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Benzoylbenzimidazole-based selective inhibitors targeting *Cryptosporidium parvum* and *Toxoplasma gondii* calcium-dependent protein kinase-1

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Abstract

Calcium-dependent protein kinase-1 (CDPK1) from *Cryptosporidium parvum* (CpCDPK1) and *Toxoplasma gondii* (TgCDPK1) have become attractive targets for discovering selective inhibitors to combat infections caused by these protozoa. We used structure-based design to improve a series of benzoylbenzimidazole-based compounds in terms of solubility, selectivity, and potency against CpCDPK1 and TgCDPK1. The best inhibitors show inhibitory potencies below 50 nM and selectivity well above 200-fold over two human kinases with small gatekeeper residues.

Keywords

Cryptosporidium parvum; *Toxoplasma gondii*; calcium-dependent protein kinase 1; enzyme inhibitor; selectivity

Cryptosporidium parvum and *Toxoplasma gondii* are apicomplexan protozoa that can infect humans and domestic animals. Infection by *C. parvum* parasites is transmitted by water and causes cryptosporidiosis.¹ It results in debilitating diarrhea and may be life-threatening for immune compromised people and malnourished children. Nitazoxanide is the only approved drug available to treat cryptosporidiosis, but it is too costly for routine use in poorer countries.² Toxoplasmosis caused by *T. gondii* is spread to humans by ingestion of undercooked meat or oocysts from cat feces in food or water. Human infection by this parasite is very common and is lifelong.³ Infection by *T. gondii* can cause retinitis in

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immune-competent persons and serious fetal abnormalities during pregnancy. Immune compromised individuals may develop fatal encephalitis. Most women of childbearing age in the United States are susceptible to acute infection.⁴ Treatment options are limited to a single first-line therapy (pyrimethamine-sulfadiazine), and the need to be given lifelong in immune compromised persons. Both *C. parvum* and *T. gondii* are listed as biodefense agents due to possible threats by food or water contamination. New therapies for treating both parasite infections are needed.

Recently, the calcium-dependent protein kinase-1 (CDPK1) found in both parasites was shown to be an attractive target for drug discovery.^{5–7} That is because *Cp*CDPK1 and *Tg*CDPK1 are not only associated with important calcium-regulated biological functions such as secretion, invasion, and gliding motility,^{8–10} they also offer unique opportunities for discovering selective inhibitors. First, these two enzymes belong to a clade of CDPKs that are present in plants but not in animals.¹¹ Second, a unique sequence and structural feature of both enzymes is the small glycine residue at the “gatekeeper” position around the ATP-binding cleft.^{5, 12} This structural feature allows for the design of selective inhibitors that take advantage of the larger ATP-binding cavity near the gatekeeper residue of the parasite enzymes as compared to human kinases, for which a larger amino acid residue occupies the gatekeeper position and leaves a smaller ATP-binding pocket.^{13, 14} As a result, we were able to show previously that pyrazolepyrimidine-based inhibitors can be designed to optimally occupy the enlarged ATP-binding pockets of these CDPK1s and selectively inhibit parasite enzymes over human kinases.^{7, 15} In this report, we describe structure-guided optimization of compounds based on a different chemical scaffold that can also selectively inhibit parasite CDPK1s.

Screening work under the Medical Structural Genomics of Pathogenic Protozoa Consortium (<http://www.msgpp.org>) identified methyl (5-benzoylbenzimidazol-2-yl)carbamate (**1**, mebendazole) from a commercial library as a binder to *Tg*CDPK1. Mebendazole **1** is an approved drug with broad spectrum anthelmintic activities. Enzyme inhibition assay confirmed that **1** was inhibitory to both parasite CDPK1 enzymes (IC₅₀: 0.67 μ M against *Tg*CDPK1, 0.92 μ M against *Cp*CDPK1). A crystal structure of *Tg*CDPK1 bound to **1** reveals that compound **1** binds at the same site as the pyrazolepyrimidine class of inhibitors to *Tg*CDPK1,¹⁶ which serves as the starting point of our structure-based optimization of benzoylbenzimidazole-based compounds.

An initial structure activity relationship (SAR) study was performed to probe the importance of functional groups in **1** for inhibition of CDPK1 activity. As shown in Scheme 1, reduction converts the ketone group in **1** to hydroxyl group in **2**, but leads to loss of inhibition towards both parasite CDPK1 enzymes (IC₅₀ >3 μ M). Compound **3**, without the carbamate moiety in **1**, retains the inhibitory potencies (IC₅₀: 0.23 μ M against *Tg*CDPK1, 0.60 μ M against *Cp*CDPK1), indicating that the carbonyl function of the carbamate is not critical for inhibition. In addition, crystal structure of **1** bound to *Tg*CDPK1 also revealed that the binding pocket of CDPK1 has more room to accommodate hydrophilic substitutions around the 2-aminobenzimidazole function of **3**, as this group is pointing towards open solvent. Therefore, a variety of alkyl, acyl, and aminoacyl substituted **3** were prepared.

Synthesis of derivatives of **3** is shown in Scheme 2. Reaction of 2-bromobenzimidazole with substituted amines gave alkylated 2-amino derivatives **4**. Reaction of **1** with an amine produced urea type of derivatives **5**, and direct acylation of **3** generated amide analogs **6**.

Enzyme inhibition data for compounds **4** – **6** is summarized in Table 1. In general, addition of a variety of hydrophilic groups through urea (**5**) or amide (**6**) linkages resulted in improvements to the inhibition of parasite CDPK1s, with several of them having IC₅₀ of less

than 100 nM (see data for **6b** – **6f**). However, selectivity over human kinases with smaller gatekeeper residues, such as Abl and Src (both with threonine gatekeeper) was minimal, and occasionally reversed undesirably (**5e**). Therefore, extension from the 2-amino group of **3** did not yield significant improvements in terms of selectivity.

In order to improve selectivity, a design approach validated in the pyrazolepyrimidine class of inhibitors was taken into consideration for the optimization of the benzoylbenzimidazoles. For pyrazolepyrimidine class of compounds, we found that selective inhibition of parasite CDPK1 can be significantly enhanced by simultaneously occupying the binding pockets close to the gatekeeper residues, and a pocket in the enzyme that recognizes the ribose moiety of ATP.¹⁶ For this reason, we designed compounds **7** (synthesized according to Scheme 3) with a variety of substituents to fill the ribose binding pocket. In addition, one example of compound **8**, which combines features of **7** and **6** was also synthesized for comparison.

As shown in Table 2, this design approach of simultaneously occupying the two pockets described above in general improve both efficacy and selectivity of the inhibitors compared to the starting compound **1** or **3**. The best inhibitors contain a piperidine or a piperazine ring bridged by 1 or 2 carbons from the benzimidazole N1 position, such as **7a**, **7c**, or **7p**. These inhibitors all have IC₅₀ below 50 nM against both parasite CDPK1s, and selectivity index over human Src or Abl of >200 folds. The substitution on N1 of **7** is not compatible to acylation on the 2-amino group as compound **8** lost inhibitory potency when compared to **7a**. In addition, the addition of hydrophilic groups into **7** in general improved the solubility of this class of inhibitors to > 50 μ M compared to ~4 μ M of the starting hit **1**.

The best inhibitors were also tested in cell-based assays for antiparasitic activity and host cell toxicity using described procedures.^{5, 15, 16} We found low toxicities against mammalian cells (EC₅₀ generally > 30 μ M). Unfortunately, none of them showed activity below 1 μ M against either *C. parvum* or *T. gondii*. The exact causes for the lack of cellular activity are still under investigation but may arise from poor cell permeability, selective export by molecular pumps, or intracellular inactivation.

In summary, using structure-based design, we synthesized a series of benzoylbenzimidazole based inhibitors of *Cp*CDPK1 and *Tg*CDPK1 that have low nM potency and good selectivity against human kinases that have small gatekeeper residues. This offers a new chemical scaffold upon which anti-cryptosporidiosis and anti-toxoplasmosis drugs may be discovered.

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Scheme 1.

Synthesis of **2** and **3** for initial SAR studies.

Reaction conditions: (a) NaBH_4 , THF, rt.; (b) BrCN , H_2O .

Scheme 2.

Synthesis of alkyl, acyl, and aminoacylated derivatives of compound 3.

Reaction conditions: (a) potassium ethyl xanthate, H₂O/EtOH, refluxing; (b) HBr/Br₂, HOAc; (c) R₁-NH₂, pyridine, microwave irradiation, 120 °C, 40 min, Boc is removed by TFA/DCM if necessary; d) R₁-NH₂, Acetonitrile, microwave irradiation, 100 °C, 2 h. Boc is removed by TFA/DCM if necessary; e) R₁-COOH, HATU DIPEA, microwave irradiation, 70 °C, 20 min, THF/DCM. Boc is removed by TFA/DCM if necessary.

Scheme 3.

Synthesis of N1-substituted compounds **7** and **8**.

Reaction conditions: (a) thionyl chloride, microwave, 85 °C, 30 min; (b) benzene, AlCl₃, microwave, 65 °C, 10 min; (c) R₁-NH₂, K₂CO₃, acetonitrile, microwave irradiation, 75 °C, 30 min; (d) Zn powder, ammonium formate, MeOH, 5 min, rt; (e) BrCN, K₂CO₃, acetonitrile, microwave irradiation, 70 °C, 20 min; (f) Boc-β-aniline, HATU, DIPEA, microwave irradiation, 70 °C, 20 min, THF/DCM; (g) TFA/DCM, 20 min, rt.

Table 1

Enzyme inhibition data for compounds 4 – 6

ID	R ₁ in Scheme 2	TgCDPK1 (IC ₅₀ , μM)	CpCDPK1 (IC ₅₀ , μM)	Human kinase (IC ₅₀ , μM)
1		0.67	0.92	5.0 (Abl) > 10 (Src)
3		0.23	0.60	~10 (Abl) >10 (Src)
4a		>3	>3	
4b		>3	>3	
5a		0.14	0.15	
5b		0.38	0.59	
5c		0.13	0.18	0.094 (Abl)
5d		0.13	0.18	0.10 (Abl)
5e		0.12	0.16	0.010 (Abl)
6a		0.18	0.13	0.55 (Abl) > 10 (Src)
6b		0.069	0.091	
6c		0.022	0.072	
6d		0.060	0.12	

ID	R ₁ in Scheme 2	TgCDPK1 (IC ₅₀ , μM)	CpCDPK1 (IC ₅₀ , μM)	Human kinase (IC ₅₀ , μM)
6e		0.041	0.11	
6f		0.018	0.082	
6g		0.16	0.13	
6h		0.52	1.41	
6i		0.14	0.32	

Table 2

Enzyme inhibition data for compounds **7** and **8**

ID	R ₁ in Scheme 3	TgCDPK1 (IC ₅₀ , μM)	CpCDPK1 (IC ₅₀ , μM)	Human kinase (IC ₅₀ , μM)
7a		0.015	0.035	>10 (Src) >10 (Abl)
7b		0.15	0.26	
7c		0.020	0.017	>10 (Src) >10 (Abl)
7d		0.030	0.026	>10 (Src) >10 (Abl)
7e		1.00	0.87	
7f		0.41	0.26	
7g		0.66	0.79	
7h		0.029	0.030	>10 (Src) >10 (Abl)
7i		0.068	0.071	
7j		0.065	0.095	

ID	R ₁ in Scheme 3	TgCDPK1 (IC ₅₀ , μM)	CpCDPK1 (IC ₅₀ , μM)	Human kinase (IC ₅₀ , μM)
7k		0.12	0.12	
7l		0.067	0.23	
7m		0.063	0.13	
7n		0.16	0.30	
7o		0.22	0.39	
7p		0.043	0.036	>10 (Src) >10 (Abl)
7q		0.18	0.17	
7r		0.19	0.95	
7s		0.26	0.83	
8		0.57	0.86	>10 (Src)