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ORIGINAL ARTICLE

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Plasma kinetics of a cholesterol-rich microemulsion (LDE) in patients with Hodgkin's and non-Hodgkin's lymphoma and a preliminary study on the toxicity of etoposide associated with LDE

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Abstract *Background*: Neoplastic diseases are often associated with low plasma low-density lipoprotein (LDL) cholesterol and diminished LDL clearance due to upregulation in cancer cells of the receptors that internalize the lipoprotein. Thus, it is possible to use LDL or cholesterol-rich microemulsions (LDE) that bind to LDL receptors as carriers of antineoplastic agents to concentrate those drugs into cancer tissues. Our aim was to determine whether LDL cholesterol concentration plus LDE increased clearance occur in lymphomas. *Patients and methods*: The LDE labeled with [^{3} H]-cholesteryl oleate was injected into four Hodgkin's and 12 non-Hodgkin's lymphoma patients and into 16 healthy control subjects and the LDE plasma residence time (RT) was determined from sequential plasma samples. Two volunteers with relapsed/refractory lymphoma were treated with 300 mg/m^2 body surface etoposide associated with LDE in six cycles at 3-week intervals. *Results*: The LDL cholesterol was lower in lymphoma patients than in controls (94 ± 52 and 115 ± 16 mg/dL, $p = 0.0362$, respectively). The LDE RT was 49% smaller in lymphoma patients than in controls (RT = 21.9 and 45.7 h; $p = 0.0134$), with positive correlation between RT and LDL cholesterol. LDE-etoposide showed no considerable toxicity in all cycles in the two treated patients and the disease remained stable during the treatment. *Conclusions*: Our results suggest that lymphomas overexpress LDL receptors that make room for using LDE as drug-targeting vehicle and that the LDE-etoposide preparation is suitable for patient use.

Keywords Cholesterol · Drug-targeting · Etoposide · Lipid emulsions · Lymphoma treatment · Nanoparticles

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Introduction

Low-density lipoprotein (LDL) is the main carrier of cholesterol in the plasma. Lipoprotein is removed from the circulation into the cells by specific receptors on the plasma membrane that recognize the LDL protein, apolipoprotein (apo) B100. The status of the LDL receptor function is the most important determinant of the serum concentration of total and LDL cholesterol [4]. In the seminal study by Ho et al. [14], it was shown that in acute myelogenous leukemia cells the activity of the LDL receptor is upregulated. This leads to increased removal of the lipoprotein from the circulation and lower concentration of both total and LDL cholesterol that was also observed in other several malignant diseases.

It was shown that a cholesterol-rich microemulsion (LDE), termed LDE, when injected into the blood stream has the ability to bind to LDL receptors. Injected in subjects, LDE showed the plasma kinetic behavior expected for the native LDL in several clinical situations such as dyslipidemias [22], under statin treatment [29], in coronary artery disease [30], in acute myelocytic leukemia [20], in multiple myeloma [15], and under the effects of aging [23]. As an alternative to the injection of the native lipoprotein, the use of LDE greatly facilitates the study of the intravascular LDL metabolism. The artificial microemulsion approach allows the injection of a single preparation in several study subjects [20, 27] and due to the much greater affinity of LDE for the receptors

compared to native LDL [12], the observation period is markedly reduced.

It was also proposed that LDE may serve as vehicle to carry anticancer drugs to the target tissues, based on the properties of the microemulsion to concentrate in tumors that overexpress LDL receptors demonstrated in acute myelogenous leukemia [20], multiple myeloma [15], ovarian [1], and breast carcinoma [10].

This study aimed to evaluate in patients with Hodgkin's and non-Hodgkin's lymphoma whether or not the pattern of increased plasma clearance-low LDL cholesterol concentration prevails in the lymphomas suggesting that the LDL receptor upregulation occurs in those neoplastic cells. The LDE labeled with [^{3} H]-cholesteryl oleate and injected intravenously was used as the device to test the LDL plasma kinetics and in vivo receptor function. The results lead us to perform a preliminary study restricted to two lymphoma patients refractory to standard chemotherapy schemes to test the feasibility of using LDE as drug carrier in the treatment of the disease.

Patients and methods

Study subjects

The study on the plasma kinetics of LDE was performed in 16 patients who had lymphoid malignancy, either Hodgkin's or non-Hodgkin's lymphoma, were selected from the outpatient clinics of the Hematology and Hemotherapy Section of the Santa Casa Medical School Hospital of São Paulo, Brazil. They were newly diagnosed or had refractory/relapsed disease. None of them were receiving chemotherapy or radiotherapy at the time of the study and their blood samples were obtained for analysis before the beginning of treatment. Histological diagnosis was confirmed in all cases and they were subclassified according to the WHO classification of neoplasms of the hematopoietic and lymphoid tissues [11]. Patient characteristics are shown in Table 1. Among the 12 non-Hodgkin's participant patients, seven had diffuse large B-cell, two small lymphocytic, one follicular, one mantle-cell, and one peripheral T-cell lymphoma. Among the four Hodgkin's lymphoma patients, three had nodular sclerosis and one mixed cellularity subtype. Sixteen healthy subjects (10 males), aged 19–80 years (47 ± 19 years), were enrolled in the study as the control group.

Table 1 Patient characteristics and type of lymphoma

	Hodgkin's lymphoma	Non-Hodgkin's lymphoma
Number of patients	4	12
Male/female	2/2	7/5
Age (years)		
Median	32	51
Range	16–76	16–72
Ann arbor stage		
I	–	1
II	4	2
III	–	3
IV	–	6
Disease status		
Newly diagnosed	2	3
Refractory/relapsed	2	9

The preliminary clinical study on the treatment with LDE-etoposide was performed in two volunteer patients, also from the outpatient clinics of the Hematology and Hemotherapy Section of the Santa Casa Medical School Hospital, with relapsed or refractory lymphoma. One with non-Hodgkin's and the other with Hodgkin's lymphoma were selected for a six-cycle LDE-etoposide oleate chemotherapeutic scheme. Both patients had active disease, confirmed by clinical examination, laboratory findings, and CTs.

Patient 1 was of female sex, aged 25 years, with body surface = 1.43 m^2. She had Hodgkin's lymphoma, nodular sclerosis subtype, stage III SB diagnosed 5 years previously. She received eight cycles of COPP-ABV and relapsed after roughly 1 year, with involvement of lung and mediastin. Two cycles of Mini-BEAM were then administered followed by autologous bone marrow transplantation. The second relapse episode occurred 1 year later, when she was treated with a Gemcitabine + Vinarelbine + Ifosfamide + Prednisone scheme that resulted in partial remission. Upon entering the study protocol, she showed multiple enlarged cervical lymphonodes that measured up to 3.0 cm as estimated by CT scan.

Patient 2 was male, aged 41 years, with 1.70 m^2 body surface. He had non-Hodgkin's lymphoma, of diffuse large B-cell subtype; stage IV B, diagnosed 3 years previously. He received eight cycles of CHOP and relapsed 16 months later. Despite three cycles of IMVP-Bleo administered as salvage therapy, disease progression was observed. Upon entering the LDE-etoposide protocol he showed enlarged left axillary lymphonodes, the greatest measuring 3.5 cm, and an infra-clavicular mass measuring up to 4.8 cm as estimated by CT scan.

Determination of plasma lipids

Plasma lipids were determined in blood samples collected after a 12 h fast just before beginning of the kinetic studies. Commercial enzymatic methods were used to determine total cholesterol (Boehringer-Mannheim, Penzberg, Germany) and triglycerides (Abbott Park, IL, USA). The HDL cholesterol was determined by the same method used for total cholesterol after lipoprotein precipitation with magnesium phosphotungstate. The LDL cholesterol was calculated by the formula of Friedewald [7].

LDE preparation

The LDE was prepared from a lipid mixture composed of 40 mg of phosphatidylcholine, 20 mg of cholesterol

ester, 1 mg of triolein, and 0.5 mg of unesterified cholesterol purchased from Nu-Check Prep (Elysian, MN, USA). [^{3} H]-cholesteryl oleate purchased from Amersham International (Amersham, UK) was added to the mixture. Emulsification of lipids by prolonged ultrasonic irradiation in aqueous media and the procedure of two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain LDE microemulsion was carried out by the method of Ginsburg et al. [8] modified by Maranhão et al. [19]. The LDE was dialized against saline solution and passed through 0.22 μm filter for injection into the patients.

LDE plasma kinetics

The participants were fasting for 12 h at the beginning of the test at approximately 9 am, but they were allowed two standard meals during the study, at approximately 12:30 pm and 7 pm. The LDE containing 37 kBq (kilo-Becquerel) of [^{3} H]-cholesteryl oleate in a total of 1 mg in a volume 100 μL was intravenously injected in a bolus. Plasma samples were collected during 24 h, in intervals of 5 min and 1, 2, 4, 6, 8, and 24 h after injection. Radioactivity in aliquots of 1.0 mL was quantified in a scintillation solution (Ultima Gold XR, Packard BioScience, Meriden, USA) using a liquid scintillation analyzer (Packard beta spectrometer, model 1600 TR).

Estimation of fractional clearance rate (FCR) and residence time (RT) of the radioisotope

For each patient the kinetic activity–time curve was fitted to the mathematical model [17, 24] defined by the sum of two exponential functions, that is, $y = A_1 e^{(-\alpha_1 \times t)} + A_2 e^{(-\alpha_2 \times t)}$. The model consists of two discrete pools, one intravascular pool in dynamic equilibrium with an extravascular pool. This model assumes that all input or exit of the radiolabeled lipid occurs from the intravascular pool. The FCR of the radiolabeled lipid was estimated as

$$\mathrm{FCR} = \frac{A_1 + A_2}{\int_0^\infty \left(A_1 e^{(-\alpha_1 \times t)} + A_2 e^{(-\alpha_2 \times t)}\right) \cdot \mathrm{dt}}$$

which is essentially the inverse of the area under the activity–time curve. The plasma LDE mean RT was calculated as the inverse of the FCR:

$$\mathrm{RT} = \frac{1}{\mathrm{FCR}}$$

Etoposide oleate synthesis and association with LDE

An oleyl group was attached to etoposide by an esterification reaction to increase the association rate of the drug to LDE and improve the stability of the complex [34]. Etoposide oleate was associated to LDE as previously described [21] by solubilizing 6.0 mg etoposide oleate powder in 10% final volume ethanol and by addition of 1.0 mL of emulsion to the etoposide oleate in ethanol solution. The solution was then sonicated for 40 min at 55°C using a Branson Sonifier 450 (Danbury, CT, USA).

Preliminary clinical study

The evaluation of the patient disease was performed less than 3 weeks before the entry into the study, and consisted of a complete physical examination, CT of the chest, abdomen, and pelvis. Complete blood cell count, chemistry profile including liver enzymes, bilirubin, serum lactate dehydrogenase, and concentration of plasma lipids, urea and creatinine were determined by standard methods prior to study entry. The two patients showed performance status ≤ 2 according to the WHO index, platelet count $\geq 100.000\ \mu L^{-1}$, neutrophil count $\geq 1.500\ \mu L^{-1}$, adequate renal (creatinine < 2.0 mg/dL) and hepatic function (bilirubin level < 1.5 mg/dL and liver enzymes $<$ double the normal value) and, finally, the presence of two-dimensionally measurable tumors. Patients were not infected with human immunodeficiency virus and had no central nervous system disease. Complete blood counts were obtained 14 days after each treatment cycle and the response to treatment was evaluated every two cycles by laboratorial and radiological examination.

The protocol design required that patients would be immediately dropped from the study if progression of disease was detected or if grade 3 or 4 clinical or laboratory toxicity appeared, according to NCI common toxicity criteria.

The LDE-etoposide oleate was infused intravenously over 2 h at a dose of 100 mg/m^2 of body surface of etoposide oleate during three consecutive days and the treatment was repeated every 3 weeks. The evaluation of the response to treatment was determined according to the guidelines of Cheson et al. [6].

Statistical analysis

The unpaired t-test was used to compare means of age, HDL, and of LDE RT obtained from patients and control subjects. Differences in the other plasma lipids (total cholesterol, LDL, VLDL, and triglycerides) between the two groups were assessed by the nonparametric Mann–Whitney test. Spearman's rank correlation coefficient (r) was used to detect strength of association between LDE RT and plasma lipids. Difference of $p < 0.05$ was considered statistically significant.

Informed consent and radiological safety

The study was approved by the institutional review boards of the participating institutions and all

participants provided written informed consent. The safety of the radioactive dose intravenously injected into the patients was assured according to the regulations of the International Commission on Radiological Protection [31] as described in our previous study [21]. The injected dose on each experiment was 0.03 mSV, well below the 50 mSV annual limit for intake of radionuclides [31].

Results

In Table 2 it is shown that in the 16 lymphoma patients, the values of both LDL ($p = 0.0362$) and HDL ($p < 0.0001$) cholesterol were lower than in the 16 control healthy subjects but the plasma fasting triglyceride values were not different.

Figure 1 shows the plasma decay curves of the LDE radioactive label obtained from the lymphoma patients and the controls. Apparently, the decay curve of the LDE label is faster in the lymphoma patients than in the control subjects. In fact, as shown in Table 2, the calculated RT of the LDE label was roughly 49% smaller in lymphoma patients than in the control group ($p = 0.0134$). Table 2 also shows the rate constant $k1$ that represents the exit from the intravascular pool to the extravascular pool and corresponds to the systemic clearance, and $k2$ that represents the exit from the extravascular to the intravascular pool. There was no statistically significant difference in those constants between the values of patients and controls. In the lymphoma patients, the RT was positively correlated with the values of plasma total ($r = 0.71$; $p = 0.0028$), and LDL ($r = 0.54$; $p = 0.0378$) (Fig. 2), as well as with triglycerides ($r = 0.90$; $p < 0.0001$).

Regarding the pilot clinical study of the treatment with LDE-etoposide, it was possible to accomplish the whole six treatment cycles scheduled for each of the two-lymphoma patients. The LDE-etoposide showed no or minimal toxicity in all cycles. In all 12 cycles, myelotoxicity, documented by platelet, leukocyte, and red blood cell counting was grade zero. Hypersensitivity reactions, including vasomotor changes and pulmonary symptoms, hepatic and renal toxicity were also grade zero. The only observed toxicity was gastrointestinal toxicity grade 1 in all cycles in the patient with non-Hodgkin's and in three cycles in the patient with Hodgkin's lymphoma. During the treatment, the patients showed performance status > 80% and well-being. By physical examination and CT scans performed at every two cycles, the disease remained stable during the treatment. In the non-Hodgkin's lymphoma patient, disease progression was observed only 2 months after the discontinuation of the treatment. The other patient was submitted to bone-marrow transplantation shortly after the last treatment cycle.

Discussion

This study suggests that in the lymphomas the LDL receptors are upregulated because it was found in the patients that LDE plasma RT was increased in the presence of increased plasma concentration of LDL cholesterol; this effect has been typically observed in other malignant neoplasias.

In man, LDL is the lipoprotein that carries most of the cholesterol in plasma. The initial event in cellular LDL metabolism involves the binding of LDL-to-LDL receptor through the apo B100 molecule. This binding exhibits saturability, high affinity, and specificity [4, 14]. The expression of the LDL receptors is the main determinant of the plasma LDL cholesterol concentration [4], so that the existence in the organism of a sufficiently large number of neoplastic cells with LDL receptor upregulation conceivably elicits decreased LDL cholesterol. In this regard, Muller et al. [25] showed that in Hodgkin's lymphoma, lower cholesterol values and more advanced or aggressive disease can best be reconciled by the assumption that cholesterol values are low because of high disease activity. Accordingly, Alexopoulos et al. [2] found that LDL cholesterol values increased after successful chemotherapy in lymphomas. However, in a study enrolling non-Hodgkin's lymphoma patients [32] and in our previous study with both Hodgkin's and non-Hodgkin's lymphoma patients [9], differences between patients and normal subjects were not documented. The low HDL cholesterol levels found here in the lymphoma patients is in agreement with previous descriptions by other authors [3, 32].

Although LDE does not contain apo B100, in contact with plasma the microemulsion acquires apo E. Because

Table 2 Plasma lipids (mg/dL) and LDE plasma kinetic parameters

	Lymphoma	Controls
Age (years)	46 ± 21	47 ± 19
Cholesterol		
Total	155 ± 69	188 ± 24
LDL	94 ± 52*	115 ± 16
HDL	26 ± 14**	50 ± 14
VLDL	28 ± 17	22 ± 6
Triglycerides	173 ± 148	104 ± 29
LDE RT	21.9 ± 20.0*	45.7 ± 65.9
LDE FCR	0.0613 ± 0.0245*	0.0417 ± 0.0168
K1	0.1818 ± 0.2292	0.1679 ± 0.1445
K2	0.1661 ± 0.1544	0.1431 ± 0.0935

* $p < 0.05$; ** $p < 0.0001$
FCR Fractional clearance rate (in h^{-1}), *RT* Residence time (in h), expressed as means ± SD

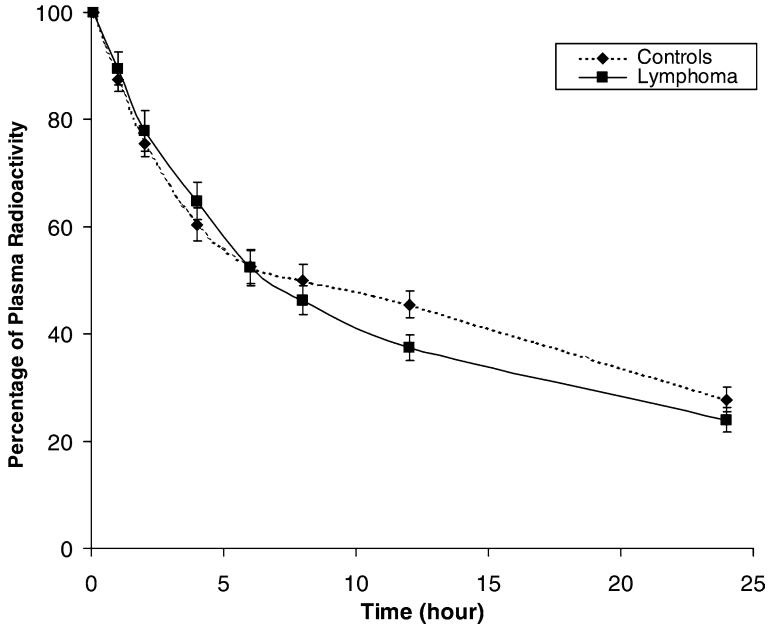

Fig. 1 Decay curves of the LDE radioactive label in lymphoma patients (*continuous line*) and control healthy subjects (*dotted line*)

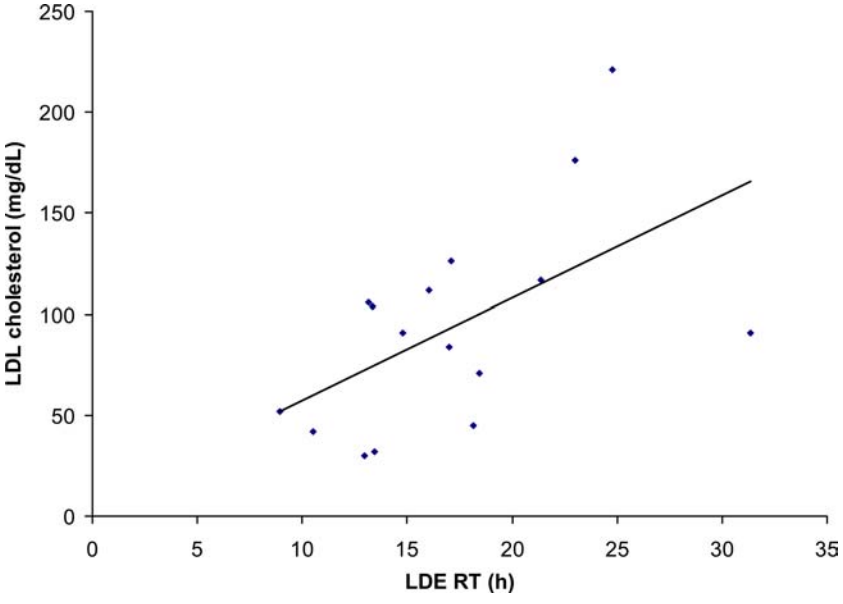

Fig. 2 Correlation between the RT of the LDE radioactive label and the LDL cholesterol values in lymphoma patients

apo E can also bind to LDL receptors with even more affinity than apo B, the affinity of LDE for the receptors is greater than that of native LDL [12, 19, 20, 22]. In view of our previous studies [3–8], it can be assumed that the faster removal of LDE in lymphoma patients found in this study indeed corresponds to faster removal of native LDL. The increased removal of LDE and for extension of LDL is conceivably due to overexpression of LDL receptors in the lymphoma cells. Overexpression of LDL receptors was shown in vitro by Yen et al. [35, 36] in Burkitt lymphoma cells of the Daudi line. In hairy cell leukemia [26] there is the suggestion that the degree of LDL receptor expression or hypocholesterolemia may be related with tumor burden. Alterations in plasma lipids and lipoprotein fractions were demonstrated in patients with acute myelocytic leukemia and non-Hodgkin's lymphoma that were related to the degree of underlying tumor burden and to the presence of bone marrow involvement. Although, in the current study it was not possible to evaluate the relationship between cholesterol levels or LDE RT and tumor burden, it can be presumed that this could have been influential in our results [16].

Two aspects of our results are worthwhile to mention that suggest that the intensity of the LDL receptor up regulation phenomenum in the lymphoma is of great magnitude. First, it was able to roughly double the values of the LDE clearance, as documented by the comparison with the control group RT values. Second, it was able to generate a negative correlation between the LDE clearance and the LDL cholesterol plasma concentration that was absent in the control group of sub-

jects. Therefore, the overall results of the LDE metabolic behavior in the lymphoma patients furnished the rationale for the utilization of the microemulsion as a vehicle of antineoplastic agents in the treatment of the disease. The preliminary clinical study of the use of LDE as vehicle for etoposide performed here in two patients confirmed the feasibility of this approach in the treatment of lymphomas.

In previous experiments it was shown that carmustine [21] and derivatized compounds of etoposide [34] and paclitaxel [28] are able to be stably associated to LDE. Association to LDE markedly decreased the toxicity to animals of those chemotherapeutic agents. Moreover, the therapeutic efficiency of those drugs was increased by association with LDE, as shown in rats and mice implanted with Walker 256 and melanoma B16 tumors [33]. In a dose-escalating study enrolling patients with advanced solid cancers [21] we observed that the association with LDE resulted in negligible side effects of carmustine, even at very high doses. In multiple myeloma patients, absence of side effects of LDE-carmustine at carmustine dose of 180 mg/m^2 body surface was documented together with a substantial reduction in the plasma levels of the disease marker gammaglobulin, while in all seven patients there was reduction in pain and improvement of the hemoglobin concentration [15].

Because the association to LDE of the original, non-modified form of etoposide is poor, an oleyl group was attached to etoposide by a sterification reaction to increase its lipophilicity and rates of stable association with LDE. The LDE-etoposide diminishes the animal toxicity but not the in vitro antineoplastic activity of the drug [34]. Among the three preparations of chemotherapeutic agents associated to LDE we developed to date, LDE-etoposide oleate was chosen to test the LDE system in the lymphomas because etoposide is often included in the chemotherapeutic schemes to treat the disease. As shown in our previous study [34], the antiproliferative activity of etoposide is preserved after the attachment of the oleyl moiety to the 4′-hydroxyl. Incidentally, Lundberg had also reported that when oleyl and linoleyl groups are attached to etoposide and teniposide the drug activity is conserved. The mechanism of action of etoposide is not known in detail. It could be rationalized that the lactone ring reacts with the ezyme topoisomerase II while the phenolic hydroxyl at 4′-position reacts with DNA. From these considerations, it was assumed by Lundberg [18] that the ester bond at the C-4′-position would have to be hydrolyzed by esterases before the fatty acid derivatives could exert their action and, in fact, a rather slow but definite intracellular hydrolysis of the fatty acid derivatives occurs [18].

The use of etoposide associated to LDE at the etoposide dose level usually employed in conventional chemotherapy in the two study patients was virtually devoid of side effects in the roughly 5-month period it was observed. Furthermore, the disease remained stable during the treatment period. This outcome encourages the performance of phase I/II studies of this preparation aiming to seek promising new approaches to treat refractory/relapsed lymphoma patients with pronounced reduction of life-threatening side effects.

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