

## Targeting of the *Brucella suis* Virulence Factor Histidinol Dehydrogenase by Histidinol Analogues Results in Inhibition of Intramacrophagic Multiplication of the Pathogen<sup>∇</sup>

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***Brucella suis* histidinol dehydrogenase (HDH) can be efficiently targeted by substrate analogues. The growth of this pathogen in minimal medium was inhibited and the multiplication in human macrophages was totally abolished in the presence of the drugs. These effects have been shown to be correlated with the previously described inhibition of *Brucella* HDH activity.**

*Brucella* is the causative agent of brucellosis (Malta fever), which is the most widespread zoonosis worldwide (7). The pathogen is capable of establishing persistent infections in humans which are difficult to eradicate even with antibiotic therapy. Moreover, this microorganism has been classified as a potential bioweapon (14). A vaccine for humans is not available, and the isolation of antibiotic-resistant strains is easily conceivable. In the case of an accident or a bioterrorism attack with such modified strains, a classical therapy would therefore be without effect.

We have undertaken a large-scale analysis of the *Brucella suis* virulome as an original approach to the identification of pathogen-restricted targets of novel antibacterial agents acting on the bacteria specifically in their replicative niche (9, 12). As a consequence, the development of the pathogen will be blocked specifically inside the host cell niche, without, however, affecting the host itself or the commensal flora. Some amino acid biosynthetic enzymes have been shown to be essential for the intracellular replication of the pathogen (2, 5, 9), therefore providing specific targets for the development of new anti-*Brucella* agents capable of restricting intracellular replication (12). We have shown previously that the virulence factor acetohydroxyacid synthase of *B. suis*, involved in biosynthesis of branched-chain amino acids, can be effectively targeted by sulfonyleureas, abolishing totally the bacterial growth in minimal medium as well as in human macrophage-like THP1 cells (2).

Recent work by our group has shown that another amino acid biosynthetic enzyme, the histidinol dehydrogenase (HDH; EC 1.1.1.23), encoded by the gene *hisD* (BR0252) in *B. suis*, is essential for intramacrophagic replication, providing a novel

target for the development of anti-*Brucella* agents (1). L-HDH is a homodimeric zinc metalloenzyme that catalyzes the last two steps in L-histidine biosynthesis, and it is found in microorganisms such as bacteria and fungi and in plants but not in mammals (13). Ten years ago, Dancer et al. reported that HDH is a suitable target for the development of potential herbicides (4). The approach developed by this group was to prepare HDH inhibitors which target the lipophilic binding pocket adjoining the active site of the enzyme. To date, no other work has been published on the inhibition of this enzyme except for a computational modeling study in 2001 (8).

Recently, we have shown that substituted benzylic ketones derived from histidine (Fig. 1) have an inhibitory effect on the activity of the purified *B. suis* HDH, the 50% inhibitory concentration (IC<sub>50</sub>) being in the nanomolar range (1). In this report, we investigated the biological effects of these drugs on the in vitro growth of *B. suis* 1330 (ATCC 23444) in minimal medium as well as on the multiplication of the pathogen in human macrophage-like cells.

**Substituted benzylic ketones derived from histidine inhibit the growth of *B. suis* in minimal medium.** Activities of HDH inhibitors in minimal medium (6) that mimicked the presumably nutrient-poor *Brucella*-containing vacuole in the macrophage have been evaluated (9–11). In order to grow under these specific conditions, brucellae have to synthesize their amino acids. The inhibition of HDH is therefore expected to abolish the capacity of this pathogen to grow in minimal medium. The results show that among the 15 drugs tested, drugs 5b, 5c, 5d, 5e, and 5n were the most effective in blocking, as growth was strongly inhibited throughout the duration of the experiment compared to what was seen for the other drugs (Fig. 2). At 96 h in the presence of these drugs, inhibition resulted in a 12- to 21-fold-reduced growth compared to what was seen for untreated *Brucella* (Fig. 2). Interestingly, these drugs were also the most active ones in inhibiting the activity of purified HDH, as they possess the lowest IC<sub>50</sub> values, ranging from 6 to 14.5 nM (1). In contrast, the drug 5i, which has been

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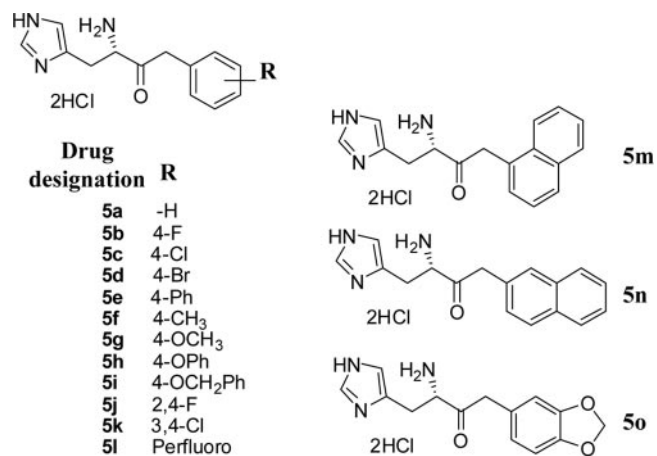


FIG. 1. Structure of the drugs active on the purified HDH from *B. suis*. (Reprinted from reference 1 with permission of the publisher.)

shown previously to possess the best inhibition profile ( $IC_{50} = 3$  nM) (1), inhibits the in vitro growth of *B. suis* to a lower extent than the drugs 5b, 5c, 5d, 5e, and 5n (Fig. 2). This result is likely due to drug 5i having a lower capability to cross the bacterial membrane.

To compare the inhibitory concentrations of drugs on *B. suis* cultures, the bacteria were incubated for 96 h with 0, 25, 50, and 100  $\mu$ M of inhibitors. Results showed that the inhibitory effect of the drugs on in vitro *Brucella* growth in minimal medium was concentration dependent (Fig. 3). Determination of bacterial viability by plating and enumeration showed that the concentration of live brucellae remained constant over this

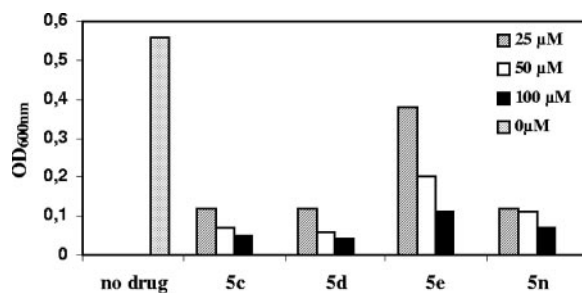


FIG. 3. Effects of various drug concentrations on the in vitro growth of *B. suis* in minimal medium. The experiments were performed as described in the legend to Fig. 2, except that *B. suis* was incubated for 96 h without or with final concentrations of 25, 50, and 100  $\mu$ M of inhibitors 5c, 5d, 5e, and 5n. One representative experiment out of three is shown.

period of time, possibly due to the consumption of remaining stocks of amino acids.

**Inhibition of *B. suis* growth in vitro is correlated with inhibition of histidine biosynthesis.** The experiments described above did not reveal whether the inhibitory effect on *B. suis* growth was correlated with the inhibition of histidine biosynthesis. To determine the specificity of the inhibitors, we first grew bacteria in rich medium (tryptic soy broth) with or without drugs (100  $\mu$ M). The presence of the drugs did not affect the growth of *B. suis* under these conditions (data not shown). The absence of any biological effect of the drugs in rich medium which contains all amino acids was expected, since the bacteria do not need an active histidine biosynthesis pathway under such conditions. In parallel, *B. suis* was grown in drug-

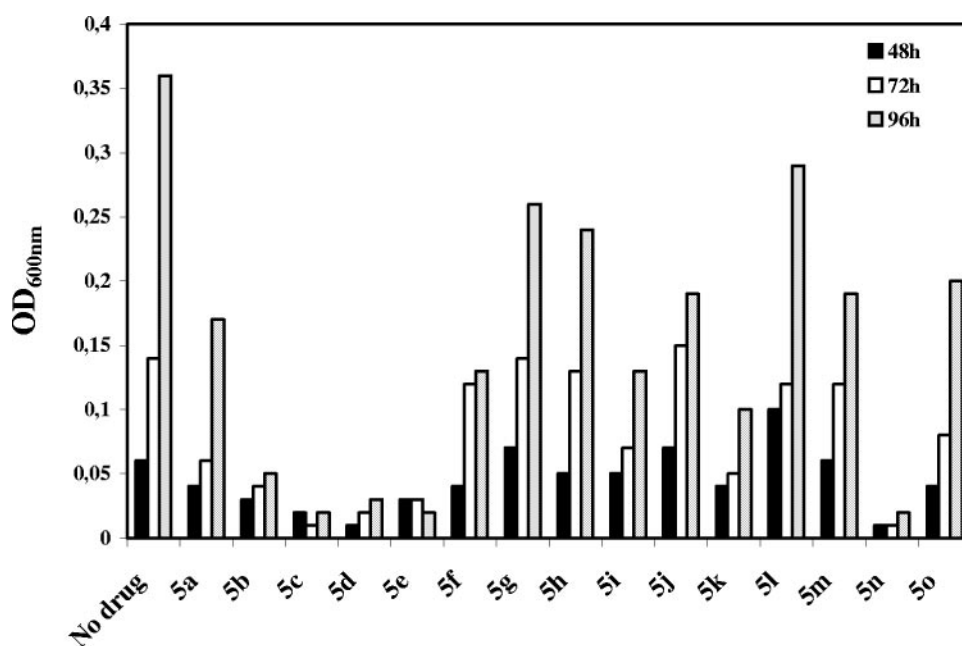


FIG. 2. Effect of HDH inhibitors on *B. suis* growth in minimal medium. The experiments were performed as follows. Bacteria ( $10^8$ /ml) from an overnight culture in tryptic soy broth were used to inoculate 3 ml of freshly prepared minimal medium. Growth was performed under shaking at 170 rpm and at 37°C in the absence or in the presence of various drugs at final a concentration of 100  $\mu$ M. The growth of bacteria was followed by measuring the optical density at 600 nm ( $OD_{600nm}$ ) at 48, 72, and 96 h of incubation. One representative experiment out of three is shown.

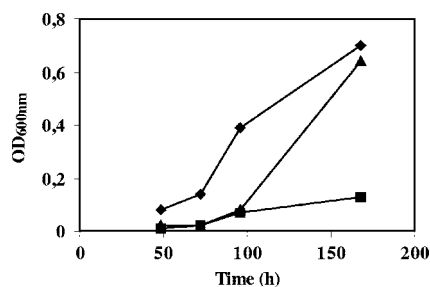


FIG. 4. Growth of *B. suis* in minimal medium in the presence of the drug 5e (100  $\mu$ M) with (▲) or without (■) added histidine (1 mM) and in the absence of drug (◆). The experiments were performed as described in the legend to Fig. 2. One representative experiment out of three is shown.

containing minimal medium with or without histidine. The results showed that bacteria grew in minimal medium containing the drug 5e only in the presence of 1 mM histidine (Fig. 4). As a control, we verified that the *hisD::Tn5* mutant, in which the gene encoding the HDH has been inactivated (9), was able to multiply only in the presence of 1 mM histidine, independently of whether it was grown with or without drugs (data not shown). Taking these data together, we concluded that the inhibitory effect of the drugs on *B. suis* growth is most likely due to the inhibitor's effect on *Brucella* HDH. The results obtained for the other drugs were all consistent with those shown in Fig. 4 (data not shown).

**Substituted benzylic ketones inhibit the intramacrophagic replication of *B. suis*.** We next measured the effects of the most active drugs identified above (5c, 5d, 5e, and 5n) on the intramacrophagic replication of *B. suis*. Macrophage infection ex-

periments were performed as described previously by using human macrophage-like THP1 cells (3). A potential toxic effect of drugs on the macrophages was excluded by trypan blue staining at 48 h postinfection (data not shown) for the drug concentrations tested in this study. The results showed that in the presence of 25  $\mu$ M of drugs 5c, 5d, 5e, and 5n, the number of viable intracellular bacteria at 24 h postinfection was lower than the number present at 90 min, whereas the pathogen multiplied 10<sup>2</sup>-fold without inhibitor (Fig. 5). More precisely, these drugs led to a 50- to 2,500-fold reduction in the intramacrophagic multiplication of *Brucella* compared to the growth of untreated cells at 24 h postinfection (Fig. 5). The inhibition of intramacrophagic growth is most likely due to the inhibition of HDH activity, as we have shown in the present study a specific biological effect of the drugs on the extracellular growth of the pathogen in minimal medium devoid of histidine, and as we know that histidine biosynthesis is essential for the intramacrophagic replication of *B. suis* (9).

Inhibition of bacterial growth in minimum medium and intracellularly signified that the drugs efficiently crossed the macrophage membrane, the membrane forming the vacuole containing *Brucella*, and the bacterial membranes, to finally reach the cytoplasmic HDH target. Surprisingly, the drug 5i, which had a lower effect on the in vitro growth, inhibited the intramacrophagic multiplication of *B. suis* to the same extent as the drugs 5c, 5d, 5e, and 5n. This increased effect onto intramacrophagic growth may be explained by an increasing drug concentration in the vacuole containing *Brucella*. The internalized drug may be entrapped inside the vacuole because of a possible protonation of the molecule in the acidic environment (15).

One potential advantage of using these drugs is that they may limit the selective pressure, i.e., the appearance of spon-

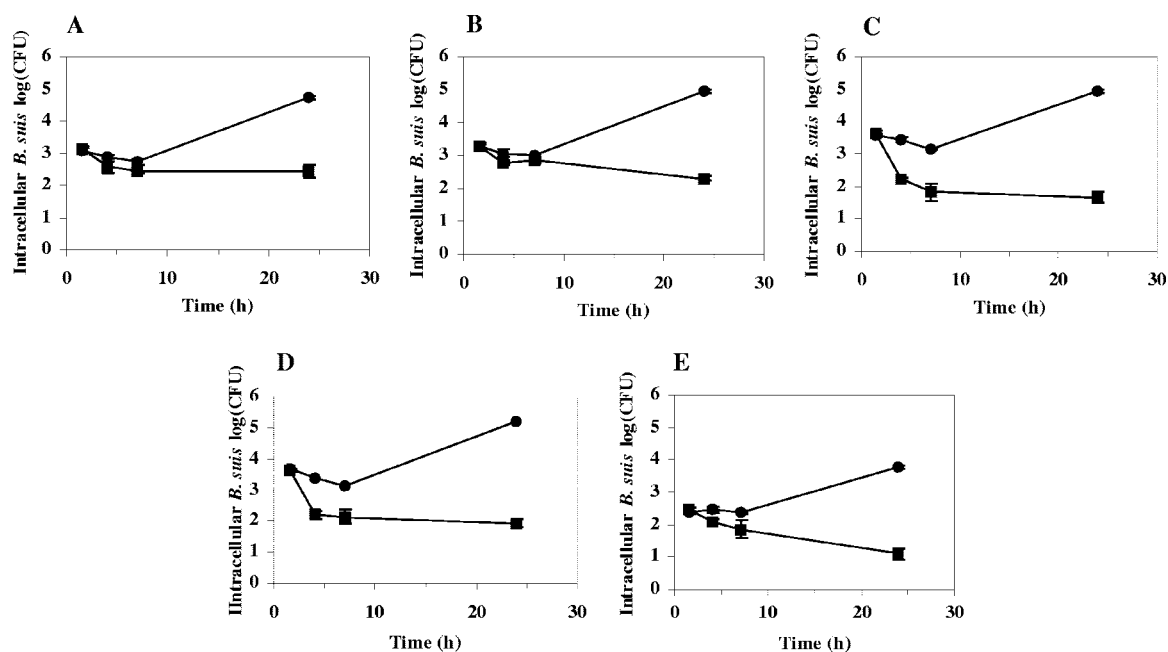


FIG. 5. Effects of drugs on the intracellular replication of *B. suis* in human macrophage-like THP1 cells. *Brucella* growth within untreated cells (●) or in the presence of 25  $\mu$ M of various drugs (■). Shown are results for drug 5c (A), drug 5d (B), drug 5e (C), drug 5i (D), and drug 5n (E). Drugs were added to the infected macrophages at 90 min postinfection. The experiments were performed in triplicate. Error bars indicate standard deviation. Student's *t* test revealed that differences were always significant at 24 h ( $P \leq 0.001$ , except for panel E [ $P < 0.05$ ]).

taneously resistant mutants, to the intracellular niche, as they act specifically on *Brucella* inside the host cell. In addition, they may cause little or no damage to the bacterial flora in comparison to the classical antimicrobials, which cause permanent, nonselective action on bacteria. We investigated the appearance of spontaneously drug-resistant mutants in the presence of 100  $\mu$ M of drugs 5d, 5e, and 5i in minimal medium, followed by plating of the bacteria on the same solid medium. To date, no spontaneously resistant mutants have been isolated from minimal medium after 18 days of growth (data not shown), indicating that enzymatic activity was incompatible with resistance to HDH inhibitors or that the spontaneous mutation rate was low ( $<10^{-8}$ ).

In a previous work, we have shown that several synthetic compounds are indeed very effective inhibitors of purified *B. suis* HDH (1). In conclusion, in the present work we have proven that these compounds are biologically active against *Brucella* by inhibiting the growth of the pathogen in minimal medium which partially mimics the intracellular environment and inside the macrophage host cell. Our data therefore suggest that HDH from *B. suis* constitutes a suitable target for novel compounds which represent valuable candidates for the potential development of an alternative, nonclassical antibacterial therapy, notably against strains resistant to conventional antibiotic treatments.

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