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ORIGINAL ARTICLE

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Clinical and pharmacologic study of tributyrin: an oral butyrate prodrug

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Abstract *Purpose*: Butyrate is a small polar compound able to produce terminal differentiation and apoptosis in a variety of in vitro models at levels above 50–100 μ*M*. Previously our group demonstrated that daily oral administration of the prodrug, tributyrin, is able to briefly achieve levels >100 μ*M*. Given in vitro data that differentiating activity requires continuous butyrate exposure, the short t1/2 of the drug and a desire to mimic the effects of an intravenous infusion, we evaluated a three times daily schedule. *Patients and methods*: Enrolled in this study were 20 patients with advanced solid tumors for whom no other therapy was available, had life expectancy greater than 12 weeks, and normal organ function. They were treated with tributyrin at doses from 150 to 200 mg/kg three times daily. Blood was sampled for pharmacokinetic analysis prior to dosing and at 15 and 30 min and 1, 1.5, 2, 2.5, 3, 3.5 and 4 h thereafter. *Results*: The patients entered comprised 15 men and 5 women with a median age of 61 years (range 30–74 years). Prior therapy regimens included: chemotherapy (median two prior regimens, range none to five), radiation therapy (one), no prior therapy (one). There was no dose-limiting toxicity. Escalation was halted at the 200 mg/kg three times daily level due to the number of capsules required. A median butyrate concentration of 52 μ*M* was obtained but there was considerable interpatient variability. No objective responses were seen. There were four patients with prolonged disease stabilization ranging from 3 to 23 months; median progression-free survival was 55 days. Two patients with chemotherapy-refractory non-small-cell lung cancer had survived for >1 year at the time of this report without evidence of progression. *Conclusion*: Tributyrin is well tolerated and levels associated with in vitro activity are achieved with three times daily dosing.

Keywords Butyrate · Histone deacetylase inhibitor · Differentiating agent

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Introduction

For over 20 years butyrate has been investigated as a potential therapeutic agent for the treatment of cancer and hematologic disorders based on its in vitro ability to cause differentiation and/or growth arrest [1]. These studies have demonstrated that this differentiating ability requires continuous exposure to butyrate. This differentiating ability is felt to be primarily a result of gene reactivation due to inhibition of histone deacetylase inhibition resulting in induction of the cyclin-dependent kinase inhibitor p27 and consequent downstream effects on Rb phosphorylation [2, 3, 4]. In addition to its differentiating abilities, butyrates have demonstrated other physiologic effects relevant to cancer therapeutics including antiangiogenic effects and induction of apoptosis [5, 6].

Development has been hindered by the requirement for large intravenous volumes of the drug as well as continuous administration needed to produce

physiologically relevant levels. In addition, the agent produces an odor felt to be unacceptable by the majority of patients. Tributyrin is a triglyceride containing three butyrate moieties esterified to glycerol (Fig. 1). Tributyrin can be hydrolyzed by either acidic to neutral aqueous solutions or by plasma esterases. We have previously reported the preliminary results of a phase I trial of daily oral tributyrin in solid tumors [7]. That study demonstrated the ability to briefly (0.5–4 h) achieve relevant levels (up to 450 μM) of butyrate and led us to evaluate three times daily dosing. This report describes our experience with three times daily oral dosing.

Materials and methods

Patient eligibility

Patients were entered if they were at least 18 years of age, had a pathologically documented solid tumor refractory to standard therapy or for which there was no standard treatment and ECOG performance status ≤ 2. Patients were required to have normal hepatic (bilirubin ≤ 1.5 mg/dl, AST and/or ALT ≤ 1.5 times normal), renal (serum creatinine ≤ 1.5 mg/dl), and hematologic (WBC $\geq 3000/\mu l$, platelet count $\geq 100{,}000/\mu l$, hemoglobin ≥ 9 g/dl) function, and a life expectancy of at least 3 months. At least 4 weeks must have elapsed since their last chemotherapy (8 weeks if nitrosoureas were utilized) or radiotherapy.

Drug supply

Tributyrin (NSC-661583) was supplied by the National Cancer Institute, Cancer Therapy Evaluation Program. Drug was supplied as white, soft gelatin capsules containing 500 mg of tributyrin without additives.

Drug treatment

Cohorts of at least three patients were entered at each dose level, as previously described [2]. As no dose-limiting toxicity was experienced with once-daily oral dosing and given the preliminary pharmacokinetic data, the schedule was changed to three times daily dosing. The results of this initial experience have been previously reported [7]. Four patients were treated at 150 mg/kg three times daily (one escalated in the third cycle to 200 mg/kg three times daily) and 16 were treated at an initial dose of 200 mg/kg three times daily.

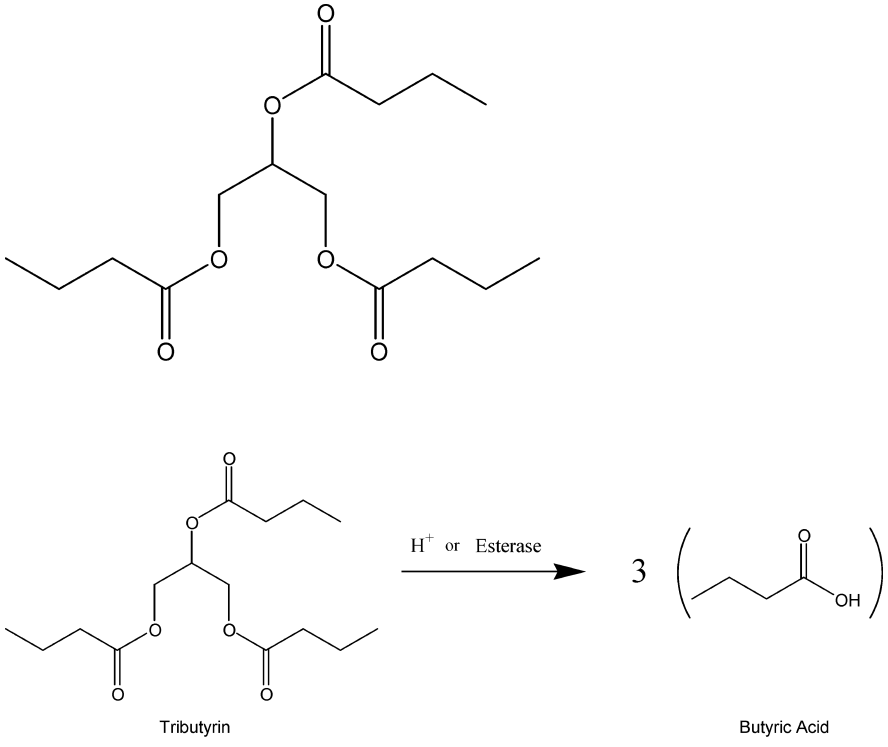
Clinical assessment

All patients underwent physical examination and a history was taken prior to entry to the study and weekly during therapy. Appropriate scans were repeated every 8 weeks. Complete blood counts and chemistry panels (including hepatic enzymes) were performed weekly. Dose-limiting toxicity was defined as a grade 3 or greater nonhematologic toxicity or sustained (> 5 days) hematologic toxicity or neutropenic fever. National Cancer Institute Common Toxicity Criteria Version 1.D were utilized. Complete response was defined as complete disappearance of all known disease without development of new lesions, partial response as a 50% or greater reduction in the sum of the perpendicular diameters of all measurable lesions, and progressive disease as a 25% or greater increase in the sum of the perpendicular diameters of all measurable lesions. Stable disease was less than a 25% reduction in the sum of the perpendicular diameters of all measurable lesions.

Pharmacokinetics

Blood samples for pharmacokinetic analysis were obtained prior to each dose and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h after the dose for each sampling period. Samples were taken after the first daily dose on days 1 and 15, and samples were also taken from three additional patients on days 2 and 16. The plasma concentrations of butyrate and the parent compound tributyrin were

Fig. 1 Tributyrin



determined using gas chromatography with flame ionization detection. Approximately 7 ml of blood per sample was collected into heparinized tubes, and plasma was obtained by centrifugation at 10,000 *g* and 4°C for 10 min. The plasma was then frozen and stored at approximately −70°C until analyzed. Butyrate and tributyrin were extracted from 100 µl of plasma by acetonitrile precipitation with the internal standard heptanoic acid. The extracted samples was dried and reconstituted with 50 µl acetonitrile. Plasma concentrations of butyrate and tributyrin were determined using gas chromatography with flame ionization detection with a carrier gas of helium at a flow rate of 8 ml/min over an Agilent HP-FFAP (polyethylene glycol-base modified) column (30 mm, 0.53 mm ID, 1.0 µm). A temperature ramp of 80°C to 280°C was used to achieve analyte separation. Heptanoic acid 5 µg/ml was used as the internal standard. The assay for both butyrate and tributyrin was validated over the range 0.30–30 µg/ml (range 3.40–340 µ*M* and 0.992–99.2 µ*M* for butyrate and tributyrin, respectively).

Pharmacokinetic parameters were determined by model-independent methods using WinNonlin Professional version 3.1 (Pharsight Corporation, Mountain View, Calif.) for parameter estimation [8]. The maximum plasma concentration (Cmax) and time of maximum plasma concentration (tmax) were the observed values. The area under the plasma concentration time curve from time zero to the time of the last quantifiable sample (AUC) was calculated using the linear trapezoidal method. The AUC was reported if at least two consecutive plasma concentrations were observed for the sampling period.

Histone acetylation

Immunocytochemical analysis of acetylated histone H3 and acetylated histone H4 was performed on samples from two patients. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll. PBMC were isolated by centrifugation on Ficoll and pelleted onto glass slides by cytocentrifugation. For detection of histones, cells were fixed in 95% ethanol/5% glacial acetic acid for 1 min. The cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature [9]. Nonspecific binding sites were blocked by incubating the cells with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h at 4°C. Polyclonal rabbit anti-acetylated H3 and acetylated H4 were detected using antibodies from Upstate Biotechnology (Lake Placid, N.Y.). Anti-acetylated histone antibody was added to the coverslips followed by incubation for 1 h at 4°C . The coverslips were washed two times for 2 min with PBS and incubated at 4°C for 1 h with Cy3-conjugated goat anti-rabbit immunoglobulin (Molecular Probes, Eugene, Ore.). After incubation, the coverslips were washed with PBS, rinsed quickly with water, air-dried, mounted using SlowFade (Molecular Probes), and imaged using a Zeiss Axiophot microscope interfaced with a CCD camera (Optronics Engineering, Goleta, Calif.). Positive controls were prepared for each assay by centrifugation of a buffy coat from normal volunteer blood donors (a generous gift from Dr. Susan Leitman, Department of Transfusion Medicine, Clinical Center, NIH) on a Ficoll-Paque Plus (Amersham, Little Chalfont, UK) gradient, isolation of the mononuclear cells, depletion of monocytes by adherence to plastic for 2 hours at 37°C and incubation with the histone deacetylase inhibitor trichostatin A (100 n*M*) for 24 h. The cells were then processed for histone hyperacetylation in the same manner as the patient samples.

Results

Patients

The patient characteristics are presented in Table 1 and are typical of a phase I population in that most subjects were heavily pretreated.

Table 1 Demographics

Number of patients	
Enrolled	20
Evaluable for toxicity	20
Evaluable for response	20
Age (years)	
Median	61
Range	30–74
Male/female	15/5
Race	
Caucasian	11
African-American	8
Other	1
Performance status	
0	8
1	9
2	3
Diagnosis	
Colon	6
Other gastrointestinal tract cancer	2
Non-small-cell lung cancer	8
Sarcoma	1
Melanoma	1
Carcinoma of unknown primary	1
Mesothelioma	1
Prior therapy	
None	1
Radiation therapy only	1
Chemotherapy	18
Number of prior chemotherapy regimens	
Median	2
Range	0–5

Table 2 Toxicity

Toxicity	Grade			
	I	II	III	IV
Hematologic				
Anemia	0	1	0	0
WBC	0	0	0	0
Plateletes	0	0	0	0
Gastrointestinal				
Nausea/vomiting	2	3	1	0
Other (includingbloating, anorexia, cramping, etc.)	6	0	0	0
Diarrhea	2	0	0	0
Fatigue	4	0	0	0
Leg cramps	1	0	0	0
Neuropathy	0	0	1	0
Hyperglycemia	0	0	1	0

Toxicity

Toxicity was minimal throughout the study and is summarized in Table 2. Only two patients experienced a grade 3 toxicity. One patient had nausea and vomiting which resolved with de-escalation to 150 mg/kg three times daily. A second patient who had had prior therapy with cisplatin, docetaxel and vinorelbine and had a history of diabetes mellitus experienced worsening neuropathy and

Table 3 Day 1 pharmacokinetic parameter estimates (*Cmax* maximum observed plasma concentration, *tmax* time of maximum observed plasma concentration, $AUC_{0\text{-}t}$ area under the plasma concentration versus time curve from time zero to the time of the last quantifiable sample)

Patient no.	Butyrate			Tributyrin[a]		
	Cmax (μ*M*)	tmax (h)	$AUC_{0\text{-}t}$ (μ*M*·h)	Cmax (μ*M*)	tmax (h)	$AUC_{0\text{-}t}$ (μ*M*·h)
1	9.56	1.5	10.5	0.33	2	0.496
2	118	3.5	72.0	–	–	–
3	545	4	153	–	–	–
4	49.0	2	65.3	14.3	3.5	28.1
5	17.7	1.5	7.25	–	–	–
6	64.7	1.5	25.1	–	–	–
7	52.0	3.5	93.9	–	–	–
8	130	2	136	–	–	–
9	19.2	1	30.2	–	–	–
10	60.8	1.5	102	–	–	–
11	15.9	0.5	20.3	–	–	–
Mean	98.4		65.1	7.31		14.30
SD	154		51.2			
Median	52	1.50			2.75	
Range	9.56–545	0.5–4			2–3.5	

[a]If no value is shown no plasma concentration was detected

hyperglycemia. A dose-limiting toxicity was not identified and the escalation was halted at 200 mg/kg three times daily as further increases were not felt to be practical due to the large number of capsules which would need to be administered (28 capsules three times daily for a typical 70-kg patient). Compliance was not formally monitored. However, patients were questioned and uniformly reported that they were able to swallow the capsules and tolerated them well.

Pharmacokinetics

Pharmacokinetics for patients receiving daily tributyrin have been previously reported [7]. Plasma was obtained from 11 patients receiving 200 mg/kg tributyrin orally three times daily. Samples were obtained on day 1 for 11 patients, day 2 for 3 patients, day 15 for 8 patients, and day 16 for 3 patients. Pharmacokinetic parameters for butyrate and tributyrin obtained on day 1 are presented in Table 3. The mean observed Cmax values on days 1, 2, 15, and 16 were 98.4 ± 154, 98.7 ± 26.0, 98.9 ± 89, and 132 ± 57 μ*M*, respectively. The median tmax values were 1.5 h (0.5–4.0 h), 3.0 h (2.5–3.0 h), 1.75 h (1–3.5 h), and 1.0 h (0.25–2.0 h) on days 1, 2, 15, and 16, respectively. The $AUC_{0\text{-}t}$ was estimated to be 65.1 ± 51.2, 116 ± 26, 81.0 ± 68.7, 157 ± 33.7 μ*M*·h on days 1, 2, 15, and 16, respectively. Plasma butyrate concentrations were erratic over the sampling period increasing and decreasing with no discernible pattern. Due to the erratic pattern exhibited by butyrate concentrations, the elimination half-life for butyrate was not determinable. The interpatient variability in Cmax was in excess of 100% on day 1 (Table 3). For those patients for whom data were available for at least three sampling periods ($n = 3$) the intrapatient coefficient of variation of the observed Cmax was greater than 65% (Table 4). The %CV for the observed Cmax was 98.1%, 67.0%, and 92.9% for patients 1, 5, and 9, respectively.

Tributyrin plasma concentrations were measurable in the plasma of four patients. Two patients had measurable tributyrin on two of the sampling days, while two patients' tributyrin concentrations were only measurable on one sampling day. The mean tributyrin Cmax was 7.31 μ*M* ($n = 2$), 6.94 ± 4.38 μ*M* ($n = 3$), and 2.11 μ*M* ($n = 1$) on days 1, 15, and 16, respectively.

Table 4 Intrapatient variability of tributyrin maximum plasma concentrations in patients receiving 200 mg orally three times daily

Patient	Cmax			Number of periods sampled
	Median	Range	Mean ± SD	
1	47.1	7.05–104	51.5 ± 50.5	4
5	163	17.7–244	147 ± 98.5	4
9	83.0	19.2–209	104 ± 96.6	3

Responses

There were no objective responses noted either during the dose escalation phase of the trial or during the 200 mg/kg three times daily portion. However, prolonged disease stabilization (> 100 days) was observed in four patients. Diagnoses in the patients with disease stabilization were heterogeneous and consisted of one colon adenocarcinoma, two non-small-cell lung cancer, and one carcinoma of unknown primary.

Survival

Median progression-free survival was 55 days. Two patients remained on therapy at the time of this report, both with non-small-cell lung cancer and stable disease at 608+ and 377+ days.

Histone acetylation

Histone acetylation in PBMC was determined before and after therapy in two patients. No detectable changes were noted.

Discussion

This study demonstrated that serum levels of butyrate consistent with in vitro biological activity are obtainable with an oral drug. Butyrate has demonstrated numerous biological properties in vitro including cell cycle arrest, gene activation, the ability to differentiate a wide variety of malignant cell lines, induce apoptosis and synergize with other differentiating agents [5, 10, 11]. Therapeutic use of this relatively nontoxic compound has been hindered by the inability to administer pharmacologically relevant concentrations. Intravenous infusions have been explored, but are limited by the volume of drug that must be administered and unacceptable odor. Despite these limitations butyrate has demonstrated a potential role in the treatment of sickle cell anemia and hemoglobinopathies through stimulation of fetal globin gene expression in human subjects [12].

Tributyrin is a prodrug of butyrate and overcomes many of the problems of the parent compound. It is a triglyceride containing three butyrate molecules esterified to glycerol (Fig. 1). Most importantly, the drug can be administered orally. Though a large number of capsules were required the agent was well tolerated with several patients remaining on therapy for months. Toxicity was minimal, making it an excellent candidate for combination therapy with other agents. Interestingly, many of the patients treated reported an improved sense of well-being, appetite and pain control. Similar effects have been noted in clinical studies of phenylbutyrate [13].

The observed concentrations of butyrate were extremely variable. Most patients also had low ($< 1\ \mu M$) but detectable levels of butyrate prior to treatment, most likely due to dietary sources [14]. The erratic pharmacokinetic behavior may have resulted from instability of the molecule or individual variation in metabolism. Supporting the concept of instability is the marked intraindividual variation in drug concentrations (Table 4). Despite these variations, a median butyrate plasma concentration of $52\ \mu M$ was obtained. This level is consistent with biological activity. It is quite possible that higher levels were in fact achieved. Interestingly, these levels are quite comparable and in fact higher than the levels achieved with intravenous administration of butyrate and several derivatives [6]. We were also able to measure the prodrug tributyrin in the plasma of four patients. Although tributyrin rapidly hydrolyses in plasma the doses administered were sufficient to produce measurable concentrations up to 4 h after administration. This detection of intact tributyrin and its instability with measurement raises the intriguing possibility that many patients have intact drug circulating. In vitro, tributyrin is more active than butyrate at differentiating leukemia cells [6].

Butyrate and related agents are considered prototypical inhibitors of histone deacetylase [15]. Disappointingly, we did not detect changes in histone acetylation in PBMC of two patients. It may have been that the levels of butyrate in these two patients ($65\ \mu M$ and $61\ \mu M$) were not sufficient to produce this effect. Interestingly, both of these patients experienced prolonged stability of disease with tributyrin. Further studies are needed in a larger cohort of patients to determine if histone acetylation is a relevant mechanism of action for tributyrin in vivo.

Numerous studies have demonstrated in vitro synergism of butyrate and retinoids [5, 16]. The availability of retinoid compounds with differential effects on the various retinoid receptors including all-*trans*-retinoic acid (RAR receptors), 9-*cis*-retinoic acid (RAR and RXR) and bexarotene (RXR) allows exploration of several potentially beneficial effects. In addition, the combination of butyrate drugs with other biological response modifiers such as the interleukins also holds the promise of improved therapeutics [17].

Although tributyrin was able to achieve butyrate levels consistent with either independent activity or which have been demonstrated to potentiate the actions of other agents such as cisplatin, radiation, interferons etc, it is clear that butyrate levels were erratic. Several other butyrate derivatives have been developed, and it is possible that these may overcome some of the problems of tributyrin [18].

In summary, the current report provides the basis for the further exploration of butyrate, in the form of tributyrin, into the practical therapy of malignancies and hemoglobinopathy.

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