

Original Article

Structural basis for 18- β -glycyrrhetic acid as a novel non-GSH analog glyoxalase I inhibitor

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Aim: Glyoxalase I (GLOI), a glutathione (GSH)-dependent enzyme, is overexpressed in tumor cells and related to multi-drug resistance in chemotherapy, making GLOI inhibitors as potential anti-tumor agents. But the most studied GSH analogs exhibit poor pharmacokinetic properties. The aim of this study was to discover novel non-GSH analog GLOI inhibitors and analyze their binding mechanisms.

Methods: Mouse GLOI (mGLOI) was expressed in BL21 (DE3) pLysS after induction with isopropyl- β -D-1-thiogalactopyranoside and purified using AKTA FPLC system. An *in vitro* mGLOI enzyme assay was used to screen a small pool of compounds containing carboxyl groups. Crystal structure of the mGLOI-inhibitor complex was determined at 2.3 Å resolution. Molecular docking study was performed using Discovery Studio 2.5 software package.

Results: A natural compound 18- β -glycyrrhetic acid (GA) and its derivative carbenoxolone were identified as potent competitive non-GSH analog mGLOI inhibitors with K_i values of 0.29 μ mol/L and 0.93 μ mol/L, respectively. Four pentacyclic triterpenes (ursolic acid, oleanolic acid, betulinic acid and tripterine) showed weak activities (mGLOI inhibition ratio <25% at 10 μ mol/L) and other three (maslinic acid, corosolic acid and madecassic acid) were inactive. The crystal structure of the mGLOI-GA complex showed that the carboxyl group of GA mimicked the γ -glutamyl residue of GSH by hydrogen bonding to the glutamyl sites (residues Arg38B, Asn104B and Arg123A) in the GSH binding site of mGLOI. The extensive van der Waals interactions between GA and the surrounding residues also contributed greatly to the binding of GA and mGLOI.

Conclusion: This work demonstrates a carboxyl group to be an important functional feature of non-GSH analog GLOI inhibitors.

Keywords: glyoxalase I; 18- β -glycyrrhetic acid; carbenoxolone; pentacyclic triterpenes; GSH binding site; crystal structure; molecular docking

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Introduction

Glyoxalase I (GLOI, EC 4.4.1.5) is the first and the rate-limiting glutathione (GSH)-dependent enzyme in the glyoxalase system, which functions as a ubiquitous detoxification pathway by catalyzing the conversion of toxic α -oxoaldehydes to non-toxic *D*-lactate^[1]. Structurally, mammalian GLOI is a zinc-containing metalloenzyme with two active sites located at the homodimer interface^[2]. Each active site of GLOI is composed of a GSH binding site (the glycyl site is characterized by Lys150A, Gly155A, Lys156A, Leu160A and Phe162A and the glutamyl site is characterized by Arg37B, Asn103B and Arg122A), a Zn^{2+} catalytic site (Zn^{2+} and the Zn^{2+} -coordinating residues Gln32, His126 and Glu172) and a hydrophobic site (Leu69B, Leu92B, Met157A, Met179A, Met183A and

Phe71B)^[3,4].

Abnormal overexpression or higher activity of GLOI has been observed in many tumors and is related to multi-drug resistance (MDR) in cancer chemotherapy^[5]. Inhibition of GLOI resulted in the accumulation of α -oxoaldehydes at cytotoxic levels^[2] and reverse MDR, and thus GLOI inhibitors have been proposed as efficient anti-tumor agents and have been recognized for their anti-protozoal and anti-bacterial purposes for many years^[6–9]. The traditional and most studied GLOI inhibitors are analogs of the coenzyme GSH, which exhibits efficient inhibition *in vitro*^[3]. However, these GSH analog inhibitors suffer from poor pharmacokinetic properties and are difficult to use as a lead structure for the further design of small molecule GLOI inhibitors due to their structural similarity to GSH^[10,11]. Moreover, these highly potent GSH analog inhibitors are not able to induce efficient cytotoxic concentrations of α -oxoaldehydes in tumor cells because of their degradation by other GSH-dependent enzymes^[12] and metabolism

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by aldose reductase *in vivo*^[13, 14]. Thus, a search of new types of scaffolds for GLOI inhibition with better pharmacokinetic properties is necessary.

At present, non-GSH analog inhibitors, such as methylgerfelin (MGI)^[15], azaindole-substituted N-hydroxypyridones^[16], flavonoids^[10], curcumin and its derivatives^[17], and nonsteroidal anti-inflammatory drugs (NSAIDs)^[5], have been identified or synthesized (see Supplementary Table S1 for representative structures and inhibition levels of GLOI inhibitors). Crystallographic studies indicate that the ability to chelate Zn²⁺ at the active center of GLOI plays an essential role for the high inhibitory activity of GSH analog inhibitors (GIP)^[3] and non-GSH analog inhibitors (MGI^[15] and azaindole-substituted N-hydroxypyridones^[16]). Docking and molecular dynamic simulation analyses also suggest that coordination with Zn²⁺ is required for the inhibition of GLOI by curcumin, NSAIDs and flavonoids^[10, 18, 19]. However, metal chelation is generally undesirable in drug development because of the high potential for off-target effects in the human body^[20, 21].

In our recent work, we determined the crystal structure of mouse glyoxalase I (mGLOI) complexed with indomethacin (inhibition constant (K_i)=18 $\mu\text{mol/L}$, PDB ID: 4KYK) and zopolrestat (K_i =1.2 $\mu\text{mol/L}$, PDB ID: 4KYH), revealing a novel inhibitor-GLOI binding mode^[22]. In these two complex structures, the carboxyl groups of indomethacin and zopolrestat mimic the glycyl and γ -glutamyl residue moieties of GSH to form hydrogen bonds with the glycyl and glutamyl sites, respectively, in the GSH binding site^[22]. In this study, we propose the existence of a novel class of non-GSH analog GLOI inhibitors with a carboxyl group as an important functional feature. To confirm our proposal, we screened a small pool of compounds containing a carboxyl group using an *in vitro* assay. Furthermore, the binding mode and molecular mechanism of potential GLOI inhibitors were explored using X-ray crystallographic analysis.

Materials and methods

Protein expression, purification and *in vitro* enzyme assay

The mGLOI plasmid was the kindly gift of Dr Hideo OKUMURA, Advanced Science Institute, RIKEN, Japan. The mGLOI was expressed in BL21 (DE3) pLysS at 25 °C after induction with 0.1 mmol/L isopropyl- β -D-1-thiogalactopyranoside and purified on a HiTrap Q column using an AKTA FPLC system. The protein concentration was determined using a NanoDrop 2000c spectrophotometer.

The activity of recombinant mGLOI was determined by monitoring the changes in absorbance at 240 nm at 30 °C with a thermostated spectrophotometer, as described previously^[18, 23]. The test compound was preincubated with 0.10 mol/L potassium phosphate (pH 7.0) containing hemithioacetal (MG-SG) for 20 min at 30 °C. Different concentrations of methylglyoxal and GSH were calculated and varied by using the equation constant K_i =3.0 mmol/L to obtain the desired concentration of MG-SG. Excess free GSH in the assay was kept at 0.10 mmol/L. The reaction was initiated by the addition of mGLOI (3.0 nmol/L) to the reaction system. The K_i val-

ues of inhibitors were evaluated using a Dixon plot showing the reciprocal of enzyme velocity against the inhibitor concentration at various concentrations of the substrate.

Protein crystallization, data collection and structure determination

The apo mGLOI crystals were obtained at 289 K in hanging droplets consisting of 2 μL of protein solution (20 mg/mL) mixed with 2 μL of reservoir solution consisting of 50 mmol/L 2-(N-morpholino)ethanesulfonic acid (MES; pH 5.8), 30%-32% PEG2000 and 0.10 mol/L or 0.15 mol/L NaCl. Apo mGLOI crystals were soaked in a saturated solution of GA containing 32.5% N,N-dimethylformamide and the corresponding reservoir solution. X-ray data were collected at 100 K on an in-house Oxford Diffraction Xcalibur Nova diffractometer. The structure was determined with CCP4^[24], Phenix^[25] and COOT^[26] (PDB ID: 4PV5; for data collection and refinement statistics, see Supplementary Table S2).

Molecular docking experiment

All docking experiments were performed using the Discovery Studio 2.5 software package (Accelrys Software Inc, San Diego, CA, USA). The protocol was generated using the CDOCKER protocol without the crystal waters. Before docking, hydrogen atoms were added to the unoccupied valence of the heavy atoms of the protein. The molecules used for docking were downloaded from the PubChem Compound database (<http://www.ncbi.nlm.nih.gov/pccompound/>). Prior to docking, the protocol was demonstrated to be reliable by redocking the inhibitor of each PDB file to its own receptor (PDB IDs: 1QIN, 2ZA0, and 4KYH) in terms of global conformation, orientation and root mean square deviation value (<1.5 Å).

Results

Discovery of GA as a potent *in vitro* GLOI inhibitor

To search for GLOI inhibitors with a carboxyl group feature, we screened a small pool of compounds containing carboxyl groups using an *in vitro* assay. Among this pool, GA was found to be a potent competitive mGLOI inhibitor with a K_i of 0.29 $\mu\text{mol/L}$ (Figure 1A), and its derivative carbenoxolone (a licensed drug) showed a slightly lower inhibitory activity with a K_i of 0.93 $\mu\text{mol/L}$ (Figure 1B). Seven other natural pentacyclic triterpenes in this pool showed only slight mGLOI inhibitory activity (ursolic acid, oleanolic acid, betulic acid and tripterine with inhibition ratios <25% at a concentration of 10 $\mu\text{mol/L}$; Table 1) or no activity (maslinic acid, corosolic acid and madecassic acid; Table 1). Although a subsequent literature review indicated that GA had been identified as human GLOI inhibitor by Masayoshi *et al* in 1986^[27], no further studies on the mechanism of inhibition or the structure-activity relationship were performed.

Structural basis for the inhibition of mGLOI by GA

To investigate the differences in GLOI inhibitory activity with the aforementioned pentacyclic triterpenes and to clarify the binding mode of GA, we determined the crystal structure

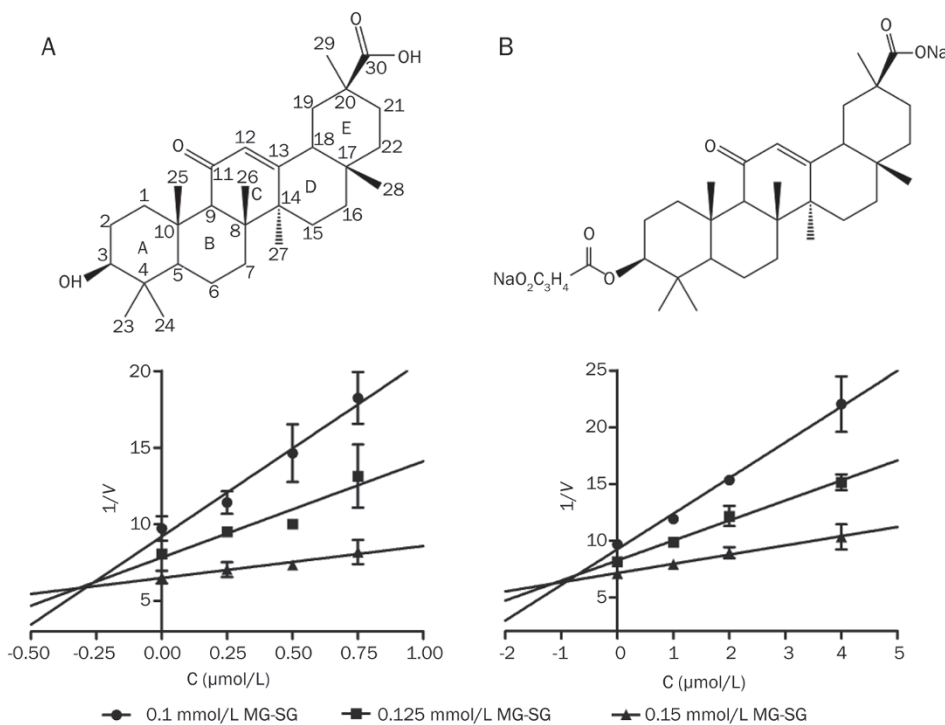


Figure 1. The Dixon plots and chemical structures of GA (A) and carboxinolone (B). Data are the mean \pm SD (error bars) of $n=3$ experiments. .

of the mGLOI-GA complex at a 2.3 Å resolution. Similar to our previously determined complex structures of mGLOI-indomethacin and mGLOI-zopolrestat, the final structural model of mGLOI-GA also contained one GA molecule in one of the active sites, which was different from the other GLOI-inhibitor complexes, where two inhibitor molecules were found in both of the active sites of GLOI (PDB IDs: 1BH5, 1FRO, 1QIN, 1QIP, 2ZA0, 3VW9, 3W0T, and 3W0U). We also determined the mGLOI-GA complex structure in a different space group using a co-crystallization method and observed only one GA molecule per GLOI dimer at the asymmetric unit (unpublished data). Superimposition of both structures revealed no noticeable backbone movement or meaningful shift of the ligand position. Therefore, this phenomenon was very likely due to the method of preparation of the crystal of the enzyme-inhibitor complex, which was controlled by the affinity and solubility of the inhibitor.

A schematic presentation of the GA-occupied active site derived from the mGLOI-GA complex crystal structure revealed that GA did not coordinate with Zn^{2+} ; instead, it nestled in the GSH binding subpocket with its hydroxyl group pointing toward the glutamyl site and established a hydrogen-bonding network with mArg38B (-O2•••Ne, 2.4 Å; -O3•••HN, 3.2 Å), mAsn104B (-O2•••HN, 3.4 Å) and mArg123A (-O2•••HN, 3.2 Å; -O2•••HN, 3.4 Å) (Figure 2). The full occupation of GA in the GSH binding subpocket also resulted in extensive van der Waals interactions with the surrounding residues mMet36B, mMet66B, mPhe68B, mThr102B, mAsn104B, mVal150A, mLys151A, mPhe163A and mTrp171A, in addition to at least three nonpolar/polar and ten nonpolar/

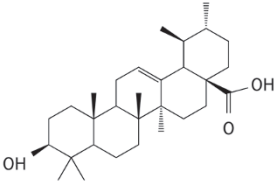
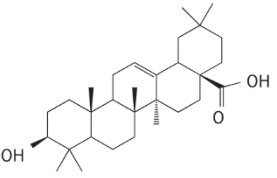
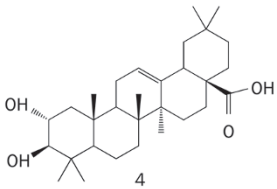
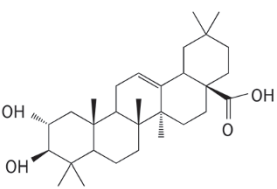
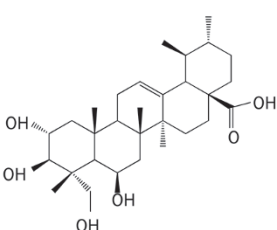
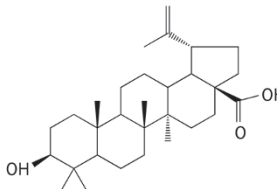
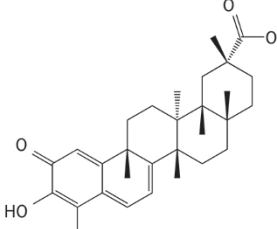
nonpolar contacts with distances shorter than 4 Å (Figure 2 and Table 2).

The binding mode of mGLOI-GA gave a rational explanation to the weak inhibitory activity of the other pentacyclic triterpenes tested here. For carboxinolone, its flexible 3-O-hemisuccinate tail hung out of the active pocket and had few or very weak interactions with any residues of mGLOI predicted by the docking experiment (Supplementary Figure S1); therefore, carboxinolone showed a similar inhibition as GA. As for ursolic acid, oleanolic acid, maslinic acid, corosolic acid, madecassic acid, and betulic acid, these six pentacyclic triterpenes showed little or no inhibition towards mGLOI due to their carboxyl groups located at the C17 position, which could not access either the glutamyl site or the glycylic site, as indicated by the structural superimposition with GA. For tripterine, the opposite stereochemistry of the carboxyl group on C30 to that of GA made it difficult to be anchored in the glutamyl site of the GSH binding site (Supplementary Figure S2). Thus, tripterine exhibited only slight inhibition of mGLOI.

Receptor model selection for a docking study with a non-GSH analog GLOI inhibitor

Notably, *in silico* GLOI inhibitor screenings published by our group and others have never identified GA as a possible candidate. In addition, our *in silico* screenings also did not identify zopolrestat or indomethacin as possible candidates. In fact, in our previously reported docking/MD study of the GLOI-indomethacin interaction mode, in which indomethacin coordinates with Zn^{2+} through its carboxyl group, a totally different result was revealed by the crystal structure^[19].

Table 1. Chemical structures of the pentacyclic triterpenes tested and their inhibition towards mGLOI. The final concentration of each test compound was 10 $\mu\text{mol/L}$. Data are the mean of triplicate experiments.

Number	Compound	Structure	Inhibition ratio (%)
1	Ursolic acid		12.5
2	Oleanolic acid		5.20
3	Maslinic acid		Inactive
4	Corosolic acid		Inactive
5	Madecassic acid		Inactive
6	Betulic acid		12.7
7	Tripterine		23.6

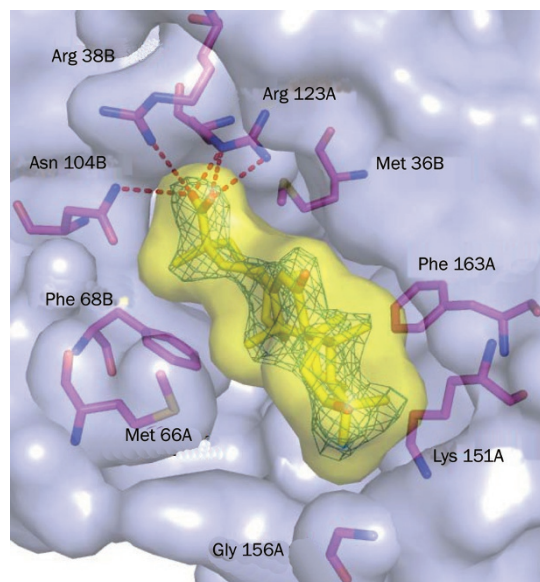


Figure 2. Structure of GA binding to mGLOI. 2Fo-Fc electron density map of GA contoured at 1.0 sigma. The protein and ligand surfaces are depicted as a transparent light blue and yellow cutaway. Key residues and ligands are shown as magenta and yellow stick models, respectively. Hydrogen bonds are shown as red dashed lines.

To investigate why GA was not identified in the *in silico* screening, we performed the docking studies with three representative GLOI-inhibitor crystal structures as receptors, *ie*, human GLOI-GIP (GSH/ Zn^{2+} -chelated inhibitor, the predominantly adopted receptor mode in *in silico* screening; PDB ID: 1QIN), mGLOI-MGI (non-GSH/ Zn^{2+} -chelated inhibitor; PDB ID: 2ZA0) and mGLOI-zopolrestat (non-GSH/non- Zn^{2+} -chelation inhibitor; PDB ID: 4KYH). The results indicated that only with the GLOI-zopolrestat structure as the receptor did the docking study produce a similar conformation of GA to that observed in the crystal structure (Figure 3).

We compared the GLOI-ligand structures in the PDB database with that of apo mGLOI (unpublished data) and found that GIP and other GSH analog inhibitors induced a significant shift ($>1.4 \text{ \AA}$) of the glycyI site toward the active center, which did not occur in the non-GSH analog GLOI inhibitors or the

Table 2. Van der Waals interactions between GA and mGLOI residues at the binding site.

Inhibitor atom	Protein atom	Number of contacts
C6	Lys151A C beta	1
C9	Phe163A C delta2, C epsilon2, Trp171 A C zeta3	3
O1	Trp171A C eta2	1
C21	Thr102B C gamma1, Met 36B C epsilon	2
C23	Lys151A C delta	1
C25	Val150A C gamma1	1
C27	Met 66B C epsilon, S delta	2
C29	Phe68B C delta1, Asn104B O delta1	2
Total		13

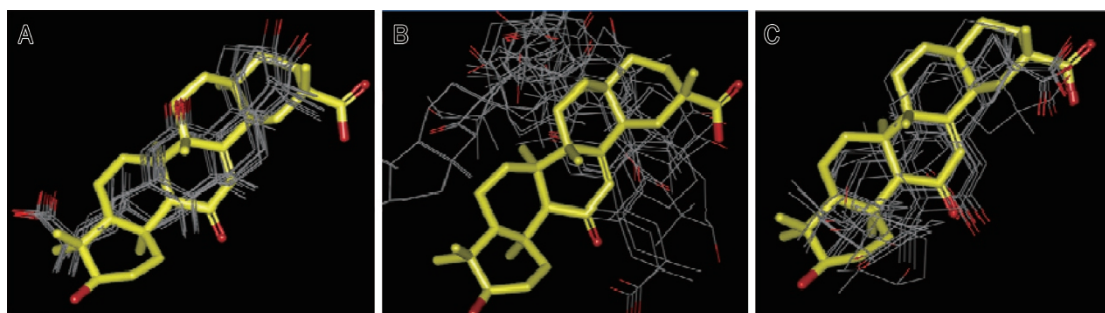


Figure 3. The binding conformations of GA derived from docking by employing crystal structure of human GLOI-GIP (PDB ID: 1QIN) (A), mGLOI-MGI (PDB ID: 2ZAO) (B) and mGLOI-zopolrestat (PDB ID: 4KYH) (C) as receptor, respectively. The crystal structure of GA derived from mGLOI-GA complex for comparison is shown as yellow sticks.

apo structure (Supplementary Figure S3). Such a shift resulted in an obvious steric clash between the human Gly155A residue of the glycol site and the methyl group at C4 of GA (1.2 Å) (Supplementary Figure S4). In contrast, such a conformational change was also likely to induce the docking programs to locate the ligands, such as indomethacin, at a deeper position in the active pocket to avoid steric interactions and therefore produce a false coordination between the ligands and Zn²⁺[19]. Because the docking method is critical for *in silico* screening and binding mode analysis, it is important to employ a suitable crystal complex structure as the receptor model when searching for non-GSH analog inhibitors.

Discussion

Since Vince *et al* proposed the concept of GLOI inhibitors as anti-tumor agents in the early 1970s[28], GSH analogs with strong *in vitro* inhibition of GLOI have been extensively studied. However, because the GSH skeleton is very difficult to work with due to its pharmacokinetic properties[5, 10, 11], non-GSH analog inhibitors could potentially lead to an alternative approach that yields a successful drug. Furthermore, considering the potential off-target effects of metal chelation[20, 21], non-GSH analog GLOI inhibitors lacking Zn²⁺-coordination may be more suitable for lead optimization in drug development. The discoveries of indomethacin, zopolrestat and GA as GLOI inhibitors and their related complex crystal structures have demonstrated the existence of a novel non-GSH/non-Zn²⁺-chelation type of GLOI inhibitor with a carboxyl group as an essential anchor to fix the inhibitors in the active site by hydrogen bonding. It is a reasonable conjecture that any compound with the ability to form strong hydrogen bonds with the glycol site and/or the glutamyl site and the capability of fitting into the active pocket of GLOI could potentially be an effective GLOI inhibitor.

In addition to the GLO system, the aldose reductase (AR) is another important enzyme responsible for the detoxification of methylglyoxal and other α -oxoaldehydes[29]. A dual-target inhibitor that could simultaneously block GLOI and AR is likely to be more effective than a single-target GLOI inhibitor. GSH analog inhibitors are unlikely candidates for dual-target inhibitors because inhibition towards both GLOI and AR

requires substituents with opposite polarities (hydrophobic and hydrophilic) on the thiol to bind to the active sites of GLOI and AR[3, 30]. In fact, a carboxyl group is a typical (and critical) functional group for most known AR inhibitors[31-33]. Both indomethacin and zopolrestat are AR inhibitors with moderate (IC₅₀=10 μ mol/L)[34] and strong (IC₅₀=3 nmol/L)[35] inhibition, respectively. Although GA is inactive towards AR[36], it is a strong inhibitor of AKR1B10 (IC₅₀ of 4.9 μ mol/L)[37], a close AR family member that also catalyzes detoxification of α -oxoaldehydes. Therefore, non-GSH/non-Zn²⁺-chelation type GLOI inhibitors containing a carboxyl group could have great potential as GLOI/AR dual-target inhibitors. Considering the large number of AR inhibitors synthesized since the 1990s, there is a strong possibility that there may be inhibitors with excellent *in vivo* anti-cancer activity in this group of compounds.

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Author contribution

Xiao-peng HU and Qing LI designed the research; Hong ZHANG performed the experiments; Qiang HUANG, Jing ZHAI and Yun-yun CHEN assisted in the experiments; and Hong ZHANG wrote the manuscript with contribution from the other authors (Yi-ning ZHAO, Li-ping ZHANG, and Ren-wei ZHANG).

Supplementary information

Supplementary information is available at Acta Pharmacologica Sinica's website.

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