

ZGDHu-1 for cancer therapy (Review)

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Abstract. N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide (ZGDHu-1) is a novel tetrazine derivative that was initially designed and produced by Professor W.X. Hu, and which has been reported by our group to exhibit antitumor activity. Accumulating evidence suggests that the anticancer mechanisms of ZGDHu-1 may be involved indifferent biological activities, particularly in acute myeloid leukemia (AML) cells. At a high concentration, ZGDHu-1 has been demonstrated to inhibit the proliferation of the leukemia cells by arresting the cell cycle at the G₂/M phase, and by inducing cell apoptosis via inducing the accumulation of reactive oxygen species, the translocation of phosphatidylserine across the plasma membrane and the loss of mitochondrial membrane potential. Furthermore, at a low concentration, it was demonstrated to induce the differentiation and degrade the AML1-eight-twenty-one fusion protein in AML cells. Finally, results from a previous study indicate that ZGDHu-1 is a potential proteasome inhibitor. Overall, our preliminary research suggests that ZGDHu-1 may be a promising anticancer drug; however, further research is warranted to identify the exact drug target and potential clinical application in leukemia cells or solid tumors. In the present review, the application of ZGDHu-1 in cancer research, in addition to the specific underlying targets of ZGDHu-1, are discussed.

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1. Introduction

Leukemia is a malignancy of the hematopoietic stem cells that severely affects human health. Currently, the main therapies available for the treatment of leukemia include chemotherapy, bone marrow transplantation, allogeneic hematopoietic stem cell transplantation, immunotherapy and targeted drug therapy (1). Despite the advances made through laboratory and clinical research, the prognosis for patients with acute myeloid leukemia (AML) remains poor. Furthermore, ~50% of patients with AML are not suitable for induction chemotherapy to achieve complete remission, while a high proportion of patients who do receive induction therapy may achieve complete remission and subsequently relapse due to the presence of chemo-refractory cells. Therefore, the development of novel therapeutic strategies to treat patients with AML is urgently required (1-3).

Tetrazine is a compound that consists of a six-membered aromatic ring containing four nitrogen atoms, with the molecular formula C₂H₂N₄. Tetrazine has three isomers: 1,2,4,5-tetrazine, 1,2,3,4-tetrazine, and 1,2,3,5-tetrazine. Temozolomide, one of the imidazole tetrazines belonging to the 1,2,3,5-tetrazine group, has been approved by the Food and Drug Administration as a novel treatment for malignant glioma, and works by inhibiting the growth and proliferation of cancer cells (4).

Among the three isomers, 1,2,4,5-tetrazines are the most stable compounds, and 1,2,4,5-tetrazine derivatives have demonstrated potential therapeutic properties, such as anti-mite (5), herbicidal (6), anti-malarial (7), antiviral (6), anti-inflammatory (6), antibacterial (8) and antitumor activities (6,9). Furthermore, 1,2,4,5-tetramethyl-3,6-bis(phenylethynyl)-1,2,4,5-tetrazine was one of the first 1,2,4,5-tetrazine derivatives to be identified as possessing antitumor activity (6).

Thus, in order to identify other antitumor tetrazine compounds, based on the novel structure of 3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine (China Invention Patent

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Abbreviations: AML, acute myeloid leukemia; ZGDHu-1, N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide; $\Delta\Psi_m$, mitochondrial membrane potential; PS, phosphatidylserine; ROS, reactive oxygen species; CLL, chronic lymphocytic leukemia; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; A/E, AML1-ETO; ATRA, all-transretinoic acid; APL, acute promyelocytic leukemia

Key words: ZGDHu-1, tetrazine, leukemia, solid tumor

no. ZL98121915.2), dozens of different structural modifications of tetrazine compounds have been synthesized by Professor W.X. Hu (Pharmaceutical College of Zhejiang University of Technology, Hangzhou, China). Among these tetrazine compounds, the derivative N,N'-di-(*m*-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide (ZGDHu-1) was identified to exhibit the highest antitumor activity (6,10-12). Its structure is shown in Fig. 1 (13). It was demonstrated that the 3,6-dimethyl group is essential for antitumor activity, and that when the 3,6-dimethyl group was substituted for an ethyl or propyl group, the antitumor activity decreased significantly (11,12). In our previous studies, the antitumor activities of ZGDHu-1 were evaluated *in vitro* in different types of tumor cells (13-22), including monocyte leukemia, t(8;21) AML, M3 leukemia, chronic lymphocytic leukemia (CLL), EBC-1 lung carcinoma and PANC-1 pancreatic cancer, as well as a xenograft lung cancer mouse model (Table I). Among them, the antitumor activity of ZGDHu-1 was highest in Kasumi-1 cells, with a 50% inhibitory concentration (IC₅₀) of 0.3 μ M (22). Furthermore, it could induce the apoptosis and differentiation of Kasumi-1 cells at different concentrations (Fig. 2). Notably, our unpublished data demonstrates that ZGDHu-1 exhibits high antitumor activity with few adverse effects, and a 50% lethal dose (LD₅₀) of 5,000 mg/kg in mice.

To date, the biological activity of ZGDHu-1 has only been investigated by our group during the past 10 years (13-22). One important characteristic demonstrated by ZGDHu-1 is its ability to arrest tumor cells at the G₂/M phase of the cell cycle (Fig. 3) (16) and, in contrast to certain other chemical compounds, ZGDHu-1 is able to induce apoptosis and inhibit the proliferation of tumor cells at a high concentration, whereas low concentrations induce leukemia cell differentiation (13-21). Finally, it may be a proteasome inhibitor (22). Therefore, we recommend that other research groups worldwide perform further studies investigate this multi-target chemical compound to fully elucidate its antitumor activity and its potential clinical utility.

2. Anti-leukemic activity of ZGDHu-1

Monocyte leukemia

Anti-proliferation activity. Over-proliferation is an essential process of cancer cells, and uncontrolled cell division is considered a defining characteristic (23). The cell cycle is a complex process that ensures the controlled replication of cells, and it can be divided into four stages: G₁ phase (first gap); S phase (DNA synthesis); G₂ phase (second gap); and M phase (mitosis). In our previous studies, SHI-1 cells were selected as a suitable model for investigating monocyte leukemia *in vitro* (24), and the effect of ZGDHu-1 was evaluated in these cells using MTT assays. Our results demonstrated that ZGDHu-1 could inhibit SHI-1 cell proliferation in a time- and dose-dependent manner; the IC₅₀ values at 48 and 72 h were 250 and 85 ng/ml, respectively (17). Notably, the majority of SHI-1 cells were arrested at the G₂/M phase (17).

Induction of apoptosis. Apoptosis refers to programmed cell death, which serves an essential role in the regulation of cell growth, and is regarded as a natural barrier to cancer cell progression (25). Resistance to apoptosis may decrease

the sensitivity of cancer cells to diverse chemotherapy agents and induce multidrug resistance. Thus, apoptosis induction is considered to be an essential strategy for the elimination of cancer cells as part of cancer treatment (26). Apoptosis is classified into two major signaling pathways, termed the extracellular (extrinsic inducers) and intracellular (intrinsic inducers) signaling pathways (26).

In SHI-1 cells, ZGDHu-1 was observed to significantly induce the apoptosis in a time- and dose-dependent manner through increasing Bcl-2-associated X, apoptosis regulator (Bax), tumor protein p53 (p53) and Fas cell surface death receptor (Fas) gene expression, and decreasing B-cell lymphoma-2 (Bcl-2) expression (20). Additionally, ZGDHu-1 was demonstrated to increase the expression of mitochondrial membrane protein apo2.7 in a dose-dependent manner, while mitochondrial membrane potential ($\Delta\Psi$ m) was significantly reduced (20).

Induction of differentiation. Following treatment with a low concentration of ZGDHu-1, SHI-1 cells were observed to be reduced in size but with an enlarged cytoplasm, resulting in a decrease in the nucleus/cytoplasm ratio. Additionally, the cells possessed no nucleolus, and exhibited thickened condensed chromatin, a small amount of azurophilic particles increased in the cytoplasm, and a shift in the location of the nucleus to one side with lobules following treatment with ZGDHu-1. Furthermore, following culturing with 2-100 ng/ml ZGDHu-1 for 3 days, the morphology of SHI-1 cells matured, as indicated by a higher rate of staining with nitro blue tetrazolium chloride (NBT), a marker for functionally differentiated myeloid cells (17). In addition, CD11b, CD14 and CD64 were upregulated, and the effect on CD14 and CD64 expression levels was dose-dependent (17). Overall, these results indicate that a low concentration ZGDHu-1 can induce SHI-1 cells to differentiate into mature monocytes.

t(8;21) acute myeloid leukemia

Anti-proliferative activity. Presently, aggressive cytosine arabinoside-based chemotherapy is the standard treatment for t(8;21) AML. However, clinical observations have demonstrated that the 5-year survival rate of patients with AML is <40% (27). Thus, the development of novel therapies is required for the treatment of patients with t(8;21) AML (27). To date, investigations of the effects of ZGDHu-1 on t(8;21) AML cells have comprised the most extensive study of ZGDHu-1 by our group (Figs. 2 and 3). Our previous study demonstrated that ZGDHu-1 could inhibit the proliferation of Kasumi-1 cells in a time- and dose-dependent manner, and the IC₅₀ values at 48 and 72 h were 450 and 300 ng/ml, respectively (22).

Arrest of the cell cycle at the G₂/M phase. The cell cycle is strictly regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) (28). Furthermore, the uncontrolled proliferation that is characteristic of human cancer cells has been associated with the dysregulation of CDKs/cyclins (28). Hyperactivation of CDK activity confers a cell growth advantage, while inactivation of tumor suppressor genes, checkpoint regulators or CKIs results in dysregulation of the cell cycle (29). Among these regulators, the CDK1 (cdc2)/cyclin B complex is a major

Table I. Effects of ZGDHu-1 on different cancer types.

Cancer type	Type of study	Molecular targets	Functions	(Refs.)
Monocyte leukemia	<i>In vitro</i>	Bax, p53, Fas, Bcl-2, $\Delta\Psi_m$	Anti-proliferative activity, induction of apoptosis, <i>in vitro</i> differentiation	(13,16)
t(8;21) acute myeloid leukemia	<i>In vitro</i>	Apo 2.7, $\Delta\Psi_m$, cyclin B1, cdc25c, CHK1, I κ B, β_5 , β_5i	Anti-proliferative activity, induction of apoptosis, inactivated NF- κ B, <i>in vitro</i> differentiation, degradation of AML-ETO, proteasome inhibition	(12,18)
Acute promyelocytic leukemia	<i>In vitro</i>	Bax, phospho-p38 MAPK	Anti-proliferative activity, induction of apoptosis, <i>in vitro</i> differentiation	(14)
Chronic lymphocytic leukemia	<i>In vitro</i>	$\Delta\Psi_m$, Bcl-2, ROS, caspase-3	Anti-proliferative activity, induction of apoptosis,	(9,10)
Pancreatic cancer	<i>In vitro</i>	I κ B, CHK1, cyclinB1, cdc2 (CDK1), Bcl-2, caspase-3, PARP	Anti-proliferative activity, induction of apoptosis	(11)
Lung cancer	<i>In vitro/ in vivo</i>	Bax, p53 and Fas, caspase-3	Anti-proliferative activity, induction of apoptosis	(15,17)

ZGDHu-1, N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial transmembrane potential; I κ B, inhibitor of NF- κ B; cdc25c, cell division cycle 25C; CHK1, checkpoint kinase 1; Bcl-2, B-cell lymphoma-2; MAPK, mitogen-activated protein kinase; PARP, poly ADP-ribose polymerase; Fas, Fas cell surface death receptor; p53, tumor protein 53; Bax, Bcl-2-associated X, apoptosis regulator.

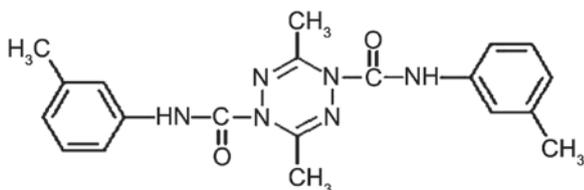


Figure 1. Molecular structure of N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide. Taken from Qiu *et al* (13).

determinant of early M phase progression, and the combination of cyclin B1 and CDK1 is essential for eukaryotic cells entering mitosis (30). Additionally, cell division cycle (cdc) 25c is a protein phosphatase that is responsible for activating and dephosphorylating CDK1 (30). At the end of this signaling cascade, CDK1/cyclin B activity is attenuated, thereby blocking entry into mitosis. Thus, inhibition of CDK1/cyclin B is achieved by the downregulation of cdc25 phosphatase and upregulation of WEE1 G2 checkpoint kinase, which together results in the accumulation of inhibitory phosphate groups on tyrosine 15 of CDK1. This checkpoint activation results in cell cycle arrest at the G₂/M phase (29).

In our study, it was revealed that treatment of Kasumi-1 cells with ZGDHu-1 results in significant dysregulation of G₂/M regulatory molecules, including the downregulation of cyclin B1, CDK1 and cdc25c, and the upregulation of checkpoint kinase 1 (CHK1), phospho-CHK1, phospho-cdc25c, phospho-p53, p27 and p53 (16). Additionally, pretreatment with a selective CHK1 inhibitor, CHIR-124, significantly abrogated G₂/M arrest via ZGDHu-1. Overall, this study indicates

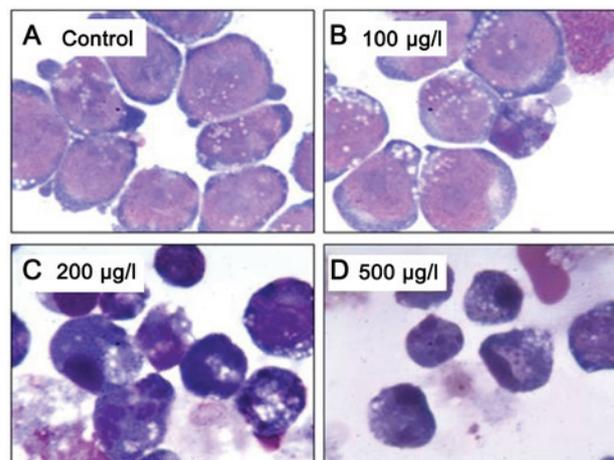


Figure 2. Wright-Giemsa staining of Kasumi-1 cells following treatment with (A) dimethyl sulfoxide (control), or ZGDHu-1 at concentrations of (B) 100, (C) 200 and (D) 500 μ g/l for 48 h (magnification, x1,000). The images illustrate that apoptotic bodies among the Kasumi-1 cells were increased with higher concentrations of ZGDHu-1, particularly at 200 and 500 μ g/l. By contrast, at 100 μ g/l ZGDHu-1, no nucleolus, chromatin condensation thickening, a small amount of azurophilic particles increased in the cytoplasm, and a shift in the location of the nucleus to one side with lobules were observed. ZGDHu-1, N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide.

that ZGDHu-1 could arrest t(8;21) AML cells at the G₂ phase through G₂/M checkpoint-associated CDKs and CKIs.

Induction of apoptosis. The mitochondrial signaling pathway serves an essential role in intracellular apoptosis. In ZGDHu-1-treated SHI-1 cells (20), changes in Apo 2.7 and $\Delta\Psi_m$

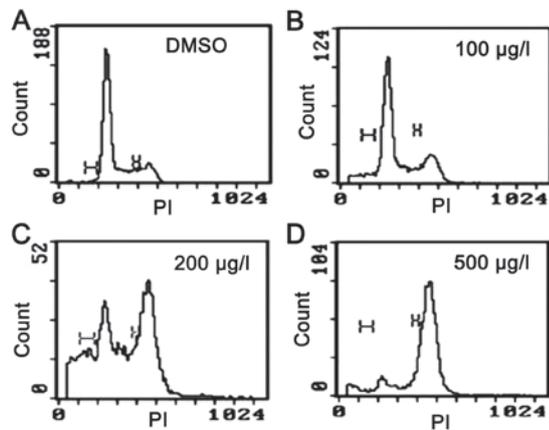


Figure 3. Cell cycle effects on Kasumi-1 cells following treatment with (A) DMSO (control), or ZGDHu-1 at (B) 100, (C) 200 and (D) 500 $\mu\text{g/l}$ for 48 h, as reported in Xia *et al.* (16). The cell cycle distribution was analyzed using a fluorescence-activated cell sorting machine, and the results demonstrated that cells were accumulated in the G_2/M phase following treatment with 200 and 500 $\mu\text{g/l}$ ZGDHu-1. DMSO, dimethyl sulfoxide; ZGDHu-1, N,N'-di-(m-methylphenyl)-3,6-dimethyl-1,4-dihydro-1,2,4,5-tetrazine-1,4-dicarboamide; PI, propidium iodide.

indicated that the integrity of the mitochondrial membrane was destroyed. Additionally, dysregulation of Bcl-2-associated agonist of cell death, Bax and Bcl-2 expression further supports the notion that ZGDHu-1 induces apoptosis through the mitochondrial signaling pathway (16). ZGDHu-1 was identified to be able to decrease caspase-3 expression and markedly increase cleaved caspase-3 expression in a dose-dependent manner, and cleaved fragments of poly ADP-ribose polymerase (PARP) were observed, which indicates that caspase-3 is activated by ZGDHu-1 treatment (16).

Inactivation of nuclear factor κB (NF- κB) over-activity. Over the last decade, studies have reported that constitutive activation of NF- κB may be observed in AML. NF- κB serves an important role in apoptosis and survival of leukemia cells, which is also dependent on Bcl-2 and Bcl-extra large (31,32). In our preliminary study, the results suggested that the expression pattern of NF- κB was significantly dysregulated by ZGDHu-1 treatment (16).

Induction of differentiation and the AML1-ETO (A/E) fusion gene target. ZGDHu-1-treated Kasumi-1 cells demonstrated reduced cell size and an increased amount of cytoplasm, resulting in the decrease in the nucleus/cytoplasm ratio. Additionally, the cells exhibited no nucleolus, chromatin condensation thickening, a small amount of azurophilic particles increased in the cytoplasm, and a shift in the location of the nucleus to one side with lobules following ZGDHu-1 treatment. Furthermore, ZGDHu-1-treated Kasumi-1 cells exhibited a significant reduction in NBT staining. Similarly to all-transretinoic acid (ATRA) treatment, ZGDHu-1 increased the percentage of CD11b⁺ and CD13⁺ cells, partially supporting the notion that ZGDHu-1 induces the differentiation of Kasumi-1 cells (22).

The A/E oncoprotein can inhibit the differentiation and enhance the self-renewal of hematopoietic stem cells, which are essential features of Kasumi-1 cells and serve a major role

in the progression of AML disease (33). In our previous study, A/E oncoprotein expression was significantly dysregulated when treated with different concentrations of ZGDHu-1 (16). The results demonstrated that A/E oncoproteins were not altered at the mRNA level, but were degraded at the protein level (16), indicating that ZGDHu-1 exerts its effect on the A/E fusion protein through a post-translational signaling pathway or other unknown mechanism.

Inhibition of proteasome activity. The ubiquitin-proteasome signaling pathway is responsible for the degradation of mutated and misfolded proteins, which involves two essential steps: The attachment of multiple ubiquitin molecules to a protein substrate, and the degradation of the tagged substrate by the 26S proteasome (34). The 26S proteasome contains a 20S catalytic core proteasome and two 19S regulatory subunits, which serve as recognition sites for proteolysis (35). The 20S core is composed of a total of 28 subunits, comprising 14 α and 14 β subunits (33).

Currently, bortezomib is the only proteasomal inhibitor for the treatment of patients with multiple myeloma or mantle cell lymphoma (36), wherein it acts as a reversible inhibitor of the 26S proteasome (37). Bortezomib can bind to the active site of the β_5 -subunit proteasome and inhibit the chymotrypsin-like activity, which is essential for cell death-inducing capability (38). Numerous important proteasome target proteins have been demonstrated to be affected, including cyclins (39,40), p53 (41), the retinoblastoma (Rb) family (42), pro-apoptotic Bax (43), CKIp27 (44), and NF- κB inhibitor α (45). In our preliminary study in Kasumi-1 cells, it was revealed that ZGDHu-1 could significantly decrease the protein expression of the β_5 and β_5 isubunits of the 20S proteasome, and partially decrease the expression of the β_1 and β_1 isubunits, but not the β_2 and β_2 isubunits (22). However, further studies are warranted in order to elucidate the association between the apoptotic effects of ZGDHu-1 as a proteasome inhibitor and the underlying signaling pathway involved.

Acute promyelocytic (M3) leukemia (APL)

Anti-proliferative activity and induction of apoptosis. AML is a heterogeneous and aggressive disease, which is characterized by the rapid growth of abnormal white blood cells in the bone marrow. Inhibition of cellular differentiation at specific stages during their development is the most prominent characteristic of AML (46). Since the success of ATRA in the treatment of APL, differentiation therapy has been regarded as a promising method for AML treatment (47), and the investigations of APL has benefited from ATRA-maturation sensitive and resistant cell lines (NB4 and UF-1) derived from leukemia cells from a patient with APL (48).

The results of our previous study revealed that ZGDHu-1 could inhibit NB4 cell proliferation; the IC_{50} values were 450 ng/ml (48 h) and 200 ng/ml (72 h) (18). Notably, the majority of NB4 cells were also arrested at G_2/M phase, and phospho-p38 and Bax expression were increased while Bcl-2 and phospho-STAT3 were unchanged when treated with ZGDHu-1 (18). Furthermore, the effect of the ZGDHu-1 on the apoptosis of NB4 cells was time- and dose-dependent, and the apoptotic effect of 100 ng/ml ZGDHu-1 on NB4 cells

was similar to the effect of 10 $\mu\text{g/ml}$ ATRA, implying that ZGDHu-1 exerts a stronger apoptotic effect compared with ATRA (18). Additionally, another APL cell line, HL-60, was also investigated. The IC_{50} values at 48 and 72 h were both 180 ng/ml, and the majority of HL-60 cells were also arrested at G_2/M phase (18).

Induction of differentiation. At a low concentration (2-100 ng/ml) of ZGDHu-1, NB4 cells exhibit more mature features after 3 days, with higher NBT positivity and CD11b and CD13 expression compared with the untreated control groups (18). In HL-60 cells, the expression levels of CD11b, CD13, CD14 and CD64 were demonstrated to be significantly upregulated following ZGDHu-1 treatment, which suggests that ZGDHu-1 induces the differentiation of Kasumi-1 cells into mature granulocytes or monocytes.

CLL. CLL is a type of cancer in which the bone marrow produces an excessive amount of lymphocytes. Combined regimens, such as fludarabine, cyclophosphamide and rituximab, have become the standard treatment for patients with CLL. However, the majority of patients with CLL are elderly and not all patients are eligible for aggressive chemoimmunotherapy (49). CLL cells are arrested at G_0/G_1 and cannot overcome the differentiation hurdle (50). Notably, impaired cell death, rather than excessive proliferation, is regarded as essential for the accumulation of CLL cells and their resistance to chemotherapy (50).

Additionally, abnormal apoptosis was demonstrated to be associated with the clinical progression of patients with CLL (51). The activated survival signaling pathways, such as the phosphoinositide 3-kinase/Akt or NF- κB pathways in CLL cells, upregulate important anti-apoptotic Bcl-2 family members, leading to chemotherapy resistance and disease progression (52,53). Furthermore, higher Bcl-2/Bax or Mcl-1/Bax ratios indicate a resistance to fludarabine and significantly shorter survival time in patients with CLL (50,54-57). Recently, ABT-199 has demonstrated the most promising clinical results of all the putative agents targeting Bcl-2 (58).

In our previous study, ZGDHu-1 dose-dependently inhibited the viability of primary CLL cells. However, this effect was not identified in the peripheral B cells of healthy people at the same concentrations, indicating that the cytotoxic effects of ZGDHu-1 are specific to CLL cells (13).

Induction of apoptosis. The results of a previous study suggest that ZGDHu-1 induces the apoptosis of malignant B lymphocytes of patients with CLL, but cannot induce the apoptosis of healthy B lymphocytes (13). This indicates that the pro-apoptotic activity of ZGDHu-1 is specifically against CLL cells, and the apoptotic effect was partially dependent on a loss of $\Delta\Psi\text{m}$, phosphatidylserine (PS) translocation across the plasma membrane, and reactive oxygen species (ROS) accumulation. Lastly, ZGDHu-1 has been demonstrated to significantly induce caspase-3 cleavage and decrease anti-apoptotic Bcl-2 expression, without affecting Bax expression (13).

ZGDHu-1 and fludarabine exert a synergistic effect on the apoptosis of CLL cells. Currently, fludarabine is widely used for the treatment of patients with CLL (59,60). It has been

demonstrated to serve multiple functions, such as interference with DNA synthesis and repair, apoptosis induction and cell cycle regulation in leukemia cells (60). However, toxic effects of fludarabine, such as severe opportunistic infections, myelosuppression and gastrointestinal toxicities (including vomiting, nausea and hepatic lesions) have been reported (60). Thus, reducing the toxicity of fludarabine by reducing its dose and the identification of novel candidate drugs are warranted. In our study, it was revealed that ZGDHu-1 and fludarabine had a synergistic effect on the cytotoxicity and apoptosis of CLL cells, and this effect was also dependent on the loss of $\Delta\Psi\text{m}$, PS translocation and ROS accumulation (14). The combination also significantly induced the cleavage of caspase-3 and significantly decreased anti-apoptotic Bcl-2 expression in CLL cells (14).

Therefore, the combination of ZGDHu-1 and fludarabine may be useful for the maintenance therapy of patients with CLL, as it can sensitize CLL cells to low doses of fludarabine without increasing the risk of long-term side effects on the immune system or other opportunistic infections (14). However, numerous important questions remain unresolved regarding what type of biomarkers that distinguish lymphoid malignancies will respond to ZGDHu-1 as a monotherapy or combination with other chemotherapy drugs, and whether relapsed or refractory patients with CLL are sensitive to the ZGDHu-1. In combination settings, the highest priority question must be to determine whether ZGDHu-1 can synergize with cytotoxic agents to overcome chemoresistance.

3. Anti-solid tumor activity of ZGDHu-1

Pancreatic cancer. Pancreatic cancer, which is characterized by early metastasis, late diagnosis, high mortality rate and <5% 5-year survival rate, is regarded as 'the king of cancer' (61). Previously, using the MTT method, it was identified that ZGDHu-1 suppressed the proliferation of PANC-1 cells in a time- and dose-dependent manner; the IC_{50} values at 48 and 72 h were 295 and 150 ng/ml, respectively (15). Furthermore, ZGDHu-1 could arrest cells at the G_2/M phase and induce the apoptosis of PANC-1 cells in a dose-dependent manner. Additionally, ZGDHu-1 was demonstrated to upregulate Bax expression and downregulate Bcl-2 expression, also activating pro-caspase-3 and PARP. Cyclin B1 and CDK1 expression levels were identified to be decreased whereas CHK1 expression was increased, all of which are G_2/M regulatory molecules (15). Overall, our research revealed that ZGDHu-1 could effectively suppress cell proliferation and induce the apoptosis of PANC-1 cells. However, further *in vivo* models are required to validate the efficacious antitumor activities of ZGDHu-1 described.

Lung cancer

Anti-proliferative activity and induction of apoptosis. ZGDHu-1 was demonstrated to inhibit cell proliferation and induce apoptosis in human lung carcinoma EBC-1 cells (21). ZGDHu-1 inhibited EBC-1 cell proliferation with an IC_{50} at 24 h of 295 ng/ml, at 48 h of 112 ng/ml and at 72 h of 23 ng/ml. Bax, p53 and Fas expression were significantly increased, whereas Bcl-2 expression was unaltered and caspase-3 expression was significantly decreased following treatment with ZGDHu-1 (21).

Induction of apoptosis of A549 cells in vitro and antitumor activity in vivo. In a previous study, ZGDHu-1 was demonstrated to inhibit A549 cell proliferation, and the majority of A549 cells were arrested at the G₂/M phase. Bax, Bax/Bcl-2 and p53 expression levels were increased significantly, with a marked decrease in Bcl-2 expression (19). Additionally, ZGDHu-1 was revealed to induce tumor cell apoptosis *in vitro* and significantly suppress the growth of a A549 xenograft tumors *in vivo* (19). When the xenograft tumor mouse models were treated with 10, 20 and 40 mg/kg ZGDHu-1 for 14 days, the tumor growth inhibition rates were 43.7, 56.9 and 60.0%, respectively (19). However, the efficacy of ZGDHu-1 has only been investigated in the A549 lung cancer xenograft mouse model, and further studies are warranted to elucidate the direct and cellular targets of the ZGDHu-1 *in vivo*. Similar to temozolomide, one of the imidazole tetrazines, which is now an orally administered alkylating agent widely used as a novel treatment for malignant glioma, ZGDHu-1 has demonstrated promising anticancer results.

4. Therapeutic perspectives and conclusions

The biological activity of ZGDHu-1 has only been investigated by our group for the past 10 years (1-14). So far, the most notable characteristic of ZGDHu-1 is its ability to arrest all tumor cells at the G₂/M phase; however, caspase-dependent and proteasome inhibitory activity has also been reported. Until now, the ZGDHu-1 appears to be an efficacious drug on tumor cell lines *in vitro*; however, its antitumor effect *in vivo* has only been investigated in the lung cancer animal model. The effect of ZGDHu-1 in other solid tumor cells and leukemia animal models are still under investigation as ZGDHu-1 is difficult to synthesize. Notably, the IC₅₀ of ZGDHu-1 in Kasumi-1 leukemia cells was 0.3 μM, which was the lowest among all other investigated tumor cells, and the drug had few adverse effects, with an LD₅₀ of 5,000 mg/kg in the mouse model. However, after 10 years of research, the exact target of ZGDHu-1 in the tumor cells remains unclear. The arrest of all tumor cells at the G₂/M phase and induction of leukemia cell differentiation at low concentrations are just some of the effects of ZGDHu-1. Further investigations into the direct or cellular targets of ZGDHu-1 are warranted. Ultimately, novel targets will be identified and may hold great promise for clinical cancer therapy.

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