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ORIGINAL ARTICLE

Use of cholesterol-rich nanoparticles that bind to lipoprotein receptors as a vehicle to paclitaxel in the treatment of breast cancer: pharmacokinetics, tumor uptake and a pilot clinical study

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Abstract
Purpose In animal experiments paclitaxel oleate associated with a cholesterol-rich nanoemulsion concentrated in the neoplastic tissues and showed reduced toxicity and increased antitumor activity compared with paclitaxel-Cremophor EL. Here, a clinical study was performed in breast cancer patients to evaluate the tumoral uptake, pharmacokinetics and toxicity of paclitaxel associated to nanoemulsions.
Methods Twenty-four hours before mastectomy [^{3}H]-paclitaxel oleate associated with [^{14}C]-cholesteryl oleate-nanoemulsion or [^{3}H]-paclitaxel in Cremophor EL were injected into five patients for collection of blood samples and fragments of tumor and normal breast tissue. A pilot clinical study of paclitaxel-nanoemulsion administered at 3-week intervals was performed in four breast cancer patients with refractory advanced disease at 175 and 220 mg/m^2 dose levels.
Results $T_{1/2}$ of paclitaxel oleate associated to the nanoemulsion was greater than that of paclitaxel ($t_{1/2}$ = 15.4 ± 4.7 and 3.5 ± 0.80 h). Uptake of the [^{14}C]-cholesteryl ester nanoemulsion and [^{3}H]-paclitaxel oleate by breast malignant tissue was threefold greater than the normal breast tissue and toxicity was minimal at the two dose levels.
Conclusions Our results suggest that the paclitaxel-nanoemulsion preparation can be advantageous for use in the treatment of breast cancer because the pharmacokinetic parameters are improved, the drug is concentrated in the neoplastic tissue and the toxicity of paclitaxel is reduced.

Keywords Nanoparticles · Emulsions · Breast cancer treatment · Drug targeting · Paclitaxel pharmacokinetics

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Introduction

It was shown previously that the particles of a cholesterol-rich nanoemulsion are taken up by the cells by the low-density lipoprotein (LDL) receptors [16]. The nanoemulsion is composed of a core of cholesterol esters and residual amounts of triglycerides surrounded by a monolayer of phosphatidylcholine with free cholesterol. The lipidic nanoemulsion is produced without proteins, but in contact with the plasma it acquires apolipoproteins (apo) E and other apos [15, 17]. Apo E endows the nanoemulsion with the ability of being recognized by the LDL receptors. Because LDL receptors are upregulated in several neoplastic cells [7, 12, 30], after injection into the blood stream the nanoemulsion concentrates in the neoplastic tissues. In patients with breast carcinoma, the nanoemulsion concentrated roughly five times more in the tumor than in the normal

contralateral mammary tissue [11] and eight times in ovarian carcinomas [1]. The nanoemulsion can thus serve as vehicle to direct chemotherapeutic agents against those tumors, decreasing the toxicity and increasing the antitumoral action of those drugs.

We recently obtained stable and high yield association to nanoemulsions of antineoplastic drugs, such as Etoposide [29] and paclitaxel [23, 24], by attaching of an oleyl group to these drugs. The association of either etoposide or paclitaxel to the nanoemulsion did not modify the biological properties of the nanoemulsion such as the binding to the LDL receptors. Compared with the formulation with Cremophor EL, the pharmacologic activity of both drugs was increased by the association to the nanoemulsion tested in melanoma B16 F10-bearing mice. In that experimental model, association of the drugs to nanoemulsions achieved greater inhibition of tumoral growth and animal survival [23, 24]. The toxicity of both etoposide and paclitaxel was reduced [24, 29].

Paclitaxel is a chemotherapeutic agent with a wide spectrum of antitumoral activity when used alone or in combination with other agents [2]. Paclitaxel is largely used in the treatment of advanced breast and ovary carcinoma and the ability of the nanoemulsion to concentrate in those tumors was previously shown [1, 11]. The preparations in current clinical use are formulated with the surfactant Cremophor EL and ethanol to allow the drug solubility in aqueous media [4]. Cremophor EL is responsible for hypersensitivity reactions as hypotension, tachycardia and dyspnea that occur during the infusion and reach 25–30% of the treated patients [31] so that pretreatment with an antihistamine and glucocorticoids is mandatory [5].

In the current study, to pave the way for future large clinical trials, the paclitaxel oleate associated to the nanoemulsion was tested in patients with breast cancer. The pharmacokinetics and the concentration of the drug in the tumor were examined. Furthermore, a drug-escalating clinical pilot study of paclitaxel oleate associated to the nanoemulsion in drug-resistant breast cancer patients was performed.

Methods

Patients

Nine volunteer women with breast cancer selected at the gynecology ward of the Medical School Hospital of the University of São Paulo participated in the study, and were allocated to two different study protocols.

Protocol 1 (paclitaxel oleate and commercial paclitexel pharmacokinetics at low dose and tumoral uptake): five postmenopausal patients aged 56–67 years with stage II or III ductal invasive breast cancer confirmed by histological analysis and scheduled for mastectomy were enrolled.

Protocol 2 (treatment with paclitaxel oleate associated to the nanoemulsion and pharmacokinetics of the drug at treatment dose): four patients aged 36–67, with unresectable, locally recurrent, or metastatic breast cancer were enrolled for treatment, and pharmacokinetics was determined in two of them. Disease was confirmed by histological analysis and they were refractory to conventional treatment, in which anthracyclines and taxanes were prescribed. All had measurable or assessable disease, a good, 0–2 performance status (WHO), and an estimated life expectancy of at least 12 weeks.

They had adequate bone marrow [absolute granulocyte count (AGC) $\geq 1.5 \times 10^9$/L, platelet count $\geq 100 \times 10^9$/L, and hemoglobin ≥ 10 g%], liver [bilirubin $\leq$1.5-fold the upper limit of normal (ULN), alanine transaminase (ALT) and aspartate transaminase (AST) $\leq$3 times ULN, or up to twofold the ULN in patients with known metastatic liver disease], and renal function (creatinine $\leq$2.5-fold above the ULN).

In both protocols, exclusion criteria included brain metastasis, cardiac arrhythmia grade two or more, active infection, dyslipidemia, presence of severe psychiatric disease, or second malignancy (except in situ carcinoma of the cervix or adequately treated basal cell carcinoma of the skin), pregnant or nursing patients.

The study was approved by the Ethics Committee of the University of São Paulo Medical School Hospital and all participants signed a written informed consent.

Preparation of the nanoemulsion and association of paclitaxel oleate

In brief, nanoemulsion was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein and 0.5 mg cholesterol. 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 the nanoemulsion were carried out by the technique described previously [8] and modified by Maranhão [15].

Paclitaxel oleate was associated to the nanoemulsion by solubilization of 6.0 mg of paclitaxel oleate in 10% final volume ethanol and by adding of 1.0 mL of nanoemulsion [24]. When necessary, trace amounts of [^{14}C]-cholesteryl oleate were added to the initial solution. After sonication the mixture was centrifuged at 3,000 rpm for 15 min to separate unbound paclitaxel oleate. In all experiments only associated to the nanoemulsion paclitaxel oleate was used. The final nanoemulsion-paclitaxel oleate preparation has a

mean diameter of 85 nm (±10), polydispersity of 0.2 and maximum loading of paclitaxel oleate of 85% (5.1 g of drug/mL of nanoemulsion) from the total mass of drug added at the proportion 1:5 drug/nanoemulsion total lipids (w/w) [24].

For the plasma kinetic studies trace amounts of [^{3}H]-paclitaxel was added to the paclitaxel cremophor-ethanol based formulation. The formulation was diluted in 1.8 mL 0.9% chloride sodium and passed through 0.22 μm pore polycarbonate filter prior to the injection into the patients.

Paclitaxel oleate associated to nanoemulsion and paclitaxel-Cremophor plasma kinetics

The association paclitaxel oleate-nanoemulsion labeled with [^{14}C]-cholesteryl oleate and [^{3}H]-paclitaxel oleate (60 mg total lipid mass and 12 mg of paclitaxel oleate, at a volume of 2 mL) or the [^{3}H]-paclitaxel-cremophor preparation (12 mg paclitaxel, at volume of 2 mL) were intravenously injected in a bolus 24 h before the beginning of the surgical procedure scheduled for the patients. Blood samples were collected at pre-established intervals during 24 h (0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 10 and 24 h). Blood was centrifuged and the radioactivity contained in 1.0 mL of plasma was measured by liquid scintillation counting (Packard 1660 TR, Meridien, CT). Removal of [^{14}C]-nanoemulsion-[^{3}H]-paclitaxel oleate or [^{3}H]-paclitaxel from the plasma was evaluated by the fractional clearance rate (FCR). The FCR was calculated according to the method described elsewhere [20], where a_1, a_2, b_1 and b_2 were estimated from biexponential curves obtained from the remaining radioactivity found in plasma after injection, fitted by least squares procedure, as $y = (a_1 \times e^{-b_1 t}) + (a_2 \times e^{-b_2 t})$ where y represents the radioactivity plasma decay.

The FCR's of the nanoemulsion [^{14}C]-cholesteryl ester and of the [^{3}H]-paclitaxel were calculated by compartmental analysis using the ANACOMP software, kindly supplied by Dr. Carlos H. Mesquita. Regarding the study performed in patients treated with paclitaxel oleate (175 mg/m^2) associated to the nanoemulsion, the pharmacokinetic parameters were calculated according to a multicompartmental model using software from PK Solutions (Ashland, OH). The log plasma concentration versus time curves was fitted by biexponential equations and the half-lives ($t_{1/2\beta}$) calculated by dividing 0.693 by the rate constant for each phase. Total plasma clearance (CL) was calculated by dividing the dose by the AUC. The volume of distribution at steady state (Vss) was estimated graphically from trapezoidal total area measurements.

Paclitaxel oleate pharmacokinetics in patient under treatment was performed by HPLC. Plasma sample were collected at the same time of the previous protocol.

The drug was extracted from the plasma by precipitation with acetonitrile and quantified by peak area ratios plotted versus nominal concentration. A weighted ($1/x^2$) linear regression was applied to generate a calibration curve at concentration of 78.12–20,000 ng/mL. Chromatographic conditions were described previously [18]. Etoposide oleate was used as internal standard (IS).

The HPLC based method used to determine the plasma kinetics of paclitaxel oleate associated to the nanoemulsion showed a relative standard deviation (RSD) <7.9% for intra-day assay and <8.1% for inter-day assay. The accuracy was between 97.6 and 104.8%. The limit of quantification (LOQ) of paclitaxel oleate was 78 ng/mL and the limit of detection (LOD) was 39 ng/mL. Therefore, the method matches the generally accepted requirements for bioanalytical methods [26].

Uptake of paclitaxel oleate associated to the nanoemulsion by tumoral and normal tissue

During the surgical procedure, at a time approximately 24 h after the injection of the preparation, the excised tissues of interest were provided by the surgeon. Fragments of 1.0 g of the tumor and of the normal tissues were stored at 4°C into 0.9% saline solution. The fragments of normal and cancer tissue were then chopped and lipids and drug of both tissues were extracted with chloroform/methanol (2:1, *v*/*v*) [6] and had the radioactivity measured in a scintillation solution.

The dose injected into each patient evaluated according to the guidelines of the International Commission on Radiological Protection [28] was well below the annual limit of ingestion dose of 50 mSv.

Treatment of advanced breast cancer patients with paclitaxel-nanoemulsion

Three patients received Paclitaxel oleate associated to nanoemulsion at the 175 mg/m^2 paclitaxel and one at the 220 mg/m^2 dose level. Paclitaxel oleate associated to nanoemulsion was diluted at 500 mL of 0.9% sodium chloride solution and administered intravenously (i.v.), on day 1 over a period of 1 h every 21-days. It was established that the treatment would be discontinued if toxicity grades three or four or disease progression occurred. Pre-medication with antihemetic, corticosteroid and antihistamine drugs was not used prior to paclitaxel oleate administration.

Baseline and treatment assessments

Assessments performed at baseline and throughout the study included history and physical examination (including weight and height), WHO performance status, and tumor

measurement of palpable or visual lesions. Radiological tests of computed tomography (CT) scan, magnetic resonance imaging (MRI), or nuclear medicine scan were used, when necessary, for tumor measurement of lesions not assessed by other imaging modalities. Chest X-rays were used in patients with chest metastasis. Full blood count (with differential and platelet counts), blood chemistries, and vital signs were done for all patients before and at regular intervals during the study. Toxicity ratings, based on NCI Common Toxicity Criteria, were assessed before the beginning of each cycle.

Statistical analysis

The differences in the FCR's were evaluated by using Graf Pad Instat, Version 3.0. The differences between the data on neoplastic and normal tissues were evaluated by Mann–Whitney test. In all analysis, a P value < 0.05 was considered significant.

Results

Figure 1 shows the decay curves of [^{14}C]-cholesteryl oleate and [^{3}H]-paclitaxel oleate obtained after the injection of the labeled paclitaxel oleate associated to the nanoemulsion into three patients 24 h before the surgery. Both curves show a biexponential aspect. It can be seen that the curve of the nanoemulsion cholesterol oleate does not substantially differ from that of the associated paclitaxel oleate: both run roughly in parallel and, accordingly, the FCR of both nanoemulsion cholesterol oleate and paclitaxel oleate did not differ (0.058 ± 0.018 and 0.052 ± 0.027, NS).

Figure 2 shows the plasma decay curves of [^{3}H]-paclitaxel oleate associated to the nanoemulsion injected into three patients and [^{3}H]-paclitaxel in Cromophor EL

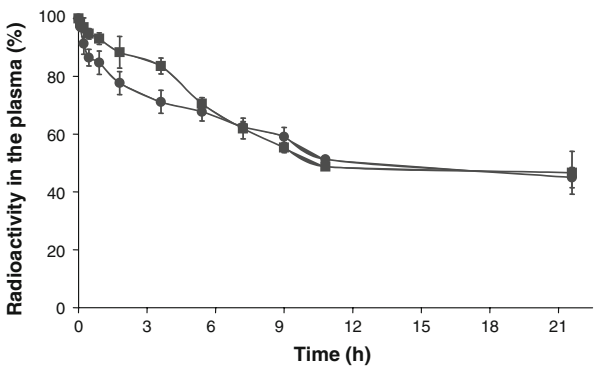

Fig. 1 Plasma decay curve of paclitaxel oleate associated to the nanoemulsion doubly labeled with [^{3}H]-paclitaxel oleate (*filled circle*) and [^{14}C] cholesteryl ester (*filled square*) following bolus injection. Results are presented as means ± SD (*bars*)

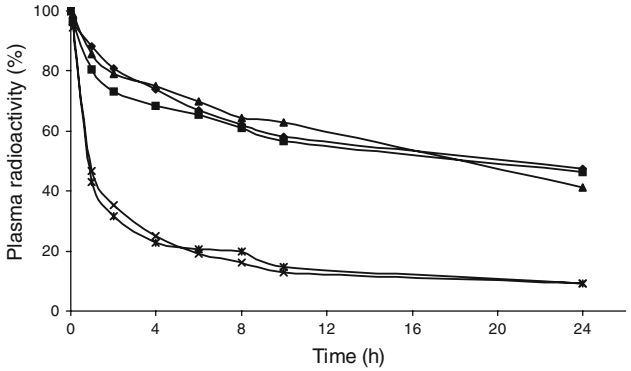

Fig. 2 Plasma decay curve of paclitaxel oleate associated to the nanoemulsion labeled with [^{3}H]-paclitaxel oleate (*filled diamond*, *filled triangle*, *filled square*) and [^{3}H]-paclitaxel in Cremophor EL (*multi symbol*, *asterisk*) following bolus injection

injected into two patients. Both curves show a biexponential aspect. The decay curve of paclitaxel oleate is markedly slower than that of paclitaxel. Tables 1 and 2 shows the pharmacokinetic parameters of paclitaxel oleate associated with the nanoemulsion and of paclitaxel-Cromophor EL, respectively, as calculated from the plasma decay curves. The paclitaxel oleate half-life is greater and the total plasma clearance is smaller than those of the paclitaxel dissolved in Cremophor EL.

Figure 3 shows the plasma decay curve of paclitaxel oleate (175 mg/m^2 body surface treatment dose) associated to the nanoemulsion obtained from two patients as determined by HPLC. The curve also shows a biexponential aspect and is clearly slower than the curve of radioactively labeled paclitaxel oleate associated with the nanoemulsion shown in Fig. 2. In Table 3 there are the pharmacokinetic parameters calculated from the curves of the two patients treated with paclitaxel oleate associated to the nanoemulsion. Pharmacokinetics was documented at the first treatment cycle. The half-life of paclitaxel oleate associated to the nanoemulsion injected at a treatment dose is greater than that obtained when a small amount of the associated drug is injected (data shown in Table 1).

Table 4 shows the data of tissue uptake of the [^{14}C]-cholesterol oleate and [^{3}H]-paclitaxel oleate of the paclitaxel oleate in association with the nanoemulsion. The uptake by the tumoral tissue of both cholesterol oleate and paclitaxel oleate was 2.4 greater than that of the normal tissues. It is clear that the nanoemulsion and the associated drug were taken-up by the tumoral tissue at the same rate.

Regarding the pilot clinical study for toxicity of the treatment with paclitaxel oleate associated to the nanoemulsion, eight cycles were evaluated in four patients. The association showed minimal toxicity. Myelotoxicity, documented by platelet, leukocyte and red blood cell count was grade zero. Hypersensitivity reactions did not occur. Hepatic

Table 1 Pharmacokinetic parameters of paclitaxel oleate following bolus infusion of [^{3}H]-paclitaxel oleate associated with the nanoemulsion

Patient	$T_{1/2\beta}$ (h)	AUC	Vd (mL/kg)	CL (mL-h/kg)
1	8.3	1,461	136.7	8.2
2	20.0	1,407	141.7	8.5
3	17.8	1,462	282.8	8.2
Mean ± SD	15.4 ± 6.2	1,443.3 ± 31.5	187.0 ± 83.0	8.3 ± 0.2

$t_{1/2\beta}$ half-life, AUC (0–t) (obs. area), area under the plasma concentration-time curve, *CL* total body clearance (obs. area), *Vd* volume of distribution at steady state

Table 2 Pharmacokinetics parameters of paclitaxel-Cremophor EL following bolus infusion of [^{3}H]-paclitaxel

Patient	$T_{1/2\beta}$ (h)	AUC	Vd (mL/kg)	CL (mL-h/kg)
4	2.7	437.6	274	27.4
5	4.2	451.3	532	26.6
Mean	3.5	444.5	403	27.0

$t_{1/2\beta}$ half-life, AUC (0–t) (obs. area) area under the plasma concentration-time curve, *CL* total body clearance (obs. area), *Vd* volume of distribution at steady state

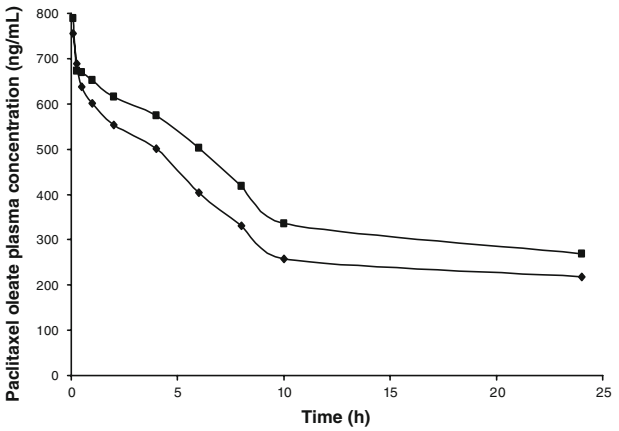

Fig. 3 Plasma decay curve of paclitaxel oleate associated to the nanoemulsion in two patients (dose = 175 mg/m^2). Plasma samples were taken over 24 h for HPLC quantification

and renal toxicity were also grade zero. Only one patient, who was treated with the lower, 175 mg/m^2 paclitaxel dose, referred nausea and anorexia classified as grade one.

Discussion

In 1992 [15] we showed by the first time that artificial, laboratory-manufactured solid particles can target neoplastic tissues. Our initial findings in patients with acute myelocytic leukemia [16, 17] were subsequently expanded to solid tumors [1, 11].

Table 3 Paclitaxel oleate pharmacokinetics in patients under treatment performed by HPLC at the dose of 175 mg/m^2 administered during 1 h

Patient	$T_{1/2\beta}$ (h)	AUC (ng-h/mL)	Vd (mL/kg)	CL (mL-h/kg)	FCR
1	59.1	8061.5	15,658.1	183.6	0.0516
2	43.5	10,129.8	10,437.3	167.3	0.0372
Mean	51.3	9,095.7	13,047.7	175.5	0.0444

$t_{1/2\beta}$ half-life, AUC (0–t) (obs. area) area under the plasma concentration-time curve, *CL* total body clearance (obs. area), *Vd* volume of distribution at steady state

In this study, it was shown that paclitaxel oleate associated to the nanoemulsion does not dissociate from the particles while in the plasma circulation of patients with breast cancer. On the other hand, the association improves the pharmacokinetic parameters by increasing the half-life of the drug in the plasma and concentrates the drug in the malignant breast tissue.

Regarding the kinetics of the paclitaxel oleate associated with the nanoemulsion, it is noteworthy that, the half-life of the treatment dose was roughly threefold greater than that of the small dose. Because paclitaxel oleate does not dissociate from the nanoemulsion while in the circulation, as shown in Fig. 1, this suggests that when administered at treatment dose, the removal from the plasma of the nanoemulsion carrying the drug is under saturation kinetics.

In pre-clinical studies in which the technique of associating paclitaxel oleate to the lipidic nanoemulsion was described it was shown that derivatization of the compound is necessary to stabilize it into the nanoparticle [24]. In those studies, the toxicity to the animals of the paclitaxel oleate associated to the nanoemulsion was diminished tenfold in comparison with the Cremophor EL formulation, as estimated by MTD (18 and 179 μmol/kg for commercial paclitaxel and paclitaxel oleate, respectively). The antitumoral action of the paclitaxel oleate associated to the nanoemulsion was also superior to that of the Cremophor EL-paclitaxel. There was a 70% reduction of tumor growth in melanoma B-16 tumor bearing mice when paclitaxel-nanoemulsion was used compared to 43% reduction resulting from treatment with the Cremophor EL formulation. The survival followed until day 40 was also comparatively increased by association with the nanoemulsion (15% for commercial paclitaxel and 25% for paclitaxel oleate associated to the nanoemulsion at the dose of 17.5 mg/kg and 33% for 70.3 mg/kg dose, related to the control group). Moreover, we had 12% tumor complete remission when the animals were treated with paclitaxel oleate associated to the nanoemulsion against 0% under the treatment with paclitaxel in Cremophor EL. Results of animal studies on toxicity and pharmacological action

Table 4 Uptake of the nanoemulsion labeled with [^{14}C]-cholesteryl oleate and paclitaxel oleate radioactivity by normal and neoplastic breast tissues

Patient	Uptake of [^{14}C]-nanoemulsion by the tissue (cpm/g)			Uptake of [^{3}H]-paclitaxel oleate by the tissue (cpm/g)		
	Tumor	Normal	Tumor/normal	Tumor	Normal	Tumor/normal
1	1,202	575	2.1	2,919	1,271	2.3
2	255	149	1.7	154	65	2.4
3	583	146	4.0	328	70	4.7

similar to those described for paclitaxel oleate associated to the nanoemulsion were obtained with the associations of carmustine [13, 19] and etoposide oleate [14, 29].

Over the 24 h follow-up of the paclitaxel oleate associated to the nanoemulsion with labeling of both nanoemulsion lipid and drug components showed that the nanoemulsion and paclitaxel oleate plasma kinetics were similar [3]. This indicates that dissociation of the drug from the nanoemulsion particles does not occur in the circulation of the patients at least at substantial amounts. This observation is of great importance, since a fundamental prerequisite for drug targeting is the delivery of the drug at its action site by the carrier, and this objective is not attained if dissociation occurs. Indeed, the drug targeting effect of the nanoemulsion was confirmed by our finding that both paclitaxel oleate and the nanoemulsion [^{14}C]-cholesteryl oleate concentrated in the breast tumor at similar rates that were much greater than those found in the normal corresponding tissue.

Once paclitaxel oleate was associated to the nanoemulsion, the pharmacokinetic profile of the carrier prevailed, resulting in a longer half-life of the drug compared with that of the formulation of paclitaxel in Cremophor EL. Paclitaxel blocks the formation of the mitotic spindle in phase M, being active only against cells in the division process [9]. By prolonging the half-life of the drug, nanoemulsions would favor the exposure of increasing numbers of susceptible cells thus conceivably increasing the paclitaxel pharmacologic action. The administration of paclitaxel in prolonged infusion schemes achieved greater responses to the treatment [27, 33].

In clinical studies enrolling patients with advanced cancers and designed to test the toxicity profile, both carmustine [13] and etoposide oleate [22] associated to the nanoemulsion were shown almost devoid of toxicity as administered at dose levels even greater than in conventional treatments. The 175 mg/m^2 body and 220 mg/m^2 surface every 3 week dose scheme used here in the four patients corresponds to the usual scheme of paclitaxel dissolved in Cremophor EL which often leads to toxicity, in which neurotoxicity, myelotoxicity and alopecia [25, 31, 32] are the most prominent features. Although the treatment was performed in only four patients, the absence of those side effects suggests that the nanoemulsion reduces the toxicity of paclitaxel in cancer patients, as previously described for the association with carmustine and etoposide.

Novel paclitaxel formulations have already been tested in clinical studies. In patients with metastatic breast cancer, albumin-bound paclitaxel (ABRAXANE®) has been shown advantageous over cremophor-based paclitaxel, with smaller side effects and improvement of overall response rate and time to tumor progression [10]. On the other hand, a paclitaxel formulation with D-mannitol, povidone C-15, and polysorbate 80 (AI-850) was shown less promising in a phase 1 study [21]. Produced from materials furnished by the chemical industry by methods that are practical and applicable at large-scale manufacturing, the lipidic nanoemulsion was shown in mice to pronouncedly decrease paclitaxel toxicity while preserving the pharmacological action. As previously shown in patients with gynecological neoplasias [3] and currently in breast carcinoma, the association with the nanoemulsion achieves great concentration of paclitaxel at the tumor sites. LDL receptors offer an open gateway for entry of nanoemulsion carried drugs into the cell.

In conclusion, the current results show that the nanoemulsion is a promising carrier for paclitaxel in breast cancer patients and encourage further clinical research to estimate MTD and to evaluate the pharmacological action aiming to introduce this new preparation in the treatment of breast cancer.

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