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Research paper

Design and synthesis of novel pyrazolo[1,5-a]pyrimidine derivatives bearing nitrogen mustard moiety and evaluation of their antitumor activity *in vitro* and *in vivo*

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ABSTRACT

A series of novel pyrazolo[1,5-a]pyrimidine derivatives bearing nitrogen mustard moiety were designed, synthesized and evaluated for their antiproliferative activities against five human cancer cell lines (A549, SH-SY5Y, HepG2, MCF-7 and DU145) *in vitro*. Among these compounds, **13b** exhibited potent inhibitory effect on the proliferation of the five tumor cells and was able to inhibit cell cycle arrest at G1 phase and induce cell apoptosis. In HepG2 HCC xenograft compound **13b** was selected for evaluating the antitumor activity *in vivo* which exhibited significant cancer growth inhibition with low host toxicity *in vivo*.

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1. Introduction

It is widely known that cancer is one leading cause of death in developed countries that may be induced by a plethora of both external and internal factors [1]. Among the anticancer drugs, Nitrogen mustard bifunctional DNA-alkylating agents which were widely used in clinical could cause DNA damage by interrupting DNA biosynthesis and in doing so caused DNA damage [2]. Many of these drugs, such as Melphalan, Chlorambucil, Cyclophosphamide and Bendamustine and so on, have revolutionized the treatment of cancers. However, these derivatives have many drawbacks, including a low specificity to target the DNA of tumor cells, an eventual loss in activity due to cellular DNA repair mechanisms, a high chemical reactivity and induced bone-marrow toxicity [3–6]. To conquer these problems of nitrogen mustards, one of the effective strategies is to link the alkylating pharmacophore with DNA-affinic molecules. To this end, numerous affinity tumor heterocyclic have been designed as carriers to transport the alkylating pharmacophore into the tumor site [7–11]. Emerging evidence shows that connecting DNA-affinic molecules to alkylating agents could improve therapeutic efficacy than the corresponding untargeted alkylating agents.

Cyclin-dependent protein kinases (CDKs) are serine/threonine kinases which are central to the appropriate regulation of cell proliferation, apoptosis, and gene expression [12–14]. Most human cancers have abnormalities in some component of CDK activity, hence, synthetic inhibitors of CDK activity present as a logical approach in the development of new cancer therapies. Pragmatically, most efforts have focused on using CDK_2 as a template to achieve the absolute selectivity, given its ready applicability to protein structure guided drug design [15]. Pyrazolo[1,5-a]pyrimidine derivatives are widely used as inhibitors of Cyclin-dependent kinase 2 [16–19], and are also found may demonstrate antitumor effect in various cancer cell lines [20–22] and other biologically

Abbreviations: MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; A549, human alveolar adenocarcinoma cell line; SH-SY5Y, human neuroblastoma cell line; HepG2, human liver carcinoma cell line; MCF-7, human breast cancer cell line; DU145, human prostate cancer cell lines; GES-1, human gastric epithelial cell line; FBS, Fetal Bovine Serum; PBS, Phosphate Buffer solution; PI, propidium iodide; RNase A, Ribonuclease A.

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activities [23–27]. Herein, we choose pyrazolo[1,5-a]pyrimidine derivatives as the carrier to selectively transport the alkylating pharmacophore into the tumor site. We coupled the aniline N-mustard residue with the pyrazolo[1,5-a]pyrimidine derivatives to afford 43 new compounds. The biological results showed that compounds **4-13a** and **4-13b** exhibited promising antitumor activity against five human tumor cells *in vitro*. In addition, cell cycle analysis and cellular apoptosis experiment indicated the excellent therapeutic efficacy of compound **13b** at the cell level. To evaluate the toxicity and *in vivo* targeting therapeutic efficacy of compound **13b**, we conducted the acute toxicity test and *in vivo* therapeutic experiment.

2. Results and discussion

2.1. Chemistry

The synthetic pathway for target compounds **4-15a**, **4-15b** and **18a-s** was illustrated in Schemes 1 and 2. 5-chloromethyl-7-chloro-3-cyanopyrazolo[1,5-a]pyrimidine (**1**) and N^1,N^1-bis(2-chloroethyl) benzene diamine **(2a-b)** were prepared by following the literature methods [28–30].

Treatment of compound **1** with N^1,N^1-bis(2-chloroethyl)benzene diamine **(2a-b)** in ethanol under reflux at nitrogen atmosphere for 2–3 h gave compounds **3a-b** in good yields. Compounds **3a-b** were then converted into target compounds **4-15a** and **4-15b** by nucleophilic substitution reaction using different amines. Compounds **16a-s** were obtained in high yield using the method similar to compounds **3a-b**. The synthesis of the key intermediates **17a-s** was achieved from compounds **16a-s** with diethanolamine in the presence potassium carbonate in N,N-Dimethylformamide at 40 °C for 2–4 h. Chlorination of the intermediates **17a-s** with thionyl chloride in dichloromethane at room temperature overnight yielded the target compounds **18a-s**.

2.2. Pharmacology

2.2.1. MTT assay

Using Sorafenib and Melphalan as the positive control, the cytotoxic activities of all target compounds **4-15a**, **4-15b** and **18a-s** have been evaluated against the A549, SH-SY5Y, HepG-2, MCF-7, DU145 cell lines using the MTT assay [31,32]. The results expressed as IC_{50} values are shown in Table 1. Compounds **4-13a** and **4-13b** showed excellent cytotoxic activity against different cancer cells, in which compounds **4-13b** and **13a** were more potent than Sorafenib and Melphalan against one or more cell lines. For example, compounds **4-5b**, **7-9b** **11-13b**, **9a** and **13a** showed stronger anti-proliferative activities against HepG-2 cell line. Compounds **4-5b**, **7-10b** and **13a-b** exhibited more promising activities against SH-SY5Y cell line. For A549 cell line, compound **9b** showed the strongest activity with the IC_{50} values of 5.122 uM. The IC_{50} values of compounds **4b** and **13b** against MCF-7 cell line were 6.538 and 6.138 uM, respectively. DU145 cells were negligibly affected by most of the compounds, with only the compounds **9b** and **13b** inducing a significant effect. However, compounds **14-15a** and **14-15b** were totally ineffective against all cancer cell lines. Analysis of compounds **4-13a** and **4-13b** revealed that the nitrogen mustard group in the para position of the benzene ring contributes more to enhancement of potency than that in the meta position. The anti-proliferative activities of derivatives **18a-s**, in which the nitrogen mustard pharmacophore was in the 5-position of the pyrazolo[1,5-a]pyrimidine ring, were almost ineffective against all the five cancer cell lines. The IC_{50} values of the most promising compound **13b** were 6.023, 0.217, 6.318, 8.317 and 6.82 uM against the A549, SH-SY5Y, HepG-2, MCF-7, DU145 cell lines, respectively, indicating that this compound was more active than Sorafenib and Melphalan. Human gastric epithelial cell line (GES-1) was treated with the potent compounds (those showing the higher cytotoxicity) and the clinical drugs Sorafenib and Melphalan to evaluate the cytotoxicity to normal cells. The result is shown in Table 2. Cytotoxicity of **4b**, **8b**, **9b** and **13b** against GES-1 was comparable to that of Sorafenib. Compounds **5b**, **6b**, **10b** and **12b** possessed smaller cytotoxicity than Sorafenib. Base on the above results, compound **13b** was selected for further study *in vitro* and *in vivo*.

2.2.2. Cell cycle inhibition

To investigate the effect of compound **13b** on cell cycle progression, cellular DNA content was measured by flow cytometry. Human liver carcinoma HepG2 cells were treated with compound **13b** at the concentrations of 1 and 3 uM for 24 h. Thereafter, the cells were collected and washed with ice-cold PBS twice, then fixed in 70% ice-cold ethanol at least for 24 h. After that, the cells were resuspended in ice-cold PBS into which RNase A was added. This solution was maintained for about 30 min. The cells were stained with propidium iodide (PI) and analyzed with a flow cytometer. The percentages of HepG2 cells in G1, S, and G2/M phases were calculated and the results were presented in Fig. 1. Cell cycle analysis showed that compound **13b** accumulated the cells at G1 phase after treatment with HepG2 for 24 h.

2.2.3. Cell apoptosis

An Annexin V-FITC&PI Apoptosis Detection Kit was used to evaluate the efficacy of compound **13b** on HepG2 tumor cell line. The result is shown in Fig. 2. The upper right section represents the cells which were in the late apoptotic or necrotic cells, and the lower right section represents the early or middle apoptosis. Compared with the control, compound **13b** increased the apoptosis percentage of HepG2 whatever the late apoptotic or the early apoptotic cells after 24 h. The results confirmed that compound **13b** could induce cell apoptosis.

2.2.4. In vivo therapeutic activity

Based on the *in vitro* cytotoxicity, Compound **13b** was selected for evaluating the antitumor therapeutic efficacy *in vivo*. The HepG2 HCC xenograft models were generated by injecting HepG2 cell in the left flank of each female nude mouse. Compound **13b** was administered *via* intraperitoneal injection every day for 15 times at the dose of 10 mg/kg and 20 mg/kg body weight. Positive control Sorafenib, Cyclophosphamide and negative control vehicle were administered in the same way as compound **13b**. The result is shown in Fig. 3. Compound **13b** showed significant tumor growth inhibition (43.88% and 58.26%, respectively) in comparison to negative control at the doses of 10 mg/kg and 20 mg/kg. The inhibition of compound **13b** was a slight lower than the positive control Sorafenib (47.90%), but higher than cyclophosphamide (42.11%) at 10 mg/kg body weight. When the dosage of compound **13b** was 20 mg/kg, the inhibition was better than the positive control Sorafenib (54.93%) and Cyclophosphamide (51.48%) at the same dose level. On the other hand, compound **13b** induced a 7.15% body-weight change at 10 mg/kg dose level during the treatment that similar to Cyclophosphamide (7.04%). Although compound **13b** induced a 9.21% body-weight change at 20 mg/kg body weight, it was better than the positive control Cyclophosphamide (10.63%). The result is shown in Fig. 4. The body-weight of mice was readily recovered after cession of treatment. Therefore, we could conclude that compound **13b** has an effective therapy *in vivo* for HepG2 HCC tumors and relatively low toxicity to the hosts.

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