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Anti-proliferative Effects of Lichen-derived Inhibitors of 5-Lipoxygenase on Malignant Cell-lines and Mitogen-stimulated Lymphocytes

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Abstract

Several lichen species have been used traditionally as medicinal plants. It has previously been shown that two low-molecular-weight lichen metabolites, lobaric acid isolated from *Stereocaulon alpinum* Laur. and protolichesterinic acid isolated from *Cetraria islandica* L. (Ach.), have in-vitro inhibitory effects on arachidonate 5-lipoxygenase. We have studied the effects of these compounds on cultured cells from man, including three malignant cell-lines (T-47D and ZR-75-1 from breast carcinomas and K-562 from erythro-leukaemia), as well as normal skin fibroblasts and peripheral blood lymphocytes.

Both test substances caused a significant reduction in DNA synthesis, as measured by thymidine uptake, in all three malignant cell-lines; the dose inducing 50% of maximum inhibition (ED50) was between 1·1 and 24·6 μg mL^{-1} for protolichesterinic acid and between 14·5 and 44·7 μg mL^{-1} for lobaric acid. The breast-cancer cell-lines were more sensitive than K-562. The proliferative response of mitogen-stimulated lymphocytes was inhibited with a mean ED50 of 8·4 μg mL^{-1} and 24·5 μg mL^{-1} for protolichesterinic acid and lobaric acid, respectively. These concentrations are of the same order of magnitude as the IC50 values in the 5-lipoxygenase assay. Significant cell death (assessed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay and trypan blue exclusion) occurred in the three malignant cell-lines at protolichesterinic acid and lobaric acid concentrations above 20 and 30 μg mL^{-1}, respectively. In K-562 morphological changes consistent with apoptosis were detected. Up to 38% cell death was observed at 20 μg mL^{-1} for protolichesterinic acid and 15 μg mL^{-1} for lobaric acid in mitogen-stimulated lymphocytes but unstimulated lymphocytes were clearly less sensitive. In contrast, the DNA synthesis, proliferation and survival of normal skin fibroblasts were not affected at doses up to 20 μg mL^{-1} for protolichesterinic acid and 30 μg mL^{-1} for lobaric acid.

We conclude that the anti-proliferative and cytotoxic effects observed might be related to the 5-lipoxygenase inhibitory activity of protolichesterinic acid and lobaric acid. These results open up the opportunity for future studies of these lichen metabolites with regard to their anti-tumour and anti-inflammatory properties.

Lichens are plants consisting of a symbiotic union between fungi and algae. Several lichens, for example *Cetraria islandica* and species belonging to the genus *Usnea*, have been used for various purposes in folk medicine. Reported applications of these species include treatment of tuberculosis and tumours (Hartwell 1971). Scientific investigations of the pharmacological properties of lichen-derived substances with regard to cytotoxic and anti-tumour properties are mostly limited to studies on lichen polysaccharides. Glucans and heteroglycans from several lichen species have been shown to inhibit the growth of sarcoma-180 implanted in mice (Fukuoka et al 1968; Nishikawa et al 1970, 1974). Usnic acid has been the most extensively studied of

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low-molecular-weight lichen constituents; it has been shown to inhibit tumour growth in several test systems (Kupchan & Kopperman 1975; Takai et al 1979; Al-Bekairi et al 1991).

In this study, the in-vitro effects of two low-molecular-weight lichen metabolites, lobaric acid isolated from *Stereocaulon alpinum* Laur. and protolichesterinic acid isolated from *Cetraria islandica* L. (Ach.), have been investigated on cells from man. Both compounds have previously been shown, in-vitro, to inhibit arachidonate 5-lipoxygenase from porcine leucocytes (IC50 values were 7·3 and 20·0 μM, respectively) without significantly inhibiting sheep seminal vesicle cyclooxygenase (Ingólfsdóttir et al 1994, 1996). Lobaric acid was also shown to inhibit cysteinyl leukotriene formation in guinea-pig taenia coli with an ED50 (the dose inducing 50% of maximum inhibition) of 5·5 μM (Gissurarson et al 1997).

Other reports of biological investigations of lobaric acid, a β-orcinol depsidone (Figure 1), are scarce. Protolichesterinic acid, an aliphatic α-methylene-γ-lactone (Figure 1), has, on the other hand, also shown activity in a number of biological assays, including in-vitro antibacterial effects against *Helicobacter pylori* (Ingólfsdóttir et al 1997), *Mycobacterium tuberculosis* and Gram-positive organisms (Stoll et al 1950); anti-tumour activity against solid type Ehrlich carcinoma in mice (Hirayama et al 1980) and in-vitro inhibitory effects against the DNA polymerase activity of HIV-1-reverse transcriptase (Pengsuparp et al 1995).

This study has investigated the effects of protolichesterinic acid and lobaric acid on growth and survival of cultured cells from man. The cell-types selected as representatives of a variety of malignant and normal cells were the breast-cancer cell-lines T-47D and ZR-75-1, the erythro-leukaemia cell-line K-562, and normal skin fibroblasts and freshly isolated peripheral blood lymphocytes from different donors. The effects of the lichen constituents on all cells were determined by microscopic examination then by analysis of DNA synthesis and proliferation and by viability tests.

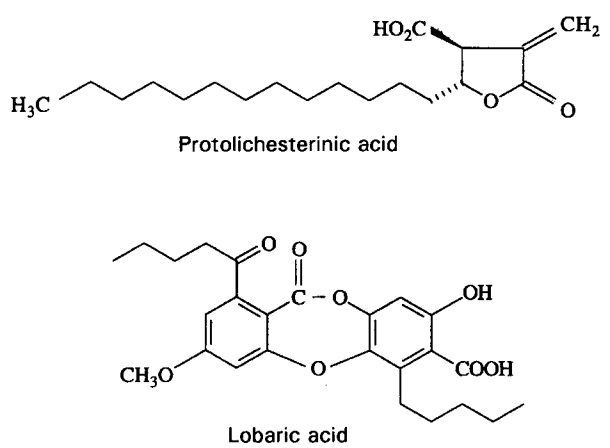

Figure 1. The chemical structures of protolichesterinic acid and lobaric acid.

Materials and Methods

Cells and chemicals

Cell-lines. The breast-cancer cell-lines T-47D and ZR-75-1 (American Type Culture Collection, Rockville, MD) and the erythro-leukaemia cell-line K-562 were cultured in RPMI 1640 medium with 10% foetal calf serum, 50 int. units mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 0·01 M HEPES-buffer and 0·2 M L-glutamine (all from Gibco, Paisley, UK), pH-adjusted with 1% bicarbonate, and 0·2 int. units mL^{-1} insulin (Sigma Diagnostics, St Louis, MO) for T-47D. The breast-cancer cell-lines were monolayer cultures but K-562 was in suspension. The cultures were incubated at 37°C in 95% humidity and 5% CO_2. Monolayer cultures were passaged once a week by trypsinization using a 1:30 dilution of standard Gibco trypsin-EDTA solution (5–2 g L^{-1}).

Normal cells. Normal fibroblasts were established as monolayer cultures from skin biopsies from healthy volunteers and maintained in the same medium as above but with 20% foetal calf serum. They were used after 5 to 12 passages in culture.

Peripheral blood mononuclear cells (PBMCs) were isolated by the standard method on a histopaque gradient (Sigma Diagnostics) from peripheral blood samples from healthy volunteers and placed in suspension culture with the same medium except that filtered foetal calf serum from HyClone Laboratories, UT, was used.

Isolation and preparation of test compounds. Lobaric acid and protolichesterinic acid were extracted and purified from *Stereocaulon alpinum* Laur. and *Cetraria islandica* L. (Ach.), respectively, as described earlier (Ingólfsdóttir et al 1994, 1996). Purity of the compounds (> 98%) was confirmed by IR, MS, ^{1}H NMR and ^{13}C NMR analysis (Ingólfsdóttir et al 1994, 1996). The substances were dissolved in pure ethanol at concentrations of 5 mg mL^{-1} for protolichesterinic acid and 2·5 mg mL^{-1} for lobaric acid and diluted for use in tissue-culture medium. All tests included controls with equivalent concentrations of ethanol.

Methods

Morphological observations. Cells were seeded in 24-well plates (Nunc, Denmark) at 2×10^4 (K-562, T-47D, ZR-75-1 and fibroblasts), 3×10^4 (fibroblasts), 5×10^4 (T-47D and ZR-75-1) and 5×10^5 (lymphocytes) cells per well. The cells were exposed to protolichesterinic acid and lobaric acid at concentrations ranging from 1·3 μg mL^{-1} to 60 μg mL^{-1}. The cultures were observed over 48 h for morphological changes and photographs were taken under a phase-contrast microscope (Leitz, Germany).

Cell viability, proliferation and apoptosis. K-562 cells were exposed to protolichesterinic acid and lobaric acid at the same concentrations as above in 24-well plates at 10^6 cells per well. Cell viability was assessed by trypan-blue (Sigma Diagnostics) exclusion at 4-h intervals for 24 h. Lymphocytes were cultured with protolichesterinic acid or lobaric acid at the same concentrations in 96-well plates at 10^5 cells per well. Cell viability was assessed by trypan-blue exclusion after 24 h of culture.

Survival and proliferation of fibroblasts was assessed after 24 and 72 h of culture in the presence of protolichesterinic acid or lobaric acid at 2500 cells per well in 96-well plates (Nunc) with the CellTiter 96TM AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Scandinavian Diagnostic Services, Falkenberg, Sweden) as described by the manufacturer. The 490-nm absorbance of formazan was measured 3 h after the addition of MTS/PMS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine methosulphate).

Survival and proliferation of T-47D and ZR-75-1 was assessed after 24 and 48 h of culture in the presence of protolichesterinic acid or lobaric acid at 10^4 cells per well in 96-well plates with the CellTiter 96TM AQ_{ueous} Non-Radioactive Cell Proliferation Assay as described above.

To demonstrate morphological changes indicating apoptosis, K-562 cells were cultured for 28 h at 10^6 cells per well as above. The cells were then precipitated on to glass slides in a cytospin centrifuge (Shandon Southern Products, Astmoor, Runcorn, Cheshire, UK), fixed for 5 min in ice-cold methanol (Microlab Aarhus A/S, Højbjerg, Denmark), stained with Giemsa (Merck, Darmstadt, Germany) in the standard way and examined under a light microscope (Leitz).

Assessment of DNA synthesis. The breast-cancer cell-lines were trypsinized, counted and placed in 96-well plates at 10^4 cells per well. The cells were cultured for 30 h and protolichesterinic acid or lobaric acid was added at the start or after 24 h of culture at the same concentrations as above. [^{3}H]Thymidine (Amersham International, Amersham, Buckinghamshire, UK) was added at 1 μCi per well after 24 h of culture and 6 h later the cells were harvested in a Mash Cell Harvester (Dynatech, Billingshurst, Sussex, UK) on to glass fibre filters (Whatman, Clifton, NJ). These were dried and the radioactivity was counted in a liquid scintillation counter (LKB-Wallac, Sweden) using toluene-based scintillation fluid containing 5 g L^{-1} 2,5-diphenyloxazole and 0·12 g L^{-1} 2,2-*p*-phenylene-bis(5-phenyloxazole) (Merck).

The erythro-leukaemia cell-line K-562 was cultured in 96-well plates at 10^4 cells per well with protolichesterinic acid or lobaric acid. The cells were exposed to the lichen metabolites after 24 h of culture along with [^{3}H]thymidine at 1 μCi per well for 6 h and then harvested as described above. To take into account cell loss during culture K-562 cells were also exposed to protolichesterinic acid or lobaric acid from the beginning of culture in lymphotubes (Nunc). The cell count was then adjusted to 10^4 viable cells per well and culture continued for 1 h in a 96-well plate with 1 μCi per well of [^{3}H]thymidine before harvesting.

The fibroblasts were trypsinized, counted and placed in 96-well plates at 6000 cells per well. Protolichesterinic acid or lobaric acid and [^{3}H]thymidine at 1 μCi per well were added after 24 h and cultures harvested 16 h later.

The PBMCs were cultured in 96-well plates at 10^5 cells per well with protolichesterinic acid or lobaric acid and stimulated with 1 μg mL^{-1} and 5 μg mL^{-1} of phytohaemaglutinin-mitogen. After 48 h [^{3}H]thymidine was added and 16 h later the cells were harvested.

Statistics

Results are presented with the standard error of the mean and measurements were compared by means of Student's *t*-test. Data for cell-lines were pooled from two or three experiments each performed in triplicate. Data for fibroblasts were very similar for six different donors and are presented and analysed as means for the whole group.

Results

Effects of ethanol on the cell-types

Administration of ethanol at the same concentrations as those present in the working dilutions of protolichesterinic acid and lobaric acid had no significant effect on any of the cell-types in any of the tests.

Morphological changes in cells exposed to lichen metabolites

Cultures of the different cell-types were exposed to protolichesterinic acid and lobaric acid at concentrations from 1·3 to 60 $\mu g\ mL^{-1}$ and observed for morphological changes. At concentrations of 20 $\mu g\ mL^{-1}$ for protolichesterinic acid and 30 $\mu g\ mL^{-1}$ for lobaric acid visible changes occurred in the two breast-cancer cell-lines and the K-562 cell-line. Normal fibroblasts changed very little and only at concentrations of 40 $\mu g\ mL^{-1}$ and 60 $\mu g\ mL^{-1}$ for protolichesterinic acid and lobaric acid, respectively. Unstimulated PBMCs were not visibly affected. Figure 2 shows the morphological effects of protolichesterinic acid on the cell-lines and fibroblasts. In the monolayer cultures cells became rounded and detached from the culture surface. ZR-75-1 also became vacuolated. K-562 cells were clearly reduced in numbers and became dull under phase-contrast microscopy.

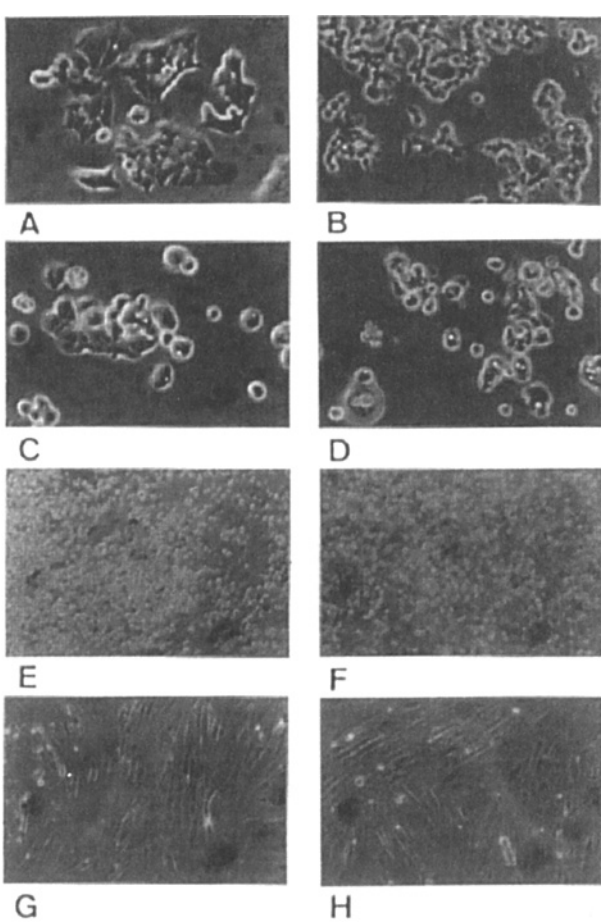

Figure 2. Morphological effects of protolichesterinic acid on T-47D (A and B), ZR-75-1 (C and D), K-562 (E and F) and fibroblasts (G and H). A, C, E and G are untreated controls; B, D, F and H are after 24 h of culture with 20 $\mu g\ mL^{-1}$ protolichesterinic acid.

Effects of lichen metabolites on DNA synthesis, growth and survival of breast-cancer cell-lines

The effects of protolichesterinic acid and lobaric acid on DNA synthesis of breast-cancer cell-lines were assessed by measuring [^{3}H]thymidine uptake. Results of experiments in which the lichen metabolites were present from the beginning of culture are shown in Figure 3. Both substances markedly inhibited [^{3}H]thymidine uptake by both cell-lines.

Protolichesterinic acid was more potent, with an ED50 of 3·8 $\mu g\ mL^{-1}$ for T-47D and 1·1 $\mu g\ mL^{-1}$ for ZR-75-1 compared with an ED50 of 21·2 $\mu g\ mL^{-1}$ for T-47D and 14·5 $\mu g\ mL^{-1}$ for ZR-75-1 for lobaric acid. ZR-75-1 was therefore somewhat more sensitive to both substances than T-47D. Late addition, after 24 h, of the two lichen metabolites to cultures of both cell-lines was less effective, ED50 values being between 25 and 50 $\mu g\ mL^{-1}$.

Figure 4 shows the effect on growth and cell survival of these two cell-lines, as assessed by the MTS assay. It is apparent that for both substances there is a marked decline in the MTS signal at the highest concentrations tested, indicating a loss of viable cells. At the lowest concentrations tested a

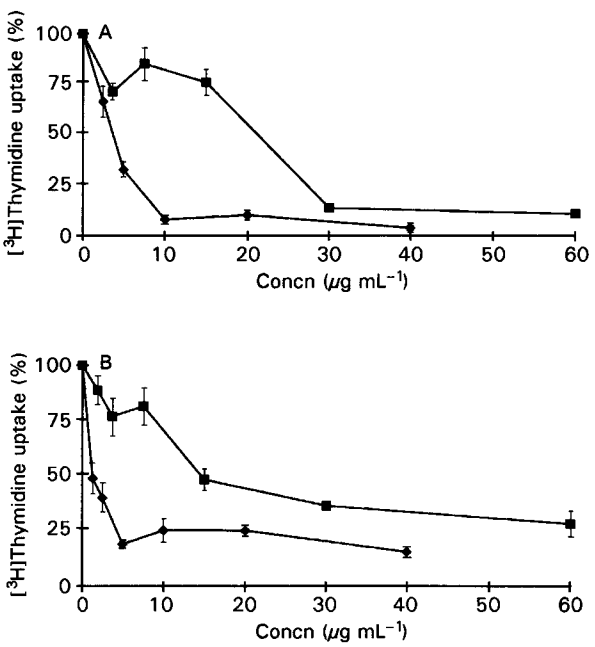

Figure 3. Effects of lichen metabolites on DNA synthesis by breast-cancer cell-lines. The effect of protolichesterinic acid (◆) and lobaric acid (■) on (A) T-47D and (B) ZR-75-1 after 30h of culture. The lichen metabolites were present from the beginning of culture in which the average 6-h uptake of [^{3}H]thymidine by untreated T-47D and ZR-75-1 controls was 55 446 and 66 517 counts min^{-1}, respectively. Results are presented as percent of control value set at 100%. Each point is the mean ± s.e.m. of results from two or three experiments, each performed in triplicate. For all results [^{3}H]thymidine uptake was reduced significantly ($P \leq 0{\cdot}05$) compared with control.

consistent and significant increase in the MTS signal was observed.

Effects on DNA synthesis, cell survival and apoptosis of K-562

The erythro-leukaemic cell-line K-562 was exposed to protolichesterinic acid and lobaric acid at the concentrations indicated above, DNA synthesis was measured and cell survival assessed by trypan-blue exclusion. As shown in Figure 5A both substances inhibited DNA synthesis by K-562; the ED50 values were 11·2 $\mu g\ mL^{-1}$ and 28·3 $\mu g\ mL^{-1}$ for protolichesterinic acid and lobaric acid, respectively. To distinguish between anti-proliferative effects and reduction in thymidine uptake caused by cell loss, K-562 was first exposed to the test substances for 24 h. Viability was then assessed and 1-h [^{3}H]thymidine uptake by viable cells determined. As shown in Figure 5B both substances inhibited DNA synthesis of remaining viable cells after 24 h with ED50 values of 24·6 $\mu g\ mL^{-1}$ and 44·7 $\mu g\ mL^{-1}$ for protolichesterinic acid and lobaric acid, respectively. Comparison with the effects on cell viability (Table 1) shows that DNA synthesis was affected at a concentration below those having a cytotoxic effect. Morphological characteristics of apoptosis were identified at higher concentrations of both substances. These included nuclear fragmentation, cytoplasmic vacuolization and blebbing.

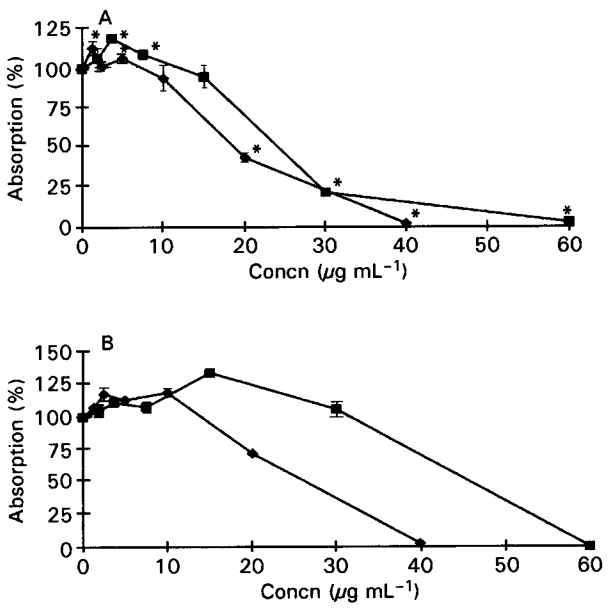

Figure 4. Effects of lichen metabolites on the viability of breast-cancer cell-lines. The effect of protolichesterinic acid (◆) and lobaric acid (■) on (A) T-47D and (B) ZR-75-1 was assessed by means of the CellTiter 96 assay after 48 h of culture in which the lichen metabolites were present from the beginning of culture. Average 3-h absorption by untreated T-47D and ZR-75-1 controls was 0·407 and 1·128, respectively. Results are presented as percent of control value set at 100%. Each point is the mean ± s.e.m. of results from two experiments, both performed in triplicate. In A, $*P \leq 0{\cdot}05$, significantly different from control; in B all results were significantly ($P \leq 0{\cdot}05$) different from control.

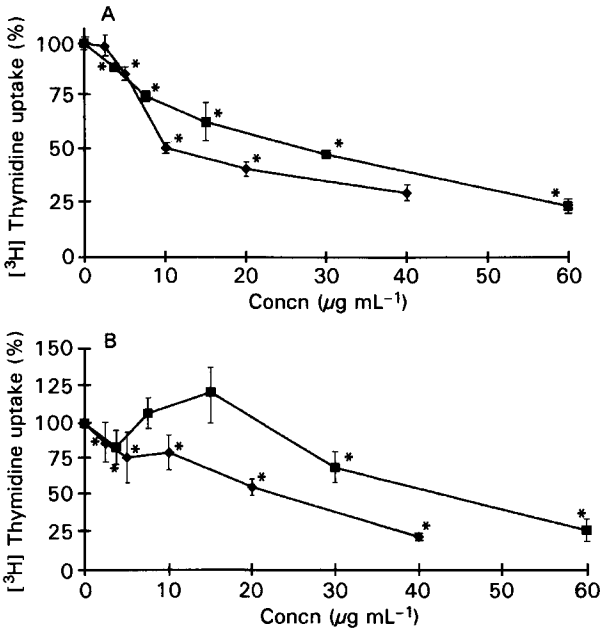

Figure 5. Effects of lichen metabolites on DNA synthesis by the erythro-leukaemia cell-line K-562. The effect of protolichesterinic acid (◆) and lobaric acid (■) on K-562 was assessed by measurement of (A) 6 h [^{3}H]-thymidine uptake by total cells after 30 h of culture in which the chemicals were added after 24 h and (B) 1 h [^{3}H] thymidine uptake by viable cells after 24 h of culture with the chemicals present from the beginning of culture. The average uptake of [^{3}H] thymidine by untreated controls was 43 070 counts min^{-1} for total cells but 7999 counts min^{-1} for viable cells. Results are presented as percent of control value set at 100%. Each point is the mean ± s.e.m. of results from two or three experiments, all performed in triplicate. $*P \leq 0{\cdot}05$, [^{3}H]thymidine uptake significantly reduced compared with control.

Effects on proliferative responses of lymphocytes to mitogen and on lymphocyte viability

The effects of protolichesterinic acid and lobaric acid on the proliferative response of lymphocytes to mitogenic stimulation by PHA were studied by measuring [^{3}H]thymidine uptake. Lymphocytes from four healthy donors were tested. All showed background responsiveness within the normal range although there were individual differences. Donor A was the highest responder showing approximately 200 000 counts min^{-1} with either dose of PHA; donor B showed an increase from 19 972 counts min^{-1} to 104 145 counts min^{-1} after stimulation with 1 $\mu g\ mL^{-1}$ and 5 $\mu g\ mL^{-1}$ PHA, respectively. Donors C and D were only tested with 5 $\mu g\ mL^{-1}$ PHA and responded with 44 734 and 145 280 counts min^{-1}, respectively. Results are shown in Table 2.

Table 1. Effects of lichen metabolites on K-562 cell viability after 4-h, 8-h and 24-h culture.

Amount of protolichesterinic acid ($\mu g\ mL^{-1}$)	Viability (%)			Amount of lobaric acid ($\mu g\ mL^{-1}$)	Viability (%)		
	4-h culture	8-h culture	24-h culture		4-h culture	8-h culture	24-h culture
0·0	100	95	90	0·0	87	98	96
1·2	ND	ND	93	1·8	ND	ND	93
2·5	94	84	86	3·7	96	91	96
5	93	84	87	7·5	88	88	96
10	88	91	82	15	94	88	86
20	84	83	80	30	100	88	71
40	58	43	25	60	10	17	4

The results for the 24 h culture are the average of two cell counts, the others are based on one cell count. ND, not determined.

Table 2. The proliferative response of peripheral blood mononuclear cells from four donors (A-D) to mitogen (PHA) in the presence of protolichesterinic acid and lobaric acid.

Donor	Amount PHA ($\mu g\ mL^{-1}$)	Response in presence of protolichesterinic acid:					
		0 $\mu g\ mL^{-1}$	1·2 $\mu g\ mL^{-1}$	5 $\mu g\ mL^{-1}$	10 $\mu g\ mL^{-1}$	20 $\mu g\ mL^{-1}$	ED50 $\mu g\ mL^{-1}$
A	1	100	ND	36	18	0	3·9
	5	100	ND	116	104	1	15·5
B	1	100	ND	31	11	0	3·6
	5	100	ND	35	18	0	3·8
C	5	100	54	33	ND	1	2·1
D	5	100	99	95	ND	0	12·3
Donor	Amount PHA ($\mu g\ mL^{-1}$)	Response in presence of lobaric acid:					
		0 $\mu g\ mL^{-1}$	1·8 $\mu g\ mL^{-1}$	7·5 $\mu g\ mL^{-1}$	15 $\mu g\ mL^{-1}$	30 $\mu g\ mL^{-1}$	ED50 $\mu g\ mL^{-1}$
A	1	100	ND	96	91	3	22·0
	5	100	ND	111	108	10	24·0
B	1	100	ND	88	70	1	19·8
	5	100	ND	88	64	1	18·7
C	5	100	86	99	ND	42	27·3
D	5	100	92	98	ND	44	28·0

Each value is the amount of [^{3}H]thymidine uptake as a percentage of that by untreated controls; each measurement was performed in triplicate. ND, not determined.

At the highest concentrations tested both protolichesterinic acid and lobaric acid caused a significant reduction in viability which was clearly more marked in stimulated cells than in resting cells (Table 3).

Effects on proliferation and viability of normal fibroblasts

Normal skin fibroblasts were exposed to protolichesterinic acid and lobaric acid for 1–3 days in culture. Effects on DNA synthesis, cell proliferation and cell viability were assessed by measurement of [^{3}H]thymidine uptake and by the MTS assay. There were no differences between results obtained from cells derived from different donors; the results are reported as an overall mean. The mean optical density for the MTS signal of untreated controls increased from 0·508 after 24 h of culture to 0·893 after 72 h of culture, indicating cell proliferation. Figure 6A shows that both substances have little effect on DNA synthesis. The results in Figure 6B show that lobaric acid had no effect on cell viability. In contrast, a small but consistent increase in the MTS signal was observed in cultures exposed to protolichesterinic acid at concentrations up to 15–20 $\mu g\ mL^{-1}$.

Discussion

In this study we have demonstrated the in-vitro inhibitory effects of two different lichen metabolites on the viability and proliferation of three malignant cell-lines. The effects on normal cells depended on cell-type and functional state. Thus normal fibroblasts remained fully viable except at the highest dose tested and showed no decrease in

Table 3. Effects of protolichesterinic acid and lobaric acid on the viability of peripheral blood mononuclear cells from donors A and B.

Donor	Amount PHA ($\mu g\ mL^{-1}$)	Viability (%)				
		Protolichesterinic acid				Lobaric acid
		0 $\mu g\ mL^{-1}$	5 $\mu g\ mL^{-1}$	10 $\mu g\ mL^{-1}$	20 $\mu g\ mL^{-1}$	15 $\mu g\ mL^{-1}$
A	0	91	85	83	70	90
	5	88	76	71	63	62
B	0	92	ND	84	ND	92
	5	82	ND	65	ND	71

Each measurement was performed in triplicate. ND, not determined.

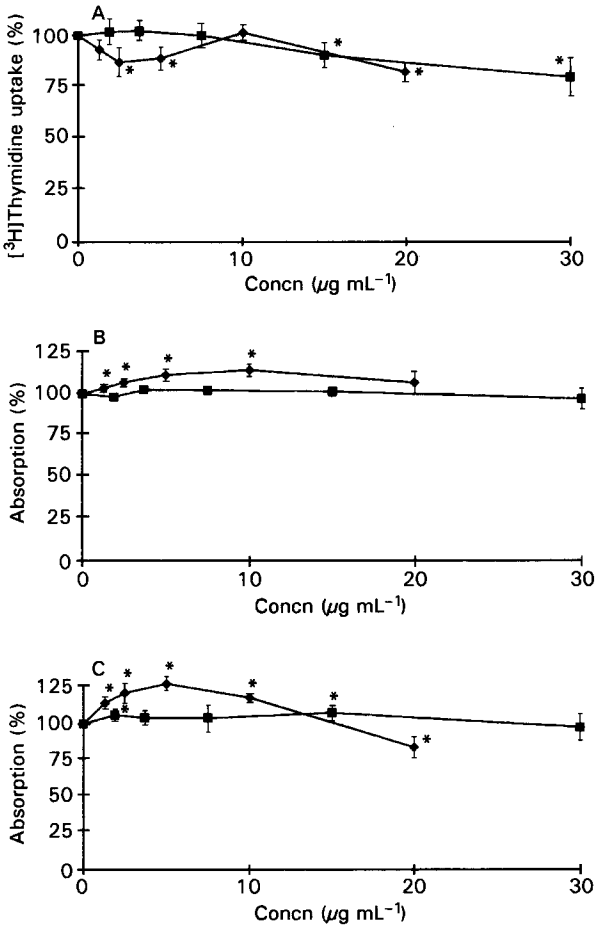

Figure 6. Effects of lichen metabolites on proliferation and viability of normal fibroblasts. A. Proliferation was assessed by measurement of 16-h uptake of [^{3}H]thymidine, added with protolichesterinic acid (◆) and lobaric acid (■) after 24 h of culture. Viability was assessed by means of the CellTiter 96 assay after 24 h (B) and 72 h (C) of culture in which the lichen metabolites were present from the beginning. The average uptake of [^{3}H]thymidine by untreated controls was 39 210 counts min^{-1} and the range of values was from 8637 to 61 210 counts min^{-1}. The average 3-h absorption by untreated controls was 0·508 and 0·893 for 24- and 72-h cultures, respectively. Results are presented as percent of control value set at 100%. Each point represents the mean ± s.e.m. of results from five or six donors, all tested in triplicate. *$P \leq 0{\cdot}05$, [^{3}H]thymidine uptake/absorption significantly different from control.

proliferative rate. In contrast, the proliferative burst of mitogen-stimulated lymphocytes was markedly inhibited at doses that had no effect on viability. Some cell death was observed at the highest doses tested but resting lymphocytes were clearly less sensitive than stimulated ones.

The inhibitory effects on DNA synthesis of the breast-cancer cells were most marked when the test substances were present from the beginning of the culture period, suggesting that early stages of the cell cycle were involved. Although there was a difference in potency both substances showed the same type of effect on cell proliferation. The observed in-vitro effects on proliferation and viability of malignant cell-lines can be related to the previously reported anti-tumour activity of protolichesterinic acid against solid type Ehrlich carcinoma in mice (Hirayama et al 1980). We have no obvious explanation of the observed increase in the MTS signal. It is unlikely that this indicates proliferation because, for the breast-cancer cell-lines, the effect occurred at a dose range that strongly inhibited DNA synthesis. Also, the effect on fibroblasts was seen after 24 h, which is before these cells start to proliferate. It might be speculated that the test substances had a direct effect on cellular metabolism, thereby increasing the levels of NADH and NADPH which are detected by the assay.

Protolichesterinic acid and lobaric acid are unrelated chemically and one clue to their cellular action is their previously demonstrated in-vitro inhibition of 5-lipoxygenase. The ED50 for inhibition of the growth of malignant cell-lines and stimulated lymphocytes was of the same order of magnitude as the IC50 values in the 5-lipoxygenase assay. Paradoxically, however, lobaric acid, which in this study had less potent anti-proliferative effects than protolichesterinic acid, is about three times more potent as an inhibitor of 5-lipoxygenase.

Leukotrienes, the products of action of lipoxygenase on arachidonic acid, are well recognized as mediators of phenomena that constitute the acute inflammatory response, including bronchoconstriction, neutrophil chemotaxis and adhesion to blood vessels, and they have also been implicated as stimulators of myelopoiesis (Henderson 1994; Hilger et al 1995). Inhibitors of 5-lipoxygenase have therefore become a target for development of new anti-inflammatory drugs (Ford-Hutchinson 1994). Lobaric acid was recently shown to inhibit leukotriene generation and contractility in smooth muscle (Gissurarson et al 1997). Cell proliferation is an essential component of specific immune responses and chronic inflammation. The 5-lipoxygenase pathway has been shown to be active in proliferation stimulation in normal and malignant human B cells (Jakobsson et al 1995) and T cells (Los et al 1995).

In a recent review (Ara & Teicher 1996) it was concluded that the inhibition of arachidonic acid metabolism through lipoxygenase or cyclooxygenase pathways can play a significant role in reducing cancer growth, promotion and metastasis. This hypothesis is supported by in-vivo and in-vitro data. Thus specific inhibitors of lipoxygenase (such as esculetin and nordihydroguaiaretic acid) were shown to have growth-inhibitory effects on the breast-cancer cell-line MDA-MB-231 (Rose & Connolly 1990) but the cyclooxygenase pathway is clearly also involved in regulation of the growth of this cell-line (Noguchi et al 1995). It must be noted, however, that Ayyub-Khan et al (1993) have pointed out a discrepancy between the effective dose range for 5-lipoxygenase inhibition by substances such as nordihydroguaiaretic acid, piriprost and MK 886 and anti-proliferative activity against malignant haemopoietic cell-lines including K-562. These findings imply that the growth-inhibitory effects of some of these test substances might not be solely attributable to their inhibition of lipoxygenase. Recent work has also implicated the 12-lipoxygenase pathway in tumour growth (Hussey & Tisdale 1996). The lichen metabolites have not yet been tested for possible effects on 12-lipoxygenase.

Higher concentrations of both compounds caused cell death and the morphological characteristics of apoptosis were identified in K-562 cells. The 5-lipoxygenase and 12-lipoxygenase pathways have recently been linked to the regulation of apoptosis (Anderson et al 1995; Tang et al 1996).

Protolichesterinic acid has been found to be a potent inhibitor of HIV-1 reverse transcriptase DNA polymerase (Pengsuparp et al 1995) in-vitro. This effect was, however, non-specific and cellular DNA polymerase β was also inhibited. This activity of protolichesterinic acid might be expected to cause a general anti-proliferative effect, irrespective of cell-type. The differences observed between the sensitivities of various cell-types tested here are more easily explained on the basis of anti-5-lipoxygenase activity. It has thus been shown that cell-types differ in their expression of 5-lipoxygenase and some might lack a necessary activating protein (Breton et al 1996).

In conclusion, we have shown that two unrelated lichen metabolites, protolichesterinic acid and lobaric acid, have marked anti-proliferative effects on three malignant cell-lines and on mitogen-stimulated lymphocytes from man. Higher doses induced cell death, probably via the apoptotic pathway. Normal fibroblasts were not affected and resting lymphocytes were less sensitive than stimulated lymphocytes to the killing effect. Because of the reported role of leukotrienes in the regulation of cell proliferation and apoptosis, the effects observed could be related to the already reported inhibition of 5-lipoxygenase by these compounds. These results reveal an opportunity for future study of the anti-tumour and anti-inflammatory properties of these lichen metabolites.

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