Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
21(2): 239-243, Mar./Apr. 2011

Search for cytotoxic agents in multiple *Laurencia* complex seaweed species (Ceramiales, Rhodophyta) harvested from the Atlantic Ocean with emphasis on the Brazilian State of Espírito Santo

Article

Erika M. Stein,[1,2] Daniel X. Andreguetti,[1] Cleidiane S. Rocha,[1] Mutue T. Fujii,[2,3] Mauricio S. Baptista,[1] Pio Colepicolo,[1] Guilherme L. Indig[*,4]

[1]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Brazil,*
[2]*Departamento de Botânica, Instituto de Biociências, Departamento de Botânica, Universidade de São Paulo, Brazil,*
[3]*Instituto de Botânica, Secretaria do Meio Ambiente, Brazil,*
[4]*Department of Chemistry and Biochemistry, University of Wisconsin, USA.*

Received 15 Dec 2010
Accepted 5 Jan 2011
Available online 22 Apr 2011

Keywords:
macroalgae
Laurencia complex
anti-cancer activity
MES-SA
MES-SA/Dx5
Hela

Abstract: The development of new anti-cancer drugs of algal origin represents one of the least explored frontiers in medicinal chemistry. In this regard, the diversity of micro- and macroalgae found in Brazilian coastal waters can be viewed as a largely untapped natural resource. In this report, we describe a comparative study on the cytotoxic properties of extracts obtained from the *Laurencia* complex: *Laurencia aldingensis, L. catarinensis, L. dendroidea, L. intricata, L. translucida,* L. sp, and *Palisada flagellifera.* All of these species were collected in the coastal waters of the State of Espírito Santo, Brazil. Four out of the twelve samples initially investigated were found to show significant levels of toxicity towards a model tumor cell line (human uterine sarcoma, MES-SA). The highest levels of cytotoxicity were typically associated with non-polar (hexane) algal extracts, while the lowest levels of cytotoxicity were found with the corresponding polar (methanol) extracts. In this report, we also describe a biological model currently in development that will not only facilitate the search for new anti-cancer drug candidates of algal origin, but also permit the identification of compounds capable of inducing the destruction of multi-drug resistant tumors with greater efficiency than the pharmaceuticals currently in clinical use.

ISSN 0102-695X
doi: 10.1590/S0102-695X2011005000069

Introduction

Out of the more than 25,000 known species of algae, only about fifteen are currently grown on a large-scale for biotechnological applications (Raja et al., 2008). Algae are considered to be a largely untapped natural resource and investigations involving them are one of the least explored frontiers of biotechnology and natural products chemistry (Pinto et al. 2000; Cardozo et al., 2007; Chakabourty et al., 2009). Significant advances in the discovery and development of new anti-cancer drugs of algal origin have been recently reported in the literature. Newly identified algal drug candidates include representatives of the terpene, steroid, and polyketide families (Mayer & Gustafson, 2008). Drug-induced over-expression of the multi-drug efflux pump P-glycoprotein (P-gp) is thought to be one of the leading causes of chemotherapy failure in clinical oncology (Higgins, 2007; Dawson & Locker, 2006; Lage, 2008; Perez-Thomas, 2006). Because P-gp can remove many unrelated chemotherapeutic agents from the target cells, including agents to which the tumor had not been previously exposed (Higgins, 2007; Dawson & Locker, 2006), these cells are referred to as multi-drug resistant (MDR) mutants.

One of the most gratifying findings that can arise from any drug screening program is the identification of new drug candidates that are capable of eliminating MDR tumor cells more efficiently than the drugs currently in clinical use. The discovery of new, more benign inhibitors of such drug efflux pumps can also be facilitated by the biological model currently in development in our

laboratories.

In this report, we describe our initial results on the search for novel anti-cancer agents in marine algae found on the coast of the Brazilian State of Espírito Santo. We also outline a biological model currently in development to guide our screening efforts. This model aids in the identification of potential new anti-cancer candidates and, at the same time, of those that should be less susceptible to the action of promiscuous therapy-induced drug-efflux pumps such as P-gp.

Materials and Methods

Reagents and cell culture media

McCoy's 5A modified medium, Dulbecco's modified Eagle's medium (DMEM), doxorubicin hydrochloride, fungizone (amphotericin B), penicillin, streptomycin, thiazolyl blue tetrazolium bromide (MTT), ethylenediamine tetraacetic acid (EDTA; disodium salt), trypsin, and Trypan Blue were obtained from Sigma-Aldrich (St. Louis, USA). Fetal bovine serum (FBS) from Vitrocell Embriolife (Campinas, Brazil). NaCl from Synth (Diadema, Brazil), sodium bicarbonate from Merck (Darmstadt, Germany), D-glucose from Becto Chemical (São Paulo, Brazil), KCl from Cetus (São Paulo, Brazil), Dulbecco's phosphate buffered saline (DPBS) from Gibco BRL (Grand Island, USA), and dimethylsulfoxide (DMSO) from Vetec Química Fina Ltda. (Duque de Caxias, Brazil) were all of high-purity grade and used as received. Water was distilled, deionized and filtered prior to use (Millipore Milli-Q system; resistivity, 18 MΩ cm).

Instruments and methods

Stock solutions of doxorubicin were prepared in water and their respective concentrations determined by absorption spectroscopy on a Shimadzu (Kyoto, Japan) Model UV-1650 PC spectrophotometer employing a molar extinction coefficient of 11,500 M^{-1} cm^{-1} at 480 nm (Zeman et al., 1998).

Algal extracts were prepared via initial sonication (30 min) of approximately 30 g of the respective powdered and dried biomass with 300 mL of hexane. After standing overnight, the mixture was filtered and the solid mass subjected to two more cycles of extraction with hexane. The hexane-extracted biomass was then further processed by performing three extraction cycles with chloroform followed by three cycles of methanol extraction. The solvent was evaporated from the filtrates on a rotary evaporator to obtain three distinct classes of algal extracts, namely the hexane extract, the chloroform extract, and the methanol extract. In selected experiments, we extracted the original algal dry powder sample only with methanol; these extracts are referred to here as "crude" methanol extracts. Stock solutions of the final algal extracts were prepared in either DMSO or ethanol and subsequently diluted in the cell growth media in order to maintain the final concentration of organic solvent in the medium below 0.5% (by volume). The species of algae investigated are listed in Table 1.

Table 1. Species of *Laurencia* complex seaweed investigated in this study. Voucher specimens were deposited in the Herbarium Maria Eneida P. Kauffman Fidalgo at the Instituto de Botânica, São Paulo, SP, Brazil.

Algae species	Collect information	Voucher number
Laurencia aldingensis Saito & Womersley	ES, Anchieta, Ponta dos Castelhanos 01/07/2007	SP 399.933
Laurencia catarinensis Cord-Mar & M.T. Fujii	ES, Anchieta, Ponta dos Castelhanos 01/07/2007	SP 400.209
Laurencia dendroidea J. Agardh	ES, Anchieta, Praia de Parati 30/06/2007	SP 400.198
Laurencia intricata J.V. Lamour	ES, Guarapari, Praia de Meaípe 04/06/2008	SP 400.793
Laurencia sp.	ES, Anchieta, Ilhote de Ubu 04/06/2008	SP 399.936
Laurencia translucida M.T. Fujii & Cord-Mar	ES, Anchieta, Praia de Parati 30/06/2007	SP 400.213
Palisada flagellifera (J. Agardh) K.W. Nam	ES, Anchieta, Ponta dos Castelhanos 03/06/2008	SP 400.203

The trypan blue dye exclusion assay (Perry et al., 1997) was routinely employed to assess the quality of our original cell cultures; in all experiments performed with cell cultures, cell viability was at least 95%. All experiments were carried out with cells in the exponential growth phase. Cell viability assays were carried out using the MTT assay (Mosmann, 1983; Garcia-Peres et al., 2010). For this purpose, 96-well flat-bottomed microtiter plates were seeded at a cell density of 5,000 cells per well (100 µL of growth medium per well) and the cells allowed to attach and grow for 24 h. The sub-culturing protocols used in this study were always in accord with the respective ATCC recommendations. The cells were subsequently exposed to growth medium containing either the standard drug (doxorubicin) or the algal extracts for a period of time that typically varied from 24 to 72 h prior to MTT analysis. The MTT assays were performed on a Model Infinite M200 multi-well plate reader from Tecan (Männedorf, Switzerland). Cells exposed to the growth medium alone or to the growth medium plus the drug/extract vehicle (DMSO or ethanol) served as the controls. Under our experimental conditions, no significant cytotoxicity was observed for the vehicle (organic solvent used to prepare the stock solutions). Cell mortality was expressed as the viability of cells treated with the drug or extract relative to that of the corresponding controls.

Cell Lines

The MES-SA human uterine sarcoma cell line (ATCC CRL-1976™) and the corresponding doxorubicin-resistant mutant (MES-SA/Dx5, ATCC CRL-1977™) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). These cells were grown in modified McCoy's 5A medium supplemented with 10% FBS, amphotericin B (0.63 µg/mL), penicillin (100 units/mL) and streptomycin (100 µg/mL). The medium used to grow the MES-SA/Dx5 mutants also contained doxorubicin (0.5 µM), as required for the maintenance of the drug-resistance phenotype (Harker, 1983; Harker & Sikic, 1985; Larroque-Lombard et al., 2010). HeLa (CCL-2™) human cervix adenocarcinoma cells were kindly provided by Dr. Mari Cleide Sogayar (Departamento de Bioquímica, Universidade de São Paulo) and grown in DMEM medium, supplemented as described above for the McCoy's medium used to grow the MES-SA cells. Cells were grown at 37 ºC in a humidified atmosphere of 5% CO_2 in air.

Results and Discussion

In this initial screening, we explored the extent to which the chemical agents extracted from a variety of *Laurencia* complex seaweed species exhibited cytotoxicity effects toward our primary model tumor cell line (MES-SA). For this purpose, we initially exposed cultures of MES-SA cells to mixtures containing substantial amounts of the algal extracts (*i.e.*, 500 µg of algal extract/mL of cell culture media), subsequently measuring the cell survival rates after 24 h of continuous exposure to the extract. Table 2 shows that substantial cytotoxicity was associated with some, but not all, of these algal extracts. The methanol extracts obtained by our sequential extraction protocol (*i.e.*, hexane/chloroform/methanol) showed the lowest levels of toxicity, even lower than that of the crude methanol extracts obtained via direct methanol extraction of the original algae dry powder samples. The somewhat higher toxicity of the crude methanol extracts relative to the sequential methanol extracts is presumably the result of the presence of less polar chemical entities in the former that are absent in the latter extract because of removal during the prior extractions with the less polar solvents. Indeed, the hexane extracts of *Laurencia catarinensis* Cord.-Mar. & M.T. Fujii, *L. dendroidea* J. Agardh, and *L. translucida* M.T. Fujii & Cord.-Mar all show high levels of cytotoxicity. Likewise, the chloroform extract of *L. dendroidea* also exhibits a high degree of cytotoxicity, but the corresponding extract of *L. aldingensis* does not (Table 2).

Table 2. Relative cytotoxic effects of algal extracts toward MES-SA cells.

Algae species	Extracts	Relative toxicicity
L. aldingensis	chloroform	-
L. aldingensis	methanol	-
L. catarinensis	hexane	++
L. catarinensis	methanol	- -
L. dendroidea	hexane	++
L. dendroidea	chloroform	++
L. dendroidea	methanol	-
L. intricata	methanol	-
L. translucida	hexane	++
L. translucida	methanol	- -
L. sp	methanol (crude)*	+
P. flagellifera	methanol (crude)*	+

Key: (++) ≥ 80% cell mortality; (+) 50-79% cell mortality; (-) 30-50% cell mortality; (--) <30% cell mortality. Cells were incubated for 24 h in the presence of algal extracts (500 µg/mL of growth medium).* See Materials and Methods.

The overall trends observed in Table 2 were further confirmed by a subsequent experiment employing the highly cytotoxic hexane extracts of *L. dendroidea* and *L. translucida* and the methanol extract of *L. translucida*. In this second study, MES-SA cells were exposed for 72 h to variable concentrations of the extracts and cell viability again measured by the MTT assay. Figure 1 confirms that the methanol extract of *L. translucida* is virtually non-toxic, while the corresponding hexane extract of the same alga is highly toxic, with an apparent IC50 of about 16 µg/mL. The IC50 associated with the hexane extract of *L. dendroidea* was ca. 5-fold higher (*i.e.*, 91 µg/mL) than that of the hexane extract of *L. translucida*. For comparative purposes, an analogous experiment carried out with doxorubicin (a classical and potent anti-cancer drug) gave an ID50 of 0.05 µM (*i.e.*, 0.03 µg/mL) for MES-SA cells (Figure 2).

The use of MES-SA cells for this screening was motivated in part by the fact that these cells grow quickly (doubling time of *ca*. 24 h), show desirable morphology, are large enough to facilitate counting/analysis of drug-induced mechanisms of damage via microscopic analysis, are highly susceptible to a large variety of anti-cancer drugs (*i.e.*, relatively easy to perform *in vitro* assays of mortality), and are also easy to manipulate and maintain (Larroque-Lombard, 2010; Lacerda et al., 2005; Belostotsky et al., 2011). The susceptibility of MES-SA cells to doxorubicin is analogous to that observed for HeLa cells (compare panels A and B in Figure 2). HeLa cells have been extensively used in pre-clinical studies and, for this reason, the inclusion of this cell line in selected screening experiments may facilitate the comparison of the

potency associated with putative new drug candidates with that of previous candidates studied with HeLa cells. On the other hand, the MES-SA line has a feature that is quite attractive in terms of the development of novel screening protocols: the existence of a commercially-available MES-SA mutant (namely the MES-SA/Dx5 line) that is also easy to work with and maintain and shows the classical multi-drug resistance phenotype (*i.e.*, over-expression of the drug efflux pump, P-gp). Therefore, the inclusion of this mutant cell line in any screening protocol permits the identification not only of novel anti-cancer agents capable of inducing the mortality of MDR tumor cells, but also of novel inhibitors of P-gp. For comparative purposes, the ID50 for doxorubicin was 0.05 µM for the wild type cell line (MES-SA), but almost two orders of magnitude higher (*i.e.*, 3.4 µM; see Figure 2, panel A) for the MDR mutant (MES-SA/Dx5). Analogous differences in the response of these cell lines to doxorubicin have been reported by others authors (Larroque-Lombard et al., 2010).

Conclusions

Our initial studies indicate that four of the twelve extracts of the *Laurencia* complex seaweeds currently under investigation exhibit significant cytotoxicity towards MES-SA cells. We are cautiously optimistic with respect to these preliminary findings and further investigations designed to isolate and identify

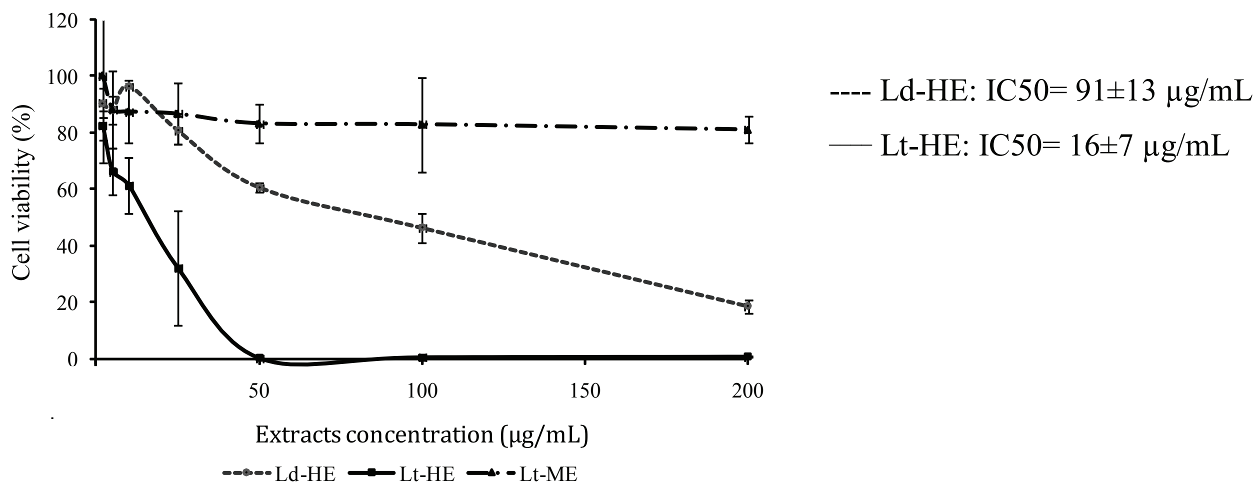

Figure 1. Cytotoxic effects of algal extracts toward human uterine sarcoma (MES-SA) cells. From the top, at 100 µg extract/ml of growth medium: Lt-ME, methanol extract of *Laurencia translucida*; Ld-HE, hexane extract of *Laurencia dendroidea;* Lt-HE, hexane extract of *Laurencia translucida.* The cell cultures were incubated for 72 h in the presence of the algal extracts.

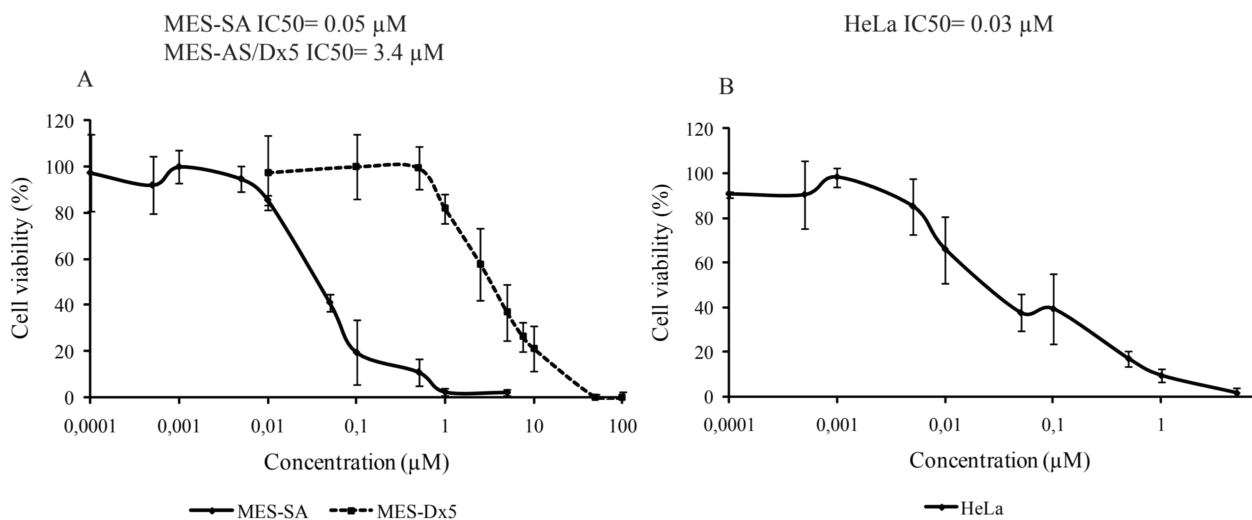

Figure 2. Cytotoxic effects of doxorubicin on the three tumor cell lines. Panel A: human uterine sarcoma (MES-SA) and its corresponding multi-drug resistant mutant (MES-SA/Dx5). Panel B: human cervix adenocarcinoma (HeLa) cells.

novel and potent anti-cancer drug candidates from these extracts are already in progress. The biological model under development here is currently being used for a wider systematic screening of algal species present in waters along the Brazilian seashore. This should expand our knowledge of the presence of cytotoxic compounds in Brazilian algal species and may identify novel candidate compounds for the more effective treatment of multi-drug resistant tumors (Higgins, 2007; Dawson & Locker, 2006; Lage, 2008; Perez-Tomas, 2006). In addition, we envision that our biological model should also facilitate the search for novel chemical inhibitors of P-gp that show positive synergism with classical anti-cancer drugs. Thus, any enhancement in MES-SA/Dx5 cell mortality (*e.g*., at ID20) in the presence of doxorubicin plus a non-toxic extract relative to that of doxorubicin alone would suggest the possible presence of P-gp inhibitors or other agents (*e.g*., pro-apoptotic factors) in the extract. Hence, in our future screening studies, even algal extracts that do not show cytotoxic properties will be tested for synergy with the standard drug doxorubicin.

Acknowledgment

This work was supported by the Brazilian research funding agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo a Pesquisa do Estado de São Paulo and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Ministério da Saúde, Ministério de Ciência e Tecnologia and CNPq-INCT-Redoxoma.

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*Correspondence

Erika M. Stein
Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo
Av. Prof. Lineu Prestes, 748, Bloco 03 superior, Cidade Universitária, 05508-900 São Paulo-SP, Brazil
glindig@uwm.ed
Tel: +1 414 229 5034
Fax: +1 414 229 5530