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Effects on Walker 256 tumour of carmustine associated with a cholesterol-rich microemulsion (LDE)

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Abstract

A cholesterol-rich microemulsion that binds to low-density lipoprotein (LDL) receptors (LDE), after injection into the bloodstream, concentrates in neoplastic tissues that over-express those receptors. LDE can thus serve as a vehicle for drug targeting. It was shown that carmustine side effects are pronouncedly reduced when the drug is associated with LDE in cancer patients. In this study, the therapeutic action of LDE associated with carmustine was compared with that of the non-associated drug in rats implanted with Walker 256 tumour. The toxicity and anti-tumour activity in rats treated with either free carmustine or carmustine associated with LDE and in control rats treated with saline solution were determined after a single intraperitoneal injection. The LD90 (90% lethal dose) of LDE–carmustine was 77 mg kg^{-1} and of free carmustine was 44 mg kg^{-1}, indicating that LDE decreases toxicity. LDE–carmustine was able to decrease tumour mass at a lower dose level than free carmustine. Tumour regression time was shorter in LDE–carmustine- than in free carmustine-treated animals. Therefore, this study shows that the association of carmustine with LDE increases the therapeutic index of carmustine.

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Introduction

Low-density lipoprotein (LDL) contains most of the cholesterol present in plasma. LDL is removed from the plasma by specific cell membrane receptors that internalize this lipoprotein into vesicles. LDL receptors are over-expressed in several cancer cell lines (Ho et al 1978; Gal et al 1981; Rudling et al 1983; Ginsberg et al 1986; Henriksson et al 1989; Ueyama et al 1990; Gueddari et al 1993), making a way for the use of the LDL receptor-mediated endocytic pathway to target anti-cancer drugs included in the lipoprotein.

In previous studies (Maranhão et al 1992, 1993, 1994, 1997) we showed that an artificially-made microemulsion, termed LDE, had the ability to bind to LDL receptors. After injection into the bloodstream, LDE concentrates in neoplastic cells with LDL receptor over-expression (Maranhão et al 1994; Ades et al 2001; Graziani et al 2002; Hungria et al 2004).

LDE is formed of quasispherical nanoparticles basically composed of a monolayer of phosphatidylcholine surrounding a core of cholesteryl esters. Small amounts of unesterified cholesterol and triglycerides are also present. LDE mimics the lipid portion of low-density lipoprotein (LDL) and when in contact with the plasma, acquires apolipoproteins (apo) from the circulating native lipoproteins. One of the proteins acquired by the microemulsion, apo E, is recognized by the LDL receptors (Hirata et al 1999). This enables the LDE particles to bind to these receptors. LDE is then removed from the circulation by LDL receptors present on the cell membrane. Once taken up by LDL receptors, LDE is internalized into the cytoplasm via LDL receptor-mediated endocytosis. As some cancer cells show LDL receptor upregulation (Ho et al 1978; Gal et al 1981; Rudling et al 1983; Ginsberg et al 1986; Henriksson et al 1989; Ueyama et al 1990; Gueddari et al 1993), LDE may target those cells and specifically deliver chemotherapeutic agents loaded into the microemulsion particles to them.

Recent studies have provided direct evidence of the selective LDE uptake by neoplastic tissues in patients with ovarian (Ades et al 2001) and breast (Graziani et al

2002) carcinoma. There is indirect evidence of uptake by neoplastic cells in acute myelocytic leukaemia and multiple myeloma (Maranhão et al 1994; Hungria et al 2004). In 43 patients with advanced cancer, the association of the lipophilic antineoplastic agent carmustine to LDE markedly diminished the toxicity of the drug (Maranhão et al 2002). LDE is conceivably the possible vehicle to use the LDL receptor mechanism to target drugs to neoplastic tissues in clinical practice.

In this study, we compared the therapeutic action of LDE associated with carmustine with that of the non-associated drug in rats implanted with Walker 256 tumour. We found that association with LDE results in a superior therapeutic index for carmustine.

Materials and Methods

Animals

Male Wistar rats (Institute of Biomedical Sciences, São Paulo, Brazil), 150–170 g, were housed in a temperature- and humidity-controlled room. Water and food were freely available. Tumour implant followed the procedure described by Fernandes et al (1990).

Briefly, ascitic tumour liquid from rats implanted intraperitonially with tumour cells was collected and viable tumour cells counted by the trypan blue exclusion method. From this, a solution with 1×10^7 viable tumour cells/mL was prepared with phosphate-buffered saline (PBS) and penicillin (150 000 U mL^{-1}). Volumes of 1 mL were injected into the left flank of each rat by the subcutaneous route. The drug was administered at 7–9 days following tumour implant, when the tumour mass was palpable. Previous studies showed that there was no difference in tumour mass of the rats 7 and 9 days after tumour implant ($P = 0.226$). Rats that had a tumour mass lower than 0.99 g were considered no-takes and were not used.

All the experiments were previously approved by the Committee for Animal Experiments of the Biomedical Sciences Institute of the University of São Paulo.

LDE preparation and association with carmustine

LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein and 0.5 mg cholesterol (Sigma Chemical Company, St Louis, MO). To study uptake of LDE by tissues, ^{3}H-cholesteryl oleate ether (Amershan Life Science, Bucks, UK) was added to the mixture. Lipid emulsification was carried out by prolonged ultrasonic irradiation in aqueous media by the method of Ginsburg et al (1982) as modified by Maranhão et al (1993).

Association of carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea; Bristol Myers Squibb, São Paulo, Brazil) to LDE was performed by co-sonication of the drug with the emulsion (1:3 w/w) for 5 min, at 27°C, using the flat tip (1 cm) of the Branson Cell Disrupter model 450 (Danbury, CT), with 20 W output and under nitrogen stream. The rate of association of carmustine with LDE under these conditions is roughly 80%. The stability of the carmustine–LDE complex had been previously defined by Maranhão et al (2002).

LDE and LDE–carmustine particle size

The size of LDE and LDE–carmustine particles was measured by light scattering using a Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY). The samples were filtered and immediately measured.

LDE uptake by the tumor, liver and muscle tissues

Groups of five rats implanted with Walker 256 tumour were intraperitoneally injected with ^{3}H-LDE and after 24 h of biodistribution were sacrificed for extraction of 1 g of liver, tumour and muscle. Each tissue was processed for total lipid extraction (Folch et al 1957) and radioactivity was counted in a β-counter analyser (Packard Bioscience Company).

Toxicity of free and LDE-associated carmustine

Male healthy Wistar rats, 150–200 g, were injected by the intraperitoneal route with single doses of 30, 60 and 80 mg kg^{-1} (body weight) of LDE–carmustine and free carmustine. All dose levels were injected into groups of 6 rats, except the 30 mg kg^{-1} dose that was injected in a group of 5.

Daily weight and status observations were performed until 15 days after drug injection. Weight variation data were plotted according to the time after drug injection. Survival data were plotted in a dose–response curve and the lethal doses (LD) of 10% (LD10), 50% (LD50) and 90% (LD90) of the rats extracted from this curve.

Tumour growth and rat survival under different treatments

Groups of ten rats implanted with Walker 256 tumour received doses of 3.5, 7 or 15 mg kg^{-1} of LDE–carmustine or free carmustine by the intraperitoneal route. The control group received saline solution (NaCl 0.9%). The protocol of tumour growth evaluation was in accordance with the National Cancer Institute guidelines (Geran et al 1972).

Tumour mass was measured with a caliper in the highest (cc) and lower (dv) axis. Three measurements in each direction were taken and the mean calculated. Each mean was used to calculate tumour mass according to the expression (Geran et al 1972):

$$\text{Tumour mass (g)} = \text{cc} \times (\text{dv})^2/2000 \quad (1)$$

These measurements were taken every two days for ten days after drug injection. Those rats that had tumour reduction in this period had the tumour mass measured until total reduction of the tumour (tumour remission). Those rats that had tumour increase in this period were followed only for survival analysis. The rats that had a

tumour mass lower than 0.99 g after treatment were considered to be in remission (Geran et al 1972).

A previous exploratory study in three rats showed that LDE alone did not cause tumour decrease. In those rats, the tumor increase was similar to the control group injected with saline solution ($P > 0.05$).

All the rats were observed daily for 60 days after drug injection to determine survival rate (Geran et al 1972).

Statistical analysis

Inferencial tests as analysis of variance, multiple comparison analysis by minimum and significant difference proceeding (Montgomery 2001) and Kaplan Meier plots were used. A significant P value of 0.05 was adopted.

Results

As measured by the light scattering technique, the average diameter of the LDE particles was 39 nm and increased to 43 nm after association with carmustine.

LDE uptake by the tumour, liver and muscle tissues

Figure 1 shows the uptake of the microemulsion labelled with ^{3}H-cholesteryl ether by the hepatic, tumour and muscle tissues, expressed in counts min^{-1} (g tissue)$^{-1}$. The uptake of the labelled microemulsion by the tumour was four times greater than that by muscle. However, the liver was the main site of uptake, the radioactivity measured being roughly sixteen times that measured in the muscle. The amount of radioactivity of the LDE label found in the plasma after 24 h (not shown in the figure) was negligible.

Toxicity of free and LDE-associated carmustine

Figure 2 shows that the association of carmustine with LDE markedly diminishes the toxicity of the drug when compared with free carmustine. With free carmustine, the dose level of 30 mg kg^{-1} was lethal to 80% of the rats; none of the rats died when injected with the same dose of LDE–carmustine.

Due to the high toxicity of the free drug, it was not possible to determine the LD10 or LD50 in the experimental dose level range. But the LD90, calculated from the curve shown in Figure 2, amounted to 44 mg kg^{-1} for free carmustine and increased 175% to 77 mg kg^{-1} after association of carmustine with LDE.

Figure 3 shows the percentage of weight variation after injection of LDE–carmustine or free carmustine at 30 and 60 mg kg^{-1} dose levels. While there was consistent decrease in weight after treatment with 30 mg kg^{-1} free carmustine, there

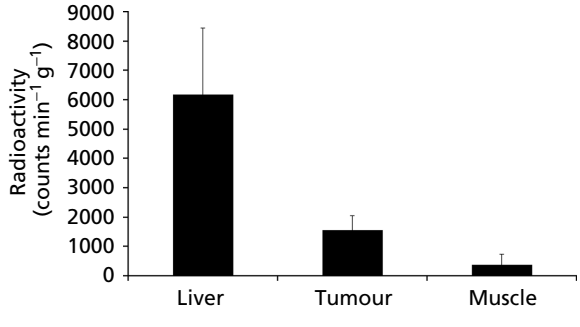

Figure 1 Tumour, liver and muscle uptake of LDE labeled with ^{3}H-cholesteryl oleate ether 24 h after intraperitoneal injection into 5 control rats (bars are means ± s.e.m.).

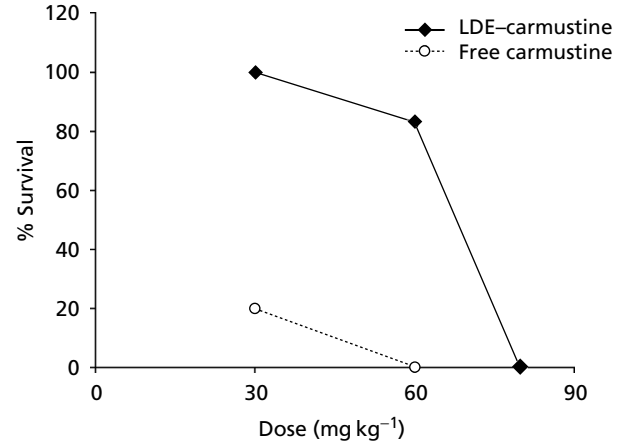

Figure 2 Percentage survival after 15 days of control rats injected with 30, 60 or 80 mg kg^{-1} single doses of LDE–carmustine or free carmustine. Each group comprises 6 rats.

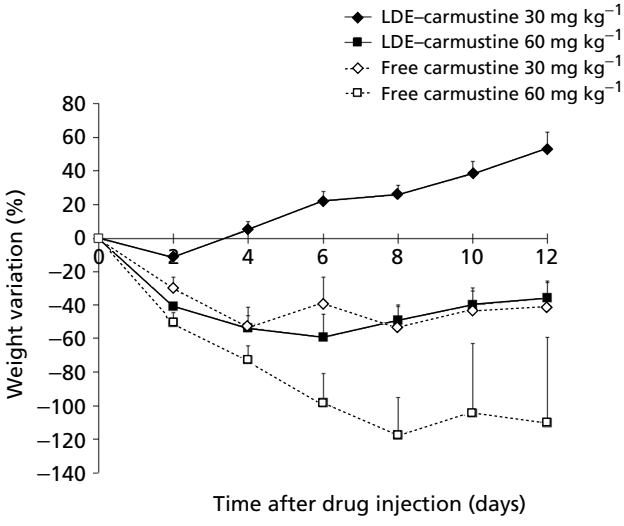

Figure 3 Percentage weight variation relative to the weight of the rats on the commencement of the treatment with 30, 60 or 80 mg kg^{-1} LDE–carmustine or free carmustine. Each group comprises 6 rats. Points are means ± s.e.m.

was, in contrast, a great weight increase with LDE–carmustine at the same dose level. At the 60 mg kg^{-1} dose, there was a decrease in weight with LDE–carmustine but the weight loss was much smaller than that observed with 60 mg kg^{-1} of free carmustine. At day 12 after drug injection, the weight decrease of rats treated with 60 mg kg^{-1} LDE–carmustine was similar to that of rats treated with 30 mg kg^{-1} free carmustine ($P > 0.766$). Therefore, association of carmustine with LDE led to a marked diminution of toxicity, as evaluated by either lethal dose or weight follow-up approaches.

Tumour growth and rat survival under different treatments

Figure 4 shows the variation in tumour mass after treatment with LDE–carmustine, free carmustine or saline solution over the ten days after drug injection. It can be seen that at a 3.5 mg kg^{-1} dose level there was a clear difference between both preparations on day 10 ($P < 0.05$). However, tumour evolution after 7 and 15 mg kg^{-1} doses of LDE–carmustine and free carmustine were not different ($P = 0.6138$). Treatment with both LDE–carmustine and free carmustine achieved total tumour regression in all rats at a dose level of 15 mg kg^{-1}. At a dose of 7 mg kg^{-1}, remission occurred in all rats treated with LDE–carmustine and in 9 of 10 rats treated with the same dose of free carmustine. Remarkably, at the lowest (3.5 mg kg^{-1}) dose, tumour remission was still achieved in all rats treated with LDE–carmustine but only in 50% of the rats receiving free carmustine treatment. As expected, after treatment with saline solution, all rats displayed tumour increase.

Figure 5 shows the Kaplan Meier plots of the remission time of the rat groups submitted to different treatments. It is apparent that rats treated with LDE–carmustine had shorter regression times than those treated with free carmustine ($P < 0.05$). In free-carmustine-treated rats, a dose–response pattern is clearly seen — the greater the dose, the shorter the time for remission to occur. In rats treated with LDE–carmustine, there is no difference between the 15 and 7 mg kg^{-1} dose, but the 3.5 mg kg^{-1} dose was less effective than the others ($P < 0.05$). No rats treated with saline solution had tumour regression.

With respect to the survival rates of the rats during the 60-day observation period, the survival in the control group was only 20%. In the rats treated with free carmustine, the group treated with a dose of 15 mg kg^{-1} showed 100% survival but the group treated with 7 mg kg^{-1} showed 90% survival and the group receiving 3.5 mg kg^{-1} showed 50% survival. In contrast, all the 3 groups of rats treated with LDE–carmustine showed a 100% survival rate. Nonetheless, these differences between LDE–carmustine and free carmustine were not statistically significant.

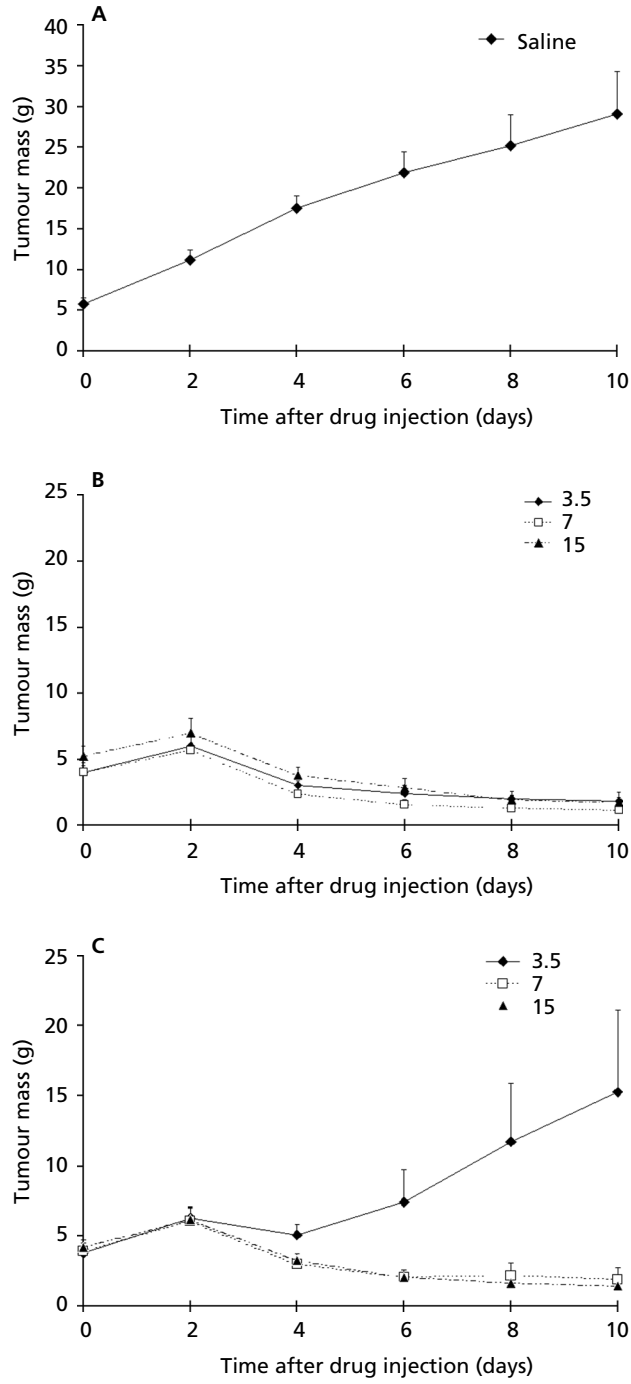

Figure 4 Tumour mass variation in Walker 256 tumour-bearing rats after injection of saline solution (NaCl 0.9%) (A) or single doses of 3.5, 7 or 15 mg kg^{-1} LDE–carmustine (B) or free carmustine (C). Each group comprises 10 rats. Points are means ± s.e.m.

Discussion

LDE–carmustine was compared with the free drug for toxicity, anti-tumour effect and survival of the treated rats. The results show that the novel preparation has a clear-cut advantage.

Similarly to native LDL, the entry of LDE into the neoplastic cells is greatly facilitated, relative to normal cells, by the phenomenon of LDL receptor over-expression. LDL

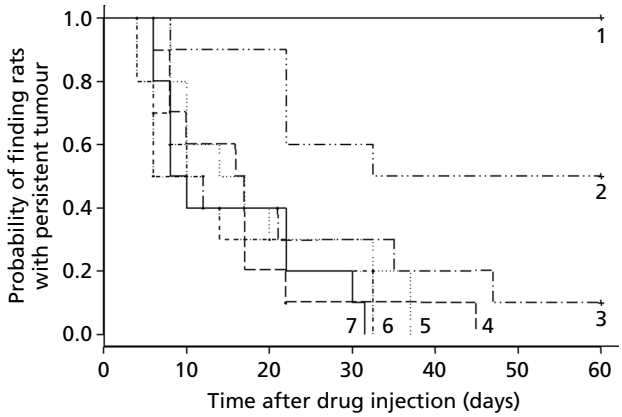

Figure 5 Kaplan Meier plots of time elapsed until the occurrence of tumour remission in Walker 256 tumour-bearing rats injected with saline solution (1), with 3.5, 7 or 15 mg kg^{-1} free carmustine doses (2, 3 and 4, respectively) or with 15, 7 and 3.5 mg kg^{-1} LDE–carmustine doses (5, 6 and 7, respectively). Each group comprises 10 rats.

receptor expression is up to 100 times greater in acute myelocytic leukaemia cells than in normal mononuclear cells (Ho et al 1978). In ovarian and breast carcinoma, the concentration of LDE after injection into patients was ten and four times greater than that measured in the normal corresponding tissue, respectively (Ades et al 2001; Graziani et al 2002). Therefore, the LDL receptor endocytic pathway offers a strong mechanism for drug targeting in cancer. Selective concentration in the tumour would conceivably diminish the side effects and increase the pharmacological action of the drug. In a previous study, we showed that the average decay curve of ^{14}C-carmustine associated with LDE in cancer patients is roughly similar to that of labelled LDE. This indicates that the drug follows the microemulsion in the circulation.

In this study, there was a marked reduction of carmustine toxicity when the drug was associated with LDE, as documented by the lethal dose values. Since these experiments were performed in control rats, in the absence of tumour, the reduction in toxicity was not due to drug-targeting but rather to the new biodistribution of the drug created by the association with the microemulsion. In this regard, hepatotoxicity would become a major concern because the liver is the main uptake site for LDE as it is for native LDL. Nonetheless, our recent report of LDE–carmustine administered to cancer patients shows that hepatotoxicity does not occur, even at very high doses of carmustine (Maranhão et al 2002). A second possible cause for the toxicity reduction is the protection conferred by the LDE association against contact of the drug with plasma proteins. Protein contact accelerates carmustine degradation (Levin et al 1978) and the degradation products attack many biological targets, not only in tumours but also in normal cells, hence the toxicity of the drug. In fact, we have observed that association of LDE prolongs the carmustine half-life (unpublished observations). This was also noticed when the drug was incorporated into native lipoproteins (Weinkam et al 1980).

Our results show that the concentration of LDE in the Walker 256 tumour, a spontaneous mammary carcinosarcoma (Dunham & Stewart 1953), was four times greater than in the adjacent normal muscle tissue. This is similar to our previous observation in mammary carcinoma patients, when the microemulsion concentrated four times more in the tumour than in the normal mammary tissue (Graziani et al 2002). In view of the observations by several authors (Ho et al 1978; Gal et al 1981; Rudling et al 1983; Ginsberg et al 1986; Henriksson et al 1989; Ueyama et al 1990; Gueddari et al 1993) that the concentration of lipoprotein particles in cancer cells is dependent on increase of LDL receptor mechanisms, the increased concentration of LDE in the Walker 256 tumour suggests that the LDL receptors are also over-expressed in that tumour. Although the liver is the greatest uptake site for the microemulsion, a substantial amount of LDE is trapped by the tumour. The high tumour/normal tissue concentration ratio shown in our previous trial (Ades et al 2001; Graziani et al 2002) and the low hepatoxicity of LDE–carmustine (Maranhão et al 2002) may account for the superior effects of LDE–carmustine compared with the free drug.

With respect to the therapeutic effect of the preparations evaluated by the percentage of rats with tumour remission after treatment, there was a clear-cut advantage of LDE–carmustine over the free drug because only the associated drug achieved 100% at the lowest (3.5 mg kg^{-1}) dose level. Therefore, regarding the % remission parameter, the LDE–carmustine preparation is already optimized at the lowest dose level, while optimization (100% remission) is attained by the free drug only two steps ahead, at the highest (15 mg kg^{-1}) dose level. The superiority of the therapeutic action of LDE–carmustine over the free drug can also be inferred from the time required for remission at the highest dose level that was shorter under LDE–carmustine treatment.

Regarding the survival time parameter, because association with LDE leads to both decrease in toxicity and increase in anti-tumour effect, it would be expected that survival time would be prolonged when LDE is used. Nonetheless, differences between the two preparations were unclear, although a 60-day observation time appears sufficient for this evaluation (Geran et al 1972). The dose administration scheme, tumour responsiveness to the treatments and the degree of life-threat posed by the tumour in the rats that did not attain tumour remission were experimental conditions that, in this study, could have obscured the differences in survival time.

Conclusion

It is apparent that the association with LDE may diminish the toxicity and improve the therapeutic action of carmustine. Currently, carmustine has limited usefulness among the anti-cancer weaponry and has been withdrawn from the Pharmacopoeia of some countries. In view of our current and previous results (Fernandes et al 1990; Maranhão et al 1992), association with LDE can extend and widen the applications of this antineoplastic agent.

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