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Supporting Information

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Prodrug Activation Gatet by a Molecular OR Logic Trigger

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Experimental

General methods. Thin layer chromatography (TLC): silica gel plates Merck 60 F_{254}: compounds were visualized by irradiation with UV light and/or by treatment with a solution of 25 g phosphomolybdic acid, 10 g $Ce(SO_4)_2 \cdot H_2O$, 60 mL conc. H_2SO_4 and 940 mL H_2O followed by heating and/or by staining with a solution of 12 g 2,4-dinitrophenylhydrazine in 60 mL conc. H_2SO_4, 80 mL H_2O and 200 mL 95% EtOH followed by heating. – Flash chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. ^{1}H NMR spectra were measured using Bruker Avance operated at 200 MHz. The chemical shifts are expressed in δ relative to TMS (δ = 0 ppm) and the coupling constants *J* in Hz. The spectra were recorded in $CDCl_3$ as solvent at room temperature unless stated otherwise. All general reagents, including salts and solvents, were purchased from Aldrich (Milwaukee, MN).

Abbreviations. Boc- t-butoxycarbonyl, CDI- Carbonyl diimidazol, DCM- Dichloromethane, DMAP- Dimethyl aminopyridine, DMF- Dimethylformamide, EtOAc- Ethyl acetate, Et_3N- Triethylamine, Hex- n-Hexane, MeOH- Methanol, PBS- Phosphate buffer saline, PNP- 4-Nitrophenyl, THF – tetrahydrofuran.

Synthesis of Compound 1:

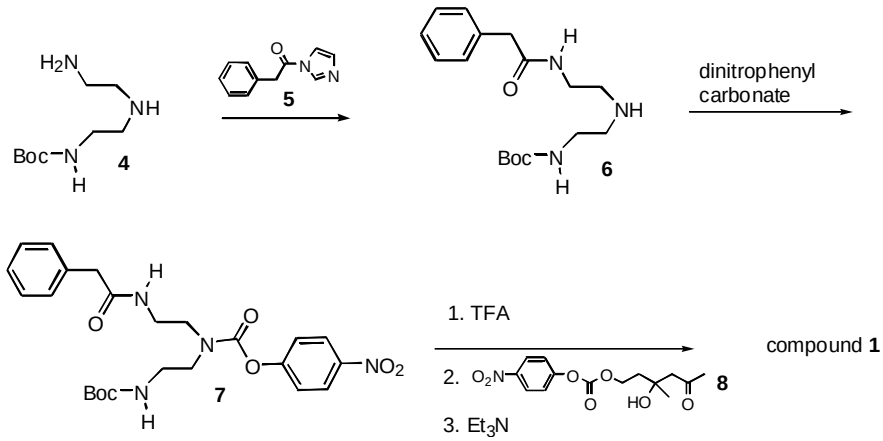

Figure 1: Synthesis of a molecular OR logic trigger with two different enzymatic substrates and 4-nitrophenol as a model drug.

Compound 6

Commercially available phenyl acetic acid (314 mg, 2.3 mmol) was dissolved in THF (10 ml). CDI (374 mg 2.3 mmol) was added and the reaction was monitored by TLC (EtOAc:Hex=1:1) for the complete disappearance of starting materials. The activated phenylacetyl imidazol amid **5** was then added dropwise to a stirred solution of tert-butyl 2-[(2-aminoethyl)-amino] ethylcarbamate **4**[1] (477 mg, 2.31 mmol) in THF (5 ml). After completion the solvent was removed under reduced pressure. The residue was dissolved in DCM and washed with water. The organic layer was dried over

magnesium sulfate, and the solvent was removed under reduced pressure. The crude product was used without further purification (677 mg, 91%).

^{1}H NMR (200MHz,$CDCl_3$): δ = 7.38-7.26 (5H, m); 3.58 (2H, s); 3.32-3.22 (4H, m); 2.72-2.61 (4H, m), 1.46 (9H, s). ^{13}C NMR (200MHz,$CDCl_3$): δ = 171.4, 156.1, 135.1, 129.4, 129.0, 127.3, 80.4, 48.8, 48.2, 43.8, 40.4, 39.3, 28.4. HRMS (CI) Calcd. for $C_{17}H_{27}N_3O_3$ 322.2131 $[MH]^+$, found 322.2130.

Compound 7

Compound **6** (660 mg, 2.1 mmol) was dissolved in DMF (4 ml). Et_3N (426 µl, 3.0 mmol) was added, followed by the addition of bis(4-nitrophenyl) carbonate (760 mg, 2.5 mmol) and the solution was stirred for 10 minutes. After completion the mixture was diluted with EtOAc (100 ml) and washed with brine. The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc) to give pure compound **7** in the form of pale yellow oil (457 mg, 46%).

^{1}H NMR (200MHz,$CDCl_3$): δ = 8.28-8.21 (2H, m); 7.35-7.25 (7H, m); 3.57-3.31 (10H, m); 1.41 (9H, s). ^{13}C NMR (200MHz,$CDCl_3$): δ = 171.8, 156.1, 154.0, 144.9, 134.9, 129.3,128.8,127.2, 125.0, 122.4, 122.1, 79.6, 48.5, 43.5, 39.1, 38.3, 29.6, 28.4.

Compound 1

Compound **7** (102 mg, 0.21 mmol) was deprotected with 2 ml TFA to remove the Boc group. The excess of the acid was removed under reduced pressure and the residue was dissolved in 2 ml DMF. Carbonate **8**[2] (100 mg, 0.31 mmol) and 0.5 ml Et_3N were added and the solution was stirred for 10 minutes. After completion the solvent was removed under reduced pressure. The crude product was purified by column

chromatography on silica gel (EtOAc) to give pure compound **1** in the form of pale yellow oil (60 mg, 51%).

^{1}H NMR (200MHz,$CDCl_3$): δ = 8.23 (2H, d, *J = 9.0 Hz*); 7.27-7.20 (7H, m); 4.22-4.12 (2H, m); 3.61-3.26 (10H, m); 2.62-2.60 (2H, m); 2.14 (3H, s); 1.82-1.75 (2H, m); 1.21 (3H, s). ^{13}C NMR (200MHz,$CDCl_3$): δ =210.5, 171.9, 156.7, 155.9, 154.0, 144.9, 134.5, 129.3, 128.9, 127.3, 125.0, 122.2, 70.4, 61.4, 52.5, 48.8, 43.6, 40.2, 38.5, 31.7, 29.6, 14.0. HRMS (MALDI) Calcd for $C_{27}H_{34}N_4O_9$ 581.2218 $[MNa]^+$, found 581.2214.

Synthesis of pro-Dox:

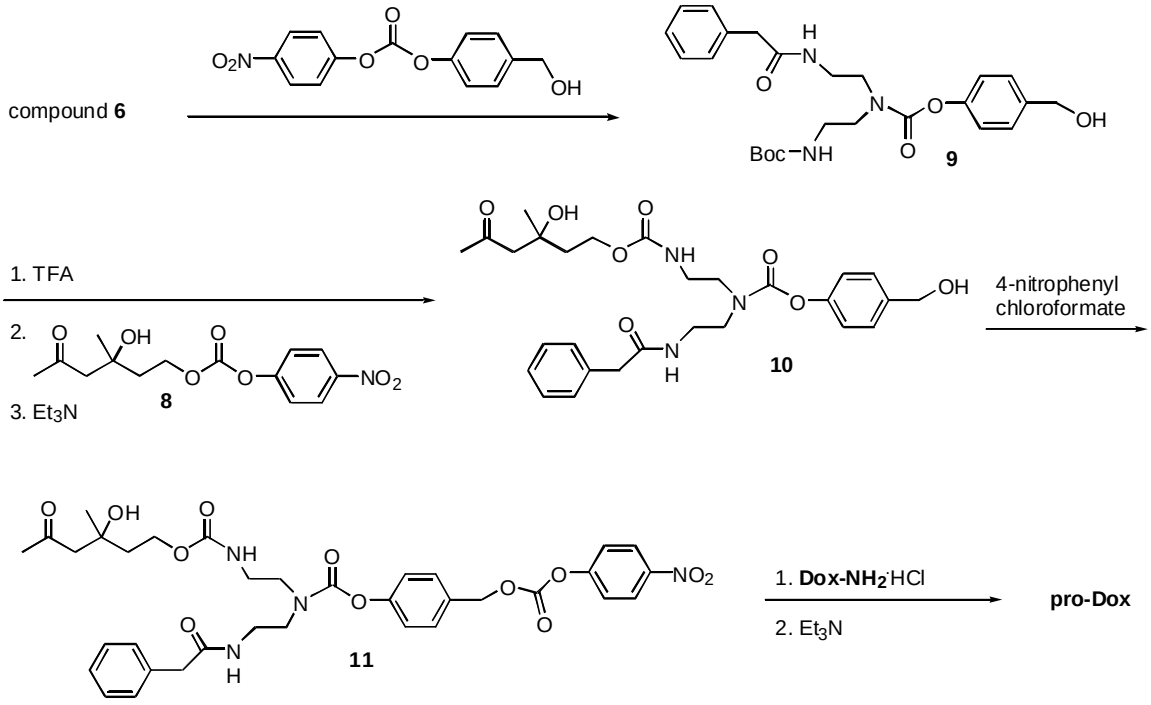

Figure 2: Synthesis of a doxorubicin prodrug with a molecular OR logic gate trigger.

Compound 9

Compound **6** (250 mg, 0.78 mmol) was dissolved in DMF (3 ml). Et_3N (162 µl, 1.2 mmol) was added, followed by the addition of Carbonic acid 4-hydroxymethyl-phenyl ester 4-nitrophenyl ester (337 mg, 1.2 mmol). The reaction was monitored by TLC (EtOAc). After completion the mixture was diluted with EtOAc (50 ml) and washed first with saturated NH_4Cl and brine. The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc) to give pure compound **9** in the form of pale yellow oil (250 mg, 68%).

^{1}H NMR (200MHz,$CDCl_3$): δ = 7.43-7.21 (7H, m); 7.06 (2H, d, *J = 8.5 Hz*); 6.47-6.18 (1H, m); 4.94 (1H, bs); 4.68 (2H, s); 3.58-3.30 (10H, m); 1.42 (9H, s). ^{13}C NMR (200MHz,$CDCl_3$): δ = 171.8, 156.2, 155.6, 150.4, 138.7, 135.0, 129.4, 128.9, 127.9, 127.2, 121.8, 79.6, 64.4, 48.5, 48.2, 43.6, 39.4, 38.7, 28.4. HRMS (ES) Calcd. for $C_{25}H_{33}N_3O_6$ 472.2442 $[MH]^+$, found 472.2451.

Compound 10

Compound **9** (124 mg, 0.26 mmol) was deprotected with 2 ml TFA to remove the Boc group. The excess of the acid was removed under reduced pressure and the residue was dissolved in 1.5 ml DMF. Carbonate **8**[2] (107 mg, 0.34 mmol) and 0.5 ml Et_3N were added and the solution was stirred for 10 minutes. After completion the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc: MeOH=9:1) to give pure compound **10** in the form of pale yellow oil (140 mg, 98%).

^{1}H NMR (200MHz,CD_3OD): δ = 7.38-7.21 (7H, m); 7.09 (2H, d, *J = 8.5 Hz*); 4.60 (2H, s); 4.17-4.07 (3H, m); 3.55-3.30 (10H, m); 2.68-2.63 (2H, m); 2.15 (3H, s); 1.87-

1.83 (2H, m); 1.22 (3H, s). ^{13}C NMR (400MHz, CD_3OD): δ =211.2, 174.4, 159.1, 156.9, 151.8, 140.1, 136.7, 130.2, 130.1, 129.6, 128.8, 128.0, 71.5, 64.6, 62.4, 54.6, 44.0, 41.5, 40.2, 38.7, 32.1, 30.7, 27.5, 14.4. HRMS (ES) Calcd. for $C_{28}H_{37}N_3O_8$ 544.2653 $[MH]^+$, found 544.2642.

Pro-Dox

Compound **10** (117 mg, 0.22 mmol) was dissolved in THF (5 ml). PNP-chloroformate (65 mg, 0.32 mmol) was added, followed by the addition of Et_3N (90 μM, 0.65 mmol) and catalytic amount of DMAP. The reaction was monitored by TLC (EtOAc: MeOH=9:1). After completion the mixture was diluted with EtOAc (20 ml) and washed first with saturated NH_4Cl and brine. The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc: MeOH=9:1) to give pure compound **11** in the form of yellow oil (39 mg, 25%). The compound was immediately used for the next step. Compound **11** (39 mg, 55 μmol) was dissolved in DMF (1.5 ml). HCl salt of doxorubicine (23 mg, 39 μmol) and 0.5 ml Et_3N were added and the solution was stirred for 10 minutes. After completion the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc: MeOH=9:1) to give pure pro-Dox in the form of red powder (35 mg, 81%).

^{1}H NMR (400MHz,CDCl3): δ = 8.02 (1H, d, *J = 8.0 Hz*); 7.78 (1H, t, *J = 8.1 Hz*); 7.38 (1H, d, *J = 8.4 Hz*); 7.28-7.20 (7H, m); 7.00 (2H, d, *J = 7.5Hz*); 5.28 (2H, s); 5.07-4.96 (2H, m); 4.75 (2H, s); 4.59 (1H, bs); 4.15-4.10 (3H, m) 4.07 (3H, s); 3.85-3.76 (2H, m); 3.54 (1H, bs); 3.47-3.24 (12H, m); 3.01 (2H, d, *J = 18.8 Hz*); 2.65-2.54 (2H, m); 2.39-2.34 (2H, m) 2.17-2.10 (5H, m); 1.28 (3H, s); 1.15 (3H, s). ^{13}C NMR

(400MHz,CDcL3): δ =216.8, 213.2, 190.0, 189.6, 174.8, 164.0, 159.1, 158.6, 158.4, 158.3, 138.7, 138.4, 137.6, 136.7, 136.6, 132.7, 132.3, 131.8, 130.2, 130.3, 124.5, 123.8, 122.8, 121.4, 114.5, 114.3, 103.6, 80.7, 73.5, 72.5, 72.3, 70.3, 68.9, 68.4, 64.3, 59.6, 55.4, 51.6, 50.0, 46.6, 43.3, 42.8, 41.6, 36.9, 34.3, 33.0, 32.6, 29.9, 19.6, 17.0. HRMS (MALDI) Calcd. for $C_{56}H_{64}N_4O_{20}$ 1135.4006 $[MNa]^+$, found 1135.3986.

4-Nitrophenol Release Analysis – General Protocol.

Compound **1** (5 μL, 10 mM) in CH_3CN was dissolved in 95 μL of PBS solutions to yield 500 μM solutions. All solutions were kept at 37^0C**.** PGA (3.5 mg/ml) and Ab38C2 (10 mg/ml) PBS solutions were used to activate compound **1**.

Reporter release was monitored by following the formation of 4-nitrophenol with visible spectroscopy at a wavelength of 405 nm.

Doxorubicin Release Analysis –General Protocol.

Pro-Dox (5 μL, 2 mM) in DMSO was dissolved in 140 μL of PBS solutions to yield 70 μM solutions. All solutions were kept at 37^0C**.** PGA (1 mg/ml) and Ab38C2 (10 mg/ml) PBS solutions were used to activate the prodrug. Drug release was monitored by an HPLC assay using C-18 column, Wavelength: 450 nm, eluent: acetonitrile:water; programmed gradient , flow rate; 1 ml/min (figures S1 and S2).

Kinetic Data.

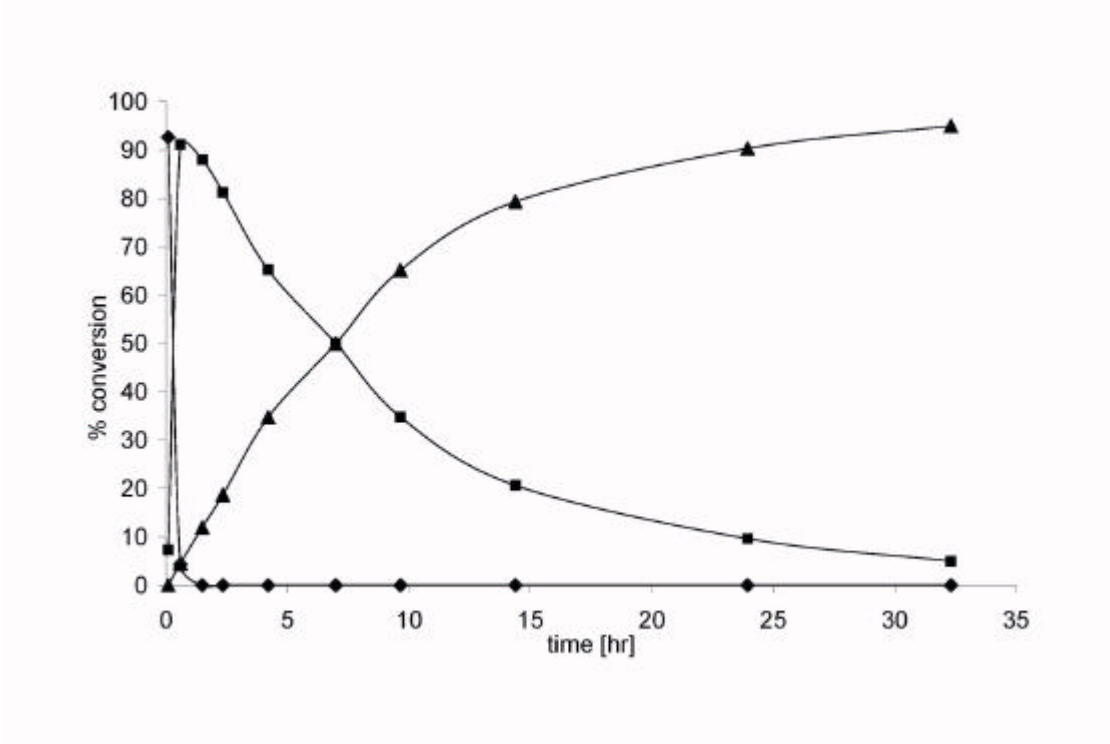

Figure 3: Release of Dox by PGA. Dox (?), pro-Dox (♦), intermediate I (¦).

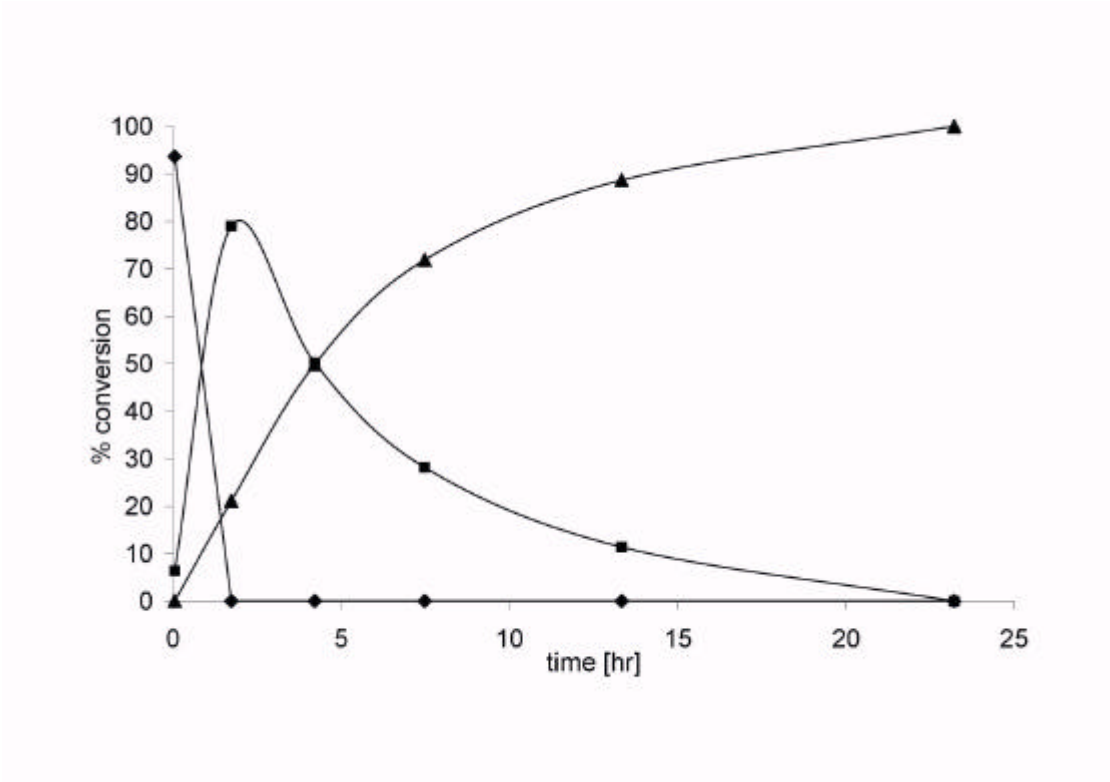

Figure 4: Release of Dox by cAb 38C2. Dox (?), pro-Dox (♦), intermediate II (¦).

Cell lines. Human T-lineage acute lymphoblastic leukemia (ALL) cell line MOLT-3, and human erythroleukemia cell line HEL were purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10 % FCS, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1 mM sodium pyruvate, and antibiotics (Gibco, Grand Island, NY).

PGA and Ab38C2 Preparation. A stock solution of 12.5 mg/mL (83.3 μM) 38C2 IgG in PBS (pH 7.4), stored at 4°C, was used. Antibody 38C2 is commercially available from Sigma-Aldrich (Steinheim, Germany). Penicillin G Amidase (PGA) was purchased from Sigma-Aldrich and used from the stock solution of 5.8 mg/mL (83.3 μM).

Cytotoxicity assays. Stock solutions of 2 mM doxorubicine (Dox) and dual-triggered prodrug (pro-Dox) in dimethylformamide were stored at 4°C. For cell-growth inhibition assays, 100 μM solutions of drugs in PBS were freshly prepared from the 2 mM stock solutions.

Cells were harvested from culture dishes, washed once with HBSS, resuspended in cell culture medium, and plated in 96-well tissue culture plate at a density of 5 x 10^3/well in 100 μL media. Drugs were further diluted in cell culture medium to yield final concentration of 50 pM -1 μM and added to the cells. For the prodrug activation experiments, 38C2 mAb or PGA at final concentration of 1 μM was mixed with prodrugs just before adding to the cells. After drug addition, the cells were incubated

for 72 h at 37°C in a humidified CO_2 incubator. [^{3}H]thymidine (ICN Radiochemicals) was added to 0.5 μCi per well (1 Ci = 37 GBq) during the last 8 h of incubation. The cells were frozen at -80°C overnight and subsequently processed on a multichannel automated cell harvester (Cambridge Technology, Cambridge, MA) and counted in a liquid scintillation beta counter (Beckman Coulter). The background was defined by running the same assay in the absence of drug. The inhibition in experiment E was calculated according to the following formula: (background - E)/background x 100%. All experiments were performed in triplicate.

For trigger titration assay, MOLT-3 cells, seeded at 5 x 10^3 per well in a 96-well tissue culture plates, were incubated with fixed at 25 nM concentration of pro-Dox in the presence of different increasing 38C2 or PGA concentrations ranging from 0.005 to 100 molar excess. Same conditions were used when HEL cells were seeded at 5 x 10^3 per well, except pro-Dox concentration was fixed at 50 nM. The cell-growth inhibition assays were performed as described above.

Assessment of apoptosis. To determine the extent of apoptosis, cells were stained with Phycoerythrin (PE)-conjugated annexin V and 7-AAD using the annexin V kit (BD PharMingen, San Diego, CA) as per manufacture's protocol. Briefly: Cells were collected at different time point, washed once with EDTA-free PBS, and then incubated for 15 min with a mixture containing annexin V-PE and 7-AAD in binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$]. After the incubation period, the supernatants were removed, and 400 μl of binding buffer was added to each sample. The fluorescence was analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) for the presence of viable (AV), and 7-AAD$^-$

), and late apoptotic/secondary necrotic (AV^+ and 7- early apoptotic (AV^+, 7-AAD AAD^+) cells.

The current model for the mechanism of action of most anticancer drugs is that treated cells die by apoptosis. To demonstrate that drug release by PGA or antibody 38C2, results in Dox-induced apoptosis of the MOLT-3 cells, annexin V/7-AAD binding experiments were performed (Figure 5). Cells were stained for annexin V/7-AAD and 7-AAD prior to flow cytometry analysis. Viable cells are negative for both markers, early apoptotic cells are annexin V positive, and late apoptotic/secondary necrotic . The annexin V/7-AAD assay confirmed that [3]cells are positive for both markers both Dox-treated and activated pro-Dox-treated cells undergo apoptosis. The majority of Dox-treated MOLT-3 cells were in either early or late apoptotic state after 24 h of incubation, whereas 48 h were required for Dox-induced apoptosis in activated pro-Dox-treated samples. Pro-Dox alone, however, was unable to induce apoptosis in MOLT-3 cells even after 72 h of incubation. The experiment was also performed in HEL cells and similar results were observed (data not presented). These data demonstrate that dual-trigger prodrug activation results in drug-induced apoptosis.

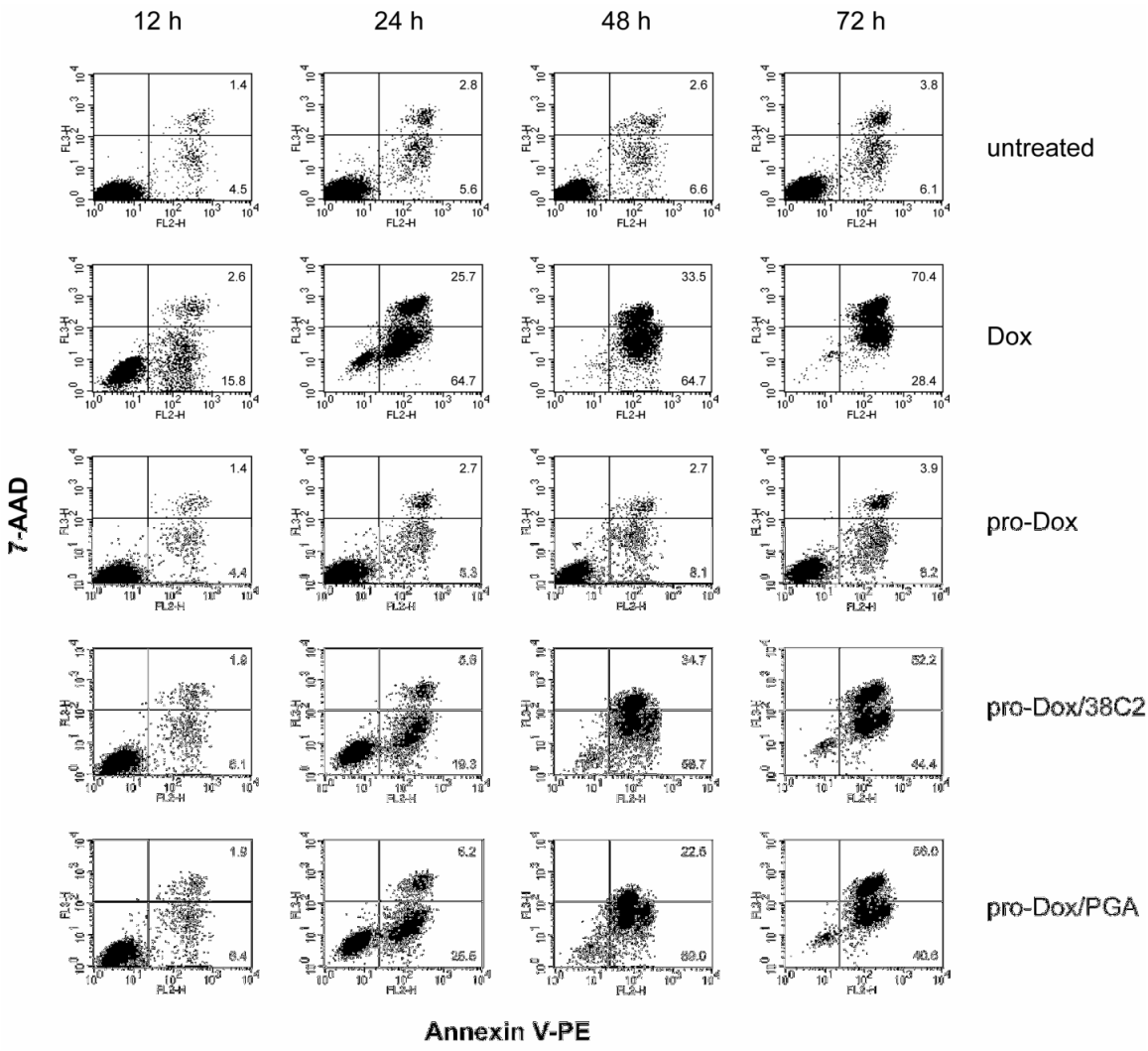

Figure 5: Two-color flow cytometry of demonstrating Dox-induced apoptosis in a leukemia cell line**.** MOLT-3 cells were treated with either 25 nM Dox or 25 nM pro-Dox in the presence or absence of 1 μM antibody 38C2 or 1 μM PGA for the periods of time described in the Methods section and were then stained for annexin V-PE and 7-AAD prior to analysis by flow cytometry. The *X* axis shows annexin V-PE fluorescence and the *Y* axis shows the 7-AAD fluorescence. The dot plots show clear separation of viable ($AV^-/7\text{-}AAD^-$; *lower left quadrant*), early apoptotic ($AV^+/7\text{-}AAD^-$; *lower right quadrant*), and late apoptotic/secondary necrotic ($AV^+/7\text{-}AAD^+$; *upper right quadrant*) cells. The percentages of cells in each quadrant are indicated.

References:

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