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Borrelidin Induces the Transcription of Amino Acid Biosynthetic Enzymes Via a *GCN4*-Dependent Pathway

Erin L. Eastwood and Scott E. Schaus*

Department of Chemistry, Metcalf Center for Science and Engineering, Boston University, 590 Commonwealth Avenue, Boston, MA 02215, USA

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Abstract—Global cellular profiling of messenger RNA levels has been used to provide insight into the effects of the angiogenesis inhibitor borrelidin on the eukaryotic model organism *Saccharomyces cerevisiae*. The most notable result of treatment with borrelidin is the induction of amino acid biosynthetic enzymes in a time-dependent fashion. We have ascertained that induction of this pathway involves the *GCN4* transcription factor. This conclusion was determined by treating a yeast strain lacking this gene and observing the absence of increased gene transcription under Gcn4p control.

The inhibition of angiogenesis by chemical agents is an approach currently under intensive study for the development of new cancer treatments.[1] Investigation of the molecular mechanisms by which these agents inhibit new capillary tube formation has led to the discovery of biological pathways through which cell proliferation occurs.[2] The macrolide antibiotic borrelidin (Fig. 1) inhibits cell proliferation in a rat aorta matrix in which growth has been induced with vascular endothelial growth factor (VEGF).[3] We sought to identify the mechanisms through which borrelidin inhibits cell proliferation using global cellular profiling techniques. Our approach was to employ whole genome transcription profiling of alterations in cellular messenger RNA levels of the eukaryotic model organism *Saccharomyces cerevisiae* following treatment with borrelidin.

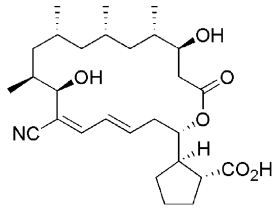

Figure 1. Borrelidin.

We were interested in understanding the mechanisms by which borrelidin affects cellular proliferation because of reports that differed in the identification of the primary cellular effects in different eukaryotic organisms. Borrelidin was first isolated from *Streptomyces rochi* and described as possessing both antibiotic and antiviral activity.[4] It was later reported that borrelidin inhibits *Escherichia coli* threonyl-tRNA synthetase.[5] The notion that threonyl-tRNA synthetase is a target of borrelidin is supported by findings that cells resistant to borrelidin, such as certain strains of yeast and cell lines of Chinese hamster ovary cells, have increased threonine concentrations as well as altered threonyl tRNA-synthetase activity.[6–9] However, a recent report illustrated that borrelidin inhibits the cyclin-dependent kinase activity of Cdc28p/Cdk2p in *S. cerevisiae* with an IC_{50} value of 24 μM.[10] In anticipation of identifying a particular pathway that is affected by the antibiotic borrelidin, we initiated experiments that would provide a global assessment of cellular responses to treatment.

We decided to use a combination of small-molecule perturbation experiments with traditional genetics in our initial studies.[11–13] *S. cerevisiae* serves as an ideal system to study basic cellular functions of eukaryotic cells in part due to the conservation of cellular processes with higher eukaryotes and metazoans.[14] Approximately 30% of genes implicated in human disease have yeast homologues.[15] Furthermore, the yeast genome is

*Corresponding author. Fax: +1-617-353-6466; e-mail: seschaus@chem.bu.edu

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completely sequenced and, as a result, significant information is available about the genes and their corresponding protein products.

The ability of borrelidin to inhibit cell proliferation of exponentially growing *S. cerevisiae* strain BY4741 (wt) was ascertained.[16] Our experimentally determined IC_{50} value was 23 μM. Global transcription profiling experiments were used to examine the changes in the mRNA levels in the wt laboratory strain treated with the concentration of borrelidin (100 μM) that inhibited continued growth of wt yeast at 75%. Aliquots of the treated culture were removed at 30-min intervals. Fluorescently-labeled cDNA probes were synthesized from messenger RNA isolated from the untreated and treated samples using cyanine-3 (green) and cyanine-5 (red) fluorescent dyes. The probes were combined for competitive hybridization to DNA microarrays constructed using optimized 70mer oligonucleotide probes for 6300 of yeast genes which included positive and negative controls for hybridization. The extent of the hybridization was determined by simultaneous fluorescent laser scanning at 532 and 635 nm. The absolute intensities at each wavelength were used to determine the relative amounts of labeled cDNA bound to the microarray.[17]

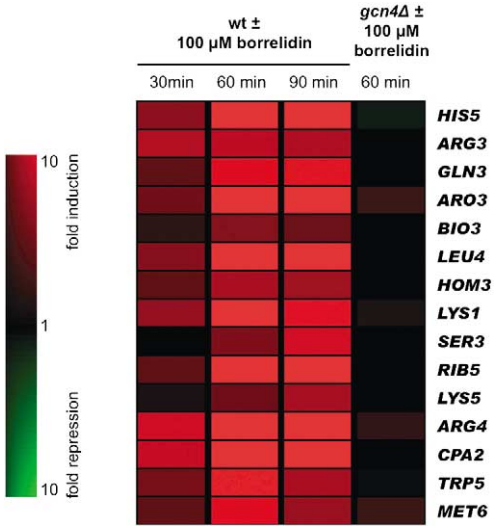
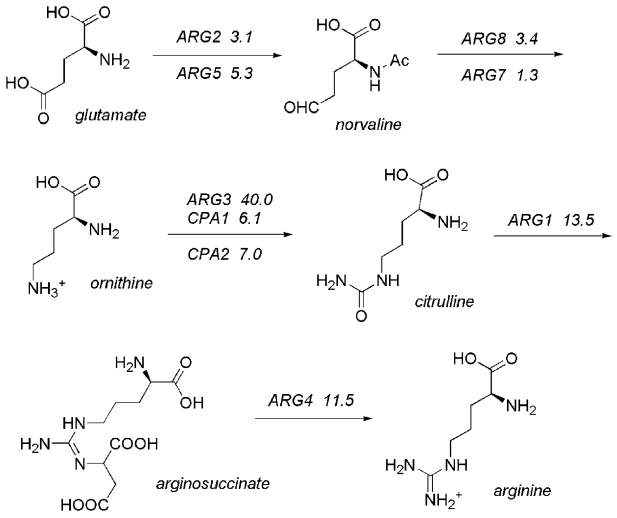


Figure 2. Color display plot of the expression ratios of genes involved in amino acid biosynthesis in the wild-type and the *gcn4*Δ strains resulting from treatment with borrelidin. The color scale at the left represents the expression ratio of the hybridization signals as compared to the control ranging from -10 (brightest green, 10-fold repressed) to 10 (brightest red, 10-fold induced). Black indicates no significant change in expression. The individual gene names are provided at the right. The graphic was generated using the GENESPRING software package.

The laboratory yeast strain treated with borrelidin showed a noticeable alteration in gene transcription in comparison to the untreated sample after 60 min. Transcripts corresponding to 8% of the yeast genome were differentially expressed by 2-fold or more. A 2-fold or greater increase in mRNA levels was observed for 300 genes. Of these, 68 genes encode proteins that are involved in amino acid metabolism. As such, the effects of borrelidin appear to extend across all amino acid biosynthetic pathways (Fig. 2), including the transcription of genes whose protein products are responsible for histidine (*HIS5*), aromatic amino acid (*ARO3*, *TRP5*), leucine (*LEU4*), aspartic acid (*HOM3*), lysine (*LYS1*, *LYS5*), serine (*SER3*), methionine (*MET6*), and arginine (*ARG3*, *ARG4*, *CPA2*) biosynthesis.[18] One of these pathways, the arginine biosynthetic pathway, is illustrated in Scheme 1 with the name of each gene whose protein product is responsible for the conversion of L-glutamate to L-arginine. It is interesting to note that mRNA levels for each gene involved in the pathway were measured to be greater than 2-fold as a result of treatment with borrelidin. We postulated that treatment with borrelidin results in the activation of a specific pathway that involves the transcription of amino acid biosynthetic enzymes.



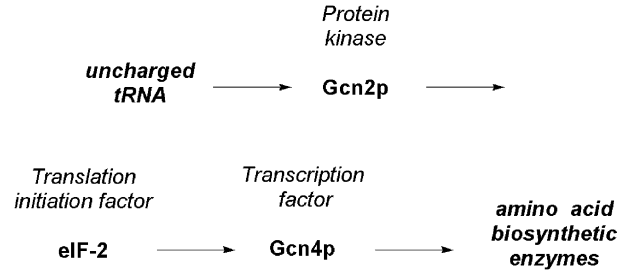
Scheme 1. Arginine biosynthetic pathway. The genes involved in each step are shown above the arrows. The numbers following the genes correspond to the measured expression ratio.

To address the question of transcriptional activation of amino acid biosynthetic enzymes resulting from treatment with borrelidin, we investigated the possibility of using traditional yeast genetics. Transcriptional regulation of amino acid biosynthetic genes is accomplished by the transcription factor of Gcn4p during amino acid starvation conditions.[19] If the pathway through which borrelidin induces the transcription of amino acid biosynthetic proteins involves Gcn4p, then the genetic deletion of this transcription factor should abrogate the observed transcriptional effects of treatment with borrelidin. A strain lacking the *GCN4* transcription factor (*gcn4*Δ) was treated with 100 μM borrelidin and the relative mRNA transcript ratios were determined using microarray analysis in order to test this hypothesis. We anticipated that the deletion strain treated with borrelidin would no longer show an increase in the mRNA abundance of the amino acid biosynthetic genes, if borrelidin was targeting the *GCN4* pathway.

Comparison of the transcription profiles obtained from the *gcn4*Δ strain treated with borrelidin with the wild-type treated with borrelidin showed significant differences. Although similar levels of transcriptional changes

were observed from treatment of the gcn4 strain, several of the genes that had increased mRNA transcript ratios upon treatment of the wild-type strain with borrelidin no longer showed increased regulation. Furthermore, many of the amino acid biosynthetic genes as well as several other Gcn4p targets,[19] including *BIO3* and *RIB5*, which are involved in the metabolism of vitamins and cofactors, and *GLN3*, a transcription factor in nitrogen metabolism, no longer displayed increased transcript ratios (Fig. 2) as determined by microarray analysis.

The observed results provide evidence that borrelidin is affecting amino acid concentrations inside cells. The *GCN4* general amino acid control pathway (Scheme 2) is responsive under amino acid starvation conditions. During amino acid starvation, the accumulation of uncharged tRNA stimulates Gcn2p protein kinase activity. The Gcn2p is a protein kinase that phosphorylates the eukaryotic translation initiation factor 2 (eIF-2), which ultimately results in increased translation of Gcn4p.[20] Taken together, these results indicate that in yeast borrelidin has the effect of reducing charged tRNA, possibly through inhibition of threonyl tRNA synthetase.



Scheme 2. Regulation of amino acid biosynthetic genes. Uncharged tRNA stimulates Gcn2p kinase activity which regulates the translation of Gcn4p through eIF-2. The translation of Gcn4p results in the transcription of 70 genes involved in amino acid biosynthesis.

In summary, we have used global transcription profiling to provide insight into the effects of the angiogenesis inhibitor borrelidin on the eukaryotic model organism *S. cerevisiae*. The most notable result of treatment with borrelidin is the induction of amino acid biosynthetic enzymes. We have ascertained that induction of this pathway involves the *GCN4* transcription factor. This conclusion was determined by treating a yeast strain lacking this gene and observing the absence of increased gene transcription under Gcn4p control. These results provide evidence of the primary cellular effects of borrelidin, and further investigation using the model organism *S. cerevisiae* and mammalian cells is ongoing.[21]

Acknowledgements

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