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Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer

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Abstract *Purpose*: D-Limonene is a natural monoterpene with pronounced chemotherapeutic activity and minimal toxicity in preclinical studies. A phase I clinical trial to assess toxicity, the maximum tolerated dose (MTD) and pharmacokinetics in patients with advanced cancer was followed by a limited phase II evaluation in breast cancer. *Methods*: A group of 32 patients with refractory solid tumors completed 99 courses of D-limonene 0.5 to 12 g/m^2 per day administered orally in 21-day cycles. Pharmacokinetics were analyzed by liquid chromatography-mass spectrometry. Ten additional breast cancer patients received 15 cycles of D-limonene at 8 g/m^2 per day. Intratumoral monoterpene levels were measured in two patients. *Results*: The MTD was 8 g/m^2 per day; nausea, vomiting and diarrhea were dose limiting. One partial response in a breast cancer patient on 8 g/m^2 per day was maintained for 11 months; three patients with colorectal carcinoma had prolonged stable disease. There were no responses in the phase II study. Peak plasma concentration (C_{max}) for D-limonene ranged from 10.8 ± 6.7 to 20.5 ± 11.2 μ*M*. Predominant circulating metabolites were perillic acid (C_{max} 20.7 ± 13.2 to 71 ± 29.3 μ*M*), dihydroperillic acid (C_{max} 16.6 ± 7.9 to 28.1 ± 3.1 μ*M*), limonene-1,2-diol (C_{max} 10.1 ± 8 to 20.7 ± 8.6 μ*M*), uroterpenol (C_{max} 14.3 ± 1.5 to 45.1 ± 1.8 μ*M*), and an isomer of perillic acid. Both isomers of perillic acid, and *cis* and *trans* isomers of dihydroperillic acid were in urine hydrolysates. Intratumoral levels of D-limonene and uroterpenol exceeded the corresponding plasma levels. Other metabolites were trace constituents in tissue. *Conclusions:* D-Limonene is well tolerated in cancer patients at doses which may have clinical activity. The favorable toxicity profile supports further clinical evaluation.

Key words D-Limonene · Natural monoterpene · Farnesyltransferase inhibitor · Pharmacokinetics

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Introduction

D-Limonene, a monocyclic monoterpene, is widely distributed as a natural non-nutritive constituent of a variety of foods and volatile oils, particularly citrus oils [3]. Interest in this compound as a potential cancer chemotherapeutic agent was stimulated by pronounced chemopreventive and chemotherapeutic efficacy in spontaneous and carcinogen-induced animal tumor models with remarkably little toxicity [19]. Dietary administration of D-limonene increases tumor latency during the promotion/progression stage of *N*-methyl-*N*-nitrosourea (NMU)-induced rat mammary tumorigenesis [17], and during both the initiation and the promotion/progression stages of 7,12-dimethylbenz-(a)anthracene (DMBA)-induced carcinogenesis [9]. In rats with advanced NMU- and DMBA-induced mammary carcinomas fed a 10% D-limonene diet, 80% of tumors regress; most regressions are complete and toxicity is minimal [2, 13].

D-Limonene selectively inhibits post-translational isoprenylation of small GTP-binding proteins including $p21^{ras}$ which regulate signal transduction and cell growth [5]. Cellular transforming activity of Ras is critically dependent on post-translational addition of a farnesyl isoprenoid moiety to the *C*-terminal tetrapeptide. The reaction is catalyzed by farnesyltransferase [15], inhibition of which may be a mechanism whereby D-limonene inhibits isoprenylation [7]. It has been postulated that such inhibition may alter signal transduction and gene expression leading to cytostasis, followed by apoptosis, cellular redifferentiation and tumor regression (reviewed in reference 11).

In human subjects and several other mammalian species, oral D-limonene is completely absorbed and undergoes extensive biotransformation to active metabolites [8, 14, 16]. The predominant circulating metabolites perillic acid, dihydroperillic acid and limonene-1,2-diol all have greater pharmacological potency in vitro than the parent compound as inhibitors of cellular proliferation and protein isoprenylation [6].

A single-center phase I clinical trial was undertaken to determine the toxicity and maximum tolerated dose (MTD) of orally administered D-limonene in patients with advanced solid tumors, and to characterize its pharmacokinetics following single and repetitive dosing. The efficacy of D-limonene in breast cancer patients and measurement of intratumoral drug and metabolite levels were evaluated in a limited pilot phase II investigation.

Patients and methods

Patient selection

Patients with histologically proven nonhematological malignancies and progressive locally advanced or metastatic disease refractory to conventional therapies were considered eligible for enrollment. Principal additional inclusion criteria were: WHO performance status 0–2 [20]; projected life expectancy ≥2 months; ≥3 weeks following completion of previous anticancer therapy (≥6 weeks for nitrosoureas or mitomycin C) and recovery from toxicity; and adequate bone marrow function (Hb ≥9.0 g/dl, WBC ≥3000/mm^3, platelets ≥100 000/mm^3), renal function (normal creatinine clearance), and hepatic function (serum bilirubin ≤2.0 mg/dl, alanine and aspartate transaminases not more than three times the upper limit of the normal range). Exclusion criteria were pregnancy, prior or concurrent primary malignancies at other sites, coexisting nonmalignant pulmonary, cardiac or metabolic disease, or positive human immnodeficiency virus serology.

Ethical considerations

All patients were required to give informed written consent in a form approved by the Research Ethics Committee of Charing Cross Hospital and by the Cancer Research Campaign (CRC) Protocol Review Committee. The trial was conducted under the auspices of the CRC Phase I/II Clinical Trials Committee and in accordance with the principles outlined in the Declaration of Helsinki.

Drug formulation

D-Limonene (97% purity by capillary gas chromatography, GC; Aldrich Chemical Corporation, Milwaukee, Wis.) was formulated in 400-mg capsules for oral administration. In preformulation studies (Aston Molecules, Aston Science Park, Birmingham, UK), D-limonene had been shown to be susceptible to photo-oxidation and temperatures ≥50 °C but to remain stable for up to 3 months under ambient conditions when protected from light. The low viscosity and high vapor pressure of D-limonene precluded simple formulation in sealed gelatin capsules. Preliminary experiments indicated that a mixture of D-limonene/hydrogenated vegetable oil E85/ethylcellulose 10NF, 1:0.08:0.12 by weight, was liquid at elevated temperatures (> 40 °C) but gelled under ambient conditions. This formulation could be filled into hard gelatin capsules using melt/fill technology and reduced D-limonene volatility to enable capsule banding thereby preventing possible leakage. A batch of 400-mg capsules was manufactured and subjected to an accelerated stability study under conditions of elevated temperature, light and oxygen. D-Limonene and degradation products were assayed by high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS). At 55 °C and in an oxygen atmosphere, there was loss of D-limonene and the appearance of degradation products (Table 1). The *cis* and *trans* isomers of 1,2-epoxy-*p*-menth-8-ene and carveol were detected and arise from oxidation of the double bonds. The effect was temperature dependent and influenced by oxygen since capsules stored in air exhibited reduced degradation. An ongoing stability study has confirmed that the capsules are stable for at least 2 years when stored in the dark under ambient conditions (25 °C), enabling phase I clinical studies to proceed.

Treatment protocol

D-Limonene was administered by mouth as a single dose on day 1 after an overnight fast. Treatment recommenced on day 4 with regular daily administration in three divided doses. Patients initially received D-limonene for 21 days. Treatment was discontinued at the end of this period in the event of disease progression, unacceptable toxicity, or patient request. Standard Union Internationale Contre le Cancer (UICC) response criteria, and National Cancer Institute (NCI) Common Toxicity Criteria (CTC) were used [18]. Patients with stable disease, or complete/partial disease response were considered suitable to continue treatment in cycles of 21 days until disease progression or toxicity supervened. Complete response was defined as complete disappearance for at least 4 weeks of all clinical disease, as assessed by physical and/or radiological

Table 1 Accelerated stability study of D-limonene formulation. Chemical stability of 400 mg D-limonene capsules stored for 1 month in an oxygen atmosphere and protected from light as assessed by GC-MS. The values for D-limonene are percentages of stated capsule content. The values for the degradation products are areas of the products expressed as a percentage of total area

	Storage temperature			
	4 °C	25 °C	37 °C	55 °C
D-Limonene	99.1	96.2	94.9	93.5
cis-1,2-Epoxy-*p*-menth-8-ene	0.017	0.023	0.027	0.045
trans-1,2-Epoxy-*p*-menth-8-ene	0.013	0.018	0.021	0.034
cis-Carveol	0.014	0.018	0.02	0.036
trans-Carveol	0.01	0.012	0.013	0.025

examination. The definition of partial response was ≥50% reduction in tumor size as determined by two measurements not less than 4 weeks apart, stable disease as < 50% tumor regression or < 25% progression, and progressive disease as ≥25% increase in tumor measurements. Patients were hospitalized for the first 72 h to facilitate clinical evaluation and sampling for pharmacokinetic analysis; thereafter treatment was continued on an outpatient basis.

Cohorts of three to five patients were entered at each of eight dose levels in an escalating schedule ranging from 0.5 to 12 g/m^2 per day. Intraindividual dose escalation was not permitted. The initial daily dose of 0.5 g/m^2 was equivalent to one-tenth the oral minimal lethal dose (LD_{10}) in mice, the most sensitive species [19], and represented 10% of a dose previously administered to healthy human volunteers without serious short-term toxicity in a single-dose study [8]. Subsequent cohorts of patients were added only after subjects in the preceding cohort had completed at least the first 21-day cycle of treatment without dose-limiting (CTC grade II or higher) toxicity attributable to D-limonene. Any patients who developed grade III/IV toxicity were withdrawn from the study and two additional patients added to that dosage cohort. When a dose was reached at which more than 50% of the patients in that cohort developed dose-limiting toxicity, the next lower dose was considered the MTD. Supportive treatment was not restricted, but concomitant administration of other investigational agents or anticancer therapies was proscribed.

Study parameters

Complete clinical assessment including performance status and measurement of tumor dimensions was undertaken in every patient prior to entering the study and at the commencement of each subsequent treatment cycle. Complete blood count, serum biochemistry and liver function tests, and urinalysis were performed at baseline and repeated at weekly intervals; tumor imaging by appropriate X-rays, computed tomography and/or ultrasound scans was performed on entry and 3-weekly thereafter for the duration of study.

Pharmacokinetic studies

Serial samples of peripheral venous blood (~7 ml) were collected into heparinized tubes (Vacutainer; Becton Dickinson, Orangeburg, N.Y.) prior to administration of D-limonene, at 10-min intervals for the first 30 min, at 1, 2, 4, 6, 12, 24, 48 and 72 h, and on days 8 and 15 of the first treatment cycle. A sampling profile from 0 (predose) to 12 h was repeated on day 21. Blood samples for the first 6 h were obtained via an indwelling peripheral venous cannula to minimize patient discomfort; thereafter sampling was by direct venepuncture. After mixing by gentle inversion, plasma was separated by centrifugation (3000 *g* for 15 min at 4 °C). Aliquots of urine (50 ml) were taken from 24-h collections obtained predosing and for 24 h after D-limonene administration. Plasma and urine samples were stored at −20 °C until analyzed. Methods for the characterization of phase I and phase II metabolites in patient samples by liquid chromatography-mass spectrometry (LC-MS) and ^{1}H-NMR spectroscopy, and quantitative analysis of circulating monoterpenes were developed in our laboratory and performed exactly as described previously [21].

Pharmacokinetic data were obtained for ten patients treated with D-limonene in doses ranging from 8 to 12 g/m^2 per day. Plasma concentration-time profiles were analyzed by noncompartmental methods. Estimates of peak plasma concentration (C_{max}) and time to peak plasma concentration (T_{max}) were obtained by inspection of individual patient profiles. Area under the curve (AUC) values from 0 to 24 h after the initial dose were calculated using the linear trapezoidal rule. Apparent plasma clearance of D-limonene was defined as dose/AUC.

Intratumoral monoterpene levels

In a preliminary analysis, D-limonene and its major metabolites were identified and quantified in metastatic lymph node tissue obtained during routine biopsy from two breast cancer patients in the phase II study receiving D-limonene 8 g/m^2 per day, and the results compared with corresponding plasma concentrations determined as described previous [21]. Briefly, each tissue sample (200 mg) was added to 2 ml 50 m*M* sodium phosphate buffer, pH 7.5, containing sodium dodecyl sulfate 0.1% (w/v) and 2 μg α-terpinene as internal standard. Samples in glass tubes were ground using a Potter homogenizer with a PTFE pestle set at 2500 rpm. After centrifugation at 2000 *g* for 15 min at 15 °C, supernatants were decanted, subjected to solid phase extraction and analyzed by LC-MS as previously described [21]. Standard curves were generated from spiked samples of rat liver (200 mg) containing known amounts of D-limonene and metabolites (from stock solutions of 1 mg/ml in methanol) to give a series of calibration standards from 1 to 25 μg/g each with 10 μg/g α-terpinene added as internal standard. Standard curves were linear over the range 0–25 μg/g ($r > 0.99$, linear regression analysis). The calibration curves were used to determine analyte concentrations in tissue, and the results compared with circulating monoterpene levels in corresponding plasma samples. Samples were analyzed in duplicate, each batch including blank and quality control samples. Between-day coefficients of variation were < 20% for all analytes. Analytical recoveries ranged from 85% to 110%.

Statistical methods

The nonparametric Mann Whitney *U*-test was used for comparative analysis of paired data. Plasma concentrations were compared by one-way ANOVA; time and group comparisons by two-way ANOVA. Statistical significance was defined at the 5% level ($\alpha = 0.05$).

Results

Patient characteristics

A group of 32 patients, 17 female and 15 male aged 35–78 (median 57) years, with locally advanced and metastatic solid tumors entered the initial phase I study. In a limited phase II investigation, accrual was subsequently expanded to a further 10 female patients with advanced breast cancer, aged 40–82 (median 57) years. The demographic characteristics of the patients are summarized in Table 2.

Toxicities

The dose escalation schedule and adverse events attributable to D-limonene are indicated in Tables 3 and 4, respectively. Of the first 32 patients enrolled in this study, 8 discontinued prematurely before completing the first cycle of treatment, 6 because of progressive disease and 2 with D-limonene-associated toxicity. One patient at the 6 g/m^2 per day dose level had subjective intolerance of mild upper gastrointestinal symptoms, and another patient on 10 g/m^2 per day withdrew from the study because of CTC grade II nausea and grade I diarrhea. Of 10 patients who completed one cycle of treatment, 8 discontinued treatment because of progressive malignancy, and 2 were unwilling to continue in the study. A further 6 patients went on to complete two cycles of D-limonene, 2 patients received three cycles of

Table 2 Patient characteristics

Patients entered/evaluable	32/32	10/10
Age (years)		
Median	57	57
Range	35–78	40–82
Sex		
Male	15	
Female	17	10
Performance status		
0	17	9
1	12	1
2	3	
Tumor type		
Breast	16	10
Colon/rectum	7	
Other[a]	9	
Prior therapy		
Surgery	29	9
Chemotherapy	28	7
Radiation therapy	23	7
Hormone therapy	20	10
Supportive therapy only	2	

[a] Metastatic adenocarcinoma of unknown primary (two patients), stomach (two patients), esophagus, pancreas, bronchus, ovary and soft-tissue sarcoma

Table 3 Dose escalation schedule

Dose of D-limonene (g/m^2/day)	No. of patients	No. of treatment cycles per patient
0.5	5	<1, 2, 2, 12, 17
1	5	1, 1, 2, 6, 10
2	5	<1, 1, 2, 3, 3
4	5	<1, 1, 1, 1, 2
6	3	<1, <1, 5
8	3	<1, 1, 21
10	3	<1, 1, 2
12	3	<1, 1, 1
8	10	<1, <1, <1, 1, 1, 2, 2, 3, 3, 3

treatment, and 6 patients completed five or more cycles; all were withdrawn from the study because of disease progression. In total, 99 treatment cycles of D-limonene were administered. The toxicity of D-limonene was limited to gastrointestinal side effects and was dose related. Grade IV or serious organ toxicities were not seen. The MTD was 8 g/m^2 per day. Three of the 32 patients (9.4%) were noncompliant and were withdrawn from the study at their own request.

Ten additional breast cancer patients received 15 cycles of D-limonene treatment at a dose of 8 g/m^2 per day. Three patients failed to complete the first cycle, of whom two had progressive malignancy and one was intolerant of CTC grade I diarrhea. Two patients with progressive disease were withdrawn from the study after completing one treatment cycle. Two patients completed two cycles and another three patients received three cycles of treatment before disease progression necessitated withdrawal from the study.

Response

One female breast cancer patient in the initial dose-escalation study showed a partial response to D-limonene at a dose of 8 g/m^2 per day. Axillary and supraclavicular lymph nodes proven to contain metastatic infiltrating ductal carcinoma on fine needle aspiration cytology were stable during the first five treatment cycles. At the start of the sixth cycle, there had been a reduction of >50% in the size of the supraclavicular lymphadenopathy, and by course 14 axillary lymphadenopathy was no longer palpable. Widespread lytic bone metastases showed increased sclerosis after 3 months, associated with substantial reduction in bone pain. The response was sustained for 11 months before disease progression in bone necessitated withdrawal from the study. Three additional patients with colorectal carcinoma showed stabilization of disease for longer than 6 months on D-limonene. One patient with locally advanced mucinous cystadenocarcinoma of the appendix had no measurable

Table 4 Toxicity attributable to D-limonene at each dose level (– no toxicity recorded)

Dose (g/m^2/day)	No. of patients	Maximum CTC grade											
		Nausea				Vomiting				Diarrhea			
		0	1	2	3	0	1	2	3	0	1	2	3
0.5	5	5	–	–	–	5	–	–	–	5	–	–	–
1	5	5	–	–	–	5	–	–	–	5	–	–	–
2	5	5	–	–	–	5	–	–	–	4	1	–	–
4	5	5	–	–	–	5	–	–	–	3	–	2	–
6	3	–	3	–	–	2	–	1	–	2	–	–	1
8	3	1	1	1	–	3	–	–	–	–	3	–	–
10	3	–	1	2	–	2	1	–	–	–	1	2	–
12	3	3	–	–	–	3	–	–	–	2	–	1	–
8	10[a]	5	3	2	–	9	1	–	–	4	4	2	–
All doses	42	29	8	5	0	39	2	1	0	25	9	7	1

[a] Additional breast cancer patients enrolled following the initial phase I study

tumor progression for 9 months at a dose of 0.5 g/m^2 per day. Another patient with presacral recurrence of an adenocarcinoma of the sigmoid colon showed a reduction of < 50% in the size of the tumor after two cycles of D-limonene at 0.5 g/m^2 per day which was maintained for a total of 12 months. A third patient with local retrovesical recurrence of colorectal adenocarcinoma had stable disease on 1 g/m^2 per day for 7.5 months.

The partial response to D-limonene in a breast cancer patient was the basis for the limited phase II study. Ten additional breast cancer patients with locally advanced or metastatic disease, heavily pretreated and refractory to conventional hormonal and cytotoxic treatment received the same dose of 8 g/m^2 per day. Seven patients completed one or more cycles of treatment, but no additional responses were observed.

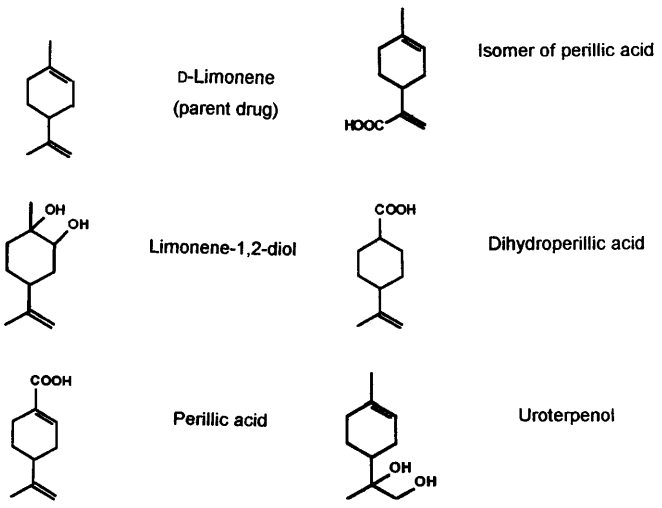

Fig. 1 Structures of D-limonene and major circulating metabolites

Pharmacokinetic studies

In addition to D-limonene, there were five major plasma metabolites: perillic acid, dihydroperillic acid, limonene-1,2-diol, an analog of perillic acid (putative identity *p*-mentha-1,8-diene-10-carboxylic acid), and uroterpenol [21] (Fig. 1). Contrary to previous speculation regarding the identification of perillic acid methyl ester and dihydroperillic acid methyl ester as putative minor metabolites in human plasma [6, 8], these compounds were not detected in any plasma sample analyzed by LC-MS in the present study, including plasma sampled from patients at the highest dose level of 12 g/m^2 per day. Furthermore, there was no evidence of in vitro methylation of the respective acids when incubated in fresh normal human plasma for up to 48 hours at 37 °C and extracted and analyzed using LC-MS as previously described [21].

Pharmacokinetic parameters are summarized in Table 5. The plasma concentration-time courses for D-limonene and metabolites in a representative patient are depicted in Fig. 2. D-Limonene was slowly absorbed following oral administration with T_{max} 1–6 h. The mean ± SD C_{max} ranged from 10.8 ± 6.7 μ*M* at 8 g/m^2 per day to 20.5 ± 11.2 μ*M* at 12 g/m^2 per day. Plasma D-limonene concentrations then fell in a monoexponential fashion. AUC values for D-limonene showed little variation with the dose administered. Perillic acid was the major circulating metabolite in most patients with mean ± SD C_{max} ranging from 20.7 ± 13.2 μ*M* (range 7.7–38.3 μ*M*) in four patients at the 8 g/m^2 per day dose level to 71 ± 29.3 μ*M* (range 40–98.3 μ*M*) in three patients receiving 10 g/m^2 per day. Plasma concentrations of the metabolites declined in parallel with D-limonene but uroterpenol appeared to be eliminated more slowly. Pharmacokinetic analysis on day 21 after repetitive dosing showed an absence of accumulation of any of the

Table 5 Plasma pharmacokinetic parameters (*AUC* area under the plasma concentration curve from time zero to 24 h, *Cl* apparent whole body clearance, *V* volume of distribution)

	D-Limonene dose (g/m^2/day)	AUC (μ*M* · h)	Cl (l/h)	V (l)
D-Limonene	8	151 (46–228)[a]	14.4 (7–40)	126 (69–352)
	10	165 (42–376)[b]	14.9 (6.2–58.8)	123 (76–170)
	12	154.5 (79–230)[c]	20.4 (13.3–27.4)	103.5 (42–165)
Perillic acid	8	277 (81–493)		
	10	976 (466–1326)		
	12	482.5 (339–626)		
Dihydroperillic acid	8	244 (111–369)		
	10	193 (171–480)		
	12	344.5 (320–369)		
Limonene-1,2-diol	8	142 (55–227)		
	10	119 (26–319)		
	12	230.5 (185–276)		
Uroterpenol	8	133.5 (86–181)		
	10	157.3 (137–335)		
	12	260 (163–357)		

[a] Median (range) in four patients
[b] Median (range) in three patients
[c] Median (range) in two patients

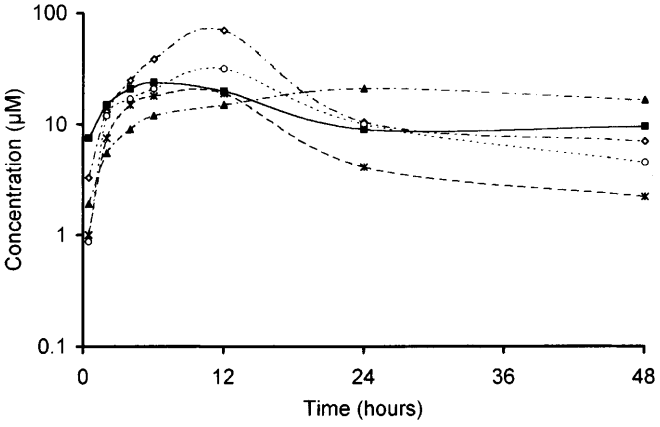

Fig. 2 Typical plasma concentration-time plot of D-limonene (■) and metabolites perillic acid (◊), dihydroperillic acid (○), limonene-1,2-diol (∇) and uroterpenol (*) at a dosage of 10 g/m^2

major plasma metabolites (Table 6). Variation in peak plasma concentrations between days 1 and 21 was evident but there was no systematic trend. The major urinary metabolites were glucuronide conjugates of perillic acid, dihydroperillic acid, uroterpenol and a monohydroxylimonene. Both isomers of perillic acid, and the *cis* and *trans* isomers of dihydroperillic acid, were detected in urine hydrolysates [21].

D-Limonene was detected in tumor tissue from two breast cancer patients at levels 1.9- and 5.5-fold greater than in plasma (Table 7). Intratumoral levels of uroterpenol were also substantially higher than circulating levels. With the exception of perillic acid in one patient, the other phase I metabolites were detectable in only trace amounts in tumor tissues.

Discussion

This is the first clinical trial of D-limonene in patients with advanced cancer. As found in preclinical and normal human volunteer studies, the drug was well tolerated in patients with heavily pretreated advanced cancer. Doses in excess of the MTD (8 g/m^2 per day) were associated with an unacceptable incidence of gastrointestinal side effects, but in all cases toxicity was reversible, and there was no serious organ dysfunction or CTC grade IV toxicity.

Several novel and important findings have emerged from the pharmacokinetic study [21]. D-Limonene is not a minor plasma constituent but circulates at concentrations similar to those of its major metabolites. A previously undescribed isomer of perillic acid was detected as a major plasma metabolite in all patients studied (putative identity *p*-mentha-1,8-diene-10-carboxylic acid); further characterization is currently underway and will form the subject of a subsequent report. Although previously identified as a phase II metabolite, uroterpenol is now known to be a major circulating metabolite. In contrast to reports suggesting that perillic acid methyl ester and dihydroperillic acid methyl ester are minor circulating metabolites in rodents and in humans, we were unable to identify these compounds in plasma, even in patients receiving doses of D-limonene threefold higher than administered in a prior study [8]. We conclude that the methyl esters of perillic acid and dihydroperillic acid previously identified by GC or GC-MS analysis were almost certainly artifacts which arose either by in vitro carboxylate methylation or by

Table 6 Peak plasma levels of D-limonene and metabolites. Values are means ± SD

Dose (g/m^2/day)	D-Limonene (μ*M*)	Perillic acid (μ*M*)	Dihydroperillic acid (μ*M*)	Limonene-1,2-diol (μ*M*)	Uroterpenol (μ*M*)
8 Day1[a]	10.8 ± 6.7	20.7 ± 13.2	16.6 ± 7.9	11.1 ± 6.1	14.3 ± 1.5
Day 21[b]	12 ± 7.3	37.4 ± 29.4	11.7 ± 9.2	5.5 ± 3.6	6.7 ± 5
10 Day 1[c]	13.8 ± 11	71 ± 29.3	18.2 ± 12.2	10.1 ± 8	17.4 ± 4.8
Day 21[d]	10.9	34.8	5.5	0.5	
12 Day 1[e]	20.5 ± 11.2	46.4 ± 29.6	28.1 ± 3.1	20.7 ± 8.6	45.1 ± 1.8
Day 21[f]	4.9 ± 2.3	40.5 ± 18	13.6 ± 6.5	3.4 ± 0.7	

[a] Four patients
[b] Three patients
[c] Three patients
[d] A single patient
[e] Two patients
[f] Two patients

Table 7 Intratumoral levels of D-limonene and metabolites

	Patient A			Patient B		
	Tissue (μg/g)	Plasma (μg/ml)	Tissue:plasma	Tissue (μg/g)	Plasma (μg/ml)	Tissue:plasma
D-Limonene	11.7	2.12	5.52	3.49	1.83	1.91
Limonene-1,2-diol	0.021	0.05	0.42	0.005	0.03	0.17
Uroterpenol	0.085	0.05	1.7	0.046	0.02	2.3
Perillic Acid	1.22	0.84	1.45	0.11	0.78	0.14
Dihydroperillic Acid	1.206	1.62	0.74	0.84	1.25	0.67
Total monoterpenes	14.23	4.68	3.04	4.49	3.91	1.15

esterification under the high temperature conditions employed in GC to desorb analytes.

It is intriguing that the only responder in the present study was a breast cancer patient who received a dose of D-limonene almost fivefold less than the minimum effective therapeutic dose predicted by extrapolation from rat mammary tumor studies [4, 10, 12]. Plasma levels of the parent compound and major metabolites were at least an order of magnitude lower than reported IC_{50} values for 21–26 kDa protein isoprenylation [5, 7] and cellular proliferation [6, 7] in vitro. Moreover, a recently developed assay based on in vitro inhibition of Ras farnesylation by human farnesyl protein transferase of placental origin, yields IC_{50} values for D-limonene of >40 m*M*, for perillic acid of 9.6 m*M*, and for perillyl alcohol of 7.6 m*M* (M.G. Rowlands, personal communication). In normal female rats given an oral dose of 8-[^{14}C]-D-limonene there is apparent concentration of D-limonene-derived material in adipose and mammary tissues [6]. If our preliminary results of intratumoral monoterpene levels in breast cancer patients are confirmed in further studies, this mechanism may account for the apparent discrepancy. However, there is a growing body of evidence that the biological activity of D-limonene and related monoterpenes cannot be attributed to inhibition of farnesyltransferase alone. Other possible mechanisms of action include inhibition of geranylgeranyltransferase I, cellular redifferentiation, transforming growth factor β activation, and enhanced apoptosis [1, 11].

The present study confirms the low toxicity of D-limonene after single and repetitive dosing for extended periods as long as 1 year in patients with advanced cancer. The biometabolism of D-limonene was characterized by LC-MS; extensive biotransformation to active monoterpenes suggests that biological activity is mediated by the phase I metabolites, but probably not solely via isoprenylation inhibition. The recognized cellular antiproliferative activity, chemopreventive and chemotherapeutic efficacy in spontaneous and carcinogen-induced animal tumor models, the absence of serious toxicity in cancer patients and the durable partial response in a breast cancer patient observed in this study, all support D-limonene as the prototype of a novel class of chemotherapeutic drugs warranting further clinical evaluation. However, the recommended phase II dose for D-limonene is still unclear. The MTD may not represent the optimum biologic dose for developing D-limonene as a cytostatic or chemopreventive agent in phase II efficacy studies. Further characterization of biological activity and correlation with pharmacokinetic data is needed.

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