Biochemical Pharmacology, Vol. 37, No. 23, pp. 4505–4512, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ENVIRONMENT-SELECTIVE SYNERGISM USING SELF-ASSEMBLING CYTOTOXIC AND ANTIMICROBIAL AGENTS*

DARRYL RIDEOUT,† JAMES JAWORSKI and RAYMOND DAGNINO, JR.
Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037, U.S.A.

(*Received* 8 *March* 1988; *accepted* 10 *May* 1988)

Abstract—Environment-selective synergistic toxicity using combinations of aldehydes and hydrazine derivatives was demonstrated in two different model systems *in vitro*. Combinations of 5-nitro-2-furaldehyde with semi-carbazide and of 2-hydrazinopyridine with pyridine-2-carboxaldehyde, which can react *in situ* to form antimicrobial hydrazones, demonstrated greater degrees of synergism against the intracellular pathogen, *Salmonella typhimurium*, at pH 5 relative to pH 7.4. Combinations are more selectively toxic at pH 5 (vs pH 7.4) than individual precursors and preformed hydrazone products because acid catalysis of hydrazone formation plays a role only for the combinations. A combination of decanal and *N*-amino, *N'*-octylguanidine (AOG) exhibited more pronounced synergistic cytolytic activity against erythrocytes in 0% serum than in 1% serum. Serum protein binding of decanal inhibited the formation of the more cytotoxic hydrazone, *N*-decylidenimino,*N'*-1-octylguanidine (DIOG), from the less cytotoxic AOG and decanal, and serum protein binding of DIOG prevented this cytotoxin from reaching the cell membrane. Because decanal binding cannot play a role in the cytotoxicity of preformed DIOG, it was less selective for cells in 0% serum than the combination of AOG and decanal. The pH 5 and 0% serum environments represent very simple models for macrophage phagolysosomal compartments and poorly vascularized solid tumor interiors respectively. If environment-selective synergism can be used as a basis for target-selective synergism in other *in vitro* model systems and *in vivo*, self-assembling combinations could provide a basis for rational introduction of target-selective synergism into chemotherapeutic drug design.

The most important factor in the chemotherapy of neoplastic and infectious diseases is not potency, but rather the selectivity of action against the target (tumor or pathogen) versus the host toxicity [1]. The target selectivity of single, intrinsically active drug molecules and conventional prodrugs can be influenced by only two factors: (a) the ratio of drug concentration which is ultimately achieved in target versus host tissues (distribution selectivity); and (b) the toxicity ratio for identical drug concentrations (biochemical and cytological selectivity [1]). Straightforward, rational combination of multiple selectivity factors in a single chemotherapeutic drug or prodrug molecule is difficult or impossible in many instances [2, 3].

For drugs containing a hydrazone moiety which were delivered as a combination of two less toxic precursors (a hydrazine derivative and an aldehyde or ketone [4]), three additional selectivity factors could be introduced: (c) ultimate distribution selectivity of the hydrazine derivative, (d) ultimate distribution selectivity of the aldehyde or ketone, and (e) the bimolecular rate constant for hydrazone formation (assembly rate constant selectivity). The result of the action of any of these three selectivity factors would be target-selective synergism between the hydrazine derivative and aldehyde (or ketone) because the toxic hydrazone would form more rapidly in the target and not in the normal tissues. Although the reaction does not require enzymatic catalysis, hydrazone formation from hydrazine derivatives and endogenous aldehydes and ketones has been observed in cultured cells [5–7], in animals [8] and in human subjects [9]. In an earlier report from this laboratory, it was demonstrated that combinations of hydrazine derivatives and aldehydes can exhibit synergistic cytotoxic and anti-microbial activity *in vitro* [4].

Selective formation of toxic chemotherapeutic agents in acidic, as opposed to neutral, environments could be of value for the achievement of pathogen-selective synergism for a number of reasons. First, several pathogens survive in acidified phagolysosomal compartments (pH near 5) after phagocytosis by macrophages and phagosome-lysosome fusion, including *Salmonella typhimurium, Yersinia pestis, Coxiella burnetti*, and protozoal parasites of the genus Leishmania [10–13]. Second, many viruses are believed to enter cells by way of acidified endosomes [14, 15]. Finally, local necrotic acidification is often characteristic of severe microbial infections [16]. Weak bases which accumulate in acidic compartments have demonstrated selective toxicity against *Leishmania mexicana* in macrophage cultures [13] and against the influenza virus [14, 15] in live mammals.

* This is Contribution No. 4954MB from the Department of Molecular Biology.

† Author to whom all correspondence should be addressed.

Selective synergistic cytolysis of mammalian cells in lower versus higher serum environments *in vitro* might be useful for the attainment of tumor-selective synergism because of the abnormally low serum levels characterizing some solid tumor interiors [17]. Serum protein binding of hematoporphyrin derivative (HPD)* by mammalian cells is believed to be related to the tumor-selective phototoxicity of HPD [17]. This inhibition is due to distribution selectivity: in normal tissues, more HPD is bound to serum albumin and less is available to enter the cells [17].

We have investigated the effects of combinations of hydrazine derivatives and aldehydes (Fig. 1) on cells in different environments in order to demonstrate, in mechanistic models, the possibility of environment-selective synergism. Comparisons were made between identical cell types in different environments in order to ascertain the effects of extracellular environment on biocidal selectivity in isolation from cell type-specific factors. Two examples involve low pH-selective inhibition of the intracellular pathogen *S. typhimurium*, which can evade the immune system in mice by surviving in the acidic macrophage phagolysosome [11, 12]. Differences in second order rate constants [18, 19] were used to provide pH selectivity (factor e described above). The third example concerns differential cell killing in low serum versus higher serum environments, using erythrocytes as a very simple but convenient and reproducible model for other mammalian cell types [4]. Aldehyde distribution selectivity (factor d) was used to enhance the selectivity of the cytolytic activity against cells in lower serum (more tumor-like) environments. Our immediate goal is the demonstration of the *principle* of environment-selective synergism. We have chosen readily interpreted model systems, as opposed to the use of more problematic and complex models which would more closely resemble disease states *in vivo*.

* Abbreviations: AOG, *N*-amino,*N'*-octylguanidine; BSA, bovine serum albumin; DIOG, *N*-decylidenimino,*N'*-1-octylguanidine; DMSO, dimethyl sulfoxide; HP, 2-hydrazinopyridine; HPD, hematoporphyrin derivative; NFA, 5-nitro-2-furaldehyde; NFZ, nitrofurazone; PBS, phosphate-buffered saline; PC, pyridine-2-carboxaldehyde; PCPH, *E*-pyridine-2-carboxaldehyde-2-pyridinylhydrazone; and SC, semicarbazide.

MATERIALS AND METHODS

Reaction kinetics. All second order rate constants were based on an average of four or more pseudo first order determinations using at least two different major component concentrations. Rates were determined by observing increases in UV or visible absorbancy using a Perkin–Elmer Lambda 4 spectrophotometer at 340 nm for PC + HP and 375 nm for SC + NFA. The rate constants for SC + NFA at pH 7.4 were measured by determining initial rates (5% reaction or less). The other rate constants were based on data collected during a time span of at least double the $T_{1/2}$, and on optimized infinity values. The medium was PBS containing divalent ions and 10 mM acetate at 37°. For the reaction between SC and NFA, the medium contained 1% DMSO as well. Errors represent 95% confidence limits.

Erythrocyte lysis studies. Ox blood in Alsevier's solution (Colorado Serum Co., Denver, CO) was stored at 4° and used within 7 days of collection. It was centrifuged and washed twice using PBS, pH 6.6, without divalent ions. All of the erythrocyte lysis experiments were carried out in PBS (pH 6.6, no divalent ions) containing 1% ethanol at 37°. In the 1% serum experiments, serum was added to the cell suspension and mixed before addition of AOG, DIOG and/or decanal stock solutions. When erythrocyte lysis was measured using hemoglobin release, aliquots of 2 ml were centrifuged in Eppendorf tubes, and the hemoglobin in the supernatant

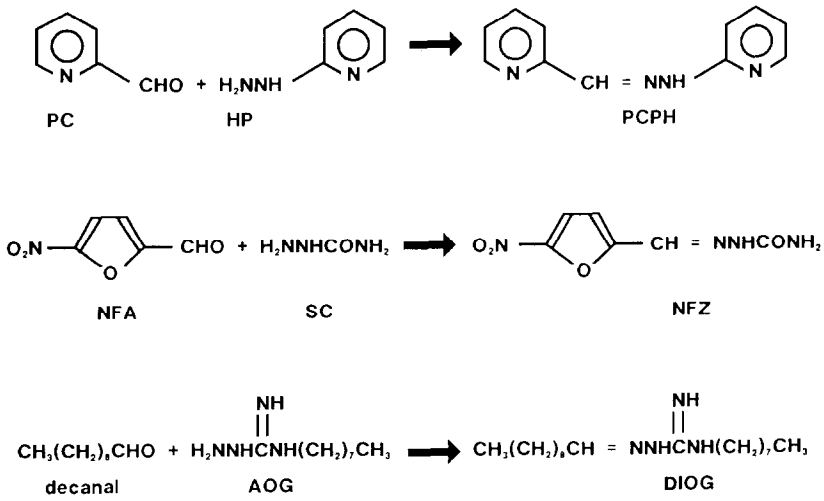

Fig. 1. Structural formulas and reaction schemes.

fraction was determined by its visible absorption at 400 nm using a Perkin–Elmer Lambda 3 or Lambda 4 spectrophotometer. Erythrocyte suspension turbidity was determined by measuring the absorption at 700 nm using a Bausch & Lomb Spectronic 20 spectrophotometer. The 1-hr time points plotted in Fig. 4 were fitted to a median effect equation [20].

Bovine serum albumin was purchased from the Sigma Chemical Co., St. Louis, MO (No. A7960). Calf serum (Sigma No. C6278) contained approximately 35 mg BSA/ml (assayed by the Sigma Chemical Co.).

Statistics. Error limits and error bars represent 95% confidence as determined using quality control methodology [21]. Nonlinear least squares fit calculations and the error limits calculated from them were determined using methodology described by Arkin and Colton [22]. The error limits reported for EC_{50} ratios represent plus or minus one standard deviation.

Chemicals. 1-Octyl-2-thiourea was purchased from Trans World Chemicals, Chevy Chase, MD. Decanal, PC, HP, NFA, and SC were purchased from the Aldrich Chemical Co., Milwaukee, WI. Decanal was purified using a silica gel chromatograph (1:1 methylene chloride/petroleum ether). PCPH was synthesized according to a literature procedure [23, 24].

To prepare *N*-amino,*N'*-1-octylguanidine (AOG) as the hydroiodide salt, 1-octyl-2-thiourea (4.12 g, 0.0219 mol) was allowed to reflux overnight with iodomethane (12.4 g, 0.0876 mol) in methanol (100 ml). After evaporating the solvent at reduced pressure, the intermediate was allowed to reflux overnight with anhydrous hydrazine (0.91 g, 0.0285 mol) in isopropanol (30 ml). After evaporating to dryness at reduced pressure, the crude AOG hydroiodide was dissolved in methylene chloride (75 ml) and filtered. After removing the solvent from the filtrate under reduced pressure, toluene (75 ml) was added and the white solid was collected by filtration. Recrystallization from hot toluene/chloroform yielded AOG hydroiodide (49%), m.p. 68–70°. Calculated for $C_9H_{23}N_4I$: C, 33.96%; H, 7.38%; N, 17.83%. Found: C, 34.4%; H, 7.34%; N, 17.95%.

N-(1-Decylidenimino), *N'*-1-octylguanidine (DIOG) was synthesized as the acetate salt by stirring a suspension of decanal (0.3 g, 1.92 mmol) and AOG hydroiodide (0.3 g, 0.96 mmol) in water (10 ml) and methanol (5 ml) for 12 hr at 25°. After adding sodium acetate (0.42 g, 5.12 mmol), the crude product was extracted with dichloromethane, dried over anhydrous sodium sulfate, and chromatographed on silica (78:20:2 dichloromethane/acetonitrile/acetic acid). The product (DIOG acetate) was obtained as a pale yellow oil in 67% yield. Mass spectrum (quadropole ion): major peak at 325 ± 0.5. Calculated molecular weight for $C_{19}H_{41}N_4^+$: 325.54.

Antibacterial assays. S. typhimurium strain 14028r was provided by F. Heffron and P. Fields. Luria broth was adjusted to pH 7.4 or pH 5.0 using hydrochloric acid and/or sodium hydroxide.

For bacteriostatic zone inhibition assays, agar plates (100 × 15 mm) were made from Luria broth of the desired pH and inoculated with 200 μl of PBS containing 1.6×10^5 viable, log phase bacteria. Viability was determined by diluting, spreading plates, and counting colonies. Sterile filter paper discs (6.3 mm diameter, Becton Dickinson, Cockeysville, MD) were soaked with 20 μl of aqueous or DMSO solutions of the bacteriostatic agents. DMSO alone had no bacteriostatic activity in these assays. Inhibition areas represent averages of three to seven experiments from at least two separate days, determined by tracing the regions with no visible bacterial growth onto graph paper, and subtracting the disc areas.

For bactericidal assays, bacteria were grown into log phase in Luria broth buffered to the appropriate pH. The bacteria were washed twice in PBS containing 10 mM acetate at the same pH. The bactericidal assay involved incubation of the bacteria in a roller drum at 37° in the PBS (10 mM acetate) containing 1% DMSO and various concentrations of bactericidal agent(s), and determination of viability by diluting aliquots of the suspensions, spreading on agar plates, and counting the resulting colonies. Cell densities were adjusted to yield control viable bacteria counts of about 2×10^7/ml. The pH value (7.4 or 5) was maintained throughout each experiment. Controls were repeated at least three times for each determination. Experimental points represent averages of at least four determinations made on at least two different days. The 1% DMSO vehicle was shown to have no significant effect on bacterial viability.

RESULTS

Reaction kinetics. Second order rate constants for the reaction between PC and HP are 2.2 ± 0.3 mol^{-1} sec^{-1} at pH 5 and 0.92 ± 0.13 mol^{-1} sec^{-1} at pH 7.4 (95% confidence limits [21]). Second order rate constants for the reaction between SC and NFA reaction were $5.55 \pm 1.8 \times 10^{-1}$ mol^{-1} sec^{-1} and $3.8 \pm 1.5 \times 10^{-3}$ mol^{-1} sec^{-1} at pH 5 and pH 7.4 respectively (1% DMSO). For reactions between AOG (28 μM) and decanal (280 μM), $T_{1/2}$ = 12–170 min in various surfactant solutions at 37°, pH 7.4 [4].

PC, HP and PCPH in bacteriostatic assays. Areas of *complete* bacterial growth inhibition (excluding disc areas) for roughly 3×10^5 log phase bacteria spread on LB agar plates (pH 5) as a PBS suspension were 0, 25 ± 25, and 150 ± 52 mm^2 after 24 hr for 4 μmol PC, 2 μmol HP, and 0.4 μmol PCPH respectively. Areas at pH 7.4 were not substantially smaller: 0, 109 ± 41, and 103 ± 5 mm^2 respectively. For 4 μmol PC plus 2 μmol HP (separate discs, 15 mm center to center), areas were 705 ± 115 mm^2 at pH 5 and 250 ± 99 mm^2 at pH 7.4 (Fig. 2). PCPH formation in the agar between the HP- and PC-treated discs was evident from the yellow color and, at high reactant concentrations, PCPH crystals.

Attenuation of bacteriostatic effects of 0.4 μmol PCPH was observed on plates containing 1 mM Zn^{2+} at pH 5 and at pH 7.4: the areas were 61 ± 15 and 33 ± 33 mm^2 respectively. For 4 μmol PC + 2 μmol HP, the areas were 265 ± 140 mm^2 at pH 5 and 253 ± 99 mm^2 at pH 7.4 with 1 mM Zn^{2+}.

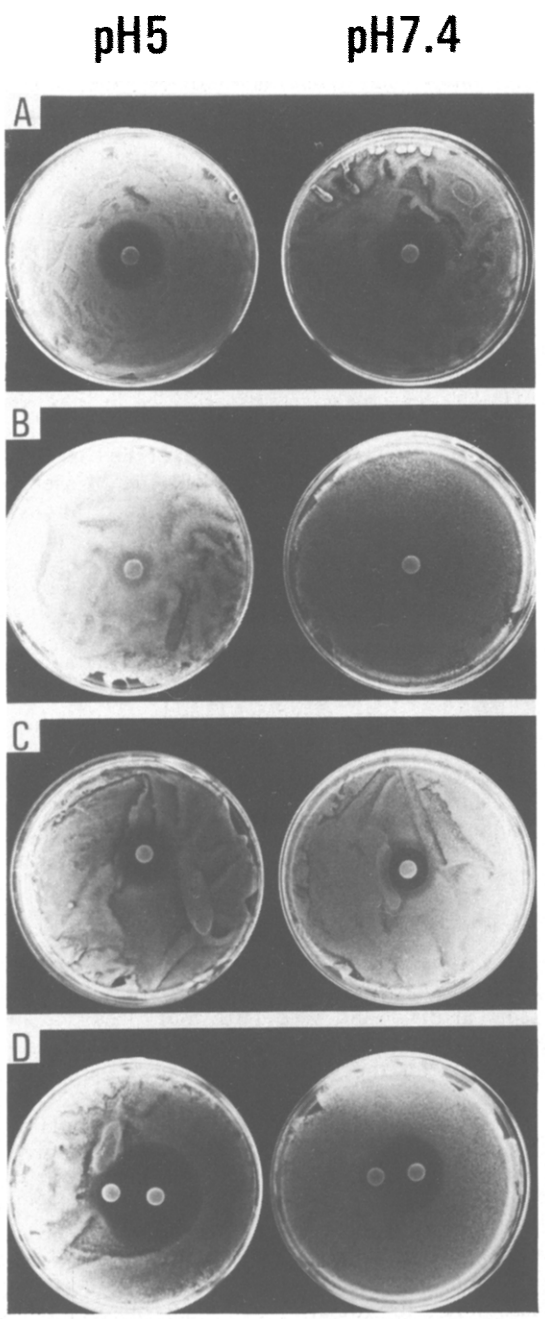

Fig. 2. Inhibition of *S. typhimurium* strain 14028r. Agar plates (100 × 15 mm, Luria broth) adjusted to pH 7.4 or pH 5 were inoculated with 200 μl PBS containing 160,000 viable, log phase bacteria. Sterile filter paper discs (6.3 mm diameter) soaked in 20 μl of solutions of the appropriate compounds in water or DMSO were placed on the surface, and plates were incubated at 37° for 24 hr. Left-hand plates: pH 5; right-hand plates: pH 7.4. Key: (A) 0.4 μmol PCPH/disc; (B) 4 μmol PC/disc; (C) 2 μmol HP/disc; and (D) 2 μmol HP on the left-hand disc on each plate; 4 μmol PC on the right-hand disc on each plate; 15 mm between disc centers.

NFA, SC and NFZ in bactericidal assays. Bactericidal activity was measured by suspending log phase bacteria in PBS containing 10 mM acetate and 1% DMSO at pH 5 or pH 7.4 for 20 hr at 37°. Viability was determined by diluting, spreading plates at the same pH, and counting colonies.

The antibacterial activity of NFZ exceeded that of NFA and SC at pH 5 (Fig. 3B). Combined, NFA and SC exhibited synergistic antibacterial activity at pH 5 (Fig. 3C). At pH 5, they were observed to react rapidly to form NFZ (Fig. 3A). No synergism was observed at pH 7.4. Bactericidal selectivity at pH 5 (the fraction surviving at pH 7.4 divided by the fraction surviving at pH 5) was substantially higher for the combinations of SC + NFA than for either SC, NFA or NFZ alone at relevant concentrations (Figs. 3B and 3C).

Erythrocyte lysis assays. The lysis of ox blood erythrocytes by the hydrazone DIOG at 37° in PBS at pH 6.6 was inhibited by bovine serum and by serum albumin (Fig. 4A). When DIOG was made to self assemble [4] from the aldehyde decanal and the hydrazine derivative AOG, the serum effect became more pronounced (Fig. 4B). Based on curves fitted to the median effect equation [20], the ratio of EC_{50} (1% serum) to EC_{50} (0% serum) was 4 ± 0.07 and the maximum difference in percentage of cells killed between erythrocytes in 0% serum versus those in 1% serum was 99.7% for decanal in the presence of 448 μM AOG (Fig. 4B). For DIOG, the respective values were 2.8 ± 0.16 and 59.5% (Fig. 4A). The presence of 448 μM AOG had no effect on the rate of lysis by DIOG.

Neither 30 μM decanal alone nor 896 μM AOG alone caused more than 10% lysis after 1 hr. Pairs of molecules closely related to AOG and decanal, which cannot combine covalently, did not exhibit substantial synergism. Thus, 1-decanal (200 μM) and a combination of 200 μM 1-decanal and 28 μM AOG both mediated 50% ox blood erythrocyte lysis in 4 hr. Dodecylguanidinium acetate at 64 μM mediated 50% lysis in 7.5 min, whereas combinations of dodecylguanidine with decanal required 10.5 min for 48 μM + 20 μM and 27.5 min for 20 μM + 48 μM respectively (37°, pH 6.6).

When AOG (448 μM) and decanal (16 μM) were allowed to prereact to form DIOG before adding them to cell suspension, lysis kinetics became monophasic (Fig. 5). Lysis kinetics were biphasic when decanal and AOG were added to cells without prereaction. Serum (1%) led to an approximate 20-fold decrease in the hemolysis rate for the combination which had been allowed to prereact. Serum caused an approximate 5-fold increase in the length of the slower phase for the studies carried out without prereaction, as well as an approximate 20-fold decrease in the rate in the faster phase.

DISSUSSION

In the first example, *S. typhimurium* was chosen as a test organism for pH-selective killing because it can survive in the acidic phagolysosomal compartment of macrophages [11, 12]. The hydrazone *E*-pyridine-2-carboxaldehyde 2-pyridinyl hydrazone (PCPH) was substantially more bacteriostatic against *S. typhimurium* than either of its two precursors, pyridine-2-carboxyaldehyde (PC) and 2-hydrazinopyridine (HP) (Figs. 1 and 2). The greater bac-

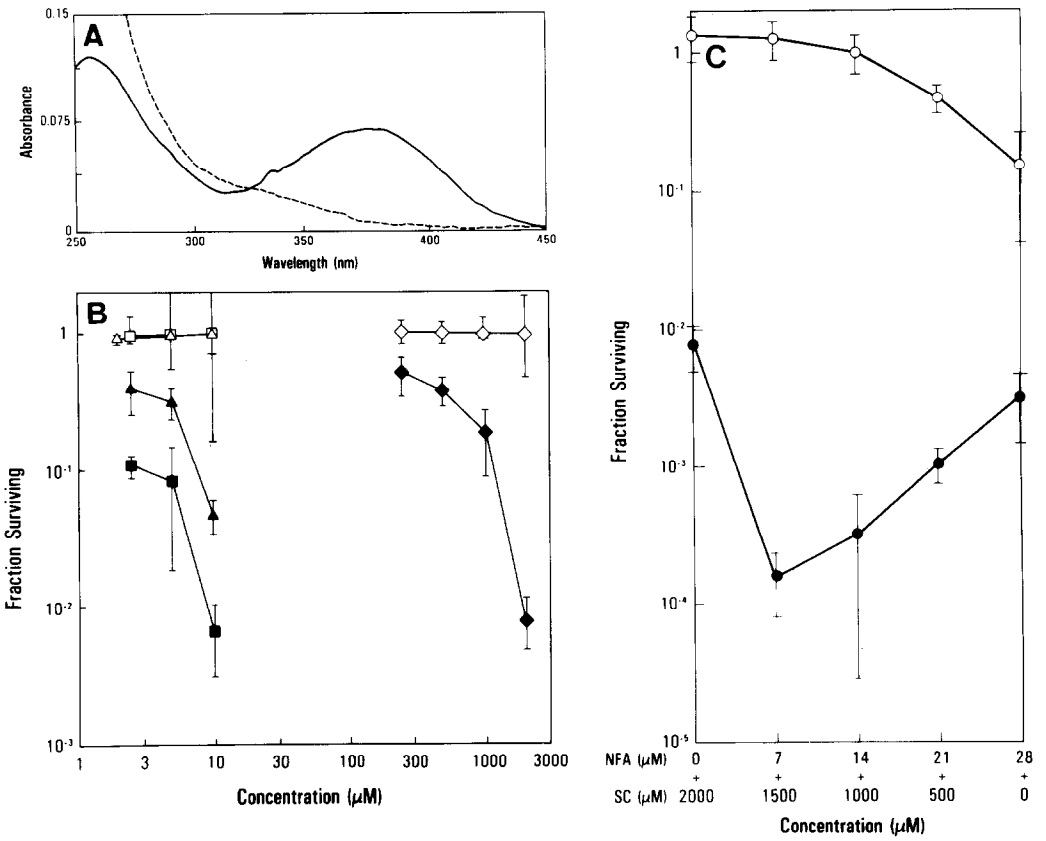

Fig. 3. Bactericidal activity against *S. typhimurium*. PBS-washed, log phase bacteria were incubated at 37° for 20 hr as a diluted suspension in PBS containing 1% DMSO with or without antimicrobials. The number of viable bacteria in controls at the end of 20 hr was close to 2×10^7/ml. (A) Effect of pH on the extent of product hydrazone (NFZ) formation from 7 μM NFA and 1500 μM SC in the presence of 2×10^7 bacteria/ml, as evidenced by the visible absorption of NFZ. After 20 hr of incubation at 37° in 1% DMSO in PBS containing magnesium and calcium, suspensions were microfiltered to remove the bacteria. The $\lambda_{max} = 375$ nm for NFZ, $\varepsilon = 1.5 \times 10^4$ at both pH 5 and pH 7.4. For SC and NFA, ε values at 375 nm were less than 10^3 for NFA and less than 6 for SC at both pH 5 and pH 7.4. Solid line: pH 5; dashed line: pH 7.4. (B) Effects of NFA, SC, and nitrofurazone (NFZ) individually on *S. typhimurium* survival at pH 5 and pH 7.4. Key: (▲) NFA, pH 5; (□) NFA, pH 7.4; (■) NFZ, pH 5; (△) NFZ, pH 7.4; (◆) SC, pH 5; and (◇) SC, pH 7.4. (C) Effect of combined nitrofuraldehyde (NFA) and semicarbazide (SC) on *S. typhimurium* survival at pH 5 and pH 7.4. Data points represent an average of five or six ratios determined during two separate sets of experiments. Key: (●) pH 5; and (○) pH 7.4.

teriostatic activity of PCPH, which is associated with metal ion chelation [23, 24], allowed synergism for combinations of PC and HP. We have chosen to compare identical bacterial cells in different environments, rather than comparing bacteria to mammalian cells, because our goal is a clear demonstration of the principle of environment-selective synergism in an unambiguous mechanistic model. (Because PC alone is inactive at 4 μmol/disc, the enhanced bacteriostatic activity observed for combinations of PC and HP is most properly termed "potentiation" or "enhancement" rather than "synergism". To avoid confusion, however, the term "synergism" will be used in place of "potentiation" and "enhancement" throughout this manuscript.)

Bacteriostatic activities of PC, HP and PCPH as single components did not differ significantly between pH 5 and pH 7.4. By contrast, if PC (4 μmol) and HP (2 μmol) were allowed to diffuse together from different discs as the bacteria were growing, the average area of the inhibition zone at pH 5 was 2.8 times larger than the area at pH 7.4 (Fig. 2). This difference is best explained as assembly rate constant specificity: the second order rate constant for PCPH formation from PC and HP was 2.4 times greater at pH 5 than at pH 7.4. At pH 5, PCPH formed from PC and HP more rapidly than at pH 7.4, achieving bacteriostatic hydrazone levels prior to observable bacterial growth on a larger portion of the plate at the lower pH. Although PCPH was more potent than the combination of PC and HP, the combination had the advantage of greater selectivity.

Also consistent with the importance of the rate constant was the lack of pH selectivity for the experiment involving PC plus HP, carried out on plates containing 1 mM Zn^{2+}. The Zn^{2+} presumably detoxified the free PCPH as it formed through chelation. Acid catalysis of PCPH formation from PC and HP led to environment-selective synergism, with the bacteria on the pH 5 plate representing the target.

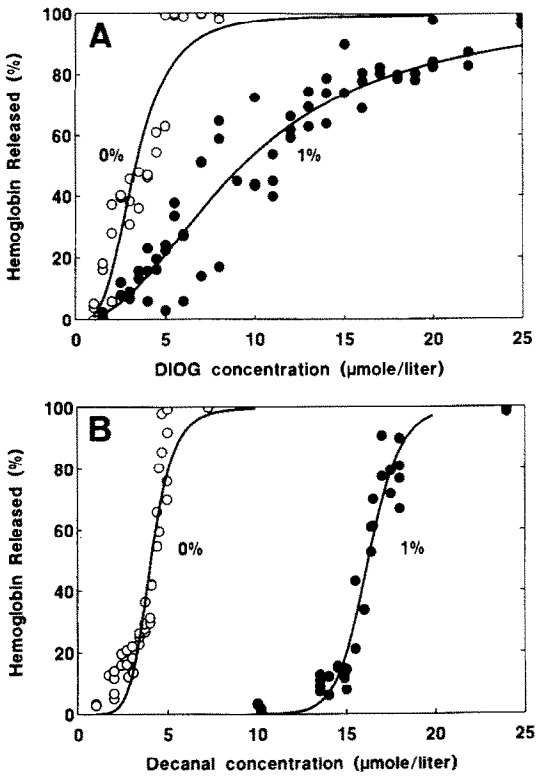

Fig. 4. Ox blood erythrocyte lysis. Ox blood stored in Alsevier's solution was used within 7 days of collection. Cells were centrifuged and washed two times with PBS. Erythrocyte lysis experiments were carried out using PBS (pH 6.6) without divalent ions with 1% ethanol. Cell density was 2×10^7 cells/ml. (A) Effect of calf serum on lysis by DIOG. Extent of lysis was determined after 1 hr at 37° by centrifuging and determining hemoglobin release in the supernatant absorbancy at 400 nm. AOG (448 μM) was present throughout in addition to the various DIOG concentrations. Results of parallel experiments without AOG did not differ significantly. Data are combined from three separate sets of experiments. The 0% serum and 1% serum curves were calculated from 29 and 58 data points respectively. Curves were calculated using a nonlinear least squares fit algorithm [22] based on a median effect equation [20]: % hemolysis = $100\%/[1 + (K/C)^{\alpha}]$, where K is a constant (median effect concentration), C is the concentration of DIOG, and α is the Hill constant. For 0% serum, $K = 3.3 \pm 0.16$ μM, $\alpha = 3.3 \pm 0.49$. For 1% serum, $K = 9.3 \pm 0.31$ μM, $\alpha = 2.1 \pm 0.16$. Key: (○) 0% serum; and (●) 1% serum. (B) Effect of serum on erythrocyte lysis at 1-hr time points in the presence of 448 μM AOG with various decanal concentrations. Conditions were as in part A. AOG alone (896 μM) or decanal alone (30 μM) caused less than 10% hemoglobin release. For the median effect equation, C = concentration of decanal (see part A). For 0% serum, the curve is based on 38 points, $K = 4.1 \pm 0.07$ μM, $\alpha = 6.4 \pm 0.7$. For 1% serum, the curve is based on 8 points, $K = 16.2 \pm 0.1$ μM, $\alpha = 18 \pm 1.8$. Key: (○) 0% serum; and (●) 1% serum.

In the second example, nitrofurazone (NFZ) was used as the hydrazone product. NFZ is a broad spectrum antibacterial and antitrypanosomal agent [25–27] which causes DNA damage [28, 29]. The combination of SC and NFA (Fig. 1) was tested for bactericidal activity against *S. typhimurium* in an

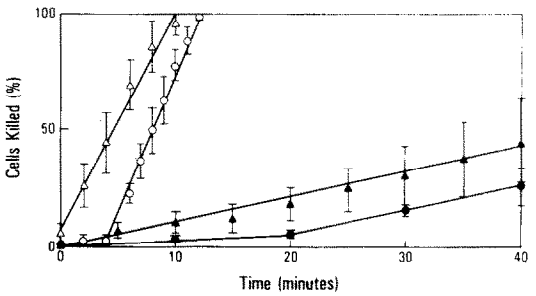

Fig. 5. Kinetics of ox blood erythrocyte lysis by decanal (16 μM) and AOG (448 μM). In preincubation experiments, 0.8 mM decanal and 22.4 mM AOG were allowed to react for 20 min at 37° in 1:1 ethanol/PBS, so that most of the decanal was already converted to DIOG by the time these reactants were diluted (50×) into the cell suspension. Lysis was followed by observing loss of light scattering at 700 nm. Key: (△) 0% serum, preincubated; (○) 0% serum, not preincubated; (▲) 1% serum, preincubated; and (●) 1% serum, not preincubated.

attempt to enhance the selectivity of action of NFZ for pH 5 (target) versus pH 7.4 by introducing the hydrazone formation step. The reaction between SC and NFA was 145 times more rapid at pH 5 as compared to pH 7.4.

The synergistic bactericidal activity apparent at pH 5 (Fig. 3C) for SC plus NFA can best be explained by invoking rapid formation of NFZ, which is more bactericidal than either precursor (Fig. 3B). The pH selectivity for combinations of NFA and SC (but not for NFZ) would be more pronounced over a wider range of NFA and SC concentrations if NFA were less bactericidal than NFZ at pH 7.4, as well as at pH 5. Unreacted NFA activity at pH 7.4 would be smaller, and the degree of selective synergism due to selective NFZ formation at pH 5 (Fig. 3A) would be more significant. The experimental results become more interesting on consideration of *in vivo* activity: NFZ can cure *Salmonella schottmulleri* and *Salmonella aertrycke* infections in mice, whereas *NFA is inactive in vivo* [27].

The pH-selective synergism exhibited by the combinations of SC plus NFA, like PC plus HP, is due to assembly rate constant specificity. By exploiting the principle of pH-selective synergism to selectively kill pathogenic microbes in acidic environments while sparing beneficial microbes in less acidic portions of the gut, it may be possible to prevent the often fatal pseudomembranous enterocolitis which can result from broad-spectrum antimicrobial treatment [30]. Selective formation of chelating agents in acidified endosomes, in contrast to the more alkaline nucleus, cytoplasm and mitochondria, could lead to a greater therapeutic index than that observed for a preformed chelating agent. Our demonstration of *environment*-selective synergistic biocidal activity in a mechanistic model provides an essential first step toward the development of *target*-selective synergism in experiments with infected macrophages in culture and infected animals.

In the third example, environment-selective synergism was introduced using aldehyde distribution

selectivity. Like the antitumor agents alkyl lysophospholipid (1-*O*-alkyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, ALP) [31] and 4-dodecylpyridine [32], DIOG is a cytotoxic detergent that can kill cells through direct membrane damage [4]. Cytotoxic selectivity after 1 hr against cells in 0% versus 1% serum (v/v in PBS, pH 6.6) was amplified when the DIOG was introduced as two self-assembling pieces, AOG and decanal (Fig. 4). Cells in 0% serum represent the simplified model target: extracellular serum levels tend to be abnormally low in solid tumor interiors [17]. (This model system is intended to provide an easily interpreted demonstration of the *principle* of environment-selective assembly and an elucidation of its mechanism. It is *not* intended to be the most suitable model for a comparison of tumor and normal cells and environments.)

A consideration of erythrocyte lysis kinetics helps clarify the origin of selectivity amplification (Fig. 5). When AOG (448 μM) and decanal (16 μM) were prereacted to form DIOG before adding them to cell suspension, lysis kinetics became monophasic. Serum (1%) caused an approximate 20-fold decrease in the hemolysis rate. Serum albumin is known to bind a wide variety of cationic detergents and other hydrophobic molecules with dissociation constants in the 20 to 100 μM range [1, 33]. The rate decrease can readily be explained by DIOG binding to albumin and other serum proteins (distribution selectivity). BSA at 0.75 mg/ml equals 1% calf serum in its ability to inhibit erythrocyte lysis by a combination of decanal (16 μM) and AOG (448 μM).

In contrast, lysis kinetics were biphasic when decanal and AOG were added to cells without prereaction (Fig. 5). Serum not only decreased the rate of the later, more rapid phase, but also lengthened the initial lag phase 5-fold. The lag phase (lysis rate of 0.5% per min) is the self-assembly period: the time period required for sufficiently cytotoxic levels of DIOG to form.

Considering the amplification in selectivity (Fig. 4), this increase of the lag phase by serum can best be explained by proposing that serum protein binding of decanal results in a decrease in decanal reactivity toward AOG. (The AOG concentration is high enough that serum protein binding of AOG would be of minor significance, at most.) Consistent with this hypothesis is the avid binding of 2-nonanone to serum albumin: 47 μM nonanone is 64% bound by 2 mg/ml BSA [1, 33]. Toxic selectivity between 0% serum and 1% serum involves distribution of both decanal and DIOG. The additional decrease in the rate at which DIOG reaches the cells (due to serum protein binding of decanal) effectively amplifies the selectivity inherent in DIOG distribution alone.

A second, more complex explanation for the selectivity amplification cannot be ruled out, using the experimental data presented here. It would require: (1) that serum proteins bind more avidly to decanal than to DIOG, and (2) that the DIOG transfer from serum proteins to erythrocyte membranes be slow enough to be rate-determining for lysis. The additional toxic selectivity would be caused by a further decrease in the rate at which DIOG reached the cell membranes. Again, decanal distribution selectivity would be responsible for environment-selective synergism involving such a mechanism.

A mechanism involving serum enzyme-promoted oxidation of decanal is highly unlikely in light of two observations: (1) erythrocyte lysis in the presence of 16 μM decanal, 448 μM AOG, and 1% serum did not become more rapid when oxygen was excluded from the suspension; and (2) purified BSA is also capable of inhibiting erythrocyte lysis.

The use of combinations of hydrazine derivatives and aldehydes (self-assembling biocides) in place of their more toxic hydrazone products made possible greater toxic selectivity *in vitro* between cells in different environments. This study demonstrates that aldehyde distribution selectivity and the second order rate constant for the bimolecular reaction can provide additional discrimination between the two environments by affecting the overall rate of biocidal hydrazone formation. Although this model system is an extreme simplification of the tumor and normal cell environment *in vivo*, its demonstration of the basic principle of environment-selective synergism in a mechanistic sense is an essential first step toward more sophisticated *in vitro* models with cultured cells and whole animal studies with solid tumors.

Our results in mechanistic model systems suggest that cytotoxin self-assembly could become a useful approach for rational development of chemotherapeutic combinations exhibiting target-selective synergism provided the target and normal tissue environments differ in ways which significantly affect hydrazone formation rates. Because selectivity factors could be introduced independently in two or more pharmacokinetically distinct precursor molecules, such an approach would provide advantages over conventional prodrug strategies. Our current efforts are aimed at developing combinations which exhibit target-selective synergism in systems involving cultured mammalian cell lines and whole animals.

Acknowledgements—We would like to thank Charles Ajioka, Peter Bonventre, Carolyn Deal, Patricia Fields, Fred Heffron, James Hicks, Donald Hilvert, Richard Lerner, Alan Schwabacher, and Magdelene So for helpful discussions concerning this project.

REFERENCES

1. Albert A, *Selective Toxicity*, 7th Edn. Chapman & Hall, New York, 1985.
2. Cates LA, Li V-S, Powell DR and Van der Helm D, Phosphorus-nitrogen compounds. 24. Phosphoramide mustard carrier derivatives. *J Med Chem* **27**: 397–401, 1984.
3. Beres J, Sagi G, Bentrude WG, Balzarini J, De Clercq E and Otvos L, Synthesis and antitumor and antiviral properties of 5-halo- and 5-(trifluoromethyl)-2′-deoxyuridine 3′,5′-cyclic monophosphates and neutral triesters. *J Med Chem* **29**: 1243–1249, 1986.
4. Rideout D, Self-assembling cytotoxins. *Science* **233**: 561–563, 1986.
5. Holta E, Korpela M and Moui T, Several inhibitors of ornithine and adenosylmethionine decarboxylases may also have antiproliferative effects unrelated to polyamine depletion. *Biochim Biophys Acta* **677**: 90–102, 1981.
6. Inoue H and Oka T, The effect of inhibitors or ornithine decarboxylase on DNA synthesis in mouse mammary gland in culture. *J Biol Chem* **255**: 3308–3312, 1980.

7. Harik SI and Snyder SH, Ornithine decarboxylase: Inhibition by α-hydrazinoornithine. *Biochim Biophys Acta* **327**: 501–509, 1973.
8. Brownlee M, Vlassana H, Kooney A, Ulrich P and Cerami A, Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* **232**: 1629–1632, 1986.
9. O'Donnell JP, Proveaux WJ and Ma JKH, High-performance liquid chromatographic studies of reaction of hydralazine with biogenic aldehydes and ketones. *J Pharm Sci* **68**: 1524–1526, 1979.
10. Mukkada AJ, Meade JC, Glaser TA and Bonventre PF, Enhanced metabolism of *Leishmania donovani* amastigotes at acid pH: An adaptation for intracellular growth. *Science* **229**: 1099–1101, 1985.
11. Moulder JW, Comparative biology of intracellular parasitism. *Microbiol Rev* **49**: 298, 1985.
12. Bloom BR, Games parasites play: How parasites evade immune surveillance. *Nature* **279**: 21–26, 1985.
13. Rabinovitch M, Zilberfarb V and Ramazeilles C, Destruction of *Leishmania mexicana amazonensis* amastigotes within macrophages by lysosomotropic amino acid esters. *J Exp Med* **163**: 520–535, 1986.
14. Mellman I, Fuchs R and Helenius A, Acidification of the endocytic and exocytic pathways. *Annu Rev Biochem* **55**: 663–700, 1986.
15. Ohkuma S and Poole B, Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc Natl Acad Sci USA* **75**: 3327–3331, 1978.
16. Cohen JJ and Kassirer JP, *Acid Base*, p. 147. Little, Brown & Co., Boston, MA, 1982.
17. Bohmer RM and Morstyn G, Uptake of hematoporphyrin derivative by normal and malignant cells: Effect of serum, pH, temperature, and cell size. *Cancer Res* **45**: 5328–5334, 1985.
18. Jencks WP, Studies of the mechanism of oxime and semicarbazone formation. *J Am Chem Soc* **81**: 475–481, 1959.
19. Young PR, Howell LG and Owen TC, Pyruvamide semicarbazone formation. Kinetics, mechanism, and pertinence to pyruvamide-dependent histidine decarboxylase. *J Am Chem Soc* **97**: 6544–6551, 1975.
20. Chou T-C and Talalay P, Applications of the median-effect principle for the assessment of low-dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. In: *New Avenues in Developmental Cancer Chemotherapy* (Eds. Harrap KR and Connors TA), pp. 37–64. Academic Press, New York, 1987.
21. Shoemaker DP, Garland CW and Steinfeld JJ, *Experiments in Physical Chemistry*, 3rd Edn. McGraw Hill, New York, 1974.
22. Arkin H and Colton RR, *Statistical Methods*. Harper & Row, New York, 1970.
23. Mihkelson AE, Reactions of PD(II) with a series of potentially tridentate nitrogen ligands—I. *J Inorg Nucl Chem* **43**: 123–126, 1981.
24. Renfrew RW, Osvath P and Weatherburn DC, Kinetic studies on the substitution reactions of (pyridine-2-carbaldehyde 2′-pyridylhydrazone)nickel (II). A study of stacking interactions in outer-sphere complexes. *Aust J Chem* **33**: 45–55, 1980.
25. Harvey SC, Antiseptics and disinfectants; Fungicides; Ectoparasiticides. In: *The Pharmacological Basics of Therapeutics* (Eds. Gilman AG, Goodman LS and Gilman A), 6th Edn. p. 979. Macmillan, New York, 1980.
26. Rollo IM, Miscellaneous drugs used in the treatment of protozoal infections. In: *The Pharmacological Basis of Therapeutics* (Eds. Gilman AG, Goodman LS and Gilman A), 6th Edn, p. 1074. Macmillan, New York, 1980.
27. Dodd MC, The chemotherapeutic properties of 5-nitro-2-furaldehyde semicarbazone (furacin). *J Pharmacol Exp Ther* **86**: 311–323, 1946.
28. Goldstein BP, Nielson F, Berti M, Bolzoui G and Silvestri LG, The mechanism of action of nitro-heterocyclic antimicrobial drugs. Primary target of 1-methyl-2-nitro-5-vinylimidazole is DNA. *J Gen Microbiol* **100**: 271–281, 1977.
29. Chatterjee SN, Ghose S and Maiti M, Cross linking of deoxyribonucleic acid in furazolidone treated *Vibrio cholerae* cell. *Biochem Pharmacol* **26**: 1453–1454, 1977.
30. Bockus HL (Ed.), *Gastroenterology*, Vol. 2, p. 686. WB Saunders, Philadelphia, PA, 1976.
31. Morris-Natschke S, Surles JR, Daniel LW, Berens ME, Modest EJ and Piantadosi C, Synthesis of sulfur analogs of alkyl lysophospholipid and neoplastic cell growth inhibitory properties. *J Med Chem* **29**: 2114–2119, 1986.
32. Firestone R, Pisano J and Bonney R, Lysosomotropic Agent. 1. Synthesis and cytotoxic action of lysosomotropic detergents. *J Med Chem* **22**: 1130–1133, 1979.
33. Helmer F, Kiehs K and Hansch C, The linear free-energy relationship between partition coefficients and the binding and conformational perturbation of macromolecules by small organic compounds. *Biochemistry* **7**: 2858–2863, 1968.