Investigating the optimal size of anticancer nanomedicine

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Nanomedicines (NMs) offer new solutions for cancer diagnosis and therapy. However, extension of progression-free interval and overall survival time achieved by Food and Drug Administration–approved NMs remain modest. To develop next generation NMs to achieve superior anticancer activities, it is crucial to investigate and understand the correlation between the physicochemical properties of NMs (particle size in particular) and their interactions with biological systems to establish criteria for NM optimization. Here, we systematically evaluated the size-dependent biological profiles of three monodisperse drug–silica nanoconjugates (NCs: 20, 50, and 200 nm) through both experiments and mathematical modeling and aimed to identify the optimal size for the most effective anticancer drug delivery. Among the three NCs investigated, the 50-nm NC shows the highest tumor tissue retention integrated over time, which is the collective outcome of deep tumor tissue penetration and efficient cancer cell internalization as well as slow tumor clearance, and thus, the highest efficacy against both primary and metastatic tumors in vivo.

Significance

Understanding the interdependency of physicochemical properties of nanomedicine (NM) in correlation to its biological response and function is crucial for additional development of anticancer NM. Here, we prepared monodisperse drug–silica nanoconjugates in three distinct sizes (20, 50, and 200 nm) with other physicochemical properties controlled to be identical to investigate size-dependent biodistribution, tumor tissue penetration and clearance, and anticancer efficacy in various tumor models. We also developed a mathematical model of the spatiotemporal distribution of NM within a tumor to gain insight into the size-dependent interaction with tumor. Our studies show clear evidence that there is an optimal size of anticancer NM and that NM with the optimal size has the highest tumor retention integrated over time.


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sizes were obtained by tuning the concentrations of tetraethyl orthosilicate, alcohol, water, and ammonia using the Stöber method (24). We conjugated camptothecin (Cpt), a cytotoxic quinoline alkaloid that inhibits the DNA enzyme topoisomerase I, to the size-controlled silica cores using a silanized derivative of Cpt (Fig. 1A), yielding the Cpt-NCs with the desired sizes of 200, 50, and 20 nm in diameter (Cpt-NC200, Cpt-NC50, and Cpt-NC20, respectively) (Fig. 1B). All three NCs had very narrow size distribution measured by scanning electron microscope (SEM) (Fig. 1B and Table 1) and dynamic light scattering (SI Appendix, Fig. S1A). The coefficient of variation values were less than 10%, indicating that the NCs were monodisperse by industrial standard (Table 1). These Cpt-NCs had similar drug loadings (15.9–16.6 wt%), release kinetics, surface PEG densities (0.22–0.24 PEG molecules/nm²), ζ-potentials, and pharmacokinetics profiles in C57BL/6 mice (t½ = 3.0–3.2 h) (Table 1 and SI Appendix, Fig. S1). Thus, the key physiochemical properties of these Cpt-NCs were identical, leaving NP size as the only variable to be studied for size impact on the interaction with biological systems.

With the highly controlled drug–silica NCs in hand, we first examined the impact of NC size on biodistribution and tumor tissue penetration as well as the outward clearance from tumors to identify the optimal size of NC for highest tumor retention. We first radiolabeled the 200-, 50-, and 20-nm silica NCs with copper-64 (64Cu) cations (64Cu-NC200, 64Cu-NC50, and 64Cu-NC20, respectively), administered them to athymic nude mice bearing s.c. human MCF-7 tumors through i.v. injection, and then, tracked their biodistribution with the PET imaging technique (Fig. 1C). Mice injected with 64Cu-NC50 had the highest radioactivity in their tumor tissues among the three NCs tested 24 h postinjection (p.i.). This observation was confirmed by quantification of the absolute radioactivity in the harvested tumor tissues using a γ-counter, which showed that the 64Cu-NC50–treated mice had 84.1% and 23.6% higher tumor accumulation than 64Cu-NC200– and 64Cu-NC20–treated mice, respectively (Fig. 1D and SI Appendix, Fig. S2). By comparing the accumulation ratio of tumor to muscle (Fig. 1E), we observed that smaller NCs (≤50 nm) could passively target tumor tissues more efficiently than larger NCs (200 nm), likely by the enhanced permeation and retention effect (18, 23). Importantly, 64Cu-NC50 showed the highest passive tumor targeting effect among the three NCs in the MCF-7 human breast tumor model.

![Fig. 1. Size-dependent biodistribution and tumor retention of drug–silica NCs. (A) Schematic illustration of the size-controlled drug–silica NCs. (B) SEM images of the Cpt-NCs. (C–E) In vivo biodistribution of 64Cu-NCs of different sizes in athymic nude mice bearing s.c. MCF-7 human breast tumors. The athymic nude mice were injected i.v. with 64Cu-NC200, 64Cu-NC50, or 64Cu-NC20 and then euthanized 24 h p.i. (n = 5). (C) Whole-body images were taken with a micro-PET/computed tomography imaging system. Yellow circles and arrows indicate the positions of tumors. (D) Mouse organs, including tumors, were collected and measured for radioactivity by γ-counter to determine the %I.D. per gram values. (E) The ratio of tumor to muscle was calculated based on the %I.D. per gram values of tumor and muscle of each mouse. All of the data are represented as the average ± SEM and analyzed by one-way ANOVA (Fisher; 0.01 < *P < 0.05; **P ≤ 0.01; ***P ≤ 0.001). (F) Monitoring of the kinetics of tumor accumulation of 64Cu-NC50 and 64Cu-NC20 from 0 to 48 h p.i. The AUC was calculated by the trapezoidal rule up to 48 h. (G) Ex vivo tumor clearance study in MCF-7 tumors. MCF-7 tumors were ex vivo cultured with Rhd-NCs of different sizes in opti-MEM for 24 h to allow the silica NCs to penetrate into the tumors passively and then immersed in fresh opti-MEM to monitor the clearance of Rhd-NCs from the tumors. After another 48 h, the tumors were sectioned and imaged with a confocal fluorescence microscope. Representative images show the retention of Rhd-NCs (red) in tumor tissues. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). (Scale bar: 50 µm.)
Table 1. Characterization of the size controlled drug-silica NCs

<table>
<thead>
<tr>
<th>Name of NC</th>
<th>D ± SD* (nm)</th>
<th>CV%†</th>
<th>PEG density‡ (no./nm²)</th>
<th>Blood half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpt-NC200</td>
<td>199.3 ± 14.8</td>
<td>7.4</td>
<td>0.237</td>
<td>3.23</td>
</tr>
<tr>
<td>Cpt-NC50</td>
<td>53.2 ± 4.9</td>
<td>9.2</td>
<td>0.239</td>
<td>3.04</td>
</tr>
<tr>
<td>Cpt-NC20</td>
<td>24.9 ± 2.3</td>
<td>9.2</td>
<td>0.222</td>
<td>3.12</td>
</tr>
</tbody>
</table>

*The sizes of NCs were measured by SEM. Average diameter (D) and standard deviation (SD) were calculated by measuring over 100 NCs in SEM images.
†Coefficient of variation (CV%) = SD/D.
‡The number of PEG molecules per nanometer² was calculated based on wt% of PEG per NC and its surface area.

To further develop insight into the size dependency of NM in tumor accumulation and retention, we developed a mathematical model of the spatiotemporal distribution of NPs within a spherically symmetrical tumor. We modeled NP diffusion into and out of the tumor spheroid, cell surface association and dissociation, and cellular internalization and externalization. Model parameters were fitted to experimental data of our silica NCs of 200, 50, and 20 nm in diameter (24), and best fit linear regression models were used to interpolate to NP sizes within this range (31–34). Full details of our model, parameter fitting, and numerical solution techniques are provided in SI Appendix. We solved our model for the spatiotemporal mass concentration of internalized NPs within a spherical tumor with a radius of 3.5 mm over a time horizon of 48 h. In Fig. 2 A–C, we illustrate the predictions of our model for NPs of diameters 200, 50, and 20 nm. The precise structure of the spatiotemporal concentration field results from the complex interplay of the NP diffusive transport and cellular uptake kinetics. The radial penetration of the NPs into the tumor spheroid is dictated in large part by the NP mobility, with the reduced diffusivity of the larger NPs resulting in a shallower depth of penetration. The temporal evolution of the profile is driven by the decay kinetics of the NPs in the bulk, but it is also controlled by the transport, surface association, and internalization of the NPs into cancer cells. The AUC of the concentration of internalized NPs within the tumor is an important parameter to compare the NP drug delivery effectiveness. In Fig. 2D, we present the model predictions of AUCs for 200, 50, and 20 nm NPs (AUC50 nm > AUC200 nm > AUC20 nm), which are in excellent agreement with the experimental results (Fig. 1 D–F). The result suggests that the diffusivity and cellular uptake kinetics of the 50-nm NPs give rise to elevated dwell time within tumor tissue relative to the 20- and 200-nm NPs.

To verify the optimal size identified by both experiment and mathematical modeling, we next investigated whether the enhanced tumor retention of 50-nm NM could directly impart...
amplified therapeutic efficacy in treating s.c.-implanted MCF-7 human breast tumors in athymic nude mice. MCF-7 is an estrogen-dependent, noninvasive human breast cancer cell line that is extensively studied as a xenograft model for ER+ /HER2+ breast cancer (35). In an acute antitumor efficacy study, the mice bearing MCF-7 tumors (initial tumor size was 49.3 mm on day 0) were treated i.v. with Cpt-NC200, Cpt-NC50, and Cpt-NC20 of 20 mg Cpt equivalent/kg three times on days 0, 4, and 8, and PBS was used as a sham negative control group (SI Appendix, Fig. S7 A and B). All mice were euthanized on day 12. The average tumor weight increased 23.5% in the PBS control group over 12 d (SI Appendix, Fig. S7 C and D). As expected, Cpt-NC200 showed very limited effect on tumor growth inhibition, and the average tumor weight on day 12 remained nearly the same as the original because of low tumor accumulation. Both Cpt-NC50 and Cpt-NC20 were substantially more efficacious than Cpt-NC200 and resulted in ~50% tumor weight reduction (SI Appendix, Fig. S7D). The tumors from all groups were excised, sectioned, and stained for Ki67 and terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end (TUNEL) to determine the proliferation index and apoptosis index. The treatment with Cpt-NCs ≤ 50 nm resulted in a significantly reduced proliferation index and an increased apoptosis index compared with Cpt-NC200 and Cpt-NC20 (Fig. 3 A–D). Cpt-NC50 reduced the proliferation index to 42.8 ± 2.6%, which was significantly lower than both Cpt-NC200 (68.6 ± 0.9%) and Cpt-NC20 (50.5 ± 1.8%) (Fig. 3 A and C). Cpt-NC50 also triggered a significantly higher apoptosis index (21.0 ± 1.8%) than Cpt-NC200 (7.2 ± 0.3%) or Cpt-NC20 (15.3 ± 1.2%) (Fig. 3 B and D). Together, Cpt-NC50 showed the highest efficacy against MCF-7 tumors with an apoptosis-to-proliferation index ratio of 61.4 ± 4.8%, which was 6.0- and 2.1-fold higher than those of Cpt-NC200 (10.2 ± 0.5%) and Cpt-NC20 (28.6 ± 2.2%) (Fig. 3E).

To evaluate the tumor growth inhibition effect by Cpt-NCs of various sizes over a longer period, a separate efficacy study was designed similarly, except for adding blank silica NP (negative control) and irinotecan (positive control) as the control groups (SI Appendix, Fig. S8 A and B). Irinotecan was administered i.p. at 100 mg/kg, which was dosed five times higher than Cpt-NCs (20 mg/kg) (36). Compared with the PBS and the blank NP negative control groups, all other treatment groups showed superior growth inhibition of the MCF-7 tumor (Fig. 3F and SI Appendix, Fig. S8C). Mice that received Cpt-NCs or irinotecan also showed improved survival probability and increased median duration of time to end point (Fig. 3 G and H). Cpt-NC50 and Cpt-NC20 improved survival times by 76.5% and 61.8%, respectively, with significantly longer time to end point than that of Cpt-NC200. Importantly, among the Cpt-NCs of three different sizes, Cpt-NC50 was the most efficacious in terms of tumor growth inhibition and survival improvement. Interestingly, Cpt-NC50 showed significantly higher efficacy compared with Cpt-NC200 starting as early as day 6 (SI Appendix, Fig. S8C), whereas the statistical significance between Cpt-NC20 and Cpt-NC200 appeared after 18 d of experimentation. Treatment with Cpt-NC50 resulted in markedly smaller mean tumor burden (107 ± 13 mm3) at day 40 compared with Cpt-NC200 (261 ± 46 mm3) and Cpt-NC20 (228 ± 42 mm3) (Fig. 3F), providing definitive evidence that Cpt-NC50 is the most effective in reducing tumor burden among all three NCs tested. There was no statistically significant difference for tumor sizes or survival rates between the Cpt-NC50 and the irinotecan groups. No significant changes of body weight and food intake were observed for the mice in all of the Cpt-NC groups (SI Appendix, Fig. S9). However, treatment with irinotecan produced higher toxicity in mice, which was evidenced by the notable body weight drop during the course of the study (SI Appendix, Fig. S9A). Thus, Cpt-NC50 was able to exert comparable anticancer activities as free irinotecan with
substantially reduced toxicity. With respect to NC size effects, Cpt-NC50 was much more efficacious than Cpt-NC200 and Cpt-NC20 against an s.c.-implanted xenograft primary tumor.

Metastatic cancer is regarded as an incurable disease by conventional methods, including surgical resection, chemotherapy, and radiation therapy, and responsible for over 90% of cancer-related death (37). NM may offer new solutions for improving the prevention or treatment of tumor metastases. Despite extensive studies on NMs for the treatment of primary tumors, very few investigations have explored the efficacy of NMs against tumor metastases, and there are no studies that characterize the size effect of NMs on the efficacy against metastatic cancer. After we showed that Cpt-NC50 was the most efficacious in a breast primary tumor model, we went on to evaluate the size-dependent efficacy of Cpt-NCs in inhibiting the 4T1 murine breast cancer metastasis to lung tissues; 4T1 tumor is highly tumorigenic and invasive and can spontaneously metastasize to distant sites, such as the lung parenchyma (38), and 4T1 cells, which were luciferase-engineered for noninvasive monitoring of tumor growth in live animals using the bioluminescence (BL) imaging technique, were injected i.v. into BALB/c mice on day 0 to establish an experimental metastatic tumor model (39). The mice were treated with different Cpt-NCs as well as irinotecan starting from day 1 to examine the efficacy of metastasis inhibition (SI Appendix, Fig. S10). The BL imaging of the control mice (PBS group) on days 8 and 12 confirmed the existence of metastatic 4T1 tumors proliferating within lung parenchyma (Fig. 4A). As shown in Fig. 4 A and C, 12 d post-tumor inoculation, the BL signal emitted from the lung tissues of mice treated with PBS, Cpt-NC200, or Cpt-NC20 intensified rapidly and progressively. There were essentially no difference in BL intensities among the Cpt-NC200 and Cpt-NC20 groups and the PBS group, suggesting very limited efficacy of these two NCs for metastasis inhibition. However, the BL signal was markedly lower in the mice treated with Cpt-NC50 and irinotecan and increased at a much slower rate, suggesting that Cpt-NC50 exerted greater anticancer activities compared with Cpt-NC20 and Cpt-NC200, achieving comparable effects as free irinotecan (Fig. 4 A and C).

To further analyze lung pathology both macroscopically and microscopically, lung tissues were harvested from inoculated mice for detailed gross and histopathological evaluation. Tumor nodules on the surface of the lungs were counted manually with the aid of dissecting microscopy (Fig. 4B and D). The results indicated that the treatment of Cpt-NC50 significantly reduced the number of macroscopic tumor nodules (77.3 ± 9.3 per lung) compared with the PBS group (108.8 ± 9.8 per lung) (Fig. 4D), which was consistent with the BL signal analysis (Fig. 4C). We also scaled the metastatic lungs based on the percentage of lung surface covered by tumor nodules (SI Appendix, Fig. S11B). The Cpt-NC50 group had an average score (3.0 ± 0.4) that was comparable with that of the irinotecan group (2.9 ± 0.2) and much lower than those of the Cpt-NC200 (3.7 ± 0.4) and Cpt-NC20 (3.8 ± 0.2) groups (SI Appendix, Fig. S11A). The results were further confirmed by sectioning the lungs and performing the histopathological analysis. Correlating with gross evaluation, there was a prominent reduction in the number of microscopic tumor nodules observed in the lung sections collected from the mice treated with Cpt-NC50 (Fig. 4E). Tumor metastases were not observed in other organs during the course of the study (SI Appendix, Fig. S12). Collectively, Cpt-NC50 outperformed Cpt-NC200 and Cpt-NC20 with respect to inhibiting the progression of 4T1 cell metastasis to lung tissues, and Cpt-NC50 showed comparable anticancer activity with that of irinotecan. In addition, the treatment of Cpt-NC50 did not cause any significant change of body weight, food intake, or histological damage to other tissues during the course of the study (SI Appendix, Figs. S12 and S13), indicating that acute toxicity associated with Cpt-NC50 in mice is clinically negligible. Similarly, as observed in...
primary MCF-7 tumors, the accumulation of 64Cu-NC50 in lungs with metastatic 4T1 tumors was 22.3% and 11.7% higher than 64Cu-NC20 and 64Cu-NC20, respectively (Fig. 4F and SI Appendix, Fig. S14). Interestingly, the accumulation of the NCs of all sizes in lungs with metastatic tumors was significantly higher than that in lungs without tumors, probably because of tumor vascularity and cancer cell internalization (Fig. 4F). In addition, Rhd-NC20 also showed increased retention in the lung tissues with metastatic 4T1 tumors, which was evidenced by the tissue section analyses compared with Rhd-NC20 and Rhd-NC200 (Fig. 4G). The enhanced tumor accumulation of 50-nm NCs in both primary and metastatic tumors likely contributes to the improved anticancer efficacy observed in both tumor models.

In conclusion, our investigation using monodisperse drug–silica NCs with discrete sizes provides clear theoretical and experimental evidence that the size of NM plays a vital role in determining its biological property and antitumor activity. The 50-nm drug–silica NC outperforms its smaller (20 nm) and larger (200 nm) analogs in overall tumor tissue accumulation and retention, and thus, shows the highest efficacy against both primary and metastatic tumors. Therefore, 50 nm could be or could be close to the optimal size of the PEG-coated anticancer drug–silica NC that balances extravasation, inward permeation through tumor tissue, tumor cell internalization, and outward diffusion and clearance from tumor to retain the highest effective drug concentrations in tumors. Many surface PEGylated anticancer NMs are in various stages of preclinical or clinical development. Because the size and the surface property of an NM likely dictate its overall biological property, PEG-coated NMs may share many similarities with respect to their size dependency of biological activities. Our study and confirmation of the existence of the optimal size of the PEG-coated drug–silica NC clearly show the importance of controlling the size and dispersion of PEGylated NMs and the potential of further improving their antitumor efficacy by identifying the optimal size against a specific cancer. However, whether the optimal size of other PEGylated NMs remains at or around 50 nm requires further study.

Materials and Methods
Cpt-NCs with controlled sizes were prepared as previously reported (24) and used for all efficacy studies. Details describing preparation and characterization of size-controlled silica NCs; size-dependent biodistribution, tumor penetration and clearance, and cellular internalization studies; size-dependent efficacy studies in a primary tumor model; size-dependent efficacy studies in a metastatic tumor model; and spatiotemporal modeling of NP uptake into tumors can be found in SI Appendix.

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