DORRIGOCINS: NOVEL ANTIFUNGAL ANTIBIOTICS THAT CHANGE THE MORPHOLOGY OF *ras*-TRANSFORMED NIH/3T3 CELLS TO THAT OF NORMAL CELLS

III. BIOLOGICAL PROPERTIES AND MECHANISM OF ACTION

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The dorrigocins are unique glutarimide antibiotics which were found to reverse the morphology of *ras*-transformed NIH/3T3 cells from a transformed phenotype to a normal one. The compounds also inhibited the release of yeast mating pheromone, a-factor. The activity of these compounds was not dependent on inhibition of prenylation or protein synthesis. Dorrigocin A was instead found to inhibit the carboxyl methylation in *K-ras* transformed cells.

The dorrigocins are novel glutarimide antifungal antibiotics discovered in the fermentation broth and mycelium of *Streptomyces platensis* subsp. *rosaceus* strain AB 1981F-75. The isolation and structural elucidation of these compounds are described in an accompanying publication[1]. Culture and fermentation properties are discussed in another accompanying paper[2]. This paper describes the biological properties and the possible mode of action of these antibiotics.

Ras-related proteins control a wide variety of cellular processes[3]. The biological role of these proteins is affected by their ability to associate with the cellular membrane. Membrane localization occurs when the C-terminus of these proteins undergoes a series of post-translational modifications[3]. The C-terminal end is modified by prenylation and proteolysis which are prerequisites to carboxyl methylation of the terminal cysteine residue. Genetic studies in yeast[4] and *in vitro* analysis of $p21^{K\text{-}ras}$ [5] indicate that carboxyl methylation augments membrane association of processed proteins. The a-factor of *Saccharomyces cerevisiae* for example, is also modified by carboxyl methylation, targeted to the membrane and eventually released into the medium where it interacts with its receptor to initiate sexual mating[4]. Both prenylation and proteolysis are essential for Ras function because they affect Ras attachment to inner surface of the plasma membrane. The role of carboxyl methylation is still unclear. However, the importance of fully processed Ras-related proteins in cell signaling suggests that carboxyl methylation may affect signal transduction[3]. In this paper, we describe the first natural product inhibitor of the carboxyl methyltransferase involved in Ras processing and show that inhibition of the enzyme can affect signal transduction in eukaryotic cells.

Materials and Methods

Cell Culture and Morphology

Normal and *K-ras* transformed NIH/3T3 mouse fibroblasts were grown in DULBECCO's minimal essential medium containing 10% fetal bovine serum and gentamicin (50 μg/ml). Transformed cells also contained G-418 (500 μg/ml) to maintain the *K-ras* containing plasmid. 6-Well Costar tissue culture plates

were inoculated with 1×10^4 cells/ml/well and incubated at 37°C in 5% CO_2 for 4 days. Test compounds were added after 24 hours of growth and the morphology of normal and transformed cells was monitored microscopically starting at 72 hours after addition of the test compounds. Transformed cell foci were counted and averaged from 6 separate fields under 50× magnification from duplicate wells.

Mating Factor Secretion Assay

The secretion of a-factor was determined as previously described[6]. Briefly wild type yeast grown in yeast potato dextrose broth (YPD), at 23°C for 2 hours were pelleted and the supernatant containing the mating factor was spotted on a lawn of test strain (*MATa sst2-4*) on YPD agar plates. The presence of a-factor triggered a sexual reproduction stage that slows down growth (growth arrest) which appears as a clear to hazy zone in the spotting area. Dorrigocins were added at a concentration of 20 μg/ml, to YPD broth, to monitor a-factor release.

Farnesyl Protein Transferase and Protein Synthesis Assays

Bovine and Yeast farnesly protein transferases (FPTase) were prepared according to SCHABER *et al.*[7]. The FPTase activity was measured with a Lamin B substrate (supplied by Amersham Inc. as part of a scintillation proximity based assay kit) according to the manufacturers instructions. Protein synthesis was estimated by measuring the incorporation of labeled [^{3}H]leucine by murine leukemia P388 cells in RPMI-1640 containing 10% fetal bovine serum in the presence of dorrigocin A or cycloheximide at various concentrations. Samples were withdrawn after 3 hours, precipitated with 10% trifluoroacetic acid, washed and the radioactivity in the precipitate was determined by liquid scintillation counting.

Carboxyl Methylation of Ras-related Proteins

Metabolic labeling of Ras-related proteins was performed as previously described[8]. Transformed NIH/3T3 cells grown in DULBECCO's modified EAGLE's medium containing G-418 and various test compounds were labeled with [^{3}H-*methyl*]methionine (350~500 μCi/ml) for 1 hour in the presence or absence of compound and harvested without trypsin. The cell pellet was quickly separated by centrifugation, lysed in electrophoresis sample buffer and the labeled proteins were analyzed by SDS-PAGE followed by autoradiography.

Results

Biological Activity

Effect of Dorrigocins on the Morphology of *K-ras* Transformed Cells

The anticancer potential of the dorrigocins A and B was determined using *K-ras* transformed NIH/3T3 mouse fibroblasts. Transformed cells were typically rounded when the mutant *ras* gene was expressed. Normal cells on the other hand were flat and adherent to the bottom of a tissue culture plate. Various concentrations of dorrigocins A and B, dissolved in DMSO, were added when the cells had uniformly adhered to the bottom of the microtiter tray and the plates were incubated at 37°C for an additional 48

Fig. 1. Morphology of *K-ras* transformed NIH/3T3 cells.

Cells cultured without dorrigocins (A). Cells cultured with dorrigocin A at 0.4 μg/ml (B). Cells cultured with dorrigocin B at 10 μg/ml (C).

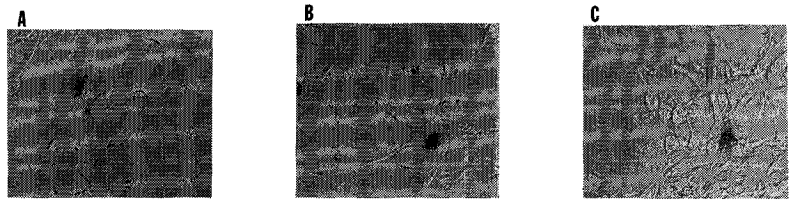
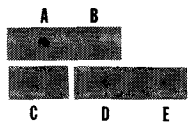


Table 1. Effect of dorrigocins A and B on the number of foci produced by *K-ras* transformed NIH/3T3 mouse fibroblasts.

Cell type	Compound (μg/ml)	Number of transformed foci (%)
NIH/3T3	None	0
NIH/3T3 *K-ras*	None	100
NIH/3T3 *K-ras*	Dorrigocin A	
	10	22
	0.4	26
	0.08	76
	Dorrigocin B	
	10	72
	0.4	96

Fig. 2. Secretion of a-factor by *Saccharomyces cerevisiae*.

(A) Complete growth arrest by a-factor released in the absence of dorrigocins. (B) Complete inhibition of growth arrest by dorrigocin A at 10 μg/ml. (C) Partial inhibition of growth arrest by dorrigocin A at 0.4 μg/ml. (D) Partial inhibition of growth arrest by dorrigocin B at 0.4 μg/ml. (E) Partial inhibition of growth arrest by dorrigocin B at 10 μg/ml.



hours. Cell foci represent transformed cells that were unaffected by the addition of compound. Transformed and reverted cells after the addition of DMSO, dorrigocins A and B are shown in Fig. 1A, B and C, respectively. Control experiments with DMSO showed no reduction in the number of transformed foci over time. The addition of dorrigocins A and B caused transformed cells to revert to a morphology resembling that of normal cells resulting in a reduction in the number of foci. This effect was dependent on the presence of the compound. Dorrigocin A was more effective than dorrigocin B in causing morphology reversion and the corresponding reduction in the number of foci. The average number of transformed foci per field under 50× magnification is shown in Table 1. At 10 μg/ml and 0.4 μg/ml dorrigocin A caused 78% and 74% reduction in transformed foci, respectively. Dorrigocin B produced a 28% reduction in transformed foci at 10 μg/ml and caused no significant effect at 0.4 μg/ml.

Inhibition of a-Factor Secretion

The addition of dorrigocins A and B to a-factor producing cells inhibited its release into the medium and produced a less pronounced zone of growth arrest as shown in Fig. 2. The addition of dorrigocin A at 10 μg/ml resulted incomplete inhibition of growth arrest when the supernate from treated cells was tested on *MATa* cells producing the a-factor receptor (Fig. 2B). Similar tests with dorrigocin B treated cells at 10 μg/ml resulted in some release as shown by evidence of a faint zone of growth arrest (Fig. 2E). At 0.4 μg/ml dorrigocin A was again more effective in blocking a-factor release than dorrigocin B as seen by the difference in the clarity of growth arrest zones (Figs. 2C and D). a-Factor released in the absence of any dorrigocin produced complete growth arrest and clear zones as shown in Fig. 2A.

Effect of Dorrigocins on FPTase and Cellular Protein Synthesis

The dorrigocins at 10 μg/ml had no significant effect on either bovine or *S. cerevisiae* FPTase activity, the activity of both enzymes was inhibited 7~10% (data not shown). When compared with cycloheximide, dorrigocin A produced insignificant inhibition of protein synthesis at concentrations below 10 μg/ml as shown in Fig. 3.

Carboxyl Methylation of Ras-related Proteins

Metabolic labeling of transformed NIH/3T3 cells with [^{3}H-*methyl*]methione, the precursor of the

Fig. 3. Effect of dorrigocin A (□) and cycloheximide (○) on *in vivo* protein synthesis.

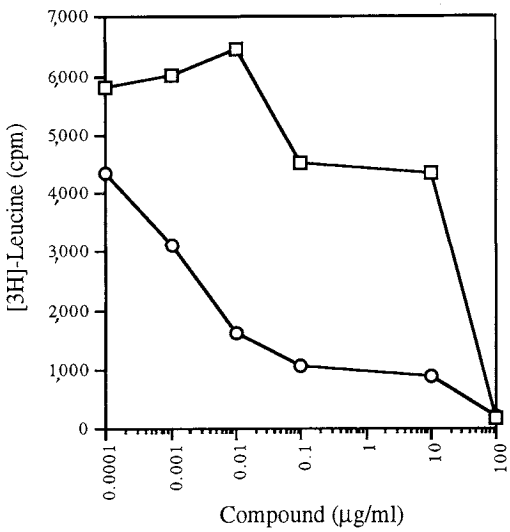
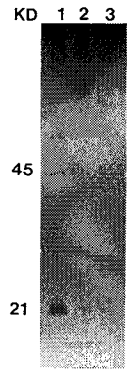


Fig. 4. Effect of dorrigocins on the metabolic labeling of *ras*-transformed NIH/3T3 carboxyl methylated, Ras-related proteins.

Lane 1, methylation on the absence of dorrigocins. Lane 2, dorrigocin A. Lane 3, dorrigocin B.



methyl donor *S*-adenosyl-L-methionine (AdoMet) followed by SDS-polyacrylamide gel electrophoresis and autoradiography showed that methylation was mainly in the 20 ~ 24-kD region consistent with the molecular mass of $p21^{ras}$ and its homologs (Fig. 4). Unlike untreated cells, exposure to dorrigocins A and B at 10 μg/ml inhibited carboxyl methylation in the 21-kD.

Discussion

Ras oncoproteins have been implicated in the pathogenesis of many types of cancers. Among colorectal and pancreatic tumors studied, 50% and 90%, respectively have *ras* oncogenes. Since membrane association is critical for the oncogenic properties of Ras proteins, several agents that block C-terminal modification and affect Ras function have been described. These include, peptides and peptidomimetics that block prenylation[9,10] and farnesylcysteine analogs that inhibit the carboxyl methylation of Ras-related proteins[11]. Among the natural products reported, limonene[12], manumycin[13], UCF1-A, B, C[14], 10′-desmethoxystreptonigrin[15], gliotoxin[16], chaetomeillic acids[17] and pepticinnamins[18] all inhibit farnesly protein transferase but are either non selective, have poor cell penetration or substantial *in vivo* toxicity. Another group of compounds including reductoleptomycin A[19], S-632-B1, B2 and C[20] and epiderstatin[21] are reported to reverse the morphology of transformed cells to that of normal cells. Of these, epiderstatin was shown to block cell cycle progression at G_0/G_1, but the mode of action of reductoleptomycin A and S-632-B1, B2 and C was not determined. The dorrigocins described here are related to the glutarimide antibiotics, epiderstatin and S-632-B1 and B2, and like these compounds also reverse the morphology of transformed cells. In addition, the dorrigocins also affect a-factor function. Since a-factor is C-terminally modified by prenylation, proteolysis and carboxyl methylation in a manner similar to the Ras-related proteins, we determined the effect of dorrigocins on bovine and *S. cerevisiae* FPTase and found that there was no significant inhibitory effect on either FPTase. Furthermore, the reversion did not appear to be due to inhibition of protein synthesis when compared with cycloheximide, a glutarimide inhibitor of eukaryotic protein synthesis. Since neither reductoleptomycin A nor S-632-C are glutarimide antibiotics and still show morphology reversion of *ras* transformed cells, the phenotypic reversion produced by the compounds described here is not likely to be dependent on the glutarimide moiety. Dorrigocin treated cells however, had an immediate effect on carboxyl methylation of proteins in the 21-kD and 45-kD region. The carboxyl methylation in the 21-kD region where $p21^{ras}$ migrates was completely inhibited by dorrigocins A and B,

while methylation in the 45-kD region was slightly reduced. The ultimate utility of the dorrigocins will depend upon their selectivity for carboxyl methyltransferase(s) involved in the processing of Ras-related proteins *versus* other methyltransferases. The availability of inhibitors should provide a valuable tool for further studies toward understanding the role of carboxyl methylation in the processing of Ras-related proteins and cellular signal transduction.

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