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Antitumour activity of 3-chlorodeoxylapachol, a naphthoquinone from *Avicennia germinans* collected from an experimental plot in southern Florida

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Abstract

As part of an ongoing collaborative effort to discover new anticancer agents from plants, an extract obtained from the leaves and twigs of *Avicennia germinans*, collected in a coastal area of southern Florida, was identified as possessing cytotoxic activity in a panel of human cancer cell lines. Fractionation of the petroleum ether partition, using cytotoxicity to guide the fractionation, led to the isolation of 3-chlorodeoxylapachol. The antitumour potential of 3-chlorodeoxylapachol was demonstrated with the in-vivo hollow fibre assay, a model of antitumour activity using human cancer cell-filled fibres implanted into mice. The possibility that this compound is an artefact of the isolation procedure was ruled out by liquid chromatography–mass spectrometry analysis of extracts prepared without the use of chlorinated solvent. In conclusion, 3-chlordeoxylapachol, a secondary metabolite obtained from the chloroform-soluble extract of a mangrove tree, was cytotoxic in a panel of human cancer cells, and active against KB human cancer cells in the murine hollow fibre antitumour model, with selectivity in KB cells for the intravenous site at lower doses, indicating possible metabolic activation.

Introduction

Natural products from vascular plants have contributed to the development of many clinically useful drugs (Newman et al 2003), and show great potential as sources of chemical diversity for drug discovery. Natural products are an apt source of evolutionarily selected, drug-like, structurally diverse molecules for drug discovery (Kinghorn 2001; Kingston & Newman 2002). The collection of plant material from areas of high biological diversity is thought to ensure the chemical diversity of compounds obtained, and thus collection in tropical and subtropical areas is of particular interest for natural product drug discovery (Kinghorn et al 2003).

As part of an ongoing collaborative effort to discover potential anticancer agents from terrestrial plant sources (Kinghorn et al 2003), plant samples from an experimental forest plot in southern Florida were collected following a plot-based selection method that ensures an unbiased collection of plant samples and permits reproducible recollection of plant material (Calderon et al 2000; Lobo-Echeverri, T., Chai, H.-B., Lee, D., Pezzuto, J.M., Swanson, S.M., Farnsworth, N.R., Kinghorn, A.D. & Soejarto, D.D., unpublished results). From these samples, a library of tannin-free chloroform extracts (Wall 1996) was prepared and screened for cytotoxicity against a panel of human cancer cell lines. In the present study, a sample of the combined leaves and twigs of *Avicennia germinans* (L.) L. was investigated by bioassay-guided fractionation in order to discover the chemical constituents responsible for the cytotoxic activity observed for this sample in the preliminary screening.

Avicennia is the sole genus in the Avicenniaceae (sometimes included in Verbenaceae), a family of mangrove shrubs and trees with erect pneumatophores and viviparous embryos (Mabberly 1997). Members of this family have adapted to the high salt levels

in the mangrove habitat by secreting salt from specialized glands in their leaves (Sobrado & Greaves 2000). Previous phytochemical and chemotaxonomic investigations within the genus have resulted in the isolation of iridoid glucoside (König et al 1987), lignan (Sharp et al 2001), phenylpropanoid (Fauvel et al 1993), and triterpenoid (Subramanian & Vedantham 1974) constituents. Itoigawa et al (2001) reported antitumour promoter activity for naphthoquinones and their analogues obtained from *Avicennia alba* and *Avicennia rumphiana*, and Sharaf et al (2000) described the isolation and identification of moderately cytotoxic flavonoids from the aerial parts of *Avicennia marina*. In the current study, 3-chlorodeoxylapachol was isolated as the cytotoxic principle from this plant sample, and the antitumour potential of 3-chlorodeoxylapachol was further evaluated in the in-vivo hollow fibre model.

The hollow fibre assay was developed by researchers at the US National Cancer Institute for the evaluation of potential anticancer agents in an in-vivo model of antitumour potential that would be less costly than tumour xenograft studies in terms of time, the number of animals required, and the quantities of test compounds needed (Casciari et al 1994; Hollingshead et al 1995). It is used in our programme as a secondary discriminator, to help prioritize selected in-vitro active compounds for additional in-vivo evaluation (Mi et al 2002; Kinghorn et al 2003). Tumour cells contained inside a semipermeable hollow fibre are implanted into immunocompromized mice at intraperitoneal and subcutaneous locations, and the test compound is injected intraperitoneally. In this way, the ability of a compound to demonstrate efficacy at the two physiological sites and against several cell lines at a number of dose levels is tested simultaneously. The assay gives a preliminary indication of how well the compound is tolerated by the host, allows estimation of the optimal dose range for subsequent in-vivo experiments, and may give a preliminary indication of compound pharmacokinetics (Hollingshead et al 1995).

Materials and Methods

General materials and equipment

Chromatographic solvents were distilled reagent grade, unless otherwise noted. Coarse (70–230 mesh) and fine (230–400 mesh) silica gel were obtained from Whatman (Clifton, NJ, USA). The mobile phase for the liquid chromatography–mass spectrometry (LC-MS) experiments consisted of acetonitrile (solvent A; Optima grade; Fisher Scientific, Fair Lawn, NJ, USA) and water (solvent B; HPLC grade; Mallinckrodt Baker, Phillipsburg, NJ, USA) with 0.1% formic acid (Laboratory grade, Fisher Scientific) added to both solvents. Indium powder, prenyl bromide, and 2,3-dichloronaphthoquinone were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethylformamide (DMF) was dried over a molecular sieve and purged of oxygen under a stream of argon.

Melting points were determined using a Fisher-Johns hot stage apparatus and are uncorrected. UV spectra were obtained on a Beckman DU-7 spectrometer. IR spectra were obtained with an ATI Mattson Fourier transform infrared spectrometer. Nuclear magnetic resonance (NMR) experiments were performed using Bruker DPX-300 and Bruker DRX-500 MHz spectrometers with tetramethylsilane as internal standard. Proton–proton correlation spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple-bond correlation spectroscopy (HMBC), and heteronuclear multiple-quantum coherence spectroscopy (HMQC) 2-D NMR experiments were carried out using default parameters. Electron-impact mass spectrometry (EIMS) and high-resolution chemical-ionization mass spectrometry (HRCIMS) data were obtained using a Finnigan MAT 95 sector field mass spectrometer. LC-MS experiments were carried out with an Agilent 1100 Series LCMSD instrument (Palo Alto, CA, USA), consisting of a single-quadrupole mass analyser with an electrospray interface coupled to an Agilent 1100 HPLC through an XTerra MS C_{18} column (2.1×150 mm, 5 μm; Waters, Milford, MA, USA), equipped with an XTerra MS C_{18} guard column (2.1×10 mm, 5 μm). Positive-ionization electrospray mass spectrometry used a N_2 gas nebulization pressure of 20 psi and a N_2 drying gas temperature of 325°C at a flow of 4.5 L min^{-1}. A positive capillary voltage of 3000 V and a fragmentor voltage of 80 V were used. The mobile phase flow rate was 200 μL min^{-1}.

Plant material and selection method

A sample of the leaves and twigs of *A. germinans* (L.) L. was collected at Matheson Hammock County Park, Miami-Dade County, Florida, USA, with permission from the Miami-Dade County Park and Recreation Department [collection permit no. 0014 (1999–2003)]. Separate samples of the leaves and the twigs were collected at the same site at a later date. Voucher specimens (TL-61 and TL-99) were deposited at the Field Museum of Natural History, Chicago, IL, USA. For the plot-based selection method, the vascular plant species in the plot were inventoried, and all trees with a diameter at breast height ≥5 cm were tagged to facilitate relocation. Samples of aerial plant parts for extraction were collected from inventoried species, and the samples were dried and processed for extraction.

Isolation procedure

The dried and milled leaves and twigs (825 g) of *A. germinans* were extracted with MeOH (3×2.5 L). The concentrated extract was suspended in aqueous MeOH (1:9 v/v, 500 mL) and partitioned with petroleum ether (5×250 mL), resulting in a dried combined petroleum ether extract (5.8 g). After concentrating the methanolic partition to a thick syrup, it was suspended in distilled, deionized water (500 mL) and partitioned with $CHCl_3$ (4×200 mL). Only the petroleum ether partition exhibited activity when tested against several human cancer cell lines (50% effective dose (ED50) = 15.8 μg mL^{-1} for LNCaP and 17.4 μg mL^{-1} for Lu1 cells). Accordingly, the petroleum ether partition was fractionated by normal-phase chromatography (260 g

coarse silica gel), eluting initially with 100% petroleum ether, then with increasing proportions of $CHCl_3$, followed by increasing proportions of MeOH in $CHCl_3$, affording 17 fractions (combined according to similarities among thin-layer chromatography profiles). Fraction 7 (89 mg, eluted with petroleum ether/$CHCl_3$, 6:4) was chromatographed (13.8 g fine silica gel) by elution with increasing proportions of ethyl acetate in petroleum ether. The combined fractions 2 and 3 (petroleum ether/ethyl acetate, 99:1) yielded 3-chlorodeoxylapachol (2 mg) after being further purified by preparative thin-layer chromatography (hexanes/ethyl acetate, 19:1; Merck, Darmstadt, Germany). 3-Chlorodeoxylapachol was the only active substance obtained from this plant part (Figure 1).

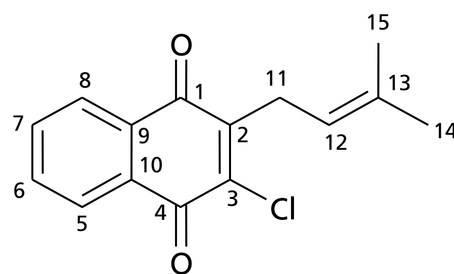

Figure 1 Structure of 3-chlorodeoxylapachol.

Cell culture and in-vitro cytotoxicity assay

Cytotoxicity assays were carried out using the following cell lines: Col2 (human colon cancer), KB (human oral epidermoid carcinoma), LNCaP (hormone-dependent human prostate cancer), Lu1 (human lung cancer), and hTERT-RPE1 (human telomerase reverse transcriptase-retinal pigment epithelium). The in-vitro cytotoxicity assays were performed following published protocols (Seo et al 2001; Mi et al 2002). In brief, the cell lines were incubated for 72 h under a controlled atmosphere in 96-well microtitre plates seeded with test compounds or chromatographic fractions. At the end of this period, the cells were fixed with aqueous trichloroacetic acid, and cell growth was measured using the sulforhodamine B protein-binding method (Skehan et al 1990). Pure compounds with ED50 values $\leq 5\,\mu g\,mL^{-1}$ ($\leq 20\,\mu g\,mL^{-1}$ for extracts and fractions) are considered sufficiently active to be considered for further evaluation (or fractionation).

In-vivo hollow fibre assay

The protocol for the hollow fibre assay has been described elsewhere (Hollingshead et al 1995) and was used in the present study with minor modification (Mi et al 2002). Briefly, the assay was performed using NCr nu/nu athymic mice (5–6 weeks old) obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD, USA). Colour-coded polyvinylidine fluoride fibres (500 000 Da cut-off, 1.0 mm i.d.; Spectrum Medical Industries, Lugman Hills, CA, USA) were filled with KB, LNCaP or hTERT-RPE1 cells and the fibres were clamped and heat-sealed. Each mouse was anaesthetized and received three fibres (one per cell line) in the intraperitoneal compartment and three in the subcutaneous compartment; the incisions were sutured with surgical staples. The mice were randomized into control and test groups. The test compound, 3-chlorodeoxylapachol, was co-precipitated with polyvinylpyrrolidone (MW 360 000; Sigma-Aldrich) to increase solubility and then dissolved in a phosphate-buffered saline solution. From Days 3–6 after implantation, the test compound was administered once daily by intraperitoneal injection to three mice per dose tested (6.25, 12.5, 25, 50 and 100 mg kg^{-1}). An additional six mice served as the vehicle control group, receiving intraperitoneal injections of phosphate-buffered saline. Bodyweights were determined daily. The mice were anaesthetized and killed on Day 7 and the fibres were removed for assessment of cell viability. The cell masses were removed from the fibres and the viable cells were quantified using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye conversion assay. The mice were handled and cared for humanely, following a protocol (ACC No. 01-124) approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago.

Statistical analysis

Statistical analysis of the effects of intraperitoneal administration of 3-chlorodeoxylapachol on growth of KB, LNCaP and hTERT-RPE1 cells contained in porous fibres at intraperitoneal and subcutaneous sites was carried out using the Kruskal–Wallis test (SPSS, Chicago, IL, USA). A post-hoc comparison of the means of individual groups was performed using the default paired comparisons method provided by the software package. A level of $P < 0.05$ denoted significance in all cases.

3-Chlorodeoxylapachol

Yellow needles (petroleum ether) m.p. 89–90°C; UV (MeOH): λ_{max} (log ϵ) = 208 (4.43), 245 (4.44), 251 (4.42), 275 (4.41), 3.35 (3.76) nm; IR (film) ν_{max} = 2918, 2853, 1665, 1589 cm^{-1}; EIMS: *m/z* (rel. int.) = 262 (13), 260 $[M]^+$ (41), 225 (100), 217 (60); HRCIMS: *m/z* = 260.0612 (calcd. for $C_{15}H_{13}ClO_2$ 260.0604); ^1H NMR: ($CDCl_3$, 500 MHz) δ = 8.15 (1H, ddd, J = 9.2, 2.4, 0.8 Hz, H-5), 8.12 (1H, ddd, J = 9.2, 2.4, 0.8 Hz, H-8), 7.74–7.76 (2H, m, H-6 and H-7), 5.12 (1H, t, J = 7.2 Hz, H-12), 3.53 (2H, d, J = 7.2 Hz, H-11), 1.82 (3H, s, H-15), 1.70 (3H, s, H-14); ^{13}C NMR (DEPT135): δ = 182.40 (s, C-1), 178.08 (s, C-4), 147.01 (s, C-2), 142.82 (s, C-3), 135.60 (s, C-13), 134.14 (d, C-7), 133.86 (d, C-6), 131.81 (s, C-9), 131.42 (s, C-10), 127.13 (d, C-8), 127.03 (d, C-5), 117.41 (d, C-12), 27.90 (t, C-11), 25.81 (q, C-14), 18.23, (q, C-15).

Synthesis of 3-chlorodeoxylapachol

3-Chlorodeoxylapachol was synthesized from prenyl bromide and 2,3-dichloronaphthoquinone (Araki et al

1991). Indium powder (233 mg, 1.98 mmol) was added to DMF (5 mL) in a 50-mL round-bottomed flask. Prenyl bromide (0.35 mL, 3.03 mmol) was added dropwise, and the funnel washed with DMF (2 mL). The mixture was stirred at room temperature for 1 h, protected from light. 2,3-Dichloronaphthoquinone (452 mg, 1.99 mmol) was added, washing with DMF (2 mL), and the mixture was stirred at −21°C for 3 h under argon, protected from light. The reaction was quenched with water (10 mL) and partitioned with diethyl ether (10 mL). The ether extract was washed with 1% brine (10 mL), dried over Na_2SO_4, and refluxed with Ag_2O (700 mg) for 1 h. The crude product was purified by column chromatography over silica gel and recrystallization from hexanes to yield (272 mg, 52%). The synthetic product was characterized as 3-chlorodeoxylapachol being identical to the natural product by comparison of spectral data and physical properties.

LC-MS analysis of crude petroleum ether extracts

Portions of the original petroleum ether partition of the combined leaves and twigs, and separate samples of similarly prepared petroleum ether partitions of the leaves and the twigs (35–40 mg each, three samples total), were extracted by sonication for 5 min with acetonitrile (5 mL, Optima grade) and the supernatant was filtered through a solvent-washed cotton plug. The residual sample was washed with acetonitrile (2 mL) and filtered. The combined filtrates were concentrated under vacuum and weighed, then dissolved in acetonitrile (2 mL, sonicating for 2 min), and filtered through a 0.45-μm PVDF syringe filter (13 mm i.d.; Alltech Associates, Deerfield, IL, USA) pre-washed with acetonitrile (2 mL). The filtered extracts were diluted to 0.1 mg mL^{-1} and analysed by LC-MS using a 21-min gradient from 60% to 80% solvent A in solvent B. Scanning in the positive mode ($m/z = 200 \rightarrow 300$) was used to identify 3-chlorodeoxylapachol with a retention time of 13.8 min and characteristic $[M+H]^+$ and $[M+2+H]^+$ ions (Figure 2). A solvent delay of 4 min was used for these analyses.

Results and Discussion

Phytochemical study of *A. germinans* leaves and twigs

A sample of the combined leaves and twigs of *A. germinans* was extracted with MeOH and sequentially partitioned between aqueous MeOH, petroleum ether and

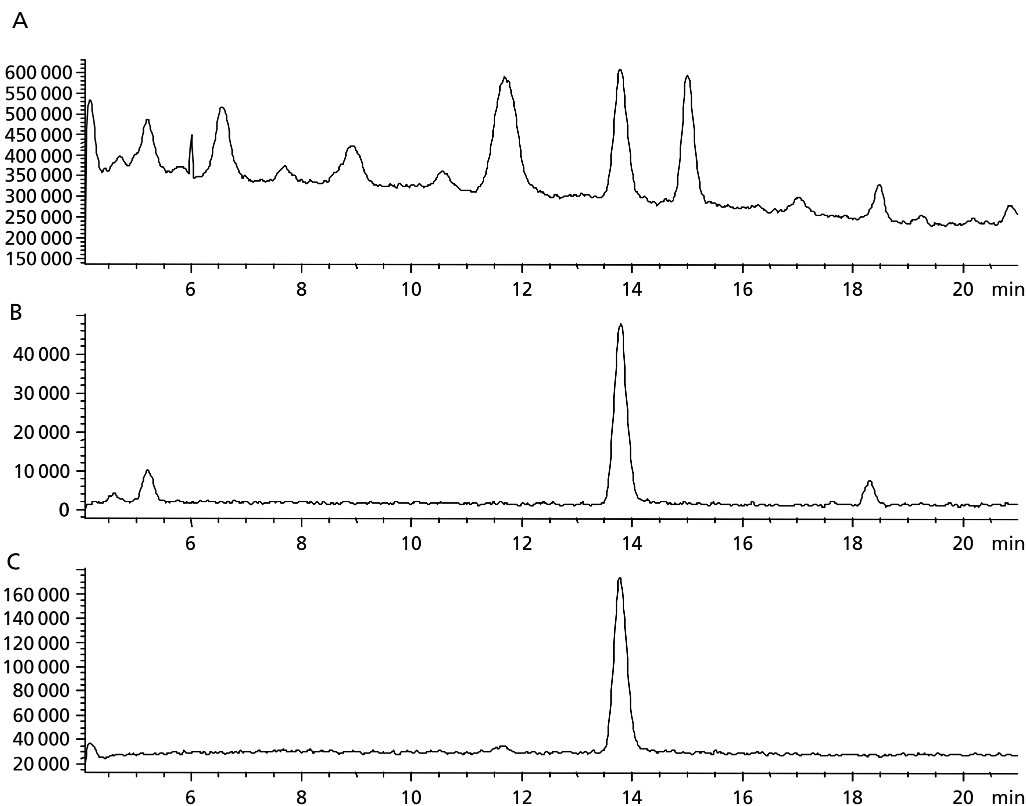

Figure 2 Positive-ion electrospray liquid chromatography-mass spectrometry analysis (monitoring after a 4-min solvent delay) of the petroleum ether extract of *Avicennia germinans* twigs. A. Total-ion current, scanning the mass range m/z = 200 → 300. B and C. Extracted-ion chromatograms at m/z = 263 and 261, showing the peak for 3-chlorodeoxylapachol at 13.8 min.

$CHCl_3$. The petroleum ether partition inhibited the growth of Lu1 (ED50 = 17.4 $\mu g\,mL^{-1}$) and LNCaP cells (ED50 = 15.8 $\mu g\,mL^{-1}$). Bioassay-guided fractionation of the petroleum ether partition by repeated column chromatography and preparative thin-layer chromatography resulted in the isolation of 3-chlorodeoxylapachol. This is the first report of the isolation of 3-chlorodeoxylapachol from a natural source. However, it was previously reported as a synthetic compound (Kapoor et al 1982; Araki et al 1991).

Structure elucidation of 3-chlorodeoxylapachol

The ^1H NMR spectrum of 3-chlorodeoxylapachol showed signals in the aromatic region of the spectrum integrating as four protons at δ_H 7.74–7.76 (2H), 8.12 and 8.15. The splitting patterns were consistent with a 1,2-disubstituted aromatic ring and the deshielding of the downfield signals suggested a quinone structure. In addition, a set of signals characteristic of a prenyl moiety were observed: a doublet integrating as two protons at δ_H 3.53 (7.2 Hz) coupled with a triplet integrating as one proton δ_H 5.12 (7.2 Hz) and two singlets integrating as three protons each at δ_H 1.70 and 1.82. Resonances for 15 carbon atoms were observed in the ^{13}C NMR spectrum, including two carbonyl carbons at δ_C 178.08 and 182.40. Thus, a C-2 prenyl naphthoquinone skeleton was proposed, representing a compound class previously reported from *Avicennia* (Itoigawa et al 2001). The ^1H-^1H COSY NMR spectrum was used to confirm the presence of two isolated spin systems, with the four aromatic protons comprising the first, and the methylene and olefinic methine of the prenyl group comprising the other. Correlations observed in the HMBC spectrum from the methyl proton signals CH_3-14 and CH_3-15 to C-12 and C-13, and from CH_2-11 to C-1, C-3, C-12 and C-13 confirmed the prenyl group structure and its placement at C-2. Additional HMBC correlations from H-8 to C-1, C-6 and C-10, and from H-5 to C-7, C-9 and C-4, in conjunction with 1-bond correlations observed in the HMQC spectrum, allowed the assignment of the ^1H and ^{13}C NMR signals. The ^1H and ^{13}C NMR resonances of 3-chlorodeoxylapachol were consistent with those of deoxylapachol (Perry et al 1991), with the exception of those associated with C-3, which in 3-chlorodeoxylapachol was a quaternary carbon at $\delta_C = 142.82$, compared with $\delta_C = 134.66$ for the olefinic methine C-3 in deoxylapachol. In contrast, the chemical shift for a hydroxy substituent in this position is $\delta_C = 152.67$ (Dawson et al 1989). EIMS gave ions at $m/z = 260$ (molecular ion), 262 (M + 2 isotope peak) and 225 (M − 35, base peak), consistent with the presence of chlorine in the molecule, and the molecular formula $C_{15}H_{13}ClO_2$ was confirmed by HRCIMS ($m/z = 260.0612$, calculated for $C_{15}H_{13}ClO_2$, 260.0604).

Biological evaluation of 3-chlorodeoxylapachol

3-Chlorodeoxylapachol was evaluated for cytotoxic potential against a panel of human cancer cell lines and found to be active against several of these (Table 1). Naphthoquinones related to 3-chlorodeoxylapachol have been extensively studied with in-vivo models, and several structure–activity relationships have been noted among lapachol derivatives based on antitumour activity, primarily with the W-256 and L1210 models (Driscoll et al 1974). Notably, loss of the hydroxyl group from lapachol results in reduced activity, as does replacement with an amino group, but the effect of replacement with chlorine has not been studied. Ham et al (1998) reported inactivation of cdk25A phosphatase by menadione (2-methyl-1,4-naphthoquinone), plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), and several other naphthoquinone analogues, including 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone. The latter compound was the most potent, and was reported to inhibit cell cycle progression through hyperphosphorylation of cdc2 (consistent with the inhibition of cdk25A phosphatase) (Ham et al 1998).

Table 1 Bioassay results for 3-chlorodeoxylapachol in a cancer cell line cytotoxicity panel

Compound	ED50 ($\mu g\,mL^{-1}$)				
	Col2	KB	LNCaP	Lu1	hTERT-RPE1
3-Chlorodeoxy-lapachol	3.7 ± 1.7	3.2 ± 2.4	4.1 ± 2.6	8.3 ± 3.3	5.0 ± 2.7

Col2, human colon cancer; KB, human oral epidermoid carcinoma; LNCaP, human hormone-dependent prostate cancer; Lu1, human lung cancer; hTERT-RPE1, human telomerase reverse transcriptase retinal pigment epithelium. Values represent the mean ± s.d. of three independent experiments.

3-Chlorodeoxylapachol was selected for evaluation in the hollow fibre assay to determine further development potential. Because the yield of 3-chlorodeoxylapachol in the original extract was low, it was synthesized to provide a sufficient amount of material for in-vivo evaluation. The three cell lines selected for the hollow fibre assay were KB, LNCaP and hTERT-RPE1, based on activity observed in cell culture. At the non-toxic dose of 25 $mg\,kg^{-1}$, 3-chlorodeoxylapachol inhibited the growth of KB cells at the intraperitoneal and subcutaneous sites (Figure 3). At doses of 6.25 $mg\,kg^{-1}$ and 12.5 $mg\,kg^{-1}$, 3-chlorodeoxylapachol inhibited growth of KB cells at the subcutaneous site. In contrast, no significant inhibition of LNCaP cells or the non-cancer hTERT-RPE1 cells was observed at either site, even at 50 $mg\,kg^{-1}$. Mi et al (2002) have reported hollow fibre assay results for two cytotoxic naphthoquinones, α-lapachone (ED50 = 3.0 $\mu g\,mL^{-1}$, SW626) and 2-(1-hydroxyethyl)naphtha[2,3-*b*]-furan-4,9-quinone (ED50 = 0.2 $\mu g\,mL^{-1}$, Lu1). No inhibition of SW626 cell growth was observed for α-lapachone at either the intraperitoneal or subcutaneous sites at any of the doses tested (31, 62 and 124 $mg\,kg^{-1}$). Similarly, the above-mentioned furanoquinone did not inhibit Lu1 cell growth at several non-lethal doses (3.75, 7.5, 15.0, 30.0 and 60.0 $mg\,kg^{-1}$). Gu et al (2004) reported hollow fibre results for plumbagin (5-hydroxymenadione) in KB,

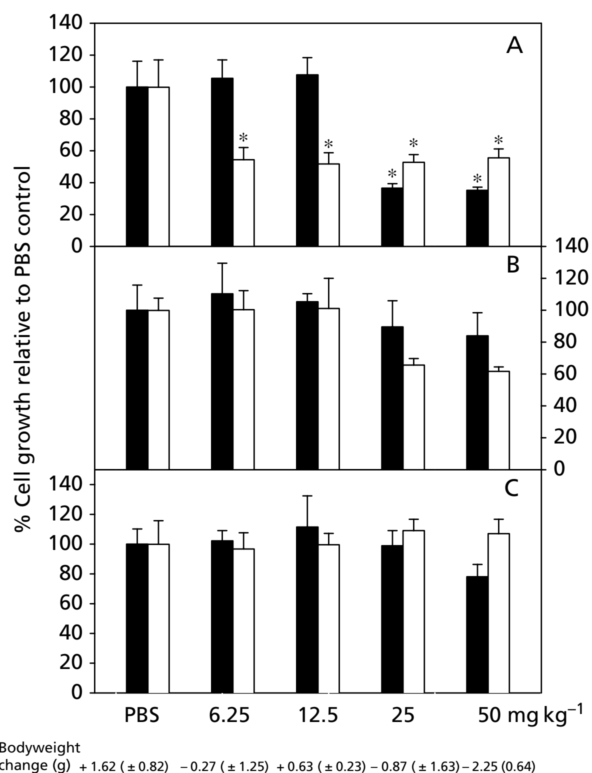

Figure 3 The effect of 3-chlorodeoxylapachol on the growth of KB (A), LNCaP (B) and hTERT-RPE1 (C) cells implanted at the intraperitoneal (closed columns) and the subcutaneous (open columns) compartments of NCr nu/nu mice. The mice were treated with phosphate-buffered saline (PBS; control) or the indicated doses of 3-chlorodeoxylapachol once daily by intraperitoneal injection from Days 3–6 after implantation. On Day 7, mice were killed, and the fibres were retrieved and analysed. Results are the mean ± s.e. (bars) percentage of cell growth relative to control. Changes in mouse bodyweight at the end of the experiment are also given (± s.e.). $*P < 0.05$, treatment groups were significantly different compared with the control group using the Kruskal–Wallis test, with $n = 6$ for the control group and $n = 3$ for treatment groups.

LNCaP and Lu1 cells. While plumbagin showed relatively strong in-vitro activity (ED50 values of 0.1, 0.8 and $0.3\,\mu g\,mL^{-1}$ in KB, LNCaP and Lu1 cells, respectively), no cell growth inhibitory activity was observed in the hollow fibre assay at doses of 1.25, 2.5, 5.0, 7.5 and $10\,mg\,kg^{-1}$, with the latter two doses proving lethal.

Although the in-vitro potency of 3-chlorodeoxylapachol was determined as being comparable with that of α-lapachone and about 10-fold less potent than plumbagin and 2-(1-hydroxyethyl)naphtha[2,3-*b*]-furan-4,9-quinone, only 3-chlorodeoxylapachol showed activity in the hollow fibre assay. The difference in hollow fibre activity between 3-chlorodeoxylapachol and the other three naphthoquinone analogues suggests that 3-chlorodeoxylapachol may possess a greater degree of in-vivo stability, or be metabolically activated, particularly in view of the activity observed at the subcutaneous site for KB cells. Based on the positive results in the hollow fibre assay, 3-chlorodeoxylapachol was submitted for evaluation in the National Cancer Institute's in-vitro 60-cell line panel. The 60-cell line panel is designed to test whether a compound has selectivity for a particular cell line or cancer type (Boyd & Paull 1995). However, 3-chlorodeoxylapachol did not show reproducible selectivity and was not recommended for further evaluation (results not shown).

LC-MS investigation of the origin of 3-chlorodeoxylapachol

The presence of chlorine in terrestrial plant metabolites is unusual, and therefore the question was raised as to whether 3-chlorodeoxylapachol might be an artefact of the isolation procedure, perhaps resulting from impurities in $CHCl_3$. To test this possibility, a portion of the petroleum ether partition from the original sample was analysed by LC-MS for the presence of 3-chlorodeoxylapachol. In addition, petroleum ether partitions obtained after MeOH extraction of separate samples of the leaves and the twigs of *A. germinans* (collected from the same plot at a later date) were analysed under the same LC-MS conditions to determine the distribution of 3-chlorodeoxylapachol between the two plant parts. 3-Chlorodeoxylapachol was determined to be present in the sample obtained from the twigs (Figure 2), but not in the sample obtained from the leaves of *A. germinans* (data not shown). It was also not detected in the original petroleum ether partition obtained from the combined leaves and twigs (data not shown), perhaps because of the low concentration in the original sample, or due to decomposition of the sample over time. These results suggest that 3-chlorodeoxylapachol is a natural product associated with the twigs, but the question of its biosynthetic origin remains uncertain. Little is known about the biosynthesis of chlorine-containing metabolites in vascular plants, but there is evidence of stress-induced chloroperoxidase activity in *Lilium maximowiczii* (Monde et al 1998).

Although organochlorine metabolites are most often associated with marine organisms and terrestrial microorganisms and fungi, there are reports of organochlorine metabolites from terrestrial plants (for reviews see Siuda & DeBernardis 1973 and Gribble 2003). The chloride-ion concentration in the xylem of *Avicennia* (and several other salt-secreting mangrove species) is reported to be as much as 100-times greater than in 'typical' vascular plant species (Scholander et al 1962). This may help to explain the presence of chlorine in 3-chlorodeoxylapachol, and raises the interesting possibility that other chlorine-containing metabolites remain to be discovered from this and other salt-secreting members of the mangrove habitat.

Conclusion

A petroleum ether-soluble extract of the leaves and twigs of the black mangrove tree, *A. germinans*, was investigated

using bioassay-guided isolation methods for the presence of potential anticancer agents. 3-Chlorodeoxylapachol was obtained as the active principle, demonstrating cytotoxic activity against several cancer cell lines. The structure of 3-chlorodeoxylapachol was determined using spectroscopic methods, including 1D- and 2D-NMR and mass spectrometry. Synthesis afforded a sufficient quantity of 3-chlorodeoxylapachol for evaluation in the murine hollow fibre model of antitumour activity, in which it demonstrated activity against KB cells, with some indication of selectivity for the subcutaneous site. In contrast, 3-chlorodeoxylapachol was not active against LNCaP or hTERT-RPE1 cells at either site at the doses tested. An LC-MS investigation of extracts prepared without the use of chlorinated solvents ruled out the possibility that the chlorine atom in 3-chlorodeoxylapachol was an artefact of the isolation procedure. Thus, 3-chlorodeoxylapachol is a natural product that demonstrates in-vitro and in-vivo potential as an anticancer agent, and may perhaps serve as a template for further synthetic modification.

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