Self-Assembling Cytotoxins

DARRYL RIDEOUT

Decanal and *N*-amino-*N'*-1-octylguanidine (AOG), combined at 28 μ*M* each, mediated erythrocyte lysis within 80 minutes under physiological conditions. By contrast, no lysis was observed after 20 hours with either decanal (56 μ*M*) or AOG (100 μ*M*) alone. The pronounced synergism observed for these chemicals and similar reactive pairs of chemicals is due to the self-assembly of more cytotoxic hydrazones in situ. Decanal and AOG also exhibit synergistic activity against cultured human cells (HeLa) and bacteria (*Escherichia coli* J96). This synergism may be useful in the design of cytotoxins that would self-assemble selectively from nontoxic precursors within tumors, while sparing normal tissue.

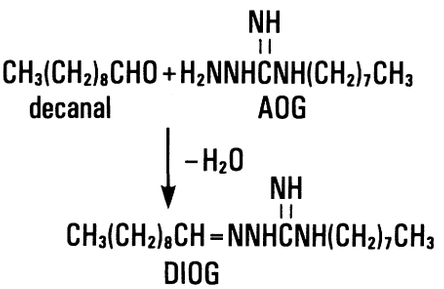

Fig. 1. Structural formulas and reaction scheme for decanal, AOG, and DIOG.

BIOCHEMICAL DIFFERENCES BETWEEN normal and tumor cells are all too often merely distinctions of degree (*1*). For this reason, existing clinical chemotherapeutic agents generally exhibit substantial cytotoxicity against normal proliferative cells at therapeutically useful concentrations (*2*). A process that could amplify subtle differences between normal and tumor cells, such as the small selectivities for tumor binding exhibited by hematoporphyrin derivative (*3*), rhodamine 123 (*4*), and sulfamethoxazole (*5*), might provide the basis for a novel, nontoxic chemotherapeutic protocol. When a biological response requires the simultaneous interaction of two or more molecules, the dose-response relationship becomes steeper and more sensitive to concentration than responses requiring individual molecules (*6*). For a self-assembling cytotoxin, formed in situ through covalent bond formation between smaller, less cytotoxic molecules, such synergism would be inherent. I shall describe here examples of self-assembling cytotoxins formed from mixtures of hydrazine derivatives and carbonyl compounds. Synergism and the steepening of the dose-response will be demonstrated in one such system to lay the foundation for the eventual development of selectivity amplification.

The formation of hydrazones from monosubstituted hydrazines and ketones or aldehydes is a reasonable candidate system for cytotoxin self-assembly in vivo. Monosubstituted hydrazines are not necessarily highly toxic or very reactive. For example, isoniazide, hydralazine, and phenelzine are all clinically useful therapeutic agents (*7*). In addition, Lucifer yellow CH (a fluorescent hydrazine derivative) retains its ability to form hydrazones after injection into living cells (*8*). Similarly, many ketones and aldehydes are relatively benign [for example, streptomycin, caproic aldehyde, progesterone, and pyruvate (*9*)].

A reaction between decanal and *N*-amino-*N'*-1-octylguanidine (*10*) (AOG) (Fig. 1) can be observed in micellar surfactant solutions. Good pseudo first-order kinetics are obtained ($r^2 > 0.999$) for reactions between 280 μ*M* decanal and 28 μ*M* AOG. The rates vary with microenvironment: $9.8 \pm 1.0 \times 10^{-4}$ sec^{-1} in 10 m*M* sodium dodecyl sulfate, $6.9 \pm 0.7 \times 10^{-5}$ sec^{-1} in 20 m*M* octyl-β-D-glucopyranoside, and $5.6 \pm 1.6 \times 10^{-4}$ sec^{-1} in 10 m*M* sodium dodecyl sulfate plus 10 m*M* octylglucopyranoside at *p*H 7.4 [all measured at 37°C in phosphate-buffered saline (PBS) plus 1% ethanol] (*11*). These surfactants were chosen in view of the relatively high negative surface charge density found in many highly metastatic tumor cells (*12*).

Combinations of AOG and decanal were tested for synergistic cytotoxicity. Human erythrocytes (*13*) exposed to a mixture of decanal and AOG (each at 28 μ*M*) in PBS lysed within 80 minutes at 37°C (Fig. 2A). By contrast, neither 56 μ*M* decanal nor 100 μ*M* AOG, by themselves, had any effect after more than 420 minutes. Decanal (28 μ*M*) combined with sodium iodide (28 μ*M*) was also inactive (no effect after 420 minutes). Similar results were obtained in two separate experiments involving the measurement of hemoglobin release and direct counting of cells (*14*).

The rate of hemolysis is sensitive to independent increases in the concentration of either component (Fig. 2B). The results can best be explained by invoking a bimolecular reaction in situ between decanal and AOG (Fig. 1) to form a more cytotoxic hydrazone, *N*-decylidenimino-*N'*-1-octylguanidine (DIOG) (*15*). As expected, DIOG can be detected by thin-layer chromatography after extraction with chloroform plus methanol (9:1 by volume) of an erythrocyte suspension lysed with decanal and AOG together at 28 μ*M*, but not in extracts of erythrocytes treated with either component alone under otherwise identical conditions. DIOG mediates complete lysis within 20 minutes at 14 μ*M*, while a mixture of 28 μ*M* AOG and 28 μ*M* decanal mediates less than 10% lysis within 20 minutes. Thus, DIOG is a sufficiently potent hemolytic agent to account for the hemolytic activity

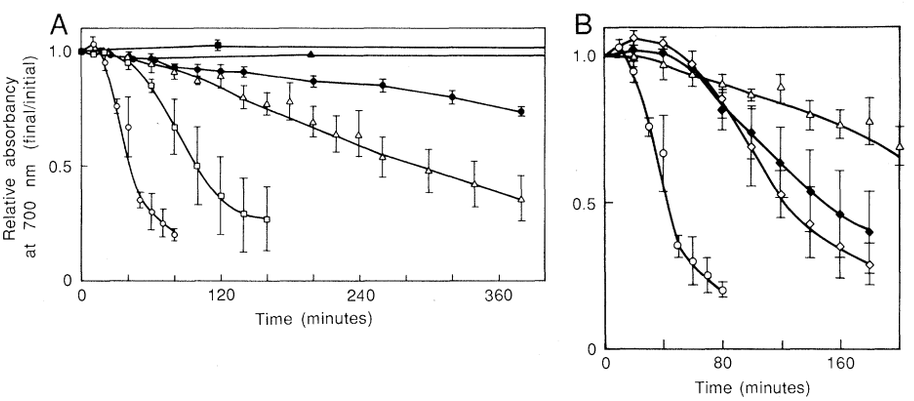

Fig. 2. Human erythrocyte lysis kinetics. Suspensions of 3×10^7 to 5×10^7 cells per milliliter in PBS (1% ethanol, no divalent cations, *p*H 7.4) were incubated at 37°C. Cell lysis was followed by observing the decrease in absorbance at 700 nm ($A_{700} \cong 1.0$ for 0% lysis, 0.2 for 100% lysis). Data points represent averages of 3 to 12 measurements. Error bars represent a range of 2 standard deviations. Control experiments (28 μ*M* and 56 μ*M* decanal; 28 μ*M*, 100 μ*M*, and 200 μ*M* AOG) exhibited less than 5% lysis after more than 400 minutes. (A) Effect of equal concentrations of decanal and AOG. (○) 28 + 28 μ*M*; (□) 20 + 20 μ*M*; (△) 14 + 14 μ*M*; (●) 10 + 10 μ*M*; (■) 56 μ*M* decanal; (▲) 100 μ*M* AOG. (B) Effect of independent variations of the concentrations of AOG and decanal on rate of hemolysis. (○) 28 μ*M* decanal plus 28 μ*M* AOG; (◇) 28 μ*M* decanal plus 14 μ*M* AOG; (◆) 14 μ*M* decanal plus 28 μ*M* AOG; (△) 14 μ*M* decanal plus 14 μ*M* AOG.

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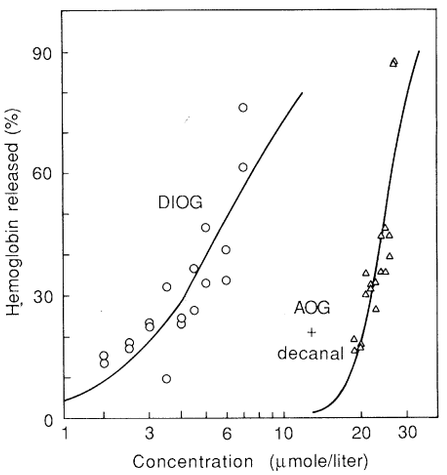

Fig. 3. Dose-response curves for hemoglobin release from human erythrocytes (*13*). After 1 hour of incubation at 37°C (PBS, 1% ethanol), the supernatant was centrifuged and monitored at 400 nm. Curves were calculated using a nonlinear least squares fitting algorithm (*18*) based on a modified Hill equation (*17*): % hemolysis = $100\%/[1+(K/C)^{\alpha}]$, where K is a constant (median effect concentration), C is the concentration of DIOG or of decanal and AOG, and α is the Hill constant. For DIOG, $K = 6.2 \pm 0.45$ μM, $\alpha = 2 \pm 0.35$. For decanal plus AOG, $K = 25 \pm 0.55$ μM, $\alpha = 6.7 \pm 1.4$. Error limits represent a range of two standard deviations.

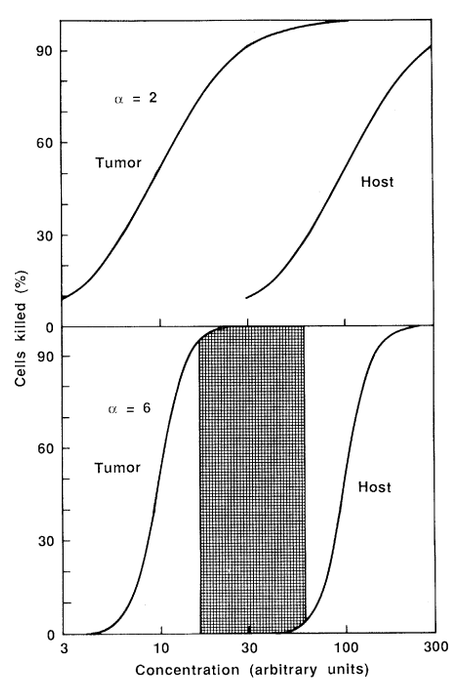

Fig. 4. Relation between dose-response cooperativity and selectivity of cytotoxicity. Both hypothetical examples shown involve molecules with a tenfold greater affinity for tumor as opposed to normal tissue. Dose-response curves are based on Hill constant values $\alpha = 2.0$ and $\alpha = 6.0$ (see also Fig. 3). The "therapeutic window" (tumor-to-host cytotoxicity differences greater than 95%) is represented by the crosshatched area. For the system with a Hill constant value of 2.0, host-to-tumor cytotoxicity differences would never exceed 82%.

of mixtures of AOG and decanal. The cytotoxicity of DIOG is presumably due to the disruptive interaction between the cationic, protonated form (*16*) and the biomembranes.

The rate of cytolysis varies sharply with concentration when equimolar decanal and AOG are used. The hemoglobin release values at 1-hour incubation times are depicted in Fig. 3 as a function of the concentrations of equimolar decanal and AOG combinations and of the adduct, DIOG. A Hill equation (*17*) was fitted to the two data sets with a nonlinear least squares algorithm (*18*). The relation, as expected (*6*), is substantially more cooperative for the combination of decanal and AOG [Hill constant (α) = 6.7] than for the adduct DIOG ($\alpha = 2$). Similar results were obtained from light scattering data for DIOG and for decanal plus AOG mixtures. A steeper dose response has been obtained by introducing the cytotoxin in two parts that self-assemble in situ.

A steeper dose response could be translated into a larger therapeutic window. In the hypothetical example (Fig. 4), two cytotoxins with different dose-response cooperativities ($\alpha = 2$ and $\alpha = 6$) are depicted. Despite identical tumor-to-host affinity ratios, greater differences between tumor and host toxicity could be attained in the more cooperative ($\alpha = 6$) system. Covalent self-assembly could thus be used as a powerful amplifier for translation of small tumor cell to normal cell selective affinities into substantial selective cytotoxicity. One would expect this effect to be even more pronounced for polymeric self-assembling cytotoxins formed from more than two precursor molecules (*6*).

Synergistic erythrocyte cytolysis effects are not restricted to combinations of decanal and AOG. I have also observed synergism in combinations of cyclohexane-1,4-dione and *N*-phenyl-*N*′,*N*″-diaminoguanidinium (*19*), although higher concentrations are required. These two compounds are cytolytic (76% lysis by cell counting) in combination at 5 mM and 0.5 mM, respectively, yet much less active (<18% lysis) by themselves at 60 mM and 3 mM, respectively (24 hours, 37°C, PBS). The erythrocyte aggregation that occurred upon exposure to equimolar concentrations of these two compounds (2.5 to 5 mM) was perhaps due to polycation formation (*20*). Another example of a self-assembling hemolytic agent is the combination of cyclohexanone and AOG (5 mM plus 0.1 mM, respectively).

Decanal and AOG also exhibited synergistic bactericidal activity against *Escherichia coli* J96 (*21*). When these bacteria were exposed to a combination of 200 μM decanal and 200 μM AOG for 20 hours (*22*), the number of colony forming units decreased approximately 180-fold compared to controls containing each component alone at 400 μM. When 400 μM decanal was combined with 400 μM AOG, no viable bacteria were observed (at least a million times decrease in viability compared to controls). Decanal and AOG also exhibited synergistic activity in Dulbecco's modified Eagle's medium against the cultured human tumor cell line HeLa (*23*) (Fig. 5). (These data were obtained in media containing divalent metal ions and are not intended for comparison with erythrocyte lysis data.)

The fact that all of the self-assembling cytotoxins described here involve the formation of cationic, detergent-like hydrazones is not meant to imply that the effect need be restricted to one mechanism of cytotoxity and one type of bimolecular reaction. A variety of cytotoxic agents (such as polyintercalators and polyalkylating agents) and reaction types (such as metal chelation and cycloaddition) might usefully be applied to cytotoxin self-assembly. The experiments

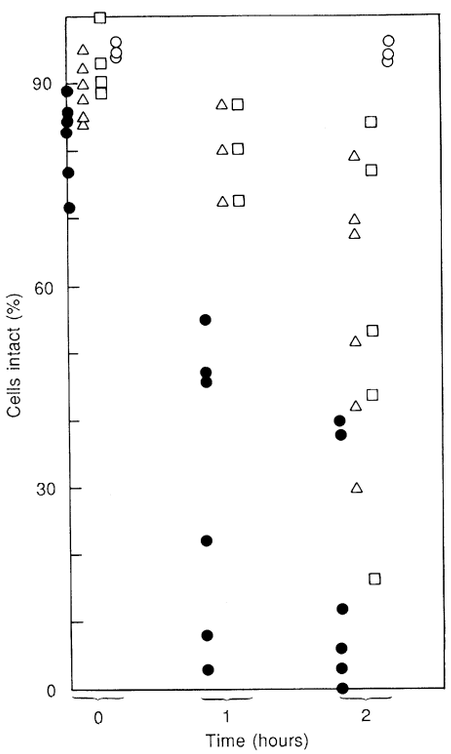

Fig. 5. HeLa cell cytotoxicity. Cells were grown in suspension in Joklik's medium supplemented with calf serum, centrifuged, and taken up in Dulbecco's modified Eagle's medium (DMEM) without serum. Cell suspensions (2×10^6 to 4×10^6 cells per milliliter) in DMEM containing 1% ethanol were incubated at 37°C. Cell membrane damage was measured using trypan blue exclusion (hand counting of cells). Symbols: ○, blank control; □, 400 μM decanal; △, 400 μM AOG; ●, 200 μM decanal plus 200 μM AOG.

described here are intended to lay the groundwork for the amplification of selectivity between neoplastic and normal cells.

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10. The synthesis of *N*-amino-*N'*-octylguanidinium iodide will be described in detail elsewhere. Decanal (Aldrich) was used as purchased without further purification. When chromatographically pure decanal was used in the erythrocyte experiments, the results were qualitatively the same.
11. Error limits represent a range of two standard deviations. The standard deviations were estimated from K_2 values: [D. P. Shoemaker, C. W. Garland, J. J. Steinfeld, Eds., *Experiments in Physical Chemistry*, (McGraw Hill, New York, ed. 3, 1974), p. 34].
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13. Blood was collected and stored with potassium EDTA at 4°C. Erythrocytes were prepared within 48 hours of collection by centrifuging three times in PBS (*p*H 7.4). Suspensions containing 3×10^7 cells/ml were prepared in PBS containing 1% ethanol. The PBS used for erythrocyte experiments did not contain divalent cations. Suspensions were incubated at 37°C with various concentrations of the cytotoxins.
14. Hemoglobin release was followed at 400 nm by monitoring the supernatant. Counting was carried out by hand with a hemacytometer. Concentrations of AOG and decanal were 20 μ*M*.
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22. Bacteria were incubated at 37°C in PBS containing magnesium and calcium with 1% ethanol and various concentrations of the cytotoxins. Reported values are based on averages of $\log_{10}$ (colonies per milliliter) for three determinations.
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Hydrogen Sulfide Oxidation Is Coupled to Oxidative Phosphorylation in Mitochondria of *Solemya reidi*

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***Solemya reidi*, a gutless clam found in sulfide-rich habitats, contains within its gills bacterial symbionts thought to oxidize sulfur compounds and provide a reduced carbon food source to the clam. However, the initial step or steps in sulfide oxidation occur in the animal tissue, and mitochondria isolated from both gill and symbiont-free foot tissue of the clam coupled the oxidation of sulfide to oxidative phosphorylation [adenosine triphosphate (ATP) synthesis]. The ability of *Solemya reidi* to exploit directly the energy in sulfide for ATP synthesis is unprecedented, and suggests that sulfide-habitat animals that lack bacterial symbionts may also use sulfide as an inorganic energy source.**

MANY MARINE INVERTEBRATE ANIMALS live in sulfide-rich habitats, including the deep-sea hydrothermal vents where geothermally produced sulfide is present (*1*), and reduced sediments where microbial degradation of organic matter leads to the reduction of sulfate to sulfide (*2*). The high sulfide concentrations of these habitats pose a significant threat to these animals because sulfide is a potent inhibitor of aerobic respiration through its effects on the cytochrome *c* oxidase system (*3, 4*). Despite its toxicity, however, sulfide is important for the energy supplies of these animals. Sulfide is a highly reduced compound that can be oxidized by bacteria to yield energy for driving net carbon dioxide fixation. Some, but not all, of the invertebrates of sulfide-rich habitats harbor symbiotic sulfur bacteria in their tissues, and these bacteria are thought to provide their hosts with a substantial source of nutrients (*2*).

The gutless clam *Solemya reidi*, which is found in reduced sediments near sewage outfall zones and pulpmill effluent sites (*5*), contains large numbers of chemolithoautotrophic sulfur bacteria in its gills (*6*) and has a high capacity for aerobic respiration (*7*); nevertheless, the initial steps in sulfide oxidation occur in the animal compartment of the symbiosis, not the bacterial (*6*). This finding led to the present study in which we report that mitochondria of gill and (symbiont-free) foot tissues of *S. reidi* can use sulfide as an inorganic energy source for oxidative phosphorylation. Sulfide oxidation in mitochondria thus protects the aerobic respiration (*6*) of the clam and allows it to exploit, without the direct assistance of bacteria, the energy of the sulfide molecule. This is the first report known to us of an animal that can use an inorganic energy source directly.

To characterize the mitochondria of *S. reidi*, we first examined their ability to use substrates previously reported for marine invertebrate mitochondria (*8*). With succinate and malate as substrates, mitochondria from both tissues respired at rates similar to those reported for mitochondria from other marine invertebrates (*8*), and showed typical mitochondrial acceptor control [stimulation of oxygen consumption by adenosine diphosphate (ADP)] (Fig. 1 and Table 1). Acceptor control, a widely used indicator of the intactness of mitochondria (*9*), is defined as the rate of oxygen consumption when ADP is present divided by the rate when ADP has been consumed. High acceptor control ratios are indicative of intact mitochondria. Our value of 2.5 with succinate as the substrate is in the range reported for marine invertebrates (*8*), which is lower than values common for vertebrates (*9*).

The addition of 20 μ*M* sulfide (*10*) as the sole substrate to mitochondria isolated from gill and foot resulted in oxygen consumption and in acceptor control (Fig. 1 and Table 1). Control experiments without substrates added showed no acceptor control, and control experiments without mitochondria present showed no consumption of oxygen when concentrations of 20 or 50 μ*M* sulfide were added to the system. No oxygen consumption was observed with thiosulfate or sulfite (sulfur compounds more oxidized than sulfide) as substrates. The acceptor control value of 1.8 for sulfide was slightly higher than the value of 1.7 reported for ascorbate plus tetramethylphenylenediamine (*11*), which, like sulfide, are coupled through site III (see below).

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