Cytotoxic activity of LCS-1 is not only due to inhibition of SOD1

Running title

Trypanocidal activity of LCS-1

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ABSTRACT

Background The cytotoxic activity of the pyridazin-3-one derivative LCS-1 was previously suggested to be due to the inhibition of superoxide dismutase 1 (SOD1). However, no direct evidence was provided that LCS-1 inhibits SOD1 within cells.

Methods In this study, we investigated the cytotoxic activity of LCS-1 against bloodstream forms of *Trypanosoma brucei*, a protozoan parasite that does not express copper/zinc-containing SOD1, but an iron-containing superoxide dismutase (FeSOD).

Results At 250 μM, LCS-1 did not inhibit the activity of FeSOD in cell lysates of bloodstream forms of *T. brucei*, confirming that the compound is a specific inhibitor of SOD1. However, LCS-1 displayed substantial trypanocidal activity with a minimum inhibitory concentration of 10 μM and a half-maximal effective concentration of 1.36 μM, indicating that the cytotoxic action of the compound cannot solely be due to inhibition of SOD1.

Conclusion The results of this study is an important finding as it shows that LCS-1 has more than one cytotoxic mode of action.

Introduction

The pyridazin-3-one derivative LCS-1 (4,5-dichloro-2-(3-methylphenyl)pyridazin-3-one; **Fig. 1**) was previously identified in a high-throughput chemical screen as an inhibitor for human lung adenocarcinoma cells [1]. Subsequent analysis provided evidence that superoxide dismutase 1 (SOD1) might be the target for LCS-1 [2]. However, direct inhibition of SOD1 within the lung adenocarcinoma H358 cells was not shown. In addition, the half-maximal inhibitory concentration (IC_{50}) for the inhibition of purified SOD1 was found to be higher than the half-maximal effective concentration (EC_{50}) for the inhibition of the growth of H358 cells (1.07 µM versus 0.8 µM [2]). This is an indication that SOD1 is probably not the only target for LCS-1. Usually, an inhibitor has a much better potency to block the activity of an isolated enzyme than to affect the growth of cells ($IC_{50} < EC_{50}$); the reasons for this are diverse. Firstly, pharmacokinetics/pharmacodynamics relationships may result in lower intracellular drug concentration. Secondly, the free efficacious drug concentration within a cell may be reduced due to nonspecific binding to intracellular proteins. Thirdly, even if the intracellular concentration of a drug is not influenced by pharmacokinetics, pharmacodynamics and nonspecific binding, and an enzyme target can be inhibited to 50% at the IC_{50} value, in most cases this would not lead to 50% growth inhibition, as the remaining active enzyme molecules are usually abundant enough to maintain the cellular functions. Consequently, enzyme inhibitors exert their cell growth inhibitory activity at a much higher concentration.

To investigate whether LCS-1 displays additional cytotoxic activity, we tested the compound for its ability to affect the growth of bloodstream forms of the protozoan parasite *Trypanosoma brucei*. In contrast to mammalian cells, *T. brucei* does not express a SOD1 (Cu/Zn-SOD) but a Fe-SOD [3]. As LCS-1 specifically inhibits SOD1 and not SOD2 (Mn-SOD) [2], and as SOD2 has a high degree of sequence and structure similarity with FeSOD [4], one would expect that LCS-1 should not inhibit Fe-SOD and therefore should not affect the growth of bloodstream forms of *T. brucei*.

Materials and Methods

Drugs and chemicals

LCS-1, pyrogallol and resazurin sodium salt were purchased from Sigma-Aldrich (Gillingham, Dorset, UK).

Cell culture

Bloodstream forms of the *T. brucei* clone 427-221a were grown in Baltz medium supplemented with 16.7% heat-inactivated bovine serum as described previously [5]. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

SOD activity assay

The activity of Fe-SOD in trypanosome cell extracts was determined indirectly by the inhibition of pyrogallol autoxidation as described previously [5, 6]. After harvesting bloodstream form trypanosomes, the cells were washed three times with PBS/1% glucose and lysed (5×10^7 cells/100 mL) in 5 mM Tris, 0.1 mM Na_4-EDTA, pH 7.8, 400 µM PMSF on ice for 10 min. To remove cell debris, the lysed cells were centrifuged at 16873 g for 5 min. Then, to 100 µL measuring buffer (100 mM Tris, 2 mM EDTA, pH 8.0), 25.5 µL water, 4.5 µL DMSO (positive control) or 4.5 µL 11.11 mM LCS-1 dissolved in DMSO (test), 50 µL cleared cell extract (2.5×10^7 cell equivalents) or 50 µL lysis buffer (negative controls) were pipetted into wells of a 96-well plate. The background absorbance was read on a microplate reader at 450 nm. Then, 20 µL of a 2 mM pyrogallol solution in 1 mM HCl was added and the increase in absorbance at 450 nm was recorded every minute over a period of 20 min.

Cell growth inhibition assay

The cell growth inhibition assay was performed as described in [5]. In brief, trypanosomes were seeded in 96-well plates in a final volume of 200 µL of Baltz medium containing various concentration of LCS-1 (tenfold dilutions from 10^{-4} M to 10^{-9} M) and 1 % DMSO. Wells containing medium and 1% DMSO served as controls. The initial cell density was 1×10^4 cells/mL. After 24 h incubation, 20 µL of 0.5 mM resazurin in PBS (sterile filtered) was added and the cells were incubated for a further 48 h. Subsequently, the absorbance was read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. The EC_{50} value (50% effective concentration, i.e., the concentration of a compound necessary to reduce the growth rate of cells by 50% to that of controls) was determined by linear interpolation. The MIC value (minimum inhibitory concentration, i.e., the concentration of the compound at which all cells were killed) was determined microscopically by inspecting each well thoroughly for the presence of motile trypanosomes.

Results

To confirm that LCS-1 is not an inhibitor of Fe-SOD, the effect of the compound on the activity of Fe-SOD in *T. brucei* cell lysates was determined using the pyrogallol autoxidation assay. As previously shown, this assay readily determines the activity and inhibition of Fe-SOD in cell extracts of bloodstream forms of *T. brucei* [6]. However, SOD activity tests have to be evaluated with care as test compounds can interfere with the assay as recently shown [5]. Therefore, we first established whether the compound LCS-1 adversely affected the autoxidation of pyrogallol. The presence of 250 µM LCS-1 did not markedly influence the autoxidation rate of pyrogallol as measured by the increase in absorbance at 450 nm (**Fig. 2a**). The time-dependent increase of absorbance in the presence and absence of LCS-1 was found to be almost identical. Next, we measured the effect of LCS-1 on the Fe-SOD activity in

trypanosome cell lysates. Results showed that the presence of 250 μM LCS-1 did not abolish the ability of the cell lysate to inhibit the autoxidation of pyrogallol (**Fig. 2a**). The observed inhibition of the autoxidation of pyrogallol by the cell lysate in the presence of LCS-1 was indistinguishable to that of the control cell lysate. The slow increase in absorbance towards the end of the measurement period is due to the fact that the hydrogen peroxide produced by the dismutation of superoxide is an inhibitor of Fe-SOD [3, 6]. Taken together, this result confirmed that LCS-1 does not inhibit the activity of Fe-SOD.

The trypanocidal activity of LCS-1 was determined with *T. brucei* bloodstream forms 427-221a using the resazurin assay [5]. The compound showed a dose-dependent effect on the growth of trypanosomes with a MIC value of 10 μM and an EC_{50} value of 1.36 μM (**Fig 2b**). Notably, the EC_{50} value of LCS-1 for its trypanocidal activity against trypanosomes did not differ much from the EC_{50} value of the compound for its cytotoxic activity against lung adenocarcinoma H358 cells (1.36 μM (this study) vs 0.8 μM [2]). Based on the considerable trypanocidal activity it can be concluded that SOD1 is most likely not the main target of LCS-1. Consequently, it can also be reasoned that the cytotoxic action of LCS-1 observed for human H358 cells is probably not just due to inhibition of SOD1.

Discussion

Pyridazin-3-one derivatives represent one of the most active class of chemical compounds displaying a wide range of biological activity [7]. For example, substituted pyridazin-3-ones have been shown to be inhibitors of stearoyl-CoA desaturase, cyclooxygenase, acetylcholine esterase and aldose reductase [7]. In light of this it is interesting to point out two herbicide compounds, chloridazon (5-amino-4-chloro-2-phenylpyridazin-3-one) and metflurazon (4-chloro-5-(dimethylamino)-2-[3-(trifluoromethyl)phenyl]pyridazin-3-one), which have very similar structure to LCS-1

(**Fig. 1**). Chloridazon has been shown to interact with cell membranes [8] while metflurazon was found to affect lipid biosynthesis [9]. As LCS-1 and both herbicides are 4-chloro-2-phenylpyridazin-3-one derivatives, it is reasonable to assume that the mode of action of LCS-1 is related to that of chloridazon and metflurazon. In this context, it is worth to mention a previous study, which reported that the mitochondria of breast cancer cells treated with LCS-1 showed increased fragmentation and dilated cristae [10]. Although the authors of the study suggested that the observed effect was due to inhibition of SOD1 by LCS-1, only indirect evidence for reduced SOD activity was provided (1.6-fold increase of mitochondrial superoxide levels) [10]. However, the observed collapse of the integrity of mitochondria could also be a direct result of an interaction of LCS-1 with the membranes of the organelles rather than due to elevated levels of superoxide. Furthermore, it is also possible that the mitochondrial fragmentation is the result of a combined effect of LCS-1. For example, increased superoxide levels could impair mitochondrial membranes so that they are more susceptible to direct damage by LCS-1 or vice versa. In any case, the exact mechanism of the cytotoxic activity of LCS-1 remains to be uncovered but this may be difficult because pyridazin-3-one derivatives have been shown to affect many cellular targets [7]. Based on the relative high lipophilicity of LCS-1, which is greater than that of chloridazon (LogP of LCS-1 = 2.85; LogP of chloridazon = 1.14), it is reasonable to assume that the compound may interact with cell membranes. This suggestion is supported by preliminary experiments. For example, incubation of bloodstream forms of *T. brucei* with LCS-1 caused fast lysis of the cells (**Fig. 1S**). In contrast, the trypanocidal drug suramin that inhibits especially trypanosomal glycolytic enzymes, did not cause any lysis of trypanosomes at the same concentration and incubation period (**Fig. 1S**). Likewise, incubation of human promyelocytic leukaemia HL-60 cells with LCS-1 resulted in fast cell lysis while the anti-cancer drug and proteasome inhibitor bortezomib did not under the same incubation conditions (**Fig. 2S**).

In conclusion, our results do not support the previous suggestion that LCS-1 exerts its cytotoxic activity solely through inhibition of SOD1. This finding is of major

relevance for future studies, as it will help to avoid misinterpretation of research results obtained with LCS-1.

Conflict of interest

The authors declare no conflict of interests.

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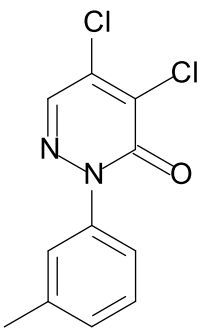
Figure legends

Fig. 1 Structures of LCS-1, and of the related compounds chloridazon and metflurazon. The PubChem Compound Identifier (CID) for each compound is also shown.

Fig. 2 Effect of LCS-1 on the activity of Fe-SOD in cell extract and on the growth of bloodstream form of *T. brucei*. (**a**) The activity of Fe-SOD in cleared trypanosome cell lysates was determined indirectly by the inhibition of pyrogallol autoxidation. To 180 µL mixture containing 2.5×10^7 cell equivalents and 50 nmol LCS-1 (closed circles) or 2.5% DMSO alone (closed squares), 20 µL of a 2 mM pyrogallol solution in 1 mM HCl was added and the increase in absorbance at 450 nm was followed photometrically. Negative controls indicate the autoxidation of pyrogallol in the absence of cell lysate but in the presence of 50 nmol LCS-1 (open circle) or 2.5% DMSO alone (open squares). A representative result from two independent experiments is shown. (**b**) Trypanosomes were incubated with varying concentrations of LCS-1. After 72 h of culture, cell viability and proliferation was determined with the colorimetric dye resazurin. Mean values ± SD of three experiments are shown.

244 **Fig. 1**



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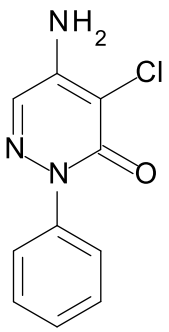

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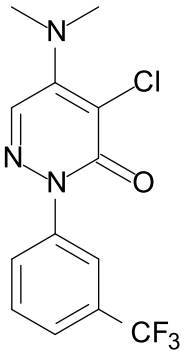

metflurazon
CID: 32011

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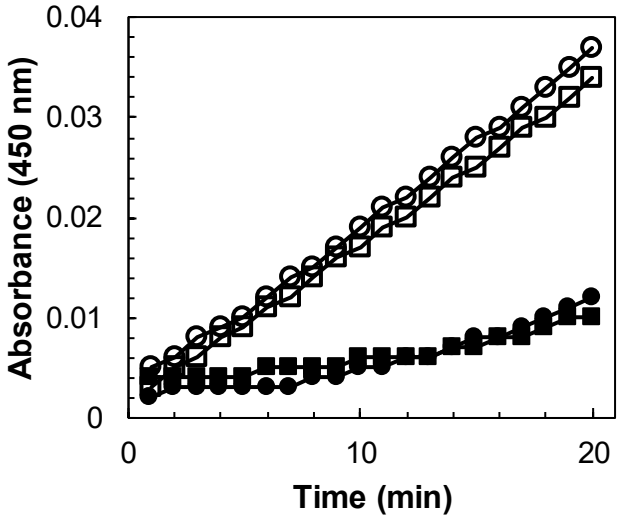
246

Fig. 2

a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10
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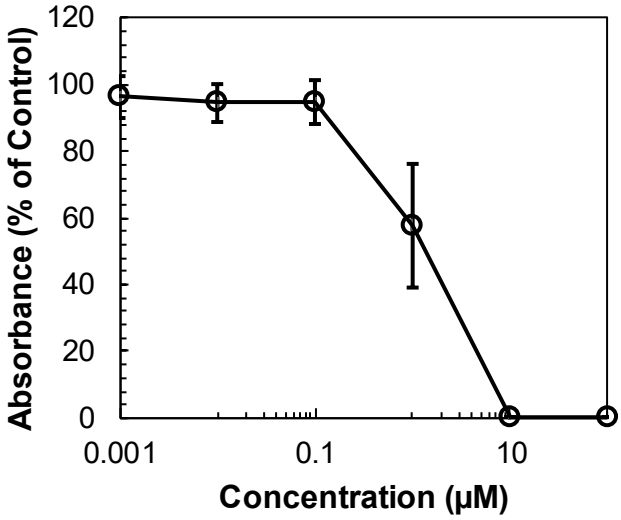
Time (min)

b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80
60
40
20
0
Absorbance (% of Control)
0.001
0.1
10
Concentration (μM)

Supporting Information

Cytotoxic activity of LCS-1 is not only due to inhibition of SOD1

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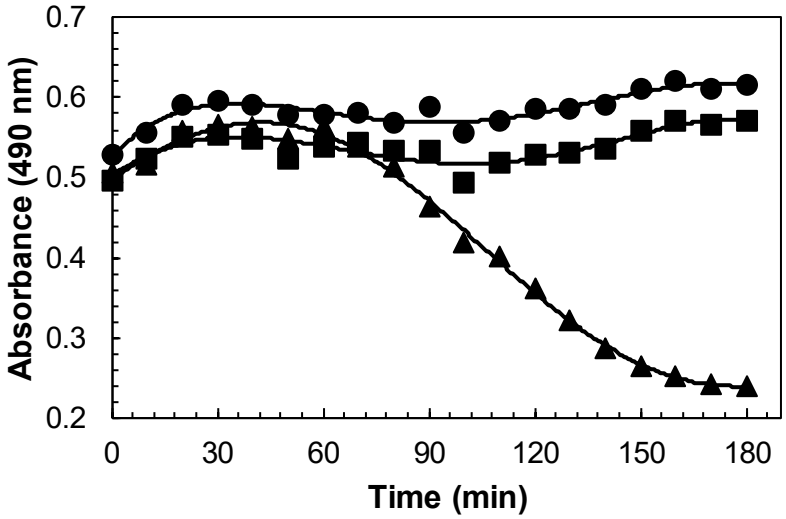

Fig. 1S Lytic effect of LCS-1 on bloodstream forms of *Trypanosoma brucei*. Lysis of trypanosomes was measured by light scattering at 490 nm. Note that a decrease in absorbance corresponds to increasing lysis of cells. Bloodstream forms of *T. brucei* (5 × 10^7 cell/ml) were incubated with 100 µM LCS-1 (triangles), 100 µM suramin (squares) or DMSO (circles) in culture medium at 37 °C in a humidified atmosphere containing 5% CO_2. The final DMSO concentration was 1%. Every 10 min, the absorbance was measured over a period of 3 h. After 1 h incubation, absorbance of the culture incubated with LCS-1 started to decrease indicating lysis of trypanosomes. After 3 h incubation, all trypanosomes were lysed. In contrast, cultures incubated with only DMSO or with the trypanocidal drug suramin showed no lysis of trypanosomes over the 3 h incubation period. A representative result from two independent experiments is shown.

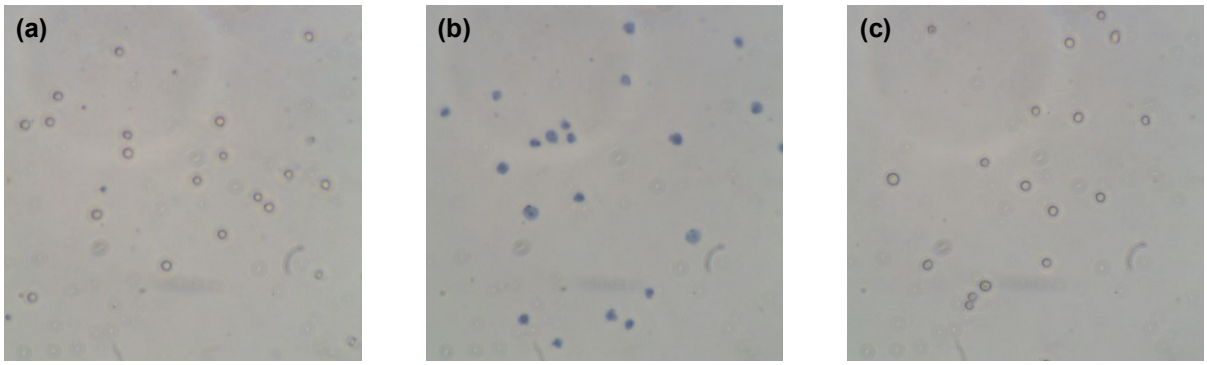

Fig. 2S Lytic effect of LCS-1 on HL-60 cells. Lysis of HL-60 cells was assessed by the trypan blue exclusion test. HL-60 cells (1×10^6 cell/ml)m were incubated with 250 µM LCS-1 (**a**), 250 µM bortezomib (**b**), or DMSO (**c**) in culture medium at 37 °C in a humidified atmosphere containing 5% CO_2. The final DMSO concentration was 2.25%. After 3 h incubation, cells were stained with trypan blue (1:1) and images were recorded using an inverted microscope fitted with a digital camera. Whereas the cytoplasm of cells incubated with only DMSO or with the anti-cancer drug bortezomib were not stained, then cytoplasm of cells treated with LCS-1 appeared bluish indicating that their membranes were not intact and therefore could not anymore exclude the dye.