eight patients with refractory ovarian cancer were treated with adriamycin plus verapamil [14]. As a consequence of that study it was proposed by the authors that verapamil should be replaced by 'less cardiotoxic calcium channel blockers, since the dose of the modifying agent appears to be a critical factor and the toxicity of some of the currently available drugs, such as verapamil, may not permit the necessary plasma concentrations (of verapamil) to be achieved'. Fendiline might be a candidate as a substitute for verapamil in such clinical trials, because 'the clinical profile of fendiline is characterized . . . by an absence of any adverse cardiac effects' [15].

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Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis

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Summary. The honeybee hive product, propolis, is a folk medicine employed for treating various ailments. Many important pharmaceutical properties have been ascribed to propolis, including anti-inflammatory, antiviral, immunostimulatory and carcinostatic activities. Propolis extracts have provided an active component identified as caffeic acid phenethyl ester (CAPE), which was readily prepared in one step. Differential cytotoxicity has been observed in normal rat/human versus transformed rat/human melanoma and breast carcinoma cell lines in the presence of CAPE.
Key words. Honeybee hive; propolis; caffeic acid ester; cytoxicity.

The popular folk medicine, propolis [1], is alleged to exhibit a broad spectrum of activities including antibiotic, anti-inflammatory and tumor growth arrest; some of the observed biological activities may be traced to identified chemical constituents such as caffeic acid [2] which is antimicrobial and anti-inflammatory [3]. Ethyl ether extracts of propolis had previously been demonstrated to be cytostatic to KB and HeLa cell lines [4] but the components responsible for this interesting activity were not defined. Guided by an Ltk^- cell growth inhibition assay, we have isolated and characterized one such biologically active component which was readily synthesized in large quantities for further investigation into its cytostatic properties.

Propolis, a gift of Mr Chaim Kalman (Bee Farm-Honey, Israel), was collected from hives located on the Carmel Mountains. It was received in the form of hard, brown lumps (~ 2 cm in diameter) which were chopped, extracted with 80% EtOH/H_2O (1.5 l, 2 d), suction-filtered and evaporated in vacuo to yield a golden brown solid. This extract displayed cytostatic activity in Ltk^- cells at 50 mg/ml. The EtOH extract was dissolved in 80% MeOH/H_2O (400 ml) and sequentially extracted with hexane (6 × 80 ml), toluene (4 × 80 ml), and EtOAc (4 × 100 ml). All organic layers were dried, evaporated and submitted for Ltk^- testing (along with the residue from the aqueous layer). The EtOAc extract exhibited at least twice the cytostatic activity of other fractions (100% inhibition at 65 mg/ml). Subsequent purifications of the EtOAc extract by preparative TLC (7% i-PrOH/CH_2Cl_2 then 4% i-PrOH/CH_2Cl_2) yielded two increasingly active fractions, with the latter exhibiting 100% Ltk^- inhibition at 40 mg/ml.

Reversed phase HPLC separation of this latter fraction (IBM-C18, 10 × 250 mm, 5 µm; MeOH/MeCN/THF/H_2O – 25:35:3.5:36.5; 1.8 ml/min; 213 nm detection) yielded a pure compound (retention time = 16.8 min; SiO_2-TLC: 4% i-PrOH/CH_2Cl_2, Rf = 0.25, 366 nm illumination – blue fluorescence) with the following Ltk^- cytostatic activities (% inhibition in parentheses) – 10 mg/ml (20%), 20 mg/ml (80%), 30 mg/ml (95%); values are semiquantitative since only minute quantities were available (see fig. 2 for more quantitative values with synthetic sample).

The structure of this active component was determined as being caffeic acid phenethyl ester (**1**, CAPE) from data shown in figure 1. Acid-catalyzed (p-toluene sulfonic acid) esterification of caffeic acid (CA) with phenethyl alcohol (molar ratios 1:15) in benzene (refluxing, 3–4 days, water removed by Dean-Stark trap) was the simplest synthetic route to CAPE. Following work-up, excess phenethyl alcohol was removed by Kugelröhr distillation (60 °C, < 0.1 mm Hg) to give pure CAPE, mp 126–128 °C, needles (benzene or H_2O), 40% yield. All properties of natural and synthetic CAPE were identical.

Cytostatic activities of synthetic CAPE were tested by observing its effect on the number of different cell types in

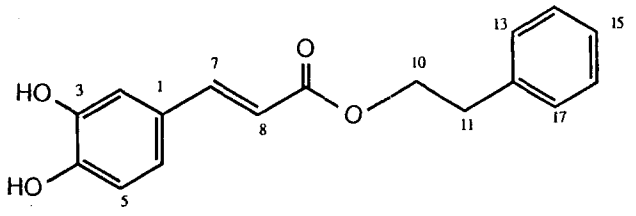

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Figure 1. UV (MeOH): 325 nm (ε 16,200), 300 (sh, 12,500), 245 (9,700), 235 (9,700); IR (KBr): 3490 cm^{-1} (OH's), 1685 (C = O); HR-MS (EI): $C_{17}H_{16}O_4$, m/z 284.1080, calcd 284.1049 (M^+), major fragments – 180, 163, 104, 91, 77; ^1H nmr (250 MHz, acetone – d_6): d 8.4–8.2 (br s, 2H, OH's), 7.52 (d, J = 15.9 Hz, 1H, 7-H), 7.31–7.15 (m, 5H, 13 ~ 17-H), 7.15 (d, J = 2.1, 1H, 2-H), 7.03 (dd, J = 8.2, 2.1 1H, 6-H), 6.86 (d, J = 8.2, 1H, 5-H), 6.26 (d, J = 15.9, 1H, 8-H), 4.34 (t, J = 7.0, 2H, 10-H), 2.98 (t, J = 7.0, 2H, 11-H); ^{13}C nmr (62.89 MHz, $CDCl_3$): d 167.8 (s), 146.5 (s), 145.2 (d), 144.0 (s), 137.8 (s), 128.9 (d), 128.5 (d), 127.6 (s), 126.6 (d), 122.5 (d), 115.5 (2d's), 114.5 (d), 65.2 (t), 35.3 (t)

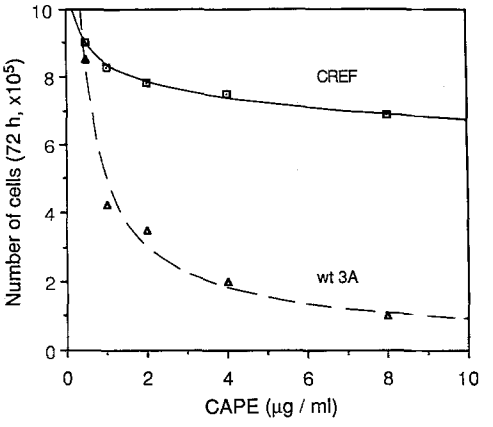

Figure 3. Effect of CAPE on the growth of CREF (normal rat) and wt3A (adenovirus transformed CREF) cells. Experimental conditions are similar to those described in figure 2.

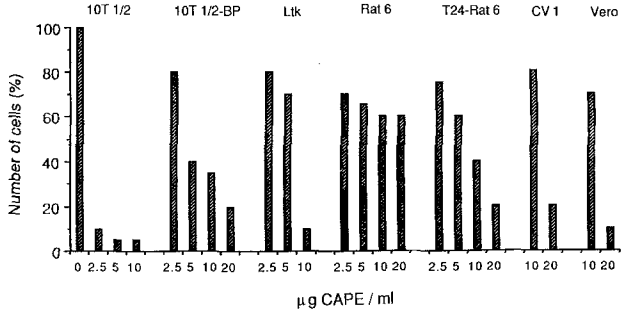

Figure 2. The effect of increasing concentrations of CAPE on various cultured cell lines. Approximately $3-4 \times 10^5$ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5–10% calf serum and maintained at 37 °C in 5% CO_2. Cells were plated on 60-mm Petri dishes. After 24 h, the cells were supplied with fresh medium containing various amounts of CAPE in ethanol or corresponding amounts of EtOH (control). After approx. 48 h, the cells on individual plates were counted by Coulter counter or fixed and stained with Giemsa.

culture. Results from various cell lines are summarized in figure 2. Mouse cells (C3H 10T1/2 and Ltk^-) appeared most sensitive, with concentrations as low as 2.5 µg/ml CAPE effectively blocking the increase in number of 10T1/2 cells. Interestingly, benzo(a)pyrene-transformed 10T1/2 cells exhibited increased resistance to CAPE action, requiring up to 20 µg/ml CAPE for 80% inhibition. In contrast, normal rat 6 cells were less sensitive to CAPE than those transformed by T24 oncogene. The growth of two monkey cell lines, CV1 and Vero, suffered severe inhibition only at concentrations of CAPE greater than 10 µg/ml.

The differential effect of CAPE on normal and transformed cells was further investigated with a cloned cell line of Fischer rat embryo fibroblasts (CREF) and its counterpart, transformed by adenovirus serotype 5 (wt3A)[5]. The transformed cells were identified by their altered morphology and the presence of adenovirus DNA sequences in their genomes[5]. The effects of various concentrations of CAPE on the growth of CREF and wt3A lines after 72 h are summarized in figure 3. At CAPE concentrations as high as 8 µg/ml, approximately 75% of the CREF cells remained unaffected, yet under the same conditions, the wt3A cells were nearly 90% inhibited. Similar effects were observed after 24 h and 48 h treatments. Hence, the presence of CAPE elicited a marked differential toxicity on the two rat cell lines which also displayed important differences in their biological properties. Unlike CREF cells, morphologically transformed cells exhibited higher saturation densities and were capable of anchorage-independent growth. Because the cytotoxic mechanism of CAPE is not understood at present, any explanation for its differential toxic effects on normal CREF and transformed wt3A cells or rat 6 and T24 oncogene transformed rat 6 cells remains speculative. This does, however, represent an interesting model system which may be useful for addressing such questions.

The effect of CAPE on human cancer cells was tested by measuring incorporation of [^{3}H]thymidine into the DNA of human breast carcinoma (MCF-7) and melanoma (SK-MEL-28 and SK-MEL-170) cell lines in culture[6]. Figure 4a reveals that 5 µg/ml CAPE inhibits incorporation of [^{3}H]T into the DNA of human breast carcinoma MCF-7 by ~ 50% and is completely blocked by concentrations of 10 µg/ml. More dramatic effects were observed with the two melanoma lines, SK-MEL-28 and SK-MEL-170. Figure 4b illustrates the effect of different concentrations of CAPE on the incorporation of [^{3}H]T into SK-MEL-28 cells. At 5 µg CAPE/ml the cells displayed minimal incorporation and were completely inhibited at 10 µg/ml. Similar inhibitions were observed for HT29 colon and renal carcinoma lines (not shown). On the other hand, the effect of CAPE on normal 1434 fibroblasts and melanocytes was significantly less. Incorporation of [3H]T into these normal cells was inhibited by only 50% at concentrations of CAPE up to ten times greater (50 µg/ml, data not shown). These differential effects were reminiscent of those observed with the normal CREF versus transformed wt3A cells or with rat 6 versus T24 transformed rat 6 cells (figs 2 and 3).

We have described the identification of a compound present in propolis which is at least partially responsible for its reported cytostatic properties. It represents the most active component as judged by the assay employed in this study (cytostatic towards Ltk^- cells). Its identification as CAPE, a compound of structural simplicity, permitted a one-step synthesis which was amenable to large-scale preparations. This in turn, allowed a more thorough investigation of CAPE's cytostatic properties in which several differential effects were uncovered. Most interestingly, human tumor cell lines displayed a significantly greater sensitivity to the action of CAPE than analogous normal lines.

Phenethyl alcohol and caffeic acid, the most obvious metabolic products of CAPE, displayed none of the aforementioned activities. Caffeic acid is known to possess several interesting pharmacological properties[3,7,8] and one possibility is that it represents the ultimate effector. Esterification

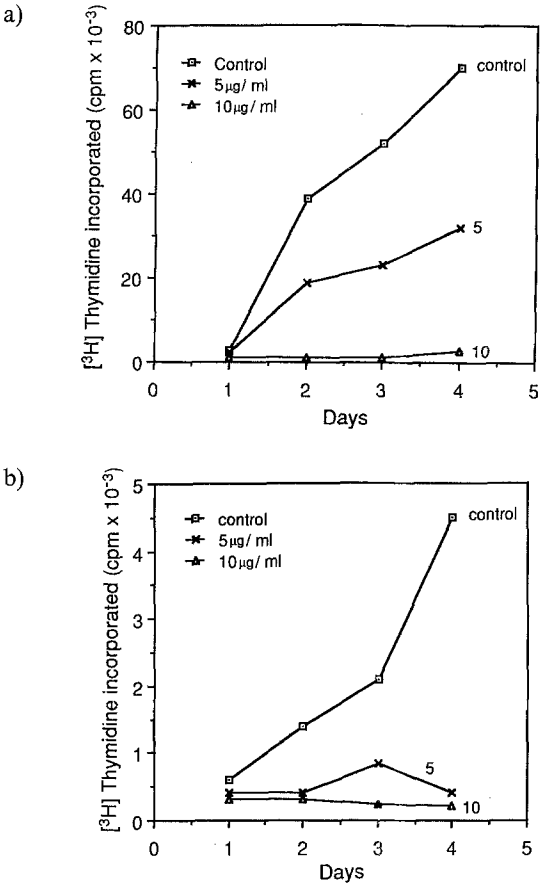

Figure 4. Effect of CAPE on the incorporation of [^{3}H]thymidine into DNA of (*a*) human MCF-7 breast carcinoma and (*b*) human SK-MEL-28 melanoma cells. Cells were maintained in Eagle's minimal essential medium (MEM) with Earle's salts and 10% fetal bovine serum. The cells were seeded in the same medium in tissue culture cluster plates (96 flat bottom wells) at 10^3 cells/well. After 24 h (day 1) cultures were washed and different concentrations of CAPE were added to each well in triplicate. Labeling of cells was accomplished on days 1–4 by incubating with 0.5 mCi [^{3}H]thymidine for 5 h. For further details see Eisinger et al.[6].

of caffeic acid with a lipophilic alcohol may simply facilitate its transport into cells, where it is hydrolyzed. Ester analogs of CAPE may be readily prepared for testing this and other possibilities.

The ready accessibility of analogs and labeled versions of CAPE will simplify further investigations into its mode of action, and may lead to an understanding of the observed differential effects on a molecular structural level. Furthermore, such studies with CAPE and other cytostatic compounds may provide a clearer insight into the molecular events responsible for the dissimilar biological properties exhibited by transformed and normal cells. Because the cytostatic action of CAPE is more dramatic on transformed cells, one may reasonably assume that it is at least partly responsible for the claimed carcinostatic properties of propolis.

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Adenosine-5′-triphosphate levels in experimental CaNT and Fib/t tumours of varying volume and degree of hypoxia

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Summary. The variation of adenosine-5′-triphosphate (ATP) content per unit mass of tumour, versus tumour volume was measured in vivo under normoxic conditions, using CaNT and Fib/t murine tumours grown in CBA and WHT mice respectively. A monotonically decreasing relation was found. Artificially induced tumour hypoxia resulting from 15 min of clamping was accompanied by reduced ATP levels.
Key words. CaNT and Fib/t tumours; degree of hypoxia; tumour volume; ATP.

Many approaches to the improvement of cancer therapy are based on the assumption that the tumour tissue contains viable hypoxic regions that are radioresistant and often chemoresistant too. Such hypoxic regions may therefore be responsible for some failures of treatment[1]. Oxygen diffuses out from the capillaries and is avidly consumed by the active metabolic activity of tumour cells. So oxygen is depleted within a distance of 150–200 µm from the capillary. Hence cells lying more than about 150 µm away from the capillary can exist in hypoxic and anoxic states[2]. Rapid proliferation of tumour cells may disorganize their blood supply to such an extent that sufficient oxygen and nutrients cannot reach all the cells and anaerobic glycolysis is necessary to provide the energy for cell growth and division[3]. These changes re-