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Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells *in vitro*

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Abstract

The trypanocidal activity of naturally occurring 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (hispidin) prompted us to examine its cytotoxic activity toward normal and cancerous cells in culture. Hispidin synthesized in our laboratory to a high degree of purity (checked by ^{1}H and ^{13}C NMR spectroscopy) was shown to be cytotoxic (between 10^{-3} mol/L and 10^{-7} mol/L) toward normal human MRC-5 fibroblasts, human cancerous keratinocytes (SCL-1 cell line), and human cancerous pancreatic duct cells (Capan-1 cell line). Interestingly, addition of hispidin in three successive doses (between 10^{-5} mol/L and 10^{-7} mol/L) led to a 100-fold increase in activity with an enhanced activity on cancer cells compared to normal cells (50%). Synthetic hispidin was found to inhibit isoform β of protein kinase C (IC_{50} of 2×10^{-6} mol/L), but not *E. coli* and placental type XV alkaline phosphatases. The enhanced activity of hispidin toward the cancerous cell lines is discussed.

Abbreviations: AP, alkaline phosphatase; IC_{50}, inhibitory concentration 50%; PKC, protein kinase C

Introduction

Considerable effort has been devoted to the search for antineoplastic drugs that have more activity toward cancer cells than toward normal ones. Unfortunately, most of the available drugs are active against both kinds of cell, although some of them have been shown to have a preferential action on highly proliferating cells. These agents include the natural or synthetic antineoplastic drugs that interfere with nucleic acids or their metabolism, such as 5-fluorouracil (5-Fu) (Hartmann and Heidelberger, 1961; Chadwich and Rogers, 1972; Ardalan and Glazer, 1981; Malet-Martino et al., 1986; Wurzer et al., 1994), daunorubicin (Tarasiuk et al., 1990), adriamycin (Blum and Carter, 1974), and cisplatin (Rosenberg et al., 1969; Roberts and Pascoe, 1972; Cohen et al., 1979). Other compounds such as colchicine

(Andreu et al., 1991; Skoufias and Wilson, 1992), vinblastine (Chang, 1983), and 2-styryl-quinazolin-4(3*H*)-ones (Jiang et al., 1990) used in chemotherapy disrupt the cytoskeleton by inhibiting the polymerization of tubulin into microtubules (Andreu et al., 1991; Skoufias and Wilson, 1992).

Most of these drugs inhibit a variety of enzymes (Hartmann and Heidelberger, 1961; Chadwich and Rogers, 1972; Jiang et al., 1990; Skoufias and Wilson, 1992), and none of them appears to be specific to an enzyme involved in many metabolic processes. The ubiquitous use of phosphorylation and dephosphorylation enzymes in the inhibition and/or activation of many key cell processes indicates that many cellular processes could be disrupted via an action on protein kinases (Bollag et al., 1986; Nishizuka, 1988; Ludt et al., 1991; Dechecchi et al., 1993; Pawson, 1994; Walsh and Van Patten, 1994; Nishizuka, 1995) and phosphatases (Ingebritsen and Cohen, 1983; Walton and Dixon, 1993; Brautigan, 1994; Cohen, 1994). Cell activity has been shown to be directly related to kinase content, and overactive cancer cells are known to be highly dependent on these enzymes (Levitzki, 1994). A compound that is active against kinases would thus be expected to interfere preferentially with cancer cells compared with current cytotoxic agents, and might therefore be of value in chemotherapy (Levitzki, 1994).

During recent work on new natural drugs, we obtained extracts from the fruitbody of *Inonitus hispidus* as well as from the mycelium culture of this polypore. These extracts showed activity against the horse trypanosome (*Trypanosoma equiperdum*) cultured *in vitro* (Bergonzi, personal communication). The structures of the two active compounds, hispidin and a decarboxylated derivative (Figure 1) were determined after purification of these extracts. Although hispidin has been described and synthesized (Edwards et al., 1961; Edwards and Wilson, 1961), its biological activity has not yet been investigated. We therefore synthesized the molecule and tested its activity on both normal and cancer cells in culture. The synthetic hispidin was found to be cytotoxic *in vitro*, and a potent inhibitor of protein kinase C isoform β.

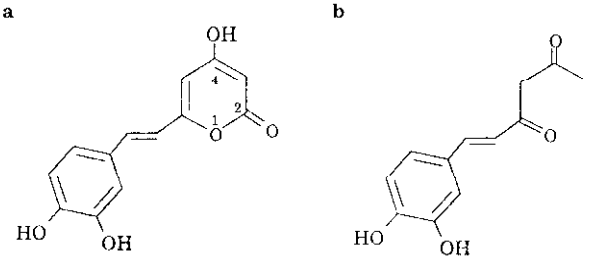

Figure 1. Hispidin (a) and the decarboxylated derivative (b), isolated by circular chromatography and active on *Trypanosoma equiperdum*.

Materials and methods

Cell lines

Three cell lines were used in this study.

(1) The MRC-5 cell line derived from healthy human pulmonary tissues, biopsied from a 14-week-old male fetus (Jacobs et al., 1970). These fibroblasts were cultured on DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) and cells were reexplanted with a mixture of 0.05% trypsin and 0.02% EDTA (Gibco).

(2) The SCL-1 cell line isolated from a facial tumor, biopsied from a human squamal carcinoma (Boukamp et al., 1982). These SCL-1 cells are slightly differentiated keratinocytes that synthesize keratin *in vitro* (Boukamp et al., 1985). The conditions of cell culture were the same as for MRC-5 cells.

(3) The Capan-1 cell line, established by Fögh, derived from a liver metastasis biopsied from a 40-year-old Caucasian patient with a pancreatic adenocarcinoma (Fögh et al., 1977). They are considered to be ductal type cells (Kyriazis et al., 1982; Hollande et al., 1990). They were obtained on the 14th passage from the American Type Culture Collection (ATCC, Rockville, MD, USA). Every 6 days, cells were maintained by passage on a 15% FCS supplemented RPMI 1640 medium (Gibco).

On each passage, MRC-5, SCL-1, and Capan-1 cells were seeded out into 25-cm^2 flasks (Nunc, Roskilde, Denmark) at a concentration of 2.5×10^5 cells/ml. Culture media contained 100 U/ml penicillin, 10^{-4} g/ml streptomycin, and 2.5×10^{-7} g/ml fungizone (Gibco) and were renewed every 2 days. All cell lines were maintained at 37°C in a 95% CO_2 atmosphere. Cultures were checked every month for contamination with mycoplasma by culture on specific media during three passages or by a PCR method (Mycoplasma PCR kits, Stratagene, La Jolla, CA, USA).

Isolation, purification, and synthesis of hispidin

Preliminary extracts were obtained after freeze-drying of the fruit bodies and of the cultured mycelium of *Inonitus hispidus*. Extractions were carried out in a Soxhlet apparatus using methanol as solvent. After concentration, the extracts were fractionated by thin-layer chromatography. Tests on *Trypanosoma equiperdum* cultures revealed two active spots (Bergonzi, personal communication). Compounds from these spots were then isolated by circular chromatography on a Chromatotron apparatus, followed by ^1H and ^{13}C NMR spectroscopy. The active compounds were shown to be hispidin (6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone) and one of its derivatives (6-(3,4-dihydroxyphenyl)-hex-5-en-2,4-dione), probably formed by opening of the lactone and decarboxylation of hispidin (Figure 1).

To study its biological activity, hispidin was synthesized using a method based on that of Edwards and Wilson (1961). The purity and structure of each intermediate was checked by ^1H and ^{13}C NMR spectroscopy. All chemicals were obtained from Aldrich (Saint Quentin Fallavier, France). 4-Hydroxymethyl-6-methyl-2-pyrone was condensed on a suitable substituted benzaldehyde (i.e., 3,4-di(methoxymethylenoxy)) in a Knoevenagel type reaction under the conditions described by Edwards and Wilson (1961). Spontaneous dehydration then leads to a styryl-pyrone methylated on C-4 and methoxymethylated on the other hydroxyl groups.

Hispidin was synthesized in the following stages:

(a) 4-Hydroxy-6-methyl-2-pyrone: 13.5 g dehydroacetic acid (0.08 mol) was heated at 120°C for 90 min in 30 ml of concentrated sulfuric acid. The reaction mixture was then added to ice-cold water and filtered; a white cake was obtained. This cake, recrystallized from water, afforded the product as fine beige needles in a 60% overall yield.

^1H NMR: (DMSO d_6) δ(ppm) 2.1 (s, 3H) CH_3; 5.2 (d, 1H, $J = 3$ Hz); 5.9 (m, 1H).

^{13}C NMR: (DMSO d_6), δ(ppm) 19.1 (C7); 88.0 (C3); 100.4 (C5); 163.6 (C6); 165.4 (C4); 171.1 (C2).

(b) 4-Methoxy-6-methyl-2-pyrone: 1.7 g 4-hydroxy-6-methyl-2-pyrone (0.0135 mol), 10 g K_2CO_3, and 1.3 ml dimethylsulfate (0.0135 mol) were refluxed for 8 h with anhydrous acetone. After filtration and evaporation of solvent, methoxylated pyrone was obtained in quantitative yield.

^1H NMR: (DMSO d_6, δ(ppm) 2.2 (dd, 3H) CH_3; 3.8 (s, 3H) OCH_3; 5.5 (d, 1H) CH; 6.0 (m, 1H) CH.

(c) 3,4-Di(methoxymethylenoxy)benzaldehyde: 6 g 3,4-dihydroxybenzaldehyde (0.0434 mol) was refluxed with 100 ml triethylamine for 1 h. 14 ml chloromethyl methyl ether (0.1878 mol) was then added dropwise and left standing for 2 h. The reaction mixture was evaporated to dryness and the residue was dissolved in dichloromethane and washed with water. A brown oil was obtained after drying and evaporation of the organic phase. This oil was dissolved in toluene and filtered through silica gel. After evaporation of the filtrate, a yellow solid was obtained and recrystallized from petroleum ether. The protected aldehyde was obtained in a yield of 77%.

^{1}H NMR: ($CDCl_3$, δ(ppm) 3.5 (s, 6H) $2CH_3O$; 5.3 (s, 2H) CH_2O; 5.3 (s, 2H) CH_2O; 7.5 (m, 3H) aromatic H; 9.9 (s, 1H) CH═O.

(d) 6-(3,4-Di(methoxymethylenoxy)styryl)-4-methoxy-2-pyrone: This product was prepared according to Edwards and Wilson (1961) with a yield of 64%.

^{1}H NMR: ($CDCl_3$), δ(ppm) 3.5 (s, 3H) CH_3O; 3.5 (s, 3H) CH_3O; 3.8 (s, 3H) CH_3O; 5.2 (s, 4H) 2 (O-CH_2-O); 5.5 (d, 1H) CH; 5.9 (d, 1H) CH; 6.4 (d, 1H, $J = 16$ Hz) H–C═; 7.2 (m, 3H) aromatic H; 7.9 (d, 1H, $J = 16$ Hz) H–C═.

^{13}C NMR: ($CDCl_3$) δ(ppm) 51.6 (C19); 51.9 (C16 and C18); 84.2 (C3); 91.0 (C15); 91.2 (C17); 96.5 (C5); 110.7 (C10); 112.1 (C13); 113.0 (C14); 118.4 (C7); 125.5 (C9); 131.0 (C8); 143.2 (C6); 144.2 (C11); 154.5 (C12); 159.7 (C4); 166.8 (C2).

(e) 6-(3,4-Dihydroxystyryl)-4-hydroxy-2-pyrone: 4-Demethylation was carried out as described by Edwards and Wilson (1961). The protecting phenol acetals were hydrolyzed by dissolving the residue in methanol and adding an acid resin (CG120, 24 h). A yellow powder was obtained after filtration and evaporation of methanol. The product was purified by silica gel chromatography ($CHCl_3$–MeOH 8:2 v/v). Hispidin had shown NMR characteristics as follows:

^{1}H NMR: (acetone d_6), 200 MHz, δ(ppm) 5.3 (d, 1H) CH; 6.0 (d, 1H) CH; 6.6 (d, 1H, $J = 16$ Hz) CH; 7.1 (d, 1H, $J = 16$ Hz) CH; 6.9 (m, 3H) aromatic H; 8.0 (s, 1H) OH; 8.3 (s, 1H) OH; 10.2 (s, 1H) OH.

^{13}C NMR: (acetone d_6), 50 MHz, δ(ppm) 90.7 (C3); 101.0 (C5); 114.7 (C10); 116.4 (C13); 117.5 (C14); 121.6 (C7); 128.8 (C9); 135.9 (C8); 146.3 (C6); 147.9 (C11); 161.2 (C12); 163.9 (C14), 170.8 (C2).

The purity of the synthetic hispidin was verified using the following methods: (i) identification of an extra signal on the ^{1}H and ^{13}C spectra; (ii) thin-layer chromatography (silica gel with $CHCl_3$–MeOH 8:2 (v/v) as solvent; (iii) identification of the molecular peak (MH^+) on mass spectrometry with chemical ionization (NH_3); (iv) determination of melting point.

Cytotoxicity tests

Hispidin was tested on the three cell lines seeded in Nunc culture plates (4 wells) at the following concentrations: 2.5×10^4 cells/ml (MRC-5), 10^5 cells/ml (SCL-1), and 1.25×10^5 cells/ml (Capan-1).

To identify the various growth stages, growth curves for each cell line were established in the presence of 1% DMSO. Hispidin was dissolved in DMSO and added to the cell culture medium on day 1 (concentrations between 10^{-3} and 10^{-7} mol/L). Control cultures were grown on medium containing the same concentration of DMSO (1%). In the first set of experiments, hispidin was added to the culture medium in a single daily dose on day 1 and the cells were counted in a Malassez hematimeter every day for 5 days. In the second set of experiments, hispidin was added to the cell cultures on days 1, 2, and 3, and cells were counted daily for a period of 8 days. Each data point is the mean of 10 experimental values.

Cytological tests

Growth of the different cell lines, with or without hispidin, was examined every day by phase contrast microscopy or after fixation with Bouin's liquid and hemalun–eosin staining. Cytotoxic alterations were observed after fixation with Baker's formol–calcium and by staining with Sudan black. All cells were cultured in Leighton tubes.

Enzyme inhibition

Protein kinase C: PKC isoform β phosphorylation assays were performed in reaction mixture (80 µl) containing histones III (Sigma, St Louis, MO, USA) (6×10^{-5} g/ml), $MgCl_2$ (10^{-2} mol/L), $CaCl_2$ (2.5×10^{-4} mol/L), phosphatidylserine (Sigma) (5×10^{-5} g/ml), diolein (Sigma) (5×10^{-6} g/ml), [γ-^{32}P]ATP (10^{-5} mol/L, 2000–4000 cpm/pmol), Tris-HCl buffer (pH 7.5), protein kinase C (Glaxo Laboratories, Les Ulis, France) (5×10^{-7} g), and inhibitors at different concentrations (Ricouart et al., 1991).

Substances were added in the following order: inhibitor, radioactive mixture containing $CaCl_2$, $MgCl_2$, substrate then phospholipids, and finally PKC. The reaction was studied under initial rate conditions; for a given inhibitor concentration, between about $t = 0$ and $t = 10$ min the inhibition was time-dependent; it was also linear with respect to the amount of enzyme present. In the absence of diolein and phospholipids, the basal activity was too low for the effect of inhibitors to be measured. Reactions were run at 30°C for 7 min and stopped by addition of trichloroacetic acid (12% w/v) in the presence of bovine serum albumin (9×10^{-4} g) as a carrier. After centrifugation (10 min at 3000 rpm), supernatant containing [γ-^{32}P]ATP and unprecipit able inhibitors was discarded and the pellet was dissolved in 1 mol/L NaOH and precipitated a second time with trichloroacetic acid. Radioactivity incorporated into histones was counted by scintillation spectrometry with Aqualyte reagent. Ninety-six percent of the activity was strictly Ca^{2+}- and phospholipid-dependent. All experiments were carried out in triplicate. The IC_{50} of hispidin was compared with that of quercetin.

Alkaline phosphatases: Two enzymes were studied, the *Escherichia coli* AP (Sigma), and a human placental type XV AP (Sigma), using a modified method of Mullivor et al. (1978). Enzymes were incubated at 25°C for various times, using different inhibitor concentrations, and in 0.2 mol/L diethylamine (DEA) buffer (pH 9) containing 0.045 mol/L $MgCl_2$ and sufficient NaCl to maintain the ionic strength at 0.15 mol/L. For each assay a control was carried out using MeOH or EtOH instead of the inhibitor. After addition of substrate (sodium *p*-nitrophenyl phosphate, 10^{-2} mol/L in DEA buffer), the appearance of *p*-nitrophenate ions was followed at 400 nm and 25°C for at least 5 min on a Perkin-Elmer Lambda 15 spectrophotometer.

Results

Purity of hispidin

The synthesized hispidin had an estimated purity of 99%. Its melting point (uncorrected) was 259°C and it gave rise to a single spot on TLC. Mass spectrophotometry (MS) demonstrated a molecular peak $m/e = 337$. No additional signal was detected on the ^{1}H and ^{13}C NMR spectra.

Cytotoxicity of synthetic hispidin

It can be seen from the growth curves (Figure 2) of the MRC-5, SCL-1, and Capan-1 cell lines that hispidin was added during the exponential growth phase. Concentrations of hispi-

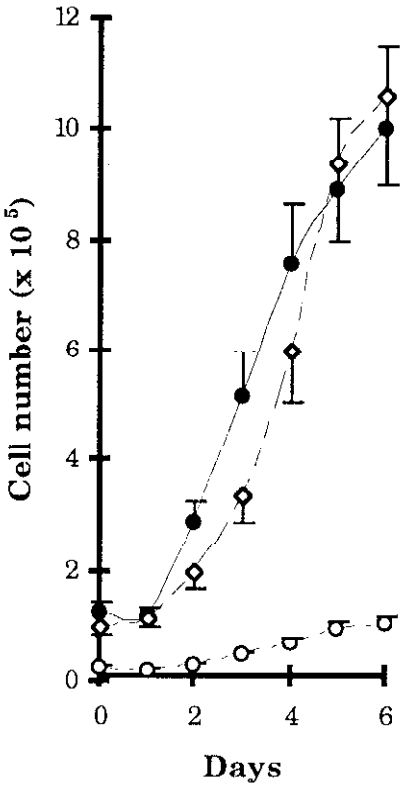

Figure 2. Growth curves of MRC 5 (○), SCL-1 (◇) and Capan-1 (●) cell lines. The number of cells is expressed in cell units of 10^5 (±SD, $n=3$). The exponential growth phase was observed to commence after a latency period of 24 h and continued beyond day 4.

din between 10^{-3} and 10^{-7} mol/L were found to be cytotoxic on the three cell lines tested (Figure 3).

Normal cells, MRC-5 line: Figure 3a shows a maximum cytotoxic effect (93%±8% cell death) 4 days after a single addition of 10^{-3} mol/L hispidin. Between 10^{-4} and 10^{-5} mol/L, moderate cytotoxicity was observed (62%± 10% and 57%±9% cell death, respectively, 2 days after addition). After either dose, the remaining cells resumed proliferation at day 4. At 10^{-6} and 10^{-7} mol/L hispidin, cytotoxicity was 27%±8% and 10%±5%, respectively, on day 2. No significant cytotoxicity was observed at doses below 10^{-7} mol/L.

Figure 3b shows the effects of addition of hispidin in three separate doses at 24-h intervals at concentrations between 10^{-5} and 10^{-7} mol/L. A cell death rate of 46%±8% was observed, and cell proliferation resumed on day 7. At 10^{-5} mol/L, 34%±8% cell death was still noted on day 8. The addition of hispidin in three separate doses led to a 300-fold increase in activity on day 5 (31% cell death with a single dose of 10^{-4} mol/L, and 35% cell death with three doses of 10^{-7} mol/L). No cytotoxicity and no structural alterations (Figure 4a) were observed in the 1% DMSO control culture, although cell doubling time increased from 36 to 50 h.

The toxicity of hispidin on MRC-5 cells was also evidenced by cytological examination. Cytoplasmic granules appeared in many treated cells, and fatty drops could be seen after staining with Sudan black. Several hours after addition of hispidin, the cytoplasmic granules were small but became larger and more abundant after 1 day in culture (Figure 4b, arrow). Large Sudanophilic zones were noted after treatment for 2 days.

Cancer cells, SCL-1 line: Maximal cytotoxicity (96%±3% cell death) was observed at 10^{-3} mol/L hispidin on day 3 (Figure 3c), with 41%±9% cell death at 10^{-4} mol/L. On day 4, the remaining cells resumed proliferation. For hispidin concentrations between 10^{-5} and 10^{-7} mol/L, cytotoxicity was between 16%±8% and 21%±7%. However, normal cell proliferation was resumed on day 5. Figure 3d shows the marked toxicity (66%±12%) after addition of hispidin in three doses of 10^{-5} mol/L at 24-h intervals, while by day 8 cell deaths of 45%± 8% and 37%±10% were observed with a similar treatment at doses of 10^{-6} and 10^{-7} mol/L, respectively. Addition of hispidin in three separate doses blocked cell proliferation and led to a 30-fold increase in cytotoxicity on day 5 (31% cell death with one dose of 10^{-4}

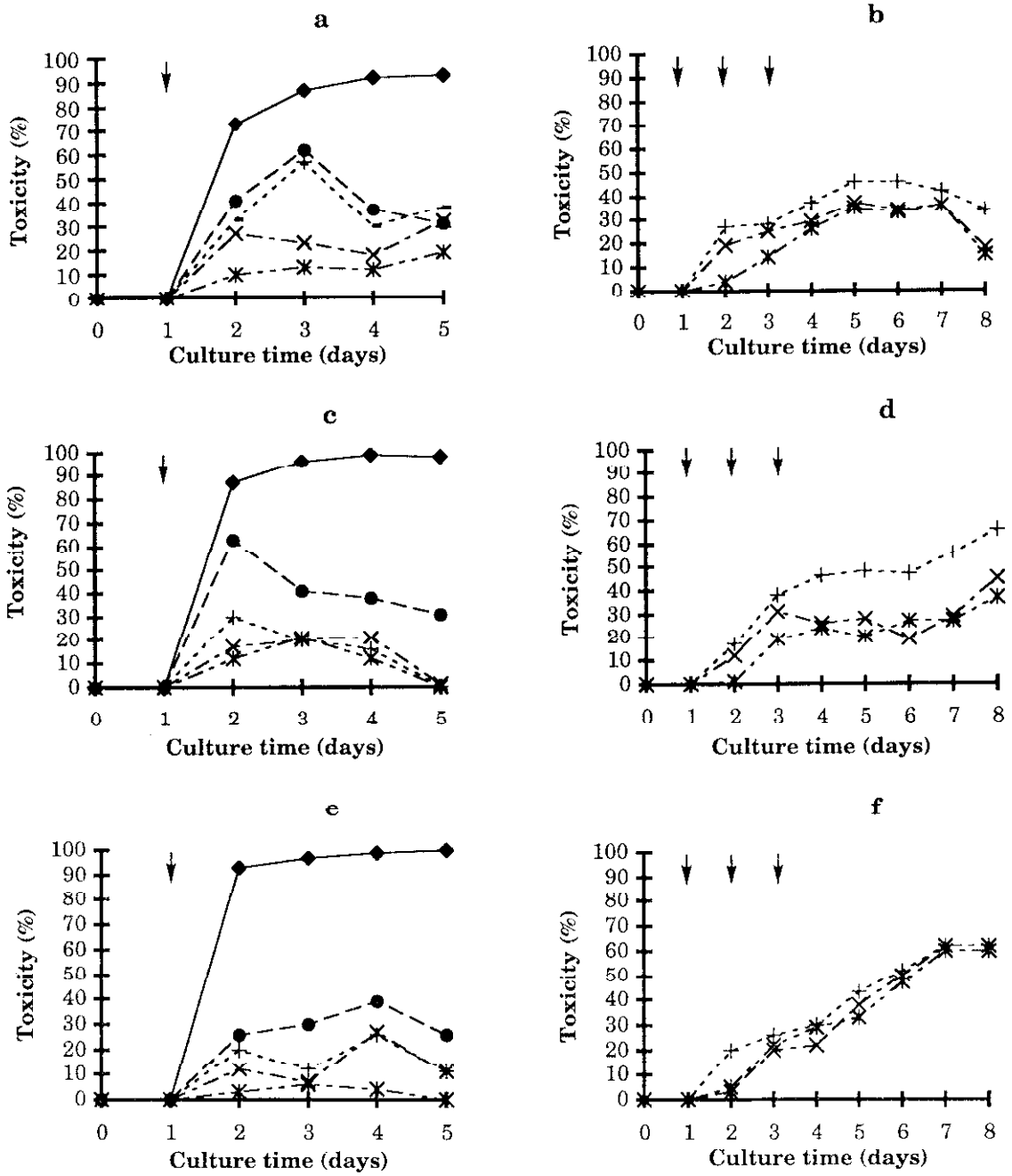

Figure 3. Cytotoxicity of hispidin during cell growth: (a,b) MRC-5, (c,d) SCL-1, and (e,f) Capan-1. The toxicity is expressed as percentage cell death compared to DMSO controls, plotted against culture time (days). (a,c,e) Cytotoxicity determined after one dose of hispidin (arrow). Note the marked toxicity on the three cell lines at a hispidin concentration of 10^{-3} mol/L (◆). Toxicity was a function of hispidin concentration (10^{-4} mol/L, ●; 10^{-5} mol/L, +; 10^{-6} mol/L, ×; 10^{-7} mol/L, ✳). (b,d,f) Cytotoxicity determined after three separate doses of hispidin (arrows) at the following concentrations: 10^{-5} mol/L (+); 10^{-6} mol/L (×), and 10^{-7} mol/L (✳). Note the increase in cytotoxicity between days 5 and 8 for cancer cells (d,f) compared with the lack of cytotoxicity on normal cells (b).

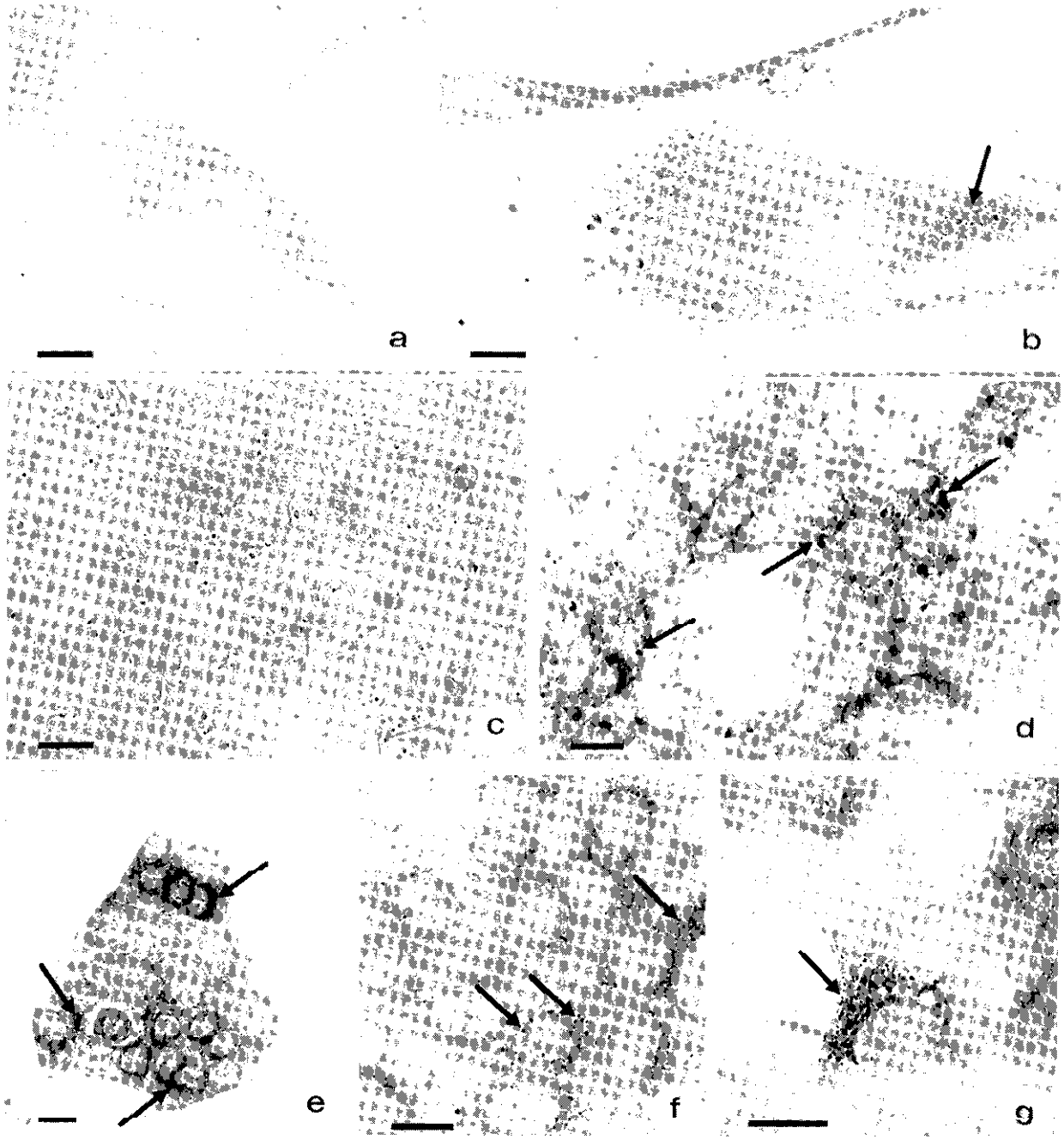

Figure 4. General aspect of cell alterations observed after Sudan black staining (bars = 20μm). (a,b) MRC-5 cells. Note lipid drops (arrow in b) when cells were cultured for 24 h with a single dose of hispidin at 10^{-4} mol/L (b) compared to 1% DMSO control cells (a). (c,d) SCL-1 cells. Note lipid drops (arrows in d) when cells were cultivated for 24 h with one dose of hispidin at 10^{-4} mol/L (d) compared to 1% DMSO control cells (c). (e,f,g) Various aspects of lipid drop accumulation (arrows) in Capan-1 cells cultivated for 24 h (e,f) and 48 h (g) with one dose of hispidin at 10^{-4} mol/L.

mol/L, and 28% cell death with three doses of 10^{-6} mol/L).

No toxicity and no structural alterations (Figure 4c) were observed in the 1% DMSO control culture, although cell doubling time increased from 24 to 28 h. Figure 4d (arrows) shows the appearance of lipid drops in the hispidin-treated cells, with an increase in size and number of drops with age in culture.

Cancer cells, Capan-1 line: Figure 3e shows a maximal cytotoxic action (98%±3% cell death) 4 days after addition of 10^{-3} mol/L hispidin. A dose of 10^{-4} mol/L led to 39%±7% cell death on day 4, while doses of 10^{-5}, 10^{-6}, and 10^{-7} mol/L hispidin led to 27%±11%, 26%±7%, and 4%+7% cell death, respectively. For hispidin concentrations between 10^{-4} and 10^{-7} mol/L, proliferation of the remaining Capan-1 cells resumed on day 5. After addition of hispidin in three separate doses at 24-h intervals, cell death of 30%±14%, 22%±9%, and 29%±12% were observed (day 4) for hispidin concentrations between 10^{-5} and 10^{-7} mol/L, respectively (Figure 3f). The cell death rate increased until day 7 (62%±7% and 60%±5%, respectively). Addition of hispidin in three separate doses led to a 300-fold increase in activity on day 5 (28% cell death with one dose of 10^{-4} mol/L, and 33% cell death with three doses of 10^{-7} mol/L).

No cytotoxicity was observed in the control cell culture (1% DMSO), although cell division time rose from 18 to 22 h.

Damaged Capan-1 cells were frequently observed 24 h after addition of the 10^{-4} mol/L dose of hispidin. Some peripheral cells of the epithelial sheet increased in volume and had lost adhesive properties. These floating cells died on subculture in fresh medium. Some degenerated Capan-1 cells, strongly granulated, remained stuck to the bottom of the flasks. Numerous granulations could be seen after staining with Sudan black (Figures 4e, 4f, and 4g), small at first (1 day after hispidin addition; Figures 4e and 4f, arrows), but increasingly gradually in size and number, with big lipid drops 2 days after treatment (Figure 4g, arrow).

Comparative cytotoxicity between cancer and normal cells: Figure 5 shows cell death rates on day 8, i.e., 5 days after the third dose of hispidin. In MRC-5 cells, 15%±9%, 18%±8%, and 34%±8% cell deaths were observed for hispidin concentrations of 10^{-7}, 10^{-6}, and 10^{-5} mol/L, respectively. Higher rates were obtained with the cancer cell lines; for SCL-1, cell death rates of 37%±10% at 10^{-7} mol/L, 45%±8% at 10^{-6} mol/L, and 66%±12% at 10^{-5} mol/L were observed; while for Capan-1 cells, 60%+5%, 62%+5%, and 62%+7% cell deaths were observed with the same doses of hispidin.

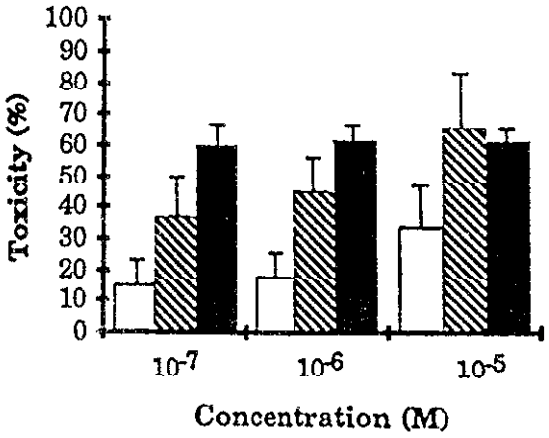

Figure 5. Cytotoxicity after addition of three separate doses (days 1, 2, and 3) of hispidin, at 10^{-5}, 10^{-6}, and 10^{-7} mol/L, observed on day 8. Toxicity is expressed as percentage cell death (±SD) compared to 1% DMSO controls. Note the low toxicity of hispidin on normal MRC-5 cells (open bars) and enhanced toxicity on SCL-1 (hatched bars) and Capan-1 (solid bars) cancer cells.

Enzyme inhibition

Protein kinase C: An IC_{50} of 2×10^{-6} mol/L was found for inhibition of this enzyme (isoform β) by hispidin, versus an IC_{50} of 3×10^{-5} mol/L for quercetin under the same conditions.

Alkaline phosphatase: No significant action of hispidin on *E. coli* and placental type XV phosphatases was observed.

Discussion

We show here that synthetic hispidin inhibits the ubiquitous isoform β of protein kinase C. Hispidin also had marked cytotoxic activity toward human epithelial cancer cells *in vitro* (pancreatic duct and human keratinocyte cell lines).

A large variety of natural (vinblastine (Chang, 1983), adriamycin (Blum and Carter, 1974), and daunorubicin (Tarasiuk et al., 1990)) and synthetic drugs (5-Fu (Hartmann and Heidelberger, 1961; Chadwich and Rogers, 1972; Ardalan and Glazer, 1981; Malet-Martino et al., 1986; Wurzer et al., 1994), cisplatin (Rosenberg et al., 1969; Roberts and Pascoe, 1972; Cohen et al., 1979)) have been tested on human cancer cell cultures (Malet-Martino et al., 1986; Wurzer et al., 1994; Blum and Carter, 1974; Chang, 1983), tumor-bearing animals (Chadwich and Rogers, 1972), or humans (Blum and Carter, 1974). Commonly used antineoplastic drugs include 5-Fu and derivatives (Hartmann and Heidelberger, 1961; Chadwich and Rogers, 1972; Ardalan and Glazer, 1981; Malet-Martino et al., 1986; Wurzer et al., 1994), cisplatin (Rosenberg et al., 1969; Roberts and Pascoe, 1972; Cohen et al., 1979), and daunorubicin (Tarasiuk et al., 1990). 5-Fu has been shown to inhibit the synthesis of DNA and RNA (Ardalan and Glazer, 1981; Wurzer et al., 1994), whereas cisplatin blocks cell multiplication by intercalating between purine and pyrimidine bases (Rosenberg et al., 1969; Roberts and Pascoe, 1972; Cohen et al., 1979). Colchicine, vinblastine, and derivatives block the polymerization of cytoskeletal tubulin into microtubules (Andreu et al., 1991; Skoufias and Wilson, 1992). The overall effect of these agents is an inhibition of cellular proliferation, although a major drawback is their low specificity as they interfere with both cancerous and normal cells. Unfortunately, both cancer and normal cells share common metabolic pathways, which hampers development of cytotoxic agents with a specific action on cancer cells. Hispidin, a natural compound extracted from the fruit body of *Inonitus hispidus* (Edwards et al., 1961), has been shown to have cytotoxic activity towards cultured trypanosomes, and might therefore be suspected of having activity toward mammalian cancer cells.

To study cytotoxicity on cell cultures, hispidin was synthesized in our laboratory using a method modified from that of Edwards and Wilson (1961) to produce a higher yield. The structures of the intermediates were determined by ^{1}H and ^{13}C NMR spectrometry, and the purity of the final product was demonstrated from its melting point, ^{1}H and ^{13}C NMR spectra, and molecular peak on MS. The results of the toxicity tests presented here were obtained with a chemically pure compound. At doses between 10^{-3} and 10^{-5} mol/L, hispidin was found to be cytotoxic on the normal cell line MRC-5 as well as on the cancerous SCL-1 and Capan-1 cell lines. The cell lysis appeared to be a result of two main effects:

(1) Alteration of the plasma membranes with a loss of adhesive properties, which released cells into suspension. A modification of membrane permeability could have induced an increase in cell volume by an accumulation of water and electrolyte in the cytoplasm.

(2) Alteration of metabolism of nutrients, which led to cell death with no increase in cellular volume. These cells accumulated light-refracting granules that were assumed to correspond to the lipid-filled spaces as indicated by their staining with Sudan black. The increase in size and number of these lipid-filled spaces with age in culture indicated that the cytoplasm of the metabolically altered cells progressively accumulates lipids. They form large pools of triglycerides, leading to cellular degeneration and eventually death of the cell.

The diverse nature of these two phenomena induced by hispidin suggests that it interferes with a variety of metabolic pathways. The fact that kinases and phosphatases are involved in numerous cellular processes, including membrane permeability (Ludt et al., 1991; Dechecchi et al., 1993; Brautigan, 1994; Nishizuka, 1995), generation of secondary messengers after receptor–ligand binding (Bollag et al., 1986; Bishop et al., 1992), formation of cytoskeleton (Gonda et al., 1990), and initiation of biochemical reactions involved in cell anabolism and catabolism (Ingebritsen and Cohen, 1983; Sander and Myatt, 1990; Brautigan, 1994; Nishizuka, 1995), prompted us to examine the action of hispidin on typical examples of a kinase and a phosphatase.

Biochemical assays showed that hispidin strongly inhibited one of the PKC isoforms (β) with an IC_{50} of around 2×10^{-6} mol/L. Hispidin was 15-fold more active than quercetin, a recognized inhibitor of these enzymes (Hagiwara et al., 1988). Other inhibitory drugs such as isoquinoline sulfonyl methylpiperazine (H 7) and related compounds inhibit kinases at concentrations of $7–10 \times 10^{-6}$ mol/L (Gali et al., 1993). Although synthetic hispidin seems a good inhibitor of PKC, it was not as active as staurosporine (Tamaki et al., 1986; Gali et al., 1993). On the other hand, hispidin had no inhibitory activity on the alkaline phosphatases tested, nor on type II and IV carbonic anhydrases (unpublished results), enzymes that are characteristic of Capan-1 cells (Mahieu et al., 1994).

Although we have not yet tested this compound on other enzymes, we conjectured that one of the effects of hispidin was an inhibition of PKC, an enzyme involved in cell permeability and metabolism. This inhibition was assumed to be irreversible as the intoxicated cells placed in fresh medium did not survive. Inhibition of PKC would thus affect the phosphorylation mechanisms required for synthesis, maturation, or mode of action of numerous intracellular factors. The toxicity of hispidin leading to cell death was attributed, at least in part, to an inhibition in the activity of protein kinases C. This would effectively block a wide variety of cellular processes. Preliminary experiments have indicated that hispidin inhibits protein kinases, including PKA and the various isoforms of PKC. However, in our current state of understanding, an action on other enzyme systems cannot be ruled out.

We found, however, that a single dose of hispidin over a range of concentrations between 10^{-6} and 10^{-7} mol/L was not sufficient to have a permanent effect on cell metabolism. On the other hand, three doses of 10^{-7} mol/L given at 24-h intervals had a comparable cytotoxic effect to that obtained after a single dose at higher concentrations (10^{-3}, 10^{-4} mol/L). The multiple doses led to a 100-fold increase in activity.

It is not yet clear why hispidin was more toxic toward epithelial cancer cells (such as pancreatic duct cells or human keratinocytes) than toward normal fibroblasts.

We found that it was at least 50% more active on cancer cells than on normal ones. This difference could perhaps be explained by the dependence of cancer cells on signal-transduction pathways (Levitzki, 1994). It is known, for example, that PKC is more abundant in cancer cells, which may make them more vulnerable to the action of hispidin.

In summary, pure synthetic hispidin was shown to be more cytotoxic toward cancerous cells (pancreatic duct and keratinocyte) than toward normal cells (fibroblast) by inhibiting at least protein kinase enzymes.

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