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Nitric Oxide Donor-Mediated Killing of Bioluminescent *Escherichia coli*

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The antimicrobial activities of two nitric oxide-releasing compounds against *Escherichia coli* were investigated by using recombinant *E. coli* cloned with a luciferase gene from *Pyrophorus plagiophthalamus*. Since luciferase uses intracellular ATP to generate visible light which can be measured from living cells in real time, we wanted to compare the extent to which cell viability parallels light emission. Results from luminescence measurements and CFU counts were in good agreement, and the decrease in light emission was shown to provide a rapid and more sensitive indication of cytotoxicity.

Many types of cells produce nitric oxide (NO) from L-arginine through an enzymatic pathway (17, 22). NO is a labile substance which accounts for the activity of endothelium-derived relaxing factor and which causes vasodilatation, inhibition of platelet aggregation, and tumor cytotoxicity (20). The NO produced by activated macrophages is known to kill *Mycobacterium avium* and *Listeria monocytogenes* (2), *Legionella pneumophila* (28), and *Ehrlichia risticii* (23). The exact mechanisms of NO-induced bacterial killing are still largely unknown.

Insect luciferase is an unusual enzyme produced by the Jamaican click beetle (*Pyrophorus plagiophthalamus*) (25) or the North American firefly (*Photinus pyralis*) (18), for instance. The enzyme uses D-luciferin, O_2, and ATP as substrates and produces AMP, oxyluciferin, PP_i, water, and light (547 to 617 nm) as end products (3). The gene coding for green light-emitting luciferase from the click beetle has been expressed in *Escherichia coli* (30) and insect *Spodoptera frugiperda* cells (9). The better-known firefly luciferase gene already has an established record of being a standard in reporter gene applications, and it has therefore been expressed in a wide variety of hosts from prokaryotic to eukaryotic cells (for a recent review, see reference 7). In each case the new host of the gene is able to produce light, which can be measured very sensitively. Therefore, many applications have been created during the last few years, for example, for the extremely sensitive detection of drug susceptibility of *Mycobacterium tuberculosis* (8). Because of its linkage to ATP metabolism, luciferase activity in vivo is a good indicator of the intracellular state. Indeed, soluble firefly luciferase is widely used to measure microbial contamination of foods, beverages, and surfaces since there has been shown to be a direct correlation between viable microbial cell count and ATP (for a review, see reference 27).

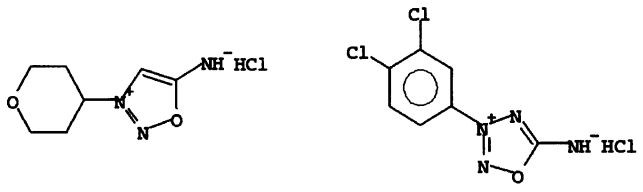
Compounds which produce NO can be used as tools to study the effects of endogenously produced NO and the mechanisms responsible for NO-mediated reactions. Recently, NO-nucleophile complexes have been shown to inhibit the in vitro proliferation of melanoma cells (16). In addition, gaseous NO, when added at high concentrations to intact human cells and to aerobic solutions of RNA, DNA, guanine, or adenine, may result in purine deamination, increased mutation frequency, DNA strand breakage, and cytotoxicity (21).

The sydnonimine 3-morpholine-sydnonimine (SIN-1), a known NO donor, undergoes an oxygen-dependent release of NO with the concomitant production of superoxide anion (4, 5). Recently, a novel group of NO-releasing compounds, mesoionic 3-aryl-substituted oxatriazole-5-imine derivatives (GEA compounds), has been used to study the effects of NO in different cell models (15, 19, 29). GEA 3162, which produces NO by a different, as yet unknown, mechanism from that of SIN-1 (15), inhibits several human neutrophil functions in vitro, with the concomitant production of cyclic GMP (19), is cytotoxic in vitro against healthy and malignant human hematopoietic cells (29), and inhibits endothelial cell-mediated oxidation of low-density lipoprotein (15).

The present work was carried out to study NO-mediated killing of bacteria by using *E. coli* cloned with the insect luciferase gene from *P. plagiophthalamus*, with a novel mesoionic oxatriazole (GEA 3162) and SIN-1 used as NO donors. We present data showing that the viabilities of cells closely parallel the light-emitting ability of *E. coli* treated with these substances.

MATERIALS AND METHODS

Chemicals. GEA 3162 and SIN-1 were provided by GEA Pharmaceutical Co., Copenhagen, Denmark, and their chemical structures are shown in Fig. 1. Immediately before use these compounds were dissolved in dimethyl sulfoxide to 10 mM stock solutions. Further dilutions were made in Hanks



SIN-1 GEA 3162

FIG. 1. Chemical structures of GEA 3162 and SIN-1.

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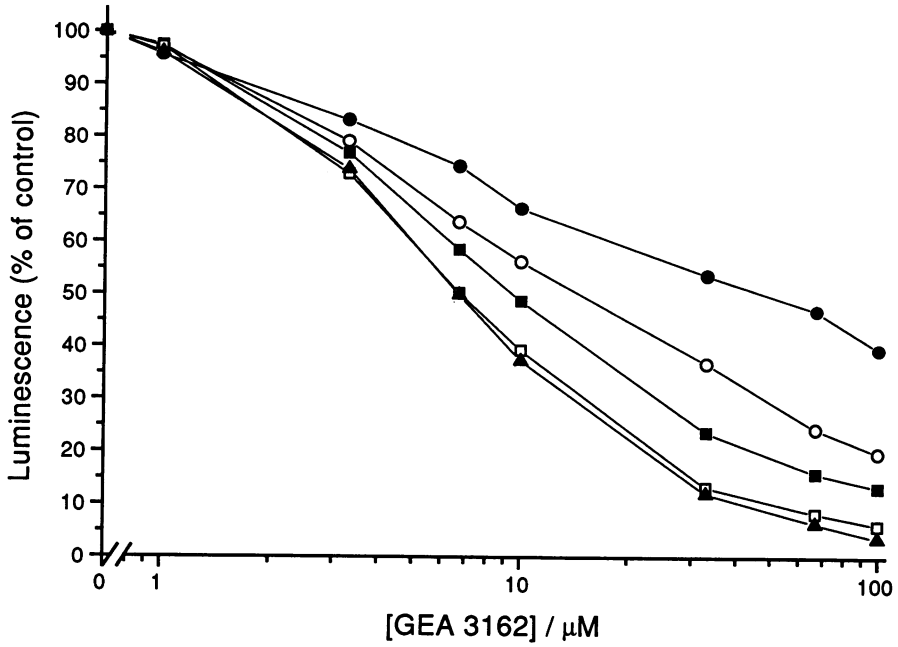

FIG. 2. Dose-response curve of GEA 3162 and effects of different incubation times on light production of *E. coli* JM101/pCSS962/pGB3. Untreated cells were used as controls. Incubation times of 10 (●), 20 (○), 30 (■), 60 (□), and 90 (▲) min were used.

balanced salt solution (HBSS; 0.14 g of $CaCl_2$, 0.4 g of KCl, 0.06 g of KH_2PO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 8.0 g of NaCl, 0.35 g of $NaHCO_3$, 0.09 g of Na_2HPO_4, and 1.0 g of glucose per liter [pH 7.4]). The luciferase substrate D-luciferin was from BioTools Oy, Turku, Finland. The other chemicals were of analytical grade.

Bacteria and cultivation. *E. coli* JM101 (*supE thi* Δ(*lac-proAB*) F'[*traD36 proAB lacI*^{q} *lacZ*ΔM15]) cloned with plasmids pCSS962 and pGB3 was used as the test strain. Plasmid pCSS962 (11) is a shuttle plasmid between *E. coli* and *Bacillus subtilis* containing the *lucGR* gene for *P. plagiophthalamus* luciferase and the *cat* gene for chloramphenicol acetyltransferase. Plasmid pGB3 (13) is a helper plasmid containing the *lacI* repressor gene and a β-lactamase gene for ampicillin resistance. Bacteria were cultivated in Luria broth medium supplemented with 30 μg of chloramphenicol and 100 μg of

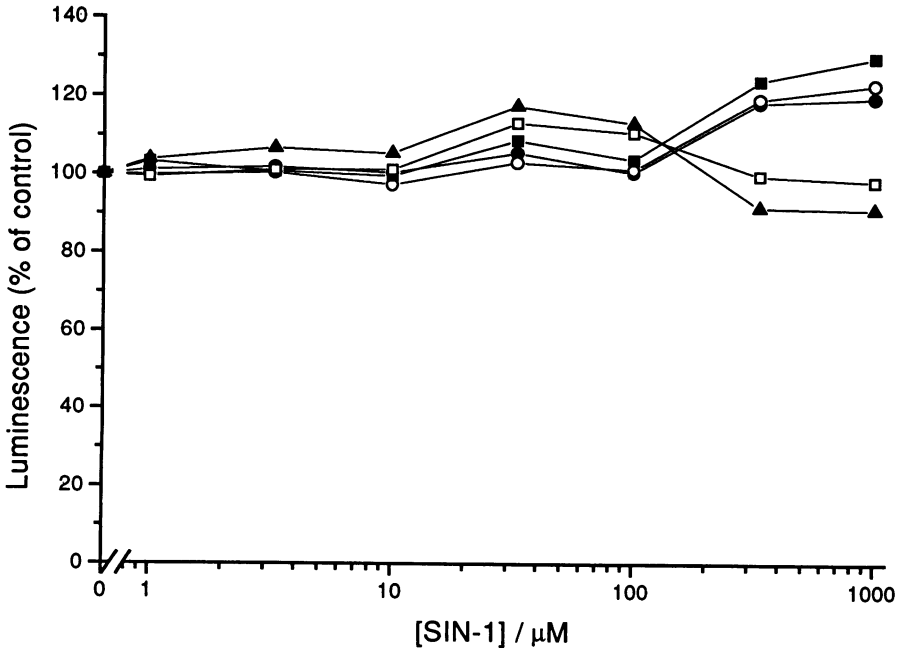

FIG. 3. Dose-response curve of SIN-1 and effects of different incubation times on light production of *E. coli* JM101/pCSS962/pGB3. Untreated cells were used as controls. See Fig. 2 legend for definitions of symbols.

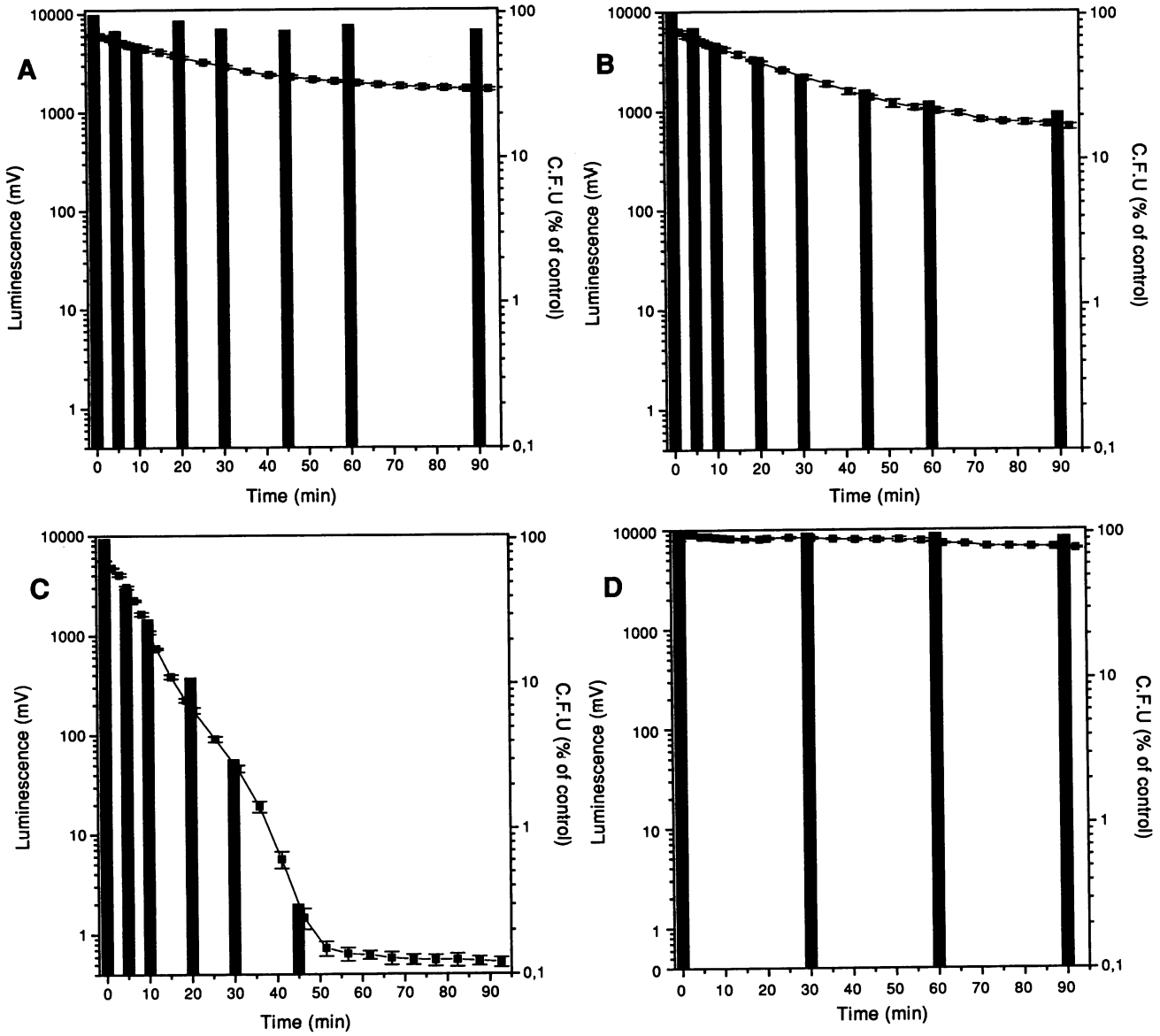

FIG. 4. Correlation of bacterial viability (CFU) and luminescence from *E. coli* JM101/pCSS962/pGB3 treated with different concentrations of NO donors. The NO donors and their concentrations were as follows: 10 μM GEA 3162 (A), 33 μM GEA 3162 (B), 100 μM GEA 3162 (C), and 1,000 μM SIN-1 (D). Light emission values are means of four independent measurements, and viability counts are from duplicate measurements. Error bars represent the standard deviations of the measurements. The bars denote the viabilities of the bacteria, and the curves denote the light emission by bacterial cells.

ampicillin per ml at 30°C with vigorous shaking to the mid-logarithmic phase, washed twice with HBSS, and kept in an ice bath until use. The light production of the cells was sufficient enough without the induction of luciferase synthesis, and thus, induction by isopropyl-β-D-thiogalactopyranoside was not used in order to keep the cells in an unstressed state.

Luminescence measurements. Luminescence was measured with an LKB Wallac 1251 automatic luminometer at a temperature of 30°C. The reaction mixture consisted of 10 μl of NO donor dilution, 15 μl of HBSS, 50 μl of 1 mM D-luciferin in HBSS, and 25 μl of cell dilution. For each measurement 10^7 cells were used. Measurements were done in quadruplicate and were started by adding bacteria to the reaction mixture exactly 15 min after diluting the NO donor in HBSS.

Viable counts. One hundred microliters of suitable dilutions of reaction mixtures similar to those used in luminescence measurements were plated onto Luria agar plates containing the same concentrations of antibiotics as those in the liquid medium used for cultivation. After overnight incubation at 30°C the colonies were counted. Viability measurements were done in duplicate.

RESULTS

Effects of GEA 3162 and SIN-1 on light production. As little as 10 min of incubation with GEA 3162 at a concentration of 10 μM reduced light production to 70% compared with that by untreated controls (Fig. 2). By using longer incubation times

and higher concentrations, the light production was effectively inhibited so that 60 min of incubation in 33 μM GEA 3162 resulted in almost 90% inhibition, which did not increase if the incubation time was increased. Incubation with 100 μM GEA 3162 for 90 min resulted in 95% inhibition. SIN-1 did not cause any reduction in light production in 90 min at a concentration of 100 μM; however, 1,000 μM SIN-1 reduced light emission to 90% of that of the control after 90 min of incubation (Fig. 3).

Correlation between light production and cell viability in GEA 3162- and SIN-1-treated *E. coli*. The effects of 100 μM GEA 3162 on light production and cell viability were very rapid (Fig. 4C). At as early as 5 min after the start of incubation there was a 50% inhibition of light production and decrease in cell viability. After 30 min of incubation no viable cells were detected. The inhibition of light production and the decrease in cell viability correlated well. Similar but less pronounced effects were found when the cells were treated with 33 μM GEA 3162. After 90 min of incubation with 33 μM GEA 3162 cell viability was decreased to 10% of that of the control (Fig. 4B). When 10 μM was used, the inhibition of light production proved to be a more sensitive indicator of cytotoxicity than counting of CFU (Fig. 4A). SIN-1 did not affect cell viability, despite slight decreases in bioluminescence, which again seems to be a more sensitive indicator of cytotoxicity (Fig. 4D).

DISCUSSION

In the present work we demonstrated the bacteriocidal effect of the novel mesoionic oxatriazole compound GEA 3162 against recombinant *E. coli* cloned with the click beetle luciferase gene. These results also emphasize the sensitivity of bioluminescence in testing antimicrobial agents. The results may also help us in the future to better understand the biochemical mechanisms of the antimicrobial effects of eukaryotic cells that produce NO because of the activation of inducible NO synthetases.

The exact mechanisms of NO-mediated bacterial killing are not known. From a biological standpoint, the important reactions of NO are those with oxygen and its various redox forms and with transition metal ions (26). In some situations NO can interact with superoxide anions to generate peroxynitrite radicals which can be decomposed to highly active hydroxyl radicals, which in turn can cause cell injury (1). NO inhibits DNA synthesis by inhibiting ribonucleotide reductase, a rate-limiting enzyme in the process (14). NO also inhibits mitochondrial respiration by reacting with aconitase (6). The novel NO donor GEA 3162 proved to be a much more effective antibacterial agent than the other NO donor, SIN-1. This difference in potency is in accordance with the results obtained with other experimental cell models, including inhibition of human neutrophil functions (19), cytotoxicity against human hematopoietic cells (29), and inhibition of endothelial cell-mediated oxidation of low-density lipoprotein (15). GEA 3162 generates NO by an as yet unknown mechanism, but the data from NO measurements by an oxyhemoglobin method (24) indicate differences between SIN-1 and GEA 3162 with respect to the mechanisms by which they generate NO (15), which may explain the different abilities of these NO donors to kill bacteria.

In the present work we demonstrated that NO donors kill recombinant *E. coli* and that the light-producing capability of *E. coli* closely parallels cell viability, as judged by plate counts. Performing plate counts is tedious and time-consuming, and as found in the present study, measurement of light gives a means of real-time analysis of cell killing which is directly correlated to the viable cell count. The luciferase reporter system relies on the measurement of the intracellular concentration of ATP, which decreases as the cell starts to die. The sensitivity of the system can clearly be seen from Fig. 4A, which shows the results when low concentrations of GEA 3162 were used to treat *E. coli*. A decrease in light emission is evident, although cell viability is not yet affected.

In several cases it has been shown that the measurement of in vivo bioluminescence from recombinant organisms directly correlates to living cell mass (10, 12). Recently, the luciferase reporter system was used for the rapid assessment of the drug susceptibilities of *M. tuberculosis* (8). In that work it was shown that antituberculosis drugs resulted in the extinction of light production and helped to reduce the time required to establish the antibiotic susceptibilities of *M. tuberculosis* strains from weeks to days and to accelerate the screening of new antituberculosis drugs. It seems evident that the luciferase reporter system is applicable to the rapid and sensitive screening of various antimicrobial agents. Expression of the luciferase gene in a target organism makes screening fast and reliable.

In conclusion, we have demonstrated that novel mesoionic oxatriazole derivatives can be used as a versatile tool to study the effects of NO in a bacterial model, as evidenced by the novel measurement of bioluminescence and its correlation to the viable cell counts of recombinant bacteria.

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