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In vitro and in vivo activity of iclaprim, a diaminopyrimidine compound and potential therapeutic alternative against *Pneumocystis* pneumonia

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Abstract

Pneumocystis pneumonia is a serious complication that may affect immunosuppressed patients. The absence of reliable and safe therapeutic alternatives to trimethoprim–sulfamethoxazole (TMP/SMX) justifies the search for more effective and less toxic agents. In this study, the in vitro and in vivo anti-*Pneumocystis jirovecii* activity of iclaprim, a diaminopyrimidine compound that exerts its antimicrobial activity through the inhibition of dihydrofolate reductase (DHFR), as does TMP, was evaluated alone or in combination with SMX. The antimicrobial activity of iclaprim was tested in vitro using an efficient axenic culture system, and in vivo using *P. carinii* endotracheally inoculated corticosteroid-treated rats. Animals were orally administered iclaprim (5, 25, 50 mg/kg/day), iclaprim/SMX (5/25, 25/125, 50/250 mg/kg/day), TMP (50 mg/kg/day), or TMP/SMX (50/250 mg/kg/day) once a day for ten consecutive days. The in vitro maximum effect (E_{max}) and the drug concentrations needed to reach 50% of E_{max} (EC_{50}) were determined, and the slope of the dose–response curve was estimated by the Hill equation (E_{max} sigmoid model). The iclaprim EC_{50} value was 20.3 μg/mL. This effect was enhanced when iclaprim was combined with SMX (EC_{50}: 13.2/66 μg/mL) ($p =$ 0.002). The TMP/SMX EC_{50} value was 51.4/257 μg/mL. In vivo, the iclaprim/SMX combination resulted in 98.1% of inhibition compared to TMP/SMX, which resulted in 86.6% of inhibition ($p =$ 0.048). Thus, overall, the iclaprim/SMX combination was more effective than TMP/SMX both in vitro and in vivo, suggesting that it could be an alternative therapy to the TMP/SMX combination for the treatment of *Pneumocystis* pneumonia.

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Introduction

Pneumocystis pneumonia (PcP) is an opportunistic infection caused by *Pneumocystis jirovecii*, which occurs when cellular immunity is depressed because of AIDS [1], malignancies [2], prolonged immunosuppressing drugs [3], IgA nephropathy [4], organ transplantation [5], or corticosteroid therapy [6]. The PcP mortality rate is high among patients with delayed diagnosis [2] and treatment, and death is due to severe respiratory failure [7].

Serological data indicate that *Pneumocystis* primary infection occurs in 70–90% of immunocompetent 2–4-year-old children from temperate or tropical regions [8, 9], and could be associated with upper respiratory infection in infants, predominantly those 1.5–4 months of age [9]. PCR survey of healthy people in Spain has revealed *P. jirovecii* in 20% of the population [10]. In addition, *Pneumocystis* organisms were detected frequently in neonatal or young children [11, 12], pregnant women [13], and patients with chronic underlying diseases [14–17]. Thus, *Pneumocystis* organisms could

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behave as a comorbidity factor [11, 15]. For instance, pulmonary carriage of *P. jirovecii* in patients with chronic obstructive pulmonary disease (COPD) could worsen the progression of this illness [15], which is the fifth most common cause of death in high-income countries [18].

Available drugs that are effective against PcP are limited. The first-line treatment and prophylaxis for *P. jirovecii* infection is trimethoprim–sulfamethoxazole (TMP/SMX) [19]; however, numerous side effects have been associated with this therapy [20–23]. Moreover, reports suggest an emergence of *P. jirovecii* resistance to sulfa drugs [24–29]. Unfortunately, the lack of cultures of *P. jirovecii* from these cases has not allowed confirmation of the cases of clinically suspected drug resistance [24]. No information about *P. jirovecii* resistane to the combination of iclaprim plus sulfa drugs is known. Efforts have been made to study putative drug resistance against TMP/SMX by sequencing the *Pneumocystis* dihydropteroate synthase (DHPS) gene from clinical *P. jirovecii* isolates. This gene is highly conserved in many organisms, and similar DHPS mutations can confer resistance to sulfa drugs in either prokaryotic (*Escherichia coli*, *Streptococcus pneumoniae*) or eukaryotic (*Plasmodium falciparum*) pathogens [30–33]. The widespread use of TMP/SMX for PcP prophylaxis could, therefore, select *P. jirovecii* strains with DHPS mutations. Consistently, DHPS mutations have been associated with both the use of sulfa drugs (TMP/SMX or dapsone, a sulfone) [29] and the duration of SMX or sulfone prophylaxis [34]. The American Thoracic Society (ATS) recommended *P. jirovecii* drug resistance as a topic of clinical research, including the association of DHPS mutations with TMP/SMX treatment failure or death [35].

The absence of suitable therapeutic alternatives to the TMP/SMX drug combination constitutes an unmet medical need; pentamidine exhibits significant toxicity [36, 37] and atovaquone is used only against mild forms of PcP [38]. Thus, deleterious side effects, limited efficacies, and emerging resistance justify the search for more effective and less toxic anti-*Pneumocystis* agents.

Iclaprim, a diaminopyrimidine compound that exerts its antimicrobial activity through the inhibition of dihydrofolate reductase (DHFR) [39, 40], may represent a new option for responding to this medical need. For these reasons, the activity of iclaprim against *Pneumocystis* was tested both in vitro, using an efficient axenic culture system [41], and in vivo, using *P. carinii* endotracheally inoculated corticosteroid-treated rats [42–45].

Materials and methods

Drugs

Iclaprim, TMP, and SMX were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, France). TMP and SMX solutions were mixed appropriately to obtain a final 1:5 combination. Then, for evaluating the in vitro anti-*Pneumocystis* activity, the drug stock solutions were diluted in Dulbecco's Modified Eagle's Medium (DMEM, BioWhittaker, France) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco BRL, France) to produce the required drug concentrations. To evaluate the in vivo anti-*Pneumocystis* activity, iclaprim, TMP, or SMX stock solutions were used. Drug solutions of 100 mg/mL were used for iclaprim and TMP, whereas a solution of 500 mg/mL was used for SMX. Then, the drug stock solutions were diluted in sterile water before gavage. Compound solutions were prepared just before use.

Source of *P. carinii*

Corticosteroid-treated conventional laboratory rats were used as the animal model to obtain *P. carinii* organisms. Ten-week-old female Wistar rats (Harlan, France) were immunosuppressed for 3 weeks with dexamethasone (Merck, France) administered in the drinking water (2 mg/L) [44]. Then, rats were inoculated with 2×10^7 of cryopreserved parasites using a non-surgical endotracheal method [46]. Dexamethasone treatment was maintained until the end of the experiment. Six to 8 weeks post-inoculation, rats were infected. Animals were allowed sterile standard food (UAR, France) and water ad libitum. The research complied with national legislation and with company policy on the care and use of animals and with the related code of practice (accreditation number: A59107, agreement number: B 59-35000). The study has been approved by the ethical committee for experiments on animals (approval number 02789.02).

Extraction, purification, and quantitation of *P. carinii*

Six to 8 weeks following inoculation, rats were euthanized and parasite extraction was performed [44]. Briefly, parasites were extracted in DMEM (BioWhittaker, France) by agitation of lung pieces with a magnetic stirrer. The resulting homogenate was poured successively through 250- and 63-μm stainless steel filters. After centrifugation, the pellet was resuspended in a hemolytic buffered solution (BioWhittaker, France). *Pneumocystis carinii* organisms were collected by centrifugation and then purified on a polysucrose gradient (Histopaque-1077, Sigma-Aldrich, France). Blood and Sabouraud dextrose agar (Difco, France) media were inoculated with purified parasites to check for the presence of eventual contaminating pathogens. *Pneumocystis carinii* was quantitated on air-dried smears stained with RAL 555 (Réactifs RAL, France), a rapid panoptic methanol Giemsa stain, which stains trophic forms, sporocytes, and cysts of *P. carinii* [41]. *Pneumocystis* was then cryopreserved by placing parasites in FCS with 10% DMSO at − 80 °C in a Nalgene 1 °C cryo-freezing container
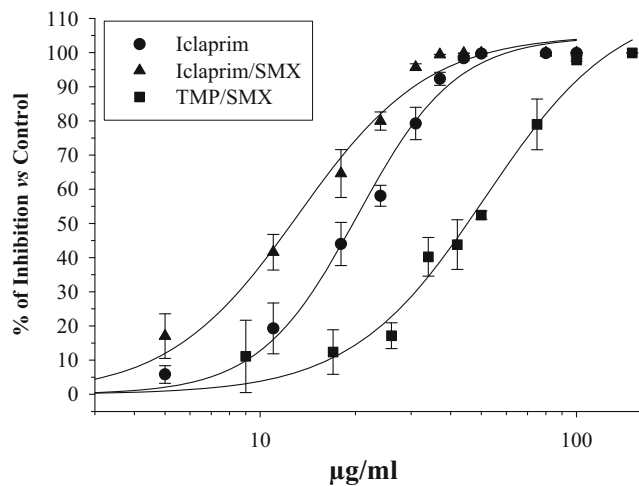
Fig. 1 Concentration–in vitro activity relationships of trimethoprim–sulfamethoxazole (TMP/SMX), iclaprim, and iclaprim/SMX combinations

(cooling rate about 1 °C/min) for 4 h [41]. The parasite samples were then stored in liquid nitrogen. Cryopreserved *P. carinii* were used for in vitro or in vivo studies.

In vitro susceptibility study

In vitro pharmacodynamic properties were determined using the Hill equation (E_{max} sigmoid model). This approach offers three parameters which can be used to describe the in vitro activity of new therapeutic compounds: the maximum effect (E_{max}) as a measure for efficacy, the 50% effective concentration (EC_{50}) as a parameter of intrinsic activity, and the slope (S) of the concentration–effect relationship. In vitro susceptibility studies were performed using the broth microdilution technique. Final drug concentrations ranged from 5 to 100 μg/mL for iclaprim, 5/25 to 100/500 μg/mL for the combination iclaprim/SMX, and 1/5 to 150/750 μg/mL for the combination TMP/SMX. All the experiments were carried out in 24-well plates with a final volume of 2 mL of DMEM supplemented with 10% FCS containing a final inoculum of 0.5×10^6 organisms per mL. Then, plates were incubated for 4 days in an atmosphere of 5% CO_2 at 37 °C. One drug-free control was included in each assay. Parasite

quantitation was performed on homogenate smears as described above. All susceptibility assays were set up in triplicate.

Analysis of in vitro results

The in vitro activity of tested compounds against *P. carinii* was expressed as a percentage of inhibition, defined as the total parasite number found in drug-treated wells in comparison with parasite counts in control wells without drug. Once all the differences between drug-treated and untreated wells were calculated, the concentration–effect relationship was established by using the Hill equation:

$$E_R = \frac{E_{R,max} \cdot C^S}{\left[(EC_{50})^S + C^S \right]}$$

where E_R is the effect of each drug concentration (C) on the percentage of inhibition estimated from experimental results; S is a parameter reflecting the steepness of the concentration–effect relationship curve; and EC_{50} is the concentration of the compound at which 50% of the maximum effect ($E_{R,max}$) is obtained. The parameters of this pharmacodynamic model were calculated by nonlinear least-squares regression techniques using commercial software (SigmaPlot, Systat Software, Inc.).

In vivo susceptibility study

An in vivo experiment with corticosteroid-treated Wistar rats endotracheally inoculated with *P. carinii* was performed in order to explore whether in vitro results reflect in vivo efficacy. Six to 8 weeks post-inoculation, animals were highly infected with *P. carinii*, as assessed by the number of *P. carinii* cysts in lung homogenates, as previously described [44]. Animals were divided into groups of three and then randomized to one of the following oral interventions: (1) iclaprim dosed at 5, 25, or 50 mg/kg; (2) TMP dosed at 50 mg/kg; (3) the combination TMP/SMX dosed at 50/250 mg/kg (diluted in DMSO); and (4) the combination iclaprim/SMX dosed at 5/25, 25/125, or 50/250 mg/kg. The drugs were given once a day for ten

Table 1 In vitro pharmacodynamic parameters of trimethoprim–sulfamethoxazole (TMP/SMX), iclaprim, and iclaprim/SMX

Drug	EC_{50} (μg/mL)	E_{max} (~ 99%) (μg/mL)	Slope	p-Value
TMP/SMX (1/5)	51.4/257	150/750	2.1	–
Iclaprim	20.3	50	2.8	0.002[a]
Iclaprim/SMX (1/5)	13.2/66	37/185	2.1	0.002[b]

[a] EC_{50} difference between iclaprim compared to TMP/SMX

[a] EC_{50} difference between iclaprim/SMX compared to TMP/SMX

consecutive days. The final concentration of DMSO in diluted drug solutions was between 1.5 and 15%. Control animals were dosed with sterile water with 15% DMSO. At the end of the experiment, therapeutic efficacy was assessed by counting *P. carinii* in lung homogenates and comparing the counts with those of the untreated controls. Lung histopathology was not analyzed. Twenty-four hours after the end of the treatment, animals were euthanized and the lung homogenized in a Stomacher 400 blender, as previously described [44]. Parasite quantitation was performed on air-dried smears stained with RAL 555 stain (trophic forms, sporocytes, and cystic forms; RAL Diagnostics, France). Therapeutic efficacy was assessed by counting *P. carinii* parasites in lung homogenates and comparing them with those of the untreated controls at the end of the experiment. For each drug concentration, the results were expressed as the percentage of inhibition versus drug-free animal controls.

Statistical analysis

Statistical analysis was performed using SPSS statistical software. *Pneumocystis carinii* parasites counts were presented as the mean and standard error. For each drug, the therapeutic efficacy was assessed by comparing the mean of the total parasite number for treated animals to that of untreated animals with a Student's *t*-test (the data distributions were found to be normal by preliminary Shapiro–Wilk tests). The comparisons of iclaprim to TMP at 50 mg/kg/day and of the combination iclaprim/SMX to the combination TMP/SMX at 50/250 mg/kg/day were similarly performed. Two-sided *p*-values < 0.05 were deemed to be statistically significant.

Results

In vitro susceptibility study

Figure 1 shows the concentration–response curves obtained after 4 days of incubation of *P. carinii* with iclaprim or the combinations iclaprim/SMX or TMP/SMX. The reduction in the number of microorganisms was gradual and concentration-dependent. TMP/SMX demonstrated an EC_{50} of 51.4/257 μg/mL (Table 1). The present work revealed a high in vitro anti-*Pneumocystis* activity of iclaprim, with an EC_{50} value of 20.3 μg/mL. Moreover, the results obtained with the iclaprim/SMX combination (proportion 1:5) showed significantly increased activity, with an EC_{50} value of 13.2/66 μg/mL ($p = 0.002$). At least 99% growth inhibition was reached with iclaprim at a concentration of 50 μg/mL and with the combination iclaprim/SMX at a concentration of 37/185 μg/mL (Table 1). Higher concentrations of TMP/SMX (150/750 μg/mL) were needed for reaching a ~ 99% inhibition.

In vivo susceptibility study

Untreated animals were highly infected at the end of the treatment period. The total number of *P. carinii* organisms per lung was $2.2 \pm 0.4 \times 10^9$ (Table 2). Iclaprim and TMP showed a similar activity at the concentration of 50 mg/kg/day ($p = 0.888$). The iclaprim/SMX combination was significantly more effective (98.1% growth inhibition) than TMP/SMX (86.6% growth inhibition) ($p = 0.048$) (Table 2).

Table 2 Efficacy of iclaprim and iclaprim/SMX combination against experimental *Pneumocystis* pneumonia in Wistar rats infected by the endotracheal route

Drug	Dose (mg/kg/day)	Total parasites: mean ± SD (10^9)	*p*-Value[a]	% Reduction of total parasites[b] ± SD
Control	–	2.180 ± 0.335	–	–
Iclaprim	5	0.721 ± 0.138	0.005	66.9 ± 7.8
	25	0.455 ± 0.372	0.008	79.1 ± 20.9
	50	0.513 ± 0.236	0.005	76.5 ± 13.3
TMP	50	0.465 ± 0.426	0.005	78.7 ± 22.6
Iclaprim/SMX	5/25	0.572 ± 0.435	0.014	73.8 ± 24.4
	25/125	0.034 ± 0.017	0.001	98.5 ± 0.9
	50/250	0.040 ± 0.022	0.001	98.1 ± 1.7
TMP/SMX	50/250	0.293 ± 0.126	0.002	86.6 ± 7.1

Rats were endotracheally infected after 2 weeks of dexamethasone administration (2 mg L^{-1} drinking water) with 20×10^6 *P. carinii* organisms. Iclaprim, iclaprim/SMX, TMP, and TMP/SMX were administered once a day by the oral route for ten consecutive days

[a] Test for equality of means of the total parasite numbers for treated and untreated animals

[b] Therapeutic efficacy was assessed by counting *P. carinii* organisms in lung homogenates of treated rats and comparing them with those of the untreated control animals

Discussion

The iclaprim/SMX combination was more active than TMP/SMX in in vitro and in vivo studies. Iclaprim alone or combined with SMX inhibited the growth of both *Pneumocystis* cysts and vegetative forms. This is an important difference with other anti-*Pneumocystis* drugs like echinocandin-derived compounds, inhibitors of β-1,3 glucan synthase, which selectively eliminates cysts in infected rats at therapeutic doses [47–49], and, thus, have been mainly utilized as salvage therapy or in combination with other antimicrobial agents [50–53].

In vitro susceptibility tests are a basic step in current pharmacological screening for new anti-infective drugs. A standard method was proposed for pathogenic yeast in 1997 ([54], but it could not be applied to *Pneumocystis* species, which do not grow well in fungal culture systems. In fact, *Pneumocystis* species are atypical fungi [55, 56]. For instance, they lack ergosterol [57], which is the target for most antifungal molecules [58]. The present results indicate that in vitro EC_{50} could be a predictive indicator of the anti-*Pneumocystis* in vivo effect, at least for iclaprim/SMX and for sordarin derivatives [43]. Pharmacodynamic parameters were calculated using the Hill equation, which has been previously used to assess the in vitro concentration–effect relationships of many anti-*Pneumocystis* drugs in the 4-day axenic *Pneumocystis* culture system used in the present work [43]. Factors such as medium composition, inoculum size, and incubation time of the in vitro susceptibility axenic system used in this work were defined previously [43, 45]. In this system, the growth of all *Pneumocystis* life cycle parasite stages is currently assessed on dry smears stained by RAL 555, a methanol Giemsa-like staining [45, 59, 60]. The in vitro strategy used in this work allows estimating the intrinsic activity against *P. carinii* of the tested molecule, and, therefore, it affords an efficient tool to perform a direct comparison among molecules.

The in vivo results were consistent with the in vitro results, showing that iclaprim/SMX is more potent than TMP/SMX against *P. carinii*. The in vivo model used the Wistar rat, which was inoculated non-surgically by the endotracheal route [46] with a suspension of *P. carinii* organisms [61]. Despite a limitation of not analyzing lung histopathology, this model did not present the drawbacks of conventional corticosteroid-induced PcP rodent models [42], which develop spontaneous PcP infections and, thus, are characterized by a marked variability in parasite infection rates and unknown origin of *Pneumocystis* strains. For these reasons, *Pneumocystis* endotracheally inoculated animal models of PcP were developed [61], such as the one used in the present study.

The present work confirmed that the activity of the combination of a diaminopyrimidine with SMX is enhanced, and showed that the activity was highest with the iclaprim/SMX combination. This combination may prevent the emergence of *P. jirovecii* resistance to sulfa drugs. Iclaprim exhibits favorable lung pharmacokinetics which will influence its utility for the treatment of pneumonia caused by various pathogens. An oral formulation of iclaprim is being developed. An iclaprim/SMX combination may enhance the favorable lung pharmacokinetics compared to iclaprim alone. A Phase 1 study investigated the tissue distribution of a single intravenous dose of iclaprim in relevant lung compartments [62]. Iclaprim concentrations were found in epithelial lung fluid and alveolar macrophages, up to 20- and 40-fold higher, respectively, than in plasma [62]. In addition, a Phase 2 study comparing the clinical cure rates of two iclaprim dosages with vancomycin in the treatment of patients with nosocomial pneumonia suspected or confirmed to be caused by Gram-positive pathogens showed iclaprim and vancomycin to have comparable clinical cure rates and safety profiles [63]. In conclusion, collectively, the current in vitro and in vivo study, and previous Phase 1 and 2 studies, support that iclaprim alone or combined with SMX could potentially offer an alternative and more potent first-line therapy to treat PcP in humans.

Compliance with ethical standards

Conflict of interest DBH is an employee of Motif BioSciences. SH is an employee of IHMA.

Ethical approval This research involved animals. All procedures in this research were in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

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