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Crude extracts of marine-derived and soil fungi of the genus *Neosartorya* exhibit selective anticancer activity by inducing cell death in colon, breast and skin cancer cell lines

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Abstract

Background: The crude ethyl acetate extracts of marine-derived fungi *Neosartorya tsunodae* KUFC 9213 (E1) and *N. laciniosa* KUFC 7896 (E2), and soil fungus *N. fischeri* KUFC 6344 (E3) were evaluated for their *in vitro* anticancer activities on a panel of seven human cancer cell lines. **Materials and Methods:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed, after 48 h treatments with different concentrations of extracts, to determine their concentration of the extract or Dox that inhibits cell viability by 50% for each cell line. The effects of the crude extracts on DNA damage, clonogenic potential and their ability to induce cell death were also assessed. **Results:** E1 was found to the void of anti-proliferative effects. E2 was shown to decrease the clonogenic potential in human colorectal carcinoma cell line (HCT116), human malignant melanoma cell line (A375), human breast adenocarcinoma cell line (MCF7), and human caucasian colon adenocarcinoma Grade II cell line (HT29) cells, whereas E3 showed such effect only in HCT116 and MCF7 cells. Both extracts were found to increase DNA damage in some cell lines. E2 was found to induce cell death in HT29, HCT116, MCF7, and A375 cells while extract E3 increased cell death in MCF7 and HCT116 cell lines. **Conclusion:** The results reveal that E2 and E3 possess anticancer activities in human colon carcinoma, breast adenocarcinoma, and melanoma cells, validating the interest for an identification of molecular targets involved in the anticancer activity.

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Full Text

Summary

The crude ethyl acetate extract of N. tsunodae (E1) did not decrease cell viability in any of the tested cell linesThe crude ethyl acetate extracts of N. laciniosa (E2) and N. fischeri (E3) decreased cell proliferation in some human cancer cell lines tested at both short- and long-termN. laciniosa (E2) induced a significant increase in the number of cell death, in part, due to the induction of DNA damageN. fischeri (E3) induce cell death but in some cell lines without induction of DNA damage detected by comet assayCrude ethyl extracts of N. laciniosa (E2) and N. fischeri (E3) exert an anticancer activity in human colon carcinoma, breast adenocarcinoma, and malignant melanoma cells.

[INLINE:1]

Abbreviations Used: A375: Human malignant melanoma cell line; A549: Human non-small lung cancer cell line; DAPI: 4,6-diamidino-2-phenylindole; DMEM: Dulbecco's Modified Eagle Medium; DMSO: Dimethylsulfoxide; Dox: Doxorubicin; E1: Neosartorya tsunodae KUFC 9213; E2: Neosartorya laciniosa KUFC 7896; E3: Neosartorya fischeri KUFC 6344; FBS: Fetal bovine serum; HCT116: Human colorectal carcinoma cell line; HEPES: (N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid]); HepG2: Human hepatocellular carcinoma cell line; HT29: Human caucasian colon adenocarcinoma Grade II cell line; IC 50 : Concentration of the extract or Dox that inhibits cell viability by 50%; MCF7: Human breast adenocarcinoma cell line; MEM: Minimum Essential Medium Eagle; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCI-H460: Human non-small lung cancer cell line; PBS: Phosphate buffered saline; PE: Plating efficiency; RPMI: Roswell park memorial institute medium; SF: Surviving fraction; U-251: Human malignant glioblastoma cell line.

INTRODUCTION

Cancer is one of the main causes of death in developed countries and is gaining lead in developing ones. [1] Considering the increasing frequency of neoplastic lesions, resistance acquired to conventional commonly used therapeutic drugs, [2] cross-resistance to structurally dissimilar and unused anticancer drugs, [3] and undesirable side effects of current chemotherapeutic drugs, new bioactive substances are needed.

The marine environment is a prosperous and underexploited resource of bioactive natural compounds. [4] Bioprospection of the oceans has brought to light numerous novel compounds with potential therapeutic applicability, for e.g., demonstrating bioactive profiles with anticancer, anti-inflammatory, anti-viral, anti-angiogenic, anti-oxidant, and anti-adhesion activity. [5],[6], [7] Until now, only a few marine-derived drugs have been approved for anticancer clinical use, although many are currently in clinical trial phases. [8],[9]

Marine-derived fungi can be considered as key relevance in the quest for biopharmaceuticals since they produce metabolites with interesting biological activities such as cytotoxic and cytostatic effects on human cancer cell lines. [10] Moreover, marine-derived fungi are also a source of compounds that may overcome the frequent limitation of production shortfall, since they have the potential to be easily produced under laboratorial/industrial conditions, and are, therefore, good candidates for compound extraction under mass-production. Additionally, compounds isolated from marine-derived fungi have been found to target different pathways involved in proliferation and cell death mechanisms and other hallmarks of cancer. [11]

Fungi of the genus Neosartorya, which are sexual forms of the Aspergillus species, especially of the Fumigati section, have recently been revealed as potential sources for potential anticancer compounds. [12],[13],[14],[15] With this rationale, the aim of this study was to assess the in vitro anticancer activity of the crude ethyl acetate extract of three species of Neosartorya, that is, the marine-derived N. tsunodae KUFC 9213 (E1) and N. laciniosa KUFC 7896 (E2), and soil N. fischeri KUFC 6344 (E3) on a panel of seven human cancer cell lines: Colon carcinoma (HT29 and HCT116), liver hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7), malignant melanoma (A375), lung carcinoma (A549), and glioblastoma

(U-251) cells. The chemical composition of the crude extracts E1, E2, and E3 has been previously analyzed by members of our research team. [13]

Materials and Methods

Reagents

Doxorubicin (Dox), Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium Eagle (MEM), Roswell Park Memorial Institute medium (RPMI), penicillin/streptomycin, trypsin solution, 4,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was purchased from AMRESCO LLC (Solon, SO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). All other reagents and chemicals used were of analytical grade.

Fungal material

N. tsunodae (KUFC 9213) was isolated from the marine sponge Aka coralliphaga, N. fischeri (KUFC 6344) was isolated from coastal forest soil and N. laciniosa (KUFC 7896) was isolated from a diseased coral (ulcerative white spotin P. lutea), as reported by Eamvijarn et al. [13]

Preparation of the fungal crude ethyl acetate extracts

Crude ethyl acetate extracts of the fungi were obtained as previously described by Eamvijarn et al. [13] Briefly, the marine-derived fungi were cultured in Petri dishes with malt extract agar (for the strains KUFC 9213 and KUFC 7896) or with potato dextrose agar (for the strain KUFC 6344) for 1-week. Then Erlenmeyer flasks containing rice and water were autoclaved, inoculated with the respective fungal mycelia, and incubated for 30 days at 28°C. The mouldy rice was macerated in ethyl acetate, filtered, and then the two layers were separated by a separating funnel, and the ethyl acetate solution was concentrated at a reduced pressure.

Cell lines

HT29, HCT116 cells were kindly provided by Prof. Carmen Jerónimo, from CI-IPO, Porto. HepG2 cells were kindly provided by Prof. Rosário Martins, from ESTSP and CIIMAR, Porto. A375, A549, U-251, and MCF7 cell lines were obtained from the European Collection of Cell Cultures. Cells were maintained as monolayer cultures in DMEM (HT29, A375 and A549) in MEM (HepG2, U-251 and MCF7) or RPMI for HCT116 cell line. Media were supplemented with 10% FBS, 1% antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethane-sulfonic acid] and 0.1 mM sodium pyruvate. Cells were maintained in a humidified incubator with 5% CO 2 at 37°C. Cells were trypsinized when approaching confluence. For experiments, the test extracts and Dox were dissolved in DMSO (final concentration <0.5%) and controls received DMSO only.

Evaluation of cell viability/proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Effects of the fungal crude extracts on cell viability/proliferation were evaluated by the MTT reduction assay. [16] Briefly, cells were seeded at a density of 0.8 × 10 4 to 1 × 10 4 cell/well in 96-well plates and incubated for 24 h in 5% CO 2 at 37°C. After adhesion, cells were exposed to fresh medium containing varying concentrations of the fungal extracts (0.1, 1, 10, 100, and 200 µg/mL) or Dox (0.001, 0.01, 0.1, 1, and 10 µM), used as a positive control. After 48 h of treatment, MTT solution at a final concentration of 0.5 mg/mL was added and incubated for 2 h at 37°C. Ethanol-DMSO (1:1) (v/v) solution was used to dissolve the formazan crystals, and the absorbance (A) was measured at 570 nm in a microplate reader (Multiskan EX, Labsystems, USA). The number of viable cells in each well was estimated by the cell's capacity to reduce MTT and produce formazan crystals. [16] The concentration of the extracts or Dox that inhibits cell viability by 50% (IC 50) was calculated by analysing dose-response data with GraphPad Prism version 5.0 software (GraphPad Software, La Jolla, CA, USA).

To evaluate the effects of the fungal crude extracts on cell proliferation, absorbance at the beginning of incubation (t = 0 h) was subtracted from all experimental conditions, including the negative control (untreated cells) at the end of treatment (t = 48 h). Cell proliferation and cell inhibition percentages were calculated according to the following equations:

Cell proliferation (%) = ([A sample − A t=0h)/(A t=48h − A t=0h)]) × 100

where A is the absorbance.

Cell inhibition (%) = 100 − Cell proliferation

Negative values for cell proliferation imply direct cytotoxic effects of the extracts; positive values (between 0 and 100%) imply inhibition of cell proliferation. The results correspond to the mean of at least six independent experiments, each carried out in duplicate. Only the fungal crude extracts that presented an IC 50 lower than the arbitrary cut-off of 200 µg/mL and without direct cytotoxic effects were used in the following assays.

Evaluation of cytostatic effect by clonogenic cell survival assay

The long-term cytostatic effect was evaluated by the clonogenic cell survival assay. Cells were seeded in 24-well plates with a density of 1 × 10 6 cells/mL, and after adhesion, cells were incubated with the fungal crude extracts and Dox at IC 50 concentrations. After 48 h of treatment, cells were harvested by trypsinization and survival cells were seeded in 12-well plates at a density of 200 cells/well. Cells were incubated with a drug-free medium for 10 days under normal culture conditions. After the incubation period, cells were washed with Phosphate Buffered Saline (PBS) and fixed in situ with 4% paraformaldehyde (in PBS) for 15 min at room temperature. Then, cells were stained with 0.05% crystal violet for 30 min at room temperature, washed with distilled water, and the plates were left to dry. In each treatment condition, colonies made of more than ~50 cells were quantified by a stereomicroscope (Leica, ZOOM 2000), and the plating efficiency (PE) and surviving fraction (SF) were calculated according to the following equations: [17],[18]

PE = number of colonies counted/number of seeded cells

SF = (PE of treated cells/PE of control) × 100.

Evaluation of genotoxic effect by single cell electrophoresis assay (comet assay)

The effect of the fungal crude extracts on DNA damage was evaluated by the single cell electrophoresis assay or comet assay. [19],[20] Briefly, cells were seeded at a density of 0.1 × 10 6 cells/ml in 24-well plates. After adhesion, cells were treated with the respective IC 50 concentrations of the fungal extracts and Dox for 4, 24 and 48 h at 37°C. By the end of each treatment, 2 × 10 4 cells were collected per sample, mixed with a 0.5% (w/v) low melting agarose, and mounted on slides previously coated with a 1% (w/v) normal melting agarose. Up to this point, all samples were maintained at 4°C to minimize further DNA repair. Slides were placed in lysis solution (2.5 M NaCl, 100 mM Na 2 , EDTA, 10 mM Tris, pH 10) plus 1% (v/v) triton X-100 for 1 h at 4°C, and then incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na 2 EDTA, pH 13) for 40 min at 4°C to allow the DNA to unwind. After a 20 min electrophoresis at 21 V, slides were washed, fixed with 100% ethanol, and dried at room temperature. Afterward, slides were stained with DAPI solution (1 µg/ml), observed under a fluorescence microscope (Olympus IX 71) and the CometScore; software (Version 1.5., TriTek Corporation, Sumerduck, VA, USA.) was used to calculate the parameter of percentage of tail intensity.

Evaluation of cell death induction by nuclear condensation assay

Effects of the fungal crude extracts on the induction of cell death were evaluated by the nuclear condensation assay. Cells were seeded at a density of 1 × 10 6 cells/mL on 24-well plates, and incubated for 24 h at 37°C. After adhesion, cells were treated with the respective IC 50 concentrations of the fungal crude extracts and Dox and incubated for 48 h. In each well, adherent and detached cells were collected, washed, centrifuged, and fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at 37°C. Samples were stored at 4°C. Prior to analysis, cells were mounted on polylysine-treated slides using a Cell Spin Cytospin centrifuge and left to air dry. Ensuing, the slides were thrice washed with PBS for 5 min each and incubated for 10 min with DAPI (1 µg/mL) for nuclei staining. The cells with condensed nuclei were observed using a fluorescence microscope (Olympus IX 71) and at least 300 cells were scored per sample. The percentage of cells with condensed nuclei was determined from the ratio between cells with nuclear condensation and the total number of cells × 100.

Statistical analysis

Results are expressed as mean ± standard deviation from at least 3 independent experiments. Statistical tests were performed by one-way ANOVA, followed by the post-hoc Newm

Keuls multiple comparison test or Dunnett's testor by the Student's t-test, using the software GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). $P \leq 0.05$ were considered statistically significant.

Results and Discussion

As a first step to ground further research, the fungal crude extracts were screened for anti-proliferative activity against HepG2, HT29, HCT116, A375, A549, MCF7, and U251 cancer cell lines. After 48 h of cell exposure to a range of concentrations of the fungal crude extracts and Dox (positive control), effects on cell viability/proliferation were measured by MTT reduction assay. Impacts of extract and Dox on cell viability were summarized in [Table 1], by IC 50 values that correspond to the concentration that was able to cause a 50% inhibition of viability. At the tested concentrations, E1 did not decrease cell viability in any of the tested cell lines (in all cases the IC 50 was higher than 200 μg/mL). Although Eamvijarn et al. [13] have recently reported the growth inhibitory activity of sartorypyrone B, isolated from the crude ethyl acetate extract of N. tsunodae, on breast adenocarcinoma (MCF7), melanoma (A375-C5), and non-small cell lung (NCI-H460) cancer cell lines, the crude ethyl acetate of this fungus (E1) did not exhibit any anti-proliferative effect on any cell lines tested in this experiment.{Table 1}

The extract of N. laciniosa (E2) was found to inhibit four of the human cancer cell lines tested, namely in HT29, HCT116, A375, and MCF7 cells. The IC 50 values for this extract were 139, 141, 179, and 200 μg/mL for HCT116, A375, MCF7, and HT29, respectively, showing that HCT116 and A375 cells were the most sensitive to E2.

The extract of N. fischeri (E3) also presented relevant inhibitory activities (IC 50 ≤ 200 μg/mL) on HCT116 and HT29 (IC 50 values of 189 μg/mL and 196 μg/mL, respectively), and MCF7 (IC 50 of 189 μg/mL), although less active than E2. Dox was used as a positive control and was shown to decrease cell viability in all the tested cell lines in a dose-dependent manner, with the IC 50 values ranging from 0.11 μM to 1.55 μM. The U251, HT29, and A549 cell lines were found to be the most resistant having the IC 50 values of 1.55, 0.87, and 0.54 μM, respectively.

The effects of the fungal crude extracts on cell proliferation have been also inferred from the MTT results. As shown in [Figure 1]a, E2 significantly inhibited cell proliferation in HT29, HCT116, A375, and MCF7 in a dose-dependent manner. E3 was also found to decrease cell proliferation, in a dose-dependent manner, in HT29, HCT116, and MCF7 cells [Figure 1]b. Both extracts were found to induce a cytotoxic effect at concentrations higher than 200 μg/mL [Figure 1]. The percentage of inhibition of cell proliferation of E2 and E3 at the same concentration (e.g., 100 μg/ml) is summarized in [Table 2]. In HCT116 and MCF7 cells, E2 inhibited cell proliferation by 45% and 69%, while E3 inhibited proliferation by 26% and 39%, respectively. On the contrary, HT29 cells showed the lowest rates of inhibition of cell proliferation for both extracts (E2 - 24% and E3 - 19%).{Figure 1}{Table 2}

Different IC 50 values and percentages of cell inhibition of the cell lines tested, suggest that the fungal crude extracts may act by different pathways, depending on the genetic characteristics of each cell line. This hypothesis is supported by the fact that the drug resistance demonstrated by several types of cancers, namely colorectal cancer, is often related with p53 mutations. [21] In this work, both extracts (E2 and E3) were found to be more active (lower IC 50 values) in cell lines with wild-type p53, however, they also showed an anti-proliferative effect in the HT29 cell line (p53 mutant) indicating that both extracts may act by p53-dependent and/or independent mechanisms.

Interestingly, E2 was found to be more active than E3 in the most sensitive cell lines (HCT116, A375 and MCF7), which could be due to the difference in the chemical constituents of the two extracts. Recently, Eamvijarn et al. [13] reported isolation of the meroditerpenesaszonapyrone A (4b) and sartorypyrone A (3), aszonalenin (1a), acetylaszonalenin (1b), 13-oxofumitremorgin B (2), 1-formyl-5-hydroxyaszonalenin (1c), and helvolic acid from the ethyl extract of K3 and aszonapyrone A (4b) and B (4a), tryptoquivaline T and L (5) and 3'-(4-oxoquinazolin-3-yl) spiro [1H-indole-3,5'-oxo-lane]-2,2'-dione from the ethyl extract of K2. [13],[22] Compounds 1 to 5 were tested for the in vitro growth inhibition against MCF7, NCI-H460, and A375-C5 cancer cell lines, with compounds 2, 3, 4b demonstrating effect in the three cell lines [13] and compound 5 in MCF7 cells. [23] Interestingly, aszonapyrone A which is a constituent of both E2 and E3, was considered to be the most potent compound with GI 50 10 μM for all the cell lines tested. Consequently, these compounds and, possibly others, may be responsible for the anti-proliferative effect of E2 and E3.

The two fungal crude extracts E2 and E3, revealed anti-proliferative effects throughout the tested concentrations, demonstrating a dose-response effect. These results are in line with morphological changes observed by phase contrast microscope after treatments either with the extracts or Dox (data not shown). The main changes were decreased cell density, increasing of rounded and detached cells (that may indicate cell death), and cell shrinkage.

Only extracts having an IC 50 ≤200 μg/mL and that did not induce direct cytotoxic effect were used in the following assays, namely to assess the clonogenic potential, the percentage of DNA damage and the ability to induce cell death. Therefore, extracts E2 and E3 were selected for HT29, HCT116, MCF7, and A375 (only for E2) cell lines.

A clonogenic assay was performed in order to assess whether the crude fungal extracts possess a long-term cytostatic effect on the ability of a single cell to proliferate into a viable colony. [24] Cells previously exposed to the extracts for 48 h were allowed to grow in fresh medium for 10 days, dyed, and afterward all colonies formed by more than 50 cells were counted. As shown in [Figure 2], E2 exhibited a significant decrease of clonogenic potential by over 90% in all tested cell lines, more specifically in HT29 (94%), HCT116 (97%), A375 (99%), and MCF7 (97%) cancer cell lines, when compared with the control (DMSO). E3 also exhibited a strong reduction of the proliferative ability of a single cell to form a viable colony in MCF7 (93%) and moderate inhibition in HCT116 (25%) cells. Dox showed nearly total inhibition of clonogenic potential in all cell lines tested. These results show that both E2 and E3 (except for E3 in HT29 cells) not only affect a short-term cell proliferation, which is in line with the MTT reduction assay, but also have an impact over indefinite proliferation mechanisms. {Figure 2}

DNA damage was determined quantitatively by a single-cell electrophoresis assay or comet assay [Figure 3]. HCT116, MCF7, and A375 cells were exposed to the IC 50 concentrations of E2 and E3 [Table 1] and Dox (as a positive control) for 4, 24, and 48 h prior to the comet assay. Most of the cancer cell lines tested presented a consistent increase in DNA strand breaks by one or more of the extracts, with an exception of HT29 colon cell line (data not shown), in which neither extract (E2 and E3) produced the effect. In the MCF7 cells, E2 (26%) and E3 (13%) significantly induced DNA damage relative to untreated control cells. Moreover, E2 also induced significant DNA damage in A375 (22%) and HCT116 cells (11%) in relation to the negative control. However, E3 did not exhibit a significant difference as to the negative control in HCT116 cells. Drugs that are able to induce consistent DNA damage may lead to cell death induction if the DNA damage is not repaired and allowed to build up. [25]{Figure 3}

In order to discern whether the anti-proliferative effect observed through the MTT reduction assay was due to the induction of cell death, nuclear condensation was analyzed after cells were subjected to a 48 h exposure to the respective IC 50 concentrations of E2 and E3 [Figure 4]. When analyzing nuclear condensation, a significant increase in the number of cell death was detected in most of the tested cell lines after treatment with E2 and E3. E3 exhibited a 19% increase in cell death in HCT116 cells, and a 10% increase in MCF7 cells in relation to the negative control. E2 presented a broader induction of cell death, having induced a significant increase of cell death in all tested cell lines. In colon carcinoma cell lines, E2 leads to an increase of 12% in HT29 cells, and 17% in HCT116 cells in relation to the negative control. Moreover, E2 presented a 13% increase in A375 cells, and 17% in MCF7 cells, in comparison to the negative control. Interestingly, E2 showed higher induction of cell death than the positive control Dox in HCT116 and MCF7 cell lines. As referred earlier, some compounds are previously isolated from E2 and E3 showed anti-proliferative effects, however their effects on cell death are still unknown.{Figure 4}

In summary, E2 was found to decreased cell proliferation in HT29, HCT116, A375, and MCF7 cells at both short- and long-term, by decreasing the proliferative ability of a single cell to form a viable colony. Besides the anti-proliferative effects, E2 also showed an induction of cell death, in part, due to the induction of DNA damage. In turn, E3 demonstrated an anti-proliferative effect at both short- and long-term, and cell death induction in HCT116 and MCF7 cells. However, the mechanisms involved in the induction of cell death seem to be somewhat different since E3 did not induce DNA damage in HCT116 cells. E3 was also found to decrease proliferation in HT29 cells, however this decrease was only observed at short-term and without effect on cell death.

CONCLUSION

These results show, for the first time that, the crude ethyl extracts of N. laciniosa (KUFC 7896) and N. fischeri (KUFC 6344) exert an anticancer activity in human colon carcinoma, breast adenocarcinoma, and malignant melanoma cells by decreasing cell proliferation and increasing cell death. The investigation of transduction pathways and molecular targets involved in the anticancer effects of these extracts should be carried out.

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Conflicts of interest

There are no conflicts of interest.

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