Two newly synthesized 5-methyltetrahydrofolate-like compounds inhibit methionine synthase activity accompanied by cell cycle arrest in G_1/S phase and apoptosis *in vitro*

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Cobalamin-dependent methionine synthase, with a cofactor of vitamin B12, catalyzes the reaction of 5-methyltetrahydrofolate and homocysteine to form methionine and tetrahydrofolate, which takes a core position in folate cycle, one-carbon-unit transfer, and sulfur amino acid pathways. The 'methyl folate trap' hypothesis suggests that methionine synthase is a potential target for anticancer drug development. ZL031 and ZL033 are 5-methyltetrahydrofolate-like compounds that have been newly synthesized as potential inhibitors of the enzyme. To identify the effect of these two compounds on methionine synthase activity, a spectrophotometric assay was used and the results proved that ZL031 and ZL033 inactivated methionine synthase in HL-60 cells with an IC_{50} dose of 10.0 and 1.4 μmol/l, respectively. Moreover, obvious inhibitory effect on proliferation of HL-60 cells was observed, leading to our further investigation of the underlying anticancer mechanism. Under the circumstances of methionine synthase deficiency and subsequent folate depletion, cell cycle was arrested in G1/S phase and apoptosis was also observed. Analysis of cell cycle regulatory proteins demonstrated that cyclin E and cyclin-dependent kinase 2 were both increased. Furthermore, reduction of caspase-3, poly (ADP-ribose) polymerase, caspase-8, and caspase-9 protein levels were observed. In all the biological experiments we have performed, ZL033 has shown a better efficacy compared with ZL031. These results suggest that ZL031 and ZL033, as novel methionine synthase inhibitors, caused G1/S phase delay and apoptosis and eventually inhibit the proliferation of HL-60 cells *in vitro*. ZL033, with a carboxylic acid substituent, might have a better potential for drug development than ZL031 with an ester substituent.

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Introduction

Cobalamin-dependent methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13), using cobalamin as the cofactor, transfers a methyl group from N^5-methyltetrahydrofolate to homocysteine yielding tetrahydrofolate and methionine, which is the significant intersection of one-carbon-unit and sulfur metabolism, and directly involved in biological methylations and polyamine and protein biosynthesis. The product, methionine, is then adenylated to form *S*-adenosylmethionine (SAM), which is a crucial methyl donor for biological reactions, including the methylation of DNA, RNA, and protein. Therefore, dysfunctional methionine synthase has been associated with macrocytic anemia [1], atherosclerosis [2], neural tube defects [3], and tumor formation [4].

Folate has a central role in one-carbon metabolism and hence is an essential factor for DNA methylation, synthesis, and repair. Substantial epidemiological and experimental studies suggest that folate depletion suppresses the progression of cancer development and enhances the sensitivity of cancer cells to chemotherapy. As methionine synthase is the only enzyme that recycles methyltetrahydrofolate to tetrahydrofolate and makes the dietary folates enter the folate cycle, decreased methionine synthase activity leads to an accumulation of methylfolate and accordingly trapping of intracellular levels of folic acid derivatives. These changes have been referred to as the 'methyl folate trap' hypothesis proposed by Noronha and Silverman [5], which has been further proven true [6,7]. The vital position of methionine synthase in the folate cycle and DNA synthesis has been recognized and considered for decades as a potential target for developing anticancer drugs [8,9]. Furthermore, inhibition of methionine synthase activity is expected to be selectively toxic to cancer cells because of their high turnover of DNA and elevated requirement for folate [5,8]. Nitrous oxide, a widely reported and studied inhibitor of methionine synthase [6,10], has been

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demonstrated to oxidize vitamin B_{12} making it no longer functional as a coenzyme. Furthermore, several animal experiments and clinical trials have provided convincing evidence for an efficacious chemotherapeutic effect of nitrous oxide alone or in combination with other antifolate agents [11–13]. Nevertheless, it could not develop as an extensively applicable chemotherapeutic approach owing to the high toxicity of nitrous oxide [14]. Another inhibitor, nitric oxide, which was reported to inhibit methionine synthase activity both *in vitro* [15] and *in vivo* [16], has been proposed to bind to the cobalt of cobalamin, as well as the donor of nitric oxide, sodium nitroprusside [17]. Other reported inhibitors are small molecules, including methyl mercury [18,19], chloroform, carbon tetrachloride [20], hydrazine [21], ethanol, and acetaldehyde [22]. However, to date, only a limited number of synthetic compounds have been reported to inhibit this enzyme, including a series of cobalamin analogs [23] and a series of benzo-fused heterocycles that are designed to link to the methyltetrahydrofolate-binding site of the enzyme and mostly act as competitive inhibitors [24]. Hence, an anticancer strategy against methionine synthase offers a promising alternative to cancer treatment and it is essential to uncover novel efficient inhibitors of the enzyme.

In the present study, two new structural compounds were synthesized and purified as inhibitors of methionine synthase. They mimic the structure of the substrate of the reaction, 5-methyltetrahydrofolate. The biological activities of these two compounds were investigated, including their anticancer properties and possible molecular mechanism. The structure–activity relationship between these two compounds was also investigated.

Materials and methods

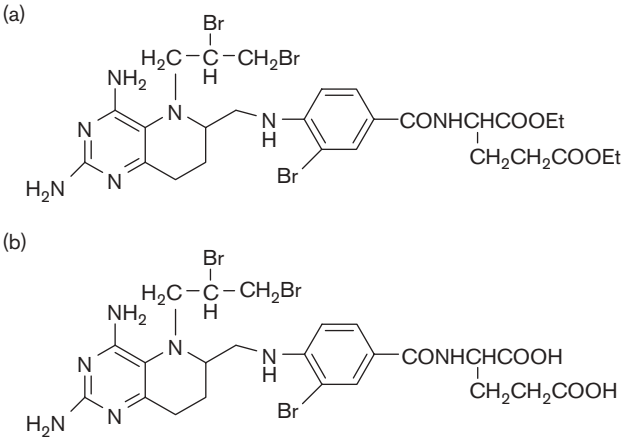
Preparation of compounds

ZL031 and ZL033 were synthesized by Zhili Zhang *et al.* (Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University) with more than 97% purity. The compounds were named as diethyl *N*-[4-{(2-[2,4-diamino-5-(2,3-dibromopropane)-5,6,7,8-tetrahydropyrido(3,2-*d*) pyrimidin-6-yl]methyl)amino}3-bromo-benzoyl]-L-glutamate (ZL031) and *N*-[4-{(2-[2,4-diamino-5-(2,3-dibromopropane)-5,6,7,8-tetrahydropyrido (3,2-*d*) pyrimidin-6-yl]methyl)amino}-3-bromo-benzoyl]-L-glutamic acid (ZL033) (Fig. 1). They were dissolved in dimethylsulfoxide to a concentration of 10 mmol/l as stock solution and were stored at −20°C. For all experiments, the compounds were diluted in PBS just before use.

Chemicals and antibodies

Sodium nitroprusside, Igepal CA-630 5-methyltetrahydrofolic acid disodium salt, SAM, DTT, L-homocysteine thiolactone, hydroxocobalamin, and monoclonal antibody for human poly (ADP-ribose) polymerase (PARP) were purchased from Sigma-Aldrich, (Sheboygen, Wisconsin, USA). Monoclonal antibody against human caspase-8 and polyclonal antibody against human caspase-3 were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Monoclonal antibodies for human cyclin E, cyclin-dependent kinase (CDK) 2, and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Fig. 1



Chemical structure of (a) ZL031 and (b) ZL033.

Cell line and culture conditions

Human promyeloleukemic cell line (HL-60) was purchased from the American Type Culture Collection (University Boulevard, Manassas, Virginia, USA). Cells were maintained in RPMI 1640 medium (Gibco/BRL, Grand Island, New York, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and grown in a humidified atmosphere of 37°C, with 5% CO_2 and 95% air.

Methionine synthase activity assay

Cells at the density of 1×10^6 cells/well in 24-well plates were serum-starved before treating with various concentrations of ZL031 or ZL033 for 3 h. Then the cells were harvested and lysed with lysis buffer (20 mmol/l HEPES pH 7.5, 3 mmol/l $MgCl_2$, 14 mmol/l NaCl, 5% glycerol, 0.5% Igepal CA-630, 1 mmol/l DTT, 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride) [25]. After centrifugation (10 000 rpm, 10 min, at 4°C), the supernatants were assayed in 96-well microplates for methionine synthase activity, following the method of Drummond *et al.* [26] and Jarret *et al.* [27] with modification. The assay solution contained 1 mol/l potassium phosphate buffer (pH 7.2) 20 μl, 1 mol/l DTT 5 μl, 4.2 mmol/l 5-methyltetrahydrofolate 12 μl, 0.76 mmol/l SAM 5 μl, 97 μl double distilled water, and 40 μl cell lysate. After adding 0.5 mmol/l

hydroxocobalamin 20 µl into the mixture, it was pre-incubated at 37°C for 5 min immediately. Then the reaction was initiated by mixing with 100 mmol/l L-homocysteine 1 µl and incubated for 10 min at 37°C. The reaction was terminated by the addition of 5 N HCl/60% formic acid 50 µl and incubated at 80°C for 10 min. The total volume in the well of a 96-well microplate is 250 µl. The plate was read at 350 nm by using FLUOstar OPTIMA microplate multidetection reader (BMG Offenburg, Germany).

Cell vitality assay

The effect of ZL031 and ZL033 on cell proliferation was determined by a standard MTT assay as previously described [28]. Briefly, exponentially growing cells were seeded in 96-well microplates at 1×10^5 cells/well and exposed to the desired concentrations of ZL031 (1.25–20 µmol/l) or ZL033 (0.312–5 µmol/l). After incubation for 24 h, 5 mg/ml MTT 20 µl were added to each well, and cells were further incubated at 37°C for 4 h. The medium was then removed and 200 µl acidified isopropanol per well (0.04 N HCl) was added to dissolve the reduced formazan product. The plate was then read at 570 nm on a FLUOstar OPTIMA microplate multidetection reader (BMG). For the time-course study, cells were exposed to 2.5 µmol/l ZL031 or ZL033 for 24 h or 48 h, respectively.

Flow cytometry analysis

Cell cycle distribution of ZL031- or ZL033-exposed cells was determined using a BECScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, California, USA) with an excitation/emission of 488/525 nm. A total of 4×10^6 cells were harvested and washed twice with PBS after treatment of ZL031 or ZL033 for 24 h and then fixed in 70% cooled ethanol at 4°C until the beginning of the next procedure. Before analysis by flow cytometry, the cells were washed in PBS. Fixed cells were pelleted and resuspended in 0.5 ml PBS containing RNase A (10 µg/ml) in 37°C for 30 min. Then 50 µg/ml propidium iodide was added to the cells. The cell cycle distribution and the percentage of apoptosis were analyzed by CellQuest and the Modfit software (Becton Dickinson).

Western blot analysis

HL-60 cells were exposed to increasing concentrations of ZL031 or ZL033 for 24 h. After washing twice with PBS, equal amounts of cells were extracted with lysis buffer containing protease inhibitors (50 mmol/l Tris–HCl pH 8.0, 150 mmol/l sodium chloride, 1% Triton X-100, 0.02% sodium azide, 100 µg/ml phenylmethylsulfonyl fluoride, and 1 µg/ml aprotinin) [29]. The protein concentration was determined by a Coomassie plus protein assay reagent (Pierce, Rockford, Illinois, USA). Equal cellular protein of control cells and treated cells were resolved by SDS-PAGE and then transferred to Hybond C membranes (Amersham, Arlington Height, Illinois, USA) electrophoretically. The membranes were blocked with 3% albumin fraction V in Tris-buffered saline Tween-20 and then incubated with specific primary monoclonal antibodies. The primary antibody complexes were then stained with either goat antimouse or goat antirabbit horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, Baltimore, Pennsylvania, USA). Protein bands were identified by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Statistics

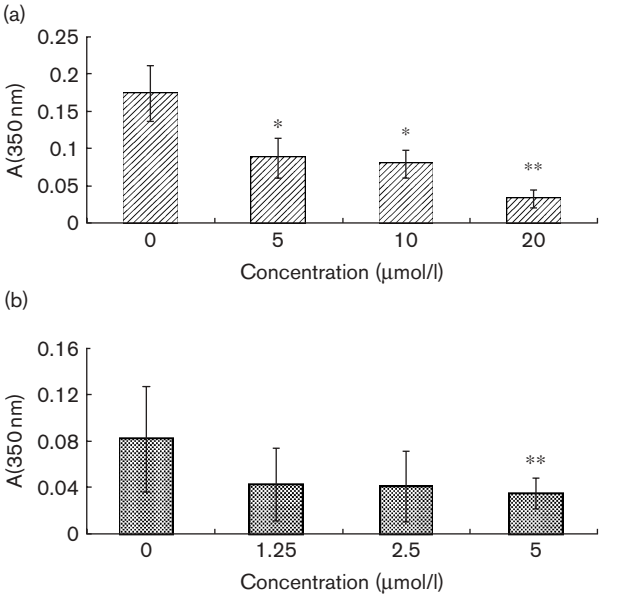
The results have been presented as mean values ± SD of at least three separate experiments. Student's *t*-test was used and $P < 0.05$ was considered significant.

Results

Effect of ZL031 and ZL033 on methionine synthase activity in HL-60 cells

To assess the effect of ZL031 or ZL033 on the activity of methionine synthase, a spectrophotometric assay in the 96-well microplate was used. The findings summarized in Fig. 2 showed that there was a significant reduction of methionine synthase activity in HL-60 cells under the treatment of either ZL031 or ZL033 for 3 h. The

Fig. 2



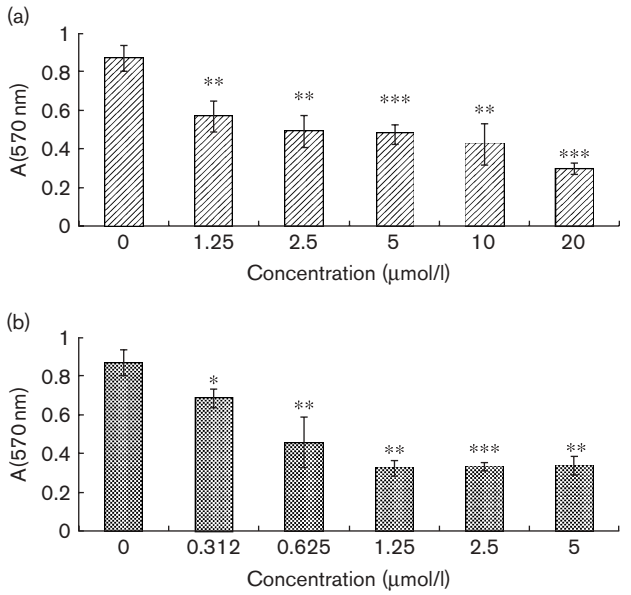
Inhibitory effect of ZL031 and ZL033 on methionine synthase activity. HL-60 cells were exposed to a serial concentration of ZL031 (5–20 µmol/l) or ZL033 (1.25–5 µmol/l) for 3 h. The inhibitory effect of methionine synthase activity was determined by a spectrophotometric assay in 96-well microplates measured at 350 nm. Data are given as the means of triplicate analyses. *$P<0.05$, **$P<0.01$ compared with control. (a) Effect of ZL031 on methionine synthase activity in HL-60 cells. (b) Effect of ZL033 on methionine synthase activity in HL-60 cells.

inhibitory rate reached more than 50% when cells were treated with 5 μmol/l ZL031 or ZL033. The IC_{50} for the inhibition was calculated as 10.0 ± 2.0 and 1.4 ± 0.4 μmol/l for ZL031 and ZL033, respectively. The degree of inhibition was almost seven-fold greater with ZL033 treatment than with ZL031 treatment, which indicated that interaction between the enzyme and the compound ZL033 is stronger and much more active than the compound ZL031.

ZL031 and ZL033 inhibit proliferation of HL-60 cells

After treating with a series of concentrations of ZL031 or ZL033, HL-60 cells proliferation was determined by using the MTT assay. Figure 3 shows that the compounds significantly inhibited the proliferation of HL-60 cells in a dose-dependent manner. When cells were treated with 1.25–20 μmol/l ZL031, the inhibitory rate ranged from 34.7 to 65.9%. In the case of ZL033, the inhibitory rate ranged from 21.1 to 61.3%. The IC_{50} value of ZL031 (5.4 ± 1.8 μmol/l) was nearly twice higher than the IC_{50} value of ZL033 (2.0 ± 1.0 μmol/l). As illustrated in Fig. 4, the inhibitory effect was enhanced in a time-dependent manner compared with control groups when the treatment of ZL031 or ZL033 was prolonged to 48 h. The results indicated that the inhibitory activity against cell proliferation of ZL033 was apparently stronger than ZL031 at both 24 and 48 h treatments.

Fig. 3

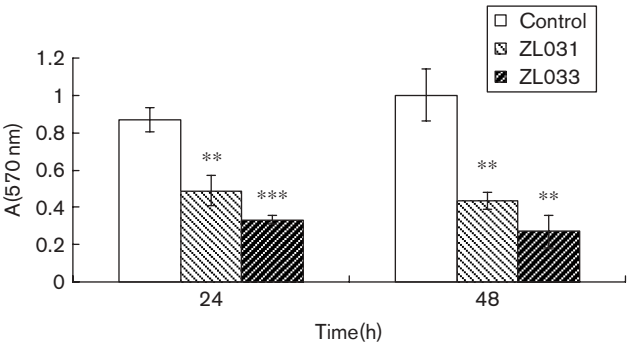


ZL031 and ZL033 inhibit HL-60 cell proliferation. HL-60 cells were exposed to increasing concentrations of ZL031 (1.25–20 μmol/l) or ZL033 (0.312–5 μmol/l) for 24 h. Then the MTT assay was performed as described. Data are given as the means of triplicate analyses. *P<0.05, **P<0.01, ***P<0.001 compared with control.
(a) Effect of ZL031 on HL-60 cells. (b) Effect of ZL033 on HL-60 cells.

Effect of ZL031 and ZL033 on cell cycle progression and related protein expression

As the inactivation of methionine synthase and the reduction of intracellular folate began in HL-60 cells, a notable change of cell cycle in HL-60 cells was observed (Tables 1, 2). The percentage of HL-60 cells in S phase was drastically increased compared with the control cells. The S phase cell accumulation started immediately after

Fig. 4



Time-dependent effect of ZL031 or ZL033 on HL-60 cell proliferation. 2.5 μmol/l ZL031 or ZL033 was used for 24 h or 48 h. Inhibition was determined by the MTT assay as described. Data are given as the means of triplicate analyses. **P<0.01, ***P<0.001 compared with control.

Table 1 Cell cycle distribution and apoptosis of HL-60 cells after being treated with ZL031 for 24 h

ZL031 concentration (μmol/l)	Cell cycle (%) G_1 (mean ± SD)	S (mean ± SD)	G_2/M (mean ± SD)	Apoptosis (%) (mean ± SD)
0	35.3 ± 2.7	48.9 ± 3.4	15.8 ± 0.9	4.5 ± 2.6
1.25	12.9 ± 8.7	76.4 ± 17.6	10.6 ± 9.2	4.9 ± 2.6
2.5	2.8 ± 3.2***	94.2 ± 4.6**	3.2 ± 5.6	6.8 ± 3.8
5	58.7 ± 6.5*	41.3 ± 6.5	0.01 ± 0.0**	11.0 ± 4.3*
10	65.0 ± 8.0*	35.0 ± 8.0*	0.02 ± 0.0**	18.4 ± 4.5*
20	73.0 ± 13.8*	26.2 ± 12.8	0.8 ± 1.4**	33.2 ± 6.8**

Cell cycle (%) and apoptosis (%) are the mean ± SD of three independent experiments.
*P<0.05, **P<0.01 compared with control, ***P<0.001 compared with control.

Table 2 Cell cycle distribution and apoptosis of HL-60 cells after being treated with ZL033 for 24 h

ZL033 concentration (μmol/l)	Cell cycle (%) G_1 (mean ± SD)	S (mean ± SD)	G_2/M (mean ± SD)	Apoptosis (%) (mean ± SD)
0	34.8 ± 1.2	50.4 ± 3.3	14.8 ± 2.0	7.1 ± 2.6
0.156	33.7 ± 2.7	52.1 ± 2.1	14.2 ± 3.3	7.0 ± 2.1
0.312	34.4 ± 2.0	58.1 ± 5.4	7.47 ± 4.9	14.0 ± 8.7
0.625	65.9 ± 7.8*	33.2 ± 8.9	0.87 ± 1.5*	42.7 ± 15.7*
1.25	78.9 ± 2.1**	20.6 ± 2.8*	0.48 ± 0.8*	54.8 ± 8.4**
2.5	82.5 ± 5.3**	17.0 ± 6.1*	0.49 ± 0.8*	59.7 ± 2.2**

Cell cycle (%) and apoptosis (%) are the mean ± SD of three independent experiments.
*P<0.05, **P<0.01 compared with control.

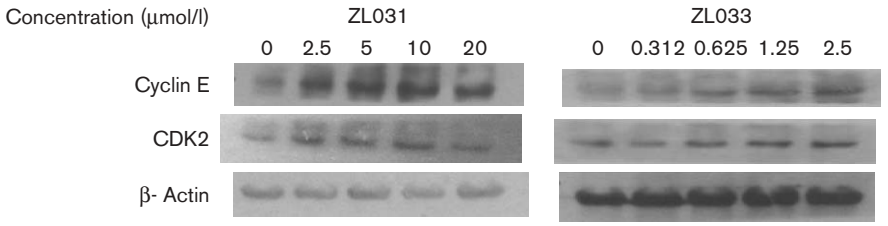
the 24 h incubation of cells with 1.25 μmol/l ZL031 or 0.312 μmol/l ZL033. When ZL031 at a concentration of 5 μmol/l or above and ZL033 at a concentration of 0.625 μmol/l or above were added to the cells, the cell cycle progression was converted to arrest in G1 phase accompanied by a decrease of S phase cells. Meanwhile, the percentage of HL-60 cells treated with either ZL031 or ZL033 in G_2/M phase was at a pronounced low level, which never exceeded the percentage of control cells. This result indicated that after treatment with the compounds, the cells were blocked in the early cell cycle progression and could not ever move on. Evidently, the ability of ZL033 to perturb cell cycle is noticeably stronger than ZL031, which is consistent with its stronger ability to inhibit methionine synthase activity and cell proliferation.

To determine the relationship between methionine synthase depression and cell cycle regulation, cyclin E and CDK2 were examined. As illustrated in Fig. 5, cyclin E and CDK2 were upregulated in a dose-dependent manner after treatment with either ZL031 or ZL033 for 24 h.

Induction of apoptosis by ZL031 and ZL033 and related proteins expression

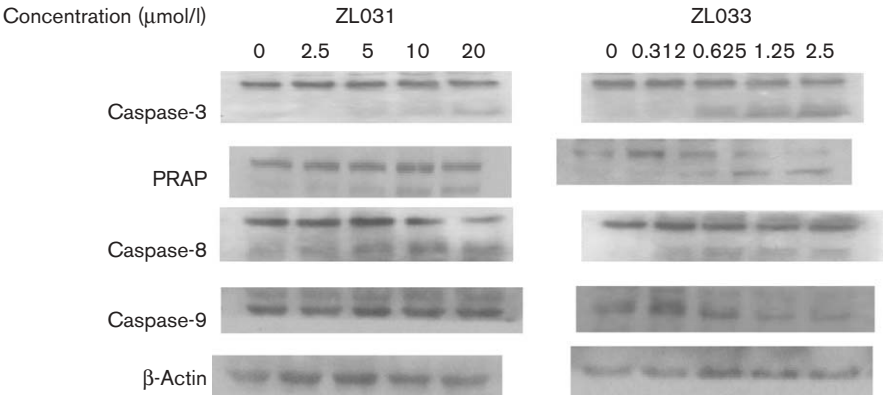
In addition to the disruption of methionine synthase activity and cell cycle progression, the compounds ZL031 and ZL033 simultaneously inducted apoptosis in a dose-dependent manner (Tables 1, 2), which was observed by flow cytometry using propidium iodide staining. To provide further evidence of apoptosis, we have initially examined the status of apoptosis-related proteins, caspase-3, and PARP, which were confirmed to be decreased accompanied by increased cleavage bonds of the proteins (Fig. 6). These findings suggested that the compounds ZL031 and ZL033 inducted apoptosis through a caspase-3 dependent pathway. No significant reduction of caspase-3 was observed until 20 μmol/l ZL031 was added to the cells for 24 h and a similar result was observed for caspase-8. On the other hand, the cleavage of PARP began to increase with 5 μmol/l of ZL031. After a 24-h exposure to ZL031, the level of caspase-9 remained almost the same, which indicated that the change in the level of caspase-8 most likely occurred before the change in caspase-9. The other

Fig. 5



Western blot analysis of cell cycle proteins cyclin E and CDK2 in HL-60 cells. HL-60 cells were treated with ZL031 or ZL033, respectively, for 24 h. Cellular lysate protein (50 μg/lane) was separated on a 12.5% SDS-PAGE and visualized by western blotting with specific antibodies against cyclin E and CDK2. CDK2, cyclin-dependent kinase 2.

Fig. 6



Induction of apoptosis and changes in related proteins in HL-60 cells. HL-60 cells were incubated with ZL031 or ZL033 for 24 h. Whole-cell lysates were extracted from cells and 50 μg protein was separated and electrophoretically blotted. Caspase-3, PARP, caspase-8, and caspase-9 were detected using specific antibodies. PARP, poly (ADP-ribose) polymerase.

compound ZL033 showed stronger effect on caspase-3, PARP, caspase-8, and caspase-9 after 24 h treatment. Caspase-3 and PARP were decreased at ZL033 concentrations greater than 0.625 μmol/l. Caspase-8 and caspase-9 were also gradually diminished as cells were treated with increasing concentrations of ZL033.

Discussion

Several enzymes involved in the folate cycle have been targeted for cancer chemotherapy, including dihydrofolate reductase and thymidylate synthase, and a large number of folate analogs have been developed [30,31]. In this project, we have focused on another enzyme related to folate metabolism, methionine synthase, and have investigated the anticancer pharmacological activity and possible mechanism.

ZL031 and ZL033 are analogs of the substrate 5-methyltetrahydrofolate and have been designed to suppress methionine synthase activity. They are expected to target the cobalt of the coenzyme cobalamin, the mechanism of which is different from the reported benzimidazoles. The interaction between the compounds and cobalamin is predicted to be a nucleophilic reaction, unlike the interaction between nitrous oxide or nitric oxide and cobalamin. Thereby, a stable covalent bond might be formed between the compounds and the enzyme, resulting in a long-term inactivation of the enzyme. Further studies to prove that the interaction is the same as predicted are in progress.

Methionine synthase is a promising target for cancer chemotherapy, which takes a critical position in de-novo biosynthesis of purines, DNA replication, and repair. To confirm the inhibitory effect of ZL031 and ZL033, we first investigated methionine synthase activity. The results showed that the activity of methionine synthase in HL-60 cells treated with ZL031 or ZL033 was significantly inhibited. Meanwhile, we observed antiproliferation effect of the compounds in HL-60 cells and found that ZL031 and ZL033 both caused proliferation inhibition in a dose-dependent and time-dependent manner. Interestingly, the inactivation of the enzyme occurred much earlier than the inhibition of cell proliferation, which might be because of the covalent bond between the compounds and the enzyme. Under long-term inactivation of methionine synthase and subsequent depletion of folate, cells undergo a series of physiological and pathological processes, which ultimately leads to proliferation inhibition.

Substantial research has confirmed that methionine synthase inactivation has some connection with folate deficiency [5] and the latter causes cell cycle dysregulation in many cases; nevertheless, little is known about cell cycle progression when intracellular folate is depleted as a result of methionine synthase inhibition. To address this question, we sought to further characterize the impact of ZL031 and ZL033 on cell cycle progression. Folate deficiency-induced cell cycle block is related to the disruption of two biochemical reactions that require folate coenzymes in pathways of DNA synthesis. One reaction is the methylation of deoxyuridylate to thymidylate in which a methylene group is supplied by the coenzyme 5,10-methylenetetrahydrofolate. In the other reaction, the coenzyme 10-formyltetrahydrofolate supplies two formate groups for the de-novo biosynthesis of purine. Folate deficiency is associated with DNA double-strand breaks, misincorporation of uracil into DNA, and impaired DNA repair and replication. It is reported that folate deficiency is always followed by cell cycle arrest in G_1 phase [32] and/or S phase [33,34], which is exactly consistent with our results. Moreover, the phenomenon that cell cycle is arrested specifically in G_1/S phase, meanwhile provides direct experimental proof that these two compounds are related to the enzymes involved in the folate cycle and eventually cause folate deficiency.

Although it is known that folate deficiency causes abnormalities in DNA synthesis and cell cycle arrest, little research has been done to explore the relationship between folate deficiency and the expression of cell cycle proteins. Cell cycle progression is delicately controlled and regulated by a number of CDKs and cyclins. The association of CDK2 with cyclin E is critical for initiation of the S phase and is required for centrosome duplication [35]. Decreased expression of cyclin E and CDK2 is frequently detected in many types of cancer [36–38], including the human leukemia HL-60 cell line [39], when cell progression is blocked in G_1 phase. In our research, HL-60 cells showed an obvious S-phase arrest after treatment with ZL031 or ZL033 in low concentrations and subsequently encountered G_1-phase arrest when treated with higher concentrations of the compounds, whereas cyclin E and CDK2 was continuously upregulated in a dose-concentration manner. It was probably because cell cycle arrest was mainly owing to the blockage of DNA synthesis caused by folate deficiency, which was before the abnormal expression of cell cycle proteins under the treatment of ZL031 or ZL033. Increased cyclin E and CDK2 were probably a result of a positive feedback of delayed G_1/S transition to promote cells into S phase. The lack of normal DNA, nevertheless, led more cells to stay in G_1 phase, despite elevated cyclin E/CDK2. These findings also implied that ZL031 and ZL033 targeted the folate cycle, but not the regulation of cell cycle-related proteins.

It is a promising strategy of cancer chemotherapy to induce apoptosis. Several lines of evidence indicate that folate depletion affects regulation of programmed cell death and induces apoptosis [32,40,41]. Furthermore, it has been observed in leukemia cell lines that apoptosis

occurred when cells were treated with N_2O followed by dysfunction of vitamin B_{12} and methionine synthase [42]. Thus, we studied the ability of ZL031 and ZL033 to induce apoptosis. The findings showed that methionine synthase activity was inhibited and apparent apoptosis occurred, which suggested that inactivation of methionine synthase might lead to apoptosis in HL-60 cells. However, under the condition of either direct or indirect folate deficiency, the specific apoptotic mechanism remains unclear and it is varied in different cell lines [43,44]. In accordance with previous reports [45], we found that the compounds ZL031 and ZL033 induced apoptosis in a caspase-3 dependent pathway resulting in increased cleavage of caspase-3 and PARP. Activation of caspase-3 occurs via two types of apoptosis, the death receptor-induced extrinsic pathway and the mitochondria apoptosome-mediated intrinsic pathway, with the respective activation of caspase-8 or caspase-9. Bid serves as a bridge between these two pathways. After being cleaved by caspase-8, truncated-Bid translocates to mitochondria and induces cytochrome c release [46–48]. With the decreased caspase-8 and slightly diminished caspase-9, our results indicated that apoptosis of HL-60 induced by compounds ZL031 and ZL033 was probably modulated by both death receptor pathway and mitochondria pathway. More drastic changes in caspase-8 level were observed than in caspase-9 at the same treatment concentration, which suggested that the extrinsic pathway through diminished caspase-8 might take priority in apoptosis caused by ZL031 and ZL033 in HL-60 cells. However, it is not clear whether caspase-8 induces the mitochondria pathway of apoptosis through Bid in the case of ZL031 or ZL033 treatment and further investigations are required.

The structural difference between ZL031 and ZL033 lies in their substituent group, ester or carboxylic acid. ZL031, with an ester substituent, easily gets hydrolyzed to form ZL033, thus it has a shorter half-life in culture medium than ZL033. However, physiological pH that is nearly neutral makes the hydrolytic reaction hardly completed. Both ZL031 and ZL033 might be present in the cytoplasm of ZL031-treated cells. The actual concentration of ZL031 might become lower during culture because of the instability of ZL031. As the substrate of the enzyme reaction, N^5-methyltetrahydrofolate, has a carboxylic acid substituent group, it is easier for ZL033, the synthetic compound with a carboxylic acid, to bind with the enzyme to suppress it owing to structural similarity. Hence, a better efficacy of ZL033 was observed. ZL031, with the hydrolysis product ZL033, has pharmacological efficacy similar to that of ZL033, whereas, a higher concentration of ZL031 is required to achieve the same effect of ZL033 in all the experiments we have performed. The pharmacological results corresponded well with the structural analysis.

Taken together, our results showed that ZL031 and ZL033 exerted significant inhibitory effect on cell proliferation, which might link to the inhibition of methionine synthase activity. Meanwhile, impaired methionine synthase also caused delay of cell cycle progression and induction of apoptosis, accompanied by changes of multiple-related proteins' expressions. Our results provided direct experimental evidences for anticancer activity of methionine synthase inhibitors. Further research *in vivo* would be necessary to determine whether these two new compounds are active anticancer agents. By comparing ZL031 and ZL033, the pharmacological results supported the chemical hypothesis, which confirmed that carboxylic acid substituent enhanced the anticancer activity of the compound *in vitro*. These results provide a fundamental basis for further studies on the structure-activity relationship of this series of compounds.

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