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TECHNICAL REPORT

ANTIRETROVIRAL ACTIVITY OF PROTEASE INHIBITORS AGAINST *Toxoplasma gondii*

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SUMMARY

The introduction of highly active antiretroviral therapy (HAART) has caused a marked reduction in the occurrence and severity of parasitic infections, including the toxoplasmic encephalitis (TE). These changes have been attributed to the restoration of cell-mediated immunity. This study was developed to examine the activity of six antiretroviral protease inhibitors (API) on *Toxoplasma gondii* tachyzoites. The six API showed anti-*Toxoplasma* activity, with IC_{50} value between 1.4 and 6.6 µg/mL. Further studies at the molecular level should be performed to clarify if the use of API could be beneficial or not for AIDS patients with TE.

KEYWORDS: *Toxoplasma gondii;* Antiretroviral protease inhibitors; Toxoplasmic encephalitis.

Toxoplasma gondii is a widely distributed apicomplexan parasite of great medical importance. Most primary infections are asymptomatic, and may affect up to one-third of the human population worldwide[7]. In immunocompromised patients, especially those with acquired immunodeficiency syndrome (AIDS), reactivation of latent infection causes necrotic lesions producing life threatening encephalitis[9]. The introduction of highly active antiretroviral therapy (HAART) has caused a marked reduction in the occurrence and clinical course of different parasitic infections, including toxoplasmic encephalitis (TE). Although these changes have been attributed to the restoration of cell-mediated immunity induced by either non-nucleoside reverse transcriptase inhibitors or protease inhibitors, in combination with at least two nucleoside reverse transcriptase inhibitors included in HAART, there are evidences that antiretroviral protease inhibitors (APIs) have a direct inhibitory effect on the proteases of some parasites[4]. Previously, experimental studies demonstrated the antiparasitic activity of APIs against *Plasmodium falciparum*[10] and *Leishmania spp*[13]. Against *T. gondii*, the *in vitro* inhibition has been observed for two protease inhibitors (indinavir and nelfinavir)[6]. The aim of this study was to investigate the *in vitro* effects against *T. gondii* of six available APIs, which are used in the treatment of Cuban patients with AIDS.

Tachyzoites of the virulent *T. gondii* RH strain maintained through serial intraperitoneal passages in Swiss mice were used. Tachyzoites were harvested from mouse peritoneal fluids 72 h post-infection and purified by centrifugation and needle extraction in the moment of use.

Female Swiss Webster mice, with a body weight of approximately 22 to 26 g, were obtained from The National Centre of Laboratory Animals Production (CENPALAB), Cuba. The animals were kept according to "Guidelines on the Care and Use of Laboratory Animals".

Atazanavir (Bristol-Myers Squibb, USA), fosamprenavir (GlaxoSmithKline, United Kingdom), indinavir (Novatec, Havana City, Cuba), nelfinavir (CIPLA, India), ritonavir (CIPLA, India) and saquinavir (Hoffmann-La Roche SA Basilea, Switzerland) were dissolved in dimethylsulfoxide (DMSO) at final concentration of 10 mg/mL. Sulfadiazine and pyrimethamine (Ipca Laboratories Ltd, India) was used as reference drug dissolved in DMSO. The products were stored at 4 ºC.

Biological evaluation: Resident macrophages were collected from the peritoneal cavities of normal Swiss Webster mice in ice-cold RPMI 1640 medium (SIGMA, St. Louis, Mo, USA) supplemented with antibiotics, and seeded at 3 x 10^5 cell/well in a 24 well plate and incubated for two hours at 37 ºC in 5% CO_2. Non-adherent cells were removed by washing with PBS, and then, *T. gondii* tachyzoites were added at a 1:3 parasite/macrophage ratio, and the cultures were incubated for one hour. Free parasites were removed by washing and the drugs were added at final concentrations between 1.25 to 10 µg/mL. The cultures were incubated for 48 h in same conditions. The cells and parasites were then fixed in absolute methanol, stained with Giemsa, and examined under light microscopy. The percentage of infected macrophage was determined by the counting of 1000 cells. The IC_{50} value was determined from the lineal equation obtained from concentration-response curves[8]. Three replicates were carried out and the results are presented as average ± standard deviation.

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*Cytotoxicity assay***:** Resident macrophages were collected and seeded as previously described in plates of 96 wells. Dilutions of the APIs were added and the cytotoxicity was determined after three days of incubation using a colorimetric assay with 15 mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA) and an additional incubation for four hours. The formazan crystals were dissolved by the addition of 100 mL of DMSO and the optical density was determined using an EMS Reader MF Version 2.4-0, at a test wavelength of 560 nm and a reference wavelength of 630 nm[15]. The 50% cytotoxic concentration (CC_{50}) was obtained from dose-response curves fit to data by means of the equation for the sigmoidal E_{max} model[2]. Three replicates were carried out and the results are presented as average ± standard deviation. Selectivity indices (SI) were then calculated by dividing the CC_{50} for peritoneal macrophage of mice by the IC_{50} for *T. gondii*[3].

Statistical analysis: The three values of IC_{50} obtained from each experiment were used and compared by Mann-Whitney test (Statistical for Windows Program, Release 4.5, StatSoft, Inc. 1993). Statistical differences were when $p < 0.05$.

The APIs were evaluated against intracellular tachyzoites resident in peritoneal macrophage from OF-1 mice. All tested APIs demonstrated anti-*Toxoplasma* activity at micromolar concentrations after 48 hours (Table I). The best activity was shown by atazanavir, which had a statistically different IC_{50} value ($p < 0.05$) compared with the other APIs. The compounds were toxic to mammalian host, with selectivity index from 6 to 15 and IC_{50} values from 15.9 to 76.8 µg/mL. Sulfadiazine and pyrimethamine were active in a similar concentrations reported[3,5]. Nevertheless, both compounds showed higher cytotoxicity compared with APIs, with selectivity index of 4 and 1, respectively.

Table 1
In vitro effects of antiretroviral protease inhibitors on *Toxoplasma gondii* RH strain OF-1 mice

Drugs	IC_{50} [a] ± SD [b] (µg/mL)	CC_{50} [c] ± SD (µg/mL)	SI[d]
	Tachyzoites	Macrophages	
Atazanavir	1.4 ± 0.3*	15.9 ± 0.6	11
Fosamprenavir	3.3 ± 0.9	51.0 ± 0.9	15
Indinavir	6.6 ± 1.5	76.8 ± 1.3	12
Nelfinavir	4.0 ± 0.2	24.8 ± 0.3	6
Ritonavir	3.4 ± 0.7	26.4 ± 0.5	8
Saquinavir	2.6 ± 0.3	24.9 ± 1.1	10
Sulfadiazine	5.8 ± 0.9	6.3 ± 0.8	1
Pyrimethamine	0.3 ± 0.01	1.1 ± 0.3	4

[a]: IC_{50}; Concentration of the compounds that caused 50% of mortality; [b]: SD; Standard deviation; [c]: CC_{50}; Concentration of the compound that caused 50% of cytotoxicity on host cell; [d]: Selectivity index: CC_{50} against macrophage / IC_{50} against tachyzoites of *T. gondii;* *: Statistical differences with respect to other evaluated compounds ($p < 0.05$)

Several studies concerning *Toxoplasma*/HIV co-infections have been published; particularly focused on diagnosis, pathogenesis and treatment. The treatment of these patients with APIs has been considered as one of the more promising studies. Currently, APIs have been also reported as potential drugs for the treatment of intracellular protozoa. All compounds studied have been used to treat HIV patients due to their potent *in vitro* antiviral activity. Specifically for *T. gondii*, all evaluated APIs exhibited *in vitro* activity and were selectivity better than other clinically used drugs such as sulfadiazine and pyrimethamine. Calculated IC_{50} were similar to those previously reported by DEROUIN & SANTILLANA-HAYAT which reported that nelfinavir and ritonavir showed an IC_{50} of 4.0 and 5.4 µg/mL, respectively[6]. The activity showed by PI, together with their cellular permeability properties could justify the further exploration of APIs as anti-*Toxoplasma* drugs in TE and ocular infections. In addition, available APIs can gain roles as treatment for toxoplasmic complicated infections due to availability of the drug and potential activity on resistant strains[6].

The mode of action of APIs on parasites, including *T. gondii*, still needs to be clarified. Nevertheless, in protozoan parasites the aspartyl protease enzymes seem to play important roles in the life cycle and display diverse functions, including the invasion of host cells and tissues, the degradation of mediators of the immune response and the hydrolysis of host proteins for nutritional purposes[11,12]. For *T. gondii*, seven genes coding for putative aspartic proteases have been identified with known human and apicomplexan proteases[13]. In this sense, SANTOS *et al.* demonstrated that nelfinavir powerfully inhibited the hydrolysis of HIV peptidase substrate by *L. amazonensis* extract[12]; while ANDREWS *et al.* reported that *in vitro* enzyme assays with plasmepsins II and IV from *P. falciparum* are both inhibited by saquinavir and ritonavir[1]. Further experiments with toxoplasma enzyme are required to back up this hypothesis.

In conclusion, our results demonstrated for the first time the potential activity of APIs (atazanavir fosamprenavir, indinavir and saquinavir) against *T. gondii*, which have not been reported previously. In this sense, *in vitro* data suggest the possibility that HIV patients receiving PI therapy might also benefit from an effect against *Toxoplasma* due to inhibition of parasite growth, and consequently limiting the development of the TE disease. Further studies about the method of the action of APIs using *T. gondii* enzymes, as well as the interaction with the co-infection HIV-parasite can help to clarify the influence of API in the development of TE in AIDS patients. In addition, new strategies can be useful in designing new PI with specific activity on protozoa parasites, as well as the exploration of their potential in patients infected only with *T. gondii*.

RESUMEN

Terapia antiretroviral de inibidores da protease contra *Toxoplasma gondii*

La introducción de la terapia antirretroviral de alta efectividad ha causada una marcada reducción en la ocurrencia y curso clínico de las infecciones parasitarias, incluyendo la toxoplasmosis encefálica (TE). Estos cambios han sido atribuidos a la restauración celular. Este estudio fue desarrollado para examinar la actividad de seis inhibidores de proteasas antirretrovirales (IPA) sobre taquizoitos de *Toxoplasma gondii*. Los seis IPA mostraron actividad anti-*Toxoplasma*, con valores de CI_{50} entre 1.4 y 6.6 µg/mL. Futuros estudios a nivel molecular deben ser realizados, los cuales podrán delucidar si el uso de IPA pudiera beneficiar o no a los pacientes que sufren de TE.

REFERENCES

1. Andrews KT, Fairlie DP, Madala PK, Ray J, Wyatt DM, Hilton PM, *et al*. Potencies of human immunodeficiency virus protease inhibitors *in vitro* against *Plasmodium falciparum* and *in vivo* against murine malaria. Antimicrob Agents Chemother. 2006;50:639-48.

2. Bodley AL, McGarry MW, Shapiro TA. Drug cytotoxicity assay for African Trypanosomes and *Leishmania* species. J Infect Dis. 1995;172:1157-9.

3. Choi KM, Gang J, Yun J. Anti-*Toxoplasma gondii* RH strain activity of herbal extracts used in traditional medicine. Int J Antimicrob Agents. 2008;32:360-2.

4. de Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. Int J Antimicrob Agents. 2009;33:307-20.

5. Derouin F. Anti-toxoplasmosis drugs. Curr Opin Investing Drugs. 2001:2:1368-74.

6. Derouin F, Santillana-Hayat M. Anti-*Toxoplasma* activities drugs and interactions with Pyrimethamine and Sulfadiazine *in vitro*. Antimicrob Agents Chemother. 2009;44:2575-7.

7. Hill D, Dubey JP. *Toxoplasma gondii*: transmission, diagnosis and prevention. Clin Microbiol Infect. 2002;8:634-40.

8. McLeod R, Bensch KG, Smith SM, Remington JS. Effects of human peripheral blood monocytes, monocyte-derived macrophages and spleen mononuclear phagocytes on *Toxoplasma gondii*. Cell Immunol. 1980;54:330-50.

9. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet. 2004;363:1965-76.

10. Parikh S, Gut J, Istvan E, Goldberg DE, Havlir DV, Rosenthal PJ. Antimalarial activity of Human Immunodeficiency Virus Type 1. Antimicrob Agents Chemother. 2005;49:2983-5.

11. Pozio E, Morales MA. The impact of HIV-protease inhibitors on opportunistic parasites. Trends Parasitol. 2005;21:58-63.

12. Santos LO, Marinho FA, Altoé EF, Vitório BS, Alves CR, Britto C, *et al.* HIV aspartyl peptidase inhibitors interfere with cellular proliferation, ultrastructure and macrophage infection of *Leishmania amazonensis*. Plos One. 2009;4:e4918.

13. Savoia D, Allice T, Tovo PA. Antileishmanial activity of HIV protease inhibitors. Int J Antimicrob Agents. 2005;26:92-4.

14. Shea M, Jakle U, Liu Q, Berry C, Joiner KA, Soldati-Favre D. A family of aspartic proteases and a novel, dynamic and cell-cycle-dependent protease localization in the secretory pathway of *Toxoplasma gondii.* Traffic. 2007;8:1018-34.

15. Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. J Immunol Methods. 1993;157:203-7.

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