J|A|C|S
ARTICLES
Published on Web 01/27/2004

Bioactivation of Self-Immolative Dendritic Prodrugs by Catalytic Antibody 38C2

Marina Shamis,[†] Holger N. Lode,[‡] and Doron Shabat*,[†]

Contribution from the School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel-Aviv University, Tel Aviv 69978, Israel, and Charité Children's Hospital, Humboldt University, Augustenburger Platz 1, 13353 Berlin, Germany

Received October 14, 2003; E-mail: chdoron@post.tau.ac.il

Abstract: Self-immolative dendrimers have recently been developed and introduced as a potential platform for a multi-prodrug. These unique structural dendrimers can release all of their tail units, through a self-immolative chain fragmentation, which is initiated by a single cleavage at the dendrimer's core. Incorporation of drug molecules as the tail units and an enzyme substrate as the trigger can generate a multi-prodrug unit that will be activated with a single enzymatic cleavage. We have synthesized the first generation of dendritic prodrugs with doxorubicin and camptothecin as tail units and a retro-aldol retro-Michael focal trigger, which can be cleaved by catalytic antibody 38C2. The bioactivation of the dendritic prodrugs was evaluated in cell-growth inhibition assay with the Molt-3 leukemia cell line in the presence and the absence of antibody 38C2. The dendritic unit was applied as a platform for a heterodimeric prodrug, which achieved a remarkable increase in toxicity with its bioactivation.

Introduction

The unique structural properties of dendrimers[1,2] increasingly entice scientists to use them for drug delivery applications.[3−7] Recently, biodegradable[8,9] and disassembled[10,11] dendritic molecules have been attracting growing attention. Several anticancer prodrugs have been designed for selective activation in malignant tissues by a specific enzyme, which is targeted[12] or secreted near tumor cells.[13] The release of the free drug by a specific enzyme only takes place upon cleavage of a prodrug protecting group. The circumstances under which a cleavage event will release one molecule of free drug may limit the total amount of the targeted drug, depending on the rate and concentration of the specific enzyme. We and others have recently reported a new class of dendritic molecules that were termed self-immolative dendrimers[14−17] (SIDs). These unique structural dendrimers can release all of their tail units through a self-immolative chain fragmentation, which is initiated by a single cleavage event at the dendrimer's core. Incorporation of drug molecules as the tail units and an enzyme substrate as the trigger can generate a multi-prodrug unit that will be activated upon a single enzymatic cleavage. Self-immolative dendritic prodrugs may open new opportunities for targeted drug delivery. In contrast to conventional dendrimers, SIDs are fully degradable and can be excreted easily from the body. The cleavage effect of a tumor-associated enzyme or a targeted one could be amplified and therefore may increase the number of active drug molecules in targeted tumor tissues.

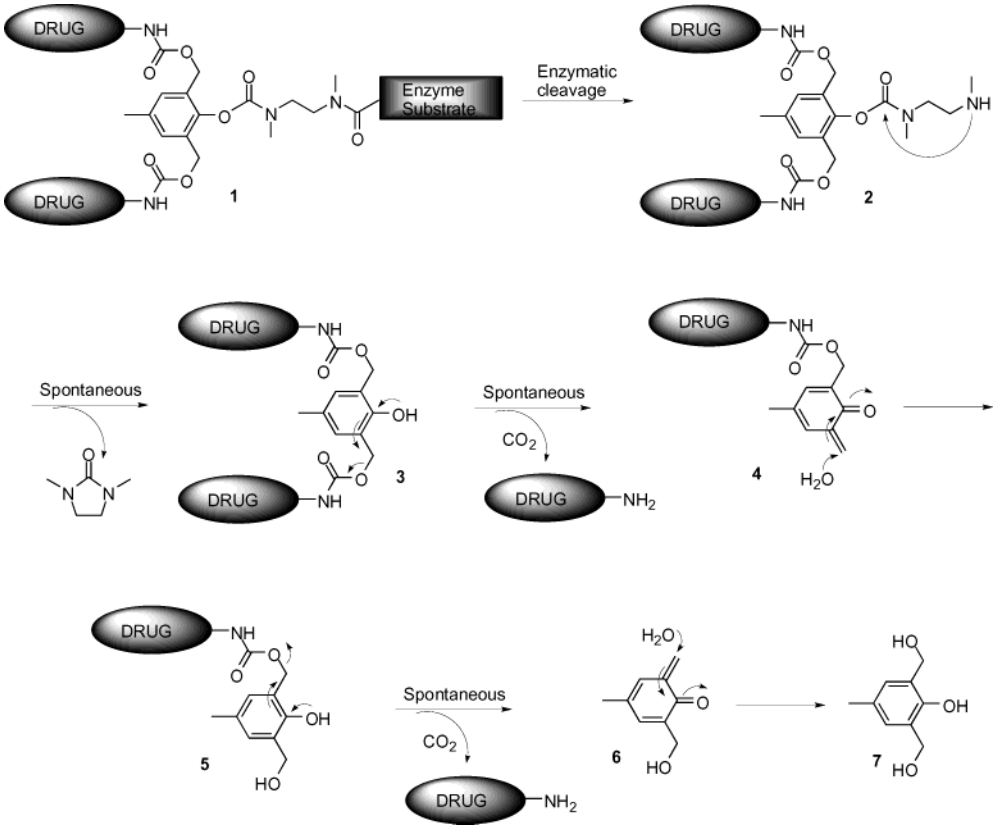
The AB_2 building unit of the dendrimer is based on 2,6-bis-(hydroxymethyl)-*p*-cresol (**7**), a commercially available compound, which has three functional groups (Scheme 1). The two hydroxybenzyl groups are attached through carbamate linkages to drug molecules, and the phenol functionality is linked to a trigger through a short spacer, *N*,*N*′-dimethylethylenediamine (compound **1**). The cleavage of the trigger initiates a self-immolative reaction, starting with spontaneous cyclization of amine intermediate **2**, to form an *N*,*N*′-dimethylurea derivative. The generated phenol **3** undergoes a 1,4-quinone methide rearrangement, followed by spontaneous decarboxylation to liberate one of the drug molecules. The quinone methide species **4** is rapidly trapped by a water molecule (from the reaction

[†] Tel-Aviv University.
[‡] Humboldt University.

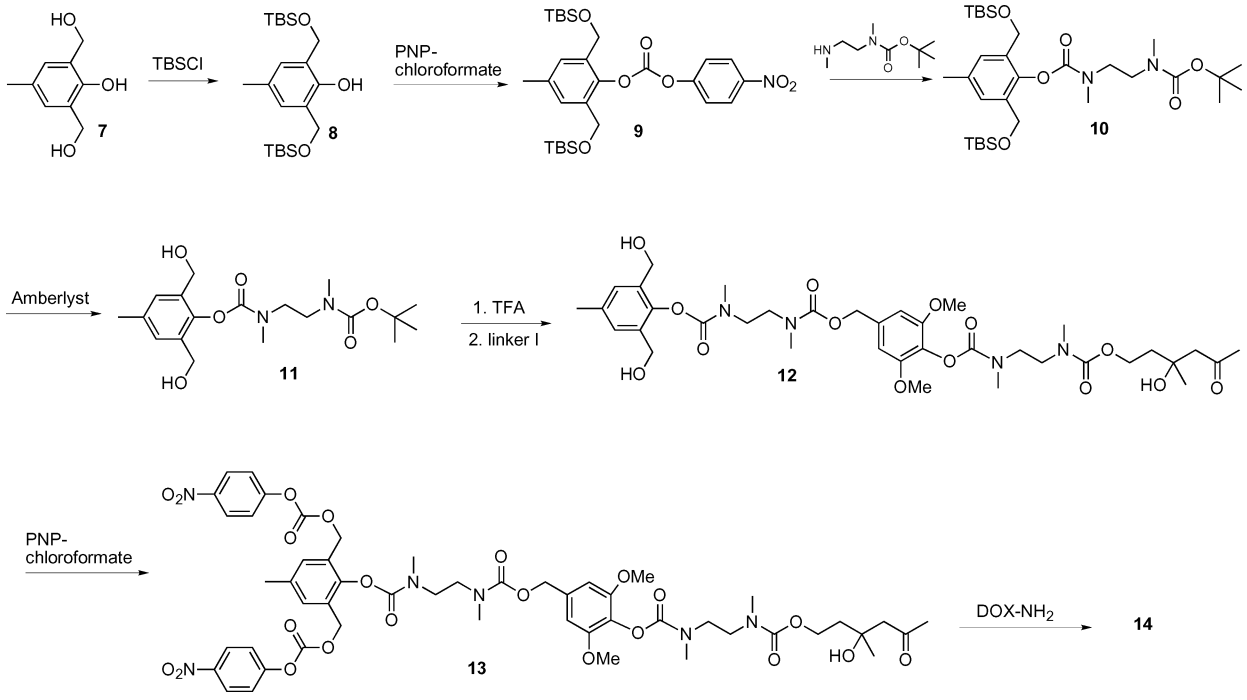
(1) Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., III. *Angew. Chem., Int. Ed. Engl.* **1990**, *102*, 119−157.
(2) Tomalia, D. A.; Frechet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 2719−2728.
(3) Stiriba, S.-E.; Frey, H.; Haag, R. *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 1329−1334.
(4) Patri, A. K.; Majoros, I. J.; Baker, J. R. *Curr. Opin. Chem. Biol.* **2002**, *6*, 466−471.
(5) Padilla De Jesus, O. L.; Ihre, H. R.; Gagne, L.; Frechet, J. M.; Szoka, F. C., Jr. *Bioconjugate Chem.* **2002**, *13*, 453−461.
(6) Kim, Y.; Zimmerman, S. C. *Curr. Opin. Chem. Biol.* **1998**, *2*, 733−742.
(7) Ihre, H. R.; Padilla De Jesus, O. L.; Szoka, F. C., Jr.; Frechet, J. M. *Bioconjugate Chem.* **2002**, *13*, 443−452.
(8) Grinstaff, M. W. *Chemistry* **2002**, *8*, 2839−2846.
(9) Seebach, D.; Herrmann, G. F.; Lengweiler, U. D.; Bachmann, B. M.; Amrein, W. *Angew. Chem., Int. Ed. Engl.* **1997**, *35*, 2795−2797.
(10) Li, S.; Szalai, M. L.; Kevwitch, R. M.; McGrath, D. V. *J. Am. Chem. Soc.* **2003**, *125*, 10516−10517.
(11) Smet, M.; Liao, L.-X.; Dehaen, W.; McGrath, D. V. *Org. Lett.* **2000**, *2*, 511−513.
(12) Bagshawe, K. D.; Springer, C. J.; Searle, F.; Antoniw, P.; Sharma, S. K.; Melton, R. G.; Sherwood, R. F. *Br. J. Cancer* **1988**, *58*, 700−703.
(13) de Groot, F. M.; Damen, E. W.; Scheeren, H. W. *Curr. Med. Chem.* **2001**, *8*, 1093−1122.
(14) Amir, R. J.; Pessah, N.; Shamis, M.; Shabat, D. *Angew. Chem., Int. Ed.* **2003**, *42*, 4494−4499.
(15) Shabat, D. U.S. provisional patent AN 60/406,958, 2002.
(16) De Groot, F. M.; Albrecht, C.; Koekkoek, R.; Beusker, P. H.; Scheeren, H. W. *Angew. Chem., Int. Ed.* **2003**, *42*, 4490−4494.
(17) Szalai, M. L.; McGrath, D. V. *Polym. Mater. Sci. Eng.* **2003**, *89*, 406−407.

10.1021/ja039052p CCC: $27.50

Scheme 1. Mechanism of Dimeric Prodrug Activation by a Single Enzymatic Cleavage



Scheme 2. Synthesis of the Homodimeric Prodrug of Doxorubicin with a Trigger That Is Activated with Catalytic Antibody 38C2



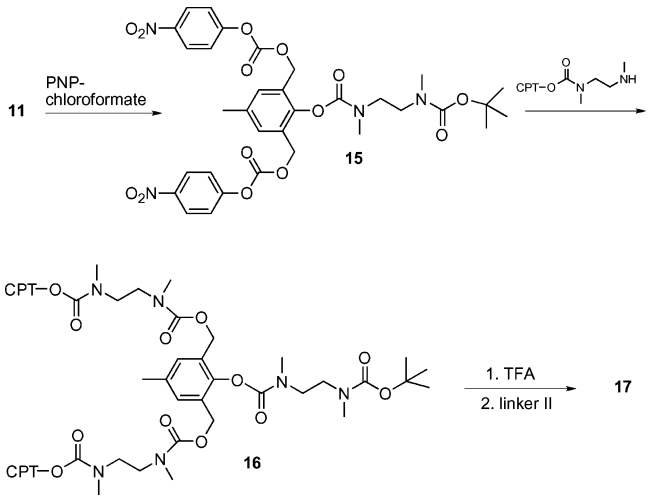
medium) to form a phenol (compound **5**), which again undergoes a 1,4-quinone methide rearrangement to liberate the second drug entity. The generated quinone methide species **6** is trapped again by a water molecule to form **7**.

Here we report on the design, synthesis, and bioactivation of the dendritic prodrug platform, which allows the release of multiple numbers of drug molecules with a single cleavage of an enzymatic trigger.

Results and Discussion

Homodimeric Prodrugs. The synthesis of the dimeric prodrugs is described in Schemes 2 and 3. Thus, commercially available **7** was reacted with 2 equiv of (TBS)Cl to afford phenol

Scheme 3. Synthesis of the Homodimeric Prodrug of Camptothecin with a Trigger That Is Activated with Catalytic Antibody 38C2



8, which was acylated with *p*-nitrophenyl chloroformate to give carbonate **9**. The latter was reacted with mono-Boc-*N*,*N*′-dimethylethylenediamine to generate compound **10**, which was deprotected in the presence of Amberlyst-15 to give diol **11**. Deprotection with TFA afforded an amine salt, which was reacted in situ with linker I (activated form of antibody 38C2 substrate; see the Supporting Information for further details) to generate compound **12**. The latter was mixed with 2 equiv of doxorubicin to afford homodimeric prodrug **14**.

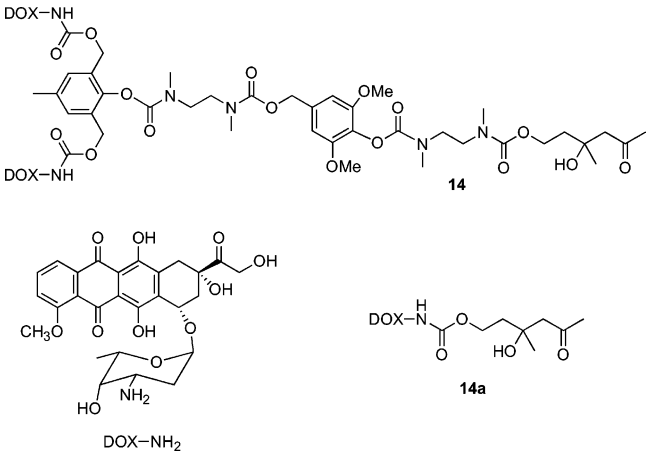
Acylation of diol **11** with 2 equiv of *p*-nitrophenyl chloroformate afforded dicarbonate **15**, which was then reacted with 2 equiv of camptothecinamine units to give compound **16**. Deprotection with TFA afforded an amine salt, which was reacted in situ with linker II (see the Supporting Information for further details) to yield homodimeric prodrug **17**.

As the first example of a dimeric prodrug, we chose to incorporate the anticancer drug doxorubicin,[18] and catalytic antibody 38C2[19] as the activating enzyme. Antibody 38C2 catalyzes a sequence of retro-aldol retro-Michael cleavage reactions, using substrates that are not recognized by human enzymes.[20] Therefore, nonspecific prodrug activation should be minimal. Furthermore, the antibody has demonstrated efficacy in activating several prodrugs in vitro and in vivo. A dramatic 75% decrease in subcutaneous (sc) tumor size has been observed in mice that received a combination of intratumoral injections of antibody 38C2 and systemic treatments with an etoposide prodrug.[21]

The retro-aldol retro-Michael substrate of antibody 38C2 was attached to the adaptor platform through a self-immolative linker with adequate length, to avoid steric hindrances accompanying the complex structure of the doxorubicin (DOX) molecule (Chart 1).

The activation of dimeric prodrug **14** was compared with that of previously reported[20] monomeric prodrug **14a**, using a cell-growth inhibition assay of the Molt-3 cell line. Solubility

Chart 1. Chemical Structure of Prodrugs **14** and **14a**



problems of the dimeric prodrug of DOX forced us to use Cremophor EL as a cosolvent agent. The in vitro assay results are shown in Figure 1.

Solvent controls show that the toxicity of Cremephor EL is about 50-fold less than that of free DOX and about the same as those of the prodrugs. Notably, there is still a 50-fold remaining difference between the IC_{50} of the prodrugs and free DOX. When catalytic antibody 38C2 was incubated with the prodrugs, it was evident that the IC_{50} of the dimeric prodrug had shifted closer to the IC_{50} of the free DOX. Importantly, the toxicities of the monomeric prodrug and the dimeric prodrug remain in a similar range, indicating the relative stability for hydrolysis of the drug molecules' linkages with the platform.

To avoid the toxicity of Cremephor EL, we synthesized a camptothecin[22] (CPT) dimeric prodrug, which was found to be much more water soluble than the DOX prodrug **14**. The CPT was attached to the platform through a short self-immolative linker. The triggering substrate was the same retro-aldol retro-Michael moiety (Chart 2). The activation of dimeric prodrug **17** was compared with that of a known monomeric CPT prodrug, **17a**, using the Molt-3 cell line growth inhibition assay (Figure 2).

The IC_{50} values of the monomeric and the dimeric prodrugs were found to be almost the same, and the prodrugs were about 200-fold less toxic than free CPT. When catalytic antibody 38C2 was added, both prodrugs were activated. However, while the activity of the monomeric prodrug had shifted to a 10-fold difference from that of free CPT, the dimeric prodrug was shown to be about 4 times more active upon addition of 38C2, meaning that more toxicity was achieved using the dimeric prodrug and 38C2 in comparison to monomeric prodrug and the same concentration of antibody.

To the best of our knowledge, these are the first reported examples of dimeric prodrug bioactivation which involve a double 1,4-quinone methide rearrangement, to release two molecules of drug in a single enzymatic event.

Heterodimeric Prodrugs. With these results in hand, we were motivated to synthesize a heterodimeric prodrug, constructed of a combination of DOX and CPT. The synthesis was performed straightforward as presented in Scheme 4. Thus, dicarbonate **13** was selectively reacted with 1 equiv of the CPT-

(18) Di Marco, A.; Cassinelli, G.; Arcamone, F. *Cancer Treat Rep.* **1981**, *65* (Suppl. 4), 3−8.
(19) Wagner, J.; Lerner, R. A.; Barbas, C. F., III. *Science (Washington, D.C.)* **1995**, *270*, 1797−1800.
(20) Shabat, D.; Rader, C.; List, B.; Lerner, R. A.; Barbas, C. F., III. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6925−6930.
(21) Shabat, D.; Lode, H.; Pertl, U.; Reisfeld, R. A.; Rader, C.; Lerner, R. A.; Barbas, C. F., III. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 7528−7533.
(22) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmar, K. H.; McPhail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3888−3890.

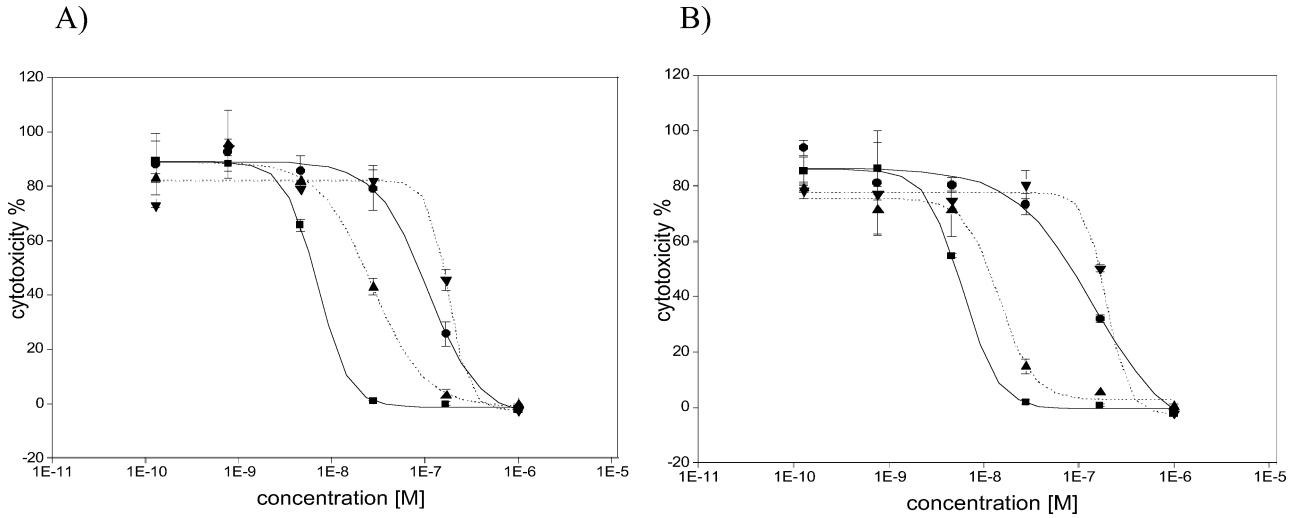


Figure 1. Growth inhibition assay of the human Molt-3 leukemia cell line, with addition of prodrugs in the presence and absence of catalytic antibody 38C2 (cells were incubated for 72 h): (A) (■) DOX, (●) pro-DOX **14a**, (▲) pro-DOX **14a** + 1 μM 38C2, (▼) solvent control; (B) (■) DOX, (●) pro-DOX **14**, (▲) pro-DOX **14** + 1 μM 38C2, (▼) solvent control.

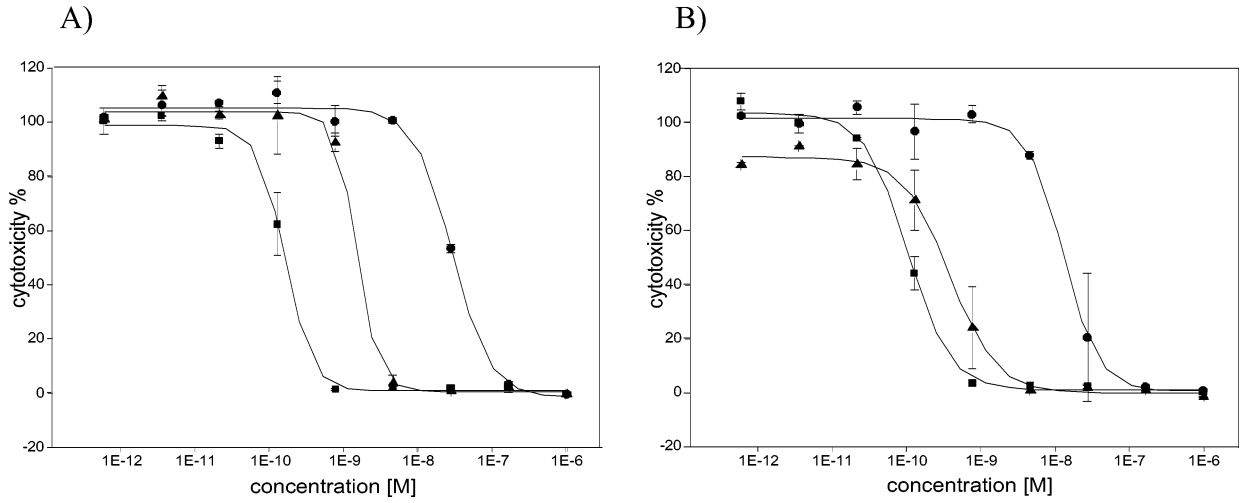
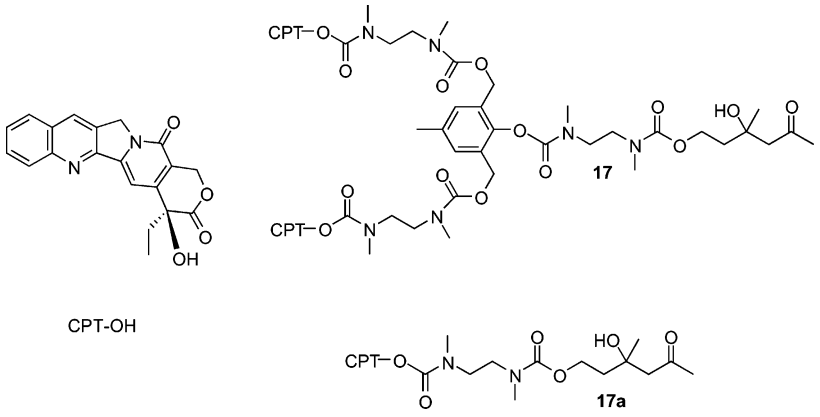


Figure 2. Growth inhibition assay of the human Molt-3 leukemia cell line, with prodrugs in the presence and absence of catalytic antibody 38C2 (cells were incubated for 72 h): (A) (■) CPT, (●) pro-CPT **17a**, (▲) pro-CPT **17a** + 1 μM 38C2; (B) (■) CPT, (●) pro-CPT **17**, (▲) pro-CPT **17** + 1 μM 38C2.

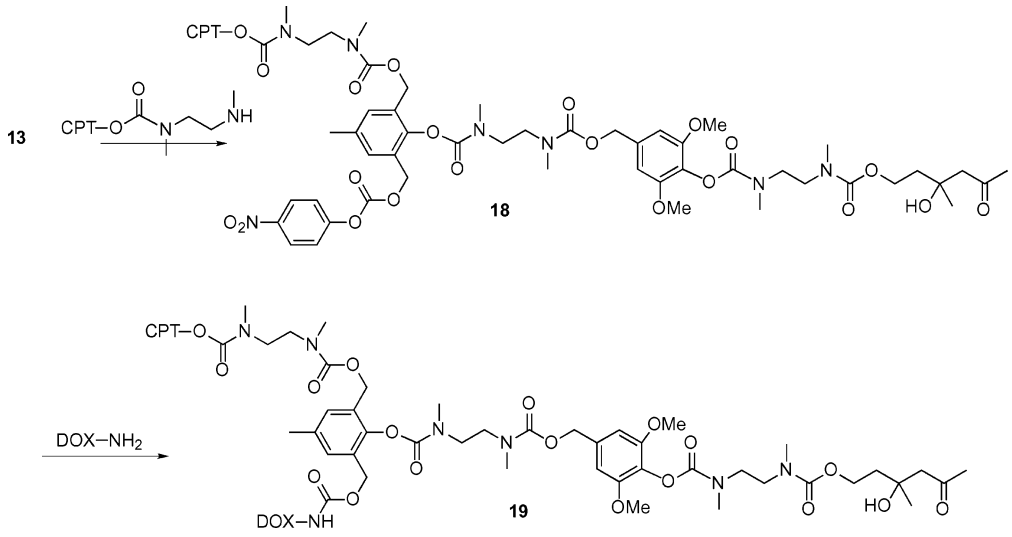
Chart 2. Chemical Structure of Prodrugs **17** and **17a**



amine unit to give compound **18**. The latter was directly reacted with 1 equiv of DOX to give the final heterodimeric prodrug **19**.

The bioactivation of dimeric prodrug **19** was compared with that of a 1:1 combination of monomeric prodrugs **14a** and **17a** at the appropriate concentrations. The in vitro results using cell-growth inhibition assay of the Molt-3 cell line are shown in Figure 3. The IC_{50} values of both prodrug **19** and the combination **14a** + **17a** are almost the same. However, in the presence of antibody 38C2, the IC_{50} of prodrugs **14a** + **17a** shifted to only 8 nM while the IC_{50} of prodrug **19** decreased to 0.17 nM. This effect is remarkable and shows a clear advantage of the

Scheme 4. Synthesis of Heterodimeric Prodrug **19**, Containing an Enzymatic Trigger Substrate of Catalytic Antibody 38C2



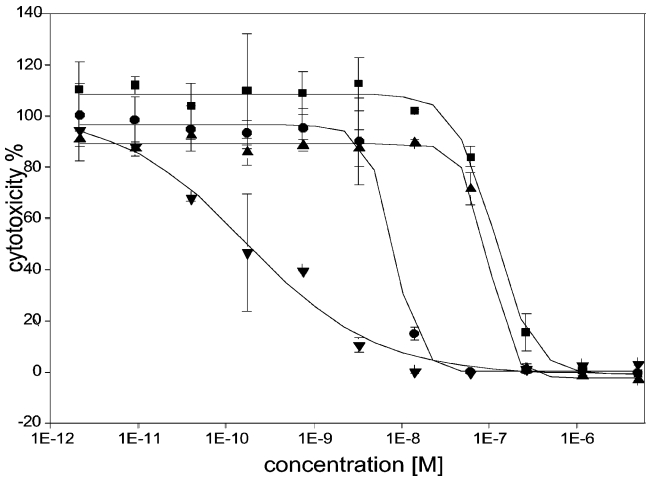


Figure 3. Growth inhibition assay of the human Molt-3 leukemia cell line (cells were incubated for 96 h): (■) pro-CPT **17a** + pro-DOX **14a**, (●) pro-CPT **17a** + pro-DOX **14a** + 1 μM 38C2, (▲) heterodimeric prodrug **19**, (▼) heterodimeric prodrug **19** + 1 μM 38C2.

Table 1. IC_{50} Values from Cell-Growth Inhibition Assays

drug/ prodrug	IC_{50}[a] (nM)	IC_{50}[b] (nM)	drug/ prodrug	IC_{50}[a] (nM)	IC_{50}[b] (nM)
DOX	6.3	6.3	17a	28.4	1.5
14	143	12.6	**14a** + **17a**[c]	112	8.0
14a	111	26.3	**19**[c]	96.5	0.17
CPT	0.13	0.13	DOX + CPT[c]	0.06	0.06
17	12.8	0.38			

[a] Cells were incubated in medium with drug/prodrug. [b] Cells were incubated in medium with drug/prodrug + 1 μM catalytic antibody 38C2. [c] Cells + prodrugs were incubated for 96 h.

heterodimeric prodrug in comparison to a combination of two monomeric prodrugs. The prodrug bioactivation is much more efficient if two drug molecules are attached to a single common masking enzymatic substrate rather than two separated substrates. Importantly, no drug release was observed when prodrug **19** was incubated in the cells' extract for 24 h.

The in vitro results of all the cell-growth inhibition assays are summarized in Table 1.

Bioactivation of homodimeric prodrugs shows a slight but clear toxicity increase over activation of the monomeric prodrugs. In the studied examples, both DOX and CPT homodimeric prodrugs (**14** and **17**) exhibit improved activity by a factor of 2−4 when compared with their monomeric counterparts (**14a** and **17a**). This effect can simply be explained by the number of enzymatic cleavages that are required to activate the prodrugs. Two cleavage events are needed for two molecules of monomeric prodrugs, while only one is needed for a homodimeric unit. This advantage can be achieved only if undesired toxicity toward normal cells of the dimeric prodrug will remain in the range of the monomeric one. To our delight, the chemical stabilities of both monomeric and dimeric prodrugs in the cell medium without the catalytic antibody were found to be relatively similar. The mild advantageous effect in bioactivation of the dimeric prodrug could be increased significantly when higher generations of dendritic prodrugs are generated. We have showed the feasibility of the synthetic design by preparing a G2-dendron prodrug of CPT with a retro-aldol retro-Michael substrate for catalytic antibody 38C2. However, the bioactivation of this dendrimer still requires additional studies.

The best results for the dendritic compounds were obtained with a heterodimeric prodrug. The toxicity for prodrug **19,** constructed of DOX and CPT, was about 50-fold higher than activity measured using a combination of two monomeric prodrugs (**14a** and **17a**) when bioactivation was performed. One reason for this effect was already discussed before and is merely related to the number of cleaving events that are required for activation. However, additional explanation is needed to clarify the significant effect. At this time, we believe that in the bioactivation of the two monomeric prodrugs (**14a** and **17a**) there may be a case of competitive substrates. Thus, if one prodrug is a better substrate than the other, the overall release of the free drug combination is inhibited in comparison with the activation of the heterodimeric prodrug. Importantly, the toxicity of the heterodimeric prodrug in the absence of catalytic antibody 38C2 was similar to that measured for the combination of the monomeric prodrugs.

Conclusions

In summary, we have demonstrated the first application of self-immolatve dendrimers as a platform for multi-prodrugs, activated by a single enzymatic cleavage. We synthesized homo-

and heterodendritic prodrugs of DOX and CPT with a triggering substrate of catalytic antibody 38C2, which functions as a model enzyme. The bioactivation of the dendritic prodrugs was evaluated by growth inhibition assays using the Molt-3 leukemia cell line, and the prodrugs showed a mild to significant increase in toxicity in comparison with the classical monomeric prodrugs. Dimeric units with an enzymatic trigger were readily activated upon addition of catalytic antibody 38C2, while the tetrameric counterpart was synthesized but thus far has failed to be a substrate for the antibody. A remarkable increased effect in toxicity was observed upon bioactivation of a heterodimeric prodrug of DOX and CPT. Different drugs can be introduced on the dendritic platform to achieve synergetic effects, and precise drug combinations may be tailored for specific types of cancer. In vivo experiments using this concept will be initiated after further optimization of the required compounds and conditions.

Acknowledgment. We thank Dr. Richard A. Lerner and Dr. Carlos F. Barbas III of The Scripps Research Institute for providing antibody 38C2. Financial support has been provided by Tel-Aviv University. Special thanks go to Dr. Michael Meijler for his helpful comments.

Supporting Information Available: Full experimental details, characterization data, and cell assay conditions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA039052P