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The Secondary Metabolite Euplotin C Induces Apoptosis-Like Death in the Marine Ciliated Protist *Euplotes vannus*

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ABSTRACT. The sesquiterpenoid euplotin C is a secondary metabolite produced by the ciliated protist *Euplotes crassus* and provides a mechanism for damping populations of potential competitors. Indeed, *E. crassus* is virtually resistant to its own product while different non-producer species representing an unbiased sample of the marine, interstitial, ciliate diversity are sensitive. For instance, euplotin C exerts a marked disruption of different homeostatic mechanisms in *Euplotes vannus*. We demonstrate by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay that euplotin C quickly decreases viability and mitochondrial function of *E. vannus* with a very high efficacy and at micromolar potency. In addition, euplotin C induces apoptosis in *E. vannus* as 4,6-diamino-2-phenylindole and terminal transferase dUTP nick end labeling staining show the rapid condensation and fragmentation of nuclear material in cells treated with euplotin C. These effects occur without detectable permeabilisation or rupture of cell membranes and with no major changes in the overall morphology, although some traits, such as vacuolisation and disorganized microtubules, can be observed by transmission electron microscopy. In particular, *E. vannus* show profound changes of the mitochondrial ultrastructure. Finally, we also show that caspase activity in *E. vannus* is increased by euplotin C. These data elucidate the pro-apoptotic role of euplotin C and suggest a mechanism for its impact on natural selection.

Key Words. Caspases, ciliates, mitochondria, nuclear morphology, programmed cell death, ultrastructure.

PROGRAMMED cell death (PCD) and apoptosis, its most frequent phenotype, are the principal mechanism by which cells are physiologically eliminated (Edinger and Thompson 2004). There is a general agreement that apoptosis plays an important role in individual development and the maintenance of tissue homeostasis in multicellular organisms, such as invertebrate and vertebrate animals and plants, but other studies have indicated that PCD can also occur in single-celled eukaryotes and prokaryotes (Ameisen 1996; Gordeeva, Labas, and Zvyagilskaya 2004; Lewis 2000). A PCD mechanism, with a phenotype resembling apoptosis, has been described in different protists: *Blastocystis* (Nasirudeen et al. 2004), *Dictyostelium* (Tatischeff et al. 2001), *Leishmania* (Das, Mukherjee, and Shaha 2001), *Plasmodium* (Al-Olayan, Williams, and Hurd 2002), *Peridinium* (Vardi et al. 1999), *Tetrahymena* (Kobayashi and Endoh 2005), *Tritrichomonas* (Mariante et al. 2003), and *Trypanosoma* (Duszenko et al. 2006). In addition, in conjugating ciliated protists most of the DNA is eliminated after the sexual pathway takes place through a process which resembles nuclear apoptosis (Gordeeva et al. 2004; Jahn and Klobutcher 2002; Santos, Lu, and Wolfe 2000).

The sesquiterpenoid euplotin C is a lipophilic secondary metabolite uniquely produced by the strains characterizing the cosmopolitan, marine, interstitial, ciliate morphospecies *Euplotes crassus* (Dini et al. 1993; Guella et al. 2004, 1994, 1996). The compound is not secreted into the medium, but its effect is mediated by cell–cell contacts (Dini et al. 1993). In particular, it has cytotoxic effects in non-producer *Euplotes* strains by altering the cell cycle, ciliary motility, and cell shape (Dini et al. 1993; Guella et al. 1994, 1996; Savoia et al. 2004). These findings suggest that euplotin C may play an ecological role, representing an adjuvant factor of the multicomponent strategy pursued by *E. crassus* in broadening its niche size. Among non-producer *Euplotes* morphospecies, *Euplotes vannus* is a representative of a sample of marine, interstitial ciliate diversity that is sensitive to euplotin C. We have recently demonstrated that euplotin C modulates the cation transport system in *E. vannus*: it increases the intracellular concentration of Ca^{2+} and Na^{+} and its membrane electrical properties without affecting the generation of reactive oxygen species (Trielli et al. 2008). These events parallel the disassembling of the ciliary structures, the inhibition of cell motility, the occurrence of aberrant cytoplasmic vacuoles, and the rapid inhibition of phagocytic activity. Euplotin C also increases lysosomal pH and decreases lysosomal membrane stability of *E. vannus* (Trielli et al. 2008). Nevertheless, the cytotoxic properties of euplotin C in *E. vannus* have not been clarified and possible involvement of euplotin C-induced PCD is currently unknown in *E. vannus* in particular and in protists, in general. Signaling mechanisms underlying pro-apoptotic effects of euplotin C have been elucidated in recent studies using neuroendocrine tumor cells of mammals (Cervia et al. 2006, 2007). Briefly, euplotin C has been shown to induce a rapid depletion of endoplasmic reticulum Ca^{2+} stores and, concomitantly, the generation of reactive oxygen species, which sustains, at least in part, the intracellular Ca^{2+} overload. The generation of reactive oxygen species and Ca^{2+} increase participate in apoptotic processes and are paralleled by endoplasmic reticulum stress and mitochondrial dysfunction. The executioner protease caspase-3 is then activated and triggers apoptosis-related morphological changes, such as nuclear condensation and fragmentation in these tumor cells.

The present study is the first attempt to investigate the relevant aspects of cellular responses to euplotin C in the non-producer *E. vannus* by dissecting the events possibly associated with cytotoxicity and apoptosis: mitochondrial activity, nuclear and cytoplasmic morphology, and protease activity. Results of this study may also help to elucidate the links, if any, between PCDs in unicellular and multicellular organisms.

MATERIALS AND METHODS

Cell culture. Marine *Euplotes* strains can be grown on microalgae or bacteria, although the former food generally has a higher nutritional value (Dini and Nyberg 1999). The reverse is true for other marine ciliates like *Euplotidium itoi*, *Aspidisca leptaspis*, and *Diophrys oligothrix*, which prefer bacteria. Thus, *Euplotes* strains were grown on the micro-alga *Dunaliella tertiolecta*,

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