RESEARCH ARTICLE

 View Article Online
 View Journal | View Issue

 Cite this: *Med. Chem. Commun.*,
 2017, 8, 1806

Structure-optimized dihydropyranoindole derivative GIBH-LRA002 potentially reactivated viral latency in primary CD4⁺ T lymphocytes of chronic HIV-1 patients†

 Qing Yang,^{‡ac} Yuyang Ding,^{‡ad} Fengling Feng,^{ae} Enxiang Pan,^{ad} Xiaozhen Fan,^{ae}
 Xiuchang Ma,^a Ling Chen,^{ac} Junling Zhao^{*b} and Caijun Sun^{id *a}

 Received 28th June 2017,
 Accepted 21st July 2017

DOI: 10.1039/c7md00327g

rsc.li/medchemcomm

Based on structure modification and a high-throughput Jurkat-Lat cell screening model, we found that GIBH-LRA002, ethyl-2-amino-3-cyano-9-methyl-4-(trifluoromethyl)-4,9-dihydropyrano[2,3-*b*]indole-4-carboxylate, effectively reactivated the latent proviruses in a Jurkat-Lat cell line and primary CD4⁺ T cells from both chronic SIV-infected rhesus macaques and HIV-1 patients but without inducing systemic activation, making this compound attractive for potentially treating HIV-1 infection.

The epidemic of human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV-1/AIDS) is still rampant throughout the world, although more than three decades have passed since the discovery of this infectious disease. Globally, an estimated 36.9 million people were living with HIV-1 in 2015, and there were 2.3 million new HIV infections annually.¹ Highly active antiretroviral therapy (HAART), usually referred to as an anti-HIV cocktail of three or more antiretroviral drugs, is currently the standard treatment for HIV-1 infection and results in effective control of HIV-1 replication in plasma to undetectable levels, but HAART fails to completely eradicate the virus. Therefore, there is frequently a rapid viremia rebound within several days following discontinuation of HAART, which makes HIV-1 patients take a life-long drug treatment.

Recent studies demonstrated that the critical barrier to HIV-1 eradication is the persistence of latent viral reservoirs, and these reservoirs represent the long-lived cell subsets of integrated HIV-1 proviruses, which include mainly resting memory CD4⁺ T cells and potentially tissue-resident dendritic cells (DC) and macrophages.² Due to the long half-life

of viral reservoirs, it has been speculated that the eradication of HIV-1 with current drugs would require over 60 years.³ The latent infected cells usually have a quiescence state of inactive transcription of viral genomes and do not express viral antigens, so they are hard to be recognized and then destroyed by immune surveillance. Therefore, the way to eliminate viral reservoirs requires firstly reactivation of the latent virus with latency-reversing agents (LRAs), and then the immune system will recognize and eradicate the activated HIV-1-antigen-expressing cells.

To date, various compounds with different structures (Fig. 1) have been tested as potential LRAs in preclinical and clinical studies. Among them, histone deacetylase inhibitors (HDACis) including valproic acid (VPA)⁴ and suberoylanilide hydroxamic acid (SAHA)⁵ were well studied and showed an extent of ability to induce latent HIV-1 expression, but failed

Fig. 1 The chemical structures of some representative LRAs. SAHA, suberoylanilide hydroxamic acid; VPA, valproic acid; PEP005, ingenol mebutate; PMA, phorbol-12-myristate-13-acetate; GIBH-LRA002, ethyl-2-amino-3-cyano-9-methyl-4-(trifluoromethyl)-4,9-dihydropyrano[2,3-*b*]indole-4-carboxylate.

^a Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences, Guangzhou 510530, China. E-mail: Sun_caijun@gibh.ac.cn

^b Institute of Medicinal Chemistry, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, P.R. China. E-mail: zhaojunl@iccas.ac.cn

^c State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China

^d University of Chinese Academy of Sciences, Beijing, 100049, China

^e School of Life Sciences, University of Science and Technology of China (USTC), Hefei, 230027, China

† Electronic supplementary information (ESI) available: Experimental procedures. See DOI: 10.1039/c7md00327g

‡ These authors contributed equally to this work.

to further proceed into clinical use due to unsatisfactory results in HIV-1-infected individuals receiving ART in clinical trials.⁶ Another issue for developing LRAs is the possible toxicity which is caused by non-specific T cell activation and release of proinflammatory cytokines. Consequently, it is of great interest to explore novel compounds to reactivate latent reservoirs but avoid global T cell activation.

The pyranindole skeleton is a pharmacology-privileged structure core in drug discovery, and a variety of pyranindole derivatives have shown anti-inflammatory, anti-cancer and anti-bacterial activities against infection.^{7–9} Recently, we have developed an efficient method for the organocatalytic enantioselective synthesis of dihydropyranindole derivatives.¹⁰ Using this efficient methodology, we synthesized a series of novel compounds bearing trifluoromethylated all-carbon-substituted stereocenters in high yields (up to 99%) with excellent enantioselectivity (up to 98% ee) (Fig. 1). However, the potential biological activities of these novel compounds are unknown.

In this study, we therefore tested those compounds in a high-throughput Jurkat-Lat cell line screening model that mimics the latent state of HIV-1 in primary CD4⁺ T cells. The Jurkat-Lat HIV-1 full length clone A10.6 cell line contains integrated but transcriptionally latent HIV proviruses, in which the green fluorescent protein (GFP)-coding gene replaces the Nef-coding sequence, and a frameshift mutation in Env. Therefore, the percentage of GFP-positive cells represents the transcriptional activation of HIV-1, and the activation efficacy can be analyzed easily in high-throughput screening using the fluorescence-activated cell sorting (FACS) technique (Fig. 2a). 24 hours after treatment with different concentrations of dihydropyranindole derivatives, the percentage of GFP-expressing cells was detected. Interestingly, we identified the elevated GFP expression in response to the stimulation of some compounds (Fig. 2b), which represented that those compounds potentially reactivated latent HIV-1 in this screening model.

A further preliminary structure–activity relationship study (Fig. 3) disclosed that: 1) the ester group (R1) is essential for maintaining the high activity of the compound. For example, a sharp drop of the activity accompanied by increasing cytotoxicity was observed when the compound without an ester group was tested (Fig. 3, compound A); 2) compounds in racemic form (Fig. 3, compound C) turned out to be more efficient than their pure chiral enantiomers (Fig. 3, compound D); 3) substituents (R2) on the nitrogen atom of the indole moiety can significantly influence the activity of the compounds, and the best result was obtained when a Me group was used (Fig. 3, compound 2, also named GIBH-LRA002).

Based on structure modification and screening, we finally confirmed that GIBH-LRA002 possesses the most optimized reactivation efficacy and lower cytotoxicity. The chemical name of GIBH-LRA002 is ethyl-2-amino-3-cyano-9-methyl-4-(trifluoromethyl)-4,9-dihydropyrano[2,3-*b*]indole-4-carboxylate, and this is a compound with a novel structure (Fig. 1). This compound GIBH-LRA002 efficiently increased the GFP-expression ratio from 5.06% to 23.0% (Fig. 4A). To clarify

Fig. 2 The reactivation efficacy towards HIV proviruses of different compounds was tested in a high-throughput cell screening model. (a) Representative FACS plots to analyze the frequency of GFP-positive cells. Jurkat-Lat A10.6 cells were incubated with different compounds, and the percentage of GFP-positive cells, which represents the transcriptional activation of HIV-1, was detected by flow cytometry. A total of 500 000 cells were acquired and analyzed using a FACSria instrument using the FlowJo software. A protein kinase C – PMA and a latent reversing agent – VPA were used as positive control in our study. The frequencies (b) of a compound pool including different kinds of compounds were screened using this Jurkat-Lat cell line. Column graphs represent statistical data for the reactivation efficacy towards HIV-1 proviruses of different compounds. These data were expressed as the mean $\pm$ SD. The representative data shown here were obtained from three independent experiments.

whether the observed reactivation was not due to the potential cytotoxicity of GIBH-LRA002, the viability of PBMC cells was measured by a CCK-8 cell proliferation assay when treated with this compound at different concentrations. There was no significant effect on cell viability when up to 500 μ M GIBH-LRA002 was added to these cells (Fig. 4B).

After confirming the activation activity of GIBH-LRA002, we subsequently tested whether it would reactivate latent proviruses in primary CD4⁺ T lymphocytes. The Jurkat T cell line model cannot reflect the genetic diversity of viral reservoir pool in primary cells which are integrated HIV proviruses in different genome sites. In this study, we firstly used primary CD4⁺ T

No.	R1 (R2=Boc; E.C.=10uM)	Multiple of activation	Multiple of cytotoxicity
A	H	0.25×	3×
B	COOCH ₃	2.1×	2.6×
C	COOBt	2.6×	1.8×
D	COOBt (pan-chirality)	1.5×	1.1×; all die at 100uM

No.	R2 (R1=COOBt; E.C.=10uM)	Multiple of activation	Multiple of cytotoxicity
1	H	2×	1.4×; all die at 100uM
2	Me	4.4×	1.3×
3	Bn	1.9×	2×; all die at 100uM
4	Boc	2.6×	1.8×

Fig. 3 Structure–activity study of pyranindole derivatives. The top table shows the activity and cytotoxicity when the R1 group was modified while R2 was fixed as the *tert*-butoxy group; the bottom table shows the activity and cytotoxicity when the R2 group was modified while R1 was fixed as the ethoxycarbonyl group. The multiple of activation or cytotoxicity was based on negative control which is equal to 1 when no compound was added to the Jurkat-Lat cell.

Fig. 4 Evaluation of the reactivation efficacy by GIBH-LRA002 stimulation in a Jurkat cell line, primary SIV-infected monkey CD4⁺ T cells and primary HIV-infected patient CD4⁺ T cells. (A) Different concentrations of GIBH-LRA002 were incubated with Jurkat cells for 24 hours and their GFP expression was analyzed by flow cytometry. (B) Different concentrations of GIBH-LRA002 were incubated with PBMC cells, and the viability of PBMCs was determined using a CCK8 kit. (C) Flowchart of determining cell-associated RNA of primary cells when stimulated with LRAs. The SIV-infected rhesus macaque or HIV-infected human PBMCs were isolated, and then CD4⁺ T cells were sorted by magnetic selection. The purified CD4⁺ T cells were incubated with different compounds for 24 hours, and cell-associated RNA were extracted from these stimulated CD4⁺ T cells. Real-time PCR was performed to analyze the copies of two kinds of transcriptional provirus RNA (msRNA and usRNA). Column graphs represent statistical data for the reactivation efficacy towards proviruses of GIBH-LRA002 in SIV-infected monkey CD4⁺ T cells (D and E) or HIV-1 infected human CD4⁺ T cells (F and G). These data were expressed as the mean $\pm$ SD. The representative data shown here were obtained from three independent experiments.

cells from chronic SIV-infected rhesus macaques to further evaluate the reactivation efficacy of GIBH-LRA002. These monkeys were infected with SIVmac239 for more than 5 years in our previously reported studies,^{11,12} and they had similar viral loads (ranging from 3.60 log to 6.33 log) in plasma to chronic HIV patients. For detecting the reactivation efficacy in primary cells, we developed quantitative assays for the level of cell-associated RNA, such as multiply spliced RNA (msRNA) and un-spliced RNA (usRNA), in LRA-treated CD4⁺ T lymphocytes (Fig. 4C). Cell-associated RNA was thought as sensitive parameters to represent the status of viral transcription and

reactivation.¹³ Interestingly, we found that valproic acid (VPA) reactivated the latent viruses in primary CD4⁺ T cells with very low efficacy (Fig. 4), though it reactivated the latent viruses in the Jurkat Lat cell line with high efficacy (Fig. 2). Notably, the levels of msRNA and usRNA copies in resting CD4⁺ T cells from both SIV-infected macaques were significantly elevated in a dose-dependent manner when different concentrations of GIBH-LRA002 were added for 24 hours (Fig. 4D and E).

Moreover, we tested the reactivation activity of GIBH-LRA002 in latent HIV-1 in primary CD4⁺ T lymphocytes from chronic HIV-1 patients. Peripheral blood mononuclear cells (PBMCs) were isolated respectively from four HIV-1 patients, who have received prolonged HAART therapy for several years and achieved effective control of viral replication in plasma (ESI[†]). CD4⁺ T cells were sorted using a magnetic cell sorting kit, and then the purified CD4⁺ T cells were incubated with GIBH-LRA002 for 24 hours. Similar to the above-mentioned quantitative assays for cell-associated RNA (Fig. 4C), the copies of msRNA and usRNA were detected in the CD4⁺ T cells of HIV-1 patients. To our surprise, we found that msRNA and usRNA were significantly elevated at a high level in response to stimulation with GIBH-LRA002, comparable to positive stimulators (PMA plus ionomycin) (Fig. 4F and G). Consistent with data in primary monkey CD4⁺ T cells, VPA did not reactivate the latent viruses in primary human CD4⁺ T cells. Consequently, these data demonstrated that GIBH-LRA002 has high potential to increase HIV-1/SIV transcription in primary CD4⁺ T cells from both chronic SIV-infected rhesus macaques and HIV-1 patients.

Hopefully, an ideal LRA candidate is expected to activate effectively the latent proviruses but without extensive T cell activation and inflammation responses. Thus, we then investigated how GIBH-LRA002 affected the global T cell activation. PBMCs were treated with GIBH-LRA002 for 24 hours, and the expressions of CD25, CD69 and CCR5 on the surface of PBMCs were detected by flow cytometry. Phorbol 12-myristate 13-acetate (PMA) was used as a positive control for T cell activation. Our data showed that no significant elevation of CD25, CD69 and CCR5 expression was found in the GIBH-LRA002-treated CD3⁺ T and CD4⁺ T cells (Fig. 5). Taken together, GIBH-LRA002 effectively reactivated the latent proviruses but without being involved in systemic activation in our study, which is consistent with the requirements of an ideal LRA.

Highly active antiretroviral therapy (HAART) only kills active HIV-1 viruses in plasma, but eradication of latent viral reservoirs from HIV-infected patients using this method is difficult. Nowadays, efforts to eradicate HIV infection have focused on reactivating latent proviruses with various compounds as latency-reversing agents (LRAs). Currently, several kinds of compounds have been tested as potential LRAs, including valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA) as histone deacetylase inhibitors (HDACis),¹⁴ BIX01294 as H3K9 histone methyltransferase inhibitors (HMTis),¹⁵ JQ-1 as BET bromodomain inhibitors,¹⁶ and PEP005 and disulfiram as protein kinase C (PKC)-NF- κ B pathway activators.¹⁷ These candidates were proven to potently reactivate HIV-1 latency under some conditions, but there

Fig. 5 Evaluation of extensive T cell activation induced with GIBH-LRA002. Monkey PBMCs were incubated with different concentrations of GIBH-LRA002 for 24 hours, and CD25, CD69 and CCR5 biomarkers of T cell activation were stained with specific monoclonal antibodies, and then detected by flow cytometry. PMA stimulation was used as the positive control.

has been no safe and effective LRA for clinical use yet. Therefore, screening and verifying potential LRAs based on novel structures is of great interest in the HIV cure field. Most importantly in this study, we found that GIBH-LRA002 showed efficacy in reactivation of HIV latency without exerting apparent cytotoxicity. Meanwhile, it was not involved in global T cell activation, thus preventing new viral infections due to excessive activation of CD4⁺ T cells.

One observation in this study is that variations on the side arms of the pyranoidole core could significantly influence the biological properties of these compounds. Our preliminary structure–activity relationship study has shown some promising implications for further structure modification, so it is likely that these compounds would be optimized to enhance their activation ability and lower cytotoxicity than that of GIBH-LRA002.

Further studies are warranted to investigate the molecular signaling mechanism to reactivate the HIV-1/SIV latency by GIBH-LRA002, and these data also inspired us to further develop GIBH-LRA002 for curing HIV-1 latency in animal infection models. Collectively, our findings may have implications for the design of optimal LRAs to purge HIV-1 latent reservoirs, and these data supported to advance GIBH-LRA002 as an attractive potential candidate for HIV cure strategies.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31370923), Science and Technology Planning Project of Guangdong Province (2017A020212005), the Municipal Health and Medical cooperation innovation Major Project of Guangzhou City (201508020256, 201704020219) and the National Science and Technology Major Project of China (2017ZX10301101-002).

Notes and references

- 1 K. Yoshimura, *J. Infect. Chemother.*, 2017, **1**, 12–16.
- 2 M. S. Dahabieh, E. Battivelli and E. Verdin, *Annu. Rev. Med.*, 2015, 407–421.
- 3 D. Persaud, P. E. Palumbo, C. Ziemniak, M. D. Hughes, C. G. Alvero, K. Luzuriaga, R. Yorgev, E. V. Capparelli and E. G. Chadwick, *AIDS*, 2012, **12**, 1483–1490.
- 4 S. M. Smith, *Retrovirology*, 2005, 56.
- 5 C. H. White, H. E. Johnston, B. Moesker, A. Manousopoulou, D. M. Margolis, D. D. Richman, C. A. Spina, S. D. Garbis, C. H. Woelk and N. Beliakova-Bethell, *Antiviral Res.*, 2015, 78–85.
- 6 T. A. Rasmussen and S. R. Lewin, *Curr. Opin. HIV AIDS*, 2016, **4**, 394–401.
- 7 A. Y. Howe, H. Cheng, I. Thompson, S. K. Chunduru, S. Herrmann, J. O'Connell, A. Agarwal, R. Chopra and A. M. Del Vecchio, *Antimicrob. Agents Chemother.*, 2006, **12**, 4103–4113.
- 8 A. Gopalsamy, K. Lim, G. Ciszewski, K. Park, J. W. Ellingboe, J. Bloom, S. Insaf, J. Upešlaciš, T. S. Mansour, G. Krishnamurthy, M. Damarla, Y. Pyatski, D. Ho, A. Y. Howe, M. Orlowski, B. Feld and J. O'Connell, *J. Med. Chem.*, 2004, **26**, 6603–6608.
- 9 J. A. May, H. H. Chen, A. Rusinko, V. M. Lynch, N. A. Sharif and M. A. McLaughlin, *J. Med. Chem.*, 2003, **19**, 4188–4195.
- 10 Q. X. Lou, Y. Y. Ding, D. F. Xu, G. K. Liu and J. L. Zhao, *Adv. Synth. Catal.*, 2017, **10**, DOI: 10.1002/adsc.201700465, (in press).
- 11 C. Sun, L. Zhang, M. Zhang, Y. Liu, M. Zhong, X. Ma and L. Chen, *J. Infect.*, 2010, **5**, 371–381.
- 12 C. Sun, Z. Chen, X. Tang, Y. Zhang, L. Feng, Y. Du, L. Xiao, L. Liu, W. Zhu, L. Chen and L. Zhang, *J. Virol.*, 2013, **10**, 5669–5677.
- 13 A. O. Pasternak, V. V. Lukashov and B. Berkhout, *Retrovirology*, 2013, 41.
- 14 Z. Klase, V. S. Yedavalli, L. Houzet, M. Perkins, F. Maldarelli, J. Brenchley, K. Strebel, P. Liu and K. T. Jeang, *PLoS Pathog.*, 2014, **3**, e1003997.
- 15 K. Imai, H. Togami and T. Okamoto, *J. Biol. Chem.*, 2010, **22**, 16538–16545.
- 16 G. Darcis, A. Kula, S. Bouchat, K. Fujinaga, F. Corazza, A. Ait-Ammar, N. Delacourt, A. Melard, K. Kabeya, C. Vanhulle, B. Van Driessche, J. S. Gatot, T. Cherrier, L. F. Pianowski, L. Gama, C. Schwartz, J. Vila, A. Burny, N. Clumeck, M. Moutschen, S. De Wit, B. M. Peterlin, C. Rouzioux, O. Rohr and C. Van Lint, *PLoS Pathog.*, 2015, **7**, e1005063.
- 17 G. Jiang, E. A. Mendes, P. Kaiser, D. P. Wong, Y. Tang, I. Cai, A. Fenton, G. P. Melcher, J. E. Hildreth, G. R. Thompson, J. K. Wong and S. Dandekar, *PLoS Pathog.*, 2015, **7**, e1005066.