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Pharmacokinetic comparison of intravenous carbendazim and remote loaded carbendazim liposomes in nude mice

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Abstract

Carbendazim is a novel anticancer agent. The aim of this study was to prepare and characterize a remote loaded liposome preparation of carbendazim, and compare its pharmacokinetic profile to that of unencapsulated carbendazim. Carbendazim was encapsulated in liposomes composed of sphingomyelin–cholesterol (3:1, w/w) by remote loading in response to a transmembrane pH gradient (pH 0.5 in/pH 4.0 out), which resulted in encapsulation of more than 95% of the available drug in preformed vesicles. High drug/lipid ratios were prepared which correspond to a molar ratio of up to 0.8. Physical isolation of the free drug and dialysis were used to determine the in vitro release of carbendazim from liposomes. The release was independent of the initial drug/lipid ratio and choice of internal buffer composition. Liposomal carbendazim (200 mg kg^{-1}) was intravenously administered to athymic nude mice and the serum levels of free carbendazim were determined by HPLC analysis after a methanol-induced protein precipitation. Administration of liposomal carbendazim to mice resulted in significant alterations in the pharmacokinetics. Serum levels of free carbendazim were approximately 10-fold greater than those achieved for the same dose of unencapsulated drug. Liposomal carbendazim showed both high C_{max}, AUC and low clearance rate. Liposomal carbendazim and unencapsulated carbendazim displayed a similar terminal half-life (43–48 min). The relatively large volume of distribution of carbendazim suggests that the compound may partially enter cells or be bound to some extravascular structures. The results indicate that the liposomal formulation of carbendazim significantly increases its blood concentrations.

Keywords: Carbendazim; Liposomes; Remote loading; Pharmacokinetics

1. Introduction

Carbendazim, classified as a representative benzimidazolic compound, was initially found to be a bioactive metabolite of the fungicide benomyl, and is widely used in crop protection as a fungi-

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cide to protect crops from decay caused by various fungal pathogens [1]. It is also used as a pesticide and herbicide for the protection of flowers and flower bulbs. Recently it has been found that subchronic administration of carbendazim induced testicular alterations, spermatogenic inactivity and embryotoxicity [2–4]. The molecular basis of reproductive toxicity appears to be related to the inhibition of microtubule assembly [5–7]. While the exact mechanism is unclear, recent findings further revealed that carbendazim induced apoptosis in a variety of cells. The apoptosis phenomena of the compound led us to our most recent findings that carbendazim inhibits both in vitro tumor cell growth and in vivo human tumor xenograft models [8]. Consequently, this agent is presently undergoing Phase 1 clinical trials in adults with advanced malignancies. However, the drug has limited aqueous solubility and low bioavailability due to the first-pass effect of liver metabolism. It has been reported that more than 95% of carbendazim was eliminated during the early phase of i.v. administration [9]. Even though carbendazim continues to be important to achieve an effective chemotherapy for the treatment of a variety of tumor models, its formulation has not been completely satisfactory. The purpose of this study was to develop a liposome formulation that has the following characteristics: (a) a high level potency of carbendazim; (b) a high encapsulation efficiency; and (c) small particle diameter (less than 0.2 μm) that can be filter-sterilized. This report describes an approach to encapsulate carbendazim into unilamellar vesicles based upon a remote loading procedure. The influence of liposome encapsulation on the pharmacokinetic properties was evaluated in a murine model in order to provide inference as to the anticipated pharmacokinetics of the formulation in humans.

2. Materials and methods

2.1. Materials

Carbendazim (F.W. 191.18) and the internal standard 2-benzimidazolyl-acetonitrile (Fig. 1) were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol and KH_2PO_4 were purchased from EM Science (Gibbstown, NJ, USA). *N*,*N*-dimethylformamide solution was bought from Allied Signal (Muskegon, MI, USA).

Stock solution of carbendazim (1 mg ml^{-1}) was prepared in *N*,*N*-dimethylformamide. Stock solution of internal standard (2 mg ml^{-1}) was prepared in acetonitrile (HPLC grade). The stock solutions were stored at −4 °C. Standard solutions of carbendazim were obtained from stock solution by diluting the solution to 1, 10 and 100 μg ml^{-1}. They were used to spike the mouse serum to prepare standard serum samples of carbendazim prior to extraction. The standard solution volume added to the serum was always less than or equal to 7% of total volume of the samples, so that the integrity of the serum was maintained.

2.2. Extraction procedure

In a 1.5-ml Eppendorf centrifuge tube, to 98 μl of carbendazim-containing mouse serum spiked with 2 μl internal standard solution (200 μg ml^{-1}), 200 μl of absolute methanol was added and serum proteins were denatured during 30 s of vortex mixing. Then all of the vials were centrifuged at 14,000 × *g* for 15 min. A 240-μl aliquot of the supernatant was transferred to an Eppendorf tube and evaporated at 45 °C to dryness. A 60-μl aliquot of mobile phase was added to the dry serum extraction. The reconstituted extraction was thoroughly mixed, and then centrifuged again at 14,000 × *g* for 15 min. A 50-μl aliquot of the supernatant was injected to a chromatographic column.

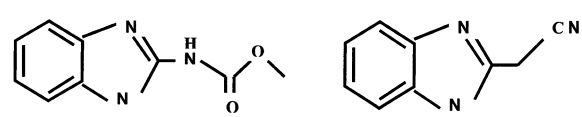

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Fig. 1. The structures of carbendazim (I) and 2-benzimidazolyl-acetonitrile (II, internal standard).

2.3. Stability and recovery

For short-term stability studies, control serum samples were spiked with 2 μg ml^{-1} of carbendazim. The samples were incubated at 37 °C and assessed at 0.5, 1, 2, 4 and 8 h by HPLC to determine the residual concentrations of carbendazim at the intervals to ensure that carbendazim did not undergo biomatrix-catalyzed degradation.

To determine the recovery of carbendazim from mouse serum, peak areas ratio of carbendazim spiked to drug-free mouse serum at final concentrations of 50, 500 and 5000 ng ml^{-1} were first determined by HPLC analysis. These peak areas were then compared to the peak areas of carbendazim at the same concentrations prepared in the supernatant of serum–methanol mixture (1:2, v/v). Each sample (50 μl) was directly injected onto the analytical column in triplicate.

2.4. Chromatographic conditions

Samples were analyzed on reversed-phase HPLC (Model HP 1100, Agilent Technologies, CA) consisted of a HP1100 autosampler. The Luna 3 μm C18 analytical column (100 × 4.6 mm I.D., Phenomenex, Torrance, CA) was maintained at 25 °C in a column block heater, and protected with a C18 octadecyl guard cartridge (4 × 3.0 mm I.D., Phenomenex). A mobile phase was composed of 20 mM phosphate buffer and acetonitrile (9:1, v/v). The mobile phase was pumped onto the column at 0.5 ml min^{-1} for 20 min. The volume injected was 50 μl. The analytes were detected at 280 nm.

2.5. Liposome formulation of carbendazim by remote loading

The approach taken to formulating carbendazim in liposomes described by Madden et al. [10] with modifications outlined here. Liposomes composed of sphingomyelin and cholesterol (3:1, w/w) were prepared by dissolving the lipid in absolute ethanol, diluting in five volumes H_2SO_4 (1.0 M) with stirring, and extruding the vesicles three times through two stacked 80 nm polycarbonate filters to generate unilamellar vesicles (110–150 nm). Carbendazim was dissolved in H_2SO_4 (0.3–1.0 M) at a concentration of 2–5 mg ml^{-1}, and mixed with the preformed liposomes at the indicated drug/lipid ratio. The preparation was diluted with 1.3-volumes of 250 mM sodium acetate and the external pH of the vesicles raised to 4.0 by the addition of NaOH (0.5 M). The generation of a transmembrane pH gradient led to the net flux of carbendazim into the vesicles, such that more than 95% of the available drug was encapsulated. Ultrafiltration of the vesicles was subsequently carried out to remove free unencapsulated material and residual solvents, and concentrate the formulation to a final drug concentration of 20 mg ml^{-1}. The formulation was then sterilized by passage through 0.22 μm filters.

Drug encapsulation efficiency was determined by passing an aliquot of the vesicles over a Microcon separation filter (MW cut-off at 30,000). Liposomes and their encapsulated contents were retained in the upper chamber, while the unencapsulated free carbendazim passed through the filters. The encapsulation efficiency was calculated by dividing the amount of carbendazim retained in the liposome fraction by the amount of total carbendazim. Control experiments demonstrated that there was no precipitated form of carbendazim presented in the formulation. The vesicle size was determined by Quasi Elastic Light Scattering using a Nicomp Particle Sizer Model 270. Analysis of carbendazim formulation was carried out by UV spectroscopy at λ_{max} 285 nm, following dilution of the samples in 70% ethanol.

2.6. Dosing and sample collection

Carbendazim and its liposomal forms were intravenously injected to athymic nude mice (200 mg kg^{-1}, $n = 3$) via the tail vein. Blood samples (0.5–0.8 ml) were withdrawn from different mice at 0.5, 5, 10, 30, 60, 180 and 360 min post dosing. The serum was obtained by centrifugation at 4000 × g for 15 min, and then mixed with twofold excess of methanol for deproteinization as described above.

2.7. Data analysis

The ratio of the peak area of carbendazim to that of internal standard was used as the assay parameter. Peak-area ratios were plotted against theoretical concentrations. Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data. Mouse serum concentrations of carbendazim were calculated from the standard calibration curves. Pharmacokinetic parameters were calculated using the computer program WinNonlin (Pharsight Co., Mountain View, CA). The serum concentration-time data of carbendazim and its formulations were fitted to a noncompartmental model based on the goodness-of-fit criteria set by the lowest standard errors of the fitted parameters, and the lowest value of Akaike Information Criterion, when comparing several models for a given set of data [11]. The WinNonlin used the trapezoidal rule to calculate the area under the serum concentration–time curves (AUC) up to the last measured serum concentration.

3. Results

3.1. Liposome characterization

The formulation appears opaque yellow liquid. The vesicle size determined by light scattering was 148 ± 57 nm. The level of carbendazim encapsulation was related to the interior/external pH gradient. For example, at a fixed drug/lipid molar ratio of 0.8, more than 96% of carbendazim is encapsulated using 300 mM H_2SO_4 (pH 0.5), whereas only 84% encapsulation was achieved at 50 mM H_2SO_4 (internal pH 1.33). The external pH was raised to 4.0 to achieve an initial transmembrane pH gradient about 3.5 units in order to accumulate the drug to high concentrations. The neutral form of carbendazim could diffuse across the membrane and subsequently become protonated in the acidic interior.

The drug content of the liposomal was governed by the initial drug/lipid ratio and the final lipid concentration. For example, at a drug/lipid molar ratio of 0.8 and lipid content 94 mg ml^{-1}, the drug content was 19.5 mg ml^{-1}, whereas, at a drug/lipid molar ratio of 0.2 and lipid content 143 mg ml^{-1}, the drug content reached 8.7 mg ml^{-1}. High drug content produced high bioavailability of carbendazim when it was administered to mice at the same dose (Fig. 2). However, changes in the drug/lipid molar ratio from 0.2 to 0.8 did not affect encapsulation efficiency (96–100%) and vesicle size.

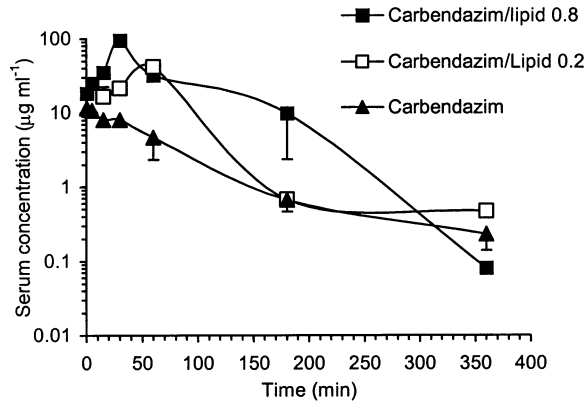

Fig. 2. Serum concentration—time profiles of intravenous carbendazim and its liposomal forms with a drug/lipid molar ratio at 0.8 and 0.2, respectively. Each point represents the mean ± SD of three nude mice.

3.2. Release characteristics of carbendazim liposomes

To determine the importance of the residual pH gradient in retaining the drug by the vesicles, we compared the release of carbendazim by dialysis in the presence and absence of ammonium acetate, which disrupts the transmembrane pH gradient. Carbendazim was loaded into vesicles formulated in 300 mM H_2SO_4 at a drug/lipid molar ratio of 0.2 and diluted to a final drug concentration of 0.13 mg ml^{-1}. The formulation was subsequently dialyzed against 145 mM NaCl, 10 mM HEPES in the presence and absence of 10 mM ammonium acetate for 24 h. Samples were taken at 0, 2, 4, 6 and 24 h during the course of dialysis for carbendazim analysis.

The release of carbendazim from liposomes at room temperature is biphasic. In the absence of ammonium acetate, approximately 40% of the

drug was released during the first 1–2 h of dialysis and subsequent release appeared slower. The extent of release observed during the first 1–2 h was related to the extent of dilution. Less than 10% of the drug remained encapsulated in liposomes after 24 h of dialysis. Carbendazim release was accelerated in the presence of ammonium acetate, indicating that drug retention is influenced by the presence of a residual transmembrane pH gradient. Control experiments demonstrated that loss of drug could not be attributed to changes in lipid concentrations. Taken together, these results indicate that the release of the drug is determined by the residual pH gradient and by the free drug concentration. There appears to be a correlation between the release observed in vitro and that observed following in vivo administration (Fig. 2).

3.3. Validation of assay performance

Observed retention times were 10.5 and 12.5 min for carbendazim and internal standard, respectively. A representative chromatogram is shown in Fig. 3A–C. No interfering peaks at the retention time of carbendazim or the internal standard were in a serum blank sample detected (Fig. 3A). In serum the peak-area ratio of carbendazim and internal standard varied linearly with concentrations ranging from 20 ng ml^{-1} to 20 μg ml^{-1}. The correlation coefficients for calibration curves of carbendazim and its liposomal form were equal to or better than 0.99. The limit of quantitation was 20 ng ml^{-1}. At this level, the mean concentration found was 19.18 ± 1.59 ng ml^{-1} (C.V., 8.3%; accuracy, 95.9%). The limit of detection was 10 ng ml^{-1}. Intra- and inter-day variabilities at concentrations of calibration standards were given in Table 1. The mean recovery of carbendazim averaged more than 90%. The result indicates a negligible effect of serum protein on the methanol extraction of carbendazim. Moreover, stability analysis revealed no significant degradation of carbendazim when incubated with serum for 8 h. We did not observe any metabolic peaks derived from the parent compound. The residual concentration of carbendazim after 8 h incubation remains almost the same as the initiation concentration: the peak area corresponding to carbendazim only decreased about 5% after 8 h incubation.

3.4. Pharmacokinetic analyses

The serum concentration versus time profile of various formulations of carbendazim are illustrated in Fig. 2. The results are a reflection of carbendazim released from liposomes at the drug/lipid molar ratio of 0.2 and 0.8. The pharmacokinetic parameters of carbendazim and its different formulations are shown in Table 2. In general, the two liposomal forms of carbendazim showed a comparable pharmacokinetic profile: both exhibited an initial raise followed by a decline in serum concentrations of carbendazim, indicating that it

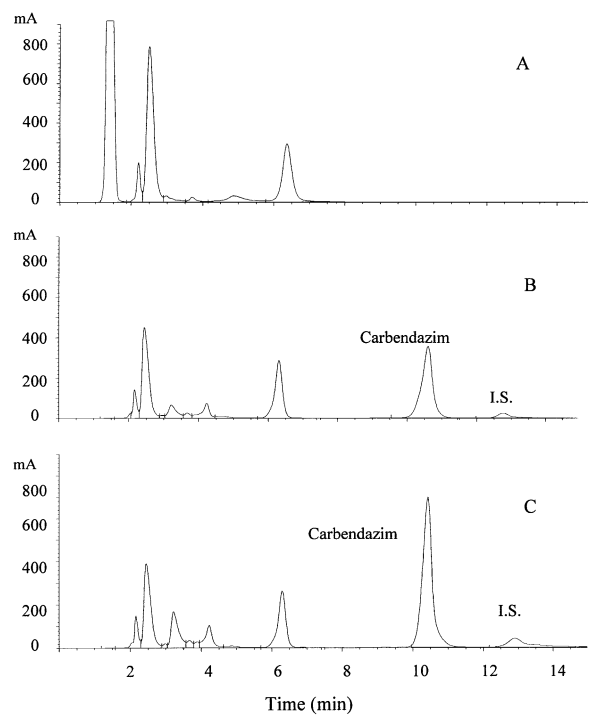

Fig. 3. Representative HPLC chromatograms of free carbendazim in mouse serum at 1 h after intravenous injection: (A) the chromatogram shows no interfering peaks at the retention times of carbendazim and I.S.; the chromatogram shows free carbendazim extracted from mouse serum after administration of non-formulated carbendazim (B) and liposome-formulated carbendazim at a drug/lipid molar ratio of 0.8 (C). The retention times at 10.5 and 12.5 min correspond to carbendazim and the internal standard, respectively.

Table 1
Inter- and intra-assay reproducibilities of the HPLC analysis of carbendazim in mouse serum

Theoretical concentration (ng ml^{-1})	Experimental concentration (Mean ± S.D.)	CV (%)
Intra-day reproducibility ($n = 3$)		
20	19.2 ± 1.59	8.28
50	61.0 ± 3.10	5.08
200	188.2 ± 6.06	3.22
500	508.3 ± 10.57	2.08
2000	2056.6 ± 11.39	0.55
Inter-day reproducibility ($n = 5$)		
200	202.4 ± 22.91	11.32
500	497.8 ± 22.73	4.56
2000	2035.0 ± 59.78	2.94

took the liposomes 30–60 min to reach a maximum release of carbendazim into the circulation system. Carbendazim serum concentration decreased at a rate of first order after the free carbendazim reached its maximum blood concentration. By comparison, unencapsulated carbendazim only showed a slow but constant elimination rate. Liposome formulation did not change the elimination half-life of carbendazim, which is generally considered as an inherent quality of a drug. The encapsulated carbendazim showed the C_{max} and AUC significantly higher than those of unencapsulated one. The more the drug/lipid molar ratio of the formulation, the higher the C_{max} and AUC were. The systemic clearance and the volume of distribution of encapsulated carbendazim were about 10-fold less than those of unencapsulated carbendazim. The high C_{max} and AUC of encapsulated carbendazim could be attributed to the low clearance rate of carbendazim-containing liposomes.

4. Discussion

The developed HPLC method proves to be useful and reliable for the determination of serum concentrations of carbendazim. The sample clean-up procedure, involving a direct deproteinization with methanol, is simple and rapid, thus avoiding degradation of the drug. This method, validated for carbendazim concentrations in serum ranging from 20 to 20,000 ng ml^{-1}, has a good reproducibility and accuracy and low limits of quantitation and detection compared to the most published methods detecting the compound in biomatrices [12–14]. Using this method, we demonstrated good in vitro stability of carbendazim when incubated with serum, in which enzymatic catalysis is most likely to happen, thus the concern that the primary metabolism occurs in the serum is greatly reduced.

Carbendazim is a small molecule with poor solubility in aqueous and organic solvents. The solubility of carbendazim is strongly influenced by protonation of the molecule, and high solubility

Table 2
Pharmacokinetic parameters of carbendazim and its liposomal forms (Lot number 5989001 and 5983501)

Parameters	Carbendazim	Liposomal carbendazim (drug/lipid, 0.2, mol mol^{-1})	Liposomal carbendazim (drug/lipid, 0.8, mol mol^{-1})
$t_{1/2}$ (min)	47	48	43
T_{max} (min)	Instant	60	30
C_{max} (μg ml^{-1})	11.4	28.1	95.8
AUC (μg ml^{-1} min)	781	2696	6914
Vz (ml)	560	74	35
Clearance (ml min^{-1})	8.2	0.96	0.56
MRT (min)	68	74	71

$t_{1/2}$, terminal half-life; T_{max}, time to maximum concentration; C_{max}, maximum serum concentration; AUC, area under the curve; Vz, volume of distribution based on the terminal phase; MRT, mean residence time.

(25 mg ml^{-1}) of the molecule can only be achieved at pH 0.5. The level of carbendazim encapsulation is related to the magnitude of the pH gradient, such that more than 95% of the drug was encapsulated for a pH gradient of 3.5 units (pH 0.5 in/pH 4.0 out). The transmembrane pH gradient can induce a corresponding concentration gradient of 1000-fold for a drug. This principle has been exploited to efficiently accumulate a wide variety of lipophilic drugs to high concentrations within liposomes [10]. In the present study, carbendazim content in the liposomes could be achieved at 20 mg ml^{-1}. Accumulation of the drug was proportional to the buffering capacity of the vesicle interior. The final drug content was also affected by the drug/lipid ratio and by the final lipid concentration.

It has been shown that ion gradients can be used to encapsulate protonated lipophilic drugs into liposomes to retain drugs. This approach, termed 'remote loading', allows drug encapsulation independent of lipid composition and vesicle size. However, this classical remote loading only suitable for molecules with a pK_a of >8.0. Carbendazim has an acidic pK_a 4.5 and low solubility at neutral pH in both aqueous and organic solvents. This precludes the use of classical remote loading approaches for formulation. In this study, we modified the remote loading approach using a liposome formulation composed of sphingomyelin–cholesterol (3:1, w/w) to reach a drug/lipid molar ratio of 0.8. This approach favorably increased the maximum drug content for carbendazim.

The prolonged blood-time course of carbendazim after its formulation may be exclusively attributed to a change in its pharmacokinetic profiles such as a slow rate of systemic clearance. The elimination rate of many small compounds and peptide drugs is rather fast [15,16]. Consequently, frequent dosing or continuous infusion is necessary to maintain efficacious blood levels of the drugs. Several approaches have been applied to decrease the elimination clearance of candidate drugs. One approach is lipid formulation, which has been used for parenteral administration of lipophilic drugs as potential drug delivery systems to either reduce the side effect, or to increase the efficacy of drugs. Sphingomyelin/cholesterol liposomes retain drugs more effectively than other phospholipid systems because they have lower permeability to solutes [17]. These formulations have been shown to have a prolonged circulation in blood and enhanced tissue distribution. It has been reported that the intravenous ^{14}C-carbendazim in rat blood showed a short period of elimination (0.25 h), within which more than 95% of the radiolabelled carbendazim was eliminated [9]. In the present study, we demonstrated that carbendazim, after encapsulated in sphingomyelin/cholesterol liposomes, acquired a clearance rate 10-fold slower than that of unencapsulated carbendazim without a significant change in its elimination half-life, an inherent quality of a compound. Therefore, the measured AUC of the free carbendazim released from the liposomes was proportionally increased, resulting in a significant improvement of the drug's blood concentrations. Besides, the relatively small liposomes (size 148 nm) containing carbendazim may be able to enter the lymphatic capillaries, and release carbendazim in the lymphatic circulation to facilitate apoptosis of tumor cells and prolong the residence time of the drug at the foci.

Of greater interest found in the present study is the large volume of distribution of carbendazim itself (Table 2). The magnitude of the volume of distribution is a useful indicator for the amount of drug outside the central compartment or in the peripheral tissues and organs. The larger the volume of distribution, the greater the amount of the drug in the extravascular compartment. We have observed that carbendazim possesses a high permeability across the lipid bilayer membrane. This characteristic facilitates the drug diffusing into the extravascular compartment, by either entering cells or being bound to some extravascular structure, and hence results in a large volume of distribution. Indeed, it has been reported that 90 min after oral administration of ^{14}C-carbendazim to rats the highest concentrations of radioactivity were found in the cytosol of hepatic fractions, and lowest in microsomes [9]. Interestingly, the liposome formulation decreases the drug's volume of distribution, indicating a prolonged residence of formulated carbendazim in the central compartment.

5. Conclusions

A unilamellar liposome formulation (sphingomyelin–cholesterol, 3:1, w/w) of carbendazim could be prepared by remote loading at drug/lipid molar ratios of 0.2 and 0.8. An initial transmembrane pH gradient (pH 0.5 in/pH 4.0 out) across the vesicles increased the encapsulation efficiency. A reversed-phase HPLC method was developed and validated to detect serum carbendazim concentration as low as 20 ng ml^{-1}. Liposomal carbendazim significantly improved the pharmacokinetic profile of the drug administered to athymic nude mice. These data may contribute to the design of the best possible formulation of this apoptosis agent.

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