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Discovery of selective 2,4-diaminopyrimidine-based photoaffinity probes for glyoxalase I†

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Yiqing Zhou,[a] Tianlin Guo,[a] Xitao Li,[a] Yi Dong,[a] Paul Galatsis,[b] Douglas S. Johnson[b] and Zhengying Pan*[a]

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Glyoxalase I (GLO-1) plays a critical role in the detoxification of 2-oxoaldehydes and is highly expressed in cancer cells. Through photo-affinity labelling and affinity pull-down approaches, a series of 2,4-diaminopyrimidine compounds were discovered to selectively bind to GLO-1 in cells. These compounds show potent inhibition of GLO-1 enzyme activity and prevent proliferation of cancer cells. The cell permeable and "clickable" photoaffinity probe **L1-Bpyne** presented here could be a valuable tool for profiling GLO-1 in live cells.

Introduction

The glyoxalase system, composed of glyoxalase I (GLO-1, EC 4.1.1.5) and glyoxalase II (GLO-2, EC 3.1.2.6), is an important and ubiquitous detoxification component of cellular metabolism in most cells.[1] Methylglyoxal (MG), a by-product in glycolysis, is cytotoxic due to its covalent binding to DNA and proteins in cells.[1,2] Newly produced MG reacts with glutathione (GSH), an abundant nucleophile in cells, to form hemithioacetal. GLO-1 catalyses the isomerisation of hemithioacetal into *S*-D-lactoylglutathione. Subsequently GLO-2 catalyses the conversion of lactoylglutathione into lactic acid and GSH *via* hydrolysis.[3] The glyoxalase system is highly expressed in cancer and inflammatory cells to rapidly remove the cytotoxic MG.[4]

Overexpression of GLO-1 has been associated with multidrug resistance in cancer chemotherapy.[5] Much effort has been made to develop inhibitors of GLO-1 as an anti-cancer therapy. Most of the early GLO-1 inhibitors are GSH derivatives, but this class of compounds has poor pharmacokinetic properties, as GSH remains charged under physiological conditions.[6] Additionally, GSH is a substrate or cofactor of many other enzymes; thus, these inhibitors show poor selectivity. In recent years, many non-GSH-based small molecule inhibitors, such as methyl gerfelin, delphinidin, curcumin and zopolrestat, have been reported to have inhibitory effects against GLO-1.[7–10] Despite advancements in the development of GLO-1 inhibitors, a selective GLO-1 labelling probe has not been reported.

The 2,4-diaminopyrimidine scaffold has been widely used in pharmaceutical agents, including the dihydrofolate reductase inhibitor pyrimethamine, and the antibiotics iclaprim and trimethoprim.[11] Recently, 2,4-diaminopyrimidine also served as a common template to generate inhibitors for a number of kinases, including c-Met,[12] MK2,[13] ALK,[14] and SYK.[15] Since 2011, several research groups have developed potent LRRK2 inhibitors based on the 2,4-diaminopyrimidine scaffold.[16–18] Of these compounds, CZC-25146 was found to attenuate Parkinson's disease-related toxicity in human neurons (Fig. 1A). While the *in vitro* selectivity of this type of kinase inhibitor can be assessed using various techniques,[19–21] the selectivity of these compounds in more complex biological systems is still unknown.[22] To examine the selectivity of this type of inhibitors

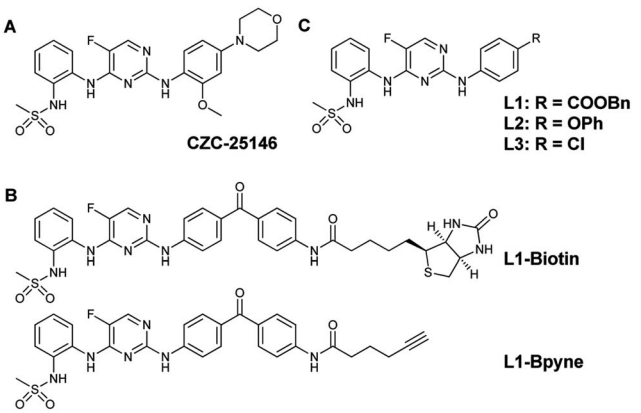


Fig. 1 Chemical structures of (A) the 2,4-diaminopyrimidine-based kinase inhibitor CZC-25146; (B) the photoaffinity probes **L1-Biotin** and **L1-Bpyne**; and (C) the analogues of the 2,4-diaminopyrimidine series compounds used in this study.

[a]*Key laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Peking University, Xili University Town, PKU Campus, Shenzhen, China, 518055. E-mail: panzy@pkusz.edu.cn; Tel: +86-755-26033072*

[b]*Neuroscience Medicinal Chemistry and Chemical Biology, Pfizer Worldwide Research and Development, Cambridge, MA 02139, USA*

† Electronic supplementary information (ESI) available: Detailed synthetic procedures, ^{1}H and ^{13}C NMR spectra, HPLC chromatograms for compounds **L1**, **L2**, **L3**, **L1-Biotin** and **L1-Bpyne**; detailed procedures for biological assays; mass spectroscopy data for GLO-1. See DOI: 10.1039/c3md00286a

in mammalian cells, we synthesised two photoaffinity probes, **L1-Biotin** and **L1-Bpyne**, to detect these compounds cellular targets (Fig. 1B). As a result, GLO-1 was identified as a major target of these 2,4-diaminopyrimidine compounds in live cells. Several synthetic analogues of CZC-25146 were further tested in *in vitro* enzyme activity and cell proliferation assays (Fig. 1C). These compounds inhibited the enzyme activity of GLO-1 and cancer cell proliferation, and the potency of these molecules depended on the glucose concentrations in culture media. Finally, these compounds were shown to cause MG accumulation in live cells and induce cell apoptosis.

Results and discussion

First, we examined the potential targets of 2,4-diaminopyrimidine compounds in cells and designed a biotinylated photoaffinity probe **L1-Biotin** to label and pull-down its binding proteins in the cellular proteome (Fig. 1B). A benzophenone group was introduced to covalently link the probe and its potential targets upon photo irradiation. Human embryo kidney 293T (HEK293T) cell lysate was pre-treated with streptavidin–sepharose beads to reduce endogenously biotinylated proteins. The cells were then incubated with 0.1 or 1 μM **L1-Biotin** with and without 50 μM compound **L1** overnight at 4 °C. After photo-crosslinking by irradiation at 365 nm for 1 hour on ice, the **L1-Biotin** binding proteins were separated by SDS-PAGE and detected by streptavidin–HRP (Fig. 2A). Alternatively, biotinylated proteins in lysates, both endogenous and those bound to the **L1-Biotin** probe, were first enriched with streptavidin–sepharose beads, then separated by SDS-PAGE and visualised by silver staining (Fig. 2B). In both experiments, the **L1-Biotin** probe bound specifically to two protein bands at approximately 28 kDa and 55 kDa. The two corresponding bands in silver staining gels were excised, destained, digested by trypsin and analysed by MALDI-TOF mass spectrometry. Both bands were identified as human glyoxalase I (see ESI†). As the literature indicates, GLO-1 exists in both monomeric and dimeric forms in cells;[2,23] the higher molecular weight band could represent the GLO-1 dimer, and the lower molecular weight band could represent the monomer. The appearance of the dimeric form band at 55 kDa in the SDS-PAGE experiments might be caused by our probe molecules and cellular modifications in HEK293T cells. These results were further confirmed by western blotting using glyoxalase I antibody (Fig. 2C). Finally, recombinant human GLO-1 protein was also successfully labelled by **L1-Biotin** (Fig. 2D) in a separate experiment. Competitive binding is clearly observed using the structurally similar compound **L1**, suggesting that GLO-1 was specifically labelled by the probe **L1-Biotin**.

Further labelling experiments were performed to evaluate the detection range of the probe **L1-Biotin** and the effect of UV irradiation time. HEK293T lysates were incubated with different concentrations of **L1-Biotin**; 0.5 to 1 μM of probe was sufficient to label GLO-1, whereas higher concentrations of the probe increased non-specific labelling (Fig. 3A). At a steady probe concentration at 0.5 μM, cell lysates were incubated with **L1-Biotin** and irradiated with UV light for various periods of time. The band intensity increased with the UV irradiation time and became saturated after approximately 1 hour (Fig. 3B).

After GLO-1 was identified as a potential target in cell lysates, we attempted to confirm it in live cells. However, the biotinylated photoaffinity probe **L1-Biotin** was unable to bind to proteins in live cells (data not shown), likely due to the low trans-membrane permeability of the biotin tag. We then designed the probe **L1-Bpyne** for labelling studies of GLO-1 in live cells (Fig. 1B). Bio-orthogonal conjugation reactions have been successfully employed to identify covalent targets of small molecules in a variety of biological systems. The copper(I) catalysed [3 + 2] azide–alkyne cycloaddition (CuAAC) reaction is

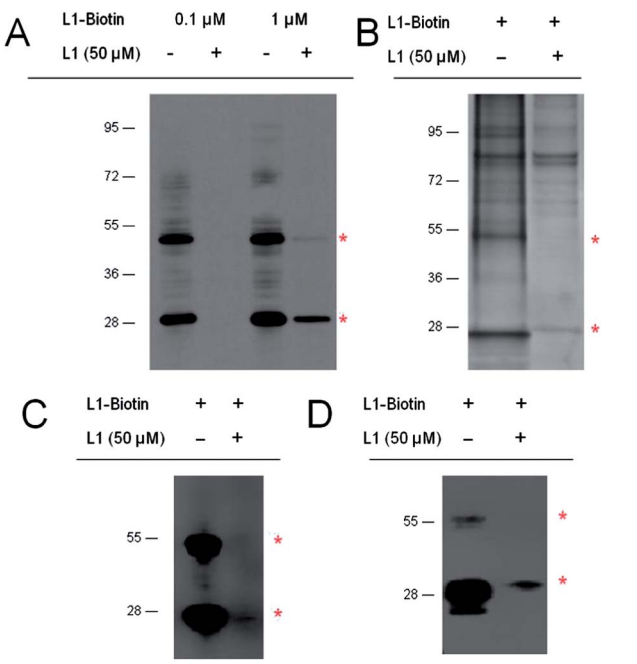


Fig. 2 (A) HEK293T cell lysate labelling by **L1-Biotin** with and without competitor **L1**; (B) silver staining of **L1-Biotin** binding proteins enriched by streptavidin–sepharose beads; (C) western blot validation with GLO-1 antibody; (D) recombinant glyoxalse-1 protein labelling by **L1-Biotin**.

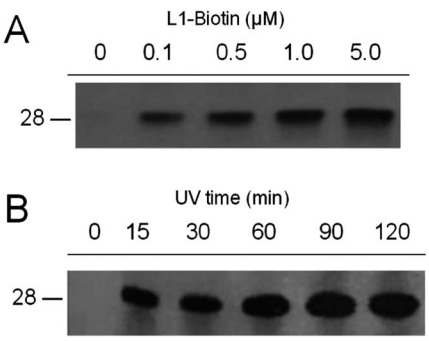


Fig. 3 (A) HEK293T cell lysates treated with different concentrations of probe **L1-Biotin**; (B) HEK293T cell lysates treated with 0.5 μM of **L1-Biotin** and irradiated by long-wave UV light for different lengths of time.

one of the most commonly used methods.[24] Thus, in the new **L1-Bpyne** probe, an alkyne tag replaced the biotin tag in **L1-Biotin** to facilitate cellular studies.

We then attempted to label GLO-1 using this new probe. For *in vitro* labelling, HEK293T lysates were incubated with 1 μM **L1-Bpyne** with and without excess compound **L1**. After UV irradiation and CuAAC conjugation with tetramethylrhodamine–azide (TAMRA–N_3), the samples were analysed by SDS-PAGE and detected by in-gel fluorescence scan. For *in situ* labelling, live HEK293T cells were incubated with 1 μM of **L1-Bpyne** with and without excess compound **L1**. The cells were irradiated by UV for 1 hour at 37 °C and washed with ice-cold PBS buffer solution to remove unbound probes. Following centrifugation and homogenisation by lysis buffer, the samples were subjected to the CuAAC reaction with the same conditions as those used for the *in vitro* labelling. Both monomeric and dimeric forms of GLO-1 were selectively labelled by the probe **L1-Bpyne** *in vitro* and *in situ*. The labelling showed specific binding competition by compound **L1** (Fig. 4A). Encouraged by the results of *in situ* labelling, the intracellular CuAAC reaction with TAMRA–N_3 was performed to show the cellular localisation of **L1-Bpyne** targets. HeLa cells were cultured in normal culture medium and treated with 20 μM **L1-Bpyne** for 2 hours with or without 100 μM compound **L1** as the competitor. Following UV irradiation, the cells were fixed, permeabilised, and subjected to CuAAC reaction with TAMRA–N_3 (Fig. 4B). Staining of the cells gave a strong signal as GLO-1 is a relatively abundant enzyme in cytosol. The labelling was significantly reduced following competitive binding by excess compound **L1**.

Because GLO-1 appeared as a major cellular target of 2,4-diaminopyrimidine probes, we next investigated whether these types of compounds could inhibit the catalytic activity of GLO-1 *in vitro*. A spectrophotometric method was employed by monitoring the increase in UV absorbance at 240 nm from the formation of *S*-D-lactoylglutathione at 25 °C.[25] The **L1-Bpyne** probe was the most potent inhibitor with an IC_{50} value of 26 nM, while other analogues **L1–L3** showed IC_{50} values of approximately 50 nM (Table 1), which suggests that the benzophenone moiety and the alkyne tag did not significantly alter the binding preference of these 2,4-diaminopyrimide compounds towards GLO-1.

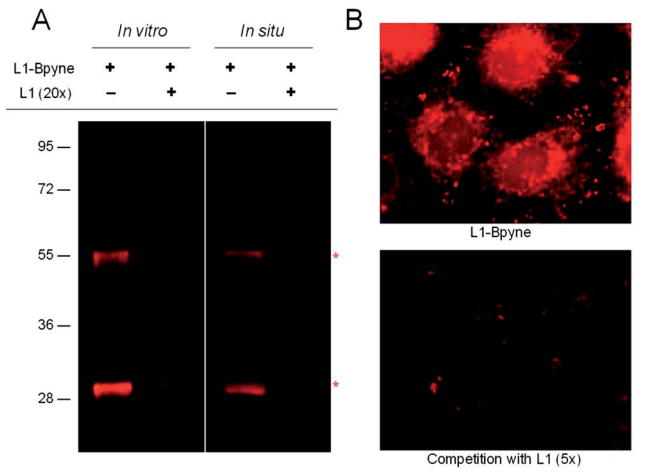


Fig. 4 (A) *In vitro* labelling of HEK293T cell lysates and *in situ* labelling of live HEK293T cells by **L1-Bpyne** (1 μM); (B) in-cell CuAAC reaction revealed the cellular distribution of **L1-Bpyne**.

Proliferation assays were performed to examine the effects of these compounds in cells. The primary cellular function of GLO-1 is MG detoxification, and the MG concentration has been reported to be elevated in high glucose conditions.[25,26] Therefore, three types of cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with two concentrations of glucose at 5 mM (approximately the normal *in vivo* glucose level) and 25 mM (high glucose level). Cell viability was assessed using a CellTiter-Glo® luminescent kit after incubating for 48 hours with various concentrations of the tested compounds. These compounds had GI_{50} values at low to mid micromolar concentrations (Table 1). In accordance with the data of the enzymatic assay, the **L1-Bpyne** probe was also the most potent inhibitor in the cell proliferation assays. Between two human lung cancer cell lines, NCI-H522 cell, which was reported to express high intrinsic GLO-1 activity, is more susceptible to the GLO-1 inhibition; A549 cell, which was reported to express relatively low intrinsic GLO-1 activity, showed about 5-fold less sensitivity towards **L1-Bpyne**.[27] HeLa cell, a human cervical cancer cell line, could also be potently inhibited by this series of inhibitors. The glucose contents in the culture media had low to moderate effects on the inhibition of tumor cell proliferation by this series of compounds. As expected, high concentrations of glucose increased cellular sensitivity to GLO-1 inhibition. The GI_{50} values of compounds **L1–L3** increased up to 2.6-fold in normal glucose medium compared to high glucose medium.

Because GLO-1 inhibition causes methylglyoxal accumulation followed by random DNA and protein labelling, we further investigated whether our compounds could induce an increase of the cellular MG concentration. HeLa cells were grown in normal or high glucose media for 3 days followed by treatment with DMSO or 5 μM of **L1-Bpyne** for 24 hours. The cells were harvested, washed with PBS buffer, resuspended in distilled water and boiled. The MG content in the supernatant was detected using the same spectrophotometric method as that in the aforementioned enzyme activity assay. The MG concentrations were slightly elevated in the high glucose medium with untreated cells but were almost doubled in the **L1-Bpyne** treated cells (Fig. 5A). This suggests that **L1-Bpyne** inhibited GLO-1 activity in HeLa cells. Cellular MG accumulation commenced upon GLO-1 inhibition.

Finally, we tested whether GLO-1 inhibition would cause cell apoptosis. HeLa cells were treated with various concentrations of **L1-Bpyne** for 24 hours, the degradation of the apoptosis marker protein caspase-3 clearly indicated cell apoptosis (Fig. 5B). Moreover, after HeLa cells were treated with 5 μM of **L1-Bpyne** for 24 hours, the cells were fixed with paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI), the cell nuclei also appeared fragmented (Fig. 5C).

MG is a highly reactive α-oxoaldehyde that reacts predominantly with the lysine or arginine residues of proteins and the guanyl residues in DNA and RNA. It may cause abnormal enzyme activity and defects during transcription.[28] Because

Table 1 Inhibition of GLO-1 enzyme activity and cancer cells proliferation by 2,4-diaminopyrimidine compounds[a]

Compound	GLO-1 (nM)	NCI-H522-HG (μM)	NCI-H522-NG (μM)	HeLa-HG (μM)	HeLa-NG (μM)	A549-HG (μM)	A549-NG (μM)
L1-Bpyne	26.4	2.9	2.7	4.6	5.7	13.3	15.5
L1	51.3	16.5	40.2	37.0	61.9	>50	>50
L2	45.8	7.3	8.3	7.3	14.0	26.9	37.4
L3	53.9	12.2	25.0	23.2	61.0	>50	>50

[a] Values are averages of at least two independent runs. HG: high glucose level; NG: normal glucose level.

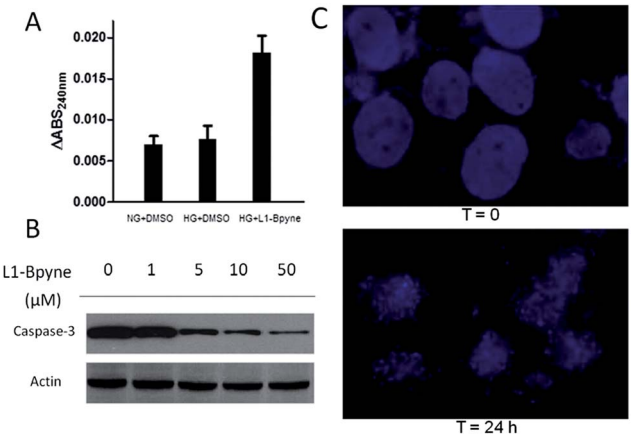


Fig. 5 (A) Intracellular MG concentration upon **L1-Bpyne** treatment; (B) HeLa cells were treated with different concentrations of **L1-Bpyne** and the intact caspase-3 levels were evaluated by western-blot; (C) HeLa cells were treated with 5 μM of **L1-Bpyne** for 24 hours and stained by DAPI.

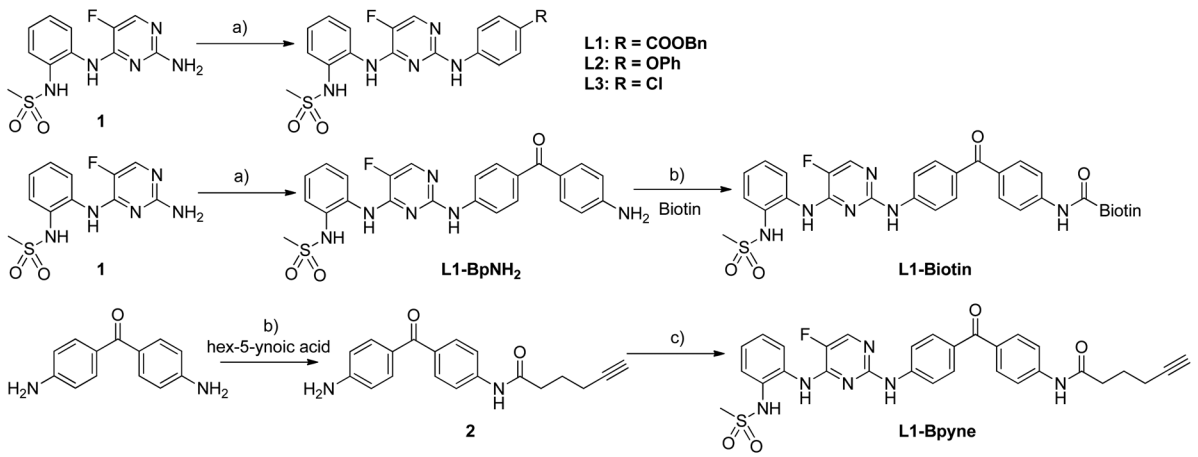
tumour tissue has higher glycolysis activity than normal tissue[29,30] and the glyoxalase system is highly activated in cancer cells,[31,32] GLO-1 inhibitors could be selectively cytotoxic towards cancer cells. Our compounds did show inhibition of proliferation of several human cancer cells, thus indicating their potential application to cancer therapy. The relative large potency shift of these compounds from the enzymatic assay to the cell proliferation assay could result from multiple factors including physiochemical properties of compounds and the network linking GLO-1 activity to cell death. A combination of GLO-1 inhibitors with compounds targeting multiple pathways may be an attractive approach to achieve significant anti-cancer effects in the clinic.

Chemistry

The syntheses of compounds **L1–L3, L1-Biotin** and **L1-Bpyne** are depicted in Scheme 1. Each was readily prepared using literature procedures with slight modifications. The common precursor **1** was coupled with substituted anilines by an S_NAr reaction to provide **L1**, **L2**, and **L3** in good yields. **L1-Biotin** was prepared from the intermediate **L1-BpNH$_2$** and biotin using standard coupling conditions for amide bond formation. Compounds **1** and **2** underwent a Hartwig–Buchwald amination reaction to furnish **L1-Bpyne** in good yield. Detailed synthetic procedures and compound characterisation data are provided in the ESI.†

Conclusions

In summary, we have discovered two photoaffinity probes, **L1-Biotin** and **L1-Bpyne**, which specifically label GLO-1 in cells. The biotinylated probe can specifically label and pull-down GLO-1 *in vitro*, while the alkyne probe is able to passively penetrate living



Scheme 1 Synthesis of GLO-1 inhibitors. *Reagents and conditions*: (a) substituted anilines, concentrated HCl, *n*-BuOH, 120 °C, 16–18 hours; (b) HATU, Hunig's base, DMF, 0 °C to rt, overnight; (c) **1**, $Pd_2(dba)_3$, DPBP, K_2CO_3, nBuOH, 100 °C, 4 hours.

cells and label GLO-1 *in situ*. Furthermore, all compounds potently inhibited the enzyme activity of GLO-1 with IC_{50} values in the range of 26 to 54 nM. These compounds prevented cancer cell proliferation, increased intracellular levels of methylglyoxal, and induced cell apoptosis. Interestingly, higher glucose content in the cell medium increased the cytotoxicity of these compounds. Taken together, this series of 2,4-diaminopyrimidine compounds directly interacts with GLO-1 in cells. Moreover, **L1-Bpyne** is both a potent inhibitor and a selective probe of GLO-1, and could be a powerful tool in studies of this enzyme. With this cell permeable probe, GLO-1 was successfully labelled both *in vitro* and *in situ*. This probe could also be utilised to label GLO-1 in tissues of animal models, though the long-wave UV light used in the photocrosslinking step could be harmful to surrounding tissues and limits the application of this probe in whole animal studies. Further research is underway to understand the binding mode of this type of probe, including the exact binding site and specific binding interactions with GLO-1, and its potential use in combination with other anti-cancer agents.

Acknowledgements

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