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Efficacy of PTX3 and Posaconazole Combination in a Rat Model of Invasive Pulmonary Aspergillosis

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Posaconazole is currently used for the prophylaxis of invasive pulmonary aspergillosis (IPA). Limitations to posaconazole usage are drug-drug interactions and side effects. PTX3 is an innate immunity glycoprotein with opsonic activity, proven to be protective in IPA animal models. This study investigated the combination of posaconazole with PTX3. The results indicate synergy between PTX3 and posaconazole against aspergillosis, suggesting that a combination of reduced doses of posaconazole with the immune response enhancer PTX3 might represent a treatment option with a higher therapeutic index than posaconazole.

Aspergillus fumigatus is a pathogenic fungus and one of the main causes of morbidity and mortality in patients with immunodeficiency, particularly after allogeneic bone marrow transplantation (1, 2). Because aspergillus spores (conidia) are ubiquitous in the environment, invasive pulmonary aspergillosis (IPA) is among the most frequent invasive fungal infections (3, 4).

Triazole antifungal drugs such as itraconazole, posaconazole (PCZ), and voriconazole are currently the mainstays of therapy in the management and prophylaxis of IPA. Posaconazole, in particular, is a new oral triazole drug with an enhanced spectrum of *in vitro* activity against a wide range of fungi, and it is currently used for the prophylaxis of IPA. However, the efficacy of PCZ might be limited by poor absorption, drug-drug interactions, and side effects, all leading to the discontinuation of treatment (5, 6, 7).

PTX3 is a member of the pentraxin superfamily, a group of acute-phase proteins highly conserved during evolution (8, 9). PTX3 functions as an opsonin and, by selective binding of microbial agents (such as conidia of *Aspergillus fumigatus*), promotes phagocytosis by several somatic cell types, thus activating various effector pathways in response to infections (10). Complement activation by the classic and lectin pathways may also contribute to the protective activity of PTX3 against *Aspergillus* and other pathogens (11). PTX3 localization in the neutrophil extracellular trap (NET) has also a key role in *Aspergillus* conidium recognition and killing (12).

PTX3 was previously shown to inhibit infection and increase survival in rat and mouse models of IPA (13, 14). We also previously demonstrated that the combination of PTX3 with first-line antifungals, such as amphotericin B and voriconazole, results in additive or synergic activities against *Aspergillus*.

On this basis, the aim of this study was to evaluate the combination of PTX3 and PCZ. The experiments were performed in a rat model of IPA as previously described (15). Briefly, Sprague-Dawley rats (Harlan) were immunosuppressed with 150 mg of cortisone acetate (CA)/kg of body weight 6 days before and then every other day up to the day of infection and maintained with 80 mg/kg every other day until the end of the experiment. The rats were infected by intratracheal deposition of *A. fumigatus* conidia (5×10^7/0.2 ml/rat). The posaconazole oral suspension was prepared from the clinical product Noxafil (Merck Sharp & Dohme) (40 mg/ml). All procedures with animals were carried out in accordance with the standards established by the Animal Ethical Committee of Takis.

The recombinant human PTX3 protein (UniProtKB accession no. P26022) was produced by a stable transfectant cell and purified to pharmaceutical grade by sequential chromatographic steps. The protein was formulated in phosphate buffer (137 mM NaCl, 10 mM phosphate [pH 7.2]) by tangential flow filtration to 2.5 mg/ml. PTX3 and PCZ were evaluated singularly in dose-response experiments in order to identify the dose range that counteracted aspergillosis and to select the doses to be used subsequently in the combination study (Table 1).

Four doses of PTX3 (0.35, 0.7, 1.5, and 3 mg/kg) were administered intraperitoneally (i.p.) in rats 3 days before the day of infection and then for an additional 3 days (Fig. 1a). PCZ was administered orally at doses of 2, 4, and 10 mg/kg on the day of infection and then once daily for an additional 8 days (Fig. 1a). Control animals were administered PCZ and PTX3 vehicles (water plus 5% glucose and phosphate-buffered saline [PBS], respectively) by the same schedules. The mortality rate was recorded every day until the end of the studies, while the fungal burden was evaluated by measuring the galactomannan index (GMI) in rat lungs by means of a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Platelia *Aspergillus*; Bio-Rad Laboratories).

The PCZ dose of 10 mg/kg resulted in the highest reductions in lung weight and GMI level but in the lowest survival percentage (Table 1). This is probably due to the potential side effects of PCZ. Consistently, upon necropsy we observed mainly gastrointestinal effects, resulting in apparent induration of the stomach and upper intestinal tract, similar to what was reported in patients (6, 7). As a consequence, the dose of 4 mg/kg of PCZ was chosen as the best compromise between survival enhancement and lung fungal bur-

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TABLE 1 Dose responses of PTX3 and PCZ in the rat model of IPA

Treatment	No. of animals	Dose (mg/kg)	Schedule (route)[a]	Survival (%)	MST (days)[b]	Lung wt (mean ± SEM) (g)	Fungal burden as lung GMI (mean ± SEM)[c]
Vehicle (PBS)	16		−3, +3 (i.p.)	12.5	9	2.1 ± 0.25	2.5 ± 0.26
PTX3	16	0.35	−3, +3 (i.p.)	50	12	1.6 ± 0.23[d]	1.6 ± 0.33[d]
PTX3	16	0.7	−3, +3 (i.p.)	50	11	1.7 ± 0.13	1.9 ± 0.34
PTX3	16	1.5	−3, +3 (i.p.)	46	11	2 ± 0.3	2 ± 0.34
PTX3	16	3	−3, +3 (i.p.)	18	8.5	2 ± 0.34	2.1 ± 0.33
Vehicle (water plus 5% glucose)	16		0, +8 (os)	6	4	2.6 ± 0.29	3.1 ± 0.26
PCZ	16	2	0, +8 (os)	38	6.5	2.1 ± 0.27	2.2 ± 0.39
PCZ	16	4	0, +8 (os)	47	11.5	1.8 ± 0.12[d]	2.2 ± 0.40[d]
PCZ	16	10	0, +8 (os)	33	6.5	1.5 ± 0.1[d]	1.1 ± 0.28[d]

[a] Numbers represent the first and last administration days, with the day of infection as day 0. i.p., intraperitoneal administration; os, oral administration.
[b] MST, mean survival time.
[c] The GMI values were calculated according to the following formula: optical density (OD) of the samples/mean cutoff control OD.
[d] Significantly different ($P < 0.05$) from comparison group according to Student's *t* test.

den reduction. The dose of 0.35 mg/kg was chosen for PTX3 because it was the one that significantly enhanced survival and reduced lung fungal burden (Table 1). Combination treatments were performed following the administration schedule for each molecule alone as described in the scheme reported in Fig. 1a. Survival rates of 37, 32, and 87% of rats were observed with PCZ, PTX3, and the PCZ-PTX3 combination, respectively. Accordingly, the mean survival times and lung fungal burden data indicated a significant synergic activity of PTX3 and PCZ (Fig. 1b and c). Evaluation of the lung levels of myeloperoxidase (MPO), a marker of neutrophil activity monitored by ELISA (Hycult Biotech catalog no. HK105), clearly confirmed the synergy of PTX3

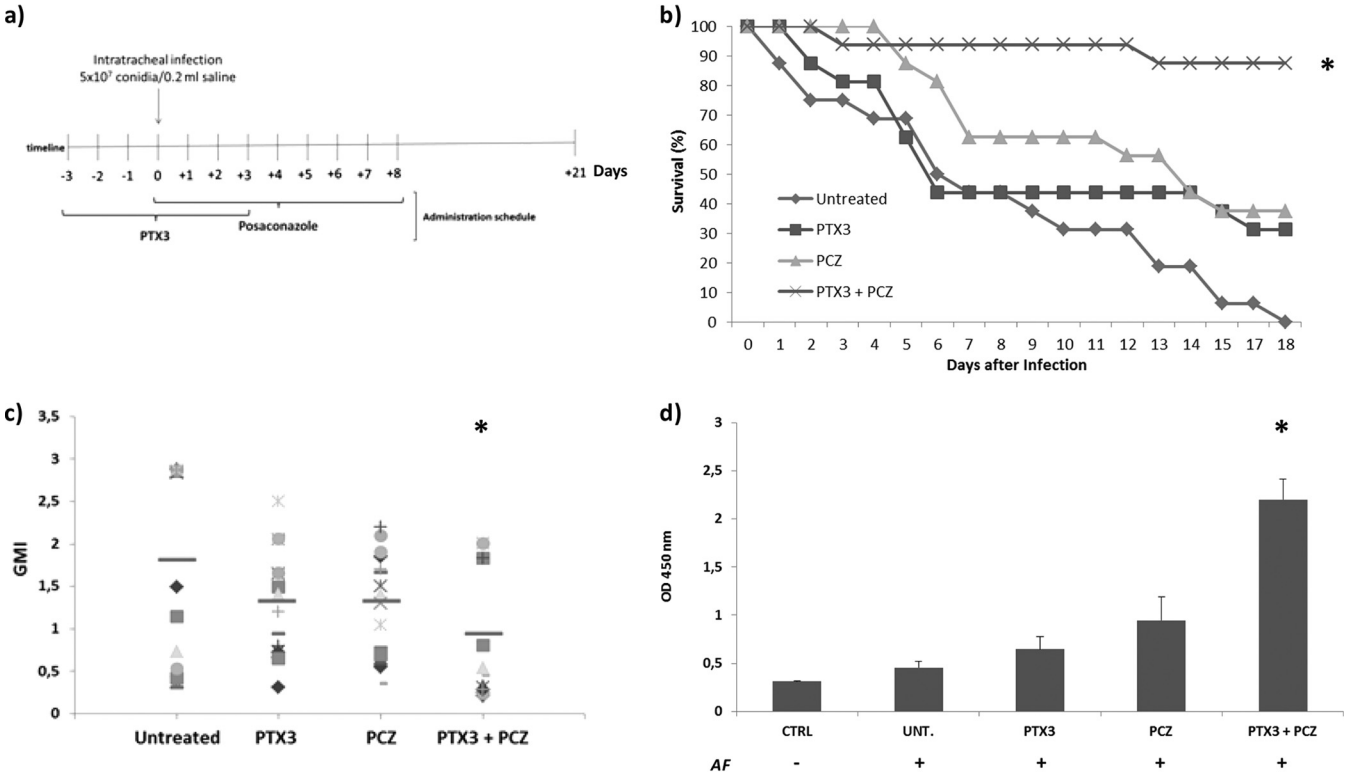

FIG 1 Combination of posaconazole and PTX3 treatments in rats immunosuppressed with cortisone acetate and infected with *Aspergillus fumigatus* conidia as described in the experimental scheme. (a) Experimental scheme for the administration schedule. (b) Survival curves for rats treated with vehicle (Untreated), posaconazole at 4 mg/kg by oral administration, PTX3 at 0.37 mg/kg by intraperitoneal administration, or a PTX3 and posaconazole combination at the same doses and administration routes used for each molecule alone. The overall survival rates (%) (mean survival times [MST]) were the following: none (MST, 7 days) for the group of rats receiving vehicle, 87% (MST, >18 days) for the rats that received a PTX3 and posaconazole combination, 32% (MST, 6.5 days) for rats that received PTX3, and 37% (MST, 14.5 days) for rats treated with posaconazole. (c) Fungal burden of treated rats. The fungal burden was measured by evaluation of the galactomannan content in the lung and was expressed as the GMI. Circles represent the GMI from a single animal. Means are represented by bars. *, $P < 0.05$ (compared to the vehicle). (d) Levels of lung myeloperoxidase (MPO) for different conditions of treatment. Rats immunosuppressed, infected, and treated as described in the experimental scheme were sacrificed on day 3 postinfection (4 rats/group), lungs were collected, and MPO levels were measured by an ELISA. The amount of MPO in the immunocompromised uninfected animals is represented by the CTRL bar. *, $P < 0.05$ (compared to the vehicle). *AF*, *Aspergillus fumigatus*; OD450 nm, optical density at 450 nm.

and PCZ in strengthening the innate immune response against the fungus (Fig. 1d). These data are in agreement with the reported binding of PTX3 to MPO (16) and suggest that trapping of MPO by PTX3 in the lung could enhance fungal clearance.

The plasma concentration of endogenous PTX3 in rat is in the range of 20 to 30 ng/ml (data not shown). In one experiment, human recombinant PTX3 administered (i.p.) at 1 mg/kg raised the plasma level in the range of 1.5 to 0.75 μg/ml 1 h after protein administration, well above the physiological level of the endogenous protein. These data suggest that the pharmacological protective activity of PTX3 against *Aspergillus* may rely on a high level of the recombinant protein in the plasma. Similarly, the physiological level of PTX3 in human plasma is around 2 ng/ml (17). Infectious diseases, including aspergillosis, raise plasma levels in the range of 20 to 150 ng/ml. Assuming a human equivalent dose in the range of 10 to 15 mg for a 70-kg patient, the plasma level of PTX3 soon after injection would be raised to 2 to 3 μg/ml, at least 1 log above the highest average concentration of PTX3 with an infection.

The pharmacokinetics (PK) of posaconazole depends on the cytochrome P450 (CYP450) enzyme pathway (18). We are not currently aware of any interaction between PTX3 and PCZ that may affect the pharmacokinetics of these two molecules. Importantly, no apparent side effects were observed in the treated rats.

Our results suggest that the cytostatic and cytotoxic activities of PCZ sustained by PTX3 with an enhanced immune-mediated clearance of the fungus might explain the observed synergic activity. This study supports the potential clinical benefit of combining PCZ and PTX3 in those clinical cases where a reduction in triazole dosage is desirable to minimize side effects or drug-drug interactions.

REFERENCES

1. **Zmeili OS, Soubani AO.** 2007. Pulmonary aspergillosis: a clinical update. Q. J. M. **100:**317–334. http://dx.doi.org/10.1093/qjmed/hcm035.
2. **Latgé JP.** 2001. The pathobiology of *Aspergillus fumigatus*. Trends Microbiol. **9:**382–389. http://dx.doi.org/10.1016/S0966-842X(01)02104-7.
3. **Burguete SR, Maselli DJ, Fernandez JF, Levine SM.** 2013. Lung transplant infection. Respirology **18:**22–38. http://dx.doi.org/10.1111/j.1440-1843.2012.02196.x.
4. **Marr KA, Carter RA, Crippa F, Wald A, Corey L.** 2002. Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. **34:**909–917. http://dx.doi.org/10.1086/339202.
5. **Dolton MJ, Ray JE, Chen SC, Ng K, Pont L, McLachlan AJ.** 2012. Multicenter study of posaconazole therapeutic drug monitoring: exposure-response relationship and factors affecting concentration. Antimicrob. Agents Chemother. **56:**5503–5510. http://dx.doi.org/10.1128/AAC.00802-12.
6. **Ullmann AJ, Lipton JH, Vesole DH, Chandrasekar P, Langston A, Tarantolo SR, Greinix H, Morais de Azevedo W, Reddy V, Boparai N, Pedicone L, Patino H, Durrant S.** 2007. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. N. Engl. J. Med. **356:**335–347. http://dx.doi.org/10.1056/NEJMoa061098.
7. **Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ, Helfgott D, Holowiecki J, Stockelberg D, Goh YT, Petrini M, Hardalo C, Suresh R, Angulo-Gonzalez D.** 2007. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N. Engl. J. Med. **356:**348–359. http://dx.doi.org/10.1056/NEJMoa061094.
8. **Introna M, Breviario F, d'Aniello EM, Golay J, Dejana E, Mantovani A.** 1993. IL-1 inducible genes in human umbilical vein endothelial cells. Eur. Heart J. **14**(Suppl K)**:**78–81.
9. **Garlanda C, Bottazzi B, Bastone A, Mantovani A.** 2005. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. Annu. Rev. Immunol. **23:**337–366. http://dx.doi.org/10.1146/annurev.immunol.23.021704.115756.
10. **Moalli F, Jaillon S, Inforzato A, Sironi M, Bottazzi B, Mantovani A, Garlanda C.** 2011. Pathogen recognition by the long pentraxin PTX3. J. Biomed. Biotechnol. **2011:**830421.
11. **Inforzato A, Doni A, Barajon I, Leone R, Garlanda C, Bottazzi B, Mantovani A.** 2013. PTX3 as a paradigm for the interaction of pentraxins with the complement system. J. Biomed. Biotechnol. Semin. Immunol. **25:**79–85.
12. **Jaillon S, Peri G, Delneste Y, Frémaux I, Doni A, Moalli F, Garlanda C, Romani L, Gascan H, Bellocchio S, Bozza S, Cassatella MA, Jeannin P, Mantovani A.** 2007. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. J. Exp. Med. **204:**793–804. http://dx.doi.org/10.1084/jem.20061301.
13. **Gaziano R, Bozza S, Bellocchio S, Perruccio K, Montagnoli C, Pitzurra L, Salvatori G, De Santis R, Carminati P, Mantovani A, Romani L.** 2004. Anti-*Aspergillus fumigatus* efficacy of pentraxin 3 alone and in combination with antifungals. Antimicrob. Agents Chemother. **48:**4414–4421. http://dx.doi.org/10.1128/AAC.48.11.4414-4421.2004.
14. **Lo Giudice P, Campo S, De Santis R, Salvatori G.** 2012. Effect of PTX3 and voriconazole combination in a rat model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. **56:**6400–6402. http://dx.doi.org/10.1128/AAC.01000-12.
15. **Lo Giudice P, Campo S, Verdoliva A, Rivieccio V, Borsini F, De Santis R, Salvatori G.** 2010. Efficacy of PTX3 in a rat model of invasive aspergillosis. Antimicrob. Agents Chemother. **54:**4513–4515. http://dx.doi.org/10.1128/AAC.00674-10.
16. **Daigo K, Yamaguchi N, Kawamura T, Matsubara K, Jiang S, Ohashi R, Sudou Y, Kodama T, Naito M, Inoue K, Hamakubo T.** 2012. The proteomic profile of circulating pentraxin 3 (PTX3) complex in sepsis demonstrates the interaction with azurocidin 1 and other components of neutrophil extracellular traps. Mol. Cell. Proteomics **11:**M111.015073.
17. **Canovi M, Lucchetti J, Stravalaci M, Valentino S, Bottazzi B, Salmona M, Bastone A, Gobbi M.** 2014. A new surface plasmon resonance-based immunoassay for rapid, reproducible and sensitive quantification of pentraxin-3 in human plasma. Sensors (Basel) **14:**10864–10875. http://dx.doi.org/10.3390/s140610864.
18. **Wexler D, Courtney R, Richards W, Banfield C, Lim J, Laughlin M.** 2004. Effect of posaconazole on cytochrome P450 enzymes: a randomized, open-label, two-way crossover study. Eur. J. Pharm. Sci. **21:**645–653. http://dx.doi.org/10.1016/j.ejps.2004.01.005.