pegg, A. E.; Tainer, J. A. DNA binding and nucleotide flipping by the human DNA repair protein AGT. *Nat. Struct. Mol. Biol.* **2004**, *11*, 714–720.
- (3) Tubbs, J. L.; Pegg, A. E.; Tainer, J. A. DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O-6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. *DNA Repair* **2007**, *6*, 1100–1115.
- (4) Pegg, A. E. Multifaceted roles of alkyltransferase and related proteins in DNA repair, DNA damage, resistance to chemotherapy, and research tools. *Chem. Res. Toxicol.* **2011**, *24*, 618–639.
- (5) Friedman, H. S.; Kokkinakis, D. M.; Pluda, J.; Friedman, A. H.; Cokgor, I.; Haglund, M. M.; Ashley, D. M.; Rich, J.; Dolan, M. E.; Pegg, A. E.; Moschel, R. C.; McLendon, R. E.; Kerby, T.; Herndon, J. E.; Bigner, D. D.; Schold, S. C. Phase I trial of O-6-benzylguanine for patients undergoing surgery for malignant glioma. *J. Clin. Oncol.* **1998**, *16*, 3570–3575.

- (6) Dolan, M. E.; Pegg, A. E. O⁶-Benzylguanine and its role in chemotherapy. *Clin. Cancer Res.* **1997**, *3*, 837–847.
- (7) Warren, K. E.; Gururangan, S.; Geyer, J. R.; McLendon, R. E.; Poussaint, T. Y.; Wallace, D.; Balis, F. M.; Berg, S. L.; Packer, R. J.; Goldman, S.; Minturn, J. E.; Pollack, I. F.; Boyett, J. M.; Kun, L. E. A phase II study of O⁶-benzylguanine and Temozolomide in pediatric patients with recurrent or progressive high-grade gliomas and brainstem gliomas: a Pediatric Brain Tumor Consortium study. *J. Neuro-Oncol.* **2012**, *106*, 643–649.
- (8) Batts, E. D.; Maisel, C.; Kane, D.; Liu, L.; Fu, P.; O'Brien, T.; Remick, S.; Bahlis, N.; Gerson, S. L. O⁶-Benzylguanine and BCNU in multiple myeloma: a phase II trial. *Cancer Chemother. Pharmacol.* **2007**, *60*, 415–421.
- (9) Apisarnthanarax, N.; Wood, G. S.; Stevens, S. R.; Carlson, S.; Chan, D. V.; Liu, L.; Szabo, S. K.; Fu, P.; Gilliam, A. C.; Gerson, S. L.; Remick, S. C.; Cooper, K. D. Phase I clinical trial of O⁶-benzylguanine and topical carmustine in the treatment of cutaneous T-cell lymphoma, mycosis fungoides type. *Arch. Dermatol.* **2012**, *148*, 613–620.
- (10) Chinnasamy, D.; Fairbairn, L. J.; Neuenfeldt, J.; Treisman, J. S.; Hanson, J. P.; Margison, G. P.; Chinnasamy, N. Lentivirus-mediated expression of mutant MGMT (P140K) protects human CD34(+) cells against the combined toxicity of O-6-benzylguanine and 1,3-bis(2-chloroethyl)-nitrosourea or Temozolomide. *Hum. Gene Ther.* **2004**, *15*, 758–769.
- (11) Bacolod, M. D.; Johnson, S. P.; Pegg, A. E.; Dolan, M. E.; Moschel, R. C.; Bullock, N. S.; Fang, Q. M.; Colvin, O. M.; Modrich, P.; Bigner, D. D.; Friedman, H. S. Brain tumor cell lines resistant to O-6-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy have O-6-alkylguanine-DNA alkyltransferase mutations. *Mol. Cancer Ther.* **2004**, *3*, 1127–1135.
- (12) McElhinney, R. S.; Donnelly, D. J.; McCormick, J. E.; Kelly, J.; Watson, A. J.; Rafferty, J. A.; Elder, R. H.; Middleton, M. R.; Willington, M. A.; McMurry, T. B. H.; Margison, G. P. Inactivation of O⁶-alkylguanine-DNA alkyltransferase. 1. Novel O⁶-(hetaryl methyl)-guanines having basic rings in the side chain. *J. Med. Chem.* **1998**, *41*, 5265–5271.
- (13) Ranson, M.; Middleton, M. R.; Bridgewater, J.; Lee, S. M.; Dawson, M.; Jowle, D.; Halbert, J. G.; Waller, S.; McGrath, H.; Gumbrell, L.; McElhinney, R. S.; Donnelly, D.; McMurry, T. B. H.; Margison, G. P. Lomeguatrib, a potent inhibitor of O⁶-alkylguanine-DNA-alkyltransferase: phase I safety, pharmacodynamic, and pharmacokinetic trial and evaluation in combination with Temozolomide in patients with advanced solid tumors. *Clin. Cancer Res.* **2006**, *12*, 1577–1584.
- (14) Fortin, S.; Berube, G. Advances in the development of hybrid anticancer drugs. *Expert Opin. Drug Discovery* **2013**, *8*, 1029–1047.
- (15) Wanner, M. J.; Koch, M.; Koomen, G. J. Synthesis and antitumor activity of methyltriazene prodrugs simultaneously releasing DNA-methylating agents and the anti-resistance drug O⁶-benzylguanine. *J. Med. Chem.* **2004**, *47*, 6875–6883.
- (16) Brahimi, F.; Rachid, Z.; Qiu, Q.; McNamee, J. P.; Li, Y. J.; Tari, A. M.; Jean-Claude, B. J. Multiple mechanisms of action of ZR2002 in human breast cancer cells: a novel combi-molecule designed to block signaling mediated by the ERB family of oncogenes and to damage genomic DNA. *Int. J. Cancer* **2004**, *112*, 484–491.
- (17) Rachid, Z.; Brahimi, F.; Domarkas, J.; Jean-Claude, B. J. Synthesis of half-mustard combi-molecules with fluorescence properties: correlation with EGFR status. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1135–1138.
- (18) Qiu, Q. Y.; Domarkas, J.; Banerjee, R.; Merayo, N.; Brahimi, F.; McNamee, J. P.; Gibbs, B. F.; Jean-Claude, B. J. The combi-targeting concept: In vitro and in vivo fragmentation of a stable combi-nitrosourea engineered to interact with the epidermal growth factor receptor while remaining DNA reactive. *Clin. Cancer Res.* **2007**, *13*, 331–340.
- (19) Qiu, Q. Y.; Dudouit, F.; Banerjee, R.; McNamee, J. P.; Jean-Claude, B. J. Inhibition of cell signaling by the combi-nitrosourea FD137 in the androgen independent DU145 prostate cancer cell line. *Prostate* **2004**, *59*, 13–21.
- (20) Domarkas, J.; Dudouit, F.; Williams, C.; Qiu, Q. Y.; Banerjee, R.; Brahimi, F.; Jean-Claude, B. J. The combi-targeting concept: Synthesis of stable nitrosoureas designed to inhibit the epidermal growth factor receptor (EGFR). *J. Med. Chem.* **2006**, *49*, 3544–3552.
- (21) Sun, G. H.; Fan, T. J.; Zhang, N.; Ren, T.; Zhao, L. J.; Zhong, R. G. Identification of the structural features of guanine derivatives as MGMT inhibitors using 3D-QSAR modeling combined with molecular docking. *Molecules* **2016**, *21*, 823.
- (22) Faustino, C.; Garcia-Rio, L.; Leis, J. R.; Norberto, F. Decomposition of N'-benzoyl-N-nitrosoureas in aqueous media. *Eur. J. Org. Chem.* **2005**, No. 1, 154–161.
- (23) Lown, J. W.; Chauhan, S. M. S. Discrimination between alternative pathways of aqueous decomposition of anti-tumor (2-chloroethyl)nitrosoureas using specific O¹⁸ labeling. *J. Org. Chem.* **1982**, *47*, 851–856.
- (24) Bobola, M.; Berger, M. S.; Silber, J. R. Contribution of O⁶-methylguanine-DNA methyltransferase to resistance to 1,3-(2-chloroethyl)-1-nitrosourea in human brain tumor derived cell lines. *Mol. Carcinog.* **1995**, *13*, 81–88.
- (25) Aida, T.; Bodell, W. J. Cellular-resistance to chloroethyl-nitrosoureas, nitrogen-mustard, and cis-diamminedichloro-platinum (II) in human glial-derived cell-lines. *Cancer Res.* **1987**, *47*, 1361–1366.
- (26) Bobola, M. S.; Blank, A.; Berger, M. S.; Silber, J. R. Contribution of O-6-methylguanine-DNA methyltransferase to alkylating-agent resistance in human brain tumor-derived cell-lines. *Mol. Carcinog.* **1995**, *13*, 70–80.
- (27) Sun, G. H.; Zhao, L. J.; Fan, T. J.; Ren, T.; Zhong, R. G. Measurement of O⁶-alkylguanine-DNA alkyltransferase activity in tumour cells using stable isotope dilution HPLC-ESI-MS/MS. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2016**, *1033*, 138–146.
- (28) Zhao, L. J.; Li, L. L.; Zhong, R. G.; Xu, J. Comparative investigation of the DNA inter-strand crosslinks induced by ACNU, BCNU, CCNU and FTMS using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Int. J. Mass Spectrom.* **2014**, *368*, 30–36.
- (29) Bai, B. Q.; Zhao, L. J.; Zhong, R. G. Quantification of meCCNU-induced dG-dC crosslinks in oligonucleotide duplexes by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2027–2034.
- (30) Li, L. L.; Zhao, L. J.; Zhong, R. G. Quantification of DNA interstrand crosslinks induced by ACNU in NIH/3T3 and L1210 cells using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2014**, *28*, 439–447.
- (31) Sun, G. H.; Zhang, N.; Zhao, L. J.; Fan, T. J.; Zhang, S. F.; Zhong, R. G. Synthesis and antitumor activity evaluation of a novel combi-nitrosourea prodrug: Designed to release a DNA cross-linking agent and an inhibitor of O⁶-alkylguanine-DNA alkyltransferase. *Bioorg. Med. Chem.* **2016**, *24*, 2097–2107.