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# A Heterodimeric Glucuronide Prodrug for Cancer Tritherapy: the Double Role of the Chemical Amplifier

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Most anticancer drugs suffer from poor selectivity leading to severe side effects due to action against normal as well as diseased tissue. Thus, the development of drug carriers designed to deliver potent therapeutic agents exclusively at the tumor site has emerged as one of the great challenges in medicinal research.[1] One promising targeting strategy relies on the use of nontoxic prodrugs that can be activated by an enzyme naturally overexpressed in the tumor microenvironment.[2] In this approach, enzymatic prodrug activation is followed by the release of the parent drug thereby restoring its antitumor activity selectively in malignant tissues. Several enzyme-responsive prodrugs have already been evaluated in vivo with encouraging results.[3] However, the relatively slow action and low concentration of all tumor-associated enzymes discovered so far represent the "Achilles' heel" of such a targeting strategy. These unfavorable enzymatic parameters limit the amount of drug liberated in targeted tissues and therefore the efficacy of the treatment. To overcome this drawback, Shabat,[4] de Groot[5] and McGrath[6] simultaneously introduced the concept of self-immolative dendrimers allowing the release of several drug units after a single triggering event, thanks to an efficient chemical amplification process. In an elegant study, the Shabat group also developed heterodimeric[7] and heterotrimeric[8] systems designed to deliver highly toxic drug cocktails with a single enzymatic activation step.

Herein, we present the novel drug delivery device **1** designed for the selective targeting of three different cytotoxic agents (Scheme 1a). This system is composed of five distinct units: an enzymatic trigger, a self-immolative linker[9] and two potent anticancer drugs articulated around a chemical amplifier. Enzymatic cleavage of the trigger–linker bond generates intermediate **2**, which then falls apart spontaneously giving rise to anticancer activity. In this study, we demonstrated for the first time that the chemical amplifier can play two crucial roles in the efficiency of this targeting strategy. First, the amplifier is responsible for signal amplification transforming a single enzymatic event into a double drug release. Second, in the course of this process, the amplifier is converted into a third highly toxic species that, combined with the two other drugs, leads to selective and potent cancer tritherapy.

The device **1** was designed to simultaneously target two established agents: the widely used doxorubicin and the well known histone deacetylase inhibitor MS-275. As illustrated in Scheme 1b, this heterodimeric prodrug includes a nitrobenzylphenoxy carbamate linker between the glucuronide trigger and amplifier unit. With this design, the enzyme substrate is located at a substantial distance from the two bulky drugs to allow easy recognition of **1** by β-glucuronidase. Thus, enzyme-catalyzed cleavage of prodrug **1** should result in the release of phenol intermediate **2**, which induces the release of aniline **3** through a 1,6-elimination process. Once turned on, the amplifier first causes the expulsion of doxorubicin via a 1,4-elimination followed by spontaneous decarboxylation. Addition of water to *ortho*-azaquinone methide **6** then generates aniline **7** thereby permitting the release of MS-275 along with the formation of derivative **8**. As azaquinone methides are potential alkylating species,[10] we anticipated that **8** could also be toxic toward cancer cells thus playing the role of a third antitumor agent.

We chosen β-glucuronidase as the triggering enzyme since it has been detected in high levels in a wide range of malignancies, such as breast, lung, colon and ovarian carcinomas, as well as melanomas.[11] Tietze[12] was one of the first researchers to propose this tumor-specific enzyme as a target for selective therapy using nontoxic glucuronide prodrugs in the course of a prodrug monotherapy (PMT[11b]). Since then, several glucuronide prodrugs[13] have been evaluated in vivo demonstrating superior efficacy compared to standard chemotherapy.[14] The efficiency of this approach is, however, limited by the reduced turnover of β-glucuronidase in the tumor microenvironment. Indeed, the optimal pH for β-glucuronidase activity is around 4, whereas the pH of tumor extracellular media is 6–7. In this context, the use of novel targeting devices such as **1** should circumvent this problem through the release of several drug units after a single enzymatic hydrolysis.

The synthesis of prodrug **1** was carried out starting from alcohol **9**,[15] previously described in the literature (Scheme 2). First, protecting groups of the carbohydrate moiety were modified via a five-step strategy to yield fully allyl-protected glucuronyl derivative **10**. Protected in this way, the glucuronide can be entirely deprotected in the course of a one-step procedure under mild conditions at the end of the synthesis.[16] Treatment of benzyl alcohol **10** with 4-nitrophenyl chloroformate and pyridine afforded activated carbonate **11** (84%). Aniline **12**[17] was introduced chemoselectively via nucleophilic

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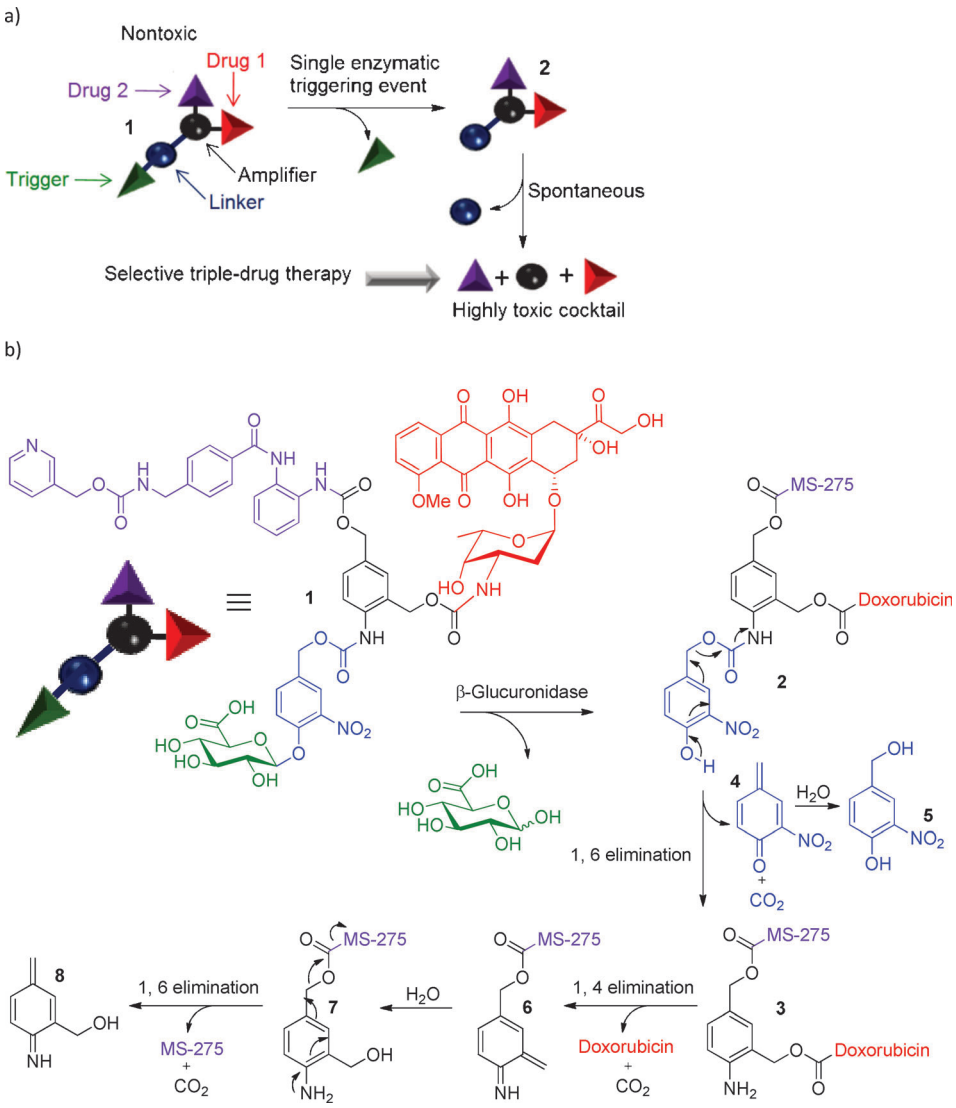



**Scheme 1.** a) A schematic representation of the principle of selective cancer tritherapy; b) The β-glucuronidase-catalyzed drug release mechanism.

substitution to give diol **13** (85%). The two primary alcohols were then activated in the presence of 4-nitrophenyl chloroformate to produce biscarbonate **14** (95%). At this stage, we were delighted to find that the aniline of MS-275 reacted preferentially with the *para*-carbonate leading to carbamate **15** (38%). Only trace amounts of the *ortho* regioisomer were detected by HPLC/MS in the reaction mixture. The structure of **15** was confirmed using two-dimensional NMR techniques (HMQC, HMBC). It is worth mentioning that the same regioselectivity was also recorded with another aniline-containing drugs, such as CI-994 (see Supporting Information). The synthesis was continued by the introduction of doxorubicin on the remaining activated *ortho*-carbonate to form protected heterodimer **16** (71%). In light of these results, biscarbonate **14** seems to be a suitable platform to allow the successive introduction of an aniline-containing drug and a second cytotoxic of a different nature. Thus, since drug combinations are usually employed in conventional cancer chemotherapy, compound **14** may permit the synthesis of the most appropriate heterodimeric prodrug to target a given tumor. Targeting device **1** was finally obtained by the cleavage of protecting groups using catalytic amount of tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) in the presence of two equivalents of aniline (80%).

The stability of prodrug **1** was examined both in phosphate buffer (pH 7) and bovine serum at 37 °C. No decomposition was observed by HPLC after 24 h under these conditions. Enzymatic hydrolysis of heterodimer **1** was then carried out using β-glucuronidase and monitored by HPLC/MS (Figure 1). When incubated with the enzyme, prodrug **1** was rapidly cleaved leading to the release of intermediate **2**. As expected, the presence of the nitrobenzylphenoxy carbamate linker allowed good recognition of the glucuronide trigger by β-glucuronidase. As soon as phenol **2** was produced in the medium, it disappeared over 3 h concomitantly with the appearance of the two drugs. Thirty minutes after the addition of β-glucuronidase, nitrophenol **5** and azaquinones **6** and **8** were also detected in the mixture (see Supporting Information). Doxorubicin was released within 5 h, whereas MS-275 was released significantly slower (within 18 h,

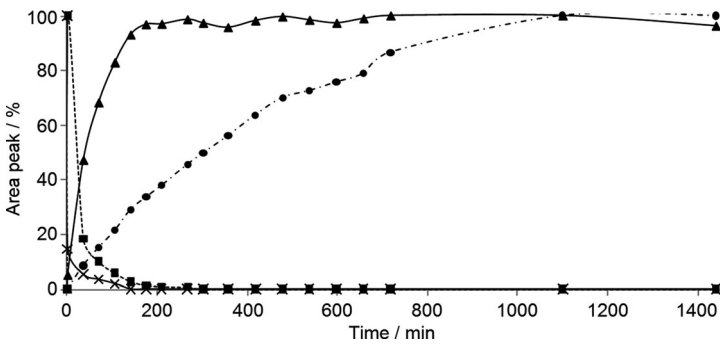
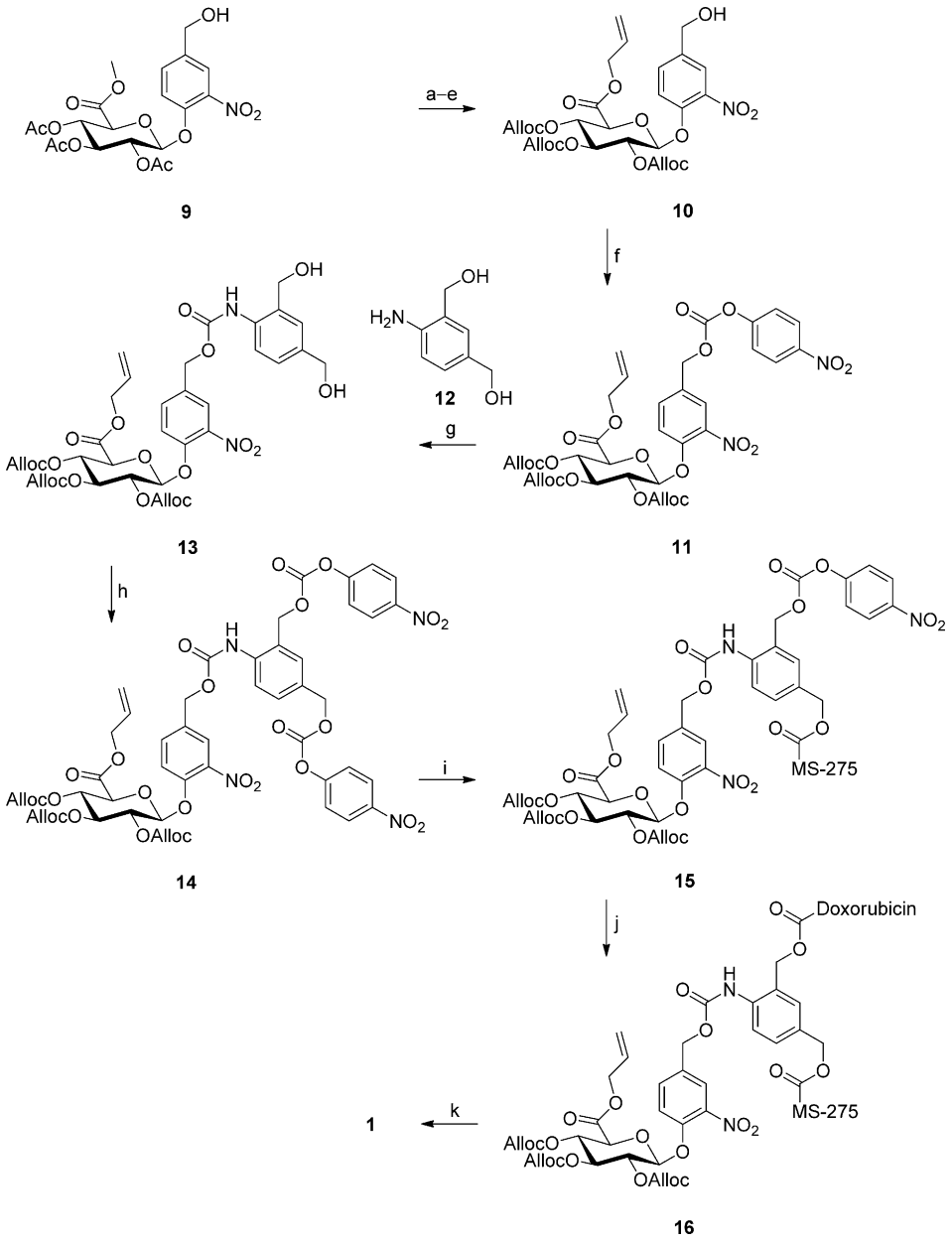



**Figure 1.** *E. coli* β-glucuronidase-catalyzed release of doxorubicin and MS-275 from prodrug **1** (phosphate buffer, pH 7, 37 °C): dox: (▲); MS-275: (●); **1**: (×); **2**: (■).




**Scheme 2.** Synthesis of prodrug **1**. *Reagents and conditions*: a) TBDMSCl, imidazole, $CH_2Cl_2$, RT, 5 h, 95%; b) MeONa, MeOH/THF (1:1), 0 °C, 2 h, 69%; c) AllONa, AllOH, RT, 1 h, 74%; d) AllocCl, pyridine, RT, 24 h, 80%; e) HF/pyridine (7:3), RT, 1 h, 95%; f) *p*-nitrophenyl chloroformate, pyridine, $CH_2Cl_2$, 0 °C→RT, 2 h, 84%; g) **12**, HOBt, DMF, 50 °C, 12 h, 85%; h) *p*-nitrophenyl chloroformate, pyridine, $CH_2Cl_2$, 0 °C→RT, 2 h, 95%; i) MS-275, HOBt, DMF, RT, 36 h, 39%; j) Doxorubicin·HCl, $Et_3N$, HOBt, DMF, RT, 3 h, 71%; k) $Pd(PPh_3)_4$, aniline, MeOH/$CH_2Cl_2$ (1:9), RT, 24 h, 80%.

Figure 1). Taken together, these results confirm that the disassembly of **1** proceeds through the self-immolative mechanism illustrated in Scheme 1. In contrast with the observations reported in a recent model study,[17] the 1,4-elimination occurred unambiguously faster than the 1,6-elimination reaction from disubstituted aniline **3**. This suggests that the nature of the amines attached at both benzylic positions via a carbamate functional group influences the kinetics of each elimination process.

Targeting device **1** was then evaluated for its antiproliferative activity against H290 lung mesothelioma cells after 48 h treatment (Figure 2). When incubated alone in the culture medium, prodrug **1** did not exhibit significant toxicity. Thus, the derivatization of the two drugs in the form of prodrug **1** markedly reduced their antiproliferative activity. As expected, the hydrophilicity imparted by the glucuronide trigger prevented passive cellular uptake and further intracellular activation of prodrug **1** by lysosomal β-glucuronidase. This hypothesis was supported by confocal microscopy experiments, made possible by the auto-fluorescence of doxorubicin. As illustrated in Figure 3b, prodrug **1** did not penetrated into H290 cells, whereas the free drug was internalized as proved by the intracellular localization of doxorubicin red fluorescence (Figure 3a).

On the other hand, addition of the enzyme in the culture medium induced a dramatic antiproliferative effect with an $IC_{50}$ value of 50 nM (Table 1). Under these conditions, β-glucuronidase-mediated release of doxorubicin was confirmed by confocal microscopy (Figure 3c) and cell-cycle arrest experiments that indicated an increase in G2/M phase cell population compared to that of the G0/G1 phase (Figure 4a). Furthermore, Western blot analysis showed that enzymatic activation of prodrug **1** restored the ability of MS-275 to inhibit histone deacetylase (Figure 4b). These results demonstrate the efficient release of

**Table 1.** $IC_{50}$ values (μM) of prodrug **1** and the individual components determined by cell growth inhibition assays.[a]

| Agent | H290 | H661 | H157 |
|---|---|---|---|
| Dox | 0.372 ± 47 | 0.342 ± 21 | 0.924 ± 91 |
| MS-275 | 0.754 ± 71 | 2.460 ± 191 | 3.285 ± 648 |
| Dox + MS-275 | 0.221 ± 19 | 0.290 ± 11 | 0.866 ± 56 |
| **1** | na | na | na |
| **1** + β-Glu | 0.048 ± 5 | 0.085 ± 4 | 0.380 ± 36 |

[a] Values represent the mean ± SEM of seven independent experiments performed in triplicate; na: no activity.

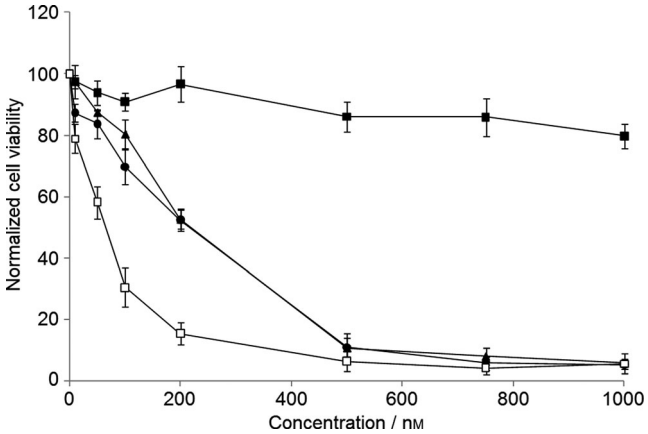



**Figure 2.** Viability of H290 lung mesothelioma cells treated for 48 h with the indicated compounds from 0 to 1000 nM. Seven experiments were performed in triplicate; standard error of the mean (SEM) values are indicated: MS-275 + Dox: (●); **17** + **18** + β-Glu: (▲); **1**: (■); **1** + β-Glu: (□).

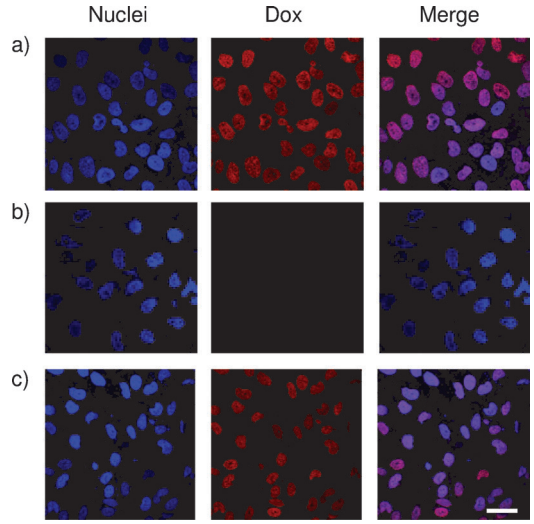



**Figure 3.** Confocal microscopy of doxorubicin (Dox) in H290 cells treated for 3 h with 10 μM of a) doxorubicin, b) prodrug **1**, c) prodrug **1** + β-glucuronidase (40 U mL$^{-1}$). Nuclei were stained with 4′,6-diamidino-2-phenyl-indole (DAPI). Scale bar: 50 μm.

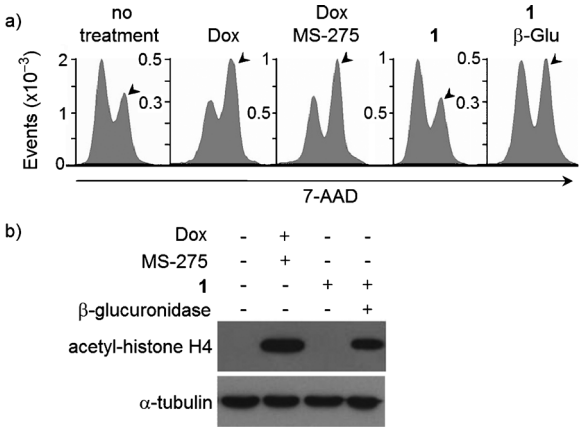
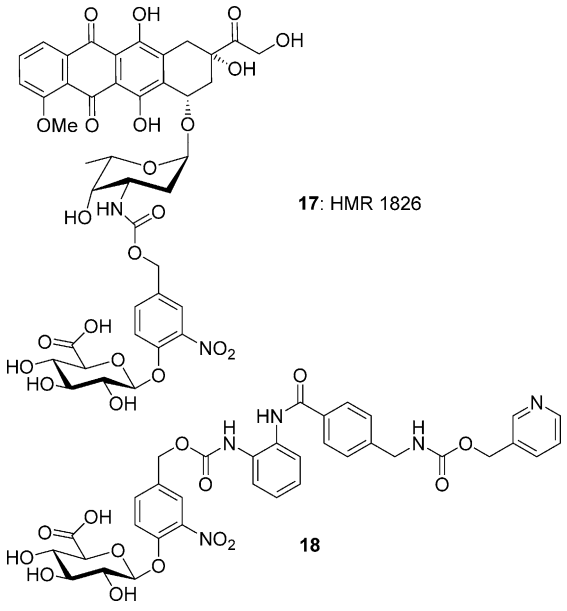



**Figure 4.** a) Cell-cycle arrest analysis. H290 cells were treated for 24 h with the indicated compounds. Arrowheads indicated cells in the G2/M phase; b) Western blot analysis of histone H4 acetylation. H290 cells were treated for 16 h with the indicated compounds, and α-tubulin was used as a loading control.

both active drugs in the culture medium. However, as activated heterodimer **1** was fivefold more toxic than the combination of the two anticancer agents ($IC_{50}$ = 50 nM vs 250 nM; Table 1), the recorded cytotoxicity cannot be solely attributed to the release of doxorubicin and MS-275. The antiproliferative activity of heterodimer **1** was further evaluated against H661 and H157 lung cancer cells. Again, upon β-glucuronidase activation, prodrug **1** was drastically more toxic than the combination of the two drugs (Table 1).

In order to examine the cause of this surprising effect, we first studied the potential toxicity of phenol **5** and aniline **12**, which can be formed by the hydrolysis of **8** in the culture cell medium. Within this framework, cell-growth inhibition assays clearly indicated that H290 cells are not sensitive at all to compounds **5** and **12** either alone or combined with the two drugs, which ruled out their implication in the observed toxicity. We next investigated the antiproliferative activity of the two monomeric glucuronide prodrugs **17** and **18**. Indeed, in the presence of β-glucuronidase, the combination of these compounds cause the release of doxorubicin, MS-275 and quinone




methide **4**, which could be responsible for the additional toxicity. However, when **17** and **18** were incubated together with the enzyme, the recorded cytotoxicity was identical to that observed for the mixture of the two drugs ($IC_{50}$ = 250 nM; Figure 2). In light of these results, it became clear that the amplifier unit was unambiguously involved in the cytotoxicity of **1** via its transformation into azaquinone methide **8**.

In conclusion, we developed the first targeting system that includes a chemical amplifier programmed both to release two

potent anticancer drugs and become cytotoxic once the amplification process is completed. Such a system is stable under physiological conditions, but allows a triple drug release in a stringently controlled fashion after a single tumor-associated triggering event. We anticipate that the amplifier unit employed in our study can be incorporated into other targeting assemblies. Thus, our findings could represent an important step in the search for efficient anticancer prodrugs for selective polychemotherapy of solid tumors.

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**Keywords:** cancer · chemical amplifiers · dendrimers · glucuronides · prodrugs

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