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Imidazo[1,2-*b*]pyridazines targeting *Toxoplasma gondii* calcium-dependent protein kinase 1 decrease the parasite burden in mice with acute toxoplasmosis

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ABSTRACT

The current therapeutic arsenal for toxoplasmosis is restricted to drugs non-specific to the parasite which cause important side effects. Development of more efficient and specific anti-*Toxoplasma* compounds is urgently needed. Imidazo[1,2-*b*]pyridazines designed to inhibit the calcium-dependent protein kinase 1 of *Toxoplasma gondii* (*Tg*CDPK1) and effective against tachyzoite growth in vitro at submicromolar ranges were modified into hydrochloride salts to be administered in vivo in a mouse model of acute toxoplasmosis. All protonated imidazo[1,2-*b*]pyridazine salts (SP230, SP231 and SP232) maintained their activity on *Tg*CDPK1 and *T. gondii* tachyzoites. Rat and mouse liver microsomes were used to predict half-life and intrinsic clearance, and the pharmacokinetic profile of the most rapidly degraded IP salt (SP230) was determined in serum, brain and lungs of mice after a single administration of 50 mg/kg. Compounds were then tested in vivo in a murine model of acute toxoplasmosis. Mice infected with tachyzoites of the ME49 strain of *T. gondii* were treated for 4, 7 or 8 days with 25 or 50 mg/kg/day of SP230, SP231 or SP232. The parasite burdens were strongly diminished (>90% reduction under some conditions) in the spleen and the lungs of mice treated with imidazo[1,2-*b*]pyridazine salts compared with untreated mice, without the need for pre-treatment. Moreover, no increases in the levels of hepatic and renal toxicity markers were observed, demonstrating no significant signs of short-term toxicity. To conclude, imidazo[1,2-*b*]pyridazine salts have strong efficacy in vivo on acute toxoplasmosis and should be further tested in a model of mouse congenital toxoplasmosis.

1. Introduction

Toxoplasmosis is an infectious disease caused by *Toxoplasma gondii*, an apicomplexan parasite infecting approximately one-third of the human global population. In the USA, toxoplasmosis is one of the five neglected parasitic infections targeted by Centers for Disease Control and Prevention for public health action (https://www.cdc.gov/parasites/toxoplasmosis). Estimation of the annual burden caused by food-borne pathogens indicated that toxoplasmosis is responsible for losses of US $ 3 billion and 32,700 quality-adjusted life years (QALYs) (Batz et al., 2012; Scallan et al., 2015). Studies on the 2000–2006 Nationwide Inpatient Sample data (http://www.hcup-us.ahrq.gov/nisoverview.jsp) estimated that parasites cause only 2% of food-borne illnesses in the USA. Nevertheless, *T. gondii* is the fourth leading cause of hospitalisation (8%) and the second leading cause of death (24%) attributable to food-borne illness (Batz et al., 2012). A recent study monitored in Europe reported that parasites are also responsible for less than 3% of the food-borne (including waterborne)

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outbreaks but in contrast to the USA, no hospitalisation or death was attributed to *T. gondii* in Europe (EFSA and ECDC, 2016).

In immunocompetent individuals, infection is usually asymptomatic, even if chorio-retinal lesions can sometimes be observed. Ocular toxoplasmosis can thus be responsible for 2–18% of uveitis cases depending on the country (Holland, 2003). However, initial exposure to the parasite leads to latent chronic infection (reservoir of tissue cysts, particularly in the brain), which can be reactivated in immunocompromised patients, resulting in severe encephalitis. The incidence rate of neurotoxoplasmosis is thus estimated at 0.04–2.6 per 100 person years (Coelho et al., 2014). Moreover, the transplacental passage of *T. gondii* tachyzoites (fast replicating forms that invade host cells) during a primary infection in pregnant women can be responsible for foetal contamination, causing abortions, or ocular and neurologic sequelae in congenitally infected newborns. The global incidence of congenital toxoplasmosis by region of the World Health Organization ranges from 0.5 per 1000 live births in western Europe to 3.4 per 1000 live births in the Americas each year (Torgerson and Mastroiacovo, 2013).

The current first-line treatments (pyrimethamine–sulfonamide), target the *T. gondii* folate pathway. Beside their moderate efficacy, these non-specific drugs require a long treatment duration and cause important side effects (e.g. allergic reactions, bone marrow toxicity). Thus, the haematological toxicity of widely used pyrimethamine requires the co-administration of folinic acid during treatment. Additionally, this molecule is not recommended during the first trimester of pregnancy due to its teratogenic properties (Meneceur et al., 2008; Julliac et al., 2010). Under these conditions, the use of the macrolide antibiotic spiramycin has been reported to decrease the frequency of vertical transmission with moderate efficacy. However, this molecule does not cross the placental barrier and is not effective in the case of foetal infection (Montoya and Remington, 2008). Second-line treatments such as clindamycin or atovaquone are available for immuno-compromised patients and/or in cases of first-line regimen contraindications. However, these agents also have significant drawbacks, and decreased effectiveness. Finally, currently available therapies are of limited effectiveness once the parasite has differentiated into the cyst form, which explains in part their inability to cure the chronic infection. There is thus a need for new short course, parasite-specific treatments with reduced toxicity.

In this context, *T. gondii* calcium-dependent protein kinase 1 (*Tg*CDPK1), identified in apicomplexans but not in animals, represents an interesting target for drug discovery (Harper and Harmon, 2005). This serine/threonine protein kinase, a specialised calcium-regulated signalling enzyme, is indeed essential for parasite survival in controlling exocytosis of micronemes, and hence cell invasion and egress (Kieschnick et al., 2001; Lourido et al., 2010; Sugi et al., 2010). Moreover, this enzyme contains a unique sequence variation in its ATP binding pocket, providing an opportunity to develop selective *Tg*CDPK1 inhibitors. Indeed, the catalytic ATP binding site is often targeted by kinase inhibitors occupying the adenine-binding region. One possible way to achieve selectivity over off-target mammalian kinases present in humans is to probe surrounding specific areas, such as the adjacent hydrophobic back pocket. This pocket size depends on an amino acid residue called the gatekeeper which is not conserved (Hui et al., 2015). The gatekeepers of the vast majority of kinases are bulky, restricting access to this hydrophobic pocket. Interestingly, crystal structures of *Tg*CDPK1 revealed that, in contrast to human kinases, this enzyme possesses a rare small gatekeeper (a glycine) in its ATP-binding cleft (Ojo et al., 2010), allowing access to a wide back pocket that can be targeted to design selective parasite-specific inhibitors (Johnson et al., 2012).

Using a structure-based design approach, we exploited this unique structural feature, and identified imidazo[1,2-*b*]pyridazines (IPs), 16a, 16b and 16f, as potent and selective ATP-competitive *Tg*CDPK1 inhibitors, with anti-*T. gondii* activity at submicromolar concentrations, and a low toxicity profile (Moine et al., 2015). Herein, we describe the biological evaluations of their respective hydrochloride salts, SP230, SP231 and SP232.

2. Materials and methods

2.1. Synthesis of imidazo[1,2-b]pyridazine (IP) salts

All solvents were anhydrous reagents from commercial sources. Unless otherwise noted, all chemicals and reagents were obtained commercially and used without purification. Melting points (Mp) were determined on a Stuart capillary apparatus and are uncorrected. [^{1}H] nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance (300 MHz) spectrometer. The chemical shifts are reported in parts per million (ppm, δ) relative to residual deuterated solvent peaks.

2.1.1. Synthesis of 2-(benzo[d][1,3]dioxol-4-yl)-6-(4-methylpiperazin-1-yl)-3-(pyridin-4-yl)imidazo[1,2-b]pyridazine bis hydrochloride salt (SP230)

Into a three-necked flask was introduced a suspension of 2-(benzo[*d*][1,3]dioxol-4-yl)-6-(4-methylpiperazin-1-yl)-3-(pyridin-4-yl)imidazo[1,2-*b*]pyridazine (209 mg, 0.50 mmol) in isopropanol (700 μL) and water (130 μL). A 37% hydrochloric acid aqueous solution (91 μL, 1.11 mmol) was added and the resulting mixture was stirred at 60 °C. The solution became limpid and acetone (960 μL) was then added dropwise. The resulting mixture was stirred for 1 h at 60 °C, cooled to room temperature and stirred for two additional hours. The formed precipitate was filtered, rinsed with acetone, and dried to give compound SP230 (228 mg, 100%) as a yellow solid. Properties were as follows: Mp 248.1 °C. [^{1}H] NMR (300 MHz, DMSO-d_6) δ 11.29 (broad signal (bs), 1H), 8.85 (doublet of doublet (dd), 2H), 8.28–8.13 (multiplet (m), 3H), 7.56 (doublet (d), 1H), 7.00 (dd, 1H), 7.10–6.96 (m, 2H), 5.80 (singlet (s), 2H), 3.35 (d, 2H), 3.47 (m, 4H), 3.16 (m, 2H), 2.79 (bs, 3H).

2.1.2. Synthesis of 2-(benzo[d][1,3]dioxol-5-yl)-6-(4-methylpiperazin-1-yl)-3-(pyridin-4-yl)imidazo[1,2-b]pyridazine bis hydrochloride salt (SP231)

The title compound was synthesised according to the general method used in Section 2.1.1 from 2-(benzo[*d*][1,3]dioxol-5-yl)-6-(4-methylpiperazin-1-yl)-3-(pyridin-4-yl)imidazo[1,2-*b*]pyridazine (118 mg, 0.29 mmol), isopropanol (350 μL), water (65 μL), hydrochloric acid (52 μL, 0.63 mmol), and acetone (480 μL). Compound SP231 was obtained (129 mg, 100%) as a bright yellow solid. Properties were as follows: Mp 282.3 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.83 (bs, 1H), 8.78 (dd, 2H), 8.15 (d, 1H), 8.00 (dd, 2H), 7.49 (d, 1H), 7.11 (d, 1H), 7.05 (dd, 1H), 6.97 (d, 1H), 6.09 (s, 2H), 4.29 (d, 2H), 3.48–3.25 (m, 4H), 3.14 (m, 2H), 2.80 (d, 3H).

2.1.3. Synthesis of 6-(4-methylpiperazin-1-yl)-2-(naphth-1-yl)-3-(pyridin-4-yl)imidazo[1,2-b]pyridazine bis hydrochloride salt (SP232)

The title compound was synthesised according to the general method used in Section 2.1.1 from 6-(4-methylpiperazin-1-yl)-2-(naphth-1-yl)-3-(pyridin-4-yl)imidazo[1,2-*b*]pyridazine (97 mg, 0.23 mmol), isopropanol (350 μL), water (65 μL), hydrochloric acid (42 μL, 0.51 mmol), and acetone (480 μL). Compound SP232 was obtained (103 mg, 98%) as a brown solid. Properties were as follows: Mp 184.7 °C. [^{1}H] NMR (300 MHz, DMSO-d_6) δ 11.80 (bs, 1H), 8.71 (d, 2H), 8.30 (d, 1H), 8.16–7.96 (m, 4H), 7.85–7.41 (m, 6H), 4.43 (d, 2H), 3.63–3.43 (m, 4H), 3.19 (m, 2H), 2.80 (d, 3H).

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