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RESEARCH PAPER

Preclinical Evaluation of the Short-Term Toxicity of 4-(*N*)-Docosahexaenoyl 2´, 2´- Difluorodeoxycytidine (DHA-dFdC)

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ABSTRACT

Purpose This study was designed to test the short-term toxicity of DHA-dFdC in a mouse model and its efficacy in a mouse model of leukemia at or below its repeat-dose maximum tolerated dose (RD-MTD).

Method A repeat-dose dose-ranging toxicity study was designed to determine the tolerability of DHA-dFdC when administered to DBA/2 mice by intravenous (i.v.) injection on a repeat-dose schedule (i.e. injections on days 0, 3, 7, 10, and 13). In order to determine the effect of a lethal dose of DHA-dFdC, mice were injected i.v. with three doses of DHA-dFdC at 100 mg/kg on days 0, 3, and 5 (i.e. a lethal-RD). The body weight of mice was recorded two or three times a week. At the end of the study, major organs (i.e. heart, liver, spleen, kidneys, lung, and pancreas) of mice that received the lethal-RD or RD-MTD were weighed, and blood samples were collected for analyses. Finally, DHA-dFdC was i.v. injected into DBA/2 mice with syngeneic L1210 mouse leukemia cells to evaluate its efficacy at or below RD-MTD.

Results The RD-MTD of DHA-dFdC is 50 mg/kg. At 100 mg/kg, a lethal-RD, DHA-dFdC decreases the weights of mouse spleen and liver and significantly affected certain blood parameters (i.e. white blood cells, lymphocytes, eosinophils, and neutrophil segmented). At or below its RD-MTD, DHA-dFdC significantly prolonged the survival of L1210 leukemia-bearing mice.

Conclusion DHA-dFdC has dose-dependent toxicity, affecting mainly spleen at a lethal-RD. At or below its RD-MTD, DHA-dFdC is effective against leukemia in a mouse model.

KEY WORDS DHA · efficacy · gemcitabine · lethal-repeated dose · leukemia · repeat dose-maximum tolerated dose

ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
BUN	Blood urea nitrogen
CPK	Creatine phosphokinase
dFdC	2`, 2`-difluorodeoxycytidine HCl
DHA	Docosahexaenoic acid
DHA-dFdC	4-(*N*)-docosahexaenoyl 2´, 2´-difluorodeoxycytidine
HCT	Hematocrit value
HGB	Hemoglobin level
i.p.	Intraperitoneally
i.v.	Intravenous
ILS	Increase in life span
Lethal-RD	Lethal-repeat dose
PSCs	Pancreatic stellate cells
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell count
RD-MTD	Repeat-dose maximum tolerated dose
T/U	The ratio of the mean survival time of treated group to the median survival time of the untreated group
WBC	White blood cells

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INTRODUCTION

Gemcitabine (2`, 2`-difluorodeoxycytidine, dFdC) is a nucleoside analogue approved for the treatment of solid tumors such as pancreatic, non-small lung, breast, and ovarian cancers (1–5). However, many factors limit its use in cancer treatment, such as its short half-life and tumor cell development of resistance (6, 7). Data from clinical studies showed a mild to moderate toxicity of gemcitabine (1, 8). The most common side effects are myelosuppression, high levels of hepatoxicity, renal toxicity, thrombocytopaenia, and anemia (1, 8, 9).

Docosahexaenoic acid (DHA) is a natural omega-3 polyunsaturated fatty acid (PUFAs) with six *cis* double bonds (10). Since DHA cannot be synthesized by mammals, it is obtained from dietary sources such as fatty cold-water fish and fish oils (10). Data from many studies suggested that DHA has anticancer activity. For example, DHA has an inhibitory effect on breast cancer cells lines (11), induces apoptosis in breast and colon cancer cell lines (12), and inhibits angiogenesis through the inhibition of important angiogenic mediators (e.g. vascular endothelial growth factor, cyclo-oxygenase 2, nitric oxide, matrix metalloproteinases, etc.) (13). In addition, there are reports that DHA is able to inhibit tumor cell invasion and metastasis (14, 15), and data from a recent study suggested that the antitumor activity of DHA may be related to its ability to increase drug transportation across tumor cell membrane (16). Toxicity study in animals and human demonstrated that the consumption of DHA is safe. For example, data from a 90-day subchronic toxicity study showed that DHA is safe in rats at 0.5 and 1.25 g/kg body weight/day (17). Clinical studies of DHA supplementation in adults did not show adverse effects in lipid levels (18, 19), platelet function (20, 21), or immune function (22, 23).

In spite of the promising results of the antitumor activity of omega-3 PUFAs in cell culture and in animal models, data from clinical studies do not support an anti-neoplastic activity by omega-3 PUFAs. Most clinical trials supported the role of PUFAs in cancer prevention, as adjuvants in improving chemotherapy, and preventing cachexia (24–30). For example, a VITAL cohort study reported that the incidence of pancreatic cancer was inversely related to the uptake of DHA (24). Another study showed that the supplementation of omega-3 PUFAs such as eicosapentaenoic acid and DHA through parental nutrition improves hepatic and pancreatic function in patients with major abdominal surgery for pancreatic or gastrointestinal cancer (31). This improvement has been associated with the role of omega-3 PUFAs in inflammation. For example, the use of gemcitabine plus i.v. omega-3 fatty acid rich lipid emulsion (e.g. Lipidem®) in pancreatic cancer patients improves their outcomes through reduction in circulating pro-angiogenic platelet-derived growth factor and fibroblast growth factor and pro-inflammatory factors (e.g. interleukin-6 and -8) (26, 27).

Previously, in an effort to exploit the antitumor activity of both gemcitabine and DHA, we synthesized a new compound, DHA-dFdC, by covalently conjugating DHA to dFdC at the 4-N position of dFdC (32). DHA-dFdC is a pale yellow waxy solid with a melting point of 96° C. It is poorly soluble in water (i.e. 25.2 ± 11.2 μg/ml) and has a log*P* value of 2.2 ± 0.3 (32). DHA-dFdC has potent cytotoxicity against a broad-spectrum of human and mouse tumor cell lines and showed significant antitumor activity in several mouse models of pancreatic cancer, including Kras-Ink4a genetically-modified mice and nude mice with orthotopically implanted human Panc-1 tumor cells (32). Importantly, in both tumor cells in culture and in animals, DHA-dFdC is significantly more effective than the molar equivalent DHA and dFdC physically mixed (32), suggesting a unique mechanism of antitumor activity by the DHA-dFdC conjugate. In the present study, we tested the short-term toxicity of DHA-dFdC in healthy DBA/2 mice by identifying its RD-MTD, a lethal-RD, and the major organ(s) and blood and serum parameters affected by DHA-dFdC at the lethal-RD. Finally, the antitumor activity of DHA-dFdC at or below its RD-MTD was also evaluated in DBA/2 mice with syngeneic leukemia to confirm that DHA-dFdC is active at or below its RD-MTD.

MATERIALS AND METHODS

Synthesis and Formulation of DHA-dFdC

DHA-dFdC was synthesized following our previously reported conjugation scheme (32). The purity of the resultant DHA-dFdC was confirmed by NMR and Mass Spectrum analyses. DHA-dFdC was dissolved in a vehicle solution that consists of Tween 80 (10%, *w*/*v*), ethanol (5.2%, *v*/*v*), and mannitol (5%, *w*/*v*) in water.

Cell Lines

L1210 cells (mouse leukemia cell line) were from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% horse serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. All cell culture medium and reagents were from Invitrogen (Carlsbad, CA).

Short-Term Repeated Dose Toxicity Studies

The animal protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin. Toxicity studies were performed using healthy DBA/2 mice (5–6 weeks) from Charles River Laboratories (Wilmington, MA). For all the toxicity studies,

a mouse weight loss of ≥20% of its initial body weight on day 0 was considered as the end point (33, 34).

Initially, a single dose acute toxicity study was completed. Mice (n = 6, half male, half female) were intravenously (i.v.) injected once with DHA-dFdC at 85 mg/kg or 100 mg/kg. Untreated mice were used as a negative control. Mice were regularly monitored for overall health, and body weight was measured on days 3 and 6. On days 6, mice in the 100 mg/kg dose group and the untreated group were euthanized; major organs (i.e. heart, kidneys, liver, spleen, pancreas, and lung) were collected and weighed. Experimental data for organ weights were expressed as the relative organ weight calculated as: (organ weight (g)/body weight (g)) x 100%. The percent of body weight change was similar in both treated groups, and there is not any significant difference in the relative organ weights in mice that received 100 mg/kg of DHA-dFdC or were left untreated (data not shown). An i.v. bolus dose of more than 100 mg/kg of DHA-dFdC was not attempted due to the limited solubility of DHA-dFdC in the vehicle solution, prompting us to focus on testing the toxicity of the DHA-dFdC on a repeat-dose schedule only.

A five-dose schedule (e.g. injection on days 0, 3, 7, 10, and 13) was adopted for short-term repeated dose toxicity studies. Mice (n = 8, half male, half female) were i.v. injected with DHA-dFdC at different doses: 70 mg/kg, 60 mg/kg, 53 mg/kg, 50 mg/kg, 40 mg/kg, or 0 mg/kg (i.e. vehicle control). An additional group of mice was left untreated as a negative control. Mice were regularly monitored for overall health, body weight change, and survival until 2 weeks after the last injection. The body weight of mice was recorded on days 0, 3, 7, 10, 13, 17, 21, 24, 28, and 31.

To identify a lethal-RD of DHA-dFdC, mice (n = 8, half male, half female) were i.v. injected with 100 mg/kg of DHA-dFdC three times on days 0, 3, and 5. Again, this dose was selected due to the limited solubility of DHA-dFdC in the vehicle solution. Mice in the control groups were left untreated or i.v. injected with vehicle solution (i.e. 0 mg/kg of DHA-dFdC). Mice were monitored every day, weighed on days 0, 2, 4, and 6, and euthanized on day 6.

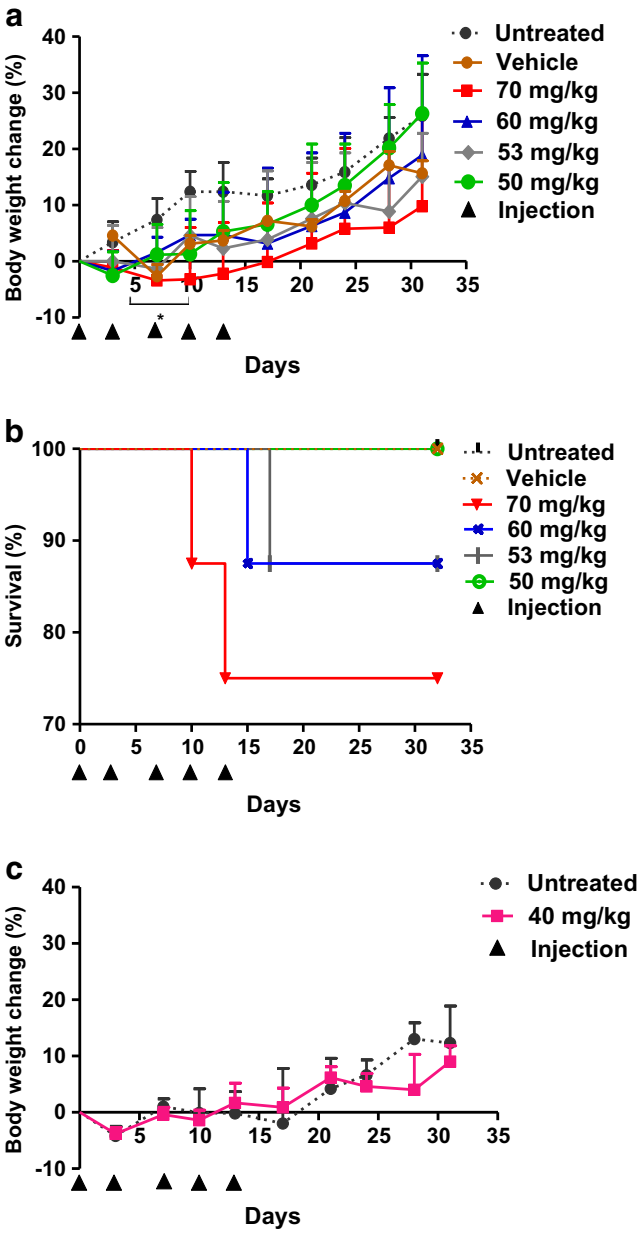

Fig. 1 Identification of the RD-MTD of DHA-dFdC following five i.v. injections (on days 0, 3, 7, 10, and 13 (▲)) in healthy DBA/2 mice (n = 8). (**a**) Percent of body weight change (* $p < 0.05$, 70 mg/kg *vs.* Untreated, one-way ANOVA followed by a Bonferroni post hoc test). (**b**) Survival curves. Log-rank (Mantel-Cox) test did not reveal any significant difference among groups. (**c**) Percent of body weight change at 40 mg/kg of DHA-dFdC. Student's t-test did not reveal any difference. In A and C, data shown are mean ± S.D.

Effect of DHA-dFdC at RD-MTD or a Lethal-RD on Major Mouse Organ Weights and Blood and Serum Parameters

Two experiments were carried out. In the first experiment, DBA/2 mice (n = 8, half male, half female) were i.v. injected with DHA-dFdC at 50 mg/kg on days 0, 3, 7, 10, and 13. As a control, another group of mice were left untreated. Five days after the last injection, mice were euthanized. Major organs were harvested, weighed, and reported as the weights relative to the body weight of individual mouse. Blood samples were collected and shipped to IDEXX Laboratories (Sacramento, CA) for blood and serum parameter analyses.

In another experiment, mice (n = 8, half male, half female) were i.v. injected with DHA-dFdC at 100 mg/kg on days 0, 3, and 5. As controls, mice were either left untreated or i.v. injected with the vehicle solution alone. On day 6 mice were euthanized. Major organs were harvested and weighed. Blood samples were collected and shipped to IDEXX Laboratories for blood and serum parameter analyses.

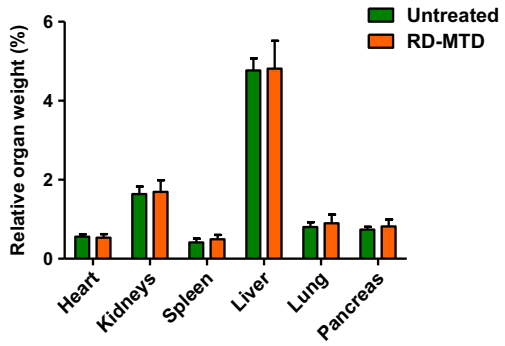

Fig. 2 Effect of DHA-dFdC at its RD-MTD on major mouse organ weights. Shown are relative organ weights (*vs.* body weights) 6 days after the completion of a schedule of five i.v. doses of DHA-dFdC at its RD-MTD. Data shown are mean ± S.D. (n = 8). Student's t-test did not reveal any significant difference between the Untreated and RD-MTD groups.

Evaluation of the Antitumor Activity of DHA-dFdC in a Mouse Model of Leukemia

Female DBA/2 mice (5–6 weeks) were intraperitoneally (i.p.) injected with the syngeneic L1210 leukemia cells (1 x 10^5) on day 0 to establish a mouse leukemia model (35, 36). Mice were then randomized into groups (n = 7) of untreated, vehicle alone, 1 x RD-MTD (i.e. 50 mg/kg), ¾ x RD-MTD (i.e. 37.5 mg/kg), and ½ x RD-MTD (i.e. 25 mg/kg), i.v. injected on days 1, 4, 8, 11, and 14, and monitored daily for overall health, weight change and survival (37). A mouse weight gain ≥60% of its initial body weight on day 0 was considered the end point (33, 34). Experimental data were reported as the mean survival time and T/U, which is defined as the ratio of the mean survival time of the treated group (T) divided by the mean survival time of the untreated group (U).

Statistical Analysis

All statistical analyses involving comparing two groups were completed using Student's t-test. For comparison of more than two groups, one-way ANOVA followed by a Bonferroni post hoc test was used to determine statistical significance between groups. Mouse survival data were analyzed using the Log-rank (Mantel-Cox) test to determine the level of significance. All of the analyses were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Data shown are mean ± S.D., and a p value of ≤0.5 is considered significant.

Table I Blood and Serum Parameters in Healthy Mice 5 Days After the Last Dose of DHA-dFdC at its RD-MTD. Data are Mean ± S.D. (n = 6–8)

Parameter	Unit	RD-MTD (50 mg/kg)	
		Untreated	DHA-dFdC
Blood			
White blood cells (WBC)	k/μl	8.0 ± 3.5	5.4 ± 2.3
Red blood cell count (RBC)	M/μl	10.1 ± 1.1	9.4 ± 1.0
Hematocrit (HCT)	%	40.0 ± 3.5	39.2 ± 4.2
Mean globular volume (MCV)	fL	39.8 ± 1.9	41.8 ± 1.7
Mean corpuscular hemoglobin (MCH)	pg	14.0 ± 0.3	14.5 ± 1.0
Mean corpuscular hemoglobin concentration (MCHC)	g/dL	35.3 ± 2.1	34.8 ± 2.7
Lymphocytes	%	79.9 ± 9.6	84.4 ± 7.8
Monocytes	%	5.5 ± 2.6	7.0 ± 3.9
Basophil	%	0.6 ± 1.0	0.2 ± 0.4
Eosinophil	%	2.9 ± 2.4	1.7 ± 1.6
Neutrophil Segmented (Neutrophil segs)	%	11.1 ± 7.6	6.8 ± 6.9
Platelet estimate		Adequate	Adequate
Serum			
Cholesterol	mg/dL	116.7 ± 17.0	127.6 ± 23.3
Glucose	mg/dL	262.5 ± 70.9	270.8 ± 39.7
Calcium	mg/dL	9.4 ± 2.2	7.0 ± 5.6
Phosphorus	mg/dL	14.6 ± 3.3	16.4 ± 1.5
Blood urea nitrogen (BUN)	mg/dL	23.5 ± 4.0	23.4 ± 1.7
Globulin	g/dL	2.8 ± 0.5	2.7 ± 0.2
Total bilirubin	mg/dL	0.3 ± 0.1	0.2 ± 0.04
Total proteins	g/dL	6.0 ± 0.6	6.0 ± 0.2
Aspartate transaminase (AST)	U/L	689.5 ± 599.5	1266.0 ± 808.9
Alanine transaminase (ALT)	U/L	116.7 ± 115.2	154.2 ± 115.2
Alkaline phosphatase (ALP)	U/L	158 ± 39.4	145.0 ± 66.3
Albumin	g/dL	3.2 ± 0.3	3.3 ± 0.2

Student's t-test did not reveal any significant difference between Untreated and DHA-dFdC groups

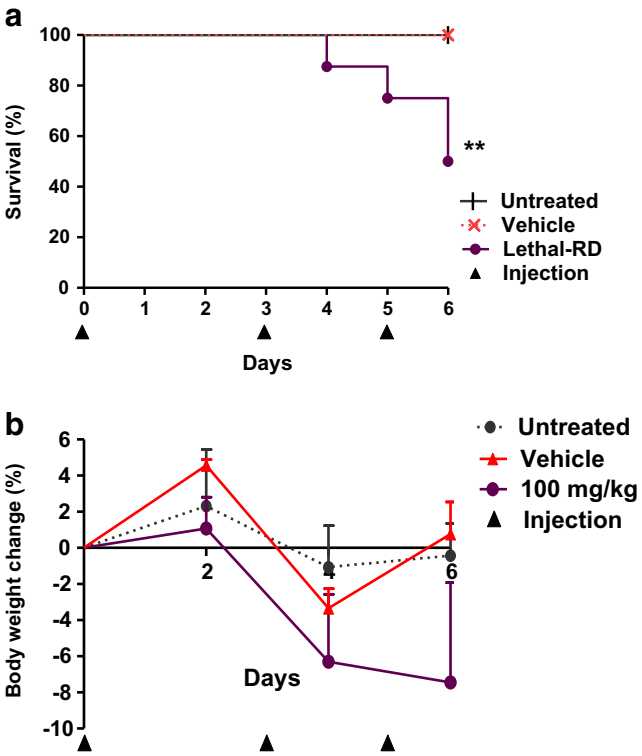

Fig. 3 Effect of a lethal-RD of DHA-dFdC following three i.v. injections (on days 0, 3, and 5 (▲)) on healthy DBA/2 mice ($n = 8$, half male, half female). (**a**) Survival curves (** $p < 0.01$, Lethal-RD *vs.* Untreated, Log-rank (Mantel-Cox) test). (**b**) Percent of body weight change. Data are mean ± S.D.

RESULTS

Identification of the RD-MTD of DHA-dFdC in Healthy Mice

A repeat-dose range finding study was performed to identify the RD-MTD of DHA-dFdC. Mice were i.v. injected with DHA-dFdC at various doses on days 0, 3, 7, 10, and 13. As shown in Fig. 1a, the highest dose tested (i.e. 70 mg/kg) caused significant body weight loss in mice, as compared with untreated groups (e.g. $p < 0.05$ *vs.* Untreated, from day 5 to day 10). In addition, in the 70 mg/kg, 60 mg/kg, and 53 mg/kg groups, some mice reached the end point (i.e. body weight loss of ≥20%) (Fig. 1b). On the other hand, DHA-dFdC at 50 mg/kg did not cause any mortality or body weight loss of ≥20% (Fig. 1a and b), and the mean mouse body weight changes did not show any difference when compared with the untreated group (Fig. 1a). Moreover, at 40 mg/kg, DHA-dFdC did not cause mortality, body weight loss of ≥20%, or any sign of toxicity in a separate experiment (Fig. 1c). Therefore, 50 mg/kg is considered the RD-MTD of DHA-dFdC in DBA/2 mice when administered intravenously.

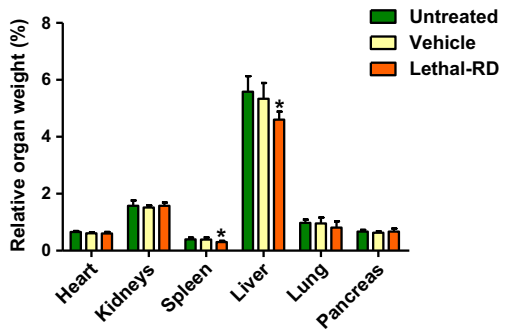

Fig. 4 Effect of DHA-dFdC at a lethal-RD on major organ weights of healthy mice. Shown are relative organ weights one day after the completion of three i.v. doses of DHA-dFdC at 100 mg/kg. Data shown are mean ± S.D. ($n = 6$–8) (* $p < 0.05$, Lethal-RD *vs.* Untreated, one-way ANOVA followed by Bonferroni post hoc test).

Effect of DHA-dFdC at Its RD-MTD on Organ Weights and Blood and Serum Parameters in Healthy Mice

DHA-dFdC was given (i.v.) to healthy mice at its RD-MTD and the weights of major organs were measured at the end of the study. As shown in Fig. 2, at RD-MTD, DHA-dFdC did not significantly affect the weight of any of the major organs tested. In addition, at RD-MTD, DHA-dFdC did not cause any significant change in the blood or serum parameters tested, as compared to mice in the untreated group (Table I).

Effect of a Lethal-RD of DHA-dFdC on Healthy Mice

In order to identify a dose of DHA-dFdC that is lethal to DBA/2 mice, healthy mice were i.v. injected with three doses of DHA-dFdC at 100 mg/kg (i.e. on days 0, 3, and 5). Following the second dose, physical signs of toxicity observed include marked piloerection, transient paralysis, and little peer interactions. One day after the third dose, 50% of mice reached the end point (i.e. mortality or body weight loss of ≥20%) (Figs. 3a and b). Therefore, 100 mg/kg in a spaced schedule is considered a lethal-RD of DHA-dFdC in DBA/2 mice.

To identify the effect of a lethal-RD of DHA-dFdC on the main organs of mice, DHA-dFdC was i.v. injected to healthy mice at 100 mg/kg on days 0, 3, and 5, and the weights of major organs were measured at the end of the study (i.e. day 6). As shown in Fig. 4, the relative weights of the spleen and liver in mice that received the DHA-dFdC at the lethal-RD were significantly lower than that of the untreated mice, whereas the vehicle in which the DHA-dFdC was dissolved in did not significantly affect the weights of any of the organs. At the lethal-RD, DHA-dFdC significantly affected some blood parameters, including the white blood cells (WBC), red blood cell count (RBC), hemoglobin level (HGB), hematocrit value (HCT), lymphocytes, eosinophil, and neutrophil segmented (i.e. neutrophil segs), as compared to mice in the untreated group (Table II). In addition, the levels of some serum

Table II Blood and Serum Parameters in Healthy Mice One Day After Three i.v. Doses of DHA-dFdC at a Lethal-RD (100 mg/kg). Data are Mean ± S.D. ($n = 8$)

Parameter	Unit	Treatment		
		Untreated	Vehicle	DHA-dFdC
Blood				
White blood cells (WBC)	k/μl	5.2 ± 1.7	5.3 ± 2.1	3.2 ± 1.4[a,b]
Red blood cell count (RBC)	M/μl	9.7 ± 0.5	9.2 ± 0.7	7.9 ± 2.0[a]
Hemoglobin (HGB)	(g/dL)	13.8 ± 0.9	13.8 ± 1.1	11.7 ± 2.5[a,b]
Hematocrit (HCT)	%	39.8 ± 3.2	38.2 ± 2.1	32.0 ± 7.5[a,b]
Mean globular volume (MCV)	fL	41.0 ± 3.8	41.4 ± 1.8	41.0 ± 2.0
Mean hemoglobin quantity (MCH)	pg	14.3 ± 1.1	14.9 ± 0.5	15.1 ± 1.2
Mean cellular hemoglobin concentration (MCHC)	g/dL	35.0 ± 4.1	36.2 ± 2.3	36.9 ± 2.5
Lymphocytes	%	69.9 ± 9.7	75.1 ± 14.4	84.7 ± 6.2[a]
Monocytes	%	9.2 ± 9.0	6.7 ± 2.0	8.5 ± 2.9
Basophil	%	1.0 ± 1.5	2.1 ± 2.6	1.4 ± 2.9
Eosinophil	%	3.8 ± 3.0	2.8 ± 1.6	0.8 ± 1.2[a,b]
Neutrophil Segmented (Neutrophil segs)	%	16.1 ± 5.6	13.4 ± 12.3	4.6 ± 3.6[a]
Platelet estimate		Adequate	Adequate	Adequate∞
Serum				
Cholesterol	mg/dL	103.7 ± 22.3	99.7 ± 9.2	94.6 ± 17.5
Glucose	mg/dL	230.4 ± 44.0	199.7 ± 37.5	210.1 ± 45.4
Calcium	mg/dL	9.9 ± 1.7	8.8 ± 3.4	9.9 ± 0.8
Phosphorus	mg/dL	14.3 ± 1.7	13.8 ± 1.5	11.9 ± 1.7[a]
Bicarbonate	(mmol/L)	28.9 ± 2.7	33.3 ± 2.8	31.6 ± 4.8
Blood urea nitrogen (BUN)	mg/dL	22.1 ± 3.0	20.5 ± 2.2	17.9 ± 1.96[a,b]
Globulin	g/dL	2.7 ± 0.1	2.7 ± 0.2	2.7 ± 0.1
Total bilirubin	mg/dL	0.3 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
Direct bilirubin	mg/dL	0.1 ± 0.04	0.1 ± 0.05	0.1 ± 0.05
Indirect bilirubin	mg/dL	0.2 ± 0.08	0.3 ± 0.1	0.2 ± 0.08
Total proteins	g/dL	5.5 ± 0.2	5.3 ± 0.4	5.1 ± 0.2
Aspartate transaminase (AST)	U/L	200.6 ± 91.6	279.9 ± 165.3	193.9 ± 69.3
Alanine transaminase (ALT)	U/L	21.3 ± 3.0	25.9 ± 8.0	18.8 ± 5.1
Alkaline phosphatase (ALP)	U/L	200.3 ± 29.1	161.9 ± 70.2	182.6 ± 21.6
Creatine phosphokinase (CPK)	U/L	1033.7 ± 609.6	1923.3 ± 1248.2	822.4 ± 451.6[b]
Albumin	g/dL	2.8 ± 0.1	2.5 ± 0.3[a]	2.4 ± 0.2[a]

Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post hoc test

[a] $p < 0.05$ *vs.* Untreated

[b] $p < 0.05$ *vs.* Vehicle; [∞] 7 adequate, 1 decreased

parameters such as phosphorus, blood urea nitrogen (BUN), and creatine phosphokinase (CPK) decreased in the lethal-RD group, as compared with the untreated group (Table II). Albumin also decreased in the lethal-RD group as compared with the untreated group, but was not different from that in the vehicle group (Table II).

Efficacy of DHA-dFdC at or below Its RD-MTD *in Vivo*

The RD-MTD identified above was in DBA/2 mice, and therefore DBA/2 mice were i.p. injected (on day 0) with the syngeneic L1210 leukemia cells to establish a mouse model of leukemia to evaluate the antitumor activity of DHA-dFdC at or below its RD-MTD. Mice were inoculated on day 0, and then received (i.v.) five doses of DHA-dFdC on days 1, 4, 8, 11, and 14. Mice in the control groups (untreated mice and vehicle alone) were sacrificed on day 18, because they reached our end point (i.e. body weight increase of ≥60%) (Figs. 5a and b). Those mice presented larger distended abdomens, urine staining, and thin body conditions. In contrast, mice treated with DHA-dFdC at or below its RD-MTD did not gain more than 10% of body weight (Fig. 5b), and they appeared normal on day 18. Mice that were treated with DHA-dFdC at or below its RD-MTD survived longer than mice in the control groups (Fig. 5a and Table III). DHA-dFdC at its RD-MTD (50 mg/kg) showed a higher T/U ratio than at 3/4 x RD-MTD (37.5 mg/kg) or at 1/2 x RD-MTD (25 mg/kg) (Table III). Furthermore, DHA-dFdC at its RD-MTD increased mouse life span by 44.4%, 27.0% at 3/4 x RD-MTD, and 21.4% at 1/2 x RD-MTD (Table III).

DISCUSSION

The repeat-dose range finding study showed that DHA-dFdC has a dose-dependent toxicity in terms of body weight and

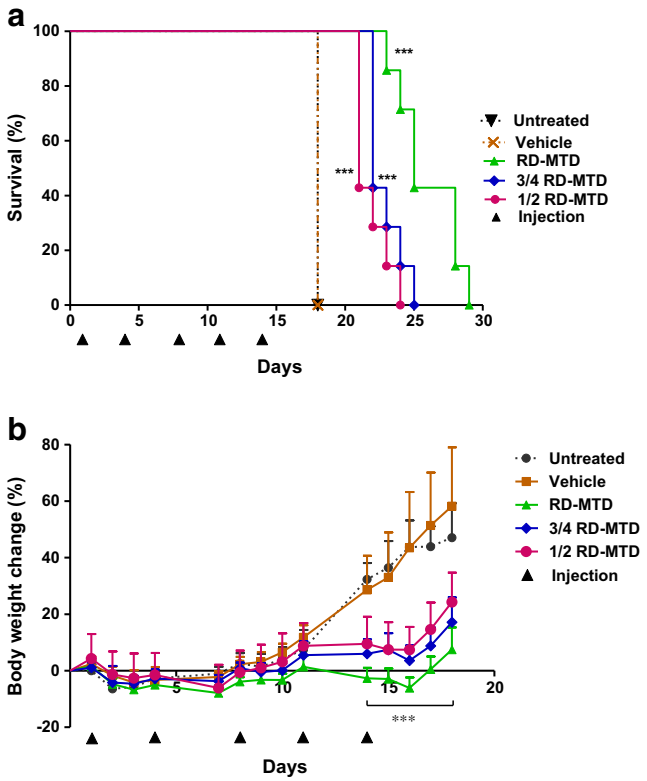

Fig. 5 Efficacy of DHA-dFdC against L1210 leukemia cells in a mouse model. Tumor cells were injected (i.p.) on day 0. On days 1, 4, 8, 11, and 14 (▲), mice ($n = 7$) were i.v. injected with DHA-dFdC at 1 x RD-MTD, ¾ x RD-MTD, or ½ x RD-MTD. Mice in control groups were i.v. injected with vehicle alone or left untreated. (**a**) Survival curves (*** $p < 0.0001$, all DHA-dFdC groups vs. Untreated, Log-rank (Mantel-Cox) test). (**b**) Percent of body weight change. Data shown are mean ± S.D. (*** $p < 0.0001$, all DHA-dFdC groups vs. Untreated, one-way ANOVA followed by Bonferroni post hoc test).

survival (i.e. weight loss of ≥20%). Its RD-MTD is 50 mg/kg (Figs. 1-2 and Table I). At or below its RD-MTD, DHA-dFdC is effective in prolonging mouse survival in a mouse leukemia model (Fig. 5). However, higher doses of DHA-dFdC following a repeat-dose schedule affected the survival and body weight of the mice significantly (Figs. 1a and b).

A single i.v. dose of DHA-dFdC at 100 mg/kg was not lethal to mice (data not shown). Due to the limited solubility of DHA-dFdC in the vehicle solution, we tested whether multiple doses of DHA-dFdC at 100 mg/kg in a spaced schedule is lethal to mice. Mice were i.v. injected with DHA-dFdC at 100 mg/kg three times on days 0, 3, and 5. At this dosing regimen, around 50% of mice reached the end point (i.e. ≥ 20% of body weight loss) as shown Figs. 3a and b. In addition, the spleen and liver weights of those mice decreased significantly in comparison to mice in the control groups (Fig. 4a). Furthermore, at this lethal-RD, DHA-dFdC showed hematological toxicity, because certain blood parameters (e.g. WBC, RBC, HGB, HCT, lymphocytes, eosinophil, and neutrophil segmented) were significantly different from that of mice in the untreated group (Table II). Gemcitabine has dose-limiting toxicities in clinics. For example, data from clinical studies showed that gemcitabine causes dose-limiting myelosuppression that is characterized mainly by thrombocytopenia (1, 8). In our study, at a lethal-RD dose, DHA-dFdC significantly decreased the levels of WBC as well as RBC, when compared to the control groups (40% and 18%, respectively) (Table II). On the other hand, our data showed that platelet estimate was adequate, indicating that DHA-dFdC may not cause significant thrombocytopenia. At the lethal-RD used, DHA-dFdC also induced a significant decrease in the levels of HGB and HCT (15% and 20%, respectively), indicating DHA-dFdC induces anemia in DBA/2 mice (38, 39). The neutrophil levels in mice that received the lethal-RD of DHA-dFdC decreased significantly as compared to the untreated group (71%), indicating an acute neutropenia. It was reported that the number of WBC decreased in healthy men when they received a diet supplemented with DHA (22). The main cause of reduction of WBC number was attributed to a reduction in the number of circulating neutrophils (22). In our study, we found that at lethal-RD, DHA-dFdC caused a decrease in the number of WBC as well as neutrophils segmented (Table II). Both the DHA and gemcitabine moieties in the DHA-dFdC may have contributed to the observed

Table III Antitumor Activity of DHA-dFdC at 1 x RD-MTD, ¾ x RD-MTD, and ½ x RD-MTD in Mice with Syngeneic L1210 Leukemia Cells. Data are Mean ± S.D. ($n = 7$)

Groups	Dose (mg/kg)	Days of treatment	Survival (day)	T/U*	% ILS+
Untreated	-	-	18	1.0	0
Vehicle	0	1, 4, 8, 11, 14	18	1.0	0
1 x RD-MTD	50	1, 4, 8, 11, 14	26.0 ± 2.3[a]	1.4 ± 0.13[a]	44.4
3/4 x RD-MTD	37.5	1, 4, 8, 11, 14	22.9 ± 1.2[a,b]	1.3 ± 0.07[a,b]	27.0
1/2 x RD-MTD	25	1, 4, 8, 11, 14	21.9 ± 1.2[a,b]	1.2 ± 0.07[a,b]	21.4

* The ratio of the mean survival time of treated group to the median survival time of the untreated group. + Increase in life span compared to untreated group

Statistical analysis was performed using one-way ANOVA followed by a Bonferroni post hoc test.

[a] $p < 0.0001$ *vs.* Control groups (i.e. Untreated and Vehicle)

[b] $p < 0.0001$ *vs.* 50 mg/kg

hematological toxicity. Some serum parameters such as phosphorus, BUN, CPK and albumin in mice that received DHA-dFdC at the lethal-RD were also decreased compared to the untreated group (Table II), but these changes were not more than 20%. According a previous study (40), small changes in some parameters in toxicity studies are due to animals having a poor tolerance to the test article. Indeed, one of the most common effects of toxicity studies in rodents is decreasing serum albumin concentration because small species have a rapid turnover of albumin (41). Finally, DHA-dFdC at the lethal-RD did not significantly affect serum levels of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP), when compared to the untreated control, in spite of the finding that it caused a decrease in the relative weight of mouse liver (Fig. 4). Data from a previous phase II clinical study showed that the liver toxicity of gemcitabine was mild and transient (1).

DHA is considered a generally recognized as safe material for inclusion in diet by the U.S. Food and Drug Administration (42). However, some side effects have been reported such as gastrointestinal disturbances, nausea, increased bleeding time, effects in the glycemic control in non-insulin-dependent diabetics, and increased levels of low-density lipoprotein cholesterol (42). Parenteral omega-3 fatty acid preparations have been used for years as part of total parenteral nutrition and are generally well tolerated (27, 43). In addition, an in vitro study reported that pancreatic stellate cells (PSCs) are highly sensitive to gemcitabine plus Lipidem® (i.e. an emulsion for infusion that contains omega-3-acid triglycerides) (44). As PSCs are responsible for therapeutic resistance of pancreatic cancer due to their contribution to secretion of extracellular matrix, the anti-proliferative and anti-invasive efficacy of gemcitabine plus Lipidem® treatment reported in PSC culture might be a good alternative to targeting pancreatic cancer.

CONCLUSION

The RD-MTD of DHA-dFdC in a spaced schedule is 50 mg/kg when intravenously injected to DBA/2 mice. DHA-dFdC mainly affects mouse spleen at a lethal-repeat dose. At or below its RD-MTD, DHA-dFdC shows anticancer activity in a mouse model of leukemia, improving the survival of leukemia-bearing mice.

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