Pharmaceutical and Biomedical Differences between Micellar Doxorubicin (NK911) and Liposomal Doxorubicin (Doxil)

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The stability and biological behavior of an *in vitro* system of doxorubicin (DXR) entrapped in NK911, polymer micelles, was examined and compared with those of DXR entrapped in Doxil, polyethylene-glycol-conjugated liposomes. The fluorescence of DXR inside micelles or liposomes in an aqueous solution is known to be strongly quenched by the outer shells of the micellar or liposo-mal formation. Thus, by measuring the fluorescence intensity of DXR released from NK911 or Doxil, we could determine the stability of the micellar or liposomal DXR formation. Furthermore, NK911 was found to be less stable than Doxil in saline solution. In drug distribution experiments using an *in vitro* solid tumor model, when spheroids formed from two human colonic cancer lines, HT-29 and WiDr, and a human stomach cancer line, MKN28, were exposed to NK911, DXR was distributed throughout the spheroids, including their center. On the other hand, when the sphe-roids were exposed to Doxil, DXR was distributed only to the surface of the spheroids. It has been suggested that Doxil can deliver DXR to a solid tumor more efficiently than NK911 via the EPR (enhanced permeability and retention) effect, because Doxil may be more stable in plasma than NK911. On the other hand, DXR packed in NK911 may be distributed by diffusion to cancer cells distant from the tumor vessel, because NK911 can leak out of the tumor vessel and may be able to release free DXR more easily than Doxil. It has been suggested that drug carrier systems such as liposomes and micelles should be selected appropriately bearing in mind the characteristics of the tumor vasculature and the tumor interstitium.

Key words: NK911 — Drug delivery system — Polymer micelles — Doxil — Doxorubicin

It has been determined from pathological, pharmacolog-ical and biochemical studies, that in general, solid tumors possess the following pathophysiological characteristics: hypervascularity, irregular vascular architecture, potential for secretion of vascular permeability factors, and absence of effective lymphatic drainage that prevents efficient clearance of macromolecules accumulated in the solid tumor tissues.[1–6] It has been suggested that these charac-teristics, which are unique to solid tumors, constitute the basis of the enhanced permeability and retention (EPR) effect.[1, 2] Moreover, macromolecules as well as small par-ticles have relatively prolonged plasma half-lives because they are too large to pass through normal vessel walls unless they are trapped by the reticuloendothelial system (RES). However, they can extravasate into and accumulate within tumor tissues through the EPR effect. On the other hand, conventional low-molecular-weight anticancer agents are usually eliminated before they reach the tumor tissue to exert their cytotoxic effects.[1, 2]

To use the EPR effect to advantage, several techniques have been developed to modify the structure of drugs and to construct carriers. Liposomes with a polyethylene gly-col (PEG) coating have proved to be very successful as a drug carrier system. PEG is electrically neutral and is not recognized by the RES in the liver or spleen; this forms the basis of the so-called "stealth effect."[7–9] Liposomal drugs exhibit reduced clearance and prolonged plasma half-lives due to the stealth effect.[10] Doxil is long-circulat-ing pegylated liposomes containing doxorubicin (DXR), and has received the Food and Drug Administration (FDA)'s approval for use in the treatment of Kaposi's sarcoma[11, 12] or ovarian cancer,[13] because its clinical bene-fits were clearly shown in recent clinical trials, including a phase-III randomized trial. Biodistribution studies using in DTPA (diethylenetriaminepentaacetic acid)-labeled pegylated liposomes have demonstrated selective tumor accumulation of the constituent drug in patients with advanced head and neck, lung or breast cancer.[14]

Polymeric micelles have also been utilized as a drug carrier system. Initially, Yokoyama *et al.* succeeded in constructing a micelle-forming polymeric drug, PEG-

poly(aspartic acid) block copolymer, conjugated with DXR.[15] PEG constituted the outer shell of the micelle, conferring the stealth property on the drug preparation. The poly(aspartic acid) chain is a hydrophobic chain and the poly(aspartic acid)-DXR conjugates form the hydrophobic core of the micelles in aqueous media. This original form of micellar DXR contained two entrapped components, DXR monomers and DXR dimers, in the inner core. It has been reported that it is the DXR monomers, and not DXR dimers, which play a major role in the antitumor activity of this drug preparation. DXR dimers, on the other hand, were considered to contribute to stabilization of the micellar DXR conformation.[16] However, it has been observed that freeze-dried samples of this micellar DXR become insoluble after prolonged storage, because of the presence of DXR dimers in this formulation.[17] To improve the solubility and stability of micellar DXR, therefore, a new type of polymeric micellar preparation, namely, NK911, containing DXR monomers alone, was generated.[17] NK911 has a small particle size, with a diameter of approximately 40 nm. After carrying out pre-clinical studies, we started a phase I clinical trial of NK911 to determine its usefulness in the treatment of various kinds of solid tumors.

Both liposomal and micellar DXR were found to have longer plasma half-life, to accumulate more efficiently in tumors due to the EPR effect and to show stronger antitumor activity in comparison with free DXR, in studies conducted in mice.[15–17] Previous studies, however, have shown that the AUC of NK911 in both plasma and the tumor is lower than that of Doxil. Therefore, in the present study, we examined the pharmaceutical and biomedical differences between NK911 and Doxil and explored the possible clinical advantages of NK911.

MATERIALS AND METHODS

Drugs The DXR-incorporating polymer micelles NK911 were procured from Nippon Kayaku Co., Ltd. (Tokyo). The structure and physical characteristics of this formulation have been described previously.[17] In brief, the micelle carrier NK911 consists of the block copolymer of PEG (MW 5000) and polyaspartic acid (about 30 units). The overall net charge of NK911 is neutral on the surface of the polymeric micellar vehicles due to the presence of PEG in the outer layer. The particle size (mean diameter 41.9 nm) and the narrow size distribution of NK911 remained unchanged after freeze-drying. The DXR-incorporating liposomes, Doxil, were purchased from ALZA Corp. (Mountain View, CA), and DXR hydrochloride ($C_{27}H_{29}NO_{11} \cdot HCl$) was purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals used were of reagent grade and were used as purchased.

Cell lines and multicellular aggregates (spheroids) The human colon cancer cell lines HT-29 and WiDr, and the human stomach cancer line MKN-28, were cultured in D-MEM (Dulbecco's modified Eagle's medium)/10% FBS (fetal bovine serum) containing 15 mg/liter gentamycin sulfate and 50 mg/liter ampicillin sodium, at 37°C in a humidified atmosphere with 5% CO_2. Each of the cell lines was seeded onto 0.5% agarose-coated 10-cm dishes containing the medium described above, and incubated under similar conditions. One week later, several sizes of spheroids were noted to have grown in the dishes. For the drug distribution experiments, 200- to 500-μm sized spheroids were used.

Stability of NK911 and Doxil in aqueous solution Free DXR, NK911 and Doxil were incubated in saline in disposable plastic cuvettes at a DXR dose-equivalent of 20, 2.0 and 0.2 μM, for 0, 0.5, 1, 3, 6, 24, 48 and 72 h at 37°C. After each incubation time, the fluorescence intensity of each solution was measured using a fluorescence spectrophotometer (F-2500, Hitachi, Tokyo) at an excitation wavelength of 480 nm and emission wavelength of 550 nm. Since the fluorescence of DXR inside micelles or liposomes is known to be strongly quenched by the outer shells of the micellar or liposomal formation, it is possible to determine the stability of the micellar or liposomal DXR formulation by measuring the fluorescence intensity of DXR released from it.

Distribution studies of free DXR, NK911, and Doxil in the spheroids Spheroids derived from HT-29, WiDr and MKN-28 were exposed to free DXR, NK911 and Doxil, at 37°C at a DXR dose-equivalent of 20, 2.0 or 0.2 μM, for 1 h or 24 h. After the drug exposure, the spheroids were washed twice with phosphate-buffered saline (PBS(−)) and divided into 3 groups. Spheroids incubated without any drug for each aforementioned incubation time were used as controls. The first group was embedded in O.C.T. Compound (TISSUE-TEK, Miles, Inc., Elkhart, IN) and frozen at −80°C until use. The frozen sections were examined under a fluorescence microscope (BX50, DP50, Olympus, Tokyo) at an excitation wavelength of 470 nm and emission wavelength of 560 nm, to evaluate the distribution of DXR in the spheroids.

The second group was mixed with 1 ml of 0.1 M ammonium chloride buffer (pH 9.0) and homogenized. Then, 1 ml of the homogenized sample was transferred to a silicone-coated glass tube (the remainder of the homogenized solution was used for protein assay), and 50 μl of 2 μg/ml daunorubicin was added as an internal standard. Then, 5 ml of CH_3Cl_3/CH_3OH (2/1,v/v) was added to the mixed solution, which was shaken vigorously in a vortex mixer for 1 min and centrifuged at 10 000 rpm, to separate the buffer from the organic material. The organic layer was collected and transferred to another tube for evaporation under nitrogen gas flow at 40°C in a water bath. After evaporation of the sample, the precipitate was dissolved in 100 μl of DMF (N,N-dimethylformamide). Then, the solu-

tion was filtered through "Ultrafree"-MC, a low-binding hydrophilic 0.4-μm PTFE membrane (Millipore Corp., Bedford, MA) and subjected to reverse-phase HPLC to detect DXR.

As samples for the standard curve, 0.5, 1.0, 5.0, 10, 50, 100, 500, 1000 μg of DXR was added to drug-free homogenized spheroid solution, and treated as described previously before being subjected to HPLC to obtain a standard curve. The reverse-phase HPLC was carried out using a Gulliver 1500 series HPLC system (JASCO Corp., Tokyo) equipped with a PU-1580 Intelligent Pump, at a flow rate of 1.0 ml/min at 40°C using a CAPCELL PAK C18 SG300 column, 4.6 mm ID×150 mm, 5 μm, Shiseido Fine Chemicals (Tokyo). The fluorescence intensity of DXR in the samples was detected using an FP-1520 (JASCO Corp.).

Two types of gradient conditions between solution A (25 mM sodium phosphate buffer, pH 4.0) and solution B (CH$_3$CN) were used. For free DXR samples and standard curve samples, solution B was graded from 22% to 30% from 0 to 10 min, 30% of solution B and 70% of solution A were maintained from 10 to 14 min, solution B was graded from 30% to 22% from 14 to 15 min, and finally, 22% of solution B and 78% of solution A were maintained from 15 to 23 min. For the samples of Doxil and NK911, solution B was graded from 22% to 30% from 0 to 10 min, 30% of solution B and 70% of solution A were maintained from 10 to 14 min, solution B was graded from 30% to 80% from 14 to 17 min, 80% of solution B and 20% of solution A were maintained from 17 to 22 min, solution B was graded from 80% to 22% from 22 to 23 min, and finally, 22% of solution B and 78% of solution A were maintained from 23 to 35 min. In the latter gradient conditions for liposomes and micelles, solution B was graded up to 80% in order to wash out the polymers from the column. Ten microliters of the homogenates (the remaining samples were used for HPLC) were put into 96-well plates with 100 μl of filtered dye, diluted Dye Reagent Concentrate (Bio-Rad Protein Assay Dye Reagent Concentrate with 4 volumes of deionized water); 500, 400, 300, 200, 100 and 50 μl/ml of bovine albumin were used for obtaining the standard curve. After a few minutes, the optical density was measured using a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) at a wavelength of 595 nm. The amount of DXR distributed in the spheroids was expressed as μg DXR per mg cell protein.

Colony formation assay for cells from spheroids treated with each drug The third group of spheroids was treated with 0.5 ml of 0.25% trypsin-0.02% EDTA/PBS(−) solution in 1.5 ml Eppendorf tubes. After incubation for a few minutes, single-cell suspensions were obtained by tapping the tube. After addition of 0.5 ml of medium, the cells in the cell suspensions were counted using a Coulter

counter (Z1, Coulter Corp., Miami, FL), and adjusted to 60 000 cells/ml by adding medium. One part of the 60 000 cells/ml cell suspension was mixed with 2 parts of medium containing 0.5% agarose to prepare 0.33% agarose gel containing 20 000 cells per ml. Then, 0.5 ml of the cell solution, containing 10 000 cells, was plated in triplicate as the upper layer in six-well plates coated with 0.55% agarose gel in the medium as the lower layer. The plates were incubated under the same incubation condi-
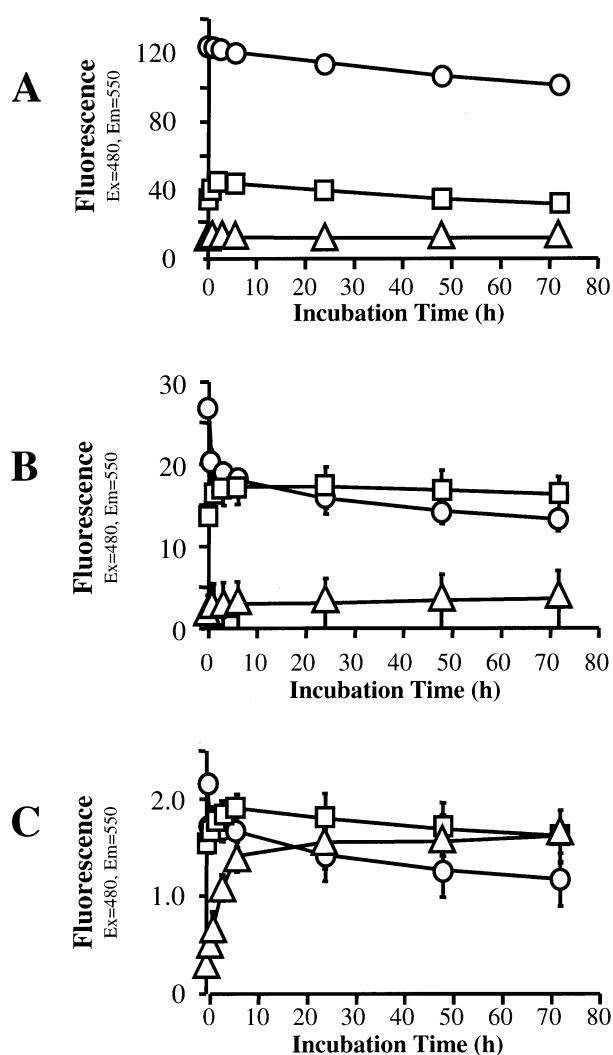

Fig. 1. Change in the fluorescence intensity of free DXR, NK911 and Doxil in PBS solution at 37°C. Free DXR (○), NK911 (□) and Doxil (△) were incubated in PBS at 37°C at a DXR dose-equivalent of 20 μM (A), 2 μM (B) and 0.2 μM (C). The fluorescence intensity of each solution was measured using a fluorescence spectrophotometer, at an excitation wavelength of 480 nm and emission wavelength of 550 nm, after 0.5, 1, 3, 6, 24, 48, and 72 h of incubation.

tions as mentioned above. One week later, colonies larger than 20–30 μm were counted in three microscopic fields. The colony formation ratio was calculated as the colony number in the test samples divided by that in the control samples×100 (%).

Statistical methods Data were compared using Fisher's PLSD test. *P* values of 0.05 or less were considered to denote statistical significance.

RESULTS

Stability of NK911 and Doxil in aqueous solution Neither NK911 nor Doxil at the DXR dose-equivalent of 20 μM released free DXR until at least 72 h in saline. At a DXR dose-equivalent of 2 μM, NK911 released almost all of the packed DXR by 3 h, while Doxil released only a very small amount of DXR. At the DXR dose-equivalent of 0.2 μM, both NK911 and Doxil released all of the DXR from each capsule by 3 h (Fig. 1).

Distribution studies of free DXR, NK911, and Doxil in the spheroids In the distribution experiments using spheroids made from HT29 cells, when the spheroids were exposed to free DXR, NK911 or Doxil for 1 h, red fluorescence originating from DXR was not clearly observed in any drug case (Fig. 2A). When the spheroids were exposed to free DXR or NK911 for 24 h, the DXR was clearly distributed throughout the spheroids, including

their center. On the other hand, when the spheroids were incubated with Doxil for 24 h, weak fluorescence of DXR was observed only on the surface of the spheroids (Fig. 2B). Similar results were obtained when spheroids derived from WiDr or MKN-28 were treated with each of the drug preparations (data not shown).

These microscopic observations were confirmed quantitatively by measuring the amount of DXR extracted by reverse-phase HPLC from each of the spheroid groups exposed to free DXR, NK911 and Doxil (Fig. 3). When the spheroids were exposed to free DXR, NK911 or Doxil for 1 h at a DXR dose-equivalent of 20 μM, the total DXR contents in the spheroids were determined to be 0.851±0.411 μg/mg protein, 0.413±0.123 μg/mg protein or 0.009±0.002 μg/mg protein, respectively. The DXR content in the spheroids following exposure to NK911 was 46-fold higher than that following exposure to Doxil (*P*=0.0242). In the case of exposure of the spheroids to free DXR, the DXR content in the spheroids was 2-fold higher and 95-fold higher than that following exposure to NK911 (*P*=0.0162) and Doxil (*P*=0.0002), respectively. The DXR contents were 0.045±0.007 μg/mg protein, 0.051±0.031 μg/mg protein and 0.002±0.001 μg/mg protein following exposure of the spheroids to free DXR, NK911 and Doxil for 1 h at the DXR dose-equivalent of 2 μM, respectively. Thus, in terms of the DXR content in the spheroids, there was no significant difference between the
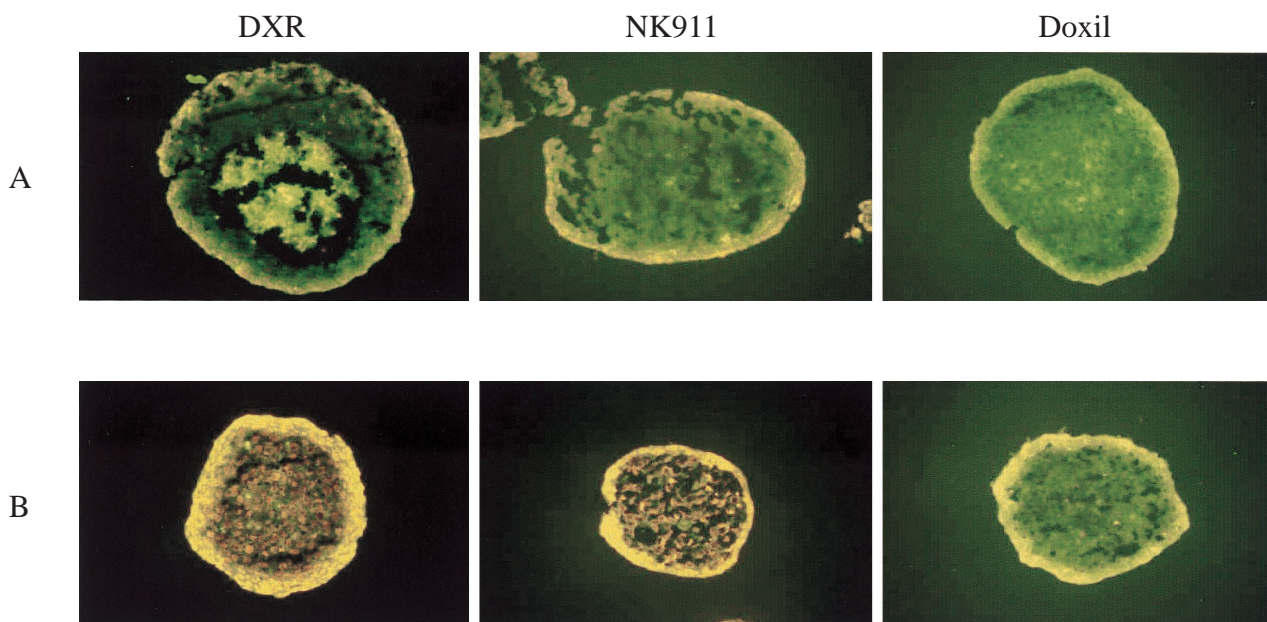

Fig. 2. DXR distribution in spheroids derived from HT-29 cells after exposure to free DXR, NK911 and Doxil. The spheroids were exposed to free DXR, NK911 or Doxil at a DXR dose-equivalent of 20 μM for 1 h (A) and 24 h (B) at 37°C. The frozen sections were examined using under a fluorescence microscope at the excitation wavelength of 470 nm and emission wavelength of 560 nm.
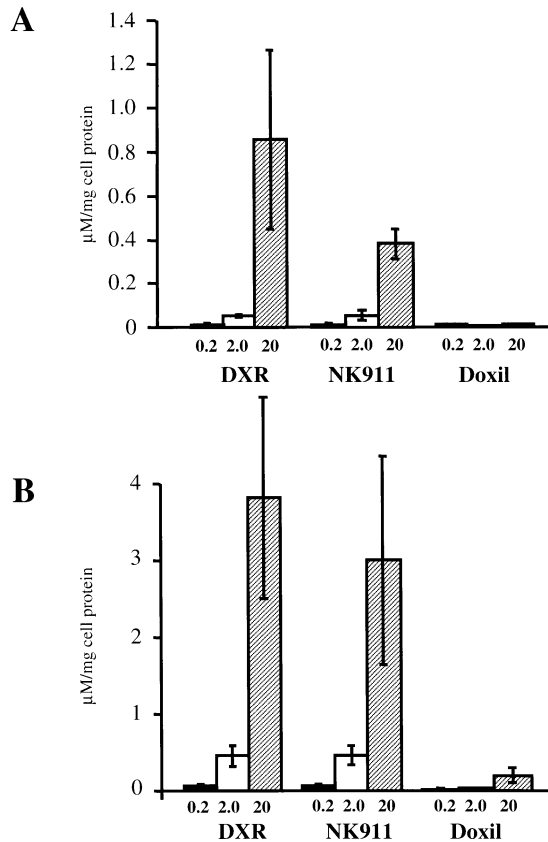
Fig. 3. Quantification of DXR distributed in spheroids derived from HT-29 after their exposure to free DXR, NK911 or Doxil. After their exposure to free DXR, NK911 or Doxil at the DXR dose-equivalent of 20 μM (☒), 2 μM (☐) and 0.2 μM (■) for 1 h (A) or 24 h (B), the spheroids were washed and homogenized. The homogenized solutions were subjected to the DXR extraction procedure and the extracts were subjected to reverse-phase HPLC to quantify the DXR content in the spheroids.
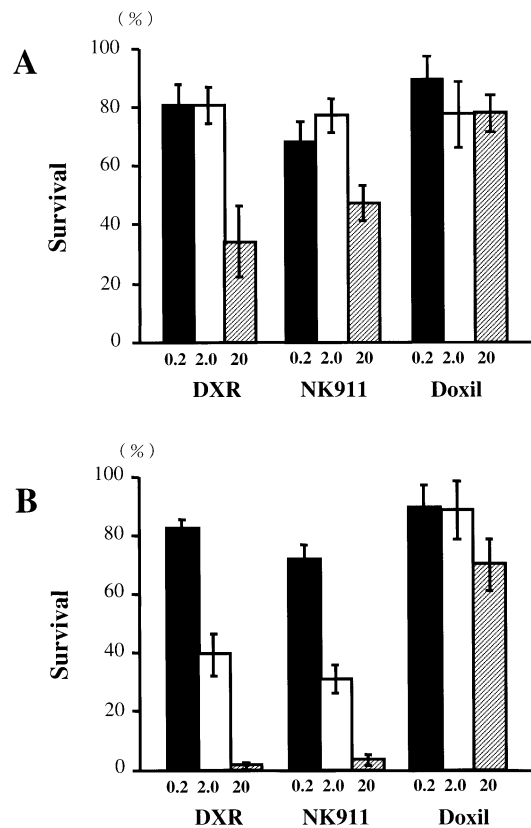

Fig. 4. Colony formation assay for cells derived from HT-29 spheroids after their exposure to free DXR, NK911 or Doxil. After exposure to free DXR, NK911 or Doxil at a DXR dose-equivalent of 20 μM (☒), 2 μM (☐) or 0.2 μM (■) for 1 h (A) or 24 h (B) the spheroids were washed and trypsinized to prepare single-cell suspensions. The cell suspensions were plated in triplicate onto soft agar. A week later, the colonies in one microscopic field were counted.

NK911 and free DXR treatment groups. However, the DXR content in the spheroids in the case of the Doxil treatment group was 23-fold and 26-fold lower than those in the free DXR and NK911 treatment groups, respectively ($P=0.0057$ for Doxil vs. DXR and $P=0.0053$ for Doxil vs. NK911). The DXR contents were 0.009±0.005 μg/mg protein and 0.010±0.002 μg/mg protein when the spheroids were exposed to free DXR and NK911 spheroids for 1 h at a DXR dose-equivalent of 0.2 μM, respectively. Almost no DXR was detected in the spheroids exposed to Doxil at this DXR dose-equivalent (Fig. 3A).

When the spheroids were treated with free DXR, NK911 and Doxil for 24 h at DXR dose-equivalents of 20 μM, 2 μM, 0.2 μM, similar results to the case of 1-h treatment were obtained. Namely, the DXR distribution was significantly higher in the case of free DXR or NK911

treatment than that in the case of Doxil treatment, but there was no significant difference between the free DXR and NK911 treatment groups at any dose of DXR (Fig. 3B). The major difference between the 1-h treatment and 24-h treatment groups was that the DXR content in the spheroids was higher following 1-h exposure to free DXR at 20 μM dose as compared to that following 1-h exposure to NK911 at a DXR dose-equivalent dose of 20 μM, but there was no significant difference between the two following exposure to a DXR dose-equivalent of 20 μM for 24 h.

Colony formation assay for cells from the spheroids treated with each drug Colony formation assay for cells from the spheroids of HT-29 was conducted to determine the cytotoxicity of free DXR, NK911 or Doxil against *in vitro* solid tumor model spheroids. As shown in Fig. 4, on

the whole, a higher cytotoxic effect was obtained in the group treated with free DXR or NK911 as compared with that treated with Doxil. The results were roughly consistent with those from the drug distribution experiments. When the spheroids were treated with each drug for 1 h, there was no significant difference among the 3 drug preparations for a DXR dose-equivalent of 0.2 μM or 2 μM. However, following 1-h exposure to a DXR dose-equivalent of 20 μM, free DXR showed the strongest cytotoxic effect, followed by NK911. In the case of 24-h exposure, while free DXR and NK911 were superior to Doxil, there was no significant difference between free DXR and NK911 treatment at any dose of DXR.

DISCUSSION

Several DDS drugs have been approved by the regulatory authorities for the treatment of cancer, including poly(styrene-maleic anhydride)-neocarzinostatin (SMANCS) in Lipiodol for the treatment of hepatoma[18] and PEG-L-asparaginase for the treatment of acute lymphocytic leukemia.[19] Regarding liposomal anthracycline formulations, extensive clinical trials of two liposomal preparations of DXR, Doxil and TLC-99,[20] and one preparation of daunorubicin, DaunoXome[21] have been conducted. Several studies have reported that liposomal preparations of anthracyclines are associated with attenuated toxicity, including reduced cardiac toxicity, while the efficacy of the parent anthracyclines is retained, or even enhanced.[20–22] Among such preparations, Doxil has already been approved for use in the treatment of Kaposi's sarcoma and ovarian cancers by the FDA in the USA. To expand the uses of liposomal preparations of anthracyclines, several clinical trials to determine their usefulness in the treatment of other cancers are currently in progress.

Ringsdorf et al. proposed that AB block copolymer-drug conjugates might form micellar structures that could improve the drug solubility.[23] Then, the utility of polymeric micelles in cancer chemotherapy was demonstrated for the first time with DXR-incorporating polymeric micelles in the early 1990's.[15] Since the size of these micellar structures was large enough to evade renal excretion and because of the covering of the outer shell of the micelle with PEG, preventing nonspecific capture by the RES, DXR-incorporating polymeric micelles have a long plasma half-life, which permits large amounts of DXR-incorporating micelles to reach the target sites and exert their EPR effect. These original DXR-incorporating polymeric micelles decreased the toxicity of DXR significantly in terms of body weight change and blood biochemical characteristics, and yet exhibited superior in vivo antitumor activity against several solid tumors in comparison with free DXR in experiments conducted in mice.[15] However, it was found that freeze-dried samples of the original

micellar DXR became water-insoluble after prolonged storage, because of the existence of the DXR dimers in the preparation. To overcome this problem, a new type of polymeric micelles was generated, containing only DXR monomer, and was found to dissolve in water easily even after prolonged storage in the freeze-dried condition. This new type of DXR-incorporating micelles, named NK911, had a shorter plasma half-life than the original micellar DXR, because NK911 was not stable in the bloodstream due to the lack of DXR dimers. Nakanishi et al. reported that the area under the concentration curve (AUC) of the DXR incorporated in NK911 in the plasma was 28.9-fold higher than that of free DXR, in experiments conducted using C-26-colon-carcinoma-bearing mice.[17] The AUC of the DXR in NK911 in the tumor of mice inoculated subcutaneously with C-26 cells was 3.4-fold higher than that of free DXR.[17] While the AUC in plasma of the DXR incorporated in Doxil was 237-fold higher than that of free DXR,[24] Vaage et al. reported that the relative values of the AUC in the tumor for free DXR and Doxil were 36.5 and 919, respectively, from experiments conducted using human prostate carcinoma (PC-3)-bearing nude mice. This represents a 25-fold increase in the concentration of the drug at the tumor site.[25] Although each study was conducted independently using different tumors and rodent models, the findings suggest that Doxil has a longer plasma half-life and higher AUC in the plasma than NK911; therefore, Doxil can accumulate in solid tumor tissue more efficiently than NK911, based on the EPR effect.

In the current study, at higher concentrations, such as a DXR dose-equivalent of 20 μM, neither NK911 nor Doxil released free DXR until at least 72 h in PBS solution. At a DXR dose-equivalent of 2 μM, NK911 released almost all the packed DXR within it by 3 h, while Doxil released only a small amount of DXR. These findings imply that NK911 is less stable than Doxil in aqueous solution. In addition, the present findings suggest that Doxil can deliver DXR to a solid tumor via the EPR effect better than NK911, probably because it is more stable in plasma than NK911.

Jain et al. extensively studied factors interfering with drug delivery to solid tumors. They reported that the convective passage of large drug molecules into the core of solid tumors could be impeded by abnormally high interstitial pressures in solid tumors. Therefore, small-molecular-weight anticancer agents (with a molecular weight lower than 2000 daltons) are superior for the treatment of solid tumors, because they can leave the tumor blood vessels and migrate into the core of the tumor by diffusion. However, low-molecular-weight anticancer agents can also be harmful to normal cells because they can leak out of normal blood vessels. Therefore, Jain suggested that one useful strategy for evading the barriers to drug dispersion

would be to inject patients with drug carriers, such as liposomes, filled with low-molecular-weight drugs. In this system, the liposomes should have time to exit from the leaky areas of blood vessels and reach reasonably high levels in the surrounding interstitium. The important thing in Jain's concept of a liposomal carrier system is that the liposomes should release the low-molecular-weight drugs packed within them gradually, so that the drug can be dispersed throughout the tumor.[26] Some interesting findings were reported by Unezaki et al.,[28] who succeeded in visualizing the extravasation of PEG-liposomes into a solid tumor using fluorescence-labeling. In a study using C-1300-bearing mice, they showed that while the fluorescence-labeled PEG-liposomes localized immediately around the tumor vessel wall after extravasation, they could not be detected in normal tissue. Thus, the PEG-liposomal system possesses the ability to deliver DXR more efficiently, but to obtain satisfactory antitumor effect, liposomes have to release free DXR at the site of extravasation.

In our current distribution study of NK911 using spheroids, when spheroids were exposed to free DXR or NK911, DXR was distributed throughout the spheroids, including their core. On the other hand, when the spheroids were exposed to Doxil, DXR was distributed only to the surface of the spheroids. These findings indicate that NK911, but not Doxil, can easily release DXR, which is then distributed throughout the spheroids by diffusion, because NK911 is not as stable as Doxil in the medium. These results were demonstrated quantitatively using HPLC, and visually by fluorescence microscopy for the spheroids in the experiment. In addition, it was confirmed by colony formation assay that NK911 exerts stronger cytotoxic effects than Doxil against spheroid models, because NK911 releases much more free DXR, and the DXR is distributed throughout the spheroids, as compared with the findings observed in the case of Doxil. Moreover, when the spheroids were exposed to each drug at a DXR dose-equivalent of 20 μM for 1 h, the best result was seen with free DXR treatment, followed by that with NK911 treatment, and the least significant result was noted with Doxil treatment, in terms of both DXR content in the spheroids and colony formation. When the spheroids were exposed to each drug at a DXR dose-equivalent of 20 μM for 24 h, the lowest DXR content in the spheroids and the lowest antitumor effect were obtained following Doxil treatment, and there was no significant difference between the results following free DXR and NK911 treatment. These results indicate that the micellar formation of NK911 was preserved during the first hours of exposure, although NK911 released DXR more gradually. However, after 24 h, the micellar formation of NK911 decayed, and the amount of DXR released was equivalent to that following free DXR exposure. Taking all these findings together, it is suggested that Doxil can deliver DXR to a solid tumor more efficiently than NK911 via the EPR effect, because it is more stable in the bloodstream and has a higher AUC in the plasma than NK911. However, DXR from NK911 is distributed more efficiently to cancer cells distant from the tumor vessel than DXR from Doxil once NK911 extravasates from the tumor vessel. Although there is no concrete evidence regarding how significant these differences between NK911 and Doxil might be clinically, NK911 may be more effective against cancers having a rough tumor vessel network, because of the presence of an abundant collagen-rich matrix. Such cancers include scirrhous stomach cancer, pancreatic cancer, inflammatory breast cancers, the so-called intractable cancers. Unfortunately, no experimental tumor models have been established as yet which can represent collagen-rich and intractable human tumors. Therefore, there is no way of determining the clinical significance of NK911 or Doxil other than conducting a clinical trial.

There are some advantages of the polymeric micellar system over the liposomal system. In the liposomal system, strongly hydrophobic drugs are preferentially retained in the lipid bilayer of liposomes, not in the inner aqueous area. The hydrophobic drugs incorporated in the lipid bilayer may destabilize the liposomal structure and so incorporation must be limited to a small amount of drug per liposome. In contrast to the liposomal system, the polymeric micelle system can incorporate hydrophobic drugs by utilizing hydrophobic interactions between the hydrophobic drug and the inner core, which is composed of the hydrophobic chain of block copolymers. In addition to DXR, cisplatin,[29] taxol[30] or KRN5500[31] have been successfully incorporated into polymeric micelles. We are currently conducting preclinical studies on these drug preparations. Kataoka et al. also succeeded in incorporating oligonucleotide into polyion complex micelles, and this suggests a possible application of the micellar system as a gene delivery system in the future.[32]

A phase I clinical trial of NK911 is currently under way to clarify its safety and pharmacokinetic profiles, in addition to evaluating its antitumor activity. This study may provide important clinical information for future clinical trials of the above-mentioned micelle carrier systems. It is emphasized here that drug carrier systems such as DXR liposomes and DXR micelles may have to be selected properly bearing in mind the tumor vascular characteristics.

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