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Reduction of Drug Toxicity Using Dendrimers Based on Melamine

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Abstract: Dendrimers based on melamine can reduce the organ toxicity of solubilized cancer drugs administered by intraperitoneal injection. Methotrexate and 6-mercaptopurine, both FDA approved anticancer drugs, are known hepatotoxins. The solubility of these molecules can be increased by mixing them with a dendrimer based on melamine. C3H mice were administered subchronic doses of methotrexate or 6-mercaptopurine with and without a solubilizing dendrimer. Forty-eight hours after dosing, the mice were sacrificed and serum was collected for biochemical analyses. The levels of alanine transaminase, ALT, were used to probe liver damage. When the drugs are encapsulated by the dendrimer, a significant reduction in hepatotoxicity is observed: ALT levels from the rescued groups (drug + dendrimer) were 27% (methotrexate) and 36% (6-mercaptopurine) lower than those of animals treated with the drug alone.

Keywords: Drug delivery; toxicity; dendrimer; melamine; methotrexate; 6-mercaptopurine

Cytotoxic drugs discriminate between normal and neoplastic cells due in large part to the rapidly dividing nature of cancerous cells. While this difference affords a therapeutic advantage, systemic and specific target organ toxicities remain limiting factors for the dose size and frequency of chemotherapeutic drugs. Strategies for reducing this toxicity without sacrificing efficacy could greatly improve treatment and quality of life issues for those besieged. To this end, we and others are exploring strategies for drug delivery.[1] The use of a macromolecular drug carrier to reduce toxicity is the focus of our work. One advantage of macromolecular drug delivery vehicles is derived from the nature of the tumor tissue vasculature.[2,3] The tumor vasculature that develops to provide rapidly dividing cells the nutrients required to sustain their growth is more permeable than the existing vasculature. The advantages of this permeability are further amplified by defects in, or complete lack of, a lymphatic drainage system within the tumor tissue. Macromolecular delivery vehicles are designed to exploit the enhanced permeability and retention (EPR) effect to gain a therapeutic advantage over free drug.[4]

Our efforts focus on dendrimers,[5] a unique class of perfectly branched macromolecules whose shape, size, and chemical functionality can be manipulated during the stepwise synthesis required for their preparation.[6] Dendrimers offer opportunities for solubilizing drugs,[7] for displaying antigens as vaccines,[8] and for delivering oligonucleotides and DNA.[9] The wealth of peripheral groups offers sites for the covalent attachment of drugs and/or biologically recognizable markers that could enhance site-specific drug delivery. These opportunities are not unique to dendrimers: micelles, polymeric micelles, liposomes, and linear polymers can be employed to similar ends.

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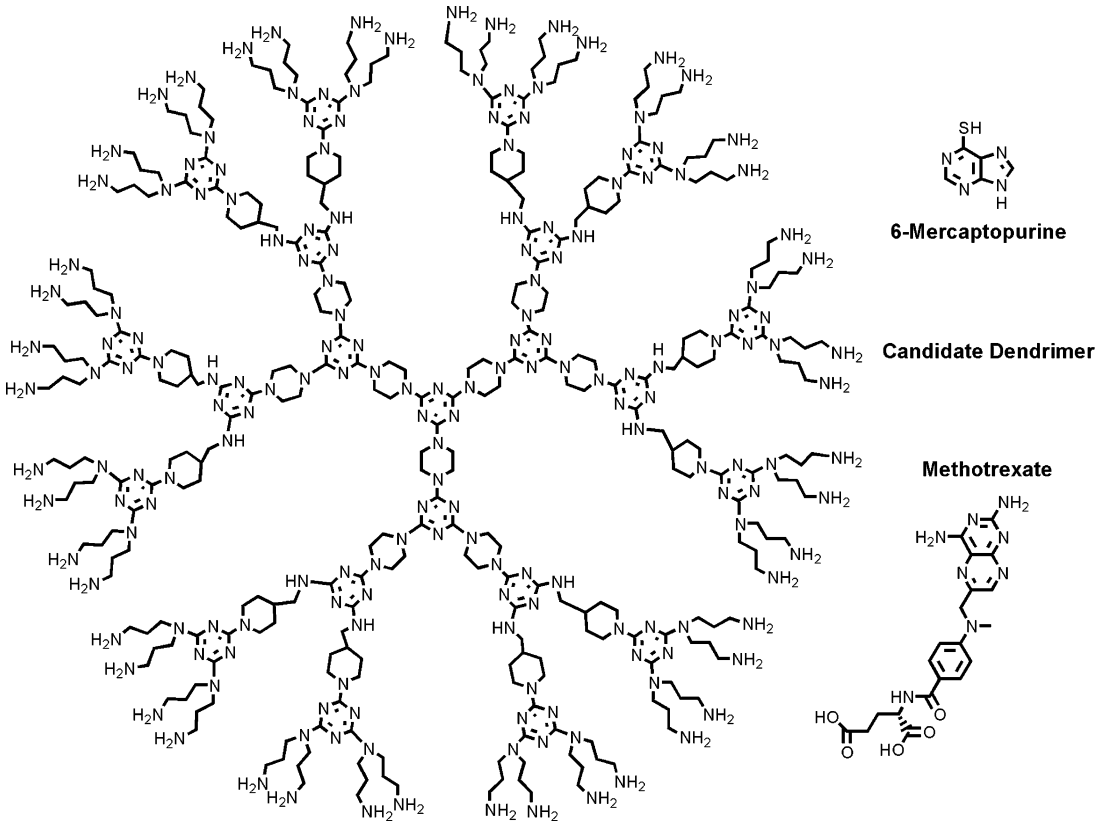
Chart 1. The Dendrimer and Drugs



A number of groups have shown that a variety of dendrimers can encapsulate a variety of hydrophobic agents including 10-hydroxycamptothecin,[10] methotrexate,[11] doxorubicin,[11] paclitaxel,[7a] and 5-fluorouracil.[12,13] Compared with the wealth of studies on in vitro solubilization of anticancer agents, we are aware of no studies that address in vivo reduction of target organ toxicity seen in these chemotherapeutic agents. Here, we report the encapsulation of the anticancer drugs methotrexate (MTX) and 6-mercaptopurine within a dendrimer based on melamine (Chart 1) and its ability to reduce the established hepatotoxicity toxicity associated with these drugs.[14−16]

The dendrimer was synthesized using previously described protocols.[17] Alanine transaminase (ALT) assay kits were purchased from Teco Diagnostics (Anaheim, CA). Methotrexate (MTX) was purchased from Sigma (St. Louis, MO), and 6-mercaptopurine (6-MP) was purchased from Acros.

Male C3H mice were purchased from Harlan (Indianapolis, IN) and housed in the Texas A&M Medical Sciences Building. Mice weighing 25 ± 5 g having free access to standard mouse chow and water were housed in metal cages and kept in a room maintained at 23 ± 2 °C with a 12 h/12 h light/dark cycle.

Methotrexate and 6-mercaptopurine were dissolved in 100 mM saline, the pH was adjusted to 8 with 1 N NaOH, and the mixture was diluted with saline.[18] The dendrimer was dissolved in saline. To solubilize the drugs with dendrimer, stock solutions of MTX and 6-MP were prepared in saline. Aliquots of these stock solutions were added to solutions of dendrimer and sonicated for 20 min until the solutions

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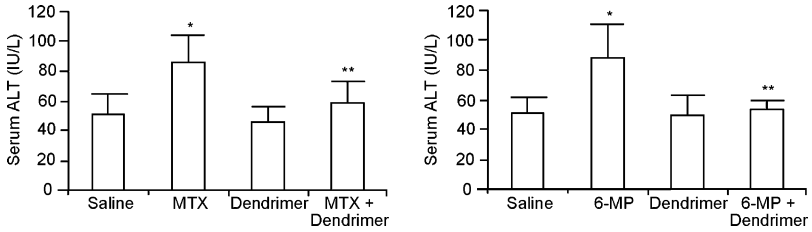

Figure 1. ALT levels from mice that were administered doses of methotrexate (left) and 6-mercaptopurine (right) and from those rescued mice (drug + dendrimer). (*) Statistically different when compared to the controls ($P < 0.05$). (**) Statistically different when compared to the drug-alone groups ($P < 0.05$).

became clear. Full details are available in the Supporting Information. The amount of drug delivered is based on administration of known amounts of these stock solutions. Dialysis to separate "bound" from "unbound" drug was not pursued.

The mice were administered 2 mg/kg MTX or 3.5 mg/kg 6-MP via ip injection for three consecutive days. Rescued mice were given the same respective amounts of MTX and 6-MP premixed with 10 mg/kg dendrimer. Controls received only saline. Forty-eight hours following the last round of injections, the mice were anesthetized and then sacrificed by cardiac puncture. Approximately 1 mL of blood was collected and processed to obtain serum for the biochemical analysis to assess liver toxicity. Blood samples from the mice were collected in heparinized syringes, and serum samples were obtained by centrifuging the whole blood at 1500 rpm for 10 min at 4 °C. Serum was used to estimate liver enzyme activity (ALT) using the standard diagnostic kit.

Statistical analysis (EpiCalc 2000)[19] of the biochemical assays was performed using analysis of variance (ANOVA). Differences between the treatment groups were determined by the Student's *t*-test. The results were presented as the mean ± SD in each group, and a statistical probability of $P < 0.05$ was considered to be significant.

The dendrimer used in this study presents 48 amine groups to the periphery which provide solubility in water. The molecule is prepared in five linear steps (nine total steps) in 56% overall yield. This macromolecule has a molecular weight of 6941 Daltons with an estimated diameter of ~2.5 nm based on gas-phase simulations. Previous studies on a related dendrimer revealed no detectable toxicity in vivo until doses of 40 mg/kg are reached.[10,20] In the current study, the mice were administered saline (controls), 2 mg/kg MTX, or 3.5 mg/kg 6-MP via ip injection for three consecutive days. Rescued mice were given the same amounts of MTX and 6-MP premixed with 10 mg/kg of dendrimer. These doses were chosen because smaller, repeated doses are more toxic than a single acute administration.[14]

The known hepatotoxic effects of methotrexate and 6-mercaptopurine can be monitored by using the levels of alanine transaminase (ALT) in the serum as an indicator. Figure 1 shows that the ALT levels that result from the subchronic dosing of both drugs alone elicit a significant increase ($P < 0.05$) in serum ALT activity when compared to the mice that received saline. The dendrimer alone shows no increase in ALT levels. However, when the dendrimer is mixed with drug and administered, two interesting observations result. First, the dendrimer−drug conjugate shows no significant difference in ALT levels compared with the control saline or the dendrimer alone groups. Second, the dendrimer−drug conjugate shows a significant decrease in serum ALT activity when compared to the drug alone.

Our results indicate that a dendrimer based on melamine can reduce the hepatotoxicity of both methotrexate and 6-mercaptopurine. The dosing used in this study corresponds to approximately 3 molecules of methotrexate (454 Da) and 15 molecules of 6-mercaptopurine (167 Da) encapsulated by each dendrimer molecule. These numbers are consistent with the molecular dimensions of these species. The diameter of the globular dendrimer in the gas phase is approximately 2.5 nm, giving a volume of approximately 5 nm^3. We expect the hydrated diameter to be slightly larger. The molecules can be approximated as blocks with volumes approximating 0.1 nm^3 for mercaptopurine and 0.4 nm^3 for methotrexate.[21] In both cases, the total volumes of drug solubilized are almost identical and correspond to 25−30% of the volume of the dendrimer.

The mechanism for reducing toxicity is not known, but presumably lies in reducing the blood levels of these drugs. The ability to reduce the cytotoxicity of these drugs creates many opportunities for these systems. To exploit the EPR effect, the drugs must remain associated with the macromolecular vehicle until the vehicle arrives at the tumor. In this study, the data suggests that the noncovalent interactions between the drug and dendrimer are significant enough to prevent all the drug from rapidly diffusing out of the vehicle. Lower systemic toxicity for an encapsulated agent suggests that higher drug dosing might be achieved using these vehicles. Such beliefs will be investigated in tumor inhibition

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(21) Estimated molecular dimensions: mercaptopurine (0.3 nm × 0.4 nm × 0.7 nm) and methotrexate (0.3 nm × 0.7 nm × 1.6 nm).

studies once vehicles more suitable for intravenous administration are identified.

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Supporting Information Available: Synthetic details and statistical analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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