molecular pharmaceutics

articles

# Nanoemulsion Preparations of the Anticancer Drug Dacarbazine Significantly Increase Its Efficacy in a Xenograft Mouse Melanoma Model

**Jean-Bosco Tagne,[†,‡,§] Srikanth Kakumanu,[†,‡] and Robert J. Nicolosi*[,†]**

*Center for Health and Disease Research and the Biomedical Engineering/Biotechnology Program, University of Massachusetts Lowell, Lowell, Massachusetts 01854*

Received May 23, 2008; Revised Manuscript Received August 21, 2008; Accepted September 5, 2008

**Abstract:** This article reports on the preparation of a water-soluble nanoemulsion of the highly lipid-soluble drug Dacarbazine (DAC). In addition, relative to suspensions of DAC, the nanoemulsion preparation demonstrated a lower $\zeta$-potential (decreased negative charge, less anionic and more cationic) which has previously been associated with influencing drug membrane permeability. This study also reports that, relative to suspensions of DAC with a mean particle size of 5470 nm, nanoemulsions of DAC having mean particle sizes of 131 nm were more efficacious. For example, in a mouse xenograft model using a human melanoma cell line, a topical application of nanoemulsions of DAC compared to the suspension preparation of DAC produced up to 10-fold greater percent (%) reductions of tumor size. The reduction in tumor size by the intramuscular (IM) injection (−61%) and topical application of the nanoemulsion preparations of DAC (−49%) appeared to be comparable in efficacy, although the former was statistically greater ($p < 0.05$). In addition, 12 weeks after DAC treatment cessation, 98% of the animals given the IM application of the nanoemulsion of DAC remained tumor-free compared to the control or untreated animals. During this drug cessation period, and compared to the suspension preparations, nanoemulsions of DAC showed 5-fold greater efficacies (73% versus 14%) in preventing tumor growth. In conclusion, in this xenograft mouse model of melanoma, nanoemulsion suspensions of DAC are more efficacious in the treatment and prevention of tumor growth.

**Keywords:** Nanoemulsion; xenograft; melanoma; Dacarbazine; cancer

## Introduction

Melanoma is a malignant tumor of melanocytes that predominantly occurs in skin but can also be found elsewhere, such as in the eye, neck, meninges, digestive tract, mucosal surfaces, or lymph nodes.[1−3] Although melanoma is less common than other types of skin cancer, it is the most serious and most lethal form of skin cancer.[4] In men, melanoma is found most often on the area between the shoulders and hips or on the head and neck, whereas in women melanoma often develops on the lower leg. It may also appear under the fingernails or toenails or on the palms or soles. The chance of developing melanoma increases with age, but it affects all age groups and is one of the most common cancers in young adults. The incidence rate for

* To whom correspondence should be addressed. Mailing address: Center for Health and Disease Research, 3 Solomont Way, University of Massachusetts Lowell, Lowell, MA 01854. Telephone: 978-934-4501. Fax: 978-934-2034. E-mail: Robert_Nicolosi@uml.edu.

† Center for Health and Disease Research.

‡ Biomedical Engineering/Biotechnology program.

§ New Address: Boston University School of Medicine, Boston, Massachusetts 02118.

(1) Little, J. W. Melanoma: etiology, treatment, and dental implications. *Gen. Dent.* **2006**, *54*(1), 61−66; quiz, 67.

(2) Murayama, K.; et al. Melanoma-associated retinopathy with unknown primary site in a Japanese woman. *Nippon Ganka Gakkai Zasshi* **2006**, *110* (3), 211–217.

(3) Lim, C. C.; Cullen, C. L.; Grahn, B. H. Choroidal melanoma in the right eye with focal retinal detachment. *Can. Vet. J.* **2006**, *47* (1), 85–86.

(4) Pasco, S.; et al. Control of melanoma cell invasion by type IV collagen. *Cancer Detect. Prev.* **2005**, *29* (3), 260–266.

(5) Scherer, H. Treatment possibilities for malignant melanomas of the palate, nasal cavity and paranasal sinuses. *Laryngol. Rhinol. Otol.* **1984**, *63* (1), 9–10.

10.1021/mp8000556 CCC: $40.75 
Published on Web 10/01/2008

melanoma (the number of new melanomas diagnosed per 100 000 people each year) has more than doubled from 5.7 to 14.3 since 1973, and the prognosis is very poor, that is, 15−17% for a 5 year survival rate.[5]

Dacarbazine (DAC), an antineoplastic chemotherapy drug used in the treatment of various cancers such as malignant melanoma and Hodgkin's lymphoma, belongs to the family of imidazole carboxamide derivatives with structural similarities to certain purines. DAC is usually administered intravenously and the selected dosing is dependent on whether the product is used in mono- or poly-chemotherapy, it is lipid soluble mostly and used intravenously as a single bolus injection via the tail vein[6] or intraperitoneal route injection (no references), and as a result, its anticancer indication is often associated with significant side effects and it is very difficult to administer. After intravenous (IV) administration, DAC is quickly distributed into tissue (unpublished data). Although DAC interferes with cell growth and impedes the formation of new tumor tissue, its primary mode of action appears to be alkylation and enhancement of nucleic acid and protein biosynthesis. As a cell cycle phase nonspecific antimelanoma drug, it is often used in combination with other cancer-fighting drugs. As with other chemotherapeutic drugs, patient responses are variable and often associated with significant degrees of toxicity and adverse side effects because it interferes with normal as well as cancer cell growth. Among the most serious possible side effects are birth defects in children conceived or carried during treatment, sterility,[7] possible permanent immune suppression with a decrease in the number of white blood cells (reduced ability of your body to fight infection or disease), and decrease in red blood cells, platelets, and appetite.[8] Similar to most powerful drugs, it may also produce more common side affects such as nausea, fatigue, headache, vomiting, flulike symptoms (joint or muscle pain), flushing or numbness of the face and temporary hair loss, and hypersensitivity with fever, hypereosinophilia, and liver dysfunction.[9]

In an attempt to overcome some of these adverse effects and increase its efficacy, a nanoemulsion system has been utilized for the delivery of DAC and tested on nude mice as for the determination of individual tumor response to chemotherapeutic agents.[10] Nanoemulsions are a class of stable emulsions formed by a monolayer of phospholipids composed of surfactant and vegetable oil suspended in water with mean particle diameters of approximately less than 100 nm.[11] The stability of nanoemulsions makes them extraordinary, and they are often referred to as "approaching thermodynamic stability".[12] It has been suggested that emulsion systems offer an appealing substitute for the formulation of poorly soluble drugs such as paclitaxel and amiodarone.[13] Compared to typical suspension preparations which can be thousands of nanometers in size, nanoemulsion delivery systems with particle sizes in the hundred nanometer range or less have been shown to increase the bioavailability and efficacy of a number of compounds such as anti-inflammatory agents, insulin, tetanus toxoid, and Dicumarol.[14]

Nanoemulsion delivery systems are fast becoming fundamental approaches for innovative strategies in the prevention and treatment of cancer. Nanoemulsion delivery systems can (1) convert fat soluble to water soluble compounds thereby allowing delivery into polar versus nonpolar matrices, potentially reducing toxicity and (2) reduce particle sizes of existing drugs that are usually thousands of nanometers in size to less than 100 nm in size, presumably resulting in more in-depth and longer penetration. During the production process, we are suggesting that the surface/volume ratios of the nanoemulsions containing the drug are increased, raising the bioavailability and efficacy of pharmaceuticals, as reported previously.[14] Support for increased efficacy of nanoemulsion delivery substances has also recently been reported by our laboratory, which demonstrated that nanoemulsions produced by the high shear forces during microfluidization and containing an antioxidant synergy formulation (ASF) dramatically reduced tumor size in a neuroblastoma-bearing xenograft mouse model compared to a suspension formulation of ASF.[15]

(6) Niederkorn, J. Y.; Sanborn, G. E.; Gamel, J. W. Suicide enzyme inhibition as a chemotherapeutic strategy for controlling metastases derived from intraocular melanomas. *Invest. Ophthalmol. Visual Sci.* **1987**, *28* (11), 1844–1850.

(7) Kumar, S. G.; et al. Dacarbazine induces genotoxic and cytotoxic germ cell damage with concomitant decrease in testosterone and increase in lactate dehydrogenase concentration in the testis. *Mutat. Res.* **2006**, *607* (2), 240–252.

(8) Ancheva, M. N.; Boeva, M. N. The effect of Biocarbazine on the indices of humoral and cellular immunity in mice. *Eksp. Onkol.* **1990**, *12* (5), 37–40.

(9) Levy, A.; et al. Hypersensitivity to dacarbazine in patients with metastatic malignant melanoma. *Ann. Dermatol. Venereol.* **2006**, *133* (2), 157–160.

(10) Povlsen, C. O.; Jacobsen, G.K. Chemotherapy of a human malignant melanoma transplanted in the nude mouse. *Cancer Res.* **1975**, *35* (10), 2790–2796.

(11) Sadurni, N.; et al. Studies on the formation of O/W nanoemulsions, by low-energy emulsification methods, suitable for pharmaceutical applications. *Eur. J. Pharm. Sci.* **2005**, *26* (5), 438–445.

(12) Tadros, T. Formation and stability of nano-emulsions. *Adv. Colloid Interface Sci.* **2004**, *108−109*, 303–318.

(13) Constantinides, P. P.; Tustian, A.; Kessler, D. R. Tocol emulsions for drug solubilization and parenteral delivery. *Adv. Drug Delivery Rev.* **2004**, *56* (9), 1243–1255.

(14) Thanos, C. G.; et al. Improving relative bioavailability of dicumarol by reducing particle size and adding the adhesive poly(fumaric-co-sebacic) anhydride. *Pharm. Res.* **2003**, *20* (7), 1093–1100.

(15) Kuo, F.; et al. A nanoemulsion of an anti-oxidant synergy formulation reduces tumor growth rate in neuroblastoma-bearing nude mice. *J. Exp. Ther. Oncol.* **2007**, *6* (2), 129–135.

## Materials and Methods

**1. Preparations of Nanoemulsions of DAC.** The DAC suspension was prepared by first dissolving it in ethanol, adding it to soybean oil, polysorbate 80, and a solution of HPLC-grade water (see below for individual component concentrations), and then homogenizing it for 60 s at 25 °C (Polytron model PT 10/35, Brinkmann Instruments, Westbury, NY). The nanoemulsion formulation of DAC was prepared from the suspension by using a microfluidizer processor model M-110EH (Microfluidics Corporation, Newton, MA). Microfluidizer processors provide high pressure and a resultant high shear rate by accelerating the product through microchannels to a high velocity for size reduction to the nanoscale range. The fluid is split in two and is pushed through microchannels with typical dimensions on the order of 75 $\mu$m at high velocities (in the range of 50−300 m/s). As the fluid exits the microchannels, it forms jets which collide with jets from opposing microchannels. In the channels,the fluid experiences high shear forces (up to $10^7$ 1/s) which are orders of magnitude greater than those of conventional technologies. Jet collisions result in mixing at the submicrometer level. Therefore, high shear and impact are responsible for particle size reduction to the nano range and mixing of multiphase fluids in the microfluidizer technology.

The viscosities of the formulations were measured using an Ubbelohde viscometer (VWR International, Boston, MA) at 25 °C. For measurement of the mean droplet size and polydispersity index (width of the particle size distribution) and ζ-potential determination, a dynamic laser light scattering Malvern Nano-S instrument (Malvern Instruments Inc., Southborough, MA) was used that is capable of measuring particle sizes between 0.6 and 6000 nm.

For the topically administered xenograft experiments, the suspension and nanoemulsion formulations of DAC were mixed together in a 1:1 ratio with a hypoallergenic cream (PCCA, Houston, TX).

For the topical experiments, 4.2 g of soybean oil, 4.0 g of polysorbate 80, 0.1 g of DAC, and 41.7 g of $H_2O$ were homogenized to prepare as a suspension and subsequently microfluidized and then added to 50 g of cream.

For the intramuscular (IM) injection experiments, 2.34 g of soybean oil, 2.12 g of polysorbate 80, 0.051 g of DAC, and 44.5 g of $H_2O$ were homogenized to prepare as a suspension and subsequently microfluidized. The average body weight of the mice was 22 g, and the concentration of each preparation was adjusted to deliver 0.1 mg/50 uL per injection into the animal.

A transmission electron microscope (Philips EM400T) was used to analyze the morphology of the nanoemulsion preparation DAC. The nanoemulsion samples were processed as previously[16] described after diluting 200-fold with distilled ionized water. Samples were placed into the carbon attached Vinylec films (Ernest F. Fullam, Inc., Latham, NY), stained with 10% phosphotungstic acid (Sigma Aldrich, St Louis, MO), allowed to stand 5 min for drying, and placed in the vacuum chambers for 30 min prior to analysis (Figures 2 and 3).

**2. Cell Line and Reagents.** The human cultured tumor line Malme 3M was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). This cancer cell line derived from a malignant melanoma of a 43 year old male was cultured in Iscove's modified Dulbecco's medium (IMDM) with 4 mM L-glutamine adjusted to contain 1.5 g/L, and 80% sodium bicarbonate, with a subcultivation ratio of 1:2 to 1:4 as indicated by the product description. The cell line was supplemented with 10% fetal bovine serum (FBS), along with 100 U penicillin, 100 $\mu$g streptomycin per mL, and 1 mM sodium pyruvate. Cells were cultured at 37 °C in a 95% $O_2$, 5% $CO_2$ incubator after subculture as indicated by the manufacturer.

**3. ζ-Potential.** The ζ-potential is a measure of the electrical force that exists between atoms, molecules, particles, and cells in a fluid. The strength of the ζ-potential determines the amount of material that fluids such as blood and lymph can carry. Increasing the electrical force in the solution allows the fluid to dissolve and hold more material. In this way, more substances can be carried throughout the body and accumulated degradation products can be removed. The measurement of ζ-potential is based on the following principle: colloidal particles of DAC dispersed in soybean oil (density 0.917 g/mL) and polysorbate 80 (density 1.064 g/mL) solutions are electrically charged due to their ionic characteristics and dipolar attributes. Each particle dispersed in the solution using the Malvern zetasizer (nano series Zen 3600, Malvern Instruments Ltd., Enigma Business Park, Grovewood Road, Malvern, Worcestershire WR14 1XZ, U.K.) is surrounded by oppositely charged ions called the fixed layer. Outside the fixed layer, there are varying compositions of ions of opposite polarities, forming a cloudlike area. This area is called the diffuse double layer, and the whole area is electrically neutral. When a voltage is applied to the solution in which the particles are dispersed, particles are attracted to the electrode of the opposite polarity, accompanied by the fixed layer and part of the diffuse double layer, or internal side of the "sliding surface". This system uses dispersion technology software (DTS v4.20) which changes to the ζ-potential measuring mode, and the sample for which the ζ-potential is measured is taken in disposable capillary cuvettes (DTS1060) equipped with electrodes. Each determination is done in triplicate.

**4. Xenograft Model.** The nude mouse, Crl: NU/NU-nuBR, used for this study is the recognized model for antitumor testing, since unlike the normal mouse it lacks a fully functional immune system and therefore does not mount a deleterious response against experimentally induced tumors on its own. Tumors were induced in 4−7 week old mice by subcutaneous injection of the human melanoma cancer cell suspension ($3 \times 10^6$ cells/mL). Mice were injected at two

(16) Zhao, X.; et al. Enhancement of transdermal delivery of theophylline using microemulsion vehicle. *Int. J. Pharm.* **2006**, *327* (1−2), 58–64.

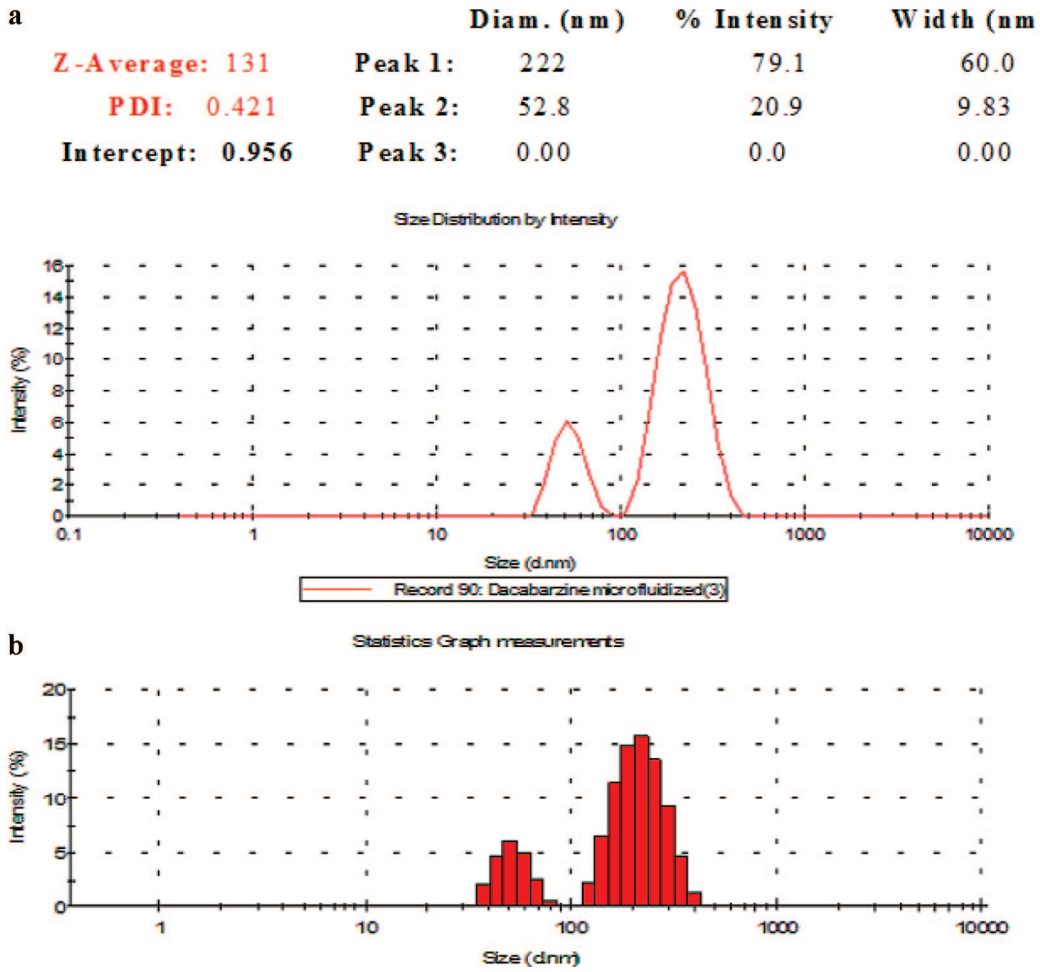


***Figure 1.*** Dynamic laser light scattering particle size analysis of nano-DAC with (a) the *Z*-average size distribution of the particle and (b) the statistics graph measurement by model distribution. As shown in both (a) and (b), microfluidization results in a dramatic decrease in particle size (a) and also demonstrates the heterogeneity of particle size even within what appears to be a homogeneous distribution (b).

sites (the left and right dorsal side of the two hind legs) with the appropriate concentration of cancer cell suspension per site and monitored every 24 h from the time of injection for the first signs of tumors. The animals were treated with suspensions or nanoemulsions of DAC at a dose of 0.1 mg/kg every 2−4 days of the drugs by IM injection and topical application and then sacrificed with $CO_2$.

Tumor size was quantified daily in accordance with the longest diameter. Tumor growth rate was reported as the change in size (mm) versus the size for the previous day, and tumor dimensions were measured using calipers. Calculation of tumor size was made using the formula $V = (W^2 \times L)/2$, where $V$ is tumor volume in $mm^3$, $L$ is the tumor length (the longest diameter of the tumor), and $W$ is the tumor width (diameter perpendicular to the length),[17] after a time course of tumor growth, measured as the average tumor size (mm) in each group at the indicated time of treatment with vehicle (control) or DAC at the indicated concentrations and tumor weights from the four individual animals in each group at the end of the treatment period (Figure 4).

**5. Preparation of Xenograft Animals and Treatment Groups.** A frozen vial (stored in a liquid nitrogen tank) of Malme 3M cells was plated in 10% FBS, and the appropriate number of plates were generated and maintained until they reached the right confluency for injection. A plate was considered ready for injection when it reached 90−100% confluency. The confluent plates of cells were trypsinized to detach the cells from the plate and spun at 3000 rpm for 1 min in 10% serum-containing media to pellet the cells. The cells were then resuspended in the appropriate volume of autoclaved phosphate-buffered saline (PBS) (1×), and this cell suspension was injected into mice subsequently. The cell suspensions from all the plates were combined into one stock suspension so that all the mice were injected with the same concentration of cells. Before injection, a hemacytometer was used to count the number of cells per milliliter of the cell suspension. The concentration of cells per 100 $\mu$L of injection was $3.5 \times 10^5$ cells. Syringes (1 mL) with 27-1/2 gauge

(17) Koziara, J. M.; et al. In-vivo efficacy of novel paclitaxel nanoparticles in paclitaxel-resistant human colorectal tumors. *J. Controlled Release* **2006**, *112* (3), 312–319.

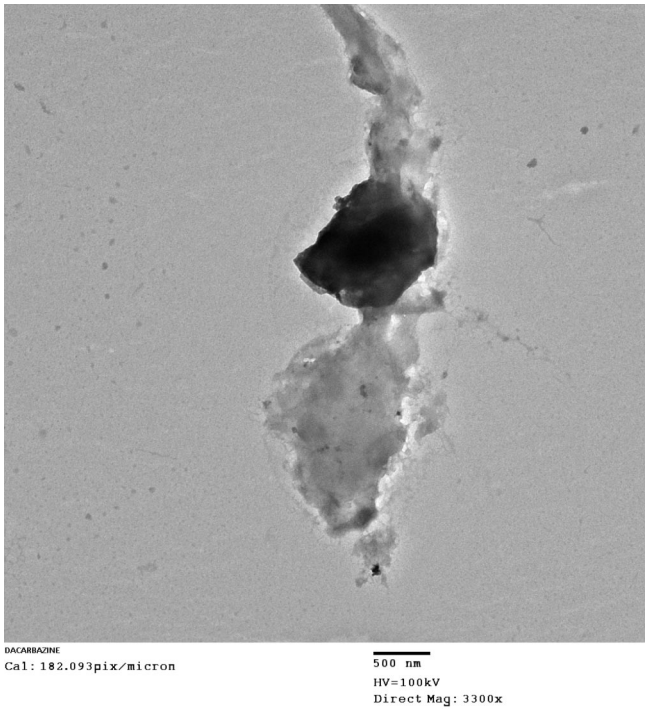


***Figure 2.*** Schematic structure of DAC by characterization of the molecule morphology using TEM (Philips EM400T). Evaluation of the morphology of the nanoemulsion preparation of DAC as shown is a large spherical particle of more than 500 nm.

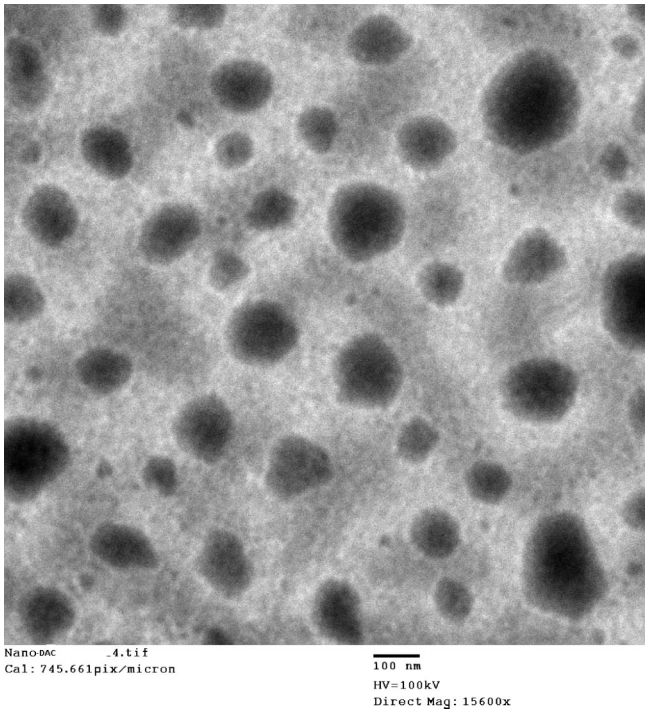


***Figure 3.*** Schematic structure of our formulation of nano-DAC by characterization of the nanoemulsion morphology using TEM (Philips EM400T). This evaluation of the morphology of the nanoemulsion preparation of DAC shows spherical particles of less than 131 nm.

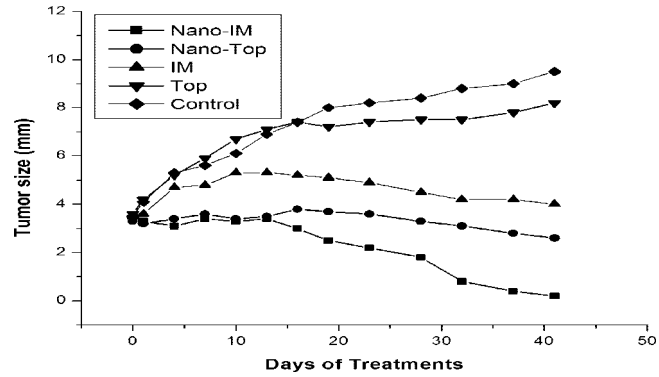


***Figure 4.*** Pattern of tumor size growth from Malme 3M xenograft mice after DAC formulation treatments for 40 days showing the significant reductions in tumor growth in nanoemulsion formulations of DAC versus suspension-treated or untreated animals. Tumor size was measured on the surface of the skin of the mouse, and tumor volume was calculated as described in the Materials and Methods section.

needles were used for the injections. The stock treatments were stored at 4 °C. The appropriate volume was aliquoted into 2 mL syringe vials which was then warmed to room temperature before injection. For every 2 mm of tumor, 20 $\mu$L of DAC was injected IM adjacent to the tumor surface. The injected site was initially cleaned with 100% alcohol, and also after the injection if any bleeding occurred. We used two injection sites for the DAC formulations per mouse (the dorsal side of the two hind legs) with 100 $\mu$L of the cell suspension per site.

For the treatment groups, 20 animals were divided into 5 groups with 4 mice in each group: (1) control (untreated mice), (2) DAC suspension for IM injection, (3) DAC suspension for topical application, (4) nanoemulsion of DAC for IM injection, and (5) nanoemulsion of DAC for topical application at a dose of 0.1 mg/kg every 2−3 days.

The mice were monitored every 24 h from the time of injection for any signs of tumor. When the tumors first appeared, they had the appearance and the size of a mosquito bite on the surface of the skin where the injection was done. Even though all the mice were injected with the same volume and concentration of cells, the tumors appeared at different times. The tumors were also of different sizes when they first appeared (2−5 mm). As soon as the tumor became visible, a caliper was used to measure the diameter of the tumor, and since the tumors were not all perfectly symmetrical, we took the measurement of the largest diameter.

**6. Statistical Methods.** Values in duplicate of pooled data were analyzed, and significant differences for all parameters measured were determined by using Student's *t*-test with $p < 0.05$ established for statistically significant differences.

All aspects of this research were approved by UMASS Lowell's Institutional Animal Care and Use Committee (IACUC). Our animal facility is routinely overseen by our university veterinarian and our departmental certified animal care technologist, and all experimentation with animals was

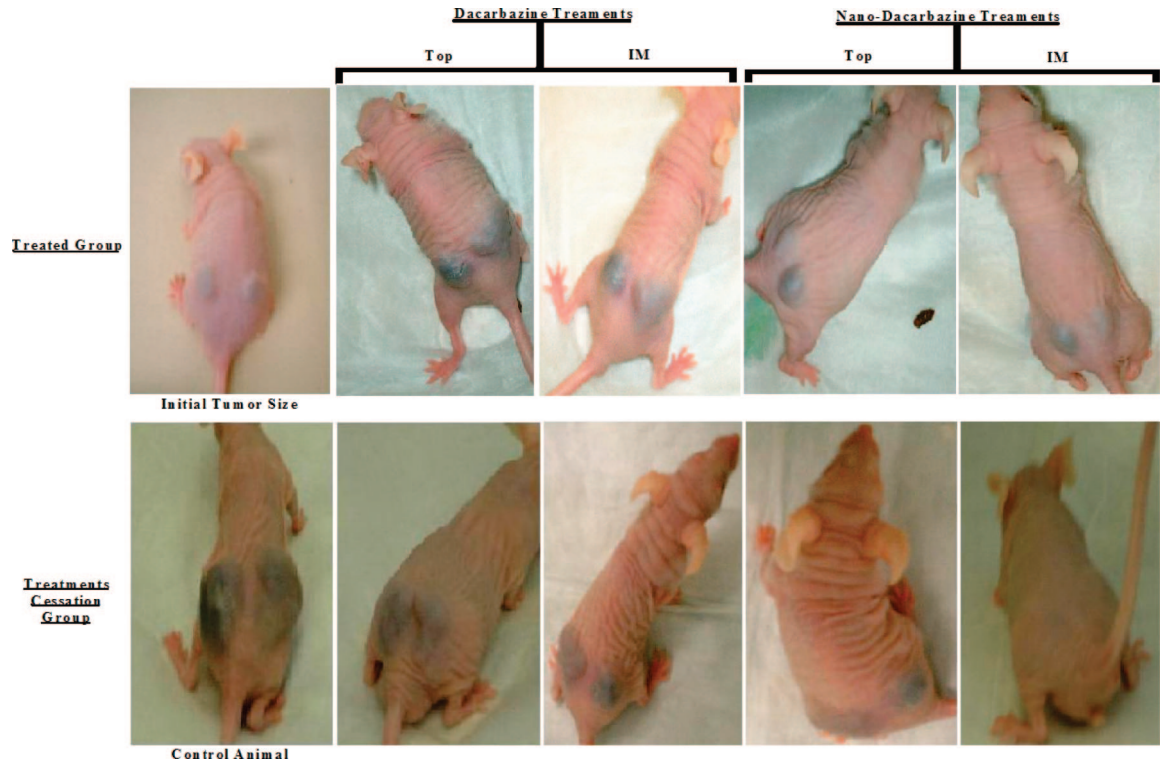


***Figure 5.*** Nude mice with melanoma Malme 3M cell line derived xenograft for further investigations of the effectiveness of the anticancer drug DAC and the novel formulate nanoparticles. This allows us to evaluate a certain drug in a more similar context as seen in human cancer patients. The mice in the upper portion were treated with both formulations Top and IM while the tumor size was measured, and the tumor sizes in the mice in the lower portion were measured 2 months after cessation of both treatments.

carried out in accordance with the Public Health Service policy on the "humane care and use of laboratory animals".

## Results

**1. Nanoemulsion Preparation and Particle Size.** The highly lipid soluble suspensions of DAC became water soluble upon preparation of the nanoemulsion of DAC. The particle size *Z*-average of DAC suspensions which was 5470 nm in size in complete cell culture media was reduced after microfluidization to 131 nm (Figure 1). The size scale of the particles can actually be visualized from the characterization of the drug suspension alone [morphology included the use of transmission electron microscopy (TEM) [Philips EM400T] (Figure 2)] to the nanoemulsion preparation of the same drug (Figure 3).

**2. Mouse Xenograft Tumor Size Reduction.** As shown in Figures 4 and 5, the growth of tumors derived from Malme 3M melanoma cells was significantly inhibited by our newly formulated nano-DAC (N-DAC) by both topical application and IM injection even after 12 weeks of treatment stoppage. The average tumor size showed an exponential size measurement from N-DAC_IM and Top treatments, DAC_IM, to the control animals as seen in Figures 4 and 5 and Table 4. Table 4 also recapitulates the significant correlation of the standard deviation (SD) from each treatment group and period at a very important cutoff value using the standard Student's *t*-test. Using the same Student's *t*-test, we were able to compare groups among them: Nano_IM versus Nano Top, DAC_IM versus DAC_Top, Nano_IM versus DAC_IM, and Nano_Top versus DAC_Top have values of 0.000646, $4.7 \times 10^{-5}$, $9.31 \times 10^{-7}$, and $8.68 \times 10^{-7}$, respectively, indicating that growth of the melanoma derived tumors was significantly inhibited by our reformulation of DAC at the intramuscular and topical administered doses.

In a mouse xenograft model injected with a human melanoma cell line, topical application of a nanoemulsion of DAC compared to the suspension preparation of DAC produced up to 10-fold greater percent reductions of tumor size ($p =$ at least $<0.05$) (Table 2). The reduction in tumor size by the IM injection ($-61\%$) and topical application of the nanoemulsion preparations of DAC ($-49\%$) appeared to be comparable in efficacy, although the former was statistically greater ($p =$ at least $<0.05$) (Table 2). In addition, 12 weeks after DAC treatment cessation, 98% of the animals given the IM application of the nanoemulsion of DAC remained tumor-free compared to the control or untreated animals ($p =$ at least $<0.05$) (Table 3). During this drug cessation period, and compared to the suspension preparations, nanoemulsions of DAC showed 5-fold greater efficacy (73% versus 14%) in preventing tumor growth ($p =$ at least $<0.05$) (Table 3).

(18) Rabinow, B. E. Nanosuspensions in drug delivery. *Nat. Rev. Drug Discovery* **2004**, *3* (9), 785–796.

***Table 1.*** Composition, Physical, and Chemical Properties of DAC and Formulations[a]

| formulation | composition | PDI | size (nm) | ζ-potential (mV) | avg mean (mV) |
|---|---|---|---|---|---|
| empty nano | SO + P80 | 0.284 | 145 | 0.304 | 0.304 |
| microemulsion | SO + P80 + DAC | 0.252 | 5470 | −1.180 | −45.41 |
| nanoemulsion | SO + P80 + DAC | 0.421 | 131 | −5.490 | −70.91 |

[a] SO, soybean oil; P80, Polysorbate 80; DAC, Dacarbazine.

***Table 2.*** Effect of Different DAC Formulations on Tumor Growth in the Xenograft Melanoma Mouse Model after 40 Days of Treatment[a]

| treatment groups | | | | |
|---|---|---|---|---|
| | (2) microemulsion of DAC | | (3) nanoemulsion of DAC | |
| (1) control | TOP | IM | TOP | IM |
| 6.9 ± 0.5 | 6.6 ± 0.4 (−4%)[a] | 4.8 ± 0.2 (−30%)[b] | 3.5 ± 0.1 (−49%)[c] | 2.7 ± 0.3 (−61%)[d] |
| | [−27%][e] | | [−23%][f] | |

[a] Values represent mean ± SEM for four animals in each group. Values in parenthesis (a−d) represent percent reduction compared to control animals. Values in brackets (e and f) represent percent reduction between IM and topical application within the micro- and nanoemulsion preparations successively. Superscripts different from each other; $p$ = at least <0.05.

***Table 3.*** Effect of Different DAC Formulations on Tumor Growth in the Xenograft Melanoma Mouse Model after 12 Weeks of Cessation of Drug Treatment[a]

| treatment groups | | | | |
|---|---|---|---|---|
| | (2) microemulsion of DAC | | (3) nanoemulsion of DAC | |
| (1) control | TOP | IM | TOP | IM |
| 9.9 ± 0.7 | 8.5 ± 0.5 (−14%)[a] | 3.9 ± 0.2 (−61%)[b] | 2.7 ± 0.1 (−73%)[c] | 0.2 ± 0.1 (−98%)[d] |
| | [−54%][e] | | [−93%][f] | |

[a] Values represent mean ± SEM for four animals in each group. Values in parenthesis (a−d) represent percent reduction compared to control animals. Values in brackets (e and f) represent percent reduction between IM and topical application within the micro- and nanoemulsion preparations successively. Superscripts different from each other; $p$ = at least <0.05.

## Discussion

Melanoma is the number one cause of cancer death in women aged 25−30. Dacarbazine (DAC), an antineoplastic chemotherapy drug used in the treatment of various cancers such as malignant melanoma and Hodgkin's lymphoma, belongs to the family of imidazole carboxamide derivatives with structural similarities to certain purines. It is lipid soluble and, as a result, is often associated with significant side effects and is very difficult to deliver. The increased water solubility of our nanoemulsion preparations of DAC would be expected to reduce adverse side effects in agreement with other findings[18] which suggested that emulsion systems offer an appealing substitute for the formulation of poorly soluble drugs such as paclitaxel and amiodarone.[13] Compared to typical suspension preparations which can be thousands of nanometers in size, nanoemulsion delivery systems with particle sizes in the hundred nanometer range or less have been shown to increase the bioavailability and efficacy of a number of compounds such as anti-inflammatory agents,[19,20] insulin,[21−23] tetanus toxoid,[24] and Dicumarol.[14] Supportive data for increased efficacy of nanoemulsion delivery substances has also recently been reported by our laboratory, which demonstrated that nanoemulsions produced by the high sheer forces during microfluidization and containing an antioxidant synergy formulation (ASF) dramatically reduced tumor size in a neuroblastoma-bearing xenograft mouse model compared to a suspension formulation of ASF.

Almost all particulate or macroscopic materials in contact with a liquid acquire an electronic charge on their surfaces. The ζ-potential is an important and useful indicator of this charge which can be used to predict and control the stability of colloidal suspensions or emulsions, for example.[25] The ζ-potential variations of our compound with surfactant addition is important because it controls the direction and magnitude of electro-osmotic permeability.[26] The greater the ζ-potential, the more likely the suspension is to be stable because the charged particles repel one another and thus overcome the natural tendency to aggregate with nanoparticles mediated by a negative ζ-potential affecting the spherical shape, narrow size distribution, and good monodispersivity of the target molecule.[27,28] Consequently, the presence or absence of charged groups on the surface of macroscopic materials such as a nanoemulsion, as revealed

(19) Kumar, R.; et al. Supramolecular Assemblies Based on Copolymers of PEG600 and Functionalized Aromatic Diesters for Drug Delivery Applications. *J. Am. Chem. Soc.* **2004**, *126* (34), 10640–10644.

(20) Cavalli, R.; et al. Solid lipid nanoparticles as carriers of hydrocortisone and progesterone complexes with beta-cyclodextrins. *Int. J. Pharm.* **1999**, *182* (1), 59–69.

(21) Attivi, D.; et al. Formulation of insulin-loaded polymeric nanoparticles using response surface methodology. *Drug Dev. Ind. Pharm.* **2005**, *31* (2), 179–189.

(22) Merisko-Liversidge, E.; McGurk, S. L.; Liversidge, G. G. Insulin nanoparticles: a novel formulation approach for poorly water soluble Zn-insulin. *Pharm. Res.* **2004**, *21* (9), 1545–1553.

(23) Pan, Y.; et al. Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres. *Acta Pharmacol. Sin.* **2002**, *23* (11), 1051–1056.

(24) Vila, A.; et al. PLA-PEG particles as nasal protein carriers: the influence of the particle size. *Int. J. Pharm.* **2005**, *292* (1−2), 43–52.

(25) Singh, B. P.; et al. Stability of dispersions of colloidal alumina particles in aqueous suspensions. *J. Colloid Interface Sci.* **2005**, *291* (1), 181–186.

(26) Kaya, A.; Yukselen, Y. Zeta potential of soils with surfactants and its relevance to electrokinetic remediation. *J. Hazard. Mater.* **2005**, *120* (1−3), 119–126.

(27) Peng, J.; et al. Influence of anions on the formation and properties of chitosan-DNA nanoparticles. *J. Nanosci. Nanotechnol.* **2005**, *5* (5), 713–717.

***Table 4.*** Statistical Analysis of the Overall Tumor Size, Tumor Size during the Treatment Period and after the 2 Month Period Describing the Mean, Plus or Minus Standard Deviation, Standard Errors Compared to the Control, and Standard Error of the Entire Treatment Group

| | N-DAC_IM | N-DAC_Top | DAC_IM | DAC_Top | control |
|---|---|---|---|---|---|
| | | overall final tumor size | | | |
| arithmetic mean | 2.1 | 3.3 | 4.6 | 7.1 | 7.6 |
| standard deviation | 1.3 | 0.4 | 0.6 | 1.4 | 1.9 |
| standard error/control | 0.7 | 0.3 | 0.6 | 0.4 | |
| standard error | 0.4 | 0.1 | 0.2 | 0.4 | 0.5 |
| *t*-test/control 1/1 | $2.22 \times 10^{-5}$ | $6.68 \times 10^{-6}$ | 0.000186 | 0.012097 | |
| | | tumor size after 40 days | | | |
| arithmetic mean | 2.7 | 3.5 | 4.8 | 6.6 | 6.9 |
| standard deviation | 0.9 | 0.2 | 0.5 | 1.1 | 1.6 |
| standard error/control | 0.6 | 0.2 | 0.6 | 0.4 | |
| standard error | 0.3 | 0.1 | 0.2 | 0.4 | 0.5 |
| | | tumor size after no treatments | | | |
| arithmetic mean | 0.2 | 2.7 | 3.9 | 8.5 | 9.9 |
| standard deviation | 0.2 | 0.1 | 0.4 | 0.9 | 1.2 |
| standard error/control | 0.08 | 0.12 | 0.03 | 0.01 | |
| standard error | 0.1 | 0.1 | 0.2 | 0.5 | 0.7 |

by their ζ-potentials, can directly affect their performance and processing characteristics.[29] Increasing the electrical force in the solution allows the fluid to dissolve and hold more material. In this way, more drugs or nutrients can be carried throughout the body and accumulated deposits of waste can be removed. ζ-Potential values can be related to the drug concentration at the lipid/aqueous media interface.[30] The reduced ζ-potential and greater cationic charge of our nanoemulsion preparations of DAC might contribute to its increased efficacy.[31,32] With the influence of the ζ-potential relative to suspensions of DAC (−1.180), the nanoemulsion preparation demonstrated a lower ζ-potential (−5.490) (decreased negative charge = less anionic and more cationic) (Table 1).

It is well-established that conventional administration vehicles may also result in chemotherapeutic agents being toxic to the patient, causing more pain and discomfort than the disease itself. Therefore, there is a practical upper limit to the amount of anticancer agent that a patient can receive. However, when a chemotherapeutic agent is delivered by a vehicle that has enhanced cellular permeability and can be locally administered to ensure uniform biodistribution, the dosage of any single drug may be lowered.[33] This is especially beneficial to the patient, since using lower levels of chemotherapeutic agents are generally safer and are associated with fewer adverse side effects for the patient. The reports that nanoemulsions are advantageous because they can deliver a wide range of dosages could address this issue and would support this notion.[34] Additionally, when anticancer agents are delivered under conditions of enhanced membrane permeability, cancer cells are less likely to generate resistance because a greater percentage of the cancer cells will be killed upon initial exposure. DAC may be activated by hepatic metabolism and spontaneous photoactivation,[35] meaning that light activation of DAC before subcutaneous penetration may have improved the effectiveness of the topical application of the drug.

In summary, we for the first time investigated the effects of newly reformulated DAC, an intravenous and intraperitoneal injected drug, by detailing its intramuscular injection, topical application, and respective effects on human melanoma xenograft tumors in nude mice after reduction of the

(28) Grosse, C.; Pedrosa, S.; Shilov, V. N. On the influence of size, zeta potential, and state of motion of dispersed particles on the conductivity of a colloidal suspension. *J. Colloid Interface Sci.* **2002**, *251* (2), 304–310.

(29) Mahato, R. I. Water insoluble and soluble lipids for gene delivery. *Adv. Drug Delivery Rev.* **2005**, *57* (5), 699–712.

(30) Matos, C.; et al. Zeta-Potential Measurements As a Tool to Quantify the Effect of Charged Drugs on the Surface Potential of Egg Phosphatidylcholine Liposomes. *Langmuir* **2004**, *20* (2), 369–377.

(31) Lockman, P. R.; et al. Nanoparticle surface charges alter blood-brain barrier integrity and permeability. *J. Drug Targeting* **2004**, *12* (9−10), 635–641.

(32) Lockman, P. R.; et al. Nanoparticle technology for drug delivery across the blood-brain barrier. *Drug Dev. Ind. Pharm.* **2002**, *28* (1), 1–13.

(33) Tagne, J. B.; et al. A Nanoemulsion Formulation of Tamoxifen Increases Its Efficacy in a Breast Cancer Cell Line. *Mol. Pharmaceutics* **2008**, *5* (2), 280–286.

(34) Lee, J. Drug nano- and microparticles processed into solid dosage forms: physical properties. *J. Pharm. Sci.* **2003**, *92* (10), 2057–2068.

(35) Reid, J. M.; et al. Metabolic activation of dacarbazine by human cytochromes P450: the role of CYP1A1, CYP1A2, and CYP2E1. *Clin. Cancer Res.* **1999**, *5* (8), 2192–2197.

particle size using the experimental design and procedures described above. We have demonstrated that a uniform nanoemulsion of Dacarbazine comprising a population of particles having maximum and minimum diameters dramatically increases its efficacy. We have also demonstrated that, 12 weeks after cessation of drug administration, only the nanoemulsion preparations continued to prevent the reoccurrence of the tumor.

In conclusion, our nanoemulsion delivery systems for drugs may provide more efficacious treatment of certain cancers and with fewer adverse side effects given our novel data indicating that DAC can be switched from its conventional IV injection to IM and/or topical application. We have also shown that delivering the said nanoemulsion to nude mice under such conditions penetrates the cell membrane and that those nanoemulsions are released intracellularly (unpublished data) due to the fact that the formulation approach allows better penetration of the skin membrane after the photoactivation of DAC, making the topical application a suitable route for delivery. This information may also be useful in guiding clinical use of reformulated therapy in different human cancers because our newly formulated nanoemulsion of DAC shows great potential in reducing tumor growth either by intramuscular injection or by transdermal topical application.

**Acknowledgment.** We would like to acknowledge the support of the Microfluidics Corp. in Newton, MA.

MP8000556