# The Protein Synthesis Inhibitors Mycalamides A and E have Limited Susceptibility Toward the Drug Efflux Network

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**ABSTRACT: The mycalamides belong to a family of protein synthesis inhibitors noted for antifungal, antitumour, antiviral, immunosuppressive, and nematocidal activities. Here we report a systematic analysis of the role of drug efflux pumps in mycalamide resistance and the first isolation of mycalamide E. In human cell lines, neither P-glycoprotein overexpression nor the use of efflux pump inhibitors significantly modulated mycalamide A toxicity in the systems tested. In** *Saccharomyces cerevisiae*, **it appears that mycalamide A is subject to efflux by the principle mediator of xenobiotic efflux, Pdr5p along with the major facilitator superfamily pump Tpo1p. Mycalamide E showed a similar efflux profile. These results suggest that future drugs based on the mycalamides are likely to be valuable in situations where efflux pump–based resistance leads to failure of other chemotherapeutic approaches, although efflux may be a mediator of resistance in antifungal applications.** © 2012 Wiley Periodicals, Inc. J Biochem Mol Toxicol 26:94–100, 2012; View this article online at wileyonlinelibrary.com. DOI 10:1002/jbt.20414

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## INTRODUCTION

The mycalamides are isolated from the sponge *Mycale hentscheli* collected from New Zealand coastal waters [1] and belong to the pederin group of compounds that includes the mycalamides, onnamides, pederin, and theopederins (Figure 1). It has long been suspected that the mycalamides function through the inhibition of protein synthesis [2–4], leading to antiviral [1] cytotoxic [5,6] and immunosuppressive [7] activities. In addition to these activities, other members of the pederin group have shown antifungal and nematocidal activities [8,9]. Mycalamide A has now been shown to bind to the E site of the large ribosomal subunit in *Haloarcula marismortui* [10].

Despite a broad range of activities and high levels of potency, compounds from the pedrin family have not been extensively developed for therapeutic purposes. However, recent advances in their syntheses [11–14] and a growing literature into structure activity relationships within this family [15,16] provide a framework for future developments. Furthermore, new insights into the biosynthesis of these interesting metabolites open the possibility of their production in large scale through biotechnological approaches [17,18] as well as the development of new analogs through biosynthesis or semisynthesis [19].

We have undertaken an extensive study of the interaction of the mycalamides with drug efflux systems as this is an important consideration for potential therapeutics. Multidrug resistance (MDR) is a phenomenon of major concern and is clinically significant in the treatment of cancer and fungal infections [20]. The ATP-binding cassette (ABC) proteins are the most important clinical mediators of MDR, along with proteins belonging to the major facilitator superfamily (MFS). The contribution of individual efflux pumps to resistance to a particular drug can be identified through the application of well-characterized pump inhibitors or through deletion or disruption of the genes for the efflux pumps or their transcriptional regulators [21,22].

In addition to providing a model organism for the antifungal activity of mycalamide A, the yeast

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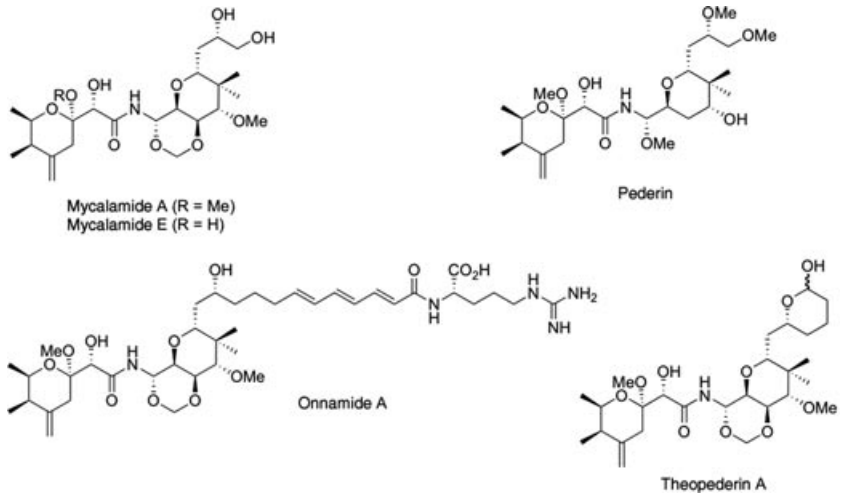


**FIGURE 1.** Structures of mycalamides A and E and related compounds.

*Saccharomyces cerevisiae* is an ideal organism to study drug efflux, having a fully sequenced genome, available gene deletion mutants and well-characterized efflux-related genes, making up a system that in yeast is called the pleiotropic drug resistance (PDR) network. In this regard, yeast possesses several orthologs and functional homologs of the genes associated with the MDR phenotype in humans. It has also been shown recently that MDR/PDR proteins influence membrane composition [23], which also impacts on drug sensitivity due to altered transport across cellular membranes.

In this particular study, we aimed to understand how mycalamide A interacts with MDR/PDR networks and to compare the impact of these interactions with that of disruption of membrane structure on mycalamide A toxicity. To achieve this, mycalamide A toxicity was tested in yeast strains harboring deletions of representative ABC and MFS transporters, PDR transcription factors, and genes involved in ergosterol biosynthesis. We have also undertaken the first isolation of mycalamide E from its natural source and performed an assessment of its interaction with selected components of the MDR/PDR system. A preliminary study on the role of drug efflux in mycalamide A toxicity in human cell lines is also reported.

## MATERIALS AND METHODS

### Materials

All reagents were purchased from Sigma-Aldrich Ltd. (MO, USA) or (Missouri, USA) except fetal bovine serum (FBS), glutamine, and penicillin-streptomycin solution, which were purchased from Invitrogen (CA, USA) or (California, USA), and yeast extract and Bacto Peptone were purchased from Becton, Dickinson and Company (NJ, USA) or (New Jersey, USA).

### Yeast Strains

The *S. cerevisiae* strains used in this study were based on the BY4742 background (*MATa/α his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0*). Strains tested for mycalamide A sensitivity included individual deletions of the following genes: *AQR1, AZR1, DTR1, ERG2, ERG3, ERG6, FLR1, HIS3, PDR1, PDR3, PDR5, PDR8, PDR10, PDR11, PDR12, SSZ1, PDR15, PDR18, QDR1, QDR2, QDR3, RDR1, SNQ2, STB5, TPO, TPO4, YOR1,* and *YRR1*. The *pdr5Δ* strain was a gift of Professor Charlie Boone, University of Toronto, all other strains were obtained from the Open Biosystems deletion strain library. The *pdr1Δpdr3Δ* strain (*MATα pdr1Δ::nat pdr3Δ::URA3*) in Y8205 background [24] was prepared by using standard methods.

### Isolation of Mycalamides A and E

Methanolic extracts of wild *Mycale hentscheli* (total 10.2 kg) collected between 2002 and 2003 from Pelorus Sound, New Zealand, were loaded onto a poly(styrene-divinylbenzene) (PSDVB) column and fractionated using acetone/water stepped gradients. The 40% acetone/water fractions were combined together and fractionated further on PSDVB (20–60% acetone/water). The 50–54% acetone/water fractions were concentrated and purified using DIOL (0–100% acetonitrile/dichloromethane). Fractions containing both mycalamides A and E were finally

purified using HPLC (C18, 4.6 × 250 mm, 32% acetonitrile/water, 1 mL/min, 210 nm detection) to afford mycalamide E ($R_T$ 9 min, 5.1 mg) and mycalamide A ($R_T$ 10 min, 25.7 mg). Mycalamide A was isolated as a colorless oil; all other data as previously described [1]. Mycalamide E was also isolated as a colorless oil; $[\alpha]_D^{25} + 94^\circ$ (*c* 0.3, $CHCl_3$); high-resolution electrospray ionisation mass spectrometry (HRESIMS) $[M + Na]^+$ observed *m/z* 512.2469, calculated 512.2466 for $C_{23}H_{39}NO_{10}Na$, $\Delta = 0.5$ ppm. The structure of mycalamide E was revealed from the analysis of one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) data and by comparison with the parent compound.

### Yeast Growth Conditions and Mycalamide Sensitivity Assays

Strains were cultured in 96-well plates containing standard liquid yeast extract peptone dextrose media (YPD) (1% (w/v) yeast extract, 2% Bacto Peptone, 2% glucose). Saturated cultures of all strains were then serially diluted 4.75-fold into fresh liquid YPD medium, and 3 μL aliquots arrayed onto YPD agar plates containing mycalamide A or E. Mycalamide E sensitivity assays were conducted only at 125 μM against *pdr1*Δ, *pdr3*Δ, *pdr1*Δ*pdr3*Δ, *pdr10*Δ, *tpo1*Δ, *tpo4*Δ, *ssz1*Δ, *erg2*Δ, and *his3*Δ strains because of limited supply of the natural product. Plates were prepared by adding the mycalamide from frozen stock solutions at 20 mM in dimethyl sulfoxide (DMSO), to the molten agar at 50°C. A control spot dilution assay was conducted onto plates containing 0.6% DMSO. Plates were grown until spots on the control plates reached an average size of 4–5 mm. Strains were scored for sensitivity as follows: Strains in which all dilution spots failed to grow were scored highly sensitive, and those that grew equivalently to the control strain harboring the functionally neutral *his3* deletion were scored as insensitive. The remaining strains were all noted to grow to within one or two spots of the control and were classified as slightly sensitive.

### Cell Culture

Cell lines HL60, HepG2, A2780, and A2780AD were cultured at 37°C in a 5% $CO_2$ atmosphere. HEPG2 and HL60 cells were cultured in RPMI 1640 with glutamine and supplemented with 10% FBS and 1% penicillin–streptomycin (complete medium). HL60 cell density was maintained between $10^5$ and $10^6$ viable cells/mL. A2780 and A2780AD cells were cultured in complete RPMI 1640 supplemented with 2% insulin. Adherent cells were passaged at 70% confluence.

### MTT Cell Proliferation Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) and the solubilizer were prepared according to the method of Hood et al. [6]. An HL60 cell suspension ($1 \times 10^6$ cells/mL) was added to twofold dilutions of mycalamide A in RPMI 1640 in sterile 96-well plates, giving final mycalamide A concentrations ranging from 22.4 μM to 0.17 nM. The volume was brought up to 100 μL with RPMI 1640. HEPG2 cells ($2 \times 10^5$cells/mL), A2780 cells ($4 \times 10^4$ cells/mL) and A2780AD cells ($1 \times 10^5$ cells/mL) were incubated overnight in sterile 96-well plates, the medium was then changed, and mycalamide A was added in fourfold dilutions, from 50 μM to 0.19 nM. Cells were grown with mycalamide A in the presence or absence of verapamil (10 μM). Cells were incubated for either 24 or 48 h at 37 °C. After the incubation, MTT (20 μL per well) was added and allowed to react for 2 h at 37 °C, solubilizer (200 μL per well) was added and the plates incubated overnight at 37 °C. The absorbance was read in a microplate reader (Versa Max, Molecular Devices) at 570 nm.

## RESULTS

### Isolation of Mycalamide E

NMR-guided fractionation of extracts of wild *Mycale hentscheli* using normal- and reversed-phase chromatography resulted in the isolation of mycalamide A and a new congener, mycalamide E. High-resolution electrospray mass spectrometry established the molecular formula of mycalamide E as $C_{23}H_{39}NO_{10}$ and signified the loss of one methyl group from mycalamide A. The structure of mycalamide E was revealed from the analysis of 1D- and 2D-NMR data and by comparison with the parent compound. Close comparison of the NMR data of the two mycalamides indicated that the compounds possessed the same carbon skeleton. The absence of an oxymethyl resonance, coupled with the presence of two sets of resonances from an epimeric centre, indicated that the pyran methoxy-acetal of mycalamide A had been replaced with a hemiacetal that is capable of existing in two forms in solution ($CDCl_3$). While mycalamide E was isolated from our sponge repository, it has been reported previously as the synthetic derivative pseudomycalamide A (6-des-*O*-methylmycalamide A) formed from the acid-catalyzed hydrolysis of mycalamide A [25]. The reported $^1H$ and $^{13}C$ NMR data for this compound are identical with that of naturally occurring mycalamide E. The current study is the first report of the compound from a natural source.

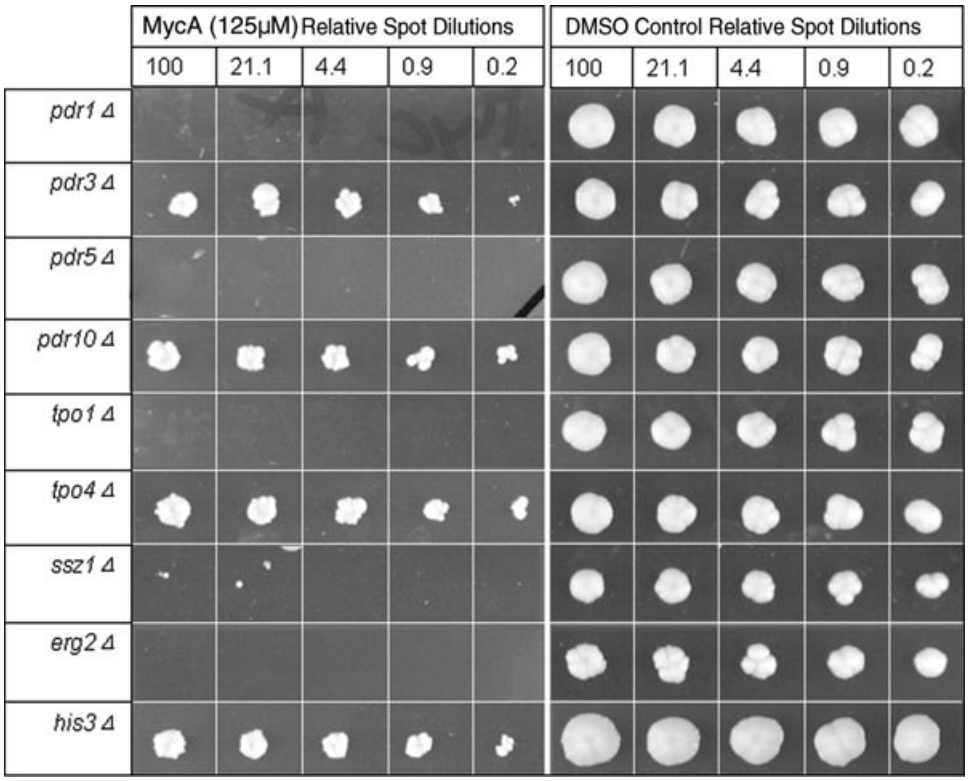


**FIGURE 2.** Representative spot dilution assays. Deletion mutant strains were spotted in serial dilutions to relative cell densities as shown, onto plates treated with mycalamide A at 125 μM or DMSO control.

### Effect of Gene Deletions on the Sensitivity of *S. cerevisiae* to Mycalamides A and E

The effects of mycalamide A treatment on deletion strains of *S. cerevisiae* are summarized in Table 1, with representative spot dilution assay results shown in Figure 2. A number of strains showed an initial growth defect with reduced growth evident at early time points, but recovered to growth levels equivalent to control by the completion of the assay. All ergosterol biosynthesis gene deletion strains tested exhibited high levels of sensitivity. Of the eight strains tested that lacked an ABC efflux pump, only the *pdr5*Δ demonstrated a high level of sensitivity, although a number of deletions caused slight sensitivity at high mycalamide A concentrations. Of the nine MFS transporter deletion strains tested, only deletion of *TPO1* led to markedly increased sensitivity and only for mycalamide A. Of the strains bearing deletion of transcription factors or gene regulators associated with the PDR network, strains bearing deletions of *PDR1* or *SSZ1* and the *pdr1*Δ*pdr3*Δ dual deletion mutant were all highly sensitive to mycalamide A, although the sensitivity of the *pdr1*Δ*pdr3*Δ strain is equivalent to that engendered by the single deletion of *PDR1*.

The sensitivity profile was unchanged at a higher mycalamide A concentration (150 μM), albeit with a higher level of sensitivity exhibited by the previously insensitive strains. At a low mycalamide A concentration (50 μM), of the strains tested only the *erg2* deletion strain remained highly sensitive.

Results for the strains tested with mycalamide E were largely equivalent to those from mycalamide A.

**TABLE 1.** Sensitivity of Yeast Deletion Strains at 125 μM Mycalamide A

| *Highly Sensitive* | *Slightly Sensitive* | *Insensitive* |
|---|---|---|
| *erg2*Δ, *erg3*Δ, *erg6*Δ, *pdr1*Δ, *pdr1*Δ*pdr3*Δ, *pdr5*Δ, *ssz1*Δ, *tpo1*Δ | *azr1*Δ, *dtr1*Δ, *qdr1*Δ, *qdr2*Δ* | *aqr1*Δ,* *flr1*Δ,* *pdr3*Δ, *pdr8*Δ,** *pdr10*Δ,* *pdr11*Δ,** *pdr12*Δ,* *pdr15*Δ,* *pdr18*Δ,* *qdr3*Δ,* *rdr1*Δ,* *snq2*Δ,* *stb5*Δ,* *tpo4*Δ, *yor1*Δ,* *yrr1*Δ* |

Strains were scored relative to the functionally neutral *his3* deletion strain; those exhibiting an initial growth defect at are marked *(slight defect) or **(greater defect). Sensitive strains were confirmed in triplicate and were retained over two different batches of mycalamide A.

Notably, in contrast to the situation with mycalamide A, the deletion of *PDR1* and *TPO4* did not lead to sensitivity and the *SSZ1* deletion strain was only modestly more sensitivity than control.

### Effect of Mycalamide A on Human Cell Lines

The inhibitory effect of mycalamide A on four different cell lines was assessed using the MTT assay. HepG2 cells were significantly more sensitive to mycalamide A than were HL60, A2780, and A2780AD cells. The effect of treatment with the drug efflux inhibitor verapamil was tested, and only the combination of A2780AD and verapamil showed a statistical significant difference from controls. The results are presented in Table 2.

## DISCUSSION

In yeast, the *PDR1/PDR3* system is considered to be the master regulator of PDR [26–28]. As *PDR1* and *PDR3* have overlapping functions in the regulation of much of the core PDR system, at least at steady state [28], the dual deletion mutant *pdr1*Δ*pdr3*Δ is generally more sensitive to drugs than the individual deletion strains. However, in this case, *pdr1*Δ*pdr3*Δ showed no higher sensitivity than the *pdr1*Δ strain for mycalamide A, potentially because mycalamide A efflux by Pdr5p alone is sufficient to generate resistance. This is significant as Pdr5p is the major mediator of active drug efflux in yeast [29] and is considered the functional homolog of the important mammalian P-glycoprotein (P-gp) efflux pump [30–32]. Unfortunately, during confirmation of results, it became apparent that there was a problem with our *pdr5*Δ library strain. Although we were able to test a validated alternative *pdr5*Δ strain with mycalamide A, limited stock prevented us from reassessing mycalamide E.

**TABLE 2.** MTT Assay $EC_{50}$ Values Obtained after 48 h incubation with mycalamide A (mycA) with and without verapamil

| | $EC_{50}$ (nM ± SE) | |
|---|---|---|
| *Cell Line* | *mycA* | *mycA + verapamil* |
| HL60 | 4.49 ± 0.6 nM | 1.04 ± 0.4 nM |
| HepG2 | 0.77 ± 0.36 nM | 0.24 ± 0.10 nM |
| A2780 | 1.06 ± 0.3 nM | 0.88 ± 0.3 nM |
| $A2780^{AD}$ | 18.90 ± 2.2 nM | 3.07 ± 0.6 nM |

Results are from three independent experiments, each performed in triplicate. The only significant difference from verapamil treatment was observed with $A2780^{AD}$ cells ($p = 0.0059$, paired $t$-test).

Other than Pdr5p, of the transporters tested, only deletion of *TPO1* led to a marked increase in sensitivity. Tpo1p is an MFS drug:proton antiporter with a broad substrate profile, which is known to be regulated by Pdr1p [21]. Although steady-state *TPO1* expression is not solely dependent on Pdr1p, its promoter is directly bound by Pdr1p facilitating its involvement in ePDR [28]. This may explain why for mycalamide E neither *pdr1* nor *tpo1* deletion mutants showed increased sensitivity. The lack of *pdr1* deletion strain sensitivity may also imply a lower susceptibility of mycalamide E to Pdr5p-mediated export.

Sensitivity engendered by deletion of *SSZ1* may arise through its regulation of Pdr1p [33] or from the loss of the Ssz1p chaperone function exacerbating disruption of protein synthesis by the mycalamides. This latter option is partially supported by the observation that *SSZ1* deletion is modestly sensitive to mycalamide E, despite the *PDR1* deletion having no effect on this compound.

While yeast and humans share a number of related ABC proteins, the major mediators of drug efflux in yeast and humans are not closely related in structural terms [30]; therefore, we assessed whether the involvement of the major ABC efflux pump in yeast was mirrored in human cell lines. Notably, P-gp overexpressing A2780AD cells were only 10-fold more resistant to mycalamide A than the parental A2780 cell line, and this was only reduced approximately fivefold by the P-gp inhibitor verapamil. In comparison, A2780AD cells have a resistance ratio for paclitaxel, that is, in excess of 1400-fold and greater than 450-fold reduction in resistance is achieved by verapamil [34]. A similar outcome was observed for *Caenorhabditis elegans* (data not shown), where no significant increase in sensitivity to mycalamide A or change in its apparent distribution was achieved by treatment with either verapamil or the MRP inhibitor probenecid.

In conclusion, although the mycalamides appear to substrates for the major efflux pump in yeast, Pdr5p, this is not mirrored in human cell line experiments, which demonstrate that mycalamide A is not a substrate for the major drug efflux system. This establishes the mycalamides, and analogously other members of the pederin family, as targets for development of therapeutic agents in areas where drug resistance is known to be problematic in higher eukaryotes, but suggests that they may have more limited application as antifungal agents.

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