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A Method to Assay Glyoxylate Cycle Inhibitors for Antifungals

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Fungi and many other prokaryotic and eukaryotic microorganisms can utilize ethanol, acetate or fatty acids as the sole carbon source. The glyoxylate cycle, which is absent in mammals, is required for the utilization of those carbon sources. The key enzymes of glyoxylate cycle are isocitrate lyase (E.C. 4.1.3.1; isocitrate glyoxylate-lyase) and malate synthase (E.C. 4.1.3.2; L-malate glyoxylate-lyase). Recently, it has been shown that the genes of glyoxylate cycle are induced when *Saccharomyces cerivisiae* and *Candida albicans* are phagocytized by macrophages, and *C. albicans* mutants lacking isocitrate lyase gene are markedly less virulent than the wild type[1]. Thus, it is expected that the glyoxylate cycle is a new target for antifungal drugs.

Current assays for each enzyme, isocitrate lyase and malate synthase, are based on UV absorption[2] and not suitable for high throughput screening. We describe here a new glyoxylate cycle assay that is in a high throughput format in order to screen for inhibitors. The experimental design of the new assay is summarized in Fig. 1. Isocitrate lyase catalyzes the reversible cleavage of isocitrate to glyoxylate and succinate. The glyoxylate molecule formed by the isocitrate reaction condenses with one acetyl-CoA (AcCoA) to produce L-malate in the subsequent step catalyzed by malate synthase. Accordingly, the amount of CoA formed is proportional to glyoxylate cycle activity, and can be determined spectrophotometrically at 412 nm by the addition of 5,5′-dithio-bis(2-nitrobenzoic acid)[3]. Thus, the activity of the two glyoxylate cycle enzymes could be measured in a single assay.

Aspergillus fumigatus ATCC 16424 was inoculated into yeast nitrogen base (YNB) medium containing 2% (w/v) potassium acetate (pH 7.0) at 2×10^6 conidia/ml (final concentration), and incubated for 24 hours at 37°C with shaking (250 rpm). Hyphae were harvested by vacuum filtration over Whatman No. 2 filter paper and disrupted by bead-beating (6×30 seconds with 2 minutes cooling between each pulse) using 0.5 mm zirconium beads in 50 mM phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. The lysates were centrifuged at 5,000 *g* for 10 minutes at 4°C. The supernatants were quick-frozen in dry ice and stored at −80°C until used. Glyoxylate cycle activity was determined in 50 mM phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$, 10 mM DL-isocitric acid, 400 μM AcCoA and 0.03 mg/ml cell lysate of *A. fumigatus* in a final volume of 50 μl. After incubation at 37°C, the reactions were terminated by addition of 50 μl of 6.4 M guanidine hydrochloride. Subsequently, 50 μl of 400 μM 5,5′-dithio-bis(2-nitrobenzoic acid) was added. After incubation for 10 minutes at room temperature, absorbance at 412 nm (A_{412}) was measured. These results are shown in Fig. 2. Note that in control mixtures lacking either isocitrate, AcCoA or cell lysate, the A_{412} did not increase during incubation. On the other hand, the formation of CoA in complete mixtures was linear for at least 60 minutes. In addition, CoA formation was dependent on the concentration of substrates and cell protein (data not shown). Accordingly, CoA was produced by the activity of the enzymes of the glyoxylate cycle. In contrast, the activity was not found in lysates prepared from the cells grown in YNB medium containing 2% glucose in place of acetate (data not shown). This is consistent with the observation that the glyoxylate cycle is induced when microorganisms are grown in medium containing C_2-

Fig. 1. A new assay for glyoxylate cycle.

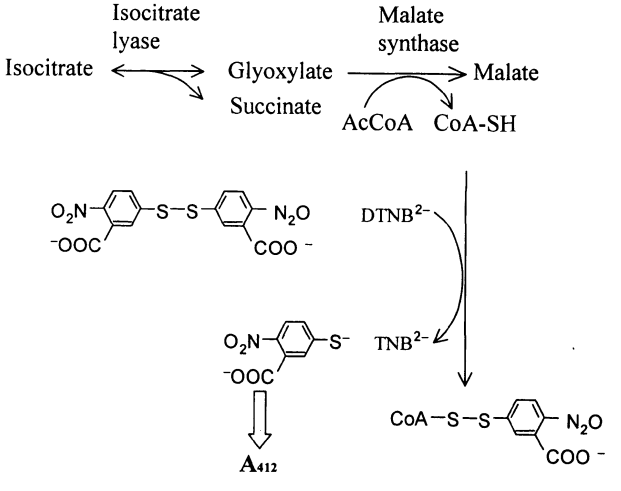

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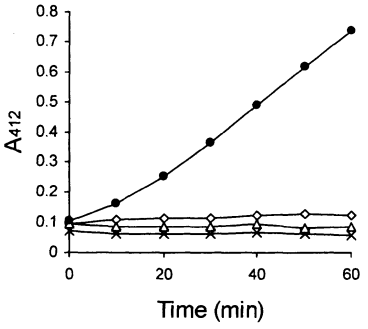
Fig. 2. The activity of glyoxylate cycle in *A. fumigatus* crude extract.



A. fumigatus lysates were prepared and used for the glyoxylate cycle assay as described in the text. Complete reaction mixtures (•) contained 10 mM DL-isocitrate, 400 μM AcCoA and 0.03 mg/ml cell lysate, while control mixtures were in the absence of DL-iscitrate (△), AcCoA (×) or cell lysate (◇). Symbols represent the average of two determinations.

Table 1. The effect of 3-nitropropionate and oxalate on glyoxylate cycle activity.

	% Inhibition					
	3-Nitropropionate (μM)			Oxalate (μM)		
	1	10	100	10	100	1000
Glyoxylate cycle*	2	46	98	23	75	92
Isocitrate lyase**	5	51	85	15	65	92
Malate synthase**	0	1	5	8	25	80

* % inhibition of the glyoxylate cycle was determined by the new assay.
** % inhibition of isocitrate lyase and malate synthase was determined by each individual assay.
The data represent the average of two determinations.

molecules (*i.e.*, acetate and ethanol) or fatty acids as a sole carbon source[4].

To confirm that we could detect glyoxylate cycle enzyme inhibitors, we tested the effects of 3-nitropropionate[5] and oxalate[6], inhibitors of isocitrate lyase and malate synthase, respectively, using this new method. In addition, we assayed the activity of each enzyme individually: isocitrate lyase activity was measured by a modified method of DIXON and KOMBERG[2], while malate synthase activity was measured by a modification of this novel assay (using 10 mM glyoxylate in place of DL-isocitrate as substrate). The results of these experiments are shown in Table 1. Note that as detected by our new method that measures the net activity of both enzymes, inhibition by 3-nitropropionate was 98, 46 and 2% at 100, 10 and 1 μM, respectively, which was similar to the inhibition of the isolated activity of isocitrate lyase as measured by an independent assay. These results clearly show that isocitrate lyase inhibitors can be detected by this assay. On the other hand, oxalate inhibited not only malate synthase activity, but also isocitrate lyase activity. Since no other specific malate synthase inhibitors are known, we could not unequivocally determine whether malate synthase inhibitors can be detected by this method. However, since malate synthase activity is downstream of isocitrate lyase activity, and this new method is based on the CoA quantification formed by malate synthase activity, it is likely that malate synthase specific inhibitors will also be detected by using this method.

We have used this novel assay to screen a number of natural product extract libraries and these results will be the

subjects of subsequent manuscripts.

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