Formulation and Antitumor Activity Evaluation of Nanocrystalline Suspensions of Poorly Soluble Anticancer Drugs

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Purpose. Determine if wet milling technology could be used to formulate water insoluble antitumor agents as stabilized nanocrystalline drug suspensions that retain biological effectiveness following intravenous injection.

Methods. The versatility of the approach is demonstrated by evaluation of four poorly water soluble chemotherapeutic agents that exhibit diverse chemistries and mechanisms of action. The compounds selected were: piposulfan (alkylating agent), etoposide (topoisomerase II inhibitor), camptothecin (topoisomerase I inhibitor) and paclitaxel (antimitotic agent). The agents were wet milled as a 2% w/v solids suspension containing 1% w/v surfactant stabilizer using a low energy ball mill. The size , physical stability and efficacy of the nanocrystalline suspensions were evaluated.

Results. The data show the feasibility of formulating poorly water soluble anticancer agents as physically stable aqueous nanocrystalline suspensions. The suspensions are physically stable and efficacious following intravenous injection.

Conclusions. Wet milling technology is a feasible approach for formulating poorly water soluble chemotherapeutic agents that may offer a number of advantages over a more classical approach.

KEY WORDS: anticancer agents; poorly water soluble agents; nanoparticles; etoposide; camptothecin; piposulfan and paclitaxel.

INTRODUCTION

Frequently in drug discovery poorly water soluble agents are identified as actives using *in vitro* assays but are discarded because they are unable to be formulated for further evaluation *in vivo*. Thus many promising agents are discarded due to poor water solubility and the lack of a generally applicable formulation approach to address this problem. Though this predicament is a likely scenario in any therapeutic/diagnostic discovery program it has been a recurring issue in cancer research. The development of a number of promising anticancer agents have been delayed or abandoned due to issues resulting from the poor water solubility of the drug (1).

Currently, the poorly water soluble chemotherapeutic agents used in the clinic and that are in various phases of development are formulated by conventional methods. Routinely, these agents are formulated using a co-solvent plus other excipients which act to solubilize the drug and provide for stability of the formulation during storage and on injection (2). Commonly, the use of co-solvents produce toxic side effects e.g., anaphylaxis, pain at the site of injection, emboli formation and paradoxically precipitation which results in poor intravenous bioavailability. For instance, etoposide (VP-16), a semisynthetic analog of podophyllotoxin, is a sparingly water soluble drug that is used to treat small-cell lung cancer and various other neoplasms (3,4). Etoposide being a poorly water soluble drug is formulated using benzyl alcohol, polyoxyethylated sorbitan ester (Tween 80) and polyethyleneglycol (PEG 300). Prior to injection the formulation is diluted then slowly infused. Even though etoposide has been used in the clinic for a number of years, formulation related toxicity issues still occur (5–7).

Extensive literature is available on other technologies concerned with delivery issues of hard to formulate chemotherapeutic agents. Although significant progress has been made in each of these areas including liposomes (8,9), emulsions (10,11) and polymeric carriers (12,13), there is a need for additional methodologies that provide a safe, effective and economical means for intravenous administration of poorly water soluble therapeutics and/or diagnostics.

In this study, a delivery system well suited for sparingly water soluble chemotherapeutic agents is described wherein the compounds are formulated as dispersible particles consisting essentially of a crystalline drug substance stabilized with a surface modifier. The methods of preparation and characterization of nanocrystalline dosage forms suitable for intravenous administration of poorly water soluble chemotherapeutic agents are presented together with preclinical efficacy data performed in the mammary 16C murine tumor model.

MATERIALS AND METHODS

Chemicals

With the exception of piposulfan which was custom synthesized at Kodak Research Laboratories, Rochester, NY., agents were obtained from the following vendors: etoposide (Sigma Chemical Co, St. Louis, MO), camptothecin (Aldrich, Milwaukee, WI) and paclitaxel (Biolyse Corporation, Port-Daniel, Que.). Pluronic and Tetronic surfactant stabilizers were obtained from BASF (Parsnippany, NJ.) and the polyoxyethylated sorbitan esters were purchased from ICI, Wilmington, DE.

Nanoparticle Formulations

Nanoparticle formulations were prepared under aseptic conditions as a drug suspension (2% wt/v) containing 1% (wt/v) surfactant stabilizer (14,15). An aqueous suspension containing drug and stabilizer was wet milled using preconditioned zirconium oxide media (Zircoa Inc., Solon, OH.) For screening, milling was performed in a 28 ml bottle using a media bead volume of 7.5 ml and 3.75 ml of the drug/surfactant slurry. The slurry was milled on a low energy mill (U.S. Stoneware, East Palestine, OH) at 57% of the critical speed. The critical speed is defined as the rotational speed of the grinding vessel when centrifuging of the grinding media occurs. Milling efficiency was dependent on a number of factors including drug substance and choice of stabilizer. The particle size of the slurry was assayed daily during milling. Routinely, the drug/surfactant slurry was milled to a final size of less than 400 nm based on

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photon correlation spectroscopy (PCS) and generally this could be achieved in a 4 day period. Milled nanosuspensions were evaluated for chemical stability, physical stability, and physical stability in plasma. Formulations with acceptable physical stability in plasma were submitted for efficacy studies. Acceptable physical stability was defined as absence of agglomeration or negligible particle size growth in the presence of plasma when the nanosuspension was incubated with plasma for 60 min at 37°C.

The stabilizers used for each of the formulations were: 1) polyoxyethylene sorbitan monooleate (Tween 80)/sorbitan monooleate (Span 80) for piposulfan; 2) Pluronic F108 for camptothecin, and 3) Pluronic F127 for etoposide and paclitaxel (Table 1).

Particle Size Analysis

Particle size analysis was determined using photon correlation spectroscopy (PCS). Prior to sizing, samples were diluted with freshly filtered deionized water. Sizing measurements were routinely performed using the Coulter Model N4MD Submicron Particle Analyzer (Coulter, Miami, Fl.) or the Malvern Zetamaster (Malvern Instruments Ltd., Worcester, England) using unimodal analysis of intensity distributions for mean particle size determination. PCS results were confirmed using scanning electron microscopy (SEM). For SEM, samples were diluted and an aliquot of the diluted preparation was dried, sputter coated and visualized using the Topco SM510 (Topcon Technologies, Inc., Pleasanton, Ca.).

Plasma Stability Assays

Stability of nanoparticle suspensions in plasma was monitored by photon correlation spectroscopy (PCS) and light microscopy. Rat plasma was delipidated prior to use by centrifugation at 150,000 × g for 1hr. The supernatant was carefully decanted and filtered through a 0.1 micron filter. The plasma was then pretested in the Coulter N4MD submicron particle analyzer to ensure that the sample was particle-free. Nanosuspensions of piposulfan, camptothecin, etoposide and paclitaxel were diluted 1:2, 1:10, and 1:100 with lipid-free plasma. Samples were vortexed and incubated at 37°C. For PCS analysis, samples were diluted with water and assayed. Presence or absence of aggregation was also monitored using the Leica DMRB optical microscope with a 100 × phase-contrast objective.

Animal Studies

Studies were performed in accordance with Wayne State University Medical School's animal use and care administrative policies. As previously described (16), tumor fragments (~30 mg) were seeded subcutaneously by trocar into 7–10 week-old female C3H mice (National Institute of Health, Bethesda, Md.). Chemotherapy of either a nanoparticle suspension or a control formulation was initiated within 6 days of tumor implantation. For chemotherapy, animals were injected via the tail vein using a multiple injection regimen. Dose and dosing schedule were selected based on the toxicity of the compound with the intention of administering the drug at its maximum tolerated dose (MTD).

To assess antitumor effectiveness, median tumor burden of treated animals was compared to median tumor burden of untreated controls. Results are expressed as a percentage determined by comparing the average weight of tumors in treated animals (expressed as "T") to the average weight of tumors in untreated controls (expressed as "C"). This value is expressed as percent T/C. A T/C = 0% is indicative of a highly active agent while a T/C >42% is considered inactive (16).

RESULTS

Particle Size Reduction

Figure 1 shows the particle size reduction profile achieved during ball milling. Generally, within the first 24 h a significant reduction in particle size is observed. Depending on the drug core and stabilizer utilized, additional milling rendered finer particle dispersions. For this study, nanosuspensions were milled for 4 days and particle size analysis was performed every 12 hrs. After four days of milling nanosuspensions of piposulfan and etoposide with a mean diameter ~200 nm were harvested. For camptothecin and paclitaxel, milling was continued for an additional 3 to 4 days to further reduce particle size. As shown in Table 1, routinely nanosuspensions were prepared with a mean particle size that averaged 200–250 nm for all four agents studied.

Characterization of Nanoparticle Drug Suspensions

In Figure 2, the particle size distribution for nano-piposulfan, nano-etoposide, nano-camptothecin and nano-paclitaxel is shown. Intensity distributions were obtained using photon correlation spectroscopy (PCS). Different drug cores with an optimal stabilizer produce a relatively uniform dispersion. The gross uniformity or homogeneity of the dispersions was verified using scanning electron microscopy (SEM). In Figure 3, the scanning electron micrographs show representative images of the nanoparticle drug suspensions. The milled nanosuspensions for each agent were homogeneous. However, the morphology of the

Table 1. Average Particle Size: Nano-Suspensions[a]

Compound (solubility)[b]	Formulation	Average Size in nm. (number of batches)
Piposulfan (125μg/ml)	2% Piposulfan, 0.33% Tween 80, 0.67% Span 80	210.2 ± 38.9 (8)
Camptothecin (50μg/ml)	2% Camptothecin, 1% F108	202.3 ± 30.5 (6)
Etoposide (200μg/ml)	2% Etoposide, 1% F127	256.2 ± 53.0 (12)
Paclitaxel (<10 μg/ml)	2% Paclitaxel, 1% F127	279.2 ± 29.60 (7)

[a] Chemotherapeutic agents were roller milled as a 2% wt/v suspension using surfactant satbilizers at a core to surfactant ratio of 2:1. Samples were sized using photon correlation spectroscopy (PCS). The average size was determined using the said number of batches.
[b] Aqueous solubility @ 25°C.

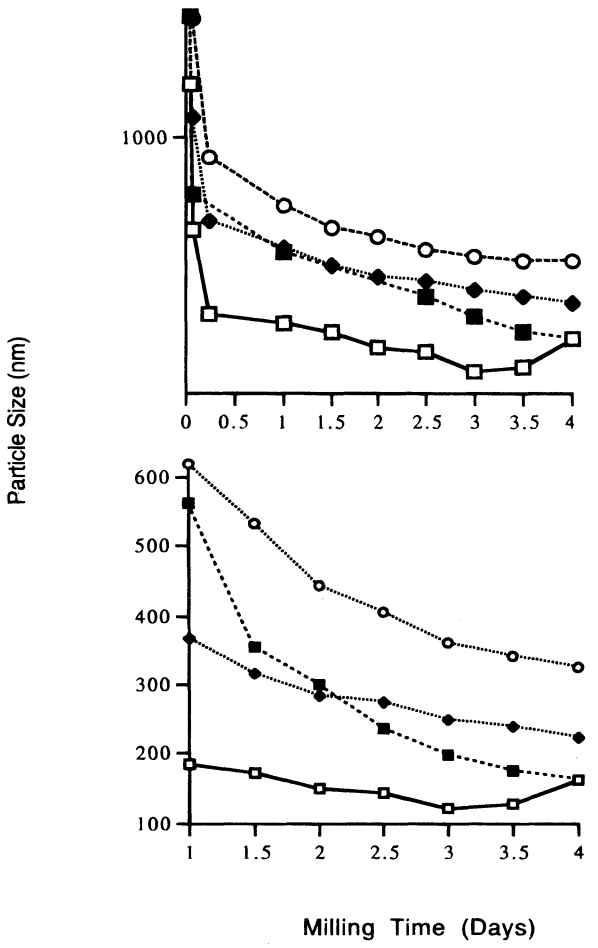

Fig. 1. Effectiveness of particle size reduction using wet milling technology to formulate water insoluble chemotherapeutic agents. Compounds (piposulfan—□; camptothecin—◆; etoposide—■; and paclitaxel—○) were roller milled using the surfactant stabilizers described in Table 1. During milling, particle size was monitored every 12 hrs. For particle size analysis samples were diluted with deionized water, vortexed and sized using photon correlation spectroscopy (PCS). Data show weight average diameters of particles in suspension.

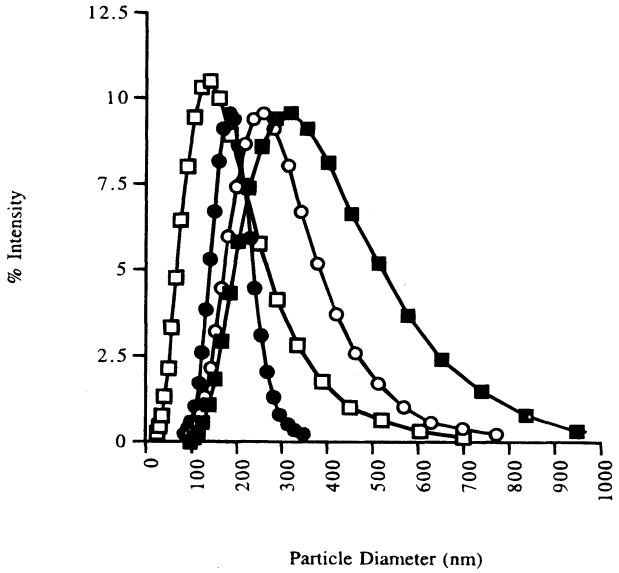

Fig. 2. Size distribution profiles of nanoparticle suspensions were obtained using the Malvern Submicron Particle Sizer. Samples were diluted with deionized water, vortexed for ~20 sec and sized. Size distribution profiles and cumulant z averages are shown for: ● nanopiposulfan (z Ave = 299.9); ○ nanocamptothecin (z Ave = 227.8; □ nanoetoposide (z Ave = 155.4); and ■ nanopaclitaxel (z Ave = 291.3).

various nanosuspensions differed from the cuboidal appearance of nanocamptothecin (Fig. 3B) to the rather elongated rod-like structures of nano-paclitaxel (Fig. 3D). Also, as monitored by both PCS and SEM, heterogeneity with respects to particle size and shape appears to be primarily governed by interactive properties of both the drug core and the surfactant stabilizer used during milling. When optimally stabilized, the suspensions did not aggregate and remained physically stable for at least four weeks post preparation (Fig. 4).

Since the intended use of these nanosuspensions is for intravenous administration, physical stability in the presence of plasma was monitored using particle size analysis and optical microscopy. Nanosuspensions were diluted with plasma and incubated at 37°C . As shown in Figure 5, for camptothecin and paclitaxel no significant change could be detected in the mean particle size of the preparations throughout a 60 min incubation period. For piposulfan and etoposide, particle growth was observed but there was no evidence of particle aggregation and/or agglomeration based on both PCS sizing and optical microscopy. Physical stability of the nanocrystalline suspensions was also not affected by time of incubation and dilution. Samples diluted 1:100 with plasma and incubated at 37°C for 24 hrs remained physically stable.

Efficacy of Nanoparticle Drug Suspensions

To evaluate efficacy, the nanocrystalline drug suspensions were tested in mice previously injected with mammary adenocarcinoma (16/C). The nanosuspensions were compared with the conventional formulation of the same agent or an agent of similar mechanism of action. Results of these studies are illustrated in Table 2. For all nanoparticle suspensions evaluated, tumor regression expressed as the percentage of tumor weight in treated animals to tumor weight in untreated controls was significant. In addition, the suspensions were suitable for intravenous bolus injection as suggested without increased incidence of acute toxicity in comparison to controls. For certain drugs, such as piposulfan, only the nanoparticle formulation was tolerated as an i.v. injectable. Control formulations were extremely toxic unless administered subcutaneously.

DISCUSSION

A technology is described for formulating water insoluble anticancer agents as nanoparticle drug suspensions that are stable. The method is versatile and suitable for many agents whose solubility is less than 200 ug/ml. The potential value of this approach was demonstrated using two well-known drugs, etoposide and paclitaxel, which are in the clinic but have reportedly been associated with formulation-related safety issues (6–8,17–19). The other agents investigated, piposulfan and camptothecin, are older drug candidates that could not be successfully formulated as an injectable. In the case of piposulfan

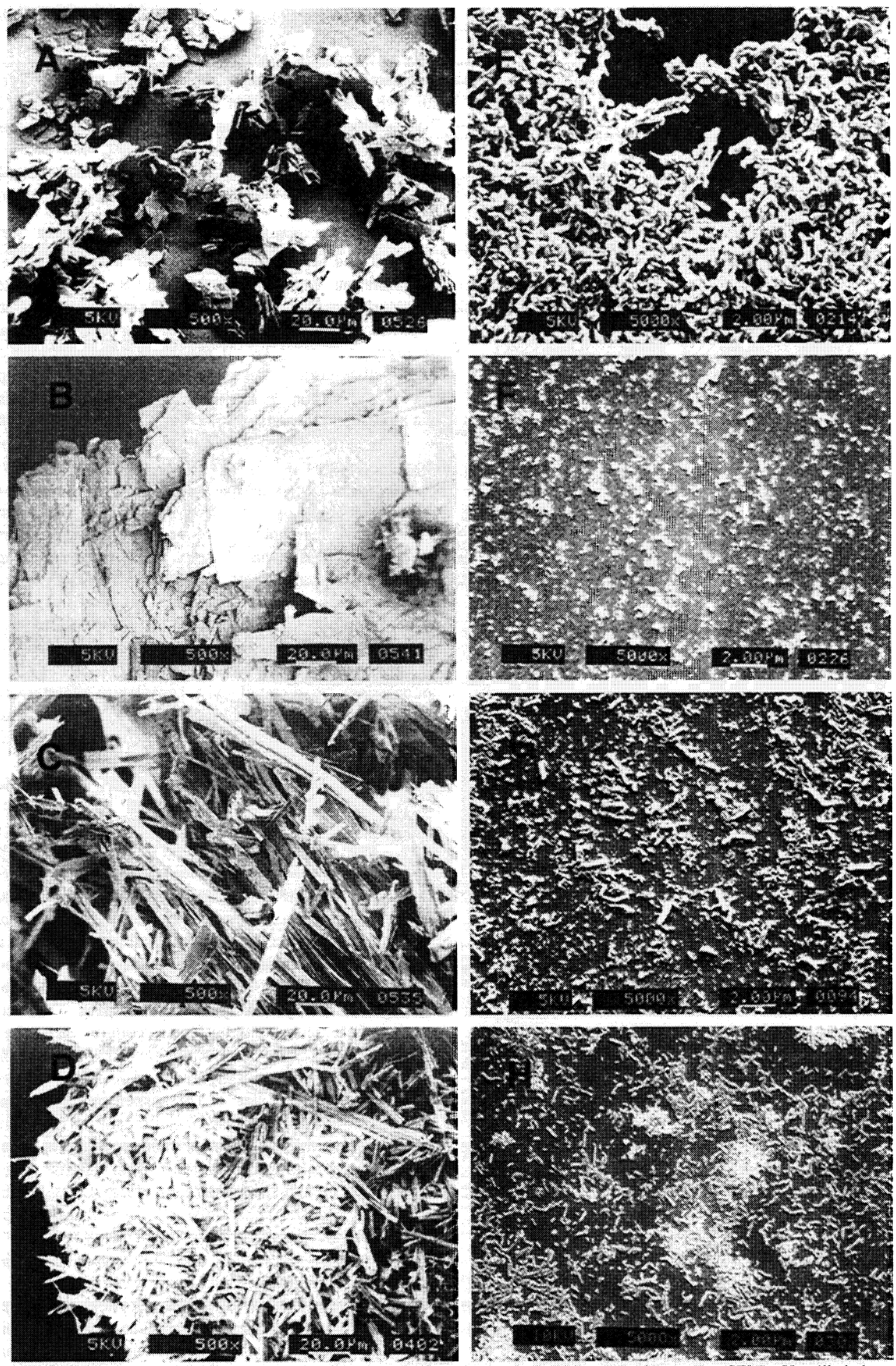

Fig. 3. Nanocrystalline drug suspensions were analyzed using scanning electron microscopy (SEM). Unmilled drug substance was visualized at 500 × magnification and is shown on the left side of the figure as: A) piposulfan; B) camptothecin; C) etoposide; and D) paclitaxel. The corresponding nanocrystalline suspensions (5,000 ×) are shown on the right side of the figure labeled as: E) nanopiposulfan; F) nanocamptothecin; G) nanoetoposide; and H) nanopaclitaxel.

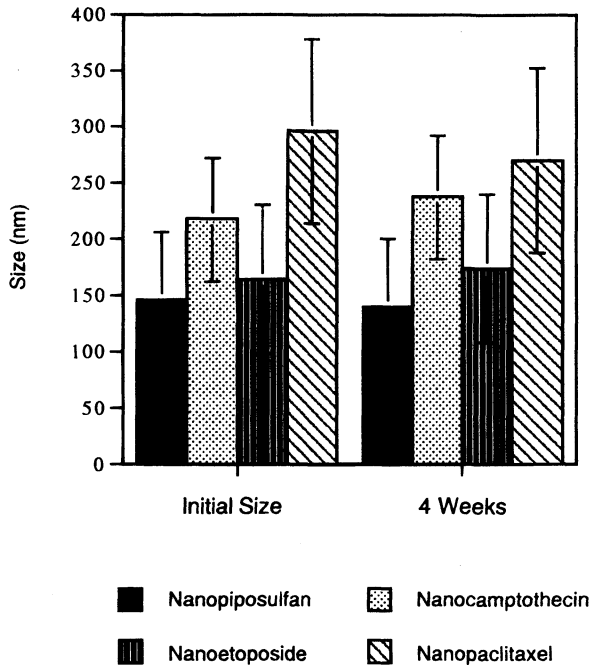

Fig. 4. Physical stability of nanocrystal suspensions were studied at ambient temperature for a four week period. The antitumor agents were roller milled as described in Table 1 and Figure 1. The suspensions were then stored at room temperature for four weeks and re-sized using PCS. The graph compares mean particle size of the preparations immediately after milling with the mean particle size of the suspension following storage at room temperature for one month.

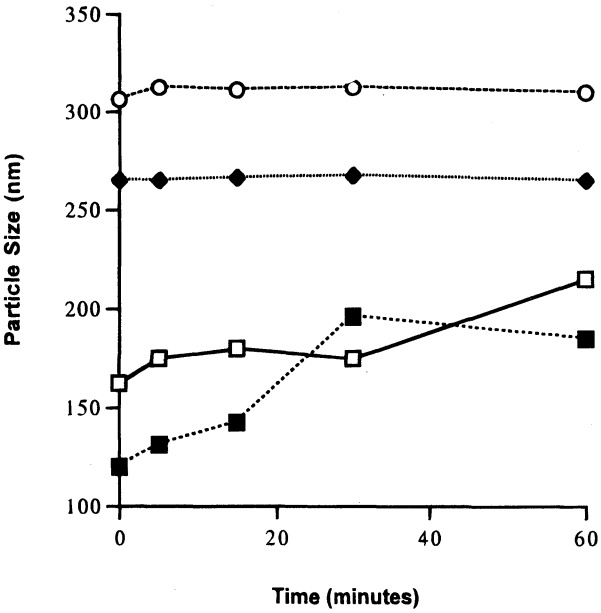

Fig. 5. The physical stability of nanocrystal drug suspensions was studied in the presence of plasma. Nanopiposulfan—□, nanocamptothecin—◆, nanoetoposide—■, and nanopaclitaxel—○, were diluted 1:2 with plasma and incubated for 60 min at 37°C. For particle size analysis samples were diluted with deionized water and sized using PCS. PCS size analysis was confirmed using optical microscopy.

drug development was abandoned, whereas, solubility issues associated with the camptothecins are being aggressively pursued via the identification of water soluble analogs (20–23). The solubility of the anticancer agents chosen for this study range from ~200 ug/ml to less than 4.0 ug/ml (24,25) and as demonstrated in this study can be readily formulated as an aqueous suspension of fine particles using a low energy wet milling process.

The nanoparticle suspensions of piposulfan, camptothecin, etoposide and paclitaxel that are described were generated using a conventional ball mill. As the data show, in the presence of the selected surfactant stabilizer(s) the procedure was effective in producing nanoparticles of pure drug substance. Generally, the higher molecular weight polymeric stabilizers were optimal for effective particle size reduction, shelf stability, and prevention of agglomeration in the presence of blood proteins. For instance, stable suspensions ~250 nm in diameter were obtained for camptothecin, etoposide and paclitaxel using the pluronic block co-polymers F108 and F127. The higher molecular weight Pluronics have been shown to be excellent stabilizers for various colloidal delivery systems (26). In addition, these surfactant coatings have been known to reduce opsonization of particulate drug carriers and enhance delivery of the desired agent to various anatomical targets which would be advantageous for passive delivery to solid tumors (27,28). The pharmacokinetic properties of these nanoparticle drug suspensions are being studied. However, since the technology described in this study is relatively new and the biodistributional properties of colloidal nanocarriers are dictated by a complexity of interactions, it would be surprising if the blood clearance of these nanosuspensions is not rapid. Currently, methodology is being developed so that properties of the nanosuspensions, e.g. size, surface characteristics and shape can be modulated to optimize delivery.

Though the effects of the comminution process on the physical state of the drug was not performed, previous studies using X-ray diffraction have shown that the dispersion process did not change the crystal structure of the compound (14). In this study the electron micrographs of the milled dispersions suggest that the process generates nanocrystalline drug particles. However, comparisons between pre and post processed materials remains to be studied.

For certain drugs such as piposulfan, generating a stable nanocrystalline suspension proved challenging. After screening a series of surfactants and surfactant combinations, the use of a Tween 80 and Span 80 mixture provided optimal physical stabilization. As shown in Figure 1 and 2, using this surfactant combination, particle size of the suspension was reduced to 250 nm in ~4 day period. For piposulfan the surfactant mixture adequately wets the drug substance and provides steric stabilization. However, as judged from the plasma stability data shown in Figure 5, though the preparation does not agglomerate in plasma, the particles apparently interact with plasma proteins resulting in an overall increase in mean particle size of the preparation. This interaction does not appear to compromise the safety and efficacy of the suspension (Table 2).

As shown in Table 2, the efficacy of oncologic agents when formulated as nanoparticles was satisfactory. For etoposide and paclitaxel, novel nanoparticle suspensions were compared to currently used clinical formulations. Etoposide is formulated using polyethylene glycol, Tween 80 and benzyl alcohol while paclitaxel is dissolved in a mixture of Cremophor EL and ethanol. To avoid acute toxicity, both formulations must be

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