Reactive oxygen species mediated apoptosis of esophageal cancer cells induced by marine triprenyl toluquinones and toluhydroquinones

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Abstract

Marine invertebrates, algae, and microorganisms are prolific producers of novel secondary metabolites. Some of these secondary metabolites have the potential to be developed as chemotherapeutic agents for the treatment of a wide variety of diseases, including cancer. We describe here the mechanism leading to apoptosis of esophageal cancer cell lines in the presence of triprenylated toluquinones and toluhydroquinones originally isolated from the Arminacean nudibranch Leminda millecra. Triprenylated toluquinone–induced and toluhydroquinone–induced cell death is mediated via apoptosis after a cell cycle block. Molecular events include production of reactive oxygen species (ROS), followed by induction and activation of c-Jun (AP1) via c-Jun-NH2-kinase–mediated and extracellular signal-regulated kinase–mediated pathways. Partial resistance to these compounds could be conferred by the ROS scavengers Trolox and butylated hydroxyanisol, a c-Jun-NH2-kinase inhibitor, and inhibition of c-Jun with a dominant negative mutant (TAM67). Interestingly, the levels of ROS produced varied between compounds, but was proportional to the ability of each compound to kill cells. Because cancer cells are often more susceptible to ROS, these compounds present a plausible lead for new antiesophageal cancer treatments and show the potential of the South African marine environment to provide new chemical entities with potential clinical significance. [Mol Cancer Ther 2007;6(9):2535–43]

Introduction

Despite the current focus on new chemical entities generated by combinatorial synthetic programs, natural products (secondary metabolites) still remain an extremely promising reservoir of chemotherapeutic lead compounds, with over 60% of currently used chemotherapeutic agents derived or developed from natural sources (1, 2). Many marine organisms (invertebrates, algae, and microorganisms) produce natural products as a chemical defense against predation or in a response to interspecies competition for limited resources (e.g., space on a reef or nutrients; ref. 3). Interestingly, the isolation and identification of marine natural products by chemists, working in tandem with pharmacologists, over the last three decades has provided important lead compounds for the pharmaceutical industry, especially in the field of new anticancer drug discovery (4–6). Marine biota are a unique reservoir of biomolecular diversity, not only because of the large number of species present in the oceans, but also because the marine environment presents very different physiological and ecological challenges compared with those experienced by terrestrial species. As a consequence of the differences between terrestrial and marine habitats, the initial expectation that marine organisms use unique biosynthetic pathways or exploit variations on established biosynthetic pathways to biosynthesize structurally unusual natural products has been validated by the complexity and novelty of the plethora of marine natural products isolated over the last 30 years (3, 7).

The southern African coastline has the potential to yield many useful compounds, as the high marine invertebrate species endemism in the coastal waters is associated with high secondary metabolite diversity (8). In our laboratory, we test compounds from a variety of sources for activity against squamous esophageal cancer cell lines (9–13). Key among these compounds are the natural products isolated from marine organisms and collected using SCUBA off the southern and east African coast (13). Squamous esophageal cancer cells were selected as a model system because this cancer is one of the most frequent cancers in males in South Africa, with an age standardized incidence rate of 16.22 per 100,000 (14), and is one of the primary causes of cancer-related deaths in South African Black males. Current therapy for esophageal cancer is complicated by the fact that the disease is normally diagnosed late once a large
tumor mass is present and begins to obstruct swallowing. Chemotherapy most often consists of a combined cisplatin/5-fluorouracil regimen, and if treatment is initiated at an early stage of the disease, complete remission occurs in 20% to 30% of treated patients (15). However, early diagnosis rarely occurs, and the 5-year survival rate is currently only 14% (16). Because other treatment modalities (radiotherapy and surgery) are not likely to improve this outlook in the near future, there is a need for more effective chemotherapeutic agents to be developed. Cervical cancer cells were included in the screening program because this disease is the leading cause of cancer deaths among women in South Africa, with a total age standardized incidence rate of 31.08 per 100,000, and is especially prevalent among Black women with an age standardized incidence rate of 38.46 per 100,000, one of the highest in the world (14). The high incidence is due to limited screening, and thus, the majority of cases of cervical cancer in South Africa, like esophageal cancer, are only diagnosed at a late stage.

An earlier natural product investigation of metabolites from South African marine organisms yielded a variety of novel chemical compounds from the endemic South African nudibranch L. millecra (17). The secondary metabolites isolated from L. millecra included a group of related triprenyl toluquinones and triprenyl toluhydroquinones, which exhibited similar cytotoxic activity to cisplatin in our culture system. These compounds induced cell cycle arrest and apoptosis in cultured cells, which was related to reactive oxygen species (ROS) production and activation of c-Jun/AP1. The limited yield of these compounds precluded further investigation; however, synthesis of these compounds and analogues is being pursued. Once an adequate supply of synthetic material is available, animal studies to test in vivo efficacy and to address the pharmacokinetic properties of these compounds will be done.

This study shows that the Southern African marine environment shows considerable promise as a source of potential chemotherapeutic lead agents for esophageal cancer.

Materials and Methods

Chemicals

The triprenylated toluquinones and toluhydroquinones used in this study were originally isolated from the nudibranch L. millecra (17), i.e., 5-methyl-2-[(2E,6′E)-3,7,11′-trimethyl-2,6′-dodecadien-9′-onyl]benzo-1,4-quinoine (KL153), 5-methyl-2-[(2E,7′Z)-3,7′,11′-trimethyl-2,7′-dodecadien-9′-onyl]benzo-1,4-quinoine (KL154), 5-methyl-2-[(2E,6′E)-3,7′,11′-trimethyl-2,6′-dodecadien-9′-onyl]-1,4-dihydroxybenzene (KL155), 5-methyl-2-[(2E,7′Z)-3,7′,11′-trimethyl-2,7′-dodecadien-9′-onyl]-1,4-dihydroxybenzene (KL156), 1-acetoxy-5-methyl-2-[(2E,7′Z)-3,7′,11′-trimethyl-2,7′-dodecadien-9′-onyl]-1,4-dihydroxybenzene (KL157), and 5-methyl-2-[(2E,6′E)-9′-hydroxy-3,7′,11′-trimethyl-2,5′,6′-dodecadienyl]-1,4-dihydroxybenzene (KL159). All test compounds were suspended in DMSO at 100 mmol/L and stored at −20°C.

Hydrocortisone, epidermal growth factor, doxycyclin, hydrochloride, cholera toxin, insulin, propidium iodide, butylated hydroxyanisol, and tocopherol were purchased from Sigma. Crystal violet was purchased from BDH. Cell culture media and supplements were purchased from Highveld Biological. Extracellular signal-regulated kinase (ERK) and β-tubulin antibodies were purchased from Santa Cruz. Phosphorylated p38, phosphorylated ERK, phosphorylated c-Jun-NH2-kinase and JNK (PhosphoPlus antibody kit 9250), and phosphorylated c-Jun and c-Jun (PhosphoPlus antibody kit 9260) antibodies were purchased from Cell Signaling. p38 antibody was purchased from Sigma. All other chemicals were analytic grade.

Cell Culture and Cell Proliferation Assays

Cells were maintained in a humidified, 37°C, 5% CO2 incubator. WHCO1 and WHCO6 cells (esophageal cancer cell lines; ref. 18), CaSki cells (a cervical cancer cell line), and TAM67 cells (CaSki cells stably transfected with the c-Jun dominant negative construct TAM67; refs. 19, 20) were cultured in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. TAM67 cells were provided as a gift from V. Leenar and M. Birrer and were maintained in media with 5 μg/mL blasticidin. ME180 cells (a cervical cancer cell line) were maintained in McCoy’s 5A medium supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. SiHa cells (a cervical cancer cell line) were maintained in MEM, 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. MCF12F cells (a benign breast epithelial cell line) were maintained in 1:1 DMEM/Hams F12 medium supplemented with 20 ng/mL epidermal growth factor, 0.1 μg/mL cholera toxin, 0.5 μg/mL hydrocortisone, and 10 μg/mL insulin.

Pictures of cells were obtained using a Zeiss Axiosvert 200 with an Axioscam camera and Axiovision 4.1 image software.

Crystal violet assays were done as previously described (13). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done using the MTT kit from Roche according to manufacturer’s instructions and as previously described (10, 12).

Cell Cycle Analysis

WHCO1 cells were seeded in 60-mm dishes at 0.25 × 10^6 cells per dish. After an overnight settling period, cells were treated with the indicated concentrations of KLM155. At appropriate time points, cells were harvested (including floating cells) and fixed in 70% ethanol at −20°C for up to 2 weeks. Cells (5 × 10^6 per mL) were incubated with 50 μg/mL RNase A in PBS for 30 min at room temperature. Staining solution (0.1% Triton X100, 2 mM/L MgCl2, 100 mM/L NaCl, 0.01 mol/L PIPES buffer, 10 μg/mL propidium iodide) was added, and cells were analyzed on a Beckman Coulter FACS-calibur flow cytometer. Analysis of cell cycle results was carried out using ModFit 3.0 (Verity Software House).

Apoptosis Assay

Caspase-3 activity in cells was used as a measure of apoptosis and was measured using the Caspase-Glo 3/7...
assay kit according to manufacturer’s instructions (Promega). Cells were seeded at 1,000 cells per well in 96-well plates in 60 μL. After 24 h incubation to allow cells to settle and, in some cases, a 2-h pretreatment with inhibitors, compounds were added as outlined and cells were incubated for the indicated time periods before processing. Luminescence was detected using a Luminoskan Ascent plate reader.

The induction of apoptosis was confirmed using a flow cytometric approach using a fluorescently labeled antibody to activate caspase-3 according to manufacturer’s instructions. Fluorescence was detected using a Beckman Coulter FACS-calibur flow cytometer.

**Necrosis Assay**

Analysis of the levels of necrosis was done using the CytoTox-ONE homogeneous membrane integrity assay (Promega) according to manufacturer’s instructions. At each time point, lysis buffer (supplied with the kit) was added to a set of wells as a positive control. Fluorescence was measured on a 96-well plate fluorimeter, and values were expressed as a percentage of the positive control.

**Measurement of ROS**

Cells were plated in 60-mm dishes at 5 × 10^5 cells per dish. After a 24-h incubation, the media was removed, cells rinsed with prewarmed Krebs-Ringer buffer [110 mmol/L NaCl, 2.6 mmol/L KCl, 1.2 mmol/L MgSO_4, 1.2 mmol/L KH_2PO_4, 25 mmol/L NaHCO_3, 11 mmol/L glucose (pH 7.4)], and 2 mL of 25 μmol/L DCF-DA in Krebs-Ringer buffer was added. After a 15-min incubation at 37 °C, 5% CO_2 in the dark, test compound was added and cells were incubated for another hour. Cells were then harvested using trypsin, centrifuged, resuspended in 1 mL Krebs-Ringer buffer, and analyzed by flow cytometry on a FACS-calibur flow cytometer.

**Western Blotting**

Cells were harvested in 200 μL of radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% Triton X100, 0.1% SDS, 10 mmol/L Tris (pH 7.5), 1% deoxycholate] with protease inhibitor (Complete tablets, Roche), and lysates were stored at −20 °C. After sonication and protein determination using the Pierce BCA kit, equal amounts of protein were loaded on a 15% polyacrylamide gel and subjected to electrophoresis at 200 V. After transfer at 100 V for 1 h, membranes were stained with Ponceau S to assess protein loading and then blocked with 5% fat-free milk in TBS with 0.1% Tween 20 for 1 h. After incubation with the indicated primary antibody and horseradish peroxidase–conjugated antirabbit or antimouse secondary antibody, detection using either LumiGLO Reserve Chemiluminescent Substrate kit (KPL) or Extended West Dura Super Signal (Pierce) was carried out. Bands were visualized by autoradiography, after which the blots were stripped and reprobed for β-tubulin as a loading control.

**Statistical Analysis**

Unless otherwise indicated, error bars indicate SD.

For ROS determination, each experimental condition was carried out in triplicate, and results are the mean ± SD and are representative of two or more independent experiments.

Apoptosis experiments and determinations of cell number by MTT assay were carried out in quadruplicate, and results are the mean ± SD and are representative of two or more independent experiments.

Each concentration for IC_{50} determination was tested in quadruplicate, and each IC_{50} determination was repeated.
IC50 results are presented as the mean of 2 or 3 independent

cisplatin 16.5 13.5 – 20.3

KLM159 32.7 32.5 – 33.0

KLM157 42.7 42.6 – 42.7

KLM156 12.9 12.5 – 13.3

KLM155 9.5 9.4 – 9.5

KLM154 83.3 82.1 – 84.5

KLM153 37.9 37.7 – 38.0

lular proliferation), higher concentrations clearly decreased

cytotoxic or cytostatic effect. Whereas lower concentrations

reduction in cell number seen in screening was due to a

with or without treatment to determine whether the

control (Table 2). KLM155 displayed cytotoxicity against

and SiHa, and the benign breast epithelial line MCF12A as a

lines WHCO1 and WHCO6, cervical cancer cell lines ME180

ref.12). The most active compound (KLM155) was evaluated

alter activity, with the most active compounds being those

Whereas the structures of these compounds are very similar,

seen at low concentrations typical of treatment with a ROS-

in several ways, with the increased growth of WHCO1 cells

initial screening and activity results confirmed this hypothesis.

Our

consideration of an autooxi-

membrane blebbing (data not shown).

Cell Death Induced by the Toluydroquinone KLM155

is Associated with a Cell Cycle Block and Apoptosis

Considering the rounded cell morphology observed in cells

treated with KLM155, cell cycle analysis was done to
determine the effect of KLM155 on the cell cycle. Treatment

with KLM155 at 20 μmol/L led to an increase in the G2

population which persisted to 48 h (Fig. 2A). This was

accompanied by a decrease in cell number of 51% at 24 h and

82% at 48 h.

We assessed the ability of KLM155 to induce apoptosis in

WHCO1 cells by measuring the activity of caspase-3/

caspase-7. Little apoptosis was observed at the lower

concentrations with apoptosis levels at 20 μmol/L

increasing to almost twice of that seen in untreated cells

at 24 h (P < 0.001; Fig. 2B). The induction of apoptosis was

confirmed using a flow cytometric approach using a

fluorescently labeled antibody to activated caspase-3

(Fig. 2C) and was similar to that observed for doxorubici-

treated cells. The lack of a sub-G1 peak of apoptotic

cells in the cell cycle analysis is most likely due to the

extended processing of cells for this assay (multiple

washes and spins), during which this population may

have been lost. We confirmed that the decrease in cell

number seen was not due to necrosis by assessing levels

of necrosis using the CytoTox-ONE homogeneous mem-

brane integrity assay and showed no significant increase

in necrosis (Fig. 2D).

Toluquinones and Toluhydroquinones Generate ROS

in Cultured Cells, and ROS Generation Is Proportional

to Cell Death

We hypothesized that the family of compounds discussed

here act by generating ROS based on their structures. Our

initial screening and activity results confirmed this hypothe-

sis in several ways, with the increased growth of WHCO1 cells

seen at low concentrations typical of treatment with a ROS-

producing compound (21) and the activity variation with

structure indicating the possible involvement of an autooxi-

dative process for the hydroquinones (22). The compounds

Table 1. IC50 values for each compound against the esophageal cancer cell line WHCO1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μmol/L)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLM153</td>
<td>37.9</td>
<td>37.7–38.0</td>
</tr>
<tr>
<td>KLM154</td>
<td>83.3</td>
<td>82.1–84.5</td>
</tr>
<tr>
<td>KLM155</td>
<td>9.5</td>
<td>9.4–9.5</td>
</tr>
<tr>
<td>KLM156</td>
<td>12.9</td>
<td>12.5–13.3</td>
</tr>
<tr>
<td>KLM157</td>
<td>42.7</td>
<td>42.6–42.7</td>
</tr>
<tr>
<td>KLM159</td>
<td>32.7</td>
<td>32.5–33.0</td>
</tr>
<tr>
<td>cisplatin</td>
<td>16.5</td>
<td>13.5–20.3</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with a range of concentrations of each compound, and final cell number was determined using the MTT assay.

Results

Treatment with Triprenyl Toluquinones and Toluydro-

quinones Induces Cell Death in Cultured Human Cancer

Cells

As part of our ongoing screening program (9–13), to

identify potential lead compounds for use in the treatment of

esophageal cancer, we tested six quinone-related com-

pounds (KLM153–KLM157, KLM159; Fig. 1A) isolated from

the nudibranch L. millecra (17) for cytotoxic activity against

the esophageal cancer cell line WHCO1. Initial results from

a crystal violet assay indicated that these compounds were

active, with all causing

95% mortality after 48 h at

50 μg/mL. At 1 μg/mL, a slight increase in cell number was

observed with some compounds (data not shown).

After the initial, promising results in the screening assay,

these compounds were evaluated for their cytotoxic activity

using the MTT assay to determine IC50 values (Table 1). Whereas

the structures of these compounds are very similar,

slight changes in substitution pattern on the ring or

geometry of the double bonds in the side chain seemed to

alter activity, with the most active compounds being those

with a nonesterified hydroquinone nucleus (e.g., KLM155

and KLM156) with activity similar to cisplatin (16.5 μmol/L; ref. 12).

The most active compound (KLM155) was evaluated

against a range of cell lines, including esophageal cancer cell

cells WHCO1 and WHCO6, cervical cancer cell lines ME180 and SiHa, and the benign breast epithelial line MCF12A as a

control (Table 2). KLM155 displayed cytotoxicity against

most of the cell lines tested, with the exception of the cervical

cancer cell line SiHa.

Cell growth (Fig. 1B) and death (Fig. 1C) were evaluated

with or without treatment to determine whether the

reduction in cell number seen in screening was due to a

cytotoxic or cytostatic effect. Whereas lower concentrations

of KLM155 served only to slow cell growth (decrease cell-

ular proliferation), higher concentrations clearly decreased

cell number to levels below that measured at the time of

treatment (indicated by the dotted line; Fig. 1C). The cyto-

toxic effect measured by MTT was associated with an

altered morphology in treated cells, including cell rounding

and membrane blebbing (data not shown).

Table 2. IC50 values for KLM155 against the esophageal cancer cell lines WHCO1 and WHCO6, the nonmalignant breast epithelial cell line MCF12, and the cervical carcinoma cell lines ME180 and SiHa

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (μmol/L)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHCO1</td>
<td>9.5</td>
<td>9.4–9.5</td>
</tr>
<tr>
<td>WHCO6</td>
<td>5.8</td>
<td>5.6–6.0</td>
</tr>
<tr>
<td>MCF12</td>
<td>32.0</td>
<td>31.6–32.3</td>
</tr>
<tr>
<td>ME180</td>
<td>33.9</td>
<td>33.7–34.1</td>
</tr>
<tr>
<td>SiHa</td>
<td>&gt;150</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with a range of concentrations of each compound, and final cell number was determined using the MTT assay.
were assessed for their ability to generate ROS in WHCO1 cells using the fluorescent probe DCF-DA. ROS induction (3.3-fold above untreated) was observed for KLM155 at 20 μmol/L (P < 0.001), but not at 5 μmol/L, and only a slight (1.3-fold) increase was observed at 10 μmol/L (P < 0.05; Fig. 3A). ROS production occurred within 15 min of treatment and increased steadily over 2 h (Fig. 3B). When the production of ROS in WHCO1 cells treated with 20 μmol/L of each compound was compared with the IC50 values obtained for this cell line, a linear relationship between the two was identified (Pearson’s correlation r = -0.818, P = 0.025; Fig. 3C). This in particular suggests that the cytotoxic activity of these compounds is directly linked to their ability to produce ROS.

Treatment with Toluhydroquinone KLM155 Results in Activation of Several Mitogen-Activated Protein Kinase Signaling Molecules

The production of ROS in cells is often associated with the activation of mitogen-activated protein kinase pathways, e.g., JNK, ERK, and p38 (21, 23). We evaluated levels of phosphorylated p38, phosphorylated ERK, phosphorylated JNK, and phosphorylated c-Jun in treated cells at 6 and 24 h posttreatment (Fig. 4A).

Phosphorylated ERK levels were increased in a concentration-dependent manner at both time points. A slight induction of phosphorylated p38 was seen after treatment, and phosphorylated JNK levels did not increase except at the highest concentration, where a slight increase was seen, and phosphorylated c-Jun levels increased substantially at the highest concentration tested, but not at lower concentrations.

As expected, total ERK and total p38 levels were not altered. However, total JNK levels increased at 20 μmol/L KLM155 at 6 h and total c-Jun levels increased in a dose-dependent manner at both time points.

Because levels of phosphorylated c-Jun corresponded with ROS production and apoptosis induction, we further characterized the role of c-Jun/AP1 in toluhydroquinone-induced cell death using a model system available in our laboratory. CaSki cells are known to express high levels of AP1. We have available a CaSki cell line stably transfected with a doxycyclin-inducible dominant negative TAM67 construct. TAM67 is a c-Jun mutant lacking amino acids 3 to 122, removing the transactivating domain. TAM67 dimerizes with wild-type c-Jun, but because it lacks the transactivation domain, the dimers comprising c-Jun and...
TAM67 are unable to transcriptionally activate genes (19, 20). This cell line (referred to from here on as TAM67), together with the parental cell line (CaSki), was used to evaluate the role of c-Jun/AP1 in cellular response to treatment with the tolhydroquinone KLM155. Cell survival after treatment was increased in both the uninduced (82% survival) and induced (100% survival) TAM67 cell line compared with the parental line (53% survival; Fig. 4B). The increase in survival compared with the parental line is significant \( P < 0.01 \) even in the case of uninduced TAM67 cells. The increased survival observed in the uninduced cells is due to the leaky expression of the TAM67 protein, as confirmed by Western blot analysis (Fig. 4B, bottom).

**ROS Scavengers and a JNK Inhibitor Blocked KLM155-Induced Apoptosis**

To test whether the cell death observed was mediated via production of ROS, we treated cells in the presence and absence of the ROS scavengers Trolox and butylated hydroxyanisol and JNK inhibitor II and assessed the cells for apoptosis (Fig. 4C) and survival (Fig. 4D).

When ROS scavengers were added to cells before treatment with KLM155, a 73% (Trolox, \( P < 0.01 \)) and 66% (butylated hydroxyanisol, \( P < 0.05 \)) reduction in caspase-3 activity induced by KLM155 was observed (Fig. 4C), and treated cells survived better (Fig. 4D). The JNK inhibitor did not have as clear an effect as it decreased levels of apoptosis (Fig. 4C) but did not significantly increase cell survival (Fig. 4D).

**Discussion**

This paper describes the anticancer activities of a family of novel compounds isolated from the nudibranch *L. millecrata* collected from Algoa Bay, South Africa (17). Compounds KLM155 and KLM156 of this family of triprenylquinones and triprenylhydroquinones (Fig. 1) displayed moderate activity against cultured esophageal cancer cells, with IC\(_{50}\) values of 9.8 μmol/L and 12.9 μmol/L, respectively.

The ability of quinone and hydroquinone structures to act as ROS producers, as well as their more traditionally accepted role as ROS scavengers has been well-documented (22, 24). The ability of these compounds to inhibit proliferation in esophageal cancer cells was directly associated with the ability of these compounds to generate ROS. Firstly, we were able to directly measure ROS production in cells treated with these agents. Secondly, there was a strong inverse correlation between the IC\(_{50}\) values of the compounds tested and ROS production. And lastly, treatment of cells with the ROS scavengers butylated hydroxyanisol and Trolox reduced the extent of apoptosis caused by treatment with KLM155 (Fig. 4). Compelling evidence in the literature shows that ROS plays a central role in the antiproliferative activity of other agents that have potential as anticancer agents, including (a) 4-HPR in head and neck squamous carcinoma cell lines (25), (b) diallyl disulfide in neuroblastoma cells (26), (c) paclitaxel in T24 urothelial carcinoma cell lines (27), and (d) heteroarotinoids in head and neck squamous cell carcinoma cell lines (28).

Our observation that ROS production is a relatively early event (within 15 min) is consistent with other reports that show early ROS production in cells treated with a variety of compounds that block cell growth and induce apoptosis through ROS-mediated pathways (25, 26). Oxidative stress is known to trigger a variety of responses in cells reflecting...
among others, the cell type studied, the level of ROS produced, and the duration of ROS exposure. ROS is known to damage various cellular components, and the cellular response to this damage is generally to attempt repair (frequently associated with a cell cycle block), followed by cell death if the damage is irreparable. The cellular events we observe after treatment with KLM155, namely cell cycle arrest and apoptosis, are characteristics of ROS-induced cell death (26, 27).

Several cellular signaling pathways may play a role in ROS-mediated cell death; among these are the mitogen-activated protein kinase pathways including Ras/Raf/MAP/ERK kinase/ERK, p38, and stress-activated protein kinase/JNK. We evaluated the levels of key molecules in these signaling pathways and identified a slight dose-dependent increase of phosphorylated p38.

Both phosphorylated ERK and c-Jun levels increased in a dose-dependent manner. However, phosphorylated c-Jun and phosphorylated JNK levels increased at only the highest concentration of KLM155 tested (20 μmol/L). The increase in these proteins correlated with the levels of ROS produced (Fig. 3A) and levels of caspase activation (Fig. 2B).

The role of ROS in activating JNK (an important upstream activator of c-Jun) through the inactivation of
endogenous JNK inhibitors has already been documented (29, 30), providing a plausible pathway for the induction of c-Jun observed here. Our results strongly implicated JNK/c-Jun signaling in mediating KLM155-induced apoptosis, considering the induction of c-Jun subsequent to treatment with KLM155 and the observation that the JNK inhibitor abrogated KLM155-induced apoptosis. It was quite intriguing that while the JNK inhibitor significantly blocked the apoptosis induced by KLM155, this inhibitor had no significant effect on the reduction in cell number caused by KLM155 (Fig. 4C and D). This should be considered in the context of the results observed for Trolox, which blocked apoptosis to the same extent as the JNK inhibitor but was associated with a 20% reduction in cell number in response to treatment with KLM155. Presumably, the block in apoptosis caused by the JNK inhibitor occurs despite the ROS-induced cellular damage inflicted by treatment of cells with KLM155. This level of cellular damage is sufficient to induce a cell cycle block as shown in Fig. 2A, effectively preventing cellular proliferation. The severely damaged cells may also self destruct through nonapoptotic pathways if denied access to apoptosis, as previously described (31). Of course, the ability of Trolox and butylated hydroxyl-sol to block both apoptosis and the reduction in cell number caused by treatment with KLM155 reflects the proximal site of action of these ROS scavengers, allowing them to protect cells from extensive ROS-induced damage and thus precluding the requirement for apoptosis or alternative cell death pathways.

Cancer cells generally are exposed to much higher levels of oxidative stress than normal cells (32). This is based on direct measurements of ROS levels in tumor tissues (33), accumulation of oxidative products in tumor tissue (34), and the presence of oxidized DNA products in the urine of cancer patients (35). Of particular interest for this study are the elevated levels of superoxide dismutase measured in squamous cell carcinomas of the esophagus relative to normal tissues (36), suggesting that esophageal tumor tissues are also under increased oxidative stress. As a result of the constant assault of oxidative stress, cancer cells have an impaired ability to deal with oxidative stress (relative to normal cells), rendering them sensitive to additional oxidative challenges. This diminished ability of cancer cells to mount an effective response to further oxidative challenges may represent a critical therapeutic window that could be exploited to develop more selective anticancer agents. The central role of ROS in mediating the cytotoxic activity of the family of triprenylquinones and triprenylhydroquinones described here underscores the value of this novel family of compounds as potential lead chemotherapeutic agents for further structure/function analysis.

In fact, the close structural similarity of this family of compounds isolated from L. millecra allows us to make some tentative structure/activity observations. Comparing the effect of the side chain on activity, the all-trans conformation confers greater activity than the trans-cis conformation in both cases (KLM153 compared with KLM154; KLM155 compared with KLM156). The substitution pattern at the 1 and 4 positions yields varying activity with (KLM156 > KLM157 > KLM154: -OH > -OAc > =O) which corresponds with previous observations (37). Although the cell lines used in this paper differ from those evaluated by Li et al. (37), this observation may be of use in rational drug design and is possibly linked to the electron-donating ability of the hydroxyl compared with the carbonyl.

The strong correlation between the levels of ROS produced by each of the compounds and their IC50 values and the structural similarity of the family of compounds described here provide an opportunity to further explore the structural elements that confer activity in these molecules. This study and others (9–11) support the hypothesis that marine benthic organisms from the coast of southern Africa represent a rich, untapped repository of novel and interesting compounds that may hold promise in the development of new chemotherapeutic agents.

Acknowledgments

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References


Molecular Cancer Therapeutics

Reactive oxygen species mediated apoptosis of esophageal cancer cells induced by marine triprenyl toluquinones and toluhydroquinones

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