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Use of a non-adherent cell culture system for testing the effect of 2',3'-dideoxyinosine against *Cryptosporidium parvum*

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Abstract

The in vitro cultivation of *Cryptosporidium parvum* in the non-adherent cell line THP-1 was evaluated for its capability as a useful additional model to investigate the effect of drugs on this parasite. The purine analog antiviral 2',3'-dideoxyinosine (ddI) was evaluated and compared to the reference molecule paromomycin in sequential 24 hour experiments beginning at 24 and 72 hour post-infection. The ability of this technique to evaluate the various parasite stages showed that ddI displayed a dose-dependent efficacy especially on the trophozoite and sexual stages. Paromomycin displayed a lower efficacy than previously reported. Both drugs induced a decrease in the number of multiparasitized cells. These results indicate that the purine salvage pathway should be a key chemotherapeutic target against *C. parvum*.

Keywords: *Cryptosporidium parvum*; In vitro; THP-1 cell; 2',3'-dideoxyinosine; Paromomycin

1. Introduction

Cryptosporidium parvum is an enteric parasite protozoan that causes life-threatening diarrheas in immunocompromised hosts. Despite real successes [1,2], it is still a difficult parasite to maintain in vitro and neither a continuous culture system or a satisfactory in vivo model are currently available. We

have successfully developed an in vitro culture model using the non-adherent myelomonocytic human cell line THP-1 which provides an enhanced versatility and allows the determination of the various parasitic stages [3]. We used this system to test 2',3'-dideoxyinosine (ddI), a purine analog active against HIV and *Toxoplasma gondii* in vitro and in vivo [4,5]. Paromomycin, an aminoglycoside reported to exert some activity against *C. parvum* in vitro [6–8], in animal models [9–11] and AIDS patients [12] was used as a positive control.

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2. Materials and methods

2.1. Cells

The myelomonocytic cell line THP-1 (ECACC #88081201, Sophia Antipolis, France) was continuously maintained in RPMI 1640 (Sigma, L'Isle d'Abeau, France) supplemented with 25 mM HEPES (Sigma H-9136), 200 U ml⁻¹ of penicillin, 200 µg ml⁻¹ of streptomycin (Sigma P-0781) and 10% fetal calf serum (DAP, Vogelgrun, France) at 37°C in a 5% CO₂ moist atmosphere. The cell suspension was diluted once a week when the cell density reached about 1 × 10⁶ ml⁻¹.

2.2. Parasites

C. parvum oocysts were passaged in neonatal calves (INRA, Nouzilly, France) and purified as previously described [3] with the following modifications: after removing the fat by ether treatment, the fecal suspension was washed 3 × for 15 min at 1500 × g in cold (4°C) tap water. The pooled fecal pellet was allowed to sediment in 25 ml cold tap water for 2 h and again for 1 h at 4°C in order to remove the larger debris. The supernatants were pooled, centrifuged 15 min at 1500 × g and suspended in 10 ml tap water. This suspension was carefully layered on the top of a chilled (4°C) Sheather solution diluted 1:2 (v/v) and centrifuged at 1500 × g for 10 min. The white layer at the top of the sucrose solution was removed and washed 3 × in tap water. The oocyst suspension was kept at 4°C in 2.5% (w/v) K₂Cr₂O₇ until required.

2.3. Percoll solutions

Sterile isotonic Percoll was prepared by mixing one part of 10 × Hanks balanced salts solution (HBSS, Sigma H-1641) with nine parts Percoll (Sigma P-4937) and stored at 4°C. Solutions of 30% and 60% (v/v) were made by diluting the isotonic Percoll stock solution with RPMI 1640. The solutions were stored at 4°C and prewarmed at 37°C prior to use.

2.4. Infection of cells with *C. parvum*

Prior to infection, the THP-1 cells were collected

and pelleted for 4 min at 160 × g. They were diluted in culture medium and kept in the incubator until required. The oocysts (approx. 30 × 10⁶) were washed 3 × and were suspended in 1 ml cold tap water. They were surface-sterilized with a 1.25% sodium hypochlorite solution on ice for 5 min, centrifuged at 1500 × g for 7 min and washed once with cold RPMI 1640. The treated oocysts were incubated for 5 min in 1 ml excystation medium (0.25% trypsin, 0.75% sodium taurocholate in PBS) at 37°C in order to facilitate excystation, and washed once in prewarmed RPMI 1640. The THP-1 cell suspension was then mixed with the oocyst pellet (oocyst to cell ratio 1:1 to 3:1) and carefully layered on top of the 60% Percoll solution and centrifuged 10 min at 1500 × g. Excystation was allowed to proceed at 37°C for 1.5 to 3 h in the parasites-cells layer at the interface between the Percoll solution and the medium. This layer was then retrieved with a Pasteur pipette and washed once in RPMI 1640 for 9 min at 600 × g. After dilution in culture medium, the parasitized cells were layered on top of a 30% Percoll solution and centrifuged at 1500 × g for 10 min. Most of the cell debris and oocysts shells stayed on top of the Percoll-medium interface. The pellet was subsequently washed once at 600 × g and 2 × at 160 × g as above and distributed in a 24-well Falcon microplate (Becton-Dickinson, Meylan, France) at an approximate density of 4 × 10⁶ cells ml⁻¹ under reduced oxygen in a candle jar. The medium was replaced twice a day until used for the experiments.

2.5. Drugs

Paromomycin sulfate (Sigma P-5057) was dissolved in RPMI 1640 as a 100 mg ml⁻¹ stock solution. 2',3'-dideoxyinosine (ddI) was kindly provided by Bristol Myers Squibb (Paris, France) and was dissolved at a 20 mg ml⁻¹ stock solution in RPMI 1640. The stock solutions were aliquoted and stored at -20°C.

2.6. Inhibition studies

The experiments were performed on 24 h and 72 h old cultures. They were washed 3 × for 5 min at 160 × g in RPMI 1640 before incubation with the drugs. Prior to use, the stock solutions were diluted

1:100 in culture medium to give concentrations of 1 mg ml^{-1} for paromomycin and $200 \text{ } \mu\text{g ml}^{-1}$ for ddI. When needed, serial dilutions (200 to $25 \text{ } \mu\text{g ml}^{-1}$) were made in complete medium and distributed under $100 \text{ } \mu\text{l}$ in a 96-well Nunclon microplate (Nunc, Denmark). Control wells received $100 \text{ } \mu\text{l}$ of culture medium. In each well, $100 \text{ } \mu\text{l}$ of the washed parasitized cell suspension (final volume: $200 \text{ } \mu\text{l}$; concentration: $4 \times 10^6 \text{ ml}^{-1}$) were added, mixed and incubated in a candle jar for 24 h at 37°C . The final concentrations were 100 to $12.5 \text{ } \mu\text{g ml}^{-1}$ for ddI and $500 \text{ } \mu\text{g ml}^{-1}$ for paromomycin. Each concentration was tested in triplicate.

2.7. Evaluation of parasite growth

The content of the wells was thoroughly mixed and centrifuged for 1 min in microtubes at $15000 \text{ revs min}^{-1}$ in a Beckman Avanti centrifuge. Thin smears were made, fixed in 100% methanol and stained with 20% Giemsa and 1% Alcian blue as previously described [3]. At least 1000 cells were counted with an oil immersion $\times 100$ objective. The percentage of cells infected with the various development stages of the parasite was assessed, as well as the number of multiparasitized cells. The inhibition percentages were calculated using the following formula: $100 - [\% \text{ of infected cells in the treated wells} / \% \text{ of infected cells in the control wells}]$ for the relevant parasite stages. The Student's *t*-test was used to assess the statistical significance of the results.

3. Results and discussion

The in vitro cultivation of *C. parvum* in the non-adherent THP-1 cells allowed an easy assessment of invasion and distribution of cell infection. Thus a good reproducibility and a standardization of the technique could be easily achieved. The use of the Percoll gradient stage provided an enhanced availability of the host cells for the excysting oocysts and a more efficient elimination of dead cells and shells. In addition, the treated and control wells originated from the same parasitized cell suspension, ensuring that there was no discrepancy in the invasion rates. This technique also allowed the sequential investigation of the effects of the drugs on various

portions of the cycle of *C. parvum* in an already established infection: the experiments were performed at 24 h post-infection (p.i.) when the asexual cycle is predominant, and at 72 h p.i., when sexual stages appear. The use of the Giemsa–Alcian blue stain allowed the enumeration of parasitized cells, the evaluation of those bearing more than one parasite and the assessment of alterations of the host cells and parasites. Finally, the reproducibility of our technique and the toxicity of the compounds could be controlled with a hemacytometer.

We have recently shown that the purine analog 2',3'-dideoxyinosine (ddI) was highly active against *T. gondii* cultured in THP-1 cells and suggested the purine salvage pathway of this parasite as a promising chemotherapeutic target [4,5]. As an Apicomplexan, *C. parvum* possesses some different metabolic pathways [13] and also lacks a de novo purine synthesis, as reported recently [14]. Paromomycin was used as a positive control because it has been consistently described as one of the few, if not the only molecule active against *C. parvum*. [6–12]. The dose of $500 \text{ } \mu\text{g ml}^{-1}$ was chosen since it has been previously reported that in 48 h tests, $100 \text{ } \mu\text{g ml}^{-1}$ produced a 69% inhibition in HCT-8 cells [15] and recently, a 72% inhibitory activity was obtained at $500 \text{ } \mu\text{g ml}^{-1}$ in vitro [16]. In addition, preliminary experiments had shown that higher doses were not more efficient. It was also of interest to determine which stages were sensitive, since this molecule does not penetrate into the cells.

There was no significant difference between the controls and the treated cultures ($4.1 \pm 0.1 \times 10^6 \text{ ml}^{-1}$), showing that neither drug had any influence on the growth rate or number of the THP-1 cells. The effect of ddI on the various stages at 24 h p.i. was dose-dependent. However, the effect rapidly reached a maximum inhibition of $14.9 \pm 9.4\%$ at $100 \text{ } \mu\text{g ml}^{-1}$ and only slightly increased with the dosage (Fig. 1). It is noteworthy that despite this rather low inhibition, the trophic stages were reduced ($26.1 \pm 6.8\%$), as well as the number of multiparasitized cells ($33.8 \pm 5.4\%$). The reference molecule paromomycin had a moderate overall activity ($12.7 \pm 5.7\%$), at the onset of parasite growth. However, the number of cells bearing more than one parasite was significantly decreased ($P < 0.05$) (Fig. 2). When added to 3 day cultures, $100 \text{ } \mu\text{g ml}^{-1}$ ddI

Fig. 1. Dose-response curve for ddI on the in vitro development of *Cryptosporidium parvum* in THP-1 cells. The drug was incubated for 24 h with 24 h old cultures. Trophozoites (□), multiparasitized cells (■), total (△). Results are expressed as percent inhibition of the control ($5.82 \pm 1.12\%$ of parasitized cells). $N=9$ for each data point, from three separate experiments with triplicate values.

triggered a marked decrease in the number of sexual stages ($73.3 \pm 10.4\%$) while paromomycin displayed a similar effect as at 24 h p.i. (Fig. 3). This correlates with paromomycin not penetrating into the host cells and hence, displaying an effect restricted to the extracellular parasites, reducing their subsequent maturation but not their invasion capability. A recent report [7] showed that paromomycin could be active

Fig. 2. Activity of $500 \mu\text{g ml}^{-1}$ of paromomycin on the development of *Cryptosporidium parvum* in THP-1 cells in 24 h old cultures. Data originate from the same experiments as Fig. 1.

Fig. 3. Inhibitory activity of $500 \mu\text{g ml}^{-1}$ of paromomycin (A) and of $100 \mu\text{g ml}^{-1}$ ddI (B) on the various stages of *Cryptosporidium parvum* in THP-1 cells in 3 day cultures. The drugs were incubated for 24 h. Results are expressed as percent inhibition of the control ($2.43 \pm 0.52\%$ of parasitized cells). $N=9$ for each data point, from three separate experiments with triplicate values.

against *C. parvum* without entering the host cell cytoplasm. Its moderate efficacy in 24 h tests in our model appears to correlate with some observed effects in vivo, especially its inefficacy reported by Brasseur et al. in a rat model [17].

Our results with ddI are consistent with an effect on the salvage pathway of the *C. parvum* purine metabolism taking place throughout the entire cycle and producing a decrease in the total parasitemia, especially when the cycle shifts towards the sexual forms. This molecule is taken up by the monocyte/macrophage populations, especially the resting cells

[18,19] where its metabolite ddATP is found, as well as ddI-derived radioactivity incorporated into the cell DNA and RNA pool [20]. In contrast to *T. gondii* where low concentrations were rapidly active [4,5], the lower inhibition of *C. parvum* probably reflects the different metabolic activity of this parasite. Furthermore, ddI must penetrate into the THP-1 cells where it may be metabolized before entering the parasite, thus reducing its subsequent inhibitory activity.

For both molecules, no significant decrease in the undifferentiated parasites could be seen compared to the control, suggesting that neither drug was active against the invasion capability of the parasite. In our system, the number of multiparasitized cells depends on both the cell density and the percentage of infection. In the present study, the multiparasitized cells usually harbored two parasites. The lower numbers of multiparasitized cells in the treated cultures are thus correlated to the global number of parasites and account for an about 10% higher inhibition.

Cultivation of *C. parvum* in THP-1 cells provides a useful additional tool for the evaluation of chemotherapeutic agents. The ability to distinguish between the parasite stages also allows insights into the mode of action of the compounds. One of its major restrictions however, is the limitation of the test duration to 24 h, but preliminary experiments with ddI for 6 consecutive days did not yield improved results. A recent report showed that some nucleoside analogs of pyrimidine structure displayed interesting activities against *C. parvum* in vitro [21]. Our results confirm the promising effects of purine analogs, indicating that the purine salvage pathway might be a key chemotherapeutic target against *C. parvum* infection.

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