THE STIMULATION OF BIOLUMINESCENCE IN *PHOTOBACTERIUM LEIOGNATHI* AS A POTENTIAL PRESCREEN FOR ANTITUMOR AGENTS

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The stimulation of bioluminescence in *Photobacterium leiognathi* has previously been described as a test for genotoxic compounds. An adaptation of this procedure has been developed which uses a dim variant of *P. leiognathi* and permits the prescreening of microbial fermentation broths for potential antitumor agents. Bioluminescence in this organism was stimulated by compounds which bind to DNA or affect DNA synthesis. Antibiotics with target sites such as protein, cell wall or RNA synthesis, did not alter bioluminescence. Fermentation broths from over 5,000 soil isolates were prescreened in this assay and 95 (1.6%) were defined as active. Further analysis of selected cultures suggested that about half produced compound(s) with DNA-binding activity. These results suggest that the photobacterium induction assay (PIA) may be useful as a prescreen for potential antitumor agents. The assay is rapid, simple and requires only microgram quantities of material for testing.

A variety of *in vitro* test systems has been developed to detect the presence of secondary metabolites in microbial fermentation broths that have potential as anticancer agents[1,2]. Active compounds selected by these prescreens are subsequently progressed to an *in vivo* screen such as a transplantable murine tumor. Most of the currently used anticancer drugs are considered to exert their cytotoxic activity *via* an effect on DNA[3]. Indeed, many are mutagenic, carcinogenic, or both in microbial and animal test systems[4,5]. Several prescreens have been based on this association between anticancer activity and a compound's ability to interact with or damage DNA, *e.g.* the biochemical induction assay[6]. A number of recent studies have focused on the effect of genotoxic agents on bacterial bioluminescence[7~10]. Of special interest in these assays are the dim variants which exhibit increased bioluminescence upon exposure to such agents. Although there is evidence that bacterial bioluminescence is an inducible system, the mechanism for chemical induction of bioluminescence in these organisms is not known[11].

In this report, we describe the development of a rapid, sensitive microbiological prescreen which uses a dim variant of *Photobacterium leiognathi* to detect potential antitumor compounds that bind to or affect DNA by means of increased bioluminescence.

Materials and Methods

Bacterial Strains

A wild type strain of *P. leiognathi* was obtained from American Type Culture Collection (ATCC 27561) and deposited in the Lederle stock collection as RC93. RC93-2 was a spontaneous dim variant of RC93 selected for use in the photobacterium induction assay (PIA).

Media

The medium used for bacterial growth (731-A) was the same as ATCC 731 except that the $CaCO_3$ was omitted. 731-A contained NaCl 30.0 g, K_2HPO_4 3.9 g, KH_2PO_4 2.1 g, NH_4Cl 5.0 g, yeast extract (Difco) 5.0 g, Bacto-Tryptone (Difco) 5.0 g, 1 M Tris buffer pH 7.5 50.0 ml, glycerol 3.0 ml, and KCl 0.75 g in 1 liter of distilled water. The pH was adjusted to 7.2 before autoclaving and 4.0 ml of $MgSO_4 \cdot 7H_2O$ (25% w/v) were added aseptically after sterilization. Agar was added to a final concentration of 1.5% (w/v) for use in culture plates and 1% for use in base layers or in seeded overlays.

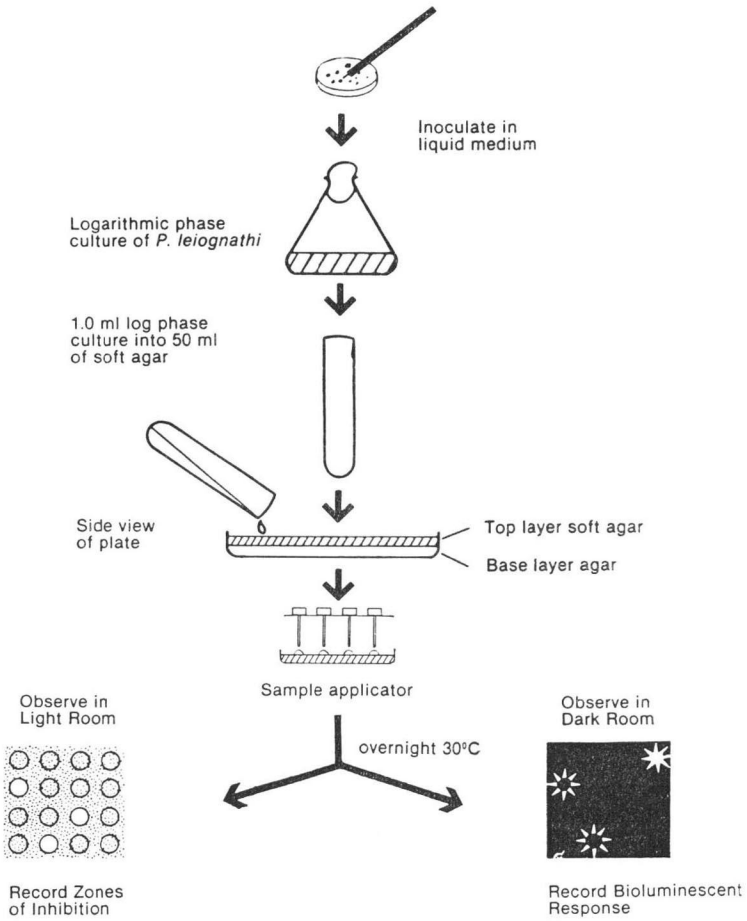
Chemicals

Acriflavine, proflavine, ethidium bromide, and nalidixic acid were obtained from Sigma Chemical Co. Antitumor antibiotics were obtained from Dr. D. BORDERS, Medical Research Division, American Cyanamid Company, Lederle Laboratories. All other compounds were purchased as Sensi-Discs from Baltimore Biological Laboratories (BBL).

Photobacterium Induction Assay (PIA)

A schematic diagram of the PIA is given (Fig. 1). Briefly, RC93-2 was grown in 731-A, diluted 1:1 with glycerol, and then stored at −70°C. A fresh, overnight culture was prepared by scraping the surface of the contents of a frozen vial, streaking the organism onto 731-A agar, and incubating the culture plate for 16~24 hours at 30°C. A single colony was used to inoculate 10 ml of 731-A broth in a 50-ml flask. The culture was aerated by shaking at 30°C, and absorbance was monitored with an

Fig. 1. Schematic representation of photobacterium induction assay.



LKB Ultrospec (Model 4050-011). At an $A_{660}=0.5$ unit, a 1.0 ml sample of this culture was used to inoculate a 50-ml overlay (45°C) which was immediately poured onto a 100-ml base layer in a 23-cm^2 Nunc Bioassay Dish (240835). After the overlay solidified, BBL Sensi-Discs or paper disks (6.35 mm; Schleicher and Schuell) containing 25 μl of sample solutions were applied to the agar surface. The plates were incubated 16~18 hours at 30°C, then scored for antimicrobial activity under normal light conditions. The plates were then examined in a light tight room and, after allowing for adjustment of one's eyesight to the dark, the bioluminescent responses were assessed relative to controls. Permanent records were obtained by photographing the plates in the absence of light using a Nikon camera with a Micro-Nikkor 55 mm 1: 2.8 lens and Tri X 400 (pushed to 1600 ASA) using an F stop= 2.8, and a 3-minute exposure.

Assay for Drug-DNA Interaction

Herring testes DNA (Sigma) was prepared as a 10 × stock in TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) and then incorporated directly into the agar at 1 mg/ml. Various compounds were then analyzed in the PIA as described above. Proflavine (25 μg/disk), which intercalates DNA base pairs, served as a positive control. Nalidixic acid (2.5 μg/disk), which does not interact with DNA directly, was used as a negative control.

Results

PIA Activity of Known Compounds

A number of compounds with different biochemical target sites was evaluated (Table 1). The concentration given for active compounds indicates the minimum amount required to stimulate bioluminescence above background. Compounds were considered inactive if they failed to stimulate bioluminescence at the maximum concentration tested. Agents which inhibited protein, RNA or

Table 1. Compounds evaluated in the PIA.

	Compound	Concentration (μg/disk)	Growth inhibition (mm)	PIA response[a]
Intercalating dyes	Acriflavine	6.3	0	+
	Proflavine	2.5	0	+
	Ethidium bromide	6.3	0	+
DNA synthesis inhibitors	Nalidixic acid	2.5	16	+
	Cinodine	0.8	0	+
	Novobiocin	30	18	−
Protein synthesis inhibitors	Clindamycin	2	0	−
	Erythromycin	15	10	−
	Tetracycline	30	23	−
	Gentamicin	10	10	−
	Streptomycin	10	10	−
	Chloramphenicol	30	27	−
Cell wall synthesis inhibitors	Penicillin	100	0	−
	Ampicillin	10	0	−
	Cefamandole	30	22	−
	Vancomycin	30	0	−
Miscellaneous antibiotics	Nystatin	100 units	0	−
	Sulfachloropyridazine	250	16	−
	Sulfathiazole	250	0	−
	Rifampin	5	15	−

[a] +: Active at minimum concentration indicated.
−: Inactive at maximum concentration tested.

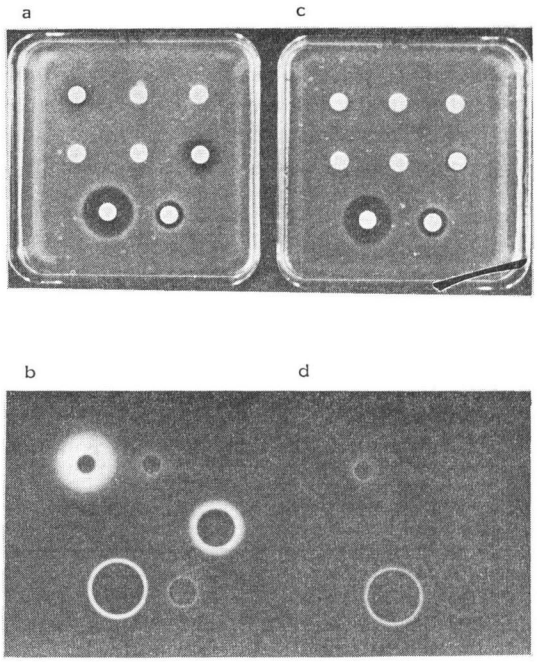
Table 2. Antitumor antibiotics evaluated in the PIA.

	Concentration (μg/disk)	Growth inhibition (mm)
Active compounds[a]		
Bleomycin	25	0
Hedamycin	25	0
Mitomycin C	1.6	8
Netropsin	1.6	0
Ravidomycin	25	13
Streptonigrin	0.02	0
Streptozotocin	0.05	0
Trioxacarcin	12.5	9
Inactive compounds[b]		
Actinomycin D	25	10
Anthramycin	25	12
Carminomycin	25	8
Chromomycin	25	0
Copiamycin	25	0
Doxorubicin	25	0
5-Fluorouracil	25	21
Largomycin	25	0
Mithramycin	25	0
Neocarzinostatin	25	0
Saframycin	25	9

[a] Active: At minimum concentration tested.
[b] Inactive: At maximum concentration tested.

Fig. 2. Effect of DNA on bioluminescent responses in the PIA.

Disks containing individual drugs were arranged left to right on the agar surface of a 9-cm^2 assay plate: top row: proflavine 25, 2.5 and 0.25 μg/disk; middle row: netropsin 0.25, 2.5 and 25 μg/disk; bottom row: nalidixic acid 2.5 and 0.25 μg/disk. The antimicrobial activities (a & c) and bioluminescent responses (b & d) in the absence (a & b) and in the presence (c & d) of exogenous DNA (1 mg/ml) are shown.



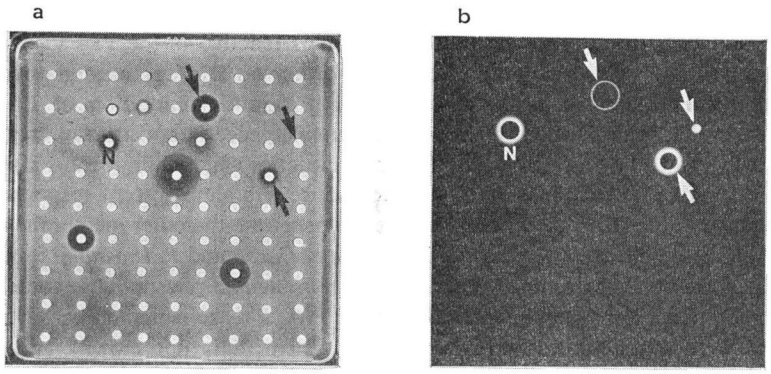
cell wall synthesis were not active in the PIA. With the exception of novobiocin, all of the tested compounds known to interact with DNA or inhibit DNA-related processes were active in the PIA. Various antitumor antibiotics were also tested in the PIA (Table 2). All of the active compounds interact directly with DNA. However, several other drugs with known DNA interactions were not detected by the PIA.

Determination of DNA-drug Interactions

A secondary assay was developed to determine if the PIA activity of an unknown compound resulted from direct or indirect interactions with DNA. Exogenous herring testes DNA was used to determine if the compounds could bind to DNA. Presumably, once bound to exogenous DNA, drugs would become unavailable and the bioluminescent response in the PIA would, therefore, be diminished or eliminated. Proflavine was selected as a positive control in the DNA-binding assay, whereas nalidixic acid served as a negative control. The amount of DNA required to effectively diminish PIA activity is determined by the number of molecules of drug which can bind per nucleotide base pair. DNA (1 mg/ml) was required to maximize the reduction of the bioluminescent response to proflavine (Fig. 2). Approximately one molecule of proflavine binds per 2.5 base pairs[3]. This molar ratio translates into a weight: weight ratio of 1:6 for proflavine: DNA. Thus, 150 μg of DNA binds 25 μg of proflavine. The PIA response to netropsin, an antitumor antibiotic that binds avidly to the

Fig. 3. Evaluation of microbial fermentation broths in the PIA.

A large assay plate containing samples of 80 fermentation broths was photographed in the light (a) and dark (b). Netropsin (N) 25 μg/disk served as a control. Three fermentation broths (arrows) appeared active on this plate.



minor groove of DNA, was also reduced by the presence of exogenous DNA[12]. As expected, the presence of DNA had no effect on the bioluminescent ring around nalidixic acid.

Evaluation of Fermentation Broths for PIA Response

Netropsin, a typical natural product and known antitumor antibiotic, was used as a positive control in the PIA when prescreening microbial fermentation broths for potential antitumor agents. A bright area of bioluminescence appeared around the netropsin disk (Fig. 3). Samples that stimulated bioluminescence to a similar degree as netropsin were considered active. Of the three active fermentation broths indicated (arrows), one was later shown to contain netropsin, whereas the active component(s) of the two remaining broths have not yet been identified.

Of the 5,760 soil isolates that were analyzed, fermentation broths from 95 (1.6%) were active in the PIA. After further analysis of the active broths, thirteen were selected for purification and chemical identification based on the reproducibility of the fermentation and bioluminescent response. Seven of the thirteen (54%) exhibited a DNA-binding activity and all of these contained netropsin. The remaining broths are currently under investigation.

Discussion

A photobacterium induction assay (PIA) has been used to prescreen microbial metabolites for potential antitumor activity. Other applications of bacterial bioluminescence have exploited different species of *Photobacterium* to identify chemicals as mutagenic or carcinogenic agents[7,9]. Dim variants of *P. phosphoreum* and *P. leiognathi* express increased bioluminescence upon exposure to genotoxic agents. The sensitivity of these assays compares favorably with that of the AMES test[4]. The presence of a highly active P-450 cytochrome system in *Photobacterium* obviated the need for microsomal activation of certain procarcinogens (*e.g.* 20-methylcholanthrene) as is required for their detection in the AMES assay[8]. Although the exact mechanisms for the increased bioluminescence is unknown, it has been suggested that exposure of the dim variant to strong mutagens (*e.g.* *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine) results in the appearance of stable, bright forms of the organism. Presumably, a permanent change in the DNA has been effected which restores the wild type level of bioluminescence. Although the mechanism for the transient, phenotypic reversion induced by intercalating dyes or inhibitors of DNA synthesis is unknown, there is evidence that suggests *de novo* protein synthesis is re-

quired for the bioluminescent response[10,13].

The analysis of known compounds in the PIA compared favorably with the sensitivity and predictability of another *in vitro* test for antitumor agents, the biochemical induction assay (BIA)[6]. In the latter, compounds which bind to DNA or inhibit DNA synthesis induce a *λplacZ* prophage in *Escherichia coli*. With the exception of netropsin, all of the tested antitumor agents that were active in the PIA are active in the BIA[2]. Of those compounds inactive in the PIA (Table 2), only 25% were inactive in the BIA. This difference in detection of known DNA active compounds, such as actinomycin D and mithramycin, may result from their preferential inhibition of RNA synthesis[14]. Although the exact mechanism of the stimulated bioluminescence is not known, continued synthesis of luciferase is believed to be required[10,13]. Hence, antibiotics which inhibit transcription in addition to their DNA damaging activity may be unable to induce a bioluminescent response. Compounds may also be inactive in the PIA due to their inability to penetrate the outer membrane of *P. leiognathi*. A recent study on the biochemistry of the cell envelopes of *E. coli* and *P. leiognathi* reveals a significant difference between the two organisms in their outer membrane and phospholipid content[15].

When fermentation broths were prescreened in the PIA, a reasonable number (1.6%) were active. These broths were subsequently analyzed in the BIA system (data not shown), and 10% were BIA positive. The preliminary characterization of several broths suggested DNA-binding activity in that their PIA activity was prevented by the addition of exogenous DNA. These data support the hypothesis that bioluminescence of the dim variant of *P. leiognathi* is stimulated by agents that interact with DNA in a direct as well as in an indirect fashion. Although bioluminescence can be quantitated, the procedures are time consuming and unable to handle a large number of samples. The ability of the PIA to prescreen thousands of samples rapidly and reproducibly makes this type of prescreen more amenable to current preclinical programs. We conclude that the PIA is a specific screen capable of detecting potential antitumor agents through their interaction with DNA. The difference in selectivity between the PIA and BIA further suggests that this test can be applied to large-scale screening for new anticancer agents that may not be detected in other currently used prescreens.

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