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In Vitro Activities of Two Antimitotic Compounds, Pancratistatin and 7-Deoxynarciclasine, against *Encephalitozoon intestinalis*, a Microsporidium Causing Infections in Humans

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The antiparasitic effect of a collection of compounds with antimitotic activity has been tested on a mammalian cell line infected with *Encephalitozoon intestinalis*, a microsporidian causing intestinal and systemic infection in immunocompromised patients. The antiparasitic effect was evaluated by counting the number of parasitophorous vacuoles detected by immunofluorescence. Out of 526 compounds tested, 2 (pancratistatin and 7-deoxynarciclasine) inhibited the infection without affecting the host cell. The 50% inhibitory concentrations (IC_{50}s) of pancratistatin and 7-deoxynarciclasine for *E. intestinalis* were 0.18 μM and 0.2 μM, respectively, approximately eightfold lower than the IC_{50}s of these same compounds against the host cells. Electron microscopy confirmed the gradual decrease in the number of parasitophorous vacuoles and showed that of the two life cycle phases, sporogony was more sensitive to the inhibitors than merogony. Furthermore, the persistence of meronts in some cells apparently devoid of sporonts and spores indicated that the inhibitors block development rather than entry of the parasite into the host cell. The occurrence of binucleate sporoblasts and spores suggests that these inhibitors blocked a specific phase of cell division.

Microsporidia are widespread obligatory intracellular parasites, apparently able to invade any cell in animals and humans (4, 16). These unicellular parasites have been increasingly recognized as opportunistic pathogens of immunodeficient patients (24). Two species cause diarrhea, malabsorption, and weight loss in AIDS patients (6). *Enterocytozoon bieneusi* is the most prevalent cause of these symptoms and is occasionally associated with hepatobiliary disease or infection of the upper respiratory tract (24). The second prevalent species, *Encephalitozoon* (*Septata*) *intestinalis*, is responsible for nephritis, bronchitis, and lytic mandibular lesions (11, 16). Rational strategies for the development of chemotherapeutic agents against microsporidia require a better understanding of the mechanisms controlling the proliferation of these parasites.

The life cycle of *Encephalitozoon intestinalis* (Fig. 1) consists of two successive developmental sequences, merogony and sporogony, both of which occur in a parasitophorous vacuole (PV) within host cells. During merogony, proliferative stages known as meronts are produced. After multiple divisions, meronts are transformed into sporonts. During sporogony, each sporont divides into two sporoblasts, which mature into spores which are approximately 2.0 by 1.2 μm. They contain a complex extrusion apparatus which ensures inoculation of the infective sporoplasm into a host cell. Meronts and sporonts are mononucleate cells which replicate by binary fission. Sometimes, the karyokinetic process is repeated before cytokinesis occurs, resulting in a ribbon-like cell containing two to four nuclei. The production of tetranucleate meronts and sporonts suggests some variability in the timing of cytokinetic cycles in *E. intestinalis* (3). Sporoblasts and spores are exclusively mononucleate, an indication that the regulation of development must be linked to the control of the cell cycle. Although very little is known about cell cycle control in microsporidia, a gene encoding a putative homologue of the cyclin-dependent kinase 1 (CDK1) has been recently identified in *Encephalitozoon cuniculi* (20), and a similar gene from *E. intestinalis* is being characterized in our laboratory (unpublished data). CDKs are major players in the progression of the eukaryotic cell cycle. Their activity is regulated by their phosphorylation status and by the association with negative (cyclin kinase inhibitors) or positive (cyclins) regulators, and by intracellular translocations. The temporary association of kinase subunits with different cyclins define time windows during which kinase activity is directed at distinct sets of substrates at the appropriate phase of the cell cycle.

Because of the importance of CDKs and their regulators in the multiplication and development of eukaryotes, these enzymes represent attractive potential targets for antiparasitic chemotherapy. The phylogenetic divergence between the parasite and its host is likely to result in divergences in the structure of their regulatory genes, as has been shown in other parasite-host systems (9, 10, 15); such divergences might confer to parasite and host differential susceptibilities to a given inhibitor. Therefore, we decided to evaluate the effect of a collection of antimitotic compounds (many but not all of which

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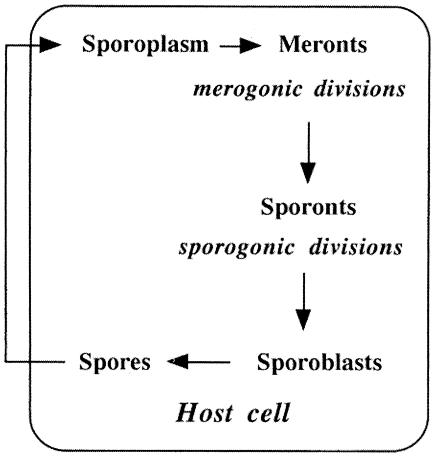

FIG. 1. Life cycle of *E. intestinalis*. Only the spores survive in the extracellular medium. The inoculation of the sporoplasm into the host cell is the initial step of the intracellular development.

are CDK inhibitors) on the course of cellular infection by *E. intestinalis.*

MATERIALS AND METHODS

Inhibitors. Pancratistatin and 7-deoxynarciclasine were extracted and purified from *Pancratium* (*Hymenocallis*) *littorale* in Hawaii (17).

Parasites. *E. intestinalis* spores were collected from monolayers of rabbit kidney cells (RK13) as described by Van Gool et al. (22). Spores were harvested every 3 days, and suspensions of parasites were centrifuged at 350 × *g* for 5 min to eliminate cellular fragments. Spores were then pelleted by centrifugation at 2,000 × *g* for 20 min and washed twice. Spores were counted with a Malassez slide and used immediately for infection of cultured cells.

Culturing of parasites and treatment. RK13 culture cells were cultivated in Lab-Tek slides, in RPMI 1640 medium (Gibco BRL, Cergy Potoise, France) supplemented with 8% heat-inactivated fetal calf serum (56°C for 30 min) (Sigma, St. Quentin-Fallavier, France), streptomycin (100 μg/ml), penicillin (100 U/ml) and L-glutamine (2 mM). Cells were adjusted to 10^5 cells/well and spores from *E. intestinalis* were added to cultured cells at a ratio of 1 spore/10 cells. The compounds were added to cultured cells at infection time. The effects of the compounds were determined by counting the PVs 48 h after infection and treatment. The detection of PVs was performed by immunofluorescence assay (IFA).

IFA. The Lab-Tek slides were fixed in ethanol at −20°C for 10 min and then incubated for 1 h at 37°C with the monoclonal antibody (MAb) $M_1.6C_1.2C_{11}$ (1) at a dilution of 1/500. This MAb is directed against a coat protein of sporogonic

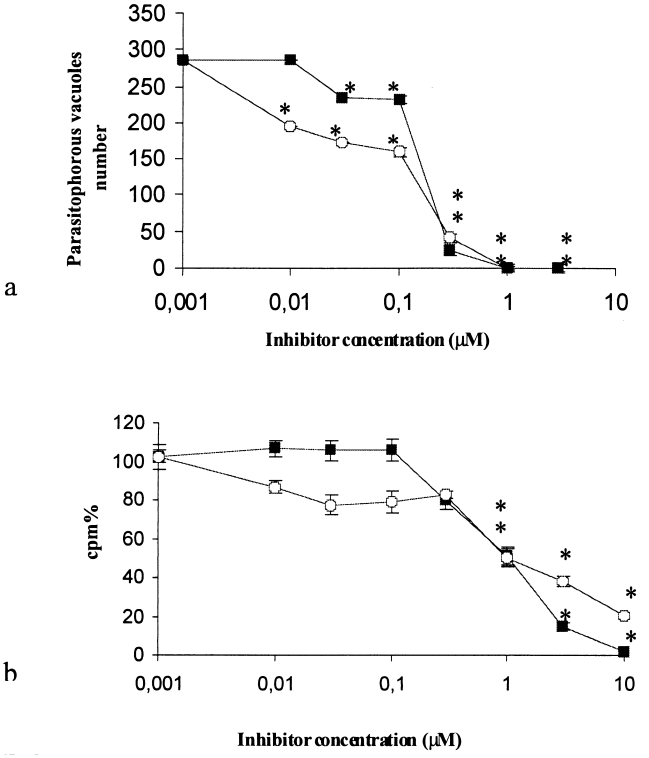

FIG. 3. (a) In vitro effect of inhibitors on *E. intestinalis* multiplication. RK13 cells infected with *E. intestinalis* spores were incubated for 48 h, in the presence of various concentrations of inhibitors. The number of PVs was determined by IFA. The data represent means ± standard deviations (error bars) of triplicate cultures. *, significant difference between the PV number obtained in treated culture and nontreated culture, as determined by Student's test ($P < 0.05$). (b) In vitro effect of inhibitors on host cells (RK13). Microculture plates prepared with RK13 cells were treated with six concentrations of inhibitors. One microcurie of [^{3}H]thymidine (5 Ci/mmol) was added to each well. Plates were incubated for 48 h, and [^{3}H]thymidine incorporation was measured. The data represent means ± standard deviations (error bars) of triplicate cultures. *, significant difference between the ^{3}H incorporation by the treated culture and nontreated culture, as determined by Student's test ($P < 0.05$).

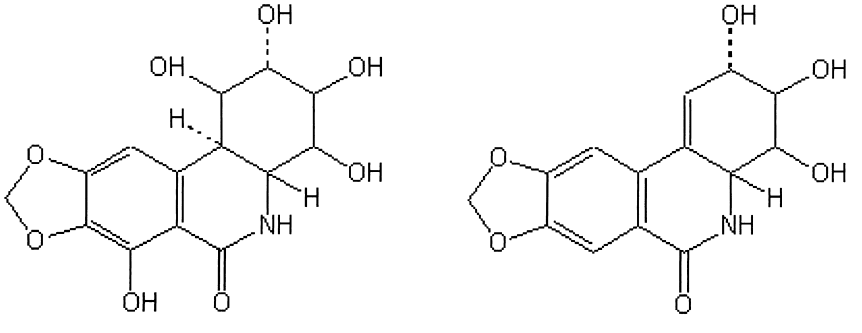

FIG. 2. Structures of pancratistatin and 7-deoxynarciclasine.

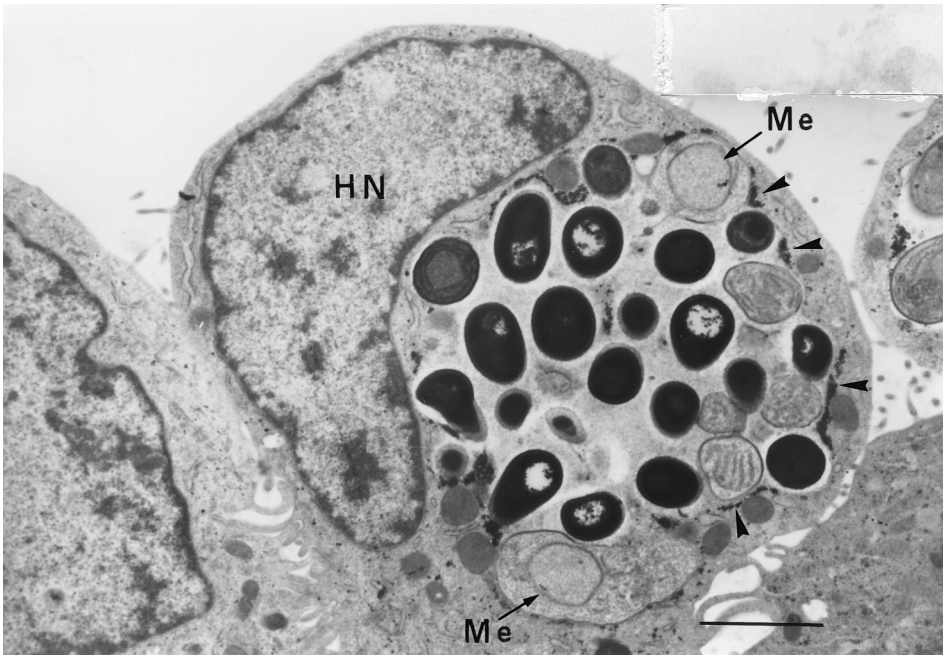

FIG. 4. In nontreated cultures and in those treated with lower doses of inhibitors, the development of the parasite results in the production of numerous spores. The electron-dense stages are sporoblasts maturing into spores. Abbreviations: HN, host cell nucleus; Me, two meronts applied to the PV membrane outlined with glycogen granules (arrowheads). Scale bar, 2 μm.

stages generated in the PVs. The slides were then washed in phosphate-buffered saline (PBS) and incubated with fluorescein-conjugated anti-mouse immunoglobulin G, diluted to 1/100 in Evans blue (1/1,000). After several washes with PBS, the slides were mounted in PBS-glycerol (50:50, vol/vol), and PVs were counted using an epifluorescence microscope. The entire surface of each well was examined at a magnification of ×500.

Determination of IC_{50}s on parasite. The concentration of inhibitor required to inhibit parasite growth by 50% (IC_{50}) was determined by IFA. The data were plotted and the IC_{50}s were determined using Cricket Graph software.

Determination of IC_{50}s on host cells. Microculture plates (96-well flat-bottom plates; Falcon) were prepared with RK13 cells. Six concentrations of each inhibitor were tested in triplicate. One microcurie of [^{3}H] thymidine (5 Ci/mmol) was added to each well. Plates were incubated for 48 h and [^{3}H]thymidine incorporation was measured with a scintillation counter (Beckman). The data were plotted and the IC_{50}s were determined using Cricket Graph software.

Electron microscopy. The effect of inhibitors on the morphology of *E. intestinalis* and RK13 cells was examined by electron microscopy. Infected monolayers were fixed at 48 h postinfection in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2) for 1 h. They were rinsed in the same buffer and postfixed in ferriosmium [OsO4 and $K_3Fe(CN)_6$ (1%, wt/vol) in cacodylate buffer] for 1 h at room temperature. After ethanolic dehydration, the samples were embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate and then examined with a JEOL TEM 100CX transmission electron microscope.

RESULTS

Effect of inhibitors on parasite growth. RK13 cells infected with *E. intestinalis* spores were incubated for 48 h in the presence of various concentrations of compounds. Since the stocks of inhibitors were dissolved in dimethyl sulfoxide (DMSO), controls were run simultaneously with the highest concentration of DMSO (0.03 μl/ml) without inhibitor. The effect of the inhibitors on parasite multiplication was evaluated from the number of PVs detected by IFA. Microsporidia are fast-growing organisms, and 48 h postinfection, PVs were detectable by IFA due to the occurrence of sporogonic stages cross-reacting with the MAb.

In the first round of screening, 526 compounds were tested at concentrations of 2 and 5 μM. As expected, most compounds were toxic to the host cells and caused cytopathic effects. The 51 compounds that showed little or no cytopathic effect on the host cells were tested again at a concentration range of 0.1 to 5 μM for antiparasitic effect. This allowed us to select two structurally related molecules with a definite effect on the number of PVs but with no apparent effect on the host cells: pancratistatin and 7-deoxynarciclasine (Fig. 2). IC_{50}s of pancratistatin and 7-deoxynarciclasine, determined using a range of concentrations between 0.01 and 3 μM (Fig. 3a), were, respectively, 0.18 and 0.2 μM. No parasite growth could be detected at the highest pancratistatin concentration (3 μM). Low concentrations of this inhibitor (0.01 μM) also significantly reduced the parasite development. Likewise, in infected cultures treated with 7-deoxynarciclasine, the number of parasites decreased markedly at higher concentrations. Thus, pancratistatin and 7-deoxynarciclasine showed very good antiparasitic activity.

Host cell IC_{50}s. To determine whether any of these two molecules may represent a useful lead compound, we next measured their effect on host cells in a standard [^{3}H]thymidine incorporation assay. RK13 cells were treated with the two inhibitors (concentrations ranging from 0.01 to 10 μM), and incorporated radiolabel was then measured (see Materials and Methods). Again, cells treated with DMSO diluted in RPMI and RPMI alone were used as controls. The IC_{50}s of pancratistatin and 7-deoxynarciclasine obtained from the graphs (Fig. 3b) were both 1.5 μM and thus were 8- and 7.5-fold higher than the parasite IC_{50}s, respectively.

Morphological effects of the inhibitors. Examination of infected cultures by electron microscopy confirmed that both

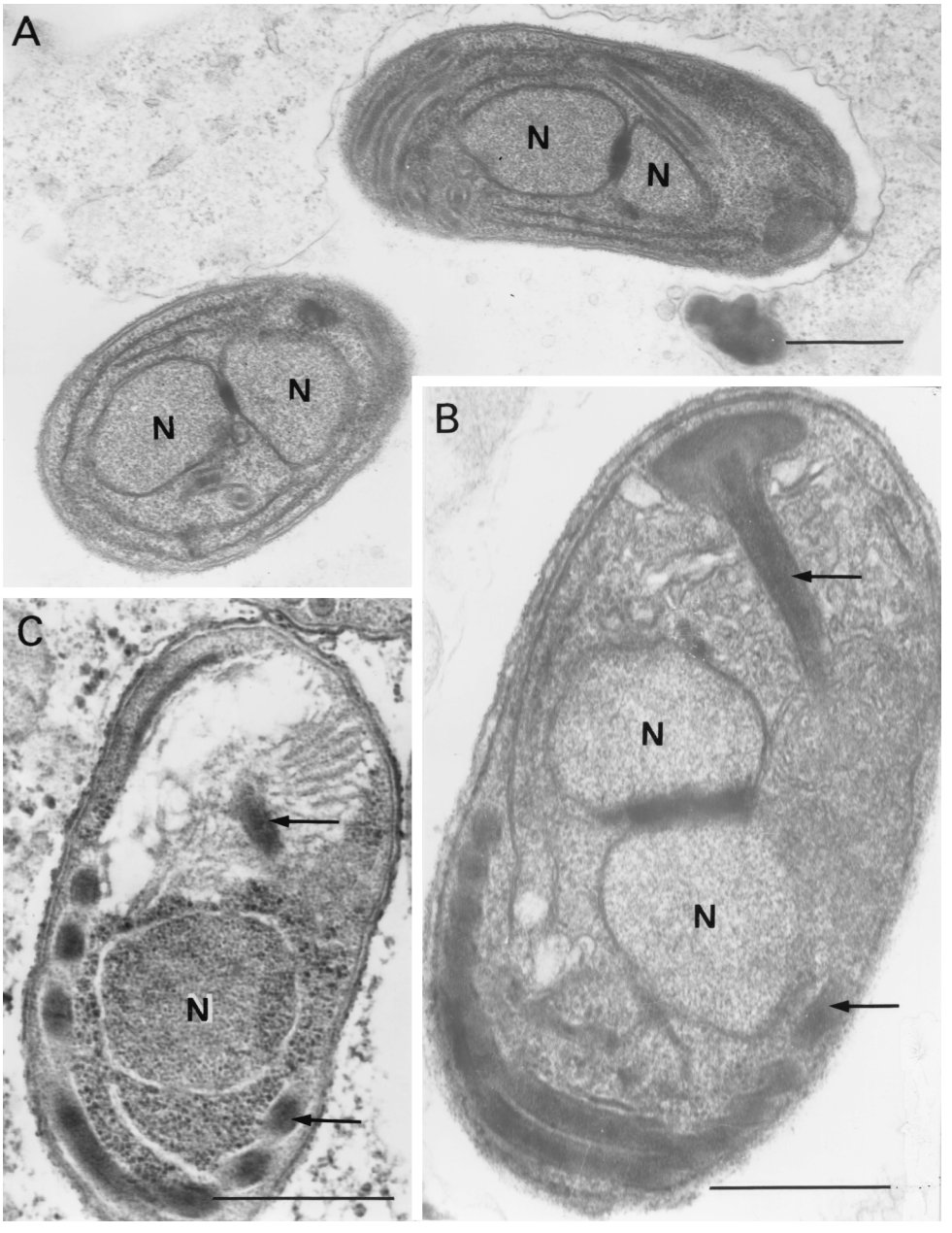

FIG. 5. Sporoblasts and spores generated in cultures treated with 0.5 μM pancratistatin contain two nuclei (N). (A) Two binucleate sporoblasts. (B) Only one polar tube (arrows) can be seen in this section of a binucleate sporoblast. The nuclei are assembled into a diplokaryon. (C) The mononucleate sporoblast of *E. intestinalis* in the absence of treatment with pancratistatin or 7-deoxynarciclasine. Scale bars, 0.5 μm.

compounds caused a gradual resorption of the infection. In cultures treated with the lower doses, most cells were infected. Normally fusiform, these cells became rounded due to the presence of a large PV (Fig. 4). Two or three PVs could be seen in some cells. All developmental stages of the parasite were present in these vacuoles, which contained up to 20 spores. At a concentration of 0.5 μM, flat cells containing small PVs with a concomitant decrease in the number of mature spores were observed. In some cells, PVs contained meronts as well as a small number of sporoblasts and spores that were larger (3 to 4 μm) than those produced in the absence of inhibitors. Additionally, some alterations were observed in the polar tube, a major component of the spore extrusion apparatus. Eight to nine coils of the polar tube were numbered in large sporoblasts and spores, instead of the five to six characteristic of the species (data not shown). Most interesting was the occurrence of two nuclei in sporoblasts and in spores (Fig. 5A and B and 6A). In most sections, these nuclei were abutted, thus displaying diplokaryotic arrangement. Used at the same concentration (0.5 μM), 7-deoxynarciclasine exerted a similar effect, although to a lesser extent.

At the highest dose (1 μM), sporogonic stages, i.e., sporonts, sporoblasts, and spores, were less frequent or even absent in PVs that, however, still contained meronts. Only one large

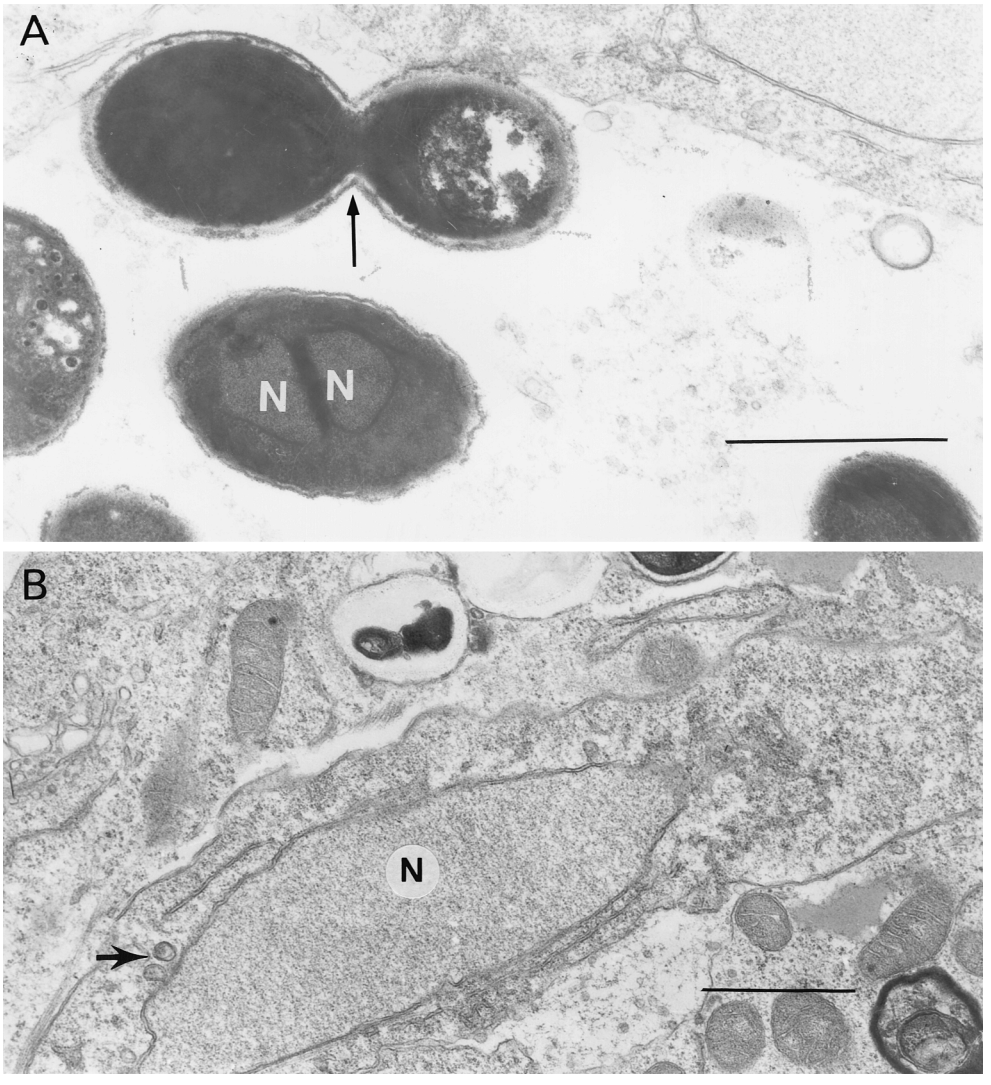

FIG. 6. Two sporogonic stages in a culture treated with 0.5 μM pancratistatin. (A) An electron dense diplokaryotic sporoblast is transforming into a mature spore. The median constriction (arrow) of the thick walled spore is another indication that this stage originates from a sporont that did not complete its division. (B) A large mononucleate meront in a cell treated with 1 μM pancratistatin. The vesicles (arrows) characteristic of microsporidia mitotic poles indicate that the nucleus is engaged into the division process. Scale bars, 1 μm.

mononucleate meront reaching up to 10 μm in diameter was observed in most cells treated with 1 μM pancratistatin (Fig. 6B). The occurrence of polar vesicles and intranuclear microtubules indicated that these meronts were engaged in the process of division at the time of sample fixation.

Deeply altered parasites embedded in an electron-dense material were also frequently observed in cells treated with any dose of the compounds (Fig. 7). No alterations were observed in RK13 cells. However, cells with an electron-dense nucleus were frequently seen in cultures (infected or not) treated with 7-deoxynarciclasine. DMSO-treated control cultures did not show any sign of morphological alteration, either in the host cell or in parasites.

DISCUSSION

Pancratistatin, 7-deoxynarciclasine, and parasite development. We have tested a small collection of compounds containing a large proportion of kinase inhibitors, especially CDK inhibitors such as purines, paullones, and indirubins. Most compounds proved inefficient in specifically inhibiting parasites while preserving the host cells. Two out of 526 compounds were found to be selective for the parasites, and, interestingly, they appeared to be closely related in their structure (Fig. 2). Pancratistatin and 7-deoxynarciclasin have been originally identified as antitumor agents extracted from *P. littorale*, a Hawaiian member of the family Amaryllidaceae, and related species (17, 18, 19). As many pancratistatin derivatives have been synthesized, we are now testing this family of compounds, with the purpose of identifying a pancratistatin analogue with improved selectivity for microsporidia.

A significant reduction of the microsporidian infection was obtained when cultures were treated with two compounds tested in this study. Both IFA and electron microscopy suggest that the molecules blocked the intracellular development

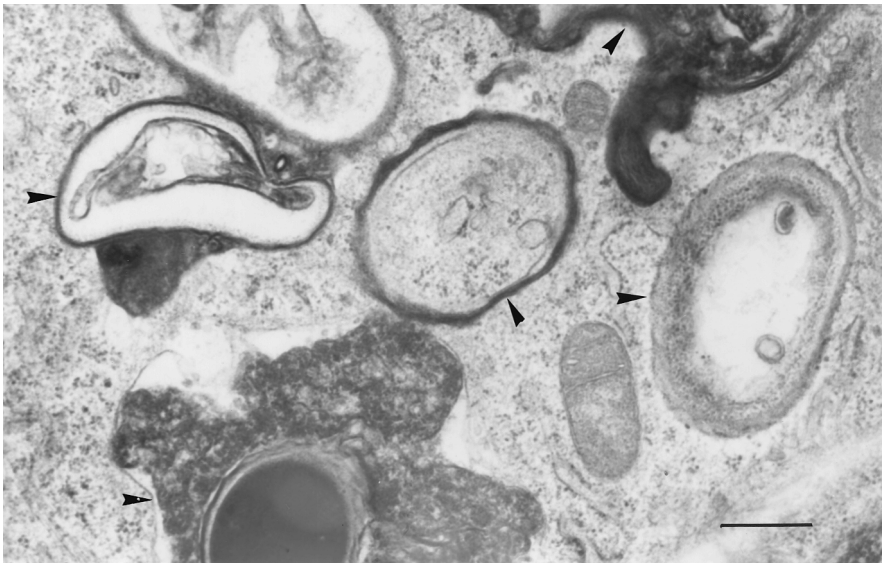

FIG. 7. Alterations of the parasite in cells treated with the inhibitors. Degenerative stages (arrowheads) are scattered in the host cell cytoplasm. Scale bar, 0.5 μm.

rather than the entry of the parasites into the host cell. Indeed, transmission electron microscope examination of apparently uninfected cells revealed the persistence of merogonic stages which were not detected by the MAb directed against a protein specific to the sporogonic stages. Furthermore, most spores generated in the PV in the presence of inhibitor lack the typical thick wall, suggesting that they were not mature enough to spread the infection through the culture. Sporogony itself was totally inhibited at the higher concentration of 1 μM while only a few merogonic stages were usually observed. These meronts were larger than those observed in cultures not treated or treated with lower doses of inhibitors. That these cells were engaged in a division process was indicated by the occurrence of vesicular structures characteristic of microsporidian mitotic poles at the surface of the nucleus (Fig. 6B). However, the large size of the nucleus and the absence of mitotic spindle suggested that the production of these giant meronts resulted from some alteration of the mitosis. A longer cytokinesis due to an alteration in the timing of cell division may cause the uncoupling of cell division and differentiation, as was demonstrated in trypanosomatid parasites (23). Thus, a major effect of the molecules consisted of abnormalities in the development of the parasite.

It is very likely that the decrease in the number of spores and their abnormally large size result from a reduction in the mitotic activity of sporonts and sporoblasts. The decrease in production of developmental stages and their concomitant enlargement were observed in the microsporidian *E. cuniculi* treated with albendazole, an inhibitor of tubulin polymerization. This effect was interpreted as a result from the alteration of the mitotic activity of the parasite (5, 21). Some effects similar to those observed in microsporidia treated with pancratistatin were caused by the protein kinase inhibitor staurosporine (which has a low specificity and inhibits kinases of several families) in *Leishmania* promastigotes. These stages were swollen and did not divide in culture, although they were, however, capable of differentiating into amastigotes (2). Staurosporine and other kinase inhibitors were shown to inhibit the invasion and intraerythrocytic development of *Plasmodium falciparum* (8).

Binucleate sporoblasts and spores induced by pancratistatin and 7-deoxynarciclasine. These molecules appear to block cell division in microsporidia in a dose-dependent manner. Morphological changes occurred gradually with the concentration. Binucleate sporoblasts and spores were seen in culture treated with 0.5 μM pancratistatin. Although less frequent, they were also observed in those treated with the same dose of 7-deoxynarciclasine. Apparently, the inhibitor caused a block in the cytodieresis of the sporonts, thus generating these binucleate sporoblasts which, however, were still able to differentiate into mature spores (Fig. 5A and B and 6A). Sporogony itself was totally inhibited at the highest dose (1 μM).

Surprisingly, the nuclei present in binucleate sporoblasts and spores display the diplokaryon arrangement observed in many microsporidian species (7). No diplokaryotic phase occurs in the life cycle of Encephalitozoonidae, but the alteration induced in *E. intestinalis* by pancratistatin, and to a lower degree by 7-deoxynarciclasine, mimics the development of other microsporidia, including polymorphic species generating monokaryotic and diplokaryotic spores alternately (14). A variety of environmental factors possibly associated with sexual processes are involved in spore polymorphism. However, the underlying molecular mechanisms have not yet been investigated.

The molecular targets of the antitumor agents pancratistatin and 7-deoxynarciclasine have not yet been identified in human cells. The electron-dense contents of the nucleus which we frequently observed in 7-deoxynarclasine-treated RK13 cells suggest an arrest of the division process occurring after prophasic condensation of chromatin. Thus, these cells were apparently dividing but some block in mitosis occurred before metaphasis. More work is needed to identify the target of the inhibitors discussed here. A possible approach would be the purification of putative targets by affinity chromatography on immobilized inhibitors (12).

The recent classification of microsporidia with the fungi (25) suggests that the different phases of their cell cycle may be controlled by a single CDK, as is the case in yeasts (13). However, some of the results reported here tend to indicate that a combination of different factors and effectors ensures the regulation of the life cycle in microsporidia. Thus, the information provided by this study is of dual interest: on one hand it points to new potential therapeutic tools which certainly deserve further characterization (notably, in terms of their effect during infection of animals), and on the other hand these molecules represent promising tools for investigating the diversity of the microsporidian life cycles.

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