Azaspiracid-1 Alters the E-cadherin Pool in Epithelial Cells

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Azaspiracids cause severe damages in the epithelium of several organs. In this study we have investigated the effects of azaspiracid-1 (AZA-1) on two epithelial cell lines. Nanomolar concentrations of AZA-1 reduced MCF-7 cell proliferation and impaired cell-cell adhesion. AZA-1 altered the cellular pool of the adhesion molecule E-cadherin by inducing a dose- and time-dependent accumulation of an E-cadherin fragment (E-cadherin-related antigen [ECRA100]), with a concentration inducing the half-maximal effect (EC50) of 0.47nM. The immunological characterization of ECRA100 revealed that it consists of an E-cadherin molecule lacking the intracellular domain, and these data showed that the effect induced by AZA-1 in MCF-7 cells is undistinguishable from that induced by yessotoxin (YTX) in the same experimental system. A comparison of toxin effects in MCF-7 and Caco 2 cells confirmed that the effects induced by AZA-1 and YTX are undistinguishable in these cells. Treatment of fibroblasts with AZA-1 did not affect the cellular pool of N-cadherin showing that the toxin effect is cadherin-specific. A comparison of the effects induced by AZA-1, YTX, and okadaic acid on F-actin and E-cadherin in MCF-7 and Caco 2 cells showed that 1nM AZA-1 did not cause significant changes in F-actin and that accumulation of ECRA100 did not correlate with decreased levels of F-actin under our experimental conditions. Matching our results with those available in literature, we notice that, when molecular effects induced by AZA-1 and YTX have been studied in the same in vitro systems, experimental data show that they are undistinguishable in terms of sensitive cellular parameters, effective doses, and kinetics of responses in several cell lines. The possibility that azaspiracids and YTXs might share their molecular mechanism(s) of action in defined biological settings should be considered.

Key Words: azaspiracid; yessotoxin; E-cadherin; N-cadherin; epithelial cells; okadaic acid.

Azaspiracids are polyether compounds containing an azaspiro ring and a carboxylic acid moiety in the molecule (Nicolaou et al., 2004a,b). These natural compounds have been originally found in edible mollusks that were cultivated in Ireland (Satake et al., 1998), and have been later detected in shellfish from France, UK, Netherlands, Spain, Italy, and Norway (Ito et al., 2002; James et al., 2002; Magdalena et al., 2003), whereas their presence in shellfish harvested in Portugal remains uncertain (Vale, 2004). Azaspiracids have been reported to be produced by the microalga Protoperidinium crassipes (James et al., 2003), and are classified among marine biotoxins because human consumption of shellfish contaminated by these compounds caused gastrointestinal symptoms (Satake et al., 1998). Furthermore, toxin administration into mice by i.p. injection has resulted in neurological symptoms and death at doses of 110–200 μg/kg body weight (Ofuji et al., 1999; Satake et al., 1998), depending on the different analogs being injected into the animals. Per os administration of azaspiracids causes damages in several organs, including small intestine, lymphoid tissues, and liver (Ito et al., 2002). Moreover, teratogenic effects of azaspiracid-1 (AZA-1) have been recorded (Colman et al., 2004). Thus, azaspiracids pose risks to consumers and, accordingly, a regulatory limit in shellfish is set by the legislation of the European Union (European Commission, 2002, 2004, 2005).

The wide and severe toxicological effects of azaspiracids have been supporting intense investigations aimed at clarifying the mechanism of action of these compounds. Initial studies with cultured cells have indicated that azaspiracids are cytotoxic (Flanagan et al., 2001), and this finding was later confirmed with other human and rodent cell lines, where the concentration inducing the half-maximal effect (EC50) related to cell viability was in the 10−7–10−8M range (Twiner et al., 2005).

The mechanism of action of azaspiracids has been investigated in lymphocytes and neuronal cells, and the data gathered so far have been showing that 10−7–10−6M concentrations of these toxins modulate cytosolic calcium concentrations (Alfonso et al., 2005; Román et al., 2002, 2004), cause an increase in cellular cyclic adenosine monophosphate (cAMP) (Román et al., 2002, 2004), and may change intracellular pH (Alfonso et al., 2006).

Azaspiracids have been also shown to alter cytoskeletal structures, as micromolar concentrations of AZA-1 led to decreased levels of F-actin in neuroblastoma cells (Román et al., 2002), whereas 10nM AZA-1 caused retraction of pseudopodia in Jurkat cells (Twiner et al., 2005).
The molecular bases of neurological symptoms of azaspiracids have been investigated in a recent functional study, where it has been shown that low nanomolar concentrations of AZA-1 inhibit the bioelectrical activity of spinal cord neuronal networks through a mechanism that should not involve voltage-gated sodium and calcium channels (Kulagina et al., 2006).

The morphological alterations caused by azaspiracids in several cellular systems (Twiner et al., 2005), and their disruptive effects on intestinal epithelia (Ito et al., 2002) have attracted our attention because we have found that several algal toxins affect the molecules responsible for cell-cell adhesion (Malaguti and Rossini, 2002; Malaguti et al., 1999; Pierotti et al., 2003; Ronzitti et al., 2004).

In this study we have investigated the effects of AZA-1 on E-cadherin, a protein responsible for cell-cell adhesive structures in epithelia (Nollet et al., 2000), and found that this toxin causes the accumulation of an E-cadherin fragment in epithelial cells, in a process that does not depend on F-actin depolymerization.

MATERIALS AND METHODS

Materials. AZA-1 was purified from contaminated blue mussel tissue (Mytilus edulis) harvested on the South West Coast of Ireland in 2001. Preparative isolation carried out at the Marine Institute followed a protocol developed by Satake et al. (1998), with some modifications. Briefly, hepatopancreas (1 kg) were dissected from 6 kg of cooked mussel flesh and extracted with methanol. The extract was dried and partitioned between hexane and 80/20 vol/vol methanol/water. The aqueous phase was collected and dried by rotary evaporation. The dry extract was then partitioned between ethyl acetate and water. The organic phase was dried again using rotary evaporation and further purified on silica gel (Merck 60, Darmstadt, Germany) in an open column (4 cm internal diameter [i.d.] × 35 cm) using a step gradient of 100% acetone followed by 100% methanol. The methanolic phase was collected and chromatographed on Sephadex LH-20 (Amersham Biosciences, UK) (1 cm i.d. × 40 cm) using methanol as mobile phase. Three-milliliter fractions were collected continuously over 90 min. Fractions containing AZA-1 were combined and dried by rotary evaporation. The sample was further cleaned using Develosil ODS lop (Phenomenex, UK) with 85/15 vol/vol methanol/water containing 0.1% acetic acid as mobile phase. Fractions (5 ml) were collected continuously for 140 min. AZA-1 containing fractions were combined and the sample was further purified on diethylaminoethyl (DEAE) (Toyopearl, Tosoh, Japan) using a step gradient of 80/20 vol/vol methanol/water and 85/15 vol/vol methanol/water containing 0.1% acetic acid. Final purification of the toxin was achieved by chromatography on an Aasahipak ODP-50 column (6 mm i.d. × 15 cm) with a linear gradient of 30/50 vol/vol methanol water for 5 min and then rising to 100% methanol over 40 min holding 100% methanol for 10 min. Fractions were collected manually. The system used for chromographic purification was a Shimadzu 10-Avp series with photo diode array detector monitoring at 200 nm and fraction collector FRC 10-A. Purity of the 800 μg AZA-1 isolated was confirmed by nuclear magnetic resonance analysis using a JEOL 600-MHz spectrometer (Dr Masayuki Satake, Tohoku University Sendai, Japan). Working solutions of AZA-1 were prepared in absolute ethanol and stored in glass vials protected from light at −20°C. Anti-E-cadherin antibodies were purchased from Alexis Corporation (HEDC-1), Santa Cruz Biotechnology (Santa Cruz, CA) (H-108), and BD Biosciences (San Jose, CA) (610182). The anti-N-cadherin antibodies were purchased from Assay Designs Inc. (Ann Arbor, MI) (91504) and BD Biosciences (610920). Peroxidase-linked anti-rabbit, anti-mouse and anti-rat Ig antibodies, and the enhanced chemiluminescence (ECL) detection reagents were from Amersham Biosciences. The pretransfected molecular mass markers used in SDS-PAGE were obtained from Sigma Aldrich (Milan, Italy). The nitrocellulose membrane Protran BA 83 was obtained from Schleicher and Schuell (Brentford, UK). Oregon green phalloidin was obtained from Molecular Probes (Eugene, OR).

Cell culture conditions and preparation of cell extracts. MCF-7 human breast cancer cells were obtained from the European Collection of Animal Cell Cultures (No. 86012803 CB No 2705) and were cultured as previously described (Ronzitti et al., 2004). Caco 2 human colon adenocarcinoma cells were obtained from the American Type Culture Collection (No. HTB-37), and were cultured as previously described (Ronzitti et al., 2004). Normal mouse fibroblasts were obtained from explants of skin from newborn normal mice and were cultured as previously reported (Rossini et al., 1999).

If not stated otherwise, cell treatments were carried out using dishes near confluence, by addition of indicated concentrations of toxins or vehicle (control samples), and incubations were carried out for the indicated times at 37°C. At the end of the incubations, MCF-7, Caco 2 cells, and fibroblasts were washed once with 20mM phosphate buffer, pH 7.4, 0.15M NaCl (phosphate-buffered saline [PBS]), harvested with PBS by scraping and recovered by low speed centrifugation. Cells were then lysed with 0.2 ml of PBS containing 1% (vol/vol) Triton X-100 and 0.1 mg/ml phenylmethylsulfonyl fluoride, with two 10-s bursts of vortexing.

Cytosoluble extracts were obtained by centrifugation of cell lysates for 30 min at 16,000 × g. The supernatants of this centrifugation were saved and the protein content of cytosoluble extracts was measured with bichinonic acid (Smith et al., 1985).

When cell proliferation was evaluated, adherent and floating cells were separately harvested and were lysed by resuspension in 20mM Tris–HCl, pH 7.5 at 2°C, 1.5M ethylenediaminetetraacetic acid (EDTA), and sonication with two 10-s bursts. The lysates were then used for DNA measurements by the procedure of Labarca and Paigen (1980).

Fractionation of proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting of cellular extracts. Cytosoluble extracts were brought to 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol, to be used for fractionation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis. Samples were fractionated by SDS-PAGE, according to Laemmll (1970), using an 8% separating gel and a 3% stacking gel. After completion of electrophoresis, proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Protran BA 83) and subjected to immunoblotting, as already reported (Ronzitti et al., 2004).

Quantitation of F-actin depolymerization. The determination of F-actin was carried out by the method of Cunningham (1995), with minor modifications. Briefly, cells were scraped in PBS, washed twice with PBS, centrifuged for 10 min at 800 × g, and the cellular sediment was resuspended in 100 μl of PBS containing 0.5% of Triton X-100 and 100mM Oregon green phallolidine. The incubation was continued for 30 min at room temperature in the dark. The cell extracts were next centrifuged for 60 min at 20,000 × g. The pellet from this centrifugation was then resuspended in 300 μl of absolute methanol and incubated for 40 h at −20°C in the dark. The content of Oregon green phallolidine of each sample was then monitored by separation on a Prodigy Column (Phenomenex 250 × 4.6 mm), using 65% methanol, 20mM potassium acetate as eluent. Twenty microliters of each sample was injected in the column and detected with a high-performance liquid chromatography fluorescence detector (HP 1046a Programmable fluorescence detector—Agilent [Santa Clara, CA]), using a λ<sub>exc</sub> = 492 nm and a λ<sub>em</sub> = 517 nm.
Calculations and data presentation. The autoradiographs obtained from immunoblotted membranes by ECL were subjected to densitometric scanning and the absorbances were recorded as the peak area of bands of two protein components, corresponding to intact E-cadherin and an E-cadherin-related antigen (ECRA100). The relative immunoreactivity of ECRA100 (IR ECRA100) was calculated as the ratio of the peak absorbance of ECRA100 and that of total absorbance (intact E-cadherin + ECRA100), and has been expressed as the percentage of total absorbance.

RESULTS

Effects of AZA-1 on Proliferation and Morphology of Epithelial Cells

Our preliminary experiments have been devoted to check whether AZA-1 could cause morphological alterations and cell death in our experimental systems. To this end, we treated human breast cancer MCF-7 cells with increasing concentrations of AZA-1 and measured the cell number and the DNA content of cultured dishes after 48 h of toxin exposure. The results we obtained are reported in Figure 1A, and show that AZA-1 concentrations higher than 1nM caused a limited, but reproducible decrease in MCF-7 cell proliferation after 2 days of treatment. At 1nM concentration, however, AZA-1 does not invariably cause cytotoxicity, as a 48-h treatment induced a loss of viability in MCF-7 cells but did not significantly alter the proliferation of Caco 2 cells (Fig. 1B). In line with these findings, gross morphological alterations were induced by 1nM AZA-1 only in MCF-7 but not in Caco 2 cells (Fig. 1C).

Effects of AZA-1 on E-cadherin

Those findings led us to check the status of the E-cadherin system in MCF-7 cells treated for different times with 1nM AZA-1. The results we obtained by immunoblotting analysis of cytosoluble extracts prepared upon cell lysis with a Triton X-100–containing buffer are reported in Figure 2, and show that AZA-1 treatment causes a time-dependent increase in the cellular content of an E-cadherin fragment with an electrophoretic mobility corresponding to a protein of about 100 kDa (ECRA100). The cellular content of intact E-cadherin was not significantly changed in the first day of AZA-1 treatment (Fig. 2A).

The effect induced by AZA-1 on E-cadherin in MCF-7 cells was undistinguishable from that we have previously observed in the same experimental system after cells had been treated with YTX (Ronzitti et al., 2004). We then carried out a parallel incubation of MCF-7 cells with increasing concentrations of either AZA-1 or YTX, and measured the accumulation of cellular ECRA100. As shown in Figure 3, the two toxins induced the same type of response with similar potencies. Based on our estimates, the EC50 of AZA-1 in inducing the accumulation of ECRA100 in MCF-7 cells is 0.47 ± 0.07nM (n = 3).

These data then prompted us to characterize the molecular alteration caused by AZA-1 on E-cadherin in MCF-7 cells.
To this end, we subjected cytosoluble extracts from MCF-7 cells treated with AZA-1 to immunoblotting using antibodies binding to epitopes involving different domains of the E-cadherin molecule (Fig. 4). Under our experimental conditions, the antibodies interacting with extracellular portions of E-cadherin (HECD-1 and H-108, see Shimoyama et al., 1989, and the information sheet of the product No. sc-7870 provided by Santa Cruz Biotechnology) allowed the detection of ECRA100, whereas the antibody 610182, whose epitope is located in the intracellular, carboxy-terminal domain of E-cadherin (see the information sheet of the product provided by BD Biosciences) did not detect this protein. Thus, AZA-1 determined the cellular accumulation of an E-cadherin fragment devoid of the intracellular portion of the molecule, further supporting the finding that the alteration of E-cadherin caused by AZA-1 is undistinguishable from that induced by YTX in MCF-7 cells (Ronzitti et al., 2004).

**The Effects of AZA-1 are Cadherin-Specific**

Our previous data have shown that YTX affects E-cadherin in several epithelial cells, including the Caco 2 colon cell line (Ronzitti et al., 2004). Since AZA-1 did not appear to cause any gross alteration of Caco 2 cells under our experimental conditions, we analyzed whether AZA-1 could alter E-cadherin in this intestinal cell line. Taking into consideration that Caco 2 cells might be relatively insensitive to AZA-1, we compared the responses that increasing concentrations of this toxin could induce in Caco 2, as compared to MCF-7 cells. The results we obtained are reported in Figure 5, and show that AZA-1 can cause the accumulation of ECRA100 in Caco 2 cells at concentrations similar to those that are effective in MCF-7 cells (Fig. 5A). Furthermore, the direct comparison of the effects induced by AZA-1 and YTX on E-cadherin in Caco 2 cells, showed that the two toxins induce the accumulation of ECRA100 to a very similar extent and over the same time-frame in this experimental system. Thus, Caco 2 cells are very sensitive to AZA-1, although this toxin did not induce extensive morphological alterations at the concentrations we used in these experiments.

Taking into consideration that cells of different histological type express distinct cadherins, we checked whether AZA-1 could alter other cadherin systems, and analyzed the effects of the toxin on N-cadherin, that represent another type I cadherin (Nollet et al., 2000). As a model system, we used normal fibroblasts that express N-cadherin (Nollet et al., 2000).
When normal mouse fibroblasts were treated with increasing AZA-1 concentrations, cell detachment from culture dishes was apparent (Fig. 6A) but proliferation of fibroblasts was not severely altered, as judged by the DNA content measured by combining floating and adherent cells (Fig. 6B).

When the N-cadherin pool was analyzed by immunoblotting using antibodies that bind to either the N-terminal (extracellular) or the C-terminal (intracellular) portion of the molecule, no qualitative or quantitative difference could be detected in extracts from control and AZA-1–treated cells (Fig. 6C). The effect of AZA-1, therefore, is cadherin specific.

The Effect of AZA-1 on E-cadherin Does Not Depend on Alterations of the Cytoskeleton

The classical model of cadherin-based cell-cell adhesion involves the interaction of the intracellular portion of cadherin molecules with the actin cytoskeleton through the participation of other proteins called catenins (Kobiela and Fuchs, 2004; Takeichi, 1990; Tepass et al., 2000). Since AZA-1 has been shown to alter the cytoskeleton in some cellular systems (Román et al., 2002; Twine et al., 2005), we have analyzed the effects of AZA-1 concentrations capable to alter the

FIG. 5. Comparative analysis of toxin potency in inducing accumulation of ECRA100 in epithelial cells. (A) MCF-7 (closed symbols) or Caco 2 (open symbols) cells have been incubated with the indicated concentrations of AZA-1 for 24 h at 37°C. At the end of the incubation, cells were processed to obtain cytosoluble extracts that have been fractionated by SDS-PAGE and subjected to immunoblotting using the HECD-1 anti-E-cadherin antibody. The relative IR ECRA100 was quantified as described under “Materials and Methods,” and the data shown in the figure have been obtained in a typical experiment. (B) Caco 2 cells were incubated with the indicated concentration of either YTX or AZA-1 for the indicated times at 37°C. At the end of the incubation, cells were processed to obtain cytosoluble extracts that have been fractionated by SDS-PAGE and subjected to immunoblotting using the HECD-1 anti-E-cadherin antibody. The panel shows the electropherogram obtained by ECL detection and autoradiography and represents the result obtained in a typical experiment. The electrophoretic mobility of β-galactosidase (116 kDa), used as marker protein, is indicated on the left.

FIG. 6. Effect of AZA-1 on mouse fibroblasts. Normal mouse fibroblasts were incubated with the indicated concentrations of AZA-1 for 24 and 48 h at 37°C. (A) Photomicrographs of control and AZA-1–treated cells. (B) Control and AZA-1–treated cultures were used for measurements of the DNA content combining adherent and floating cells, as described under “Materials and Methods.” Values have been expressed as percentages of those found in control cultures and represent the data obtained in a typical experiment. (C) Control and AZA-1–treated cells were processed to prepare cytosoluble extracts that have been fractionated by SDS-PAGE and subjected to immunoblotting using the 610920 (C-terminal) and 91504 (N-terminal) anti-N-cadherin antibodies. The panels show the electropherogram obtained by ECL detection and autoradiography. The electrophoretic mobilities of β-galactosidase (116 kDa) and pyruvate kinase (64 kDa) subunits, used as marker protein, are indicated on the left.
E-cadherin pool on other cellular parameters that could be related to alterations of cell adhesive structures. Furthermore, in order to distinguish between toxin-related and process-related effects, we compared the responses of MCF-7 and Caco 2 cells to different phycotoxins. Thus, we extended our analyses to the effects induced by cell treatment with OA that has been shown to be cytotoxic (reviewed in Rossini, 2000), and to alter both the E-cadherin system (Malaguti and Rossini, 2002) and the F-actin cytoskeleton (Leira et al., 2001; Macias-Silva and Garcia-Sainz, 1994) in cultured cells. We then treated MCF-7 and Caco 2 cells with 1nM YTX, 1nM AZA-1, or 50nM OA for 24 h. At the end of the treatments, we recorded the gross morphology of treated cells, and measured the F-actin content as well as the cellular pool of E-cadherin in those cells (Fig. 7).

The cell treatment for 24 h with either 1nM YTX or AZA-1 did not result in any gross morphological change of cultured cells, as expected, whereas OA caused extensive cell damage and death, resulting in cultures containing many rounded cells, detached from culture dishes (Fig. 7A). In keeping with the morphological alterations we have detected and the results obtained by others (Leira et al., 2001), cell treatment with OA led to a 30–40% decrease in F-actin in MCF-7 and Caco 2 cells (Fig. 7B). YTX treatment, in turn, induced decreased F-actin in MCF-7 but not in Caco 2 cells, confirming that alteration of cell cytoskeleton by this toxin is a cell-related effect (Leira et al., 2003; Pérez-Gomez et al., 2006). Cell treatment with 1nM AZA-1, instead, did not lead to detection of any significant change in the cellular levels of F-actin, in line with the observations that higher AZA-1 concentrations are needed to cause detectable cytoskeletal alterations (Román et al., 2002; Twiner et al., 2005).

When E-cadherin was analyzed in extracts from toxin-treated cells, we confirmed that YTX and AZA-1 cause the accumulation of ECRA100 in MCF-7 and Caco 2 cells, and that OA causes a 30–50% loss of cellular E-cadherin (Malaguti and Rossini, 2002) and the accumulation of the 135-kDa E-cadherin precursor protein without any change in the levels of ECRA100 (Rossini, 2002) in MCF-7 cells (Fig. 7C). The treatment of Caco 2 cells with OA, in turn, did not lead to any relevant qualitative or quantitative change of the cellular E-cadherin pool (Fig. 7C).

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Our findings, therefore, showed that the accumulation of ECRA100 induced by AZA-1 is independent of extensive destruction of F-actin, and its detection in AZA-1–treated cells is not a consequence of decreased F-actin.

**DISCUSSION**

The treatment of epithelial cells with AZA-1 has resulted in the accumulation of a 100-kDa E-cadherin fragment comprising the extracellular portion of the molecule and lacking its intracellular domain. These two portions of the E-cadherin molecule play distinct roles in maintaining proper cell-cell adhesion, as the extracellular domain is involved in establishing the homophilic association between adjacent cells that provides the tight contacts typical of epithelia (Shimoyama et al., 1989), whereas the intracellular domain contains the binding sites for β- and γ-catenin (plakoglobin) (Ozawa et al., 1989) that would link the E-cadherin molecule to the cytoskeleton and provide the mechanical strength of cell-cell adhesion (Chitaev and Troyanovsky, 1998; Shore and Nelson, 1991).

These cell adhesive structures have roles in embryonic development and maintenance of tissue architecture (Takeichi, ...
EFFECTS OF AZASPIRACID ON E-CADHERIN

The mechanism of action of azaspiracids remains uncertain. Azaspiracids have been shown to modulate cytosolic calcium concentrations (Alfonso et al., 2005; Román et al., 2002, 2004), increase cellular cAMP (Román et al., 2002, 2004), and change intracellular pH (Alfonso et al., 2006) in human lymphocytes. It is presently undetermined whether these three effects might have a role in the accumulation of ECRA100 induced by AZA-1 in epithelial cells but the great differences in effective doses of AZA-1 inducing those responses would indicate that the effect on E-cadherin should not depend on those processes. In fact, AZA-1 induces the accumulation of ECRA100 at concentrations in the $10^{-10}$–$10^{-8}$M range, whereas responses in lymphocytes were found with $10^{-7}$–$10^{-6}$M AZA-1 (Alfonso et al., 2005, 2006; Román et al., 2002, 2004). Further investigations will then clarify the details of the mechanism of action and the effects triggered by AZA-1 in different systems.

Overall, the effects induced by AZA-1 (Figs. 2–7) are undistinguishable from those of YTX in the same cell lines (Ronzitti et al., 2004). If this is considered in the light of available data on the effects induced by these two toxins in cultured cells, it can be observed that both AZA and YTX induce (1) a slight increase in the cytosolic calcium concentrations in lymphocytes at concentrations in the $10^{-7}$–$10^{-6}$M range (Alfonso et al., 2005; Pérez-Gomez et al., 2006; Román et al., 2002, 2004); (2) an increase in cAMP in lymphocytes at concentrations in the $10^{-7}$–$10^{-6}$M range (Román et al., 2002, 2004); (3) the accumulation of ECRA100 at concentrations in the $10^{-10}$–$10^{-8}$M range in epithelial cells (this paper and Ronzitti et al., 2004); and (4) a decrease in the cellular content of F-actin at concentrations above $10^{-9}$M with some degree of cell-specificity (Leira et al., 2003; Pérez-Gomez et al., 2006; Román et al., 2002; Twiner et al., 2005). Furthermore, the kinetics of those responses are undistinguishable when matching the findings obtained with AZA and YTX (Alfonso et al., 2005; Román et al., 2002, 2004; Ronzitti et al., 2004; Twiner et al., 2005; this paper).

Available experimental data then pose the question of whether azaspiracids and YTXs might share their molecular mechanism(s) of action in some target cells and/or biological settings, and it seems important that future investigations will approach a comparative analysis aimed at clarifying this aspect.

The relevance of this evaluation stems from toxicological and risk management considerations, and would be particularly informative in mechanistic terms. In fact, if the two classes of toxins share their mechanisms of action, it should be determined whether the receptorial component(s) in sensitive systems is(are) shared by azaspiracids and YTXs as well. This aspect is particularly relevant in the light of the extensive differences existing between the chemical properties of the two classes of compounds.

A more thorough clarification of identities and differences in the mechanisms of action, as well as the possible agonistic/antagonistic roles reciprocally played by azaspiracids and

FIG. 8. Effect of AZA-1 on F-actin and E-cadherin in MCF-7 cells. Cells were treated with the indicated AZA-1 concentrations for 48 h at 37°C, before being used for measurement of F-actin (top) and analysis of E-cadherin by SDS-PAGE and immunoblotting (bottom), as described under “Materials and Methods.” Values of F-actin have been expressed as the percentage of the levels found in control cells in a typical experiment and represent means ± SD of data obtained in replicate samples. The bottom panel shows the electrophoretogram obtained by ECL detection and autoradiography. The electrophoretic mobility of β-galactosidase (116 kDa) subunit, used as marker protein, is indicated on the left.

1990; Tepass et al., 2000), and alterations of E-cadherin structure and/or cellular levels have been linked to tumor spreading and metastasis formation in several human cancers, so that E-cadherin represents a tumor suppressor (Christofori and Semb, 1999; Vleminckx et al., 1991).

The accumulation of ECRA100 induced by AZA-1 in MCF-7 and Caco 2 cells would then indicate the risk that consumption of shellfish contaminated by azaspiracids could also favor tumor spreading and metastasis formation in vivo.

This type of risk deserves proper attention because the EC50 of AZA-1 with regard to the effects exerted on E-cadherin in vitro is lower than $10^{-9}$M (Fig. 3), and repeated oral administration of AZA to mice has led to the detection of lung tumors in 10–30% of the animals used in the treatments (Ito et al., 2002).

If the results of this study are considered as a whole, they show that AZA-1 perturbs cell adhesion in both epithelial cells and fibroblasts. The molecular systems involved in cell-cell adhesion in those lines, however, are not equally sensitive to the toxin, as AZA-1 affects E-cadherin in epithelial cells but not N-cadherin in fibroblasts. Thus, our results imply that AZA-1 alters both cell-cell and cell-substrate interactions. The molecules involved in AZA-1 alteration of cell-substrate interaction, however, remain(s) to be identified.

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YTXs under some biological settings, would provide a major contribution to the understanding of their toxicity both in functional and chemical terms, and such a knowledge would be important for the rational management of the risks posed by those algal toxins to consumers.

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