Original Article

Discovery of novel PDE10 inhibitors by a robust homogeneous screening assay

Qun-yi LI¹, Ming-kai XU¹, Gang LIU², Claus Tornby CHRISTOFFERSEN³, Ming-wei WANG^{1, *}

¹The National Center for Drug Screening and the State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China; ²Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing 100050, China; ³H Lundbeck A/S, DK-2500 Copenhagen, Denmark

Aim: To develop a homogeneous assay for high-throughput screening (HTS) of inhibitors of phosphodiesterase 10 (PDE10).

Methods: Purified human PDE10 enzyme derived from *E coli*, [³H]-cAMP and yttrium silicate microbeads were used to develop an HTS assay based on the scintillation proximity assay (SPA) technology. This method was applied to a large-scale screening campaign against a diverse compound library and subsequent confirmation studies. Preliminary structure-activity relationship (SAR) studies were initiated through limited structural modifications of the hits.

Results: The IC₅₀ value of the control compound (papaverine) assessed with the SPA approach was comparable and consistent with that reported in the literature. Signal to background (S/B) ratio and Z' factor of the assay system were evaluated to be 5.24 and 0.71, respectively. In an HTS campaign of 71 360 synthetic and natural compounds, 67 hits displayed reproducible PDE10 inhibition, of which, 8 were chosen as the scaffold for structural modifications and subsequent SAR analysis.

Conclusion: The homogeneous PDE10 SPA assay is an efficient and robust tool to screen potential PDE10 inhibitors. Preliminary SAR studies suggest that potent PDE10 inhibitors could be identified and developed through this strategy.

Keywords: phosphodiesterase 10 (PDE10); papaverine; high-throughput screening; psychosis

Acta Pharmacologica Sinica (2013) 34: 1116–1120; doi: 10.1038/aps.2013.25; published online 22 Apr 2013

Introduction

The cyclic nucleotides, cyclic-adenosine monophosphate (cAMP) and cyclic-guanosine monophosphate (cGMP), function as intracellular second messengers regulating a vast array of biological processes. Given the important role of cyclic nucleotides in signal transduction and physiological pathways, therapeutic benefits may be derived from the use of compounds that affect the regulation of cyclic nucleotide signaling^[1]. Phosphodiesterases (PDEs), which regulate cyclic nucleotide signaling by hydrolyzing cAMP and cGMP, may play pivotal roles in cell signaling. To date, 11 known families of PDEs encoded by 21 different genes have been identified^[2]. The PDE families are distinguished functionally based on cyclic nucleotide substrate specificity, mechanisms of regulation and sensitivity to inhibitors. Due to differential expression and functionality throughout the organism, different PDE isozymes exert distinct physiological functions^[3].

PDE10 is recognized as a unique family based on its primary

amino acid sequence and distinct enzymatic activity. It displays dual activities on hydrolysis of both cAMP and cGMP, with an affinity for cAMP ($K_m=0.05 \mu\text{mol/L}$) higher than that for cGMP ($K_m=3 \mu\text{mol/L}$)^[4, 5]. Its mRNA is highly expressed in the testis and brain^[6]. Loss of PDE10 activity in the striatum of transgenic mice led to dysregulation of cAMP and cGMP levels, thereby implying an important role in Huntington's disease^[7]. Furthermore, Siuciak and colleagues revealed that PDE10A is involved in the regulation of striatal output, possibly by reducing the sensitivity of medium spiny neurons to glutamatergic excitation^[8]. These results support the hypothesis that PDE10A inhibition may present a novel treatment for psychosis.

Small chemical modulators for PDE10 are of great value in both investigating enzyme properties and developing therapeutics, as exemplified by the PDE10 inhibitor, papaverine albeit it is not PDE10 selective. It was found that conditioned avoidance response in rats and mice as well as PCP- and amphetamine-stimulated locomotor activity in rats could be suppressed by papaverine^[9]. Moreover, PDE10A inhibition caused by papaverine effectively improved executive function deficits associated with experimental schizophrenia in

* To whom correspondence should be addressed.

E-mail wangmw@mail.shcnc.ac.cn

Received 2013-01-23 Accepted 2013-02-27

rats^[10]. Selective PDE10A inhibitors TP-10 and MP-10 have been reported to decrease psychomotor activity, reverse deficits in prepulse inhibition and inhibit conditioned avoidance response (CAR) in rodents, implying potential antipsychotic activities^[11, 12]. These compounds also improved cognitive performance in domains impaired in schizophrenia and negative symptoms in rodents^[11–13]. As mentioned above, it is believed that novel PDE10 inhibitors would serve not only as potential drug candidates to treat psychosis, but also as important tools to elucidate the mechanisms of action related to PDE10.

To discover and characterize novel chemical modulators for PDEs from large compound libraries, approaches amenable for high-throughput screening (HTS) are necessary. Obviously, there is a strong need to seek new assay systems that could offer advantages such as high throughput, robustness and low cost.

In the present study, we describe the development and validation of a robust scintillation proximity assay (SPA) for identification and characterization of human PDE10 inhibitors. A HTS campaign of 71 360 synthetic and natural compounds using this assay system was carried out. As a result, a number of novel inhibitors structurally distinct from the compounds known to modulate human PDE10 function were discovered. Eight initial hits with similar structures were chosen as the scaffold for structural modifications and subsequent structure-activity relationship (SAR) analyses.

Materials and methods

Reagents

Papaverine was provided by H Lundbeck A/S. HEPES was obtained from Invitrogen (Carlsbad, CA, USA). $MgCl_2$ and Tween 20 were purchased from Shanghai Chemical Reagents Co, Ltd (Shanghai, China). Isoplate™ microtiter plate was the product of PerkinElmer (Boston, MA, USA). SPA yttrium silicate beads and [3H]-cAMP (59 Ci/mmol) were procured from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

PDE10 assay

The SPA assay for PDE10 was modified according to the method described previously^[14]. Briefly, compounds (2 μ L per well) dissolved in 100% dimethylsulphoxide (DMSO) were pre-plated onto Isoplate™ microtiter plates. The wells were incubated in 48 μ L PDE10 solution containing 50 mmol/L HEPES, 10 mmol/L $MgCl_2$, 0.03 % Tween 20, pH=7.6. The volume of PDE10 used was determined by enzymatic activity that provides a high signal window but does not deplete the substrate.

After 10 min incubation at room temperature, 50 μ L [3H]-cAMP (20 nmol/L) per well was added to the assay plates. The plates were then placed at a shaking table (450 r/min $\times$ 10 min) and incubated for 40 min at room temperature. Fifty microliter STOP-mixture (SPA beads dissolved in water to a concentration of 10 mg/mL) was added to each well thereafter and incubated for 10 min with shaking at 450 r/min before counting on the Microbeta scintillation counter (PerkinElmer).

HTS campaign

The compound library used for the screening of human PDE10 inhibitors consisted of 71,360 pure synthetic and natural compounds. Samples dissolved in 100% DMSO were applied to the primary screening, with an average final concentration of 3.3 μ mol/L for each compound. In each 96-well assay plate, 6 wells were used as IC_{100} controls (10 μ mol/L papaverine), 4 wells as IC_{50} controls (300 nmol/L papaverine), and 6 wells as IC_0 controls (2% DMSO). Compounds showing greater than 70% inhibition relative to IC_0 were considered as “hits”.

Data analysis

Data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Non-linear regression analyses were performed to generate dose-response curves and to calculate IC_{50} values. Percentage responses of hits were compared with that of 10 μ mol/L papaverine, which was defined as 100%. Z' factor, which estimates the suitability of the SPA assay to HTS, was assessed as previously described^[15]. The equations of $S/B = M_{\text{signal}}/M_{\text{background}}$ and $CV = 100 \times (SD/M)$ were applied to calculate the signal/background ratio (S/B) and the coefficient of variation (CV) values of the assay, respectively.

Results

Assay validation

We first used various amounts of the PDE10 enzyme extract to assess the optimal protein concentration for the SPA assay. Both signal (IC_0 ; using DMSO) and background (IC_{100} ; using 10 μ mol/L papaverine) were investigated, and the optimal PDE10 amount was determined to be 5 nL/well that resulted in a signal to background (S/B) ratio of 6.2 (Figure 1). The effects of reaction time on the assay signal and background are shown in Figure 2. Not only did the signal but also the background increase along with prolonged [3H]-cAMP incubation time. As a result, a substrate incubation time of 40 min led to a better S/B ratio (5.36) and was thus used in the HTS campaign. DMSO is the most commonly employed solvent to dissolve organic compounds in HTS. At concentrations of up to 2% DMSO did not affect the assay performance (data not shown). Under these optimized conditions, the IC_{50} value of the PDE10 inhibitor papaverine was 364 nmol/L (Figure 3).

Figure 1. Reactivity characteristics of papaverine with different amounts of PDE10. Serial titration of PDE10 was made to determine the optimal protein concentration.

Figure 2. Effects of different substrate incubation time on signal and background readout in the scintillation proximity assay system with 2% DMSO or 10 $\mu\text{mol/L}$ papaverine present. Data (mean $\pm$ SEM) are representative of three independent experiments.

Figure 3. Dose-response curve of papaverine on PDE10 activity measured by the scintillation proximity assay with optimal conditions, from which IC_{50} value was calculated ($n=3$, mean $\pm$ SEM).

Assay performance

In order to apply the SPA assay system to HTS, both signal evoked by [^3H]-AMP, the product of PDE10 enzyme reaction, and background (10 $\mu\text{mol/L}$ papaverine) were studied. As shown in Figure 4, the coefficient of variation (CV) values were 5.5% for total signal and 12.5% for background, respectively. The Z' factor for the assay was 0.71 with an S/B ratio of 5.24. These characteristics indicate that the system is of high quality and well-suited to HTS^[15].

Identification of PDE10 inhibitors

The result of the HTS campaign for PDE10 inhibitors is shown in Figure 5. Of the 71 360 samples initially screened for potential inhibitory effect on PDE10, 692 hits (0.97%) displaying greater than 70% positive modulation activity relative to papaverine were discovered. In the HTS campaign, the Z' factors varying between 0.69 and 0.74 conferred the assay quality requirement of above 0.5. In the secondary confirmation study (initial hits in duplicates), 67 compounds demonstrated consistently inhibition compared to papaverine (9.68% of the initial hits or 0.09% final hit rate). Among them, 8 synthetic chemicals with similar structures displayed IC_{50} values below 0.4 $\mu\text{mol/L}$ (Table 1). The scaffold of these novel PDE inhibi-

Figure 4. Z' factor of the scintillation proximity assay on PDE10 was determined at optimized conditions. Forty-eight replicates of signal (2% DMSO, black circle) and background (10 $\mu\text{mol/L}$ papaverine, white circle) were investigated.

Figure 5. HTS campaign of 71 360 compounds using the scintillation proximity assay. Results are expressed as relative response compared to 10 $\mu\text{mol/L}$ papaverine by the number of hit compounds at each 5% interval.

tors was chosen as the starting point for medicinal chemistry efforts.

Structural modifications

Limited medicinal chemistry efforts were made to modify the structures of the initial hits. Of the 25 analogues made^[16], 7 were tested for inhibitory properties on PDE10 and none of which showed any improvements in terms of bioactivity (Table 2).

Discussion

Due to its large variety of biological activities, studies on PDE10 have become an emerging area that attracts scientists from different disciplines. Apart from potential value of using PDE10 as a drug target, exploration of small molecule inhibitors of the enzyme could provide powerful tools in further understanding of the physiological roles of this phosphodiesterase subtype. Here we report a rational approach to discover novel PDE10 inhibitors by use of a robust SPA assay.

According to our prior experience in the development of SPA-based receptor binding HTS methods^[17, 18], efforts were

Table 1. Novel small molecule inhibitors of the human PDE10 identified by a scintillation proximity assay-based high-throughput screening campaign.

Compound	Structure	M_w	IC_{50} (nmol/L)
A-021-011 (d)		480.52	17.1
A-095-011 (d)		523.54	129.9
096-0a5-011		435.53	65.4
096-0a5-020		437.50	106.8
A8 (558) 046-SH2-011		511.65	307.1
D7 (544) 064-SH2-053		518.55	125.7
IMMLG-5061		524.65	91.0
IMMLG-5178		496.59	84.3

 M_w , molecular weight.**Table 2.** Structural modifications and inhibition on human PDE10.

Compound	Structure	M_w	IC_{50} (nmol/L)
5g		448.49	40
lw-IV-28-1		468.56	45
lw-IV-30-2		504.43	403
5f		425.50	353
10c		544.99	410
5c		470.54	180
lw-V-40		448.49	162

 M_w , molecular weight.

made to expand its application to PDE10, one of the phosphodiesterases. The assay is based on the observation that linear nucleotides bind preferentially to SPA yttrium silicate beads compared to cyclic nucleotides in the presence of zinc sulphate. The conversion of [3 H]-cAMP to [3 H]-AMP catalyzed by PDE10 results in an increase in radioactivity bound to the beads thereby enhancing the signal.

In order to apply the SPA assay to a HTS setting, many factors should be considered to maximize the signal and the S/B ratio. As shown in Figure 1, varying the amount of PDE10 per well influenced the total signal and background of this assay. The substrate incubation time of 40 min was chosen for the HTS campaign as it resulted in a robust total signal and the highest S/B ratio (Figure 2). DMSO tolerance should also be examined as it can limit the maximum concentration

of a compound to be examined. In this case, up to 2% DMSO exhibited no obvious effect on the assay performance. The Z' factor is a useful indicator for assessing the quality of HTS assays. In general, a Z' value above 0.5 suggests that an assay is robust enough for use in HTS settings. The SPA system described in this paper consistently displayed a Z' value equal or above 0.69. This, and in conjunction with other parameters such as S/B ratio and CV values, suggest that the assay system employed is well suited to HTS. Subsequent HTS campaign using this system led to the discovery of 67 confirmed hits. Among them, 8 with similar structures displayed consistent inhibitory effects on PDE10 with IC_{50} values below 0.4 $\mu\text{mol/L}$. Limited structural modifications did not yield more potent analogues but this medicinal chemistry efforts in still ongoing.

In summary, we have developed and validated a SPA-based assay to identify novel PDE10 inhibitors. The approach may also be applicable to identify and evaluate modulators of other PDEs. Moreover, the newly discovered PDE10 inhibitors have distinct structural features that differ from those reported elsewhere. Indeed, knowledge obtained from the present studies will facilitate the pursuit to develop new PDE10 inhibitors.

Acknowledgements

This study was supported in part by grants from H Lundbeck A/S and the Ministry of Science and Technology of China (2009ZX09302-001, 2012ZX09304011 and 2013ZX09507002), Shanghai Science and Technology Development Fund (11DZ2292200). We are indebted to Dale E MAIS for critical review of this manuscript.

Author contribution

Qun-yi LI, Ming-kai XU, Gang LIU, and Claus Tornby CHRISTOFFERSEN performed experiments; Qun-yi LI and Ming-wei WANG analyzed the data; Qun-yi LI and Ming-wei WANG wrote the paper.

References

- 1 Zaccolo M, Movsesian MA. cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 2007; 100: 1569–78.
- 2 Vezzosi D, Bertherat J. Phosphodiesterases in endocrine physiology and disease. *Eur J Endocrinol* 2011; 165: 177–88.
- 3 Chan S, Yan C. PDE1 isozymes, key regulators of pathological vascular remodeling. *Curr Opin Pharmacol* 2011; 11: 720–4.
- 4 Loughney K, Snyder PB, Uher L, Rosman GJ, Ferguson K, Florio VA. Isolation and characterization of PDE10A, a novel human 3',5'-cyclic nucleotide phosphodiesterase. *Gene* 1999; 234: 109–17.
- 5 Soderling SH, Bayuga SJ, Beavo JA. Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc Natl Acad Sci U S A* 1999; 96: 7071–6.
- 6 Fujishige K, Kotera J, Omori K. Striatum- and testis-specific phosphodiesterase PDE10A: isolation and characterization of a rat PDE10A. *Eur J Biochem* 1999; 266: 1118–27.
- 7 Hebb AL, Robertson HA, Denovan-Wright EM. Striatal phosphodiesterase mRNA and protein levels are reduced in Huntington's disease transgenic mice prior to the onset of motor symptoms. *Neuroscience* 2004; 123: 967–81.
- 8 Siuciak JA, McCarthy SA, Chapin DS, Fujiwara RA, James LC, Williams RD, *et al*. Genetic deletion of the striatum-enriched phosphodiesterase PDE10A: evidence for altered striatal function. *Neuropharmacology* 2006; 51: 374–85.
- 9 Siuciak JA, Chapin DS, Harms JF, Lebel LA, McCarthy SA, Chambers L, *et al*. Inhibition of the striatum-enriched phosphodiesterase PDE10A: a novel approach to the treatment of psychosis. *Neuropharmacology* 2006; 51: 386–96.
- 10 Rodefer JS, Murphy ER, Baxter MG. PDE10A inhibition reverses subchronic PCP-induced deficits in attentional set-shifting in rats. *Eur J Neurosci* 2005; 21: 1070–6.
- 11 Grauer SM, Pulito VL, Navarra RL, Kelly MP, Kelley C, Graf R, *et al*. Phosphodiesterase 10A inhibitor activity in preclinical models of the positive, cognitive, and negative symptoms of schizophrenia. *J Pharmacol Exp Ther* 2009; 331: 574–90.
- 12 Menniti FS, Chappie TA, Humphrey JM, Schmidt CJ. Phosphodiesterase 10A inhibitors: a novel approach to the treatment of the symptoms of schizophrenia. *Curr Opin Investig Drugs* 2007; 8: 54–9.
- 13 Schmidt CJ, Chapin DS, Cianfrogna J, Corman ML, Hajos M, Harms JF, *et al*. Preclinical characterization of selective phosphodiesterase 10A inhibitors: a new therapeutic approach to the treatment of schizophrenia. *J Pharmacol Exp Ther* 2008; 325: 681–90.
- 14 Coste H, Grondin P. Characterization of a novel potent and specific inhibitor of type V phosphodiesterase. *Biochem Pharmacol* 1995; 50: 1577–85.
- 15 Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999; 4: 67–73.
- 16 Liang W, Liu G. Synthesis of mono- and di-fluorinated benzimidazoles. *Chin J Chem* 2011; 29: 983–90.
- 17 Meng T, Su HR, Binkert C, Fischli W, Zhou L, Shen JK, *et al*. Identification of non-peptidic neuromedin U receptor modulators by a robust homogeneous screening assay. *Acta Pharmacol Sin* 2008; 29: 517–27.
- 18 Liu Q, Zhang YY, Lu HL, Li QY, Zhou CH, Wang MW. Rhodanine derivatives as novel peroxisome proliferator-activated receptor gamma agonists. *Acta Pharmacol Sin* 2007; 28: 2033–9.