Effect of peroxisomicine A$_2$ and T 544 of the genus *Karwinskia* on peroxisomes of *Candida boidinii*

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**Abstract**

Dimeric anthracenones have been isolated from toxic plants of the genus *Karwinskia* (Rhamnaceae). T 514 or peroxisomicine A$_1$ is one of the anthracenonic compounds which produce irreversible and selective damage on the peroxisomes of yeast cells in vivo. In this paper we describe the effect of two structurally related anthracenones on cell viability and on the peroxisomes of the methylotrophic yeast *Candida boidinii*. As has been described for peroxisomicine A$_1$, peroxisomicine A$_2$ and T 544 caused a decrease in the viability of *C. boidinii* at all concentrations tested, and disruption of the peroxisomal membrane, T 544 showing the strongest effect. In *C. boidinii* cell death and peroxisomal damage seem to be independent events. © 1998 Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Dimeric anthracenone; *Karwinskia*; Methylotrophic yeast; Peroxisome; *Candida boidinii*

1. Introduction

*Karwinskia humboldtiana* is a poisonous plant that grows in Mexico, the southwestern United States and in some parts of Central America [1]. Dreyer et al. [2] isolated and characterized for the first time the toxic components from the seed of this plant. These compounds were identified as dimeric 9,10-dihydroxy anthracenones and according to their molecular mass were called T 496, T 514, T 516 and T 544. It has been demonstrated that T 514 causes severe damage to lung, kidney and liver [3]. This compound exhibits selective toxicity in vitro on tumor cells [4], and also, at nonlethal doses, produces irreversible and selective damage of yeast peroxisomes in vivo [5]. For this reason we renamed it peroxisomicine A$_1$. Recently, we reported the inhibitory effect in vitro of peroxisomicine A$_1$ and structurally related anthracenones on liver catalase activity, using the purified enzyme [6]. In contrast to the described findings in vitro, peroxisomicine A$_1$ is not able to inhibit in vivo the activity of catalase in methylotrophic yeasts with peroxisomal damage induced by the toxin [5], either in hepatic fragments.

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incubated with this toxin (in situ) or in intoxicated mice (in vivo) [7]. Considering the reported effect of peroxisomicine A₁ on peroxisomes of methylotrophic yeast cells, and the structural similarity of other dimeric anthracenones, we were interested in investigating whether this effect is exclusive to peroxisomicine A₁. In the present work, we analyzed the effect of two dimeric anthracenones isolated from plants of the genus *Karwinskia*, peroxisomicine A₂ and T 544, on cell viability as well as on the peroxisomes of *Candida boidinii*, including peroxisomicine A₁ as a positive control (Fig. 1). All these studies were carried out in vivo.

2. Materials and methods

2.1. Anthracenonic compounds

T 514 or peroxisomicine A₁, diastereoisomer 514 or peroxisomicine A₂ from fruits of *K. parvifolia*, and T 544 from fruits of *K. humboldtiana* were isolated, purified and identified by procedures already described [8,9].

2.2. Experimental procedure

*C. boidinii* (ATCC 32195) was grown in shake flask cultures in mineral medium supplemented with yeast extract [10], at 30°C, on glucose (0.25%) or methanol (0.5%). The anthracenonic compounds peroxisomicine A₁, peroxisomicine A₂ and T 544 were added to the cultures at 0.5, 2, 10, 20 and 50 mg ml⁻¹, after the cultures had reached the mid-exponential growth phase on both glucose and methanol media. As positive and negative controls cultures with peroxisomicine A₁ and untreated cells, respectively, were used. Samples were taken at 2 h after adding the compounds. The viability of the exposed cells was estimated after inoculation on YPD and YPM agar plates (YPD: 1% yeast extract, 2% peptone, 2% glucose; YPM: 1% yeast extract, 2% peptone, 0.5% methanol). The cell morphology was evaluated by phase contrast microscopy. For the experiments in which the peroxisomal integrity was studied, induction conditions for peroxisome proliferation were chosen. The anthracenonic compounds were added at 2 μg ml⁻¹ (nonlethal dose) to cultures in methanol (0.5%) at the mid-exponential growth phase. Samples for electron microscopy and immunocytochemistry were taken at 30 min, 1, 2, 4, 8 and 24 h after adding the compounds. The effect on yeast peroxisomes was also investigated by the measurement of catalase activity in crude extracts of cells from 24-h methanol cultures.

2.3. Biochemical analysis

Crude extracts were prepared as follows: 0.4 g cells (wet weight) from *C. boidinii* grown 24 h in methanol (0.5%) were disrupted by shaking with 3.5 mg glass beads (0.5 mm diameter) for 3 min (6 × 30 s) in a Vortex. The homogenate was removed from the beads and flask, and centrifuged at 10000 × g in a microfuge for 2 min to remove debris and unlysed cells. In the supernatant (crude extract) catalase activity and protein were determined by procedures already described [11,12].

2.4. Ultrastructural analysis

For ultrastructural studies, whole cells were fixed in KMnO₄ as described previously [13]. Ultrathin sections were obtained with an ultramicrotome Ultratome LKB V and examined under a Carl Zeiss EM 109 electron microscope. For the detection of alcohol oxidase by immunocytochemical procedures intact cells were fixed in glutaraldehyde, and embedded in Lowicryl K4M. Immunogold labeling was performed on ultrathin sections using anti-alcohol oxidase antibody and IgG conjugated to colloidal gold particles.

2.5. Statistical analysis

All experiment data are shown as mean ± S.D. Student’s t-test was used to analyze the results of both cell viability and determinations of catalase activity.

3. Results

The growth experiments indicate that peroxisomicine A₁ (positive control), peroxisomicine A₂ and T 544 produced a decrease in the viability of *C. boidi-
in glucose as well as in methanol liquid cultures (Fig. 2).

Morphological observations by phase contrast microscopy of the methanol-grown cells showed cellular disorganization from 10 μg ml⁻¹, proportional to the peroxisomicine A₁ concentrations. The changes consisted of enlarged vacuoles containing peroxisomal clusters (Fig. 3). At the highest concentration (50 μg ml⁻¹) damage was more evident and a mass of peroxisomes inside the vacuole was detected. C. boidinii cells exposed to peroxisomicine A₂ showed no effect on cell organization; in the case of T 544, only at 50 μg ml⁻¹ was a similar damage as seen at low concentrations of the positive control found. In general, C. boidinii cells grown in glucose showed no response to the different concentrations with all anthracenones tested, including the positive control (data not shown). C. boidinii cells grown in methanol exposed to 2 μg ml⁻¹ of each of the anthracenone compounds showed damage on the peroxisomal membrane from the first incubation time (30 min) (Fig. 4). The damage on the peroxisomal membrane was observed as holes or membrane disruptions; peroxisomes to be degraded were first sequestered from the cytoplasm by pre-autophagic membranes, followed by their uptake into the vacuole and further degradation of the peroxisomal contents. The intensity of the damage was proportional to the incubation times. At the longest incubation time (24 h), most of the peroxisomes were inside large autophagic vacuoles. C. boidinii cells exposed to T 544 showed

![Fig. 1. Chemical structure of dimeric anthracenones from plants of the genus Karwinskia.](image)

![Fig. 2. Survival rates of C. boidinii cells, grown in liquid cultures on either 0.5% glucose (A) or 0.5% methanol (B), after administration of several concentrations of peroxisomicine A₁ (positive control), peroxisomicine A₂ and T 544 in the mid-exponential growth phase (A₆₀₀ approx. 2.0). After 2 h of incubation, cells were washed once with distilled water and inoculated on YPD (glucose) and YPM (methanol) agar plates. Data are presented as percentages of surviving cells, compared to untreated controls and represent the average of three independent experiments. (*Significant difference by Student’s t-test, P < 0.005).](image)
the strongest damage on the peroxisomal membrane at all incubation times tested. A complete loss of the peroxisomal membrane and the presence of peroxisomes of various sizes suggested that this toxin caused a more intensive damage on peroxisomes than the other anthracenones. The immunocytochemistry experiments confirmed indirectly the damage on the peroxisomal membrane produced by the three anthracenonic compounds analyzed. At short incubation times, alcohol oxidase was found both inside and outside the peroxisomes, and in some cases inside the vacuole (Fig. 5). After 24 h the enzyme was found more frequently inside the vacuoles. Damage to other cellular membranes (e.g., nuclei, mitochondria, and vacuoles) was not detected under these conditions. In spite of the peroxisomal damage described above, no change in catalase activity was found in any sample analyzed.

4. Discussion

The effect of peroxisomicine A1 was described for the first time by Sepúlveda et al. [5] in two methylotrophic yeasts: Hansenula polymorpha and C. boidinii. The results of this work demonstrate that at least in C. boidinii, the selective and irreversible effect on yeast peroxisomes is not exclusive to peroxisomicine A1. Other structurally related dimeric anthracenones, such as peroxisomicine A2 and T 544, are also able to produce disruption of the peroxisomal membrane at a nonlethal dose (2 μg ml⁻¹). Regarding the effect...
on cell viability, peroxisomicine A\textsubscript{1} caused the lowest survival percentages in glucose as well as in methanol C. boidinii cultures (Fig. 2). It is very interesting that observations by phase contrast microscopy showed an effect on the cellular organization of C. boidinii cells grown in methanol, exposed to the three anthracenonic compounds, while in glucose-grown cells no effect was found at any concentrations of the compounds tested. These findings suggest that the carbon source could play a role in the effect of the anthracenonic compounds. As previously described for peroxisomicine A\textsubscript{1}, peroxisomicine A\textsubscript{2} and T 544 also produced disruption of the peroxisomal membrane, T 544 being the most active of the three compounds. We have previously reported the inhibitory effect in vitro of structurally related anthracenones, among them peroxisomicine A\textsubscript{1}, peroxisomicine A\textsubscript{2} and T 544, on liver catalase activity [6]. Recently, we also demonstrated that peroxisomicine A\textsubscript{1} was not able to inhibit the catalase activity in intoxicated mice, in vivo [7]. This is in agreement with the findings described by Sepúlveda et al. [5], which indicate that catalase activity is not modified in methylotrophic yeasts with peroxisomal damage induced by peroxisomicine A\textsubscript{1} in vivo. The fact that peroxisomicine A\textsubscript{1}, peroxisomicine A\textsubscript{2} and T 544 produce peroxisomal damage, but do not inhibit catalase activity in vivo, strongly suggests that catalase is not directly involved in the selective effect on peroxisomes of the anthracenonic compounds. The effect of T 544 on C. boidinii could be considered a very attractive system to study peroxisomal function, considering that T 544 produces intensive peroxisomal damage, without causing toxic effects on this yeast. If the effect on peroxisomes is based on an interaction between a component of the membrane and the anthracenonic compound, it is likely that a common structural element of the three toxins is involved in the peroxisome interaction. Currently, studies are in progress to elucidate the molecular mechanism involved in the toxin-peroxisome interaction.

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