Prodrug Activation

Prodrug Activation Gated by a Molecular "OR" Logic Trigger**

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Molecular logic gates are increasingly important in attributing chemical reactivity to molecular devices.[1–5] Specific input signals of basic logic gates can be programmed into single molecules that generate readable output signals, such as fluorescence[6] or UV/Vis light.[7] Here we show that the release of an active drug molecule through a prodrug activation process is a viable output signal. The term "prodrug" is used to indicate that a chemical derivatization has been applied to alter the physicochemical properties of a drug.[8,9] The prodrug is converted into the active drug in vivo by metabolic processes or environmental conditions. Masking of a functional group in a targeted drug with a simple linker which contains two moieties that can be cleaved by different mechanisms can generate a molecular "OR" logic gate trigger. The gate is activated upon a cleavage signal from either of the two input ports. The signal will be translated into a bond cleavage that releases the active drug molecule.

Several anticancer prodrugs have been designed for selective activation in malignant tissues by a specific enzyme secreted within the proximity of the tumor.[10] A prodrug with a molecular "OR" logic gate triggering device could potentially target two different cancerous tissues with different enzyme-expression patterns. There are several examples for enzymes that are present in elevated levels in malignant tissues.[11] The substrates of two of these enzymes could be introduced in the molecular "OR" logic trigger to generate an agent for dual-prodrug monotherapy. Prodrug strategies are sometimes based on the assumption that a particular enzyme is elevated in the tumor tissues and that activation of a prodrug in the tumor tissue will result in a significantly improved therapeutic index. While there is much evidence for overexpression of particular enzymes in tumors, consistent patterns of expression are elusive. Consequently, attempts to develop drugs activated by tumor-specific enzymes in general have not been very successful. Prodrugs with two modes of activation could offer an advantage over single-triggered prodrugs, particularly in circumstances where two different enzymes are present in elevated levels in various regions of the tumor. Herein, we report the design, synthesis, and bioactivation of a molecular "OR" logic trigger which is used to activate a prodrug with dual susceptibility to enzymatic activity.

Classical "OR" logic gates have two input ports and one output port.[12] An activating signal, which operates on either one of the input ports, activates the output signal of the gate (Figure 1). Obviously, positive input signals from both input ports should also activate the gate.

Diethylenetriamine has previously been introduced in a multitriggering mechanism[13] and is used as a linker in the construction of the molecular "OR" logic trigger. The central secondary amine is attached to a drug molecule while the two primary amines are linked to different enzymatic substrates (triggers 1 and 2). The enzymatic cleavage of either one of the substrates generates a free-amine intermediate (intermediate 1 or 2) that initiates an intracyclization reaction to release the free drug unit (Figure 2).

To provide a proof of concept for the "OR" triggering release mechanism suggested in Figure 2, we prepared **1**, which contains two different enzymatic substrates and 4-nitrophenol, as a model compound that represents a potential drug. The two substrates were a phenylacetamide moiety, which is the substrate for penicillin G amidase[14] (PGA), and a retro-aldol retro-Michael substrate that is cleaved by catalytic antibody (Ab) 38C2.[15–17] According to the proposed pathway presented in Figure 3, cleavage by antibody 38C2 or by PGA should catalyze the formation of intermediates **2** or **3**, respectively, and subsequent intracyclization will release 4-nitrophenol.

The "OR" logic trigger substrate **1** was incubated with either antibody 38C2 or PGA in phosphate-buffered saline (PBS) solution (pH 7.4) at 37 °C. The formation of 4-nitro-

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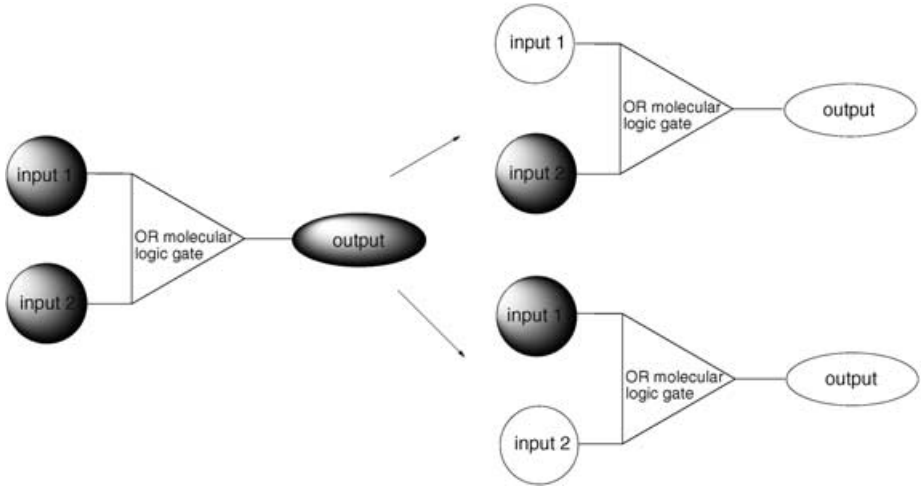

Figure 1. Schematic illustration of an "OR" molecular logic gate. Activation of the gate is indicated by a color change from black to white.

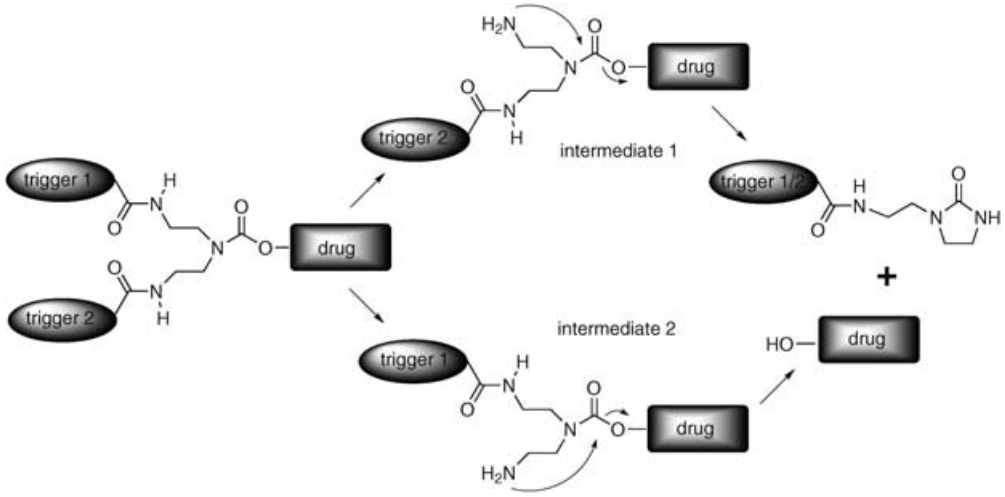

Figure 2. Diethylenetriamine as an "OR" molecular logic trigger.

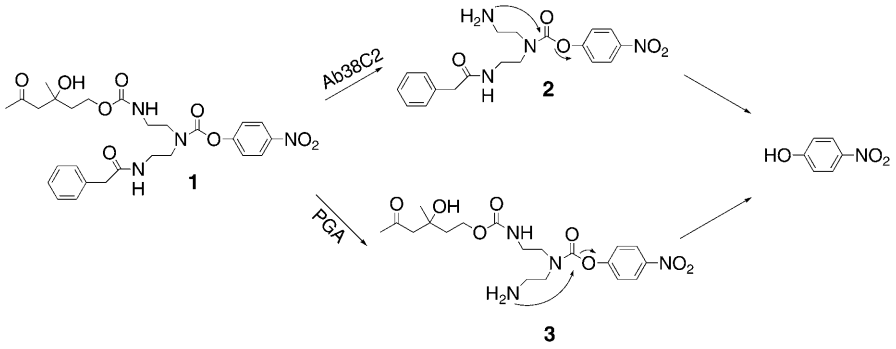

Figure 3. Activation of a molecular "OR" logic trigger by a dual-triggering mechanism with PGA or catalytic antibody (Ab) 38C2.

phenol was monitored by UV/Vis spectroscopy at a wavelength of 405 nm. Figure 4 clearly shows that either the enzyme or the catalytic antibody initiates the activation of compound **1** to release 4-nitrophenol. The reaction was faster with PGA than with antibody 38C2. No release was observed when the substrate was incubated in buffer alone.

Next, we prepared an actual prodrug (pro-Dox) by equipping the "OR" molecular logic trigger with the anticancer drug doxorubicin (Dox). 4-Hydroxybenzyl alcohol was used as a linker between the amino group of Dox and diethylenetriamine (Figure 5). As with the model compound, the two substrates were a phenylacetamide and a retro-aldol retro-Michael substrate. Cleavage by either antibody 38C2 or PGA should result in removal of the 4-hydroxybenzyl alcohol and release of free Dox (through 1,6-elimination).

The prodrug was incubated with either PGA or catalytic antibody 38C2, and the release of free Dox was monitored by reverse-phase HPLC. The HPLC chromatograms in Figure 6 show that upon incubation of PGA (Figure 6a) or antibody 38C2 (Figure 6b) with pro-Dox, intermediates 1 and 2 (shown in Figure 2) were generated and intracyclization liberated the active drug Dox. No release of the drug was observed in the absence of PGA or the antibody.

The "OR" logic gate prodrug was assessed in cell growth inhibition assays. We evaluated the ability of the prodrugs to inhibit cell proliferation in the presence of PGA or catalytic antibody 38C2 using two different cell lines: the human T-lineage acute lymphoblastic leukemia (ALL) cell line MOLT-3 and the human erythroleukemia cell line HEL. The data are shown graphically in Figure 7, and the results are summarized in Table 1. Both PGA and antibody 38C2 could activate the prodrug pro-Dox, and cell growth was inhibited with IC_{50} values close to that of the parent drug.

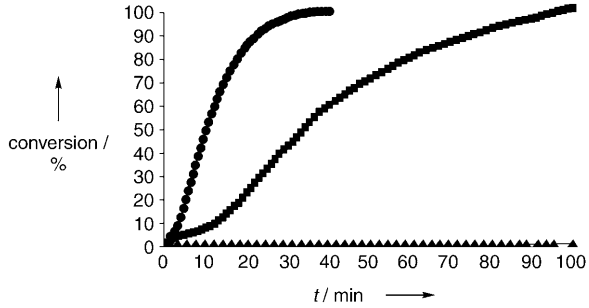

Figure 4. UV/Vis Absorbance at $\lambda = 405$ nm as a function of time in the activation of "OR" molecular logic trigger **1**. ● = **1** + PGA, ■ = **1** + Ab38C2, ▲ = **1** in PBS (pH 7.4). Concentration of substrate is 500 μM; concentrations of PGA or Ab38C2 are 50 μM each.

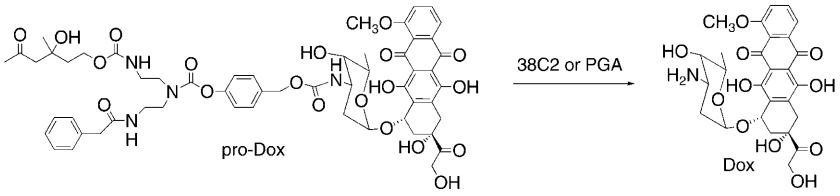

Figure 5. Activation of doxorubicin prodrug by a molecular "OR" logic trigger with antibody 38C2 or PGA.

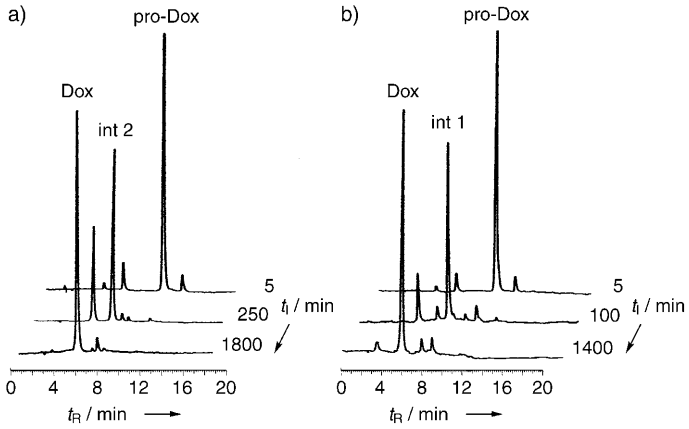

Figure 6. Reverse-phase HPLC chromatograms of a) pro-Dox (70 μM) with PGA (4 μM) in PBS (pH 7.4) and b) pro-Dox (70 μM) with catalytic antibody 38C2 (20 μM) in PBS (pH 7.4). t_R = retention time; t_I = incubation time.

Table 1: IC_{50} values from cell growth inhibition assays.

Drug/Prodrug	IC_{50} [nM]	
	MOLT-3 cells	HEL cells
Dox	3.0	20
pro-Dox	80	200
pro-Dox/38C2	6.5	24
pro-Dox/PGA	7.0	28

A prodrug with a molecular "OR" logic trigger substrate could be used as an efficient tool to evaluate the catalytic activity of the triggering enzymes. In fact, the system allows a

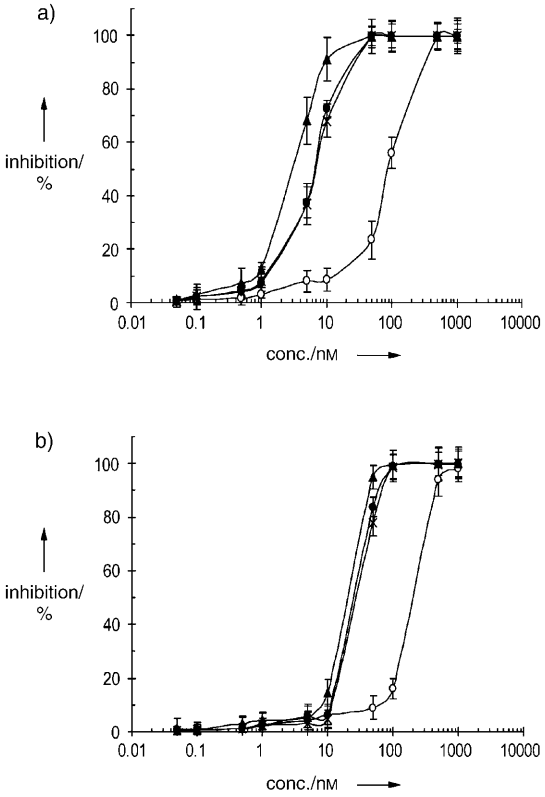

Figure 7. Growth inhibition activity of pro-Dox in the presence and absence of antibody 38C2 and PGA. The growth response of leukemia cell lines MOLT-3 (a) and HEL (b) cells to incubation (72 h) with increasing concentrations of the prodrug in the presence and absence of 38C2 catalytic antibody (1 μM; ●) or PGA (1 μM; ×) was analyzed by using a standard ^3H thymidine proliferation assay. Dox (▲) and pro-Dox (○) were also evaluated. Data points and error bars represent mean values ± standard deviation, respectively.

direct comparison to be performed between the activities of PGA and antibody 38C2 by using the same prodrug molecule. We used a fixed concentration of the pro-Dox compound (50 nM) and varying concentrations of antibody 38C2 or PGA. The results in Figure 8 show that PGA was about 50-fold more

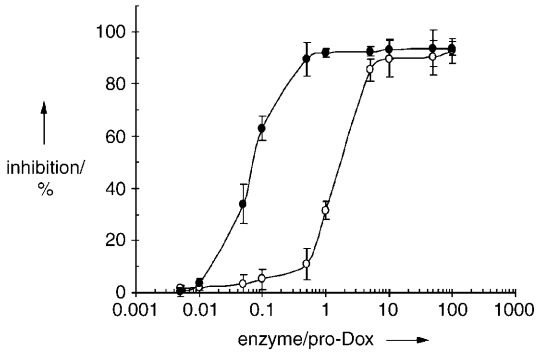

Figure 8. Growth inhibition of HEL cells in the presence of pro-Dox (50 nM) and increasing concentrations of antibody 38C2 (○) or PGA (●), shown as the ratio of enzyme to pro-Dox. Data points and error bars represent mean values ± standard deviation, respectively.

active in growth inhibition of HEL cells than antibody 38C2. Similar results were obtained with MOLT-3 cells (data not shown).

In summary, we have designed and synthesized a molecular "OR" logic trigger operated by two different enzymes. The "smart" linker that is used to mask the doxorubicin amine functionality acts as a dual-input "OR" logic trigger. The input signals are enzymatic cleavages by antibody 38C2 or penicillin G amidase, and the output is the release of the active drug. Our current efforts are focused on the design and synthesis of prodrugs that are activated with a molecular "AND" logic trigger.

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