Mechanism of Action of 4-Phenoxyphenoxyethyl Thiocyanate (WC-9) against Trypanosoma cruzi, the Causative Agent of Chagas’ Disease†

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We investigated the molecular basis of the activity of 4-phenoxyphenoxyethyl thiocyanate (WC-9) against Trypanosoma cruzi, the etiological agent of Chagas’ disease. We found that growth inhibition of T. cruzi epimastigotes induced by this compound was associated with a reduction in the content of the parasite’s endogenous sterols due to a specific blockade of their de novo synthesis at the level of squalene synthase.

There is an urgent need for safer and more potent drugs for the specific treatment of Chagas’ disease, the largest parasitic disease burden in Latin America. Currently available drugs have serious limitations due to limited efficacy, particularly in the chronic stage of the disease, and frequent toxic side effects (25). The etiological agent of Chagas’ disease, the kinetoplastid protozoan Trypanosoma cruzi, has a complex life cycle with proliferative and infective stages in both its insect (Reduviidae) vectors and mammalian hosts, where the parasite develops intracellularly, leading to tissue damage compounded by the ensuing inflammatory response. There are 16 to 18 million people already infected in Latin America. Most of them are in the chronic stage of the disease, in which 30 to 40% will develop serious, often lethal, cardiac and gastrointestinal tract lesions (7, 25).

We have recently described the potent and selective in vitro activity of 4-phenoxyphenoxy and aryloxyethyl derivatives against both the extracellular epimastigote and the clinically relevant intracellular amastigote forms of T. cruzi, but the molecular mechanisms of these effects remained unclear (4, 8). T. cruzi and related trypanosomatid parasites have a strict requirement for specific endogenous sterols (ergosterol and analogs) for survival and growth and cannot use the abundant supply of cholesterol present in their mammalian hosts (14–17). We have shown that ergosterol biosynthesis inhibitors with potent in vitro activity and special pharmacokinetic properties in mammals (large volumes of distribution and long half-lives) can induce radical parasitological cure in animal models of both acute and chronic experimental Chagas’ disease (14–18).

We decided to investigate the possible effect of 4-phenoxyphenoxyethylthiocyanate (WC-9), the most potent member of this group of compounds, on the de novo sterol biosynthesis in intact T. cruzi epimastigotes, because previous work indicated interference by this type of compounds with steroid biogenesis in mammals (22, 23).

WC-9 induced a dose-dependent effect on growth of the epimastigote form of the EP strain of parasite (Fig. 1). When the EP strain was grown in liver infusion tryptose (LIT) me-

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† We dedicate this paper to the memory of Zigman Brener, pioneer in experimental chemotherapy studies of Chagas’ disease.

FIG. 1. Effects of compound WC-9 on the proliferation of T. cruzi epimastigotes. Epimastigotes were cultured in LIT medium at 28°C with strong aeration. An arrow indicates the time the drug was added at the indicated concentrations. Cell densities were measured by turbidity at 560 nm and by direct counting with a hemocytometer. Experiments were carried out in triplicate, and each bar represents 1 standard deviation.
(6), the MIC for the organism (defined as the minimal concentration required to inhibit growth by >99% after 96 h) was 1 μM, in agreement with previous results with the Y strain (4). We analyzed the free sterol contents of control and treated cells by capillary gas-liquid chromatography coupled with mass spectrometry (18–21). We found (Table 1) that the growth-inhibitory effects of WC-9 were associated with a depletion of the parasite’s endogenous sterols, ergosterol and its 24-ethyl analog, and a concomitant increase in the relative proportion of cholesterol, which is taken passively from the growth medium by the epimastigotes (18–21). At the MIC, an almost complete disappearance of the parasite’s sterols was observed, with no accumulation of sterol intermediates (Table 1) (see reference 21 for a detailed description of the sterol biosynthesis pathway in T. cruzi epimastigotes) or precursors, such as lanosterol or squalene (not shown). These facts indicated a blockade of the biosynthetic pathway at a presqualene level (18).

To test this hypothesis, we investigated the effects of compound WC-9 on two key enzymes of the poly-isoprenoid biosynthetic pathway: farnesyl diphosphate synthase (FPPS [EC 5.3.3.2]) and squalene synthase (SQS [EC 2.5.1.1]). The prod-

### TABLE 1. Free sterols and precursors present in T. cruzi epimastigotes (EP stock) grown in the presence or absence of WC-9

<table>
<thead>
<tr>
<th>Sterol or precursor</th>
<th>Structure</th>
<th>Retention time (min)</th>
<th>Mass %</th>
<th>WC-9 concn</th>
<th>Control</th>
<th>0.1 μM</th>
<th>0.3 μM</th>
<th>1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exogenous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td>24.7</td>
<td>32.4</td>
<td>31.0</td>
<td>57.1</td>
<td>83.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Endogenous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-Methyl-5,7,22-cholesta-trien-3β-ol (ergosterol)</td>
<td></td>
<td>26.9</td>
<td>32.1</td>
<td>31.5</td>
<td>17.4</td>
<td>16.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-Ethyl-5,7,22-cholesta-trien-3β-ol</td>
<td></td>
<td>29.6</td>
<td>13.5</td>
<td>13.2</td>
<td>15.8</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ergosta-5,7,24(24′)-trien-3β-ol</td>
<td></td>
<td>28.4</td>
<td>9.6</td>
<td>12.0</td>
<td>4.3</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ergosta-5,7-dien-3β-ol</td>
<td></td>
<td>28.7</td>
<td>8.7</td>
<td>9.6</td>
<td>3.1</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ergosta-7,24(24′)-dien-3β-ol</td>
<td></td>
<td>28.9</td>
<td>3.7</td>
<td>2.7</td>
<td>2.3</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
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</table>

* Sterols were extracted from T. cruzi epimastigotes cultured in LIT medium for 120 h in the presence or absence of the indicated concentrations of WC-9; they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas-liquid chromatography and mass spectrometry (18–21).
The dose-response curves were consistent with noncompetitive inhibition, with $K_i = IC_{50}$; these $K_i$ values are 2 to 3 orders of magnitude lower than the $K_m$ of the substrates (18). This suggested that WC-9, with its electrophilic sulfur center linked to the relatively nonpolar (hydrophobic) 4-phenoxophenoxyethyl moiety, could act by mimicking the carbocational transition state of the reaction, leading to formation of the cyclopropylcarbinyll intermediate presqualene diphosphate (1, 9, 11). A similar rationale has been advanced to explain the potent anti-SQS activity of aryl-quinuclidine derivatives against both mammalian SQS and T. cruzi SQS (3, 13, 18, 24). Based on this hypothesis, it should be possible to design new and more potent SQS inhibitors, using WC-9 as lead structure (i.e., by increasing the electrophilic character of the 4-phenoxophenoyethyl substituent).

In conclusion, our results indicate that a primary mechanism of the antiproliferative effects of WC-9 against T. cruzi is the depletion of essential endogenous sterols by a specific blockade of their de novo biosynthesis at the level of SQS. This is the first explanation at a molecular level of the mechanism of action of 4-phenoxophenoyethyl derivatives against this parasite, and it suggests that this and related compounds could represent a new class of SQS inhibitors with potential antiparasitic and cholesterol-lowering activity in humans.

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FIG. 2. Effects of WC-9 on activity of T. cruzi glycosomal (A) and mitochondrial (B) SQS in the presence of saturating substrate concentrations. The dose-response curves were fitted by nonlinear regression to the equation $V = V_o (1 + ([I]/K_i)^n)^{-1}$, where $V$ is the measured enzyme activity, $V_o$ is the control (uninhibited) activity, and $K_i$ is the apparent inhibition constant. The results were $K_i = 88 \pm 4$ nM and $n = 1.05 \pm 0.04$ for the glycosomal enzyme and $K_i = 129 \pm 6$ nM and $n = 0.99 \pm 0.03$ for the mitochondrial enzyme, consistent with noncompetitive inhibition. Insets show residuals of the regression.


