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Activity-based protein profiling reveals GSTO1 as the covalent target of piperlongumine and a promising target for combination therapy for cancer†

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Through systematic target identification for piperlongumine, a cancer-selective killing molecule, we identified GSTO1 as its major covalent target for cancer cell death induction. We also reveal that GSTO1 inhibition is a promising combination strategy with other anti-cancer agents by drug combination screening in which piperlongumine exhibits broad-spectrum synergistic effects with a large proportion of the tested anti-cancer agents, especially with PI3K/Akt/mTOR pathway inhibitors.

Given that cancer is the most severe current public health problem, it is always urgent to discover new molecular targets and develop effective anti-cancer agents.[1] Piperlongumine (**PL-1**, Fig. 1a), an alkaloid natural product from *Piper longum*, is reported to kill a large variety of cancer cells while remaining nontoxic to normal cells.[2] The cancer cell death induction effect of **PL-1** was demonstrated in *in vivo* xenograft models of numerous cancer cell types, indicating that **PL-1** is a potential anti-cancer lead compound. Given its effects on multiple tumor types, **PL-1** must function by inhibiting a cellular target common to many cancers; this is very promising for anti-cancer drug development.[2,3] **PL-1** was shown to interact with glutathione S-transferase *pi* 1 (GSTP1) and carbonyl reductase 1 (CBR1).[2a] However, **PL-1** has only a modest effect on GSTP1 inhibition, and the overexpression of GSTP1 and CBR1 showed no rescue effect on **PL-1** induced cell death.[2a] These reported phenomena suggest that the selective cancer killing effect of **PL-1** might result from its interaction with cellular targets other than GSTP1 and CBR1. Thus, the functional target and precise molecular mechanism responsible for **PL-1**'s selective cancer killing effects still remain unclear.

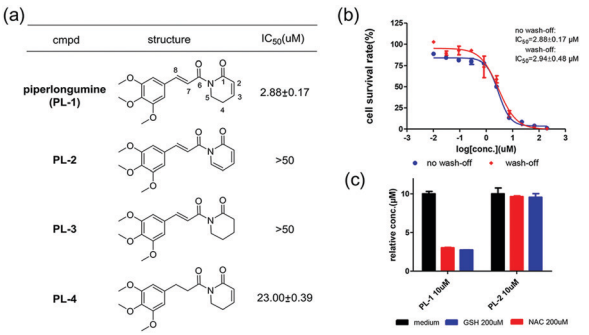

Fig. 1 **PL-1** functions in an irreversible mode. (a) Structure of **PL-1–4** and their cellular activities on NCI-H1975 cells. (b) Cytotoxic activity of **PL-1** in wash/no-wash assays. Under the wash-off condition, NCI-H1975 cells were incubated with **PL-1** for 3 h and then washed with buffer. Cell viability was measured 24 h after **PL-1** administration. (c) The reactivity of **PL-1** and **PL-2** with GSH and NAC. NAC/GSH (200 μM) was added to **PL-1**/**PL-2** (10 μM) in medium (physiological conditions) and incubated for 3 h. The concentration of **PL-1**/**PL-2** was detected by LC-MS/MS before and after the reaction.

Click-reaction-assisted activity-based protein profiling (ABPP) has been developed as a powerful approach to help identify the cellular targets of bioactive molecules.[4] Typically, the probe molecule is designed to have a terminal alkyne inserted into the bioactive parent molecule that facilitates Cu(I)-catalyzed click reactions with azido-linked affinity tags. Here, we describe the findings from the first systematic target identification of **PL-1** using click-reaction-assisted ABPP.

Consistent with previous reports, **PL-1** treatment of a variety of cancer cell lines induced cell death with an IC_{50} at the low micromolar level (Table S1, ESI†).[2] Since the olefins of **PL-1** have strong electrophilicity, we speculated that **PL-1** might covalently bind its target proteins through reaction with nucleophilic residues, most likely cysteine thiols. To test this hypothesis, we conducted wash/no-wash experiments and observed that the IC_{50} of **PL-1** remained the same under both conditions (Fig. 1b), indicating that **PL-1** functions irreversibly. Ablation of the C2–C3

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olefin in compounds **PL-2** and **PL-3** resulted in a significant loss of cytotoxic activity, while ablation of the C7–C8 olefin in compound **PL-4** only resulted in a decreased cytotoxic activity (Fig. 1a and Table S1, ESI†), similar to a previous report.[2b] These results suggest that the electrophilicity of the C2–C3 olefin in **PL-1** is essential for its cytotoxic activity. Similarly, incubating **PL-1** with either glutathione (GSH) or *N*-acetyl-L-cysteine (NAC) caused a significant decrease in the **PL-1** concentration and resulted in the formation of Michael addition products under physiological conditions (Fig. 1c and Fig. S1, ESI†), while **PL-2** remained inert to both GSH and NAC (Fig. 1c). This further confirmed that **PL-1** acts *via* covalent interactions with its targets at its C2–C3 site.

Although numerous studies have explored the targets of **PL-1** in order to explain the mechanism through which it selectively kills cancer cells, few have considered its covalent binding character.[2,3c,e,5] **PL-1**'s reported binding targets GSTP1 and CBR1 were actually identified in affinity purification experiments using solid-phase matrices conjugated with **PL-1** as the bait.[2a] However, this method precludes the identification of candidate targets that covalently bind **PL-1**, which could possibly explain why **PL-1**'s binding to GSTP1 and CBR1 apparently only made a limited contribution to its cytotoxic activity.[2a]

In order to design an active alkynylated analogue of **PL-1** as the probe molecule for click-reaction-assisted ABPP, we conducted the structure–activity relationship (SAR) study of **PL-1** to find a suitable site for incorporation of an alkyne group. The SAR study on the phenyl ring position of the PL structure indicates that this position is able to tolerate structural diversity to some extent (Table S2, ESI†). We incorporated a terminal alkyne at the 4-position of the benzyl ring and removed the $-OCH_3$ groups at the 3- and 5-positions, thereby obtaining **PL-5** (Fig. 2a) as the probe molecule. **PL-5** also functions irreversibly (Fig S2, ESI†) and retains low micromolar activity for cancer cell death induction (Fig. 2a and Table S3, ESI†).

We next performed click-reaction-assisted ABPP with **PL-5** as the probe and **PL-1** as the competitor molecule on NCI-H1975 cells (Fig. S3, ESI†). To identify PL candidate targets that were enriched in the **PL-5** labeled samples and were competed out by **PL-1**, we filtered the protein MS results (>3-fold enrichment of the probe-labeled samples *versus* the out-competed samples; four independent experiments). Glutathione S-transferase omega-1 (GSTO1) was the top-ranking hit in all four experiments and was thus identified as the most likely cellular target of **PL-1**/**PL-5** (Table S4, ESI†). We also observed an obvious **PL-5** mediated enrichment of GSTO1 in western blots and confirmed that this enrichment could be efficiently out-competed by **PL-1** (Fig. 2b).

GSTO1 is a member of the omega class of glutathione S-transferases (GST); these enzymes are responsible for cellular xenobiotic detoxification based on glutathione conjugation.[6] Recently, GSTO1 was reported to play a role in chemotherapeutic resistance and was associated with enhanced aggressiveness of cancer cells, together making GSTO1 an attractive target for anticancer drug development.[7] GSTO1 is atypical among GSTs because it harbors a cysteine residue (Cys32 in human) in its catalytic center; canonical GSTs have characteristic serine or

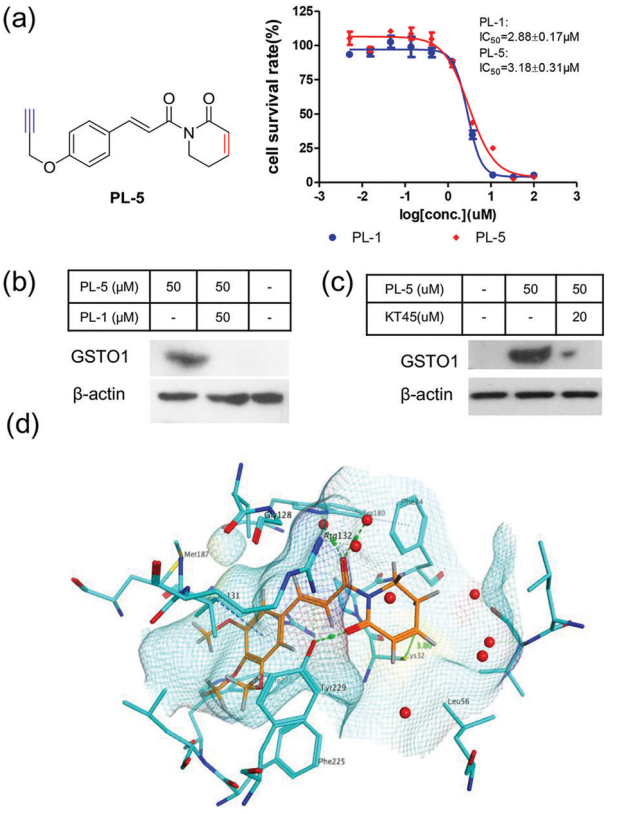

Fig. 2 Identification of GSTO1 as the covalent binding target of **PL-1**. (a) Structure of the ABPP probe **PL-5** and the cytotoxic activity of **PL-5** on NCI-H1975 cells. (b) Western blotting results of ABPP-enriched components using **PL-5** (50 μM) as the probe and **PL-1** (50 μM) as the competitor molecule on NCI-H1975 cells. (c) Western blot showing that the known GSTO1 inhibitor KT45 (20 μM) out-competed **PL-5** (50 μM) for binding with GSTO1 in an ABPP experiment with NCI-H1975 cells. (d) Molecular docking simulation of **PL-1** within the catalytic pocket of GSTO1.

tyrosine residues at this position.[7c] Two series of GSTO1-inhibitor compounds have been discovered to date, and both achieve GSTO1 inhibition through binding to the active Cys32 residue.[8] We found here that KT45—one of the reported GSTO1 inhibitors—showed micromolar activity in cell death induction in all of the cancer cell lines that we tested (Fig. S4 and Table S5, ESI†).[8a] KT45 could also out-compete the binding of **PL-5** with GSTO1 in a follow-up ABPP experiment (Fig. 2c), suggesting that **PL-1** and **PL-5** might share the same binding site as KT45.

Our MS/MS analysis showed that a 5,6-dihydro-2(1*H*)-pyridinone modification was present on the C32 site of GSTO1 in the **PL-5** pulldown samples (Fig. S5, ESI†). Labeling experiments using recombinant GSTO1 with either **PL-1** or **PL-5** also yielded the aforementioned pyridinone modification at the C32 site (Fig. S6, ESI†). These results further supported our previous speculation that **PL-1** covalently binds its target through C2–C3 olefin. We next performed a molecular docking simulation study using the crystal structure of GSTO1 (PBD: 4YQV).[8b] **PL-1** fits snugly in the catalytic pocket of GSTO1 (Fig. 2d): the carbonyl group at the C1 site of **PL-1** forms a hydrogen bond with Tyr229; the –SH group of Cys32 is 3.8 Å from the C3 atom

of the C2–C3 olefin in **PL-1**, and ready for nucleophilic attack; the Ile131 can form an H–π interaction with the phenyl ring of **PL-1**; and the 3,4,5-tri-OCH_3 substituted phenyl ring moiety dives into the pocket, which still has space for structural extension, fully consistent with our SAR results.

Compounds **PL-1** and **PL-5** but not **PL-2** or **PL-3** displayed inhibition of GSTO1 enzyme activity in cell lysates and did not affect the cellular level of the *GSTO1* protein (Fig. 3a, b and Fig. S7, ESI†). We next evaluated how *GSTO1* knockdown affects the viability of cancer cell lines that are sensitive to **PL-1**. Treatment of NCI-H1975, U2OS, HeLa, and A549 cells with *GSTO1* siRNA significantly decreased the viability of each cell type (Fig. 3c), indicating the necessity of GSTO1 for maintaining the survival of these tested cancer cell lines. Consistently, the overexpression of GSTO1 reduced the cell death induction effects of **PL-1** (Fig. 3d). Thus, we can conclude that the cancer cell death induction effect of **PL-1** mainly occurs through its binding and inhibition of GSTO1 and that decreased levels of GSTO1 are deleterious to cancer cell viability.

Both *GSTO1* knockdown and inhibition of GSTO1 by **PL-1** caused increased accumulation of cellular reactive oxygen species (ROS) (Fig. S8, ESI†). However, addition of ROS scavenger NAC after **PL-1** or KT45 treatment could not attenuate the cell death induction caused by these molecules (Fig. S9a, ESI†), suggesting that the observed increase in ROS is not the direct cause of cell death. This is in accordance with our result that pretreatment with NAC failed to rescue cells from death induced by *GSTO1* knockdown (Fig. S9b, ESI†).

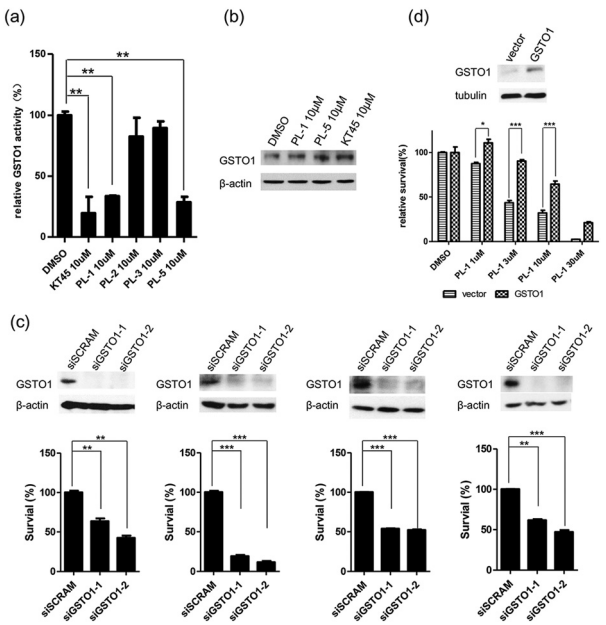

Fig. 3 Cancer cell death is induced *via* **PL-1**'s inhibition of GSTO1. (a) Effects of **PL-1**/**2**/**3**/**5** on GSTO1 catalysed 4-nitrophenacyl glutathione reduction activity in cell lysate (NCI-H1975 cell). DMSO and KT45 were applied as negative and positive controls, respectively. (b) Western blotting of GSTO1 after compound incubation of NCI-H1975 cells for 3 h. (c) Effects of *GSTO1* knockdown with two different GSTO1 siRNAs (10 nM) on survival of NCI-H1975, HeLa, U2OS and A549 cells (left to right). Top: Western blot of GSTO1 after *GSTO1* siRNA transfection. Bottom: Cell survival rate measured after *GSTO1* siRNA transfection. (d) Effects of GSTO1 overexpression on U2OS cells. Left: Western blot of GSTO1 after transfection. Right: Cell survival rate measured after **PL-1** treatment. (control: transfected with empty plasmid.) *: *P* value < 0.05; **: *P* value < 0.01; ***: *P* value < 0.001.

The overexpression of GSTO1 has been found in various types of cancer, including melanomas, lymphomas, and colorectal cancer, among others.[8b] There have also been reports that GSTO1 inhibition may sensitize cancer cells to cell death induced by the popular chemotherapy agent cisplatin.[7a,b] Consistently, several studies have noted that **PL-1** can enhance the effects of other anti-cancer agents (*e.g.*, cisplatin, gemcitabine, *etc.*).[3b,9] Since drug combinations are being applied more and more widely in cancer therapy regimens—especially to overcome drug-resistance and to reduce side-effects—we were very interested in investigating the potential for conceptualizing GSTO1 as a potentially sensitive target for combination cancer therapies. Thus, an anti-cancer library (Table S6, ESI†) composed of 540 compounds that comprised both chemotherapeutic agents and targeted small molecule inhibitors were screened for their combination effect with **PL-1** using Jurkat, NCI-H1975, and HCT116 cells.

In order to investigate **PL-1**'s combination effects with compounds that showed anti-cancer activities, we applied Chou–Talalay analysis to calculate a drug combination index (CI) value.[10] Strikingly, **PL-1** exhibited synergism with a large proportion (average of ~44%) of the active compounds for each of the three cell lines (Table S7, ESI†). For the NCI-H1975 cells, **PL-1** demonstrated synergism for cell death induction with 52.3% of the active compounds, and most of the combination displayed clear (CI < 0.7) or even strong (CI < 0.3) synergistic effects (Tables S7 and S8, ESI†). Similar results were obtained with the Jurkat and HCT-116 cell lines (42% and 38%, Tables S7, S9 and S10, ESI†). This broad-spectrum synergistic effect of **PL-1** with other anti-cancer compounds highlights that GSTO1 inhibition can be viewed as a promising new approach for combination therapies to treat multiple types of cancers.

Most of the compounds identified that could synergize with **PL-1** (100% in NCI-H1975 cells and HCT-116 cells and 98% in Jurkat cells) targeted small molecule anti-cancer agents rather than chemotherapeutic agents (Tables S8–S10, ESI†). Hit compounds that target the PI3K/Akt/mTOR pathway appeared most frequently for each of the three cell lines (27% in NCI-H1975 cells, 19% in Jurkat cells, and 20% in HCT-116 cells) (Fig. 4a and Tables S8–S10, ESI†). We selected the PI3K inhibitor pictilisib as an example compound to evaluate the synergism in detail.[11] **PL-1** significantly sensitized NCI-H1975 and Jurkat cells to pictilisib: in the absence of **PL-1**, pictilisib had an IC_{50} value of 0.16 μM for NCI-H1975 cells, whereas the addition of **PL-1** reduced its IC_{50} value to 0.015 μM, a 10-fold increase in its activity (Fig. 4b). Similarly, experiments with Jurkat cells showed that combination of pictilisib with **PL-1** resulted in an 18-fold increase in activity *vs.* pictilisib alone (Fig. S10a, ESI†). Moreover, the sensitizing effect of **PL-1** was also apparent on Akt phosphorylation monitored with western blotting: although **PL-1** itself did not inhibit phosphorylation of Akt and mTOR (Fig. S11, ESI†), the presence of **PL-1** increased the extent of

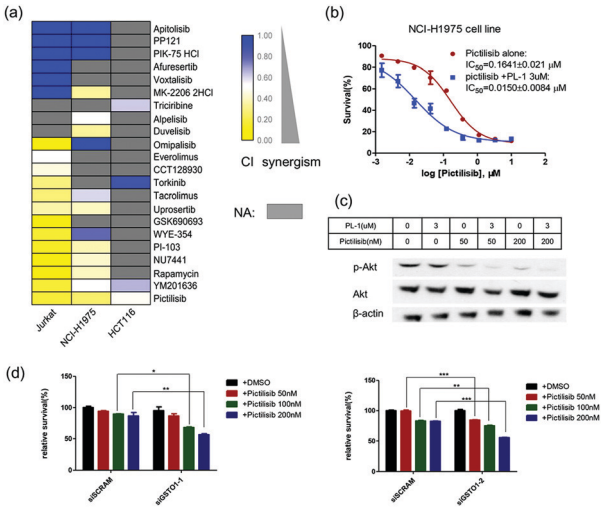

Fig. 4 Both **PL-1** treatment and *GSTO1* knockdown synergize with PI3K/Akt/mTOR pathway inhibitors to induce cancer cell death. (a) Profiling of PI3K/Akt/mTOR pathway inhibitors from screening hits that have synergistic effects in combination with **PL-1**. (b) **PL-1** synergized with pictilisib in cell death induction of NCI-H1975 cells. (c) **PL-1** enhanced the effect of pictilisib in the inhibition of Akt phosphorylation in NCI-H1975 cells. (d) *GSTO1* knockdown synergized with pictilisib in cell death induction in NCI-H1975 cells. *: *P* value < 0.05; **: *P* value < 0.01; ***: *P* value < 0.001.

pictilisib's inhibition on Akt phosphorylation in both NCI-H1975 and Jurkat cells (Fig. 4c and Fig. S10b, ESI†).

To determine whether the observed synergistic effect of **PL-1** with pictilisib resulted from GSTO1 inhibition, we evaluated the combined effect of *GSTO1* knockdown with pictilisib. Just as with the pictilisib and **PL-1** combination treatment, *GSTO1* knockdown in NCI-H1975 cells clearly enhanced the effect of pictilisib on both induction of cell death and inhibition of Akt phosphorylation (Fig. 4d and Fig. S12, ESI†), indicating that GSTO1 inhibition does indeed account for the synergistic effect of **PL-1**. The PI3K/Akt/mTOR pathway is essential for the regulation of cell survival, proliferation, growth, and metabolism, and this signaling pathway is often aberrantly activated in human cancers.[12] Despite extensive research into many inhibitors of this pathway in recent decades, clinical results with these compounds have to date been somewhat lackluster.[12b,c] Therefore, it is possible that our discovery of synergism between **PL-1**'s inhibition of GSTO1 and many PI3K/Akt/mTOR inhibitors can help to reassess and better exploit the therapeutic potential of these compounds to finally deliver on their initial promise as treatments for cancers.

In conclusion, using the click-reaction-assisted ABPP method, our study established that **PL-1**'s covalent binding and inhibition of GSTO1 can explain its strong cytotoxic effect against multiple cancer types. We also revealed the promising prospect of GSTO1 inhibition in combination therapies for cancer treatment by showing that **PL-1** exhibits quite broad-spectrum synergistic effects with many anti-cancer drugs, especially with PI3K/Akt/mTOR pathway inhibitors. Therefore, as GSTO1 may be a universal target for many different types of cancers, researchers can now focus on exploring the potential application of inhibiting GSTO1 alone as well as in combination with other anti-cancer agents as a promising strategy for cancer treatment.

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Conflicts of interest

There are no conflicts to declare.

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