The immunosuppressive drug leflunomide affects mating-pheromone response and sporulation by different mechanisms in *Saccharomyces cerevisiae*

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Abstract

Leflunomide (LFM) is a novel anti-inflammatory and immunosuppressive drug, and inhibits the growth of cytokine-stimulated lymphoid cells in vitro. The effect of LFM on haploid and diploid cells of *Saccharomyces cerevisiae* was investigated to elucidate the molecular mechanism of action of the drug. Using a halo assay, LFM was shown to enhance the cell cycle arrest of haploid cells induced by mating pheromone α-factor. LFM also inhibited sporulation of diploid cells completely. *S. cerevisiae* genes which were cloned to suppress the anti-proliferative effect when present in increased copy number were introduced and examined for their activity to suppress the effect of LFM. Out of them, MLF4/SSH4, was found to suppress the sporulation-inhibitory effect of LFM. However, MLF4 failed to suppress the enhancing effect of LFM on pheromone response. Thus, LFM is suggested to act on haploid and diploid cells by different mechanisms. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Leflunomide (LFM), an isoxazol derivative, is a novel anti-inflammatory and immunosuppressive drug that has been shown to be effective in preventing and treating autoimmune diseases in animals and the reaction leading to organ transplantation rejection [1]. Many of the immunosuppressive effects of LFM can be attributed to its inhibitory effect on the activity of many cytokines, most likely through receptor expression and signal transduction [2].

The immunosuppressants cyclosporin A (CsA), FK506, and rapamycin inhibit the intermediate steps in signal transduction leading to T-cell activation [3,4]. CsA and FK506 block the activation of quiescent T-cells (transition of the G0 to the G1 phase of the cell cycle) by interfering with the T-cell receptor-mediated signal transduction pathway [5,6]. Rapamycin inhibits subsequent progression from the G1 to the S phase of the cell cycle by blocking the response to the lymphokine interleukin-2. The action of the immunosuppressants is dependent on the formation of a complex with intracellular receptors termed immunophilins [6].

In yeast, CsA and FK506 inhibit recovery from mating pheromone α-factor-induced cell cycle arrest through interactions strikingly similar to those involved in the inhibition of T-cell activation [4,7]. Rapamycin arrests the *Saccharomyces cerevisiae* cell cycle in the G1 phase by binding to a highly conserved FK506 binding protein (FKBP) [8,9] and then by inhibiting TOR1, the yeast homolog of phosphatidylinositol-3-phosphate kinase [10]. FK506 also blocks the import of amino acids in yeast cells [11]. Recently rapamycin was reported to induce sporulation but inhibit tetrad formation of diploid cells of *S. cerevisiae* [12]. My earlier study showed that *S. cerevisiae* was susceptible to LFM and led to the understanding that LFM blocked the cell division in the G1 phase [13].

I report here the effect of LFM on haploid and diploid cells of *S. cerevisiae*. The study suggests that LFM may inhibit sporulation of diploid cells by interacting with a Shr3 protein but enhance mating-pheromone response of haploid cells via a different mechanism.
2. Materials and methods

2.1. Yeast strains and culture conditions

*S. cerevisiae* strains used in this study were: DH17-1C (*MATa* ura3 leu2 his4 trp1 gal2 his4), XF79-6D (*MATa ade2 ura3 leu2 trplhis6 stsl-2) and DH42 (*MATa/MATa ura3/ura3 leu2/leu2 trpl+/+ /his4*). Cultivation and maintenance of cells were carried out in YEPD (1% yeast extract, 2% peptone, and 2% glucose) as described previously [14]. Sporulation was performed on SP medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose) [14]. The selective medium for drug sensitivity was SD (2% glucose, 0.67% yeast nitrogen base without amino acids), supplemented with bases and amino acids according to [14] and containing 50 μg of LFM per ml. Media were solidified by addition of 2% agar.

2.2. Halo assay for mating pheromone α-factor-induced cell cycle arrest

Cells of XF79-6D were cultured in YEPD medium for 3 days at 30°C and spread onto plates as described previously [15]. Synthetic α-factor was purchased from Sigma chemicals. 5 μl of α-factor solution was spotted onto the plates and incubated for 3 days at 30°C, followed by photography.

2.3. Sporulation procedure

To induce sporulation, diploid strain DH42 was cultured in YEPD at 30°C overnight, transferred to SP medium, and incubated for 3 days. To test sporulation of DH42 with plasmids, the cells were cultured in leucine-deficient synthetic medium for 2 days, transferred to SP medium, and incubated for an additional 3 days. Asci were counted under the phase-contrast light microscope (Nikon).

2.4. Plasmids

Plasmid DNA was amplified in *Escherichia coli* DH5

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<td>Effect of LFM on sporulation of diploid cells</td>
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<td>Percentage of ascis formed</td>
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DH42 cells were tested for sporulation in the presence or absence of 20 μg of LFM per ml. Sporulation is expressed as percentage of ascii formed. The results show an average of three different experiments (n = 200).

and purified using columns (Qiagen) according to the manufacturer. Plasmids p17B1, p17L3, p17L4, and p17H4 harbor the *S. cerevisiae MLF1/FUR4*, *MLF2/URA3*, *MLF3/YNL074C*, and *MLF4/SSH4* genes, respectively [16–18]. YEp213 was used as a control vector. Plasmids were introduced into yeast cells by genetic transformation [19].

3. Results

3.1. Effect of LFM on cell cycle arrest induced by mating pheromone

Since the immunosuppressants CsA and FK506 inhibit recovery from cell cycle arrest by mating pheromone α-factor and enhance α-factor arrest, the effect of LFM on α-factor arrest was investigated. Three different amounts of synthetic α-factor were spotted onto plates lawn with cells of XF79-6D, and the halo zones formed were observed. Larger sizes of halo zones formed in the presence of LFM compared with in the absence of LFM (Fig. 1). This suggests that LFM may enhance the α-factor response or inhibit recovery from α-factor arrest as in the case of CsA and FK506.

3.2. Effect of LFM on sporulation

Rapamycin inhibits tetrad formation in sporulation of yeast cells [12]. To elucidate the molecular mechanism of action of LFM, the effect of LFM on sporulation was investigated. When diploid strain DH42 was cultivated on SP medium containing LFM, there was a dramatic inhibition of sporulation. A decrease in sporulation was observed in the presence of LFM (Table 1). This suggests that sporulation may be controlled by a process which is sensitive to LFM.

![Fig. 1. Effect of LFM on mating pheromone α-factor-induced cell cycle arrest of haploid cells. Cells of XF79-6D were spread on each plate as described in Section 2. The amounts of synthetic α-factor were 62.5 ng, 125 ng, and 250 ng, respectively. A: No LFM. B: LFM (50 μg ml⁻¹).](https://academic.oup.com/femsle/article-abstract/191/1/57/631581/The-immunosuppressive-drug-leflunomide-affects)
3.3. Suppression by yeast genes of pheromone response-enhancing or sporulation-inhibitory effects of LFM

Genes of *S. cerevisiae* which suppress the anti-proliferative effect of LFM when in increased copy number have been cloned [16–18]. Four genes (*MLF1*, 2, 3, and 4) were introduced into DH42 diploid cells, and resultant *leu*<sup>+</sup> transformants were allowed to sporulate in the presence of LFM to evaluate the roles of these genes in blocking the inhibition of sporulation. The *MLF4* gene was observed to suppress the sporulation-inhibitory effect of LFM (Table 2). Other genes showed no suppressing activity.

To clarify whether the enhancing action of LFM on α-factor response is attributable to the anti-proliferative activity or not, the same four genes were also introduced in XF79-6D haploid cells and their suppressive activities examined. Identical sizes of halo zones were formed, independent of the presence or absence of the *MLF4* gene when in increased copy number (Fig. 2). The other three genes were also unable to suppress the enhancing action of LFM (data not shown). In this experiment, mutants arose in the case of the over-expressed *MLF4* gene within the zone of inhibition that did not appear in the control (Fig. 2).

### Discussion

LFM not only enhances α-factor-induced cell cycle arrest of haploid cells but also inhibits sporulation of diploid cells of *S. cerevisiae*. The present study has also shown that the *MLF4* gene suppresses the sporulation-inhibitory action of LFM but fails to suppress the pheromone response-enhancing action of LFM.

Since the *MLF4* gene is a suppressor of the *shr3* mutation which results in the accumulation of amino acid permeases in the endoplasmic reticulum (ER) [20], the fact that the inhibitory effect of LFM was suppressed by the *SSH4* gene suggests that LFM may block the sporulation possibly by affecting the Shr3 protein. The *SHR3* gene product exhibits no homology with known proteins including cyclophilins and FKBP<sub>s</sub>, but encodes an integral membrane protein with four transmembrane domains localized to the ER membrane. Heitman et al. [11] suggested that both Shr3 and a putative FK506-sensitive FKBP possibly participate in folding or chaperoning amino acid transporters during secretion in yeast cells.

The present finding may evoke the idea that sporulation might be controlled by amino acid import and could be blocked by affecting amino acid permeases with LFM or FK506. However, Zheng and Schreiber reported that FK506, which affects the amino acid import, did not block sporulation of diploid cells in contrast to the case of rapamycin [12]. Therefore, inhibition of sporulation by LFM may not be attributed to the block of amino acid import. Gimeno et al. reported that the *shr3* mutation enhanced the pseudohyphal phenotype [21]. Inhibition of sporulation by LFM might therefore be correlated with the possible inhibition of the developmental phase of diploid cells.

Further biochemical analysis may lead to the elucidation of the possible target(s) of LFM responsible for the inhibition of sporulation and for the enhancement of the pheromone response.

### References


