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Synthesis and pharmacological evaluation of bis-3-(3,4-dichlorophenyl) acrylamide derivatives as glycogen phosphorylase inhibitors

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ABSTRACT

During our research using a high-throughput screening system for discovery of a new class of human liver glycogen phosphorylase *a* (hLGPa) inhibitors, a series of 3-(3,4-dichlorophenyl)acrylamide derivatives were synthesized, and their inhibitory activities toward hLGPa were evaluated. Among the derivatives, (2*E*,2′*E*)-*N*,*N*′-pentane-1,5-diylbis[3-(3,4-dichlorophenyl)acrylamide] (**6c**) inhibited hLGPa with an IC_{50} value of 0.023 μM. An X-ray crystallographic study of the enzyme–**6c** complex showed that the inhibitor is bound at the dimer interface site, where the 3,4-dichlorophenyl moiety interacts hydrophobically with the enzyme.

1. Introduction

Diabetes is a chronic and a severe metabolic disorder that occurs when the body does not produce or properly use insulin. There are several types of this disease, with Type 2 being the most common form. Type 2 diabetes is defined by chronic elevated blood glucose levels characterized by peripheral insulin resistance and pancreatic defects associated with insulin secretion.[1,2]

The hepatic glucose output (HGO) system plays an important role in Type 2 diabetic therapy.[3,4] The HGO rate in diabetic patients is significantly higher than that in normal individuals, and the current study indicates that the degradative phosphorylation of glycogen (glycogenolysis) is an important contributor to HGO.[5–7] Human liver glycogen phosphorylase *a* (hLGPa, the active form of human liver GP) is a homodimeric enzyme that catalyzes glycogenolysis. This enzyme has been an attractive molecular target through which HGO can be controlled for the treatment of diabetes, and several kinds of compounds have been discovered.[8–18]

To find novel GP inhibitors, our compound library was subjected to high-throughput screening (HTS). One compound (**1**), which had a symmetric structure containing two 3-(3,4-dichlorophenyl)acrylamide moieties, was found to be a potent inhibitor of hLGPa (IC_{50} = 0.42 μM) and act as a glucose sensor. In the presence of glucose, the enzyme activity of **1** increased to about 10 times (IC_{50} = 0.043 μM) that under glucose free conditions, which indicates that the risk of hypoglycemia can be minimized. Therefore, we considered **1** to be the lead compound in the search to find much more potent compounds via structural modification, such as a mono-amide analogue, bond analogues (at the *trans* double bonds), and a C*n*- or heteroatom-containing chain instead of the C4 chain in **1** (Fig. 1).

It is noteworthy that GP inhibitors that may be structurally related to **1** have also been reported. CP-526423 (**2**) has a symmetrical bis-indole structure, and a single molecule of this compound is situated at the dimer interface in a complex with hLGPa.[19] Compound **3**, from GSK research group, has a 3-(3,4-dichlorophenyl)acrylamide moiety and showed inhibitory activity toward hLGPa in the presence of glucose; however, the binding site of this compound in the enzyme was unrevealed.[20] FR258900 (**4**), which was isolated from the culture broth of a fungal strain and has a bis-3-phenylacrylester structure, showed both enzymatic and in vivo activity.[21,22] The X-ray crystallographic study of the rMGPa in complex with **4** showed that the compound binds not at the dimer interface site, but at the allosteric site on the enzyme.[23] These reports piqued our interest in the binding site on **1** (or its derivatives) considerably (Fig. 2).

In this paper, we report the structure–activity relationships (SARs) and binding site as well as binding mode for these compounds determined via X-ray crystallographic analysis of the enzyme–ligand complex.

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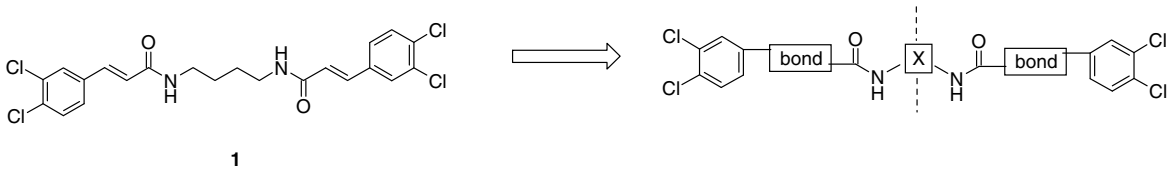

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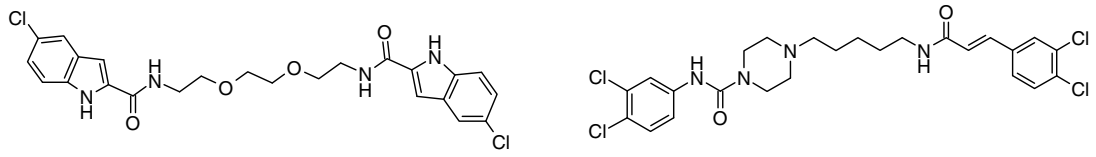
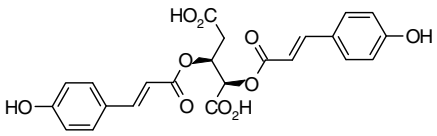
Figure 1. Design of amide derivatives based on **1**.



2: CP-526423

3



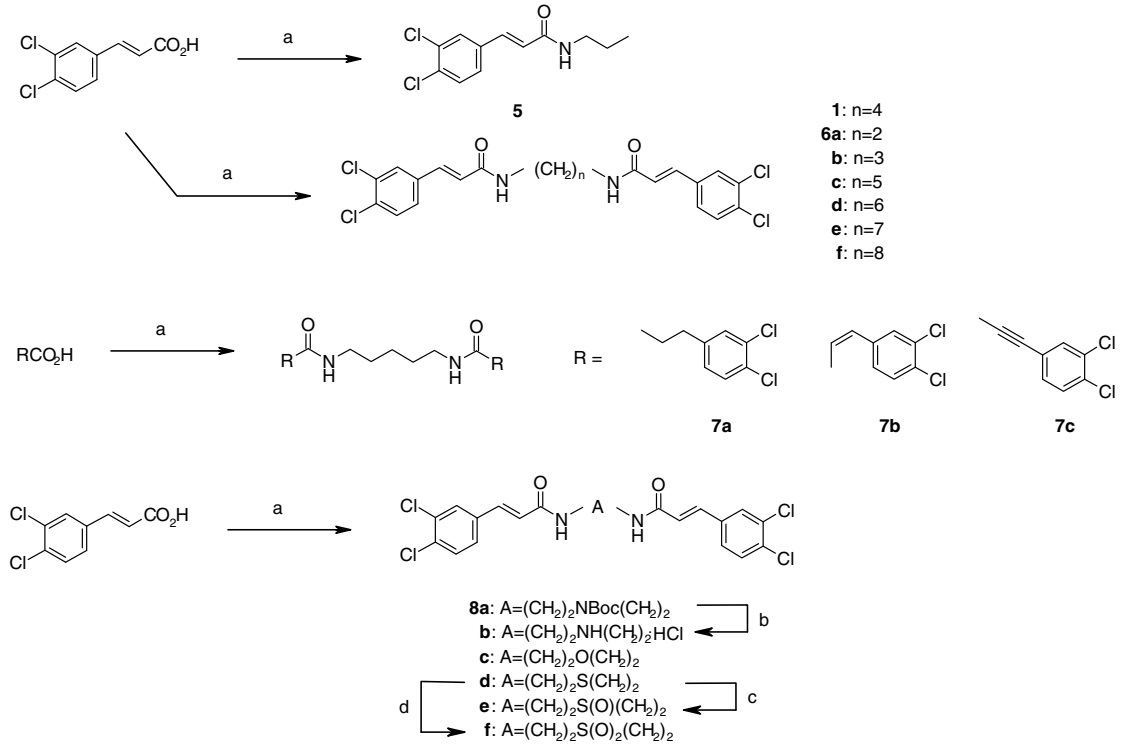
4: FR258900

Figure 2. Examples of GP inhibitors.

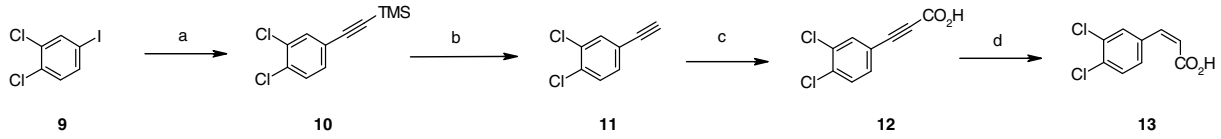
2. Chemistry

Compounds **1, 5, 6a–f, 7a–c**, and **8a–f** were synthesized via coupling reactions between carboxylic acids and various amines, in some cases followed by the conversion of the substituent parts, as shown in Scheme 1.

Carboxylic acids were prepared as the starting material for the *cis* double and triple bond analogues of **6c** shown in Scheme 2.



Scheme 1. Synthesis of compounds **1**, **5**, **6a–f**, **7a–c**, and **8a–f**. Reagents and conditions: (a) amine, WSC·HCl, HOBt, DMF; (b) 4 M HCl/AcOEt, EtOH; (c) MCPBA (1.0 equiv), CH_2Cl_2, 0 °C; (d) MCPBA (4.0 equiv), CH_2Cl_2, rt.



Scheme 2. Synthesis of carboxylic acids **12** and **13**. Reagents and conditions: (a) TMSCCH, $PdCl_2(PPh_3)_2$, CuI, $NHEt_2$; (b) KOH, MeOH, H_2O; (c) *n*-BuLi, CO_2, THF; (d) H_2/Pd–$BaSO_4$, quinoline, MeOH.

The coupling reaction between 1,2-dichloro-4-iodobenzene (**9**) with trimethylsilylacetylene, followed by deprotection under basic conditions, yielded 1,2-dichloro-4-ethynylbenzene (**11**).[24] The carboxylation of **11**, followed by partial hydrogenation, afforded **12** and **13**, respectively.

3. Results and discussion

Compounds were evaluated for their inhibitory activity toward hLGPa. The structures and activities are listed in Tables 1–3.

The bis-amide structure in **1** seemed to be critical to inhibitory activity, because the mono-amide compound (**5**) showed no activity. Shortening the butylene chain into an ethylene (**6a**) or propylene chain (**6b**) caused a decline in inhibitory activity, while the presence of a pentylene (**6c**) chain caused it to increase twofold in both the absence and presence of glucose (−Glu and +Glu). The hexylene (**6d**), heptylene (**6e**), and octylene (**6f**) analogues were not tolerated. Therefore, the length of the chain between the two amide parts was strictly limited, with the five-carbon chain being preferable (Table 1). In addition, the *trans* double bonds in the acrylamide part were clearly essential moieties since the activity with the single bond analogue (**7a**), *cis* double bond analogue (**7b**), and triple bond analogue (**7c**) were all dramatically less (Table 2). When heteroatoms were introduced into the pentylene chain in **6c**, a nitrogen atom in the center of the chain (**8a**,**b**) led to a loss of inhibitory activity, but both an oxygen atom (**8c**) and a sulfur atom (**8d**–**f**) were tolerated (Table 3).

To determine the binding site and binding mode of the compounds mentioned above, the crystal structure of the hLGPa–**6c** complex was elucidated at a resolution of 2.45 Å (Fig. 3A, B). The structure showed that **6c** existed not at the allosteric site where **4** binds, but at the dimer interface site. In addition, one molecule of **6c** acted as a bridge between the two dimers, which would stabilize the less active conformational state of the enzyme and lead to inhibitory activity.

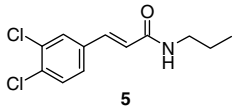
The structures also indicate that the 3,4-dichlorophenyl moieties interact with a hydrophobic pocket formed by amino acid residues such as Arg60, Val64, Trp67, Asp227, Pro229, Pro188, Trp189, Glu190, Lys191, Phe37′, Thr38′, and Val40′, where the prime (′) indicates the second subunit. No significant hydrophilic interactions between the enzyme and either of the two amide moieties were observed.

The role of the *trans* double bond in the inhibitory activity of the 3-(3,4-dichlorophenyl) acrylamide moieties could not be clearly determined from this structure. However, it probably has the ability to form a favorable conformation because the other bond analogues (**7a–c**) do not show potent activity.

The reason for the inactivity of the amine analogues (**8a,b**) could not be definitively determined from this structure either; however, it was deduced that the Boc moiety in **8a** contains steric hindrance, and the nitrogen atom in **8b** might have undesirable electrostatic interactions with the hydrophilic regions in the dimer interface, which would lead to an inappropriate bridge form.

Since the position of **6c** in hLGPa was very similar to that of indole-type inhibitors, the superposition of the crystal structure of **6c** and 5-chloro-*N*-[4-(1,2-dihydroxyethyl)phenyl]-1*H*-indole-2-car-

Table 1
SAR of compounds **1**, **5**, and **6a–f**[a]



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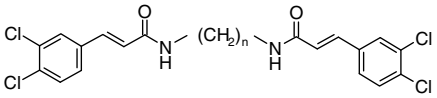
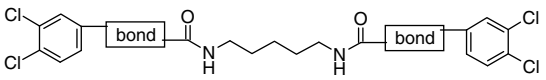


1, 6a-f

Entry	Compound	*n*	hLGPa (−Glu) IC_{50} (μM)	hLGPa (+Glu) IC_{50} (μM)
1	**5**		0%	NT
2	**6a**	2	20	NT
3	**6b**	3	55	NT
4	**1**	4	0.42	0.043
5	**6c**	5	0.26	0.023
6	**6d**	6	18	NT
7	**6e**	7	11	NT
8	**6f**	8	33	NT

[a] Inhibitory activities are expressed as the IC_{50} or as % inhibition at a concentration of 10 μM.

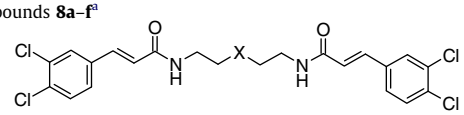
Table 2
SAR of compounds **7a–c**[a]



Entry	Compound	Bond	hLGPa (−Glu) IC_{50} (μM)
1	**7a**	—CH_2-CH_2—	45
2	**7b**		36%
3	**7c**	—C≡C—	59

[a] Inhibitory activities are expressed as the IC_{50} or as % inhibition at a concentration of 10 μM.

Table 3
SAR of compounds **8a–f**[a]



Entry	Compound	X	hLGPa (−Glu) IC_{50} (μM)	hLGPa (+Glu) IC_{50} (μM)
1	**8a**	NBoc	48%	NT
2	**8b**	NH·HCl	51%	NT
3	**8c**	O	0.43	0.026
4	**8d**	S	0.45	0.012
5	**8e**	SO	0.31	0.026
6	**8f**	SO_2	1.5	NT

[a] Inhibitory activities are expressed as the IC_{50} or as % inhibition at a concentration of 10 μM.

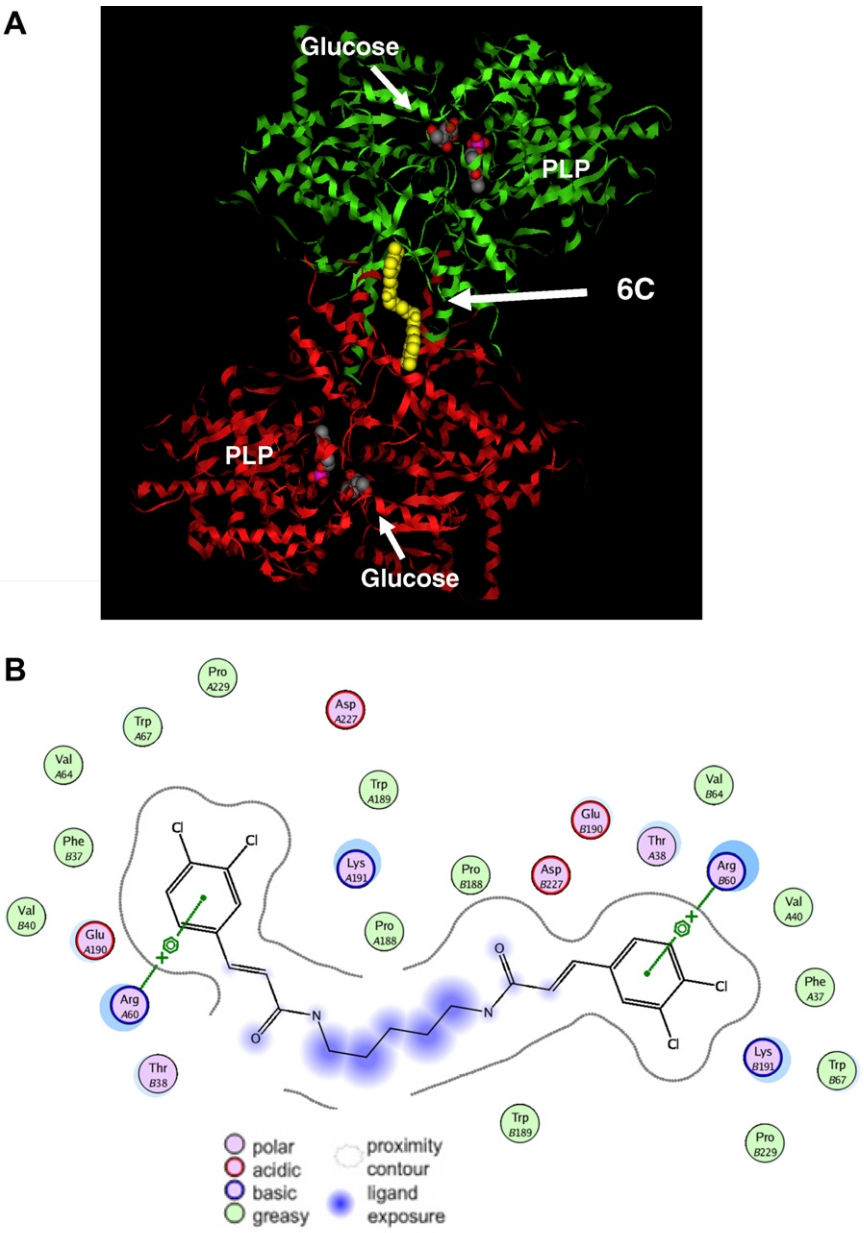


Figure 3. (A) Crystal structure of **6c** bound to the hLGPa dimer interface. Glucose and the essential cofactor pyridoxal 5′-phosphate (PLP) are situated at the catalytic site. (B) Schematic diagram of the interaction of **6c** with hLGPa as revealed by crystallographic analysis. The protein residues of the two hLGPa molecules in the dimer are labeled as A and B, respectively. The figure was prepared using the ligand interactions application in MOE.[25]

boxamide (**14**) was examined in order to compare their binding styles (Fig. 4).

As shown in Figure 4, the planar 3,4-dichlorophenyl moiety is well-situated in proximity to the center of the indole ring in **14**, and the 3,4-dichlorophenyl ring itself seems to interact hydrophobically with hLGPa, as would a 5-chloroindole ring. It should be noted that, in the case of **14**, the hydrophobic interactions between the 5-chloroindole moiety and the enzyme, as well as the hydrophilic interactions between the amide moiety and the two hydroxyl groups with the enzyme, were both important to inhibitory activity.[26] This indicates that **6c** and **14** utilize the same binding site on GP, but their means of inhibition are different.

The activity of **2** and the derivative mentioned above were then compared. According to the literature, **2** has very potent inhibitory activity against hLGPa (IC_{50} = 6 nM),[19] while the activity of the *C*8 derivative (**6f**), which has the same chain length as **2**, is very weak. It has been suggested that this is due to the lack of interaction between the amide moieties and the enzyme. The alkylene chain on **6f** is too flexible to link with the dimer in an inactive conformation; therefore, the shorter chain seems to be necessary. Binding with the *C*5 derivative (**6c**) resulted in tight control of the dimer and potent activity.

Unfortunately, this series of compounds did not inhibit glucagon-induced glucose output in cultured primary hepatocytes, probably because of their poor solubility and/or membrane permeability. Efforts to modify the structures of these compounds to achieve cell-based activity will continue.

4. Conclusion

To summarize, a series of bis-3-(3,4-dichlorophenyl)acrylamide derivatives were synthesized as a new class of hLGPa inhibitors.

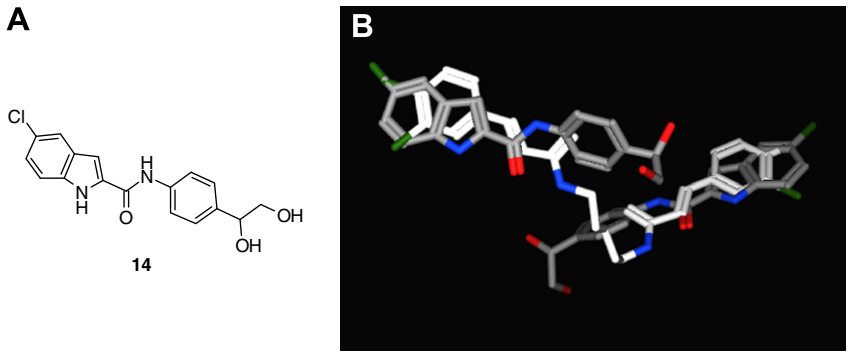


Figure 4. (A) Chemical structure of **14**. (B) An overview of the docking model for **6c** with **14**.

The bis-amide structures and the presence of a *trans* double bond in the acrylamides were found to be essential. The length of the carbon chain connecting the two amide moieties was also found to be important to activity. An X-ray crystallographic study of the enzyme–**6c** complex suggested that this compound is the first example of interaction with the dimer interface site on an enzyme, except for that seen with indole-type compounds or related derivatives. The hydrophobic interaction between the 3,4-dichlorophenyl moiety and hLGPa was also important to inhibitory activity.

5. Experimental

5.1. Chemistry

^{1}H NMR spectra were recorded on a JEOL JNM-LA300 or JEOL JNM-EX400 spectrometer, with the chemical shifts expressed in δ (ppm) using tetramethylsilane as an internal standard (NMR abbreviations: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, and br = broad peak). Mass spectra were recorded on a JEOL JMS-700T or the micromass Q-T of an Ultima API spectrometer. The elemental analyses were performed with a Yanaco MT-5 microanalyzer (C, H, N), and were within ±0.4% of theoretical values. During the work-up, organic solutions were dried over anhydrous $MgSO_4$ or Na_2SO_4.

5.1.1. (2*E*,2′*E*)-*N*,*N*′-Butane-1,4-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (1)

1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (210 mg, 1.10 mmol) and 1-hydroxybenzotriazole hydrate (170 mg, 1.11 mmol) were added to a solution of (2*E*)-3-(3,4-dichlorophenyl)acrylic acid (217 mg, 1.00 mmol) and 1,4-butylenediamine (44 mg, 0.50 mmol) in DMF (5 mL), and this mixture was stirred at room temperature for 18 h. H_2O (20 mL) was added to the reaction mixture, and the resulting precipitate was collected and washed with H_2O (20 mL) and EtOH/AcOEt (1:1, 10 mL) to yield **1** (206 mg, 85%) as a colorless solid: ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.90–2.10 (4 H, m), 3.15–3.25 (4H, m), 6.68 (2H, d, J = 15.6 Hz), 7.37 (2H, d, J = 16.1 Hz), 7.55 (2H, dd, J = 8.3, 1.9 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 1.9 Hz), 8.13 (2H, t, J = 5.3 Hz). FABMS *m/z*: 487 (M+1)$^+$. Anal. Calcd for $C_{22}H_{20}Cl_4N_2O_2$: C, 54.35; H, 4.15; N, 5.76. Found: C, 54.21; H, 3.95; N, 6.08.

5.1.2. (2*E*)-3-(3,4-Dichlorophenyl)-*N*-propylacrylamide (5)

This compound was prepared in the same manner as **1** using 1-propylamine instead of 1,4-butylenediamine (93% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.88 (3H, t, J = 7.4 Hz), 1.42–1.52 (2H, m), 3.14 (2H, q, J = 6.8 Hz), 6.69 (1H, d, J = 15.6 Hz), 7.39 (1H, d, J = 15.6 Hz), 7.56 (1H, dd, J = 8.8, 2.0 Hz), 7.67 (1H, d, J = 8.3 Hz), 7.84 (1H, d, J = 2.0 Hz), 8.10 (1H, t, J = 5.4 Hz). FABMS *m/z*: 258 (M+1)$^+$. Anal. Calcd for $C_{12}H_{13}Cl_2NO$: C, 55.83; H, 5.08; N, 5.43. Found: C, 55.83; H, 4.91; N, 5.43.

5.1.3. (2*E*,2′*E*)-*N*,*N*′-Ethane-1,2-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (6a)

This compound was prepared in the same manner as **1** using 1,2-ethylenediamine instead of 1,4-butylenediamine to obtain a quantitative yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.27–3.33 (4H, m), 6.68 (2 H, d, J = 15.6 Hz), 7.42 (2H, d, J = 16.2 Hz), 7.57 (2H, dd, J = 8.3, 2.0 Hz), 7.67 (2H, d, J = 8.3 Hz), 7.85 (2H, d, J = 1.9 Hz), 8.20–8.27 (2H, m). FABMS *m/z*: 459 (M+1)$^+$. Anal. Calcd for $C_{20}H_{16}Cl_4N_2O_2$: C, 52.43; H, 3.52; N, 6.11. Found: C, 52.35; H, 3.42; N, 6.20.

5.1.4. (2*E*,2′*E*)-*N*,*N*′-Propane-1,3-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (6b)

This compound was prepared in the same manner as **1** using 1,3-propylenediamine instead of 1,4-butylenediamine (76% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.60–1.70 (2H, m), 3.23 (4H, q, J = 6.8 Hz), 6.69 (2H, d, J = 15.6 Hz), 7.40 (2H, d, J = 16.1 Hz), 7.56 (2H, dd, J = 8.3, 1.9 Hz), 7.67 (2H, d, J = 8.8 Hz), 7.85 (2H, d, J = 1.9 Hz), 8.15 (2H, t, J = 5.3 Hz). FABMS *m/z*: 473 (M+1)$^+$. Anal. Calcd for $C_{21}H_{18}Cl_4N_2O_2$: C, 53.42; H, 3.84; N, 5.93. Found: C, 53.26; H, 3.81; N, 6.11.

5.1.5. (2*E*,2′*E*)-*N*,*N*′-Pentane-1,5-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (6c)

This compound was prepared in the same manner as **1** using 1,5-pentylenediamine instead of 1,4-butylenediamine (42% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.30–1.40 (2H, m), 1.43–1.55 (4H, m), 3.18 (4H, q, J = 6.3 Hz), 6.68 (2H, d, J = 15.6 Hz), 7.38 (2H, d, J = 15.6 Hz), 7.54 (2H, dd, J = 8.3, 1.9 Hz), 7.65 (2H, d, J = 8.3 Hz), 7.82 (2H, d, J = 1.5 Hz), 8.10 (2H, t, J = 5.4 Hz). FABMS *m/z*: 501 (M+1)$^+$. Anal. Calcd for $C_{23}H_{22}Cl_4N_2O_2$: C, 55.22; H, 4.43; N, 5.60. Found: C, 54.99; H, 4.32; N, 5.54.

5.1.6. (2*E*,2′*E*)-*N*,*N*′-Hexane-1,6-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (6d)

This compound was prepared in the same manner as **1** using 1,6-hexylenediamine instead of 1,4-butylenediamine (86% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.25–1.37 (4H, m), 1.40–1.53 (4H, m), 3.10–3.22 (4H, m), 6.68 (2H, d, J = 15.6 Hz), 7.38 (2H, d, J = 15.6 Hz), 7.55 (2H, dd, J = 8.3, 1.5 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 1.5 Hz), 8.10 (2H, t, J = 5.3 Hz). FABMS *m/z*: 515 (M+1)$^+$. Anal. Calcd for $C_{24}H_{24}Cl_4N_2O_2$: C, 56.05; H, 4.70; N, 5.45. Found: C, 55.96; H, 4.50; N, 5.72.

5.1.7. (2*E*,2′*E*)-*N*,*N*′-Heptane-1,7-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (6e)

This compound was prepared in the same manner as **1** using 1,7-heptylenediamine instead of 1,4-butylenediamine (72% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.25–1.35 (6H, m), 1.35–1.50 (4H, m), 3.17 (4H, q, J = 6.3 Hz), 6.68 (2H, d, J = 15.6 Hz), 7.38 (2H, d, J = 15.6 Hz), 7.55 (2H, dd, J = 8.3, 2.0 Hz), 7.68 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 2.0 Hz), 8.09 (2H, t, J = 5.9 Hz). FABMS m/z: 529 (M+1)$^+$. Anal. Calcd for $C_{25}H_{26}Cl_4N_2O_2$: C, 56.84; H, 4.96; N, 5.30. Found: C, 56.62; H, 4.98; N, 5.38.

5.1.8. (2*E*,2′*E*)-*N*,*N*′-Octane-1,8-diylbis[3-(3,4-dichlorophenyl)-acrylamide] (6f)

This compound was prepared in the same manner as **1** using 1,8-octylenediamine instead of 1,4-butylenediamine (81% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.25–1.32 (8H, m), 1.40–1.50 (4H, m), 3.16 (4H, q, J = 6.3 Hz), 6.68 (2H, d, J = 16.1 Hz), 7.38 (2H, d, J = 15.6 Hz), 7.55 (2H, dd, J = 8.3, 1.9 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 1.9 Hz), 8.09 (2H, t, J = 5.9 Hz). FABMS m/z: 543 (M+1)$^+$. Anal. Calcd for $C_{25}H_{26}Cl_4N_2O_2$: C, 57.58; H, 5.20; N, 5.17. Found: C, 57.47; H, 5.24; N, 5.17.

5.1.9. *N*,*N*′-Pentane-1,5-diylbis[3-(3,4-dichlorophenyl)-propanamide] (7a)

This compound was prepared in the same manner as **1** using 3-(3,4-dichlorophenyl)propionic acid instead of (2*E*)-3-(3,4-dichlorophenyl)acrylic acid (84% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.05–1.14 (2H, m), 1.25–1.35 (4 H, m), 2.36 (4H, t, J = 7.4 Hz), 2.81 (4H, t, J = 7.4 Hz), 2.97 (4H, q, J = 6.8 Hz), 7.18 (2H, dd, J = 8.3, 1.9 Hz), 7.44–7.53 (4H, m), 7.76 (2H, t, J = 5.4 Hz). FABMS m/z: 505 (M+1)$^+$. Anal. Calcd for $C_{23}H_{26}Cl_4N_2O_2$: C, 54.78; H, 5.20; N, 5.56. Found: C, 55.14; H, 5.22; N, 5.65.

5.1.10. (2*Z*,2′*Z*)-*N*,*N*′-Pentane-1,5-diylbis[3-(3,4-dichlorophenyl)acrylamide] (7b)

This compound was prepared in the same manner as **1** using (2*Z*)-3-(3,4-dichlorophenyl)acrylic acid instead of (2*E*)-3-(3,4-dichlorophenyl)acrylic acid (30% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.20–1.27 (2H, m), 1.40–1.45 (4H, m), 3.08 (4H, q, J = 6.3 Hz), 6.09 (2H, d, J = 12.7 Hz), 6.65 (2H, d, J = 12.7 Hz), 7.56–7.59 (4H, m), 8.00 (2 H, d, J = 1.5 Hz), 8.21 (2H, t, J = 5.8 Hz). FABMS m/z: 501 (M+1)$^+$. Anal. Calcd for $C_{23}H_{22}Cl_4N_2O_2$: C, 55.22; H, 4.43; N, 5.60. Found: C, 55.08; H, 4.36; N, 5.59.

5.1.11. *N*,*N*′-Pentane-1,5-diylbis[3-(3,4-dichlorophenyl)prop-2-ynamide] (7c)

This compound was prepared in the same manner as **1** using 3-(3,4-dichlorophenyl)prop-2-ynoic acid instead of (2*E*)-3-(3,4-dichlorophenyl)acrylic acid (8.3% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.25–1.35 (2H, m), 1.40–1.55 (4H, m), 3.14 (4H, q, J = 6.4 Hz), 7.56 (2H, dd, J = 6.4, 1.9 Hz), 7.72 (2H, d, J = 8.3 Hz), 7.87 (2H, d, J = 1.9 Hz), 8.83 (2H, t, J = 5.3 Hz). FABMS m/z: 497 (M+1)$^+$. Anal. Calcd for $C_{23}H_{18}Cl_4N_2O_2$: C, 55.67; H, 3.66; N, 5.65. Found: C, 55.39; H, 3.71; N, 5.60.

5.1.12. *tert*-Butyl bis(2-{[(2*E*)-3-(3,4-dichlorophenyl)prop-2-enoyl]amino}ethyl)carbamate (8a)

This compound was prepared in the same manner as **1** using *tert*-butyl bis(2-aminoethyl)carbamate instead of 1,4-butylenediamine (42% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.37 (9H, s), 3.24–3.36 (8H, m), 6.68 (2H, d, J = 15.6 Hz), 7.40 (2H, d, J = 15.6 Hz), 7.55 (2H, dd, J = 8.3, 2.0 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 2.0 Hz), 8.14–8.30 (2H, m). FABMS m/z: 602 (M+1)$^+$. HRMS Calcd for $C_{27}H_{29}Cl_4N_3O_4$ (M+1)$^+$: 600.0985. Found: 600.0992.

5.1.13. (2*E*,2′*E*)-*N*,*N*′-(Iminodiethane-2,1-diyl)bis[3-(3,4-dichlorophenyl)acrylamide] hydrochloride (8b)

A HCl solution in AcOEt (4 M, 5 mL) was added to a solution of **8a** (1.03 g, 1.71 mmol) in EtOH (20 mL), and this mixture was stirred at room temperature for 4 h. The resulting mixture was concentrated in vacuo and the residue was washed with AcOEt to yield **8b** (700 mg, 76%) as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.05–3.17 (4H, m), 3.51 (4H, q, J = 5.9 Hz)), 6.71 (2H, d, J = 15.6 Hz), 7.43 (2H, d, J = 15.6 Hz), 7.55 (2H, dd, J = 8.3, 1.9 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.83 (2H, d, J = 1.9 Hz), 8.54 (2H, t, J = 5.9 Hz), 8.94 (2H, br s). FABMS m/z: 502 (M+1)$^+$. As free base. Anal. Calcd for $C_{22}H_{21}Cl_4N_3O_2$·HCl: C, 49.14; H, 4.12; N, 7.81. Found: C, 48.99; H, 4.15; N, 7.64.

5.1.14. (2*E*,2′*E*)-*N*,*N*′-(Oxydiethane-2,1-diyl)bis[3-(3,4-dichlorophenyl)acrylamide] (8c)

This compound was prepared in the same manner as **1** using 2,2′-oxydiethanamine instead of 1,4-butylenediamine (87% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.36 (4H, q, J = 5.9 Hz), 3.51 (4H, t, J = 5.8 Hz), 6.74 (2H, d, J = 15.6 Hz), 7.39 (2H, d, J = 16.1 Hz), 7.53 (2H, dd, J = 8.3, 2.0 Hz), 7.63 (2H, d, J = 8.3 Hz), 7.81 (2H, d, J = 1.5 Hz), 8.15 (2H, t, J = 5.4 Hz). FABMS m/z: 503 (M+1)$^+$. Anal. Calcd for $C_{22}H_{20}Cl_4N_2O_3$·0.25H_2O: C, 52.15; H, 4.08; N, 5.53. Found: C, 52.08; H, 3.87; N, 5.64.

5.1.15. (2*E*,2′*E*)-*N*,*N*′-(Thiodiethane-2,1-diyl)bis[3-(3,4-dichlorophenyl)acrylamide] (8d)

This compound was prepared in the same manner as **1** using 2,2′-thiodiethanamine instead of 1,4-butylenediamine (92% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.67 (4H, t, J = 6.8 Hz), 3.38 (4H, q, J = 6.4 Hz), 6.71 (2H, d, J = 15.6 Hz), 7.40 (2H, d, J = 15.6 Hz), 7.56 (2H, dd, J = 8.3, 2.0 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.84 (2H, d, J = 2.0 Hz), 8.28 (2H, t, J = 5.8 Hz). FABMS m/z: 519 (M+1)$^+$. Anal. Calcd for $C_{22}H_{20}Cl_4N_2O_2S$: C, 50.98; H, 3.89; N, 5.41. Found: C, 50.77; H, 3.83; N, 5.69.

5.1.16. (2*E*,2′*E*)-*N*,*N*′-(Sulfinyldiethane-2,1-diyl)bis[3-(3,4-dichlorophenyl)acrylamide] (8e)

3-Chloroperbenzoic acid (70%, 123 mg, 0.50 mmol) was added to a stirred solution of **8d** (259 mg, 0.50 mmol) in CH_2Cl_2 (50 mL) at 0 °C, and this mixture was stirred at 0 °C for 50 min. The resulting solids were collected and washed with CH_2Cl_2 to yield **8e** (177 mg, 66%) as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.85–2.93 (2H, m), 3.00–3.06 (2H, m), 3.48–3.66 (4H, m), 6.68 (2H, d, J = 15.6 Hz), 7.41 (2H, d, J = 15.6 Hz), 7.55 (2H, dd, J = 8.3, 1.9 Hz), 7.66 (2H, d, J = 8.3 Hz), 7.84 (2H, d, J = 2.0 Hz), 8.43 (2H, t, J = 5.9 Hz). FABMS m/z: 535 (M+1)$^+$. Anal. Calcd for $C_{22}H_{20}Cl_4N_2O_3S$·0.2H_2O: C, 49.12; H, 3.82; N, 5.21. Found: C, 48.97; H, 3.61; N, 5.25.

5.1.17. (2*E*,2′*E*)-*N*,*N*′-(Sulfonyldiethane-2,1-diyl)bis[3-(3,4-dichlorophenyl)acrylamide] (8f)

3-Chloroperbenzoic acid (70%, 247 mg, 1.00 mmol) was added to a stirred solution of **8d** (259 mg, 0.50 mmol) in CH_2Cl_2 (50 mL) at 0 °C, and this mixture was stirred at room temperature for 2 h. The resulting solid was collected and washed with CH_2Cl_2. *m*-CPBA (70%, 247 mg, 1.00 mmol) was added to a suspension of this solid in CH_2Cl_2 (100 mL), and this mixture was stirred at room temperature for 1 h. The resulting solids were collected and washed with CH_2Cl_2 followed by heated EtOH/AcOEt to yield **8f** (33 mg, 12%) as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.37 (4H, t, J = 6.3 Hz), 3.60 (4H, t, J = 6.3 Hz), 6.68 (2H, d, J = 15.7 Hz), 7.39 (2H, d, J = 16.1 Hz), 7.53 (2H, dd, J = 8.3, 1.5 Hz), 7.65 (2H, d, J = 8.3 Hz), 7.81 (2H, d, J = 1.5 Hz), 8.38 (2H, t, J = 5.9 Hz). FABMS m/z: 551 (M+1)$^+$. Anal. Calcd for $C_{22}H_{20}Cl_4N_2O_4S$: C, 48.02; H, 3.66; N, 5.09. Found: C, 48.06; H, 3.76; N, 5.16.

5.1.18. [(3,4-Dichlorophenyl)ethynyl](trimethyl)silane (10)

Bis(triphenylphosphine)palladium dichloride (480 mg, 0.684 mmol) and copper iodide (33 mg, 0.173 mmol) were added

to a mixture of 1,2-dichloro-4-iodobenzene (9.34 g, 34.2 mmol) and trimethylsilylacetylene (4.03 g, 41.0 mmol) in diethylamine (100 mL), and this mixture was stirred at 45 °C for 2 h. The resulting mixture was concentrated in vacuo and the residue was partitioned between ether (300 mL) and H_2O (300 mL), and the ether layer was washed with saturated NaCl solution, and then dried and concentrated in vacuo. The residue was purified via column chromatography on silica gel (*n*-hexane) to yield **10** (8.26 g, 99%) as a slightly yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.24 (9H, s), 7.27 (1H, dd, J = 8.3, 2.0 Hz), 7.37 (1 H, d, J = 8.3 Hz), 7.54 (1H, d, J = 1.8 Hz). EIMS *m/z*: 242 $(M)^+$.

5.1.19. 1,2-Dichloro-4-ethynylbenzene (11)

1 M KOH (18 mL) was added to a solution of **10** (4.00 g, 16.4 mmol) in MeOH (40 mL), and this mixture was stirred at room temperature for 3 h. The resulting mixture was concentrated in vacuo, and the residue was partitioned between *n*-hexane (150 mL) and 0.5 M HCl (100 mL). The *n*-hexane layer was washed with saturated NaCl solution, and then dried and concentrated in vacuo to yield **11** (2.55 g, 91%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.14 (1H, s), 7.30 (1H, dd, J = 8.3, 1.8 Hz), 7.40 (1H, d, J = 8.3 Hz), 7.57 (1H, d, J = 1.8 Hz). EIMS *m/z*: 170 $(M)^+$.

5.1.20. 3-(3,4-Dichlorophenyl)prop-2-ynoic acid (12)

n-BuLi (1.56 M solution in *n*-hexane, 17 mL, 26.5 mmol) was added to a solution of **11** (3.95 g, 23.1 mmol) in THF (50 mL) at 0 °C in an argon atmosphere. This mixture was stirred at room temperature for 1 h, then CO_2 (solid, 5.00 g) was added at 0 °C. The resulting mixture was stirred at room temperature for 1 h, then concentrated in vacuo and the residue was partitioned between AcOEt (200 mL) and 0.5 M HCl (200 mL). The AcOEt layer was washed with saturated NaCl solution, and then dried and concentrated in vacuo. The residue was washed with *n*-hexane to yield **12** (2.06 g, 41%) as a slightly yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.63 (1H, dd, J = 8.4, 2.0 Hz), 7.75 (1H, d, J = 8.4 Hz), 7.97 (1H, d, J = 1.8 Hz). FABMS *m/z*: 213 $(M-1)^+$.

5.1.21. (2*Z*)-3-(3,4-Dichlorophenyl)acrylic acid (13)

Quinoline (25 μL) and Pd/$BaSO_4$ (5% w/w, 30 mg) were added to a solution of **12** (302 mg, 1.40 mmol) in MeOH (5 mL), and this mixture was stirred in a hydrogen atmosphere for 2.5 h. The catalyst was then filtered through Celite and the filtrate was concentrated in vacuo to yield **13** (260 mg, 86%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.03 (1H, d, J = 12.6 Hz), 6.94 (1H, d, J = 12.6 Hz), 7.42–7.44 (2H, m), 7.69 (1H, s). EIMS *m/z*: 216 $(M)^+$.

5.2. Pharmacology

5.2.1. Glycogen phosphorylase activity (in the presence of glucose)

The activity of recombinant hLGPa in the forward direction was measured by determination of NADPH according to the methods of Pesce et al. (modified).[27] The enzyme activity was assayed at pH 6.8 in a phosphate buffer containing KH_2PO_4 (45 mM), β-NADP (22.5 μM), α-glucose 1,6-bisphosphate (4×10^{-4}%), glucose-6-phophate dehydrogenase (385 U/L), phosphoglucomutase (77 U/L), glycogen (0.24%), and glucose (10.85 mM). The test compounds were added as 10 μL of solution in DMSO prior to the addition of the enzyme. The basal rate of hLGPa enzyme activity in the absence of inhibitors (control) was determined by adding 10 μL of DMSO. The rate of hLGPa enzyme activity was fully inhibited after adding 10 μL of 200 mM of the positive control test substance, caffeine. Afterwards, 216.5 μL of phosphate buffer was added, and the reaction was started by adding 23.5 μL hLGPa solution (40 mM β-glycerophosphate and 80 mM cysteine, pH 6.8). After the reaction was conducted at room temperature, the conversion of oxidized β-NADP to reduced β-NADPH was measured at 340 nm for 2 h. The hLGPa inhibition IC_{50} values were calculated using the logistic regression method with SAS software.

5.3. Crystallization and data collection

The crystal of hLGP complexed with **6c** was obtained at 4 °C using the hanging drop vapor diffusion method. The hanging drop consisted of an equal volume of the concentrated protein solution (20 mg/mL) and a reservoir solution containing 100 mM NaMES (pH 6), 22.5% MPD, 60 mM D-glucose, and 0.9 mM **6c**. The crystal belongs to the trigonal space group $P3_1$, with cell dimensions of $a = b = 124.1$ Å and $c = 123.6$ Å. The diffraction data set was 99.8% complete to 2.43 Å. The molecular replacement method was used to solve the structure, using the published hLGP/CP-403700 complex structure (PDB code 1EXV) as the starting model.[10] The CNX[28] program was used for refinement to 2.45 Å resolution with a final *R*-factor of 0.241 (R_{free}: 0.279).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.08.010.

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