Efficacy of nitazoxanide, tizoxanide and tizoxanide glucuronide against Cryptosporidium parvum development in sporozoite-infected HCT-8 enterocytic cells

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The effects of nitazoxanide and its metabolites, tizoxanide and tizoxanide glucuronide, on the development of the asexual and sexual stages of Cryptosporidium parvum in differentiated human enterocytic HCT-8 cells were evaluated in a quantitative alkaline phosphatase immunoassay. Nitazoxanide, tizoxanide and tizoxanide glucuronide were inhibitory for up to 46 h when added after sporozoite invasion (MIC\textsubscript{50} 1.2, 22.6 and 2.2 mg/L, respectively). Tizoxanide had only limited activity, but nitazoxanide and tizoxanide glucuronide strongly inhibited asexual and sexual stages, respectively.

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C. parvum sporozoites were obtained from the faeces of experimentally infected calves (kindly provided by Dr Naciri, Laboratoire de Pathologie Aviaire, INRA, Nouzilly, France) as previously described. Briefly, C. parvum oocysts were concentrated on a sucrose gradient and then excysted using a 1.5% taurocholate solution. Culture medium was removed from 24 h HCT-8 cultures and 100 μL per well of BHK 21 medium containing 1.5–2 × 10^5 freshly isolated sporozoites was added to cell monolayers for 2 h at 37°C. Two hundred microlitres of BHK 21 medium supplemented with paraminoenzoic acid 4 mg/L (Sigma, St Louis, MO, USA), ascorbic acid 35 mg/L (Sigma), glucose 25 mmol/L (Merck, Darmstadt, Germany), insulin 100 IU/L (Novo Nordisk Pharmaceutique, Boulogne-Billancourt, France), HEPES 15 mmol/L (Sigma), streptomycin 500 mg/L (Gibco BRL), penicillin 10^5 IU/L (Gibco BRL) and fetal calf serum 20% (v/v) was added to each well, and cells were cultured for an additional 46 h.

Enzyme immunoassay (EIA) for the detection of C. parvum in HCT-8 cultures was performed as previously described using rat polyclonal antibodies against Cryptosporidium and biotin–SP-conjugated anti-rat IgG and IgM (heavy and light chain) goat F(ab')2 fragments 2 mg/L (Jackson Immunoresearch, Westgrove, PE, USA) as secondary antibody revealed using alkaline phosphatase-coupled avidin (ABC Reagent; Vector Laboratories, Burlingame, CA, USA).

Inhibitory activities of agents are expressed as percentages. The per cent inhibition was defined as

\[
\frac{(A_{405} \text{ in infected cells with agent}) - (A_{405} \text{ in uninfected cells with agent})}{(A_{405} \text{ in infected cells without agent})} \times 100 - (A_{405} \text{ in uninfected cells without agent})
\]

The MIC\text{50} was defined as the concentration (in mg/L of culture) of agent that resulted in a 50% inhibition of C. parvum development.

The cytotoxicities of nitazoxanide, tizoxanide and tizoxanide glucuronide were determined using rat polyclonal antibodies against C. parvum (heavy and light chain) goat F(ab')2 fragments 2 mg/L (Sigma), streptomycin 500 mg/L (Gibco BRL), penicillin 10^5 IU/L (Gibco BRL) and fetal calf serum 20% (v/v) was added to each well, and cells were cultured for an additional 46 h. Results were expressed as the decrease in absorbance (A\text{405}) expressed as percentages of the A\text{405} values in control cultures. From preliminary studies we verified that HCT-8 cell cytotoxicity was minimal (decrease of A\text{405} from 0 to 14%) for all controls and at all concentrations of agents, except nitazoxanide at 25 and 50 mg/L (A\text{405} decrease of 36% and 39%, respectively).

A\text{405} values in control cultures in which heat-inactivated sporozoites were added instead of viable sporozoites, were not significantly different from A\text{405} values in the absence of heat-inactivated sporozoites. For uninfected wells, one S.D. accounted for <10% and 15% of the mean A\text{405} for each microplate and for the pooled data of five microplates, respectively. For infected wells, one S.D. accounted for <10% and 21% of the mean A\text{405} for each microplate and for the pooled data of five microplates, respectively.

In the EIA, background A\text{405}, resulting from the intense colour of the solutions, was higher in uninfected wells exposed to drugs than in control wells without agent, and for this reason they were used as controls for infected wells containing the same agent concentration. In addition, disruption of infected monolayers and/or peeling off of HCT-8 cells occurred during EIA in wells containing tizoxanide or tizoxanide glucuronide at 10–50 mg/L. For tizoxanide and tizoxanide glucuronide, high background A\text{405} was observed at concentrations of >50 mg/L, preventing any conclusions from being drawn.

The effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the various stages of the C. parvum life cycle were studied by adding the tested compounds, at concentrations ranging from 1 to 50 mg/L, at the start of the culture (sporozoite stage), 2 h after adding sporozoites (trophozoite stage) and 18 h after adding sporozoites (sexual stages).

**Results**

The effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the asexual and sexual stages of parasite development were investigated by exposure to the agents for 46 h starting 2 h after adding sporozoites (Figure 1 and Table). To test the effects of agents on penetration of sporozoites into HCT-8 cells, the agents were added at the

![Figure 1. Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the development of the asexual and sexual stages of C. parvum in HCT-8 cells. Agents were added to cultures 2 h after the addition of 1.5–2 × 10^5 sporozoites/well and incubated for 46 h. Results are expressed as mean (± S.D.) inhibition percentages. Pooled data from 20 independent experiments. ——, Nitazoxanide; ••••, tizoxanide; •••••, tizoxanide glucuronide.](image)
Anti-<i>C. parvum</i> activity of nitazoxanide and metabolites

**Table.** MIC<sub>50</sub> (mg/L) of nitazoxanide, tizoxanide and tizoxanide glucuronide on complete development (asexual and sexual stages), sporozoite, asexual and sexual stages of <i>Cryptosporidium parvum</i> in HCT-8 cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Asexual and sexual stages</th>
<th>Sporozoite stage</th>
<th>Asexual stages</th>
<th>Sexual stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitazoxanide</td>
<td>1.2</td>
<td>5.8</td>
<td>4.7</td>
<td>11.8</td>
</tr>
<tr>
<td>Tizoxanide</td>
<td>22.6</td>
<td>8.6</td>
<td>&gt;50</td>
<td>9</td>
</tr>
<tr>
<td>Tizoxanide glucuronide</td>
<td>2.2</td>
<td>45.2</td>
<td>11.7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Figure 2. Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the penetration of <i>C. parvum</i> sporozoite into HCT-8 cells. Agents were added to cultures at the same time as 1.5–2 × 10<sup>5</sup> sporozoites/well, and incubated for 2 h. Results of EIA detection, performed after a further 46 h incubation, are expressed as mean (± S.D.) inhibition percentages. Pooled data from five independent experiments. Key as in Figure 1.

Figure 3. Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the asexual development of <i>C. parvum</i> in HCT-8 cells. Agents were added to cultures 2 h after the addition of 1.5–2 × 10<sup>5</sup> sporozoites/well and incubated for 4 h. Results are expressed as mean (± S.D.) inhibition percentages. Pooled data from three independent experiments. Key as in Figure 1.

Figure 4. Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the sexual development of <i>C. parvum</i> in HCT-8 cells. Agents were added to cultures 18 h after the addition of 1.5–2 × 10<sup>5</sup> sporozoites/well and left in culture for 4 h. Results are expressed as mean (± S.D.) inhibition percentages. Pooled data from seven independent experiments. Key as in Figure 1.

Discussion

In this study, nitazoxanide was found to be highly effective in vitro against the development of the asexual and sexual
stages of *C. parvum*. This is in agreement with a previous study which found that nitazoxanide inhibits *C. parvum* development. Our findings, which indicate that tizoxanide glucuronide inhibits parasite development, suggest that nitazoxanide activity is at least in part due to this metabolite. Interestingly, nitazoxanide and tizoxanide appear to be more active against the extracellular sporozoite stage, whereas tizoxanide glucuronide acts primarily on intracellular development. This was confirmed using simultaneous immunofluorescent assays (data not shown). Whether this is because tizoxanide glucuronide is better at penetrating the cell membrane requires additional investigation.

Previous clinical studies have shown that nitazoxanide is clinically active against cryptosporidiosis. This compound has a wide range of antimicrobial activities, particularly against intestinal protozoa and helminths. Present data underline the potential interest in nitazoxanide metabolites as candidates for *in vivo* studies. Interestingly, studies in an immunosuppressed rat model suggest that relapses are less frequent after treatment with nitazoxanide than with the non-absorbable paromomycin and sinefungin (Li, X., unpublished results). Taken together, these data indicate the need for additional studies on the biliary clearance of nitazoxanide metabolites and their potential activity on biliary tract cryptosporidiosis.

References


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