# BACTERIAL DNA REACTIVITY OF THE NOVEL ANTITUMOR ANTIBIOTICS PD 114,759 AND PD 115,028 (VERACTAMYCINS A AND B)

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The novel antitumor antibiotics PD 114,759 and PD 115,028 were evaluated for their ability to cause repairable DNA damage and the induction of SOS functions in bacterial systems. PD 114,759 and PD 115,028 were preferentially toxic to DNA repair-defective *Escherichia coli* WP100 *uvrA recA* in comparison to wild-type *E. coli* WP2 at concentrations of 10~30 μg/ml in agar diffusion assays. Both compounds were inducers of cell filamentation and prophage λ (two *E. coli* SOS functions) at concentrations of 0.1~1 μg/ml. In addition, the ability of PD 114,759 and PD 115,028 to retain their filamentation-inducing effects under both aerobic conditions and anaerobic conditions suggests that a bioreductive, rather than an oxygen-requiring, mechanism is involved in the DNA-reactive effects of these agents.

PD 114,759 (veractamycin A) and PD 115,028 (veractamycin B) are two major components of an antibiotic complex produced by *Actinomadura verrucosospora* subsp. *veractimyces* ATCC 39363[1~3]. These antibiotics have marked antimicrobial activity, with minimal inhibitory concentrations of <6 pg/ml against several bacteria[1]. More importantly, they have marked antitumor effects *in vitro* against a variety of murine and human tumor cell lines, and *in vivo* against murine L1210 leukemia, B16 melanoma, M5076 sarcoma, Ridgway osteosarcoma, and the MX-1 human mammary xenograft[1,4]. With respect to the mechanism of action of these antibiotics, FRY *et al.* have shown that PD 114,759 is capable of causing significant single strand DNA breakage in L1210 cells *in vitro*[4]. The purpose of this paper is to demonstrate that PD 114,759 and PD 115,028 are DNA-reactive antitumor antibiotics based upon their ability to cause repairable DNA damage and the induction of SOS functions in *Escherichia coli*.

## Materials and Methods

### Chemicals

PD 114,759 and PD 115,028 were isolated as described by BUNGE *et al.*[2]. Each compound was solubilized in dimethyl sulfoxide, and fresh solutions were prepared for each assay. Bleomycin, daunomycin, mitomycin C and streptonigrin were obtained from the Warner-Lambert/Parke-Davis chemical repository.

### Microorganisms

*Escherichia coli* B derivatives WP2 (wild-type) and WP100 *uvrA recA* were obtained from E. WITKIN, Rutgers University, New Brunswick NJ, U.S.A. *E. coli* K-12 strain PQ37 (relevant genotype, *rfa uvrA sulA*:: *lacZ*) was provided by M. HOFNUNG, Institut Pasteur, Paris, France. *E. coli* K-12 strains

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BR513 (relevant genotype, *envA uvrB* λ:: *lacZ*) and BR339 (relevant genotype, *env*$^+$ *mal uvrB lexA* λ:: *lacZ*) were obtained from R. ELESPURU, Frederick Cancer Research Facility, Frederick, MD, U.S.A.

*Escherichia coli* WP2/WP100 *uvrA recA* DNA Repair Assay

*E. coli* DNA repair (differential toxicity) assays were performed as well-agar diffusion assays based on methods described by MAMBER *et al.*[5]. Either 12 or 7 mm diameter wells were cut in minimal glucose agar plates supplemented with casein hydrolysate (500 mg/liter) and L-tryptophan (20 mg/liter) seeded with either *E. coli* WP2 or *E. coli* WP100 in a soft (1%) agar overlay. Samples (80 μl/12 mm well or 50 μl/7 mm well) of a given test agent at varying concentrations were dispensed in triplicate vs. each strain. After incubating plates at 37°C for 24 hours, the diameters of the zones of growth inhibition were measured. Differential inhibition values (mean zone size difference) at each concentration

Table 1. Activity of PD 114,759 and PD 115,028 in the *Escherichia coli* WP2/WP100 DNA repair assay.

| Compound | Concentration (μg/ml) | *E. coli* WP100 *uvrA recA* mean inhibition zone diameter (mm) | *E. coli* WP2 mean inhibition zone diameter (mm) | Mean inhibition zone size difference (mm)[a] |
|---|---|---|---|---|
| PD 114,759[b] | 1 | 14.3 | 13.3 | 1.0 |
| | 3 | 16.3 | 14.7 | 1.7 |
| | 10 | 17.7 | 15.3 | 2.3 |
| | 30 | 19.3 | 16.0 | 3.3 |
| | 100 | 20.0 | 16.3 | 3.7 |
| PD 115,028[b] | 1 | 14.3 | 12.7 | 1.7 |
| | 3 | 15.3 | 13.3 | 2.0 |
| | 10 | 18.3 | 14.0 | 4.3 |
| | 30 | 19.7 | 16.0 | 3.7 |
| | 100 | 20.0 | 16.0 | 4.0 |
| Bleomycin[c] | 1 | 7.0 | 7.0 | 0.0 |
| | 5 | 7.0 | 7.0 | 0.0 |
| | 10 | 7.0 | 7.0 | 0.0 |
| | 50 | 11.3 | 11.2 | 0.1 |
| | 100 | 13.5 | 14.0 | −0.5 |
| Daunomycin[c] | 1 | 7.0 | 7.0 | 0.0 |
| | 5 | 7.0 | 7.0 | 0.0 |
| | 10 | 7.0 | 7.0 | 0.0 |
| | 50 | 11.5 | 7.0 | 4.5 |
| | 100 | 12.7 | 7.0 | 5.7 |
| Mitomycin C[c] | 1 | 8.0 | 7.0 | 1.0 |
| | 5 | 12.5 | 7.0 | 5.5 |
| | 10 | 16.5 | 7.0 | 9.5 |
| | 50 | 21.2 | 7.0 | 14.2 |
| | 100 | 24.5 | 8.2 | 16.3 |
| Streptonigrin[c] | 1 | 9.7 | 7.0 | 2.7 |
| | 5 | 15.3 | 7.0 | 8.3 |
| | 10 | 18.5 | 7.0 | 11.5 |
| | 50 | 21.0 | 11.5 | 9.5 |
| | 100 | 23.2 | 15.7 | 7.5 |
| Chloramphenicol | 30[d] | 29.8 | 30 | −0.2 |
| Tetracycline | 30[d] | 31.5 | 32.5 | −1.0 |

[a] Mean inhibition zone size difference=(WP100 mean inhibition zone diameter)−(WP2 mean inhibition zone diameter). A difference exceeding 3 mm was considered to be positive for repairable DNA damage. Values represent the means of 3 replicate samples.

[b] 12 mm wells cut in agar. [c] 7 mm wells cut in agar. [d] μg/disk (6.35 mm paper disks (Difco)).

were calculated as the mean inhibition zone vs. strain WP100 minus the mean inhibition zone vs. strain WP2. A mean zone size difference exceeding 3 mm for at least one test concentration was considered to be positive for repairable DNA effects.

*E. coli* K-12 SOS Chromotest

*E. coli* K-12 SOS chromotest agar spot tests were performed using methods described by MAMBER *et al.*[6]. Briefly, 20 $\mu$l aliquots of samples of varying concentrations were spotted directly onto the surface of LB agar trays or plates seeded with *E. coli* PQ37. After incubation at 37°C for 18~24 hours, a colorimetric indicator overlay containing 6-bromo-2-naphthol-$\beta$-D-galactopyranoside (Sigma Chemicals, St. Louis, MO, U.S.A.) and fast blue RR salt (Sigma) was prepared and poured onto the agar surface. Positive results were manifested by the presence of a red color (indicative of $\beta$-galactosidase activity) at the site of sample application. The size of the colored zone and the color intensity were used to obtain a semiquantitative measure of DNA effects.

To investigate the effects of aerobic vs. anaerobic incubation of chromotest plates on the DNA reactivity of PD 114,759 and PD 115,028, a comparative chromotest was performed using plates incubated under normal (aerobic) conditions and plates spotted with identical samples but incubated in Gaspak anaerobic jars (BBL, Cockeysville, MD). After incubation at 37°C for 18~24 hours, colorimetric overlays were prepared and poured onto all plates.

*E. coli* K-12 Bacteriophage Induction Assay (BIA)

Assays for prophage induction in *E. coli* K-12 strains BR513 and BR339 were performed using agar spot test methods[6,7]. Briefly, 20 $\mu$l aliquots of samples of varying concentrations were spotted in duplicate onto LBE + ampicillin (10 $\mu$g/ml) agar trays seeded with a given tester strain. The cell wall of strain BR339 was made more permeable to test agents by treating the cells with Trizma-HCl (Sigma) prior to seeding the agar[6,8]. After incubation at 37°C for 4 hours, a colorimetric indicator overlay (same as that for the chromotest) was prepared and poured onto the surface of the agar. The size of the colored zone ($\beta$-galactosidase activity) and intensity of the red color surrounding the site of application of a given sample were used to evaluate DNA effects.

## Results

Both PD 114,759 and PD 115,028 showed DNA reactivity in all microbial tests. In the differential

Fig. 1. Differential toxicity of PD 114,759 (○), PD 115,028 (●), bleomycin (△), daunomycin (▲), mitomycin C (□) and streptonigrin (■) in the *Escherichia coli* WP2/WP100 *uvrA recA* DNA repair assay.
Inhibition zone size difference=(inhibition zone for strain WP100)−(inhibition zone for strain WP2). Each point represents the mean of 3 replicate samples spotted against each tester strain.

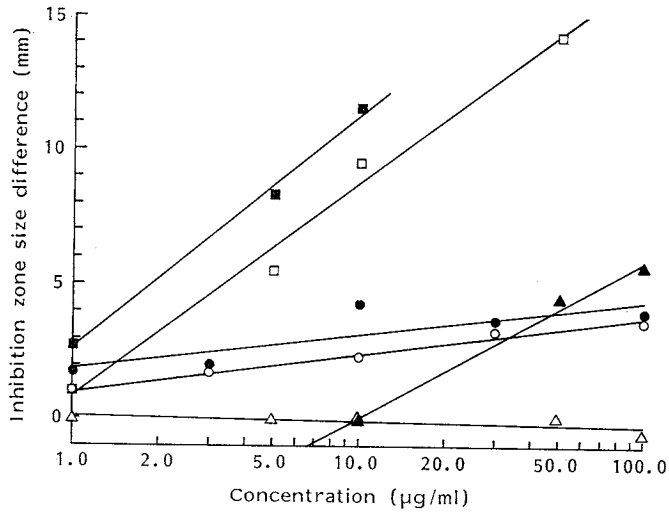

Table 2. Activity of PD 114,759 and PD 115,028 in the *Escherichia coli* K-12 SOS chromotest with tester strain PQ37.

| Compound | Aerobic incubation | | Anaerobic incubation | | Ratio of anaerobic/ aerobic MACs |
|---|---|---|---|---|---|
| | MAC ($\mu$g/ml) | OAC ($\mu$g/ml) | MAC ($\mu$g/ml) | OAC ($\mu$g/ml) | |
| PD 114,759 | 0.1 | 1 | 0.1 | 1 | 1 |
| PD 115,028 | 0.1 | 3 | 0.1 | 3 | 1 |
| Bleomycin | 0.1 | 3 | 10 | >10 | 100 |
| Daunomycin | 0.3 | 3 | 3 | 10 | 10 |
| Mitomycin C | 0.3 | 3 | 0.3 | 10 | 1 |
| Streptonigrin | 0.1 | 3 | 3 | >10 | 30 |

MAC: Minimal active concentration (lowest concentration showing activity).
OAC: Optimal active concentration (lowest concentration showing the greatest activity).

Table 3. Activity of PD 114,759 and PD 115,028 in the *Escherichia coli* K-12 bacteriophage induction assay (BIA) with tester strains BR513 and BR339.

| Compound | *E. coli* BR 513 | | *E. coli* BR 339 | |
|---|---|---|---|---|
| | MAC ($\mu$g/ml) | OAC ($\mu$g/ml) | MAC ($\mu$g/ml) | OAC ($\mu$g/ml) |
| PD 114,759 | 1 | 3 | 0.1 | 1 |
| PD 115,028 | 1 | 3 | 1 | 3 |
| Bleomycin | 1 | 3 | Negative | Negative |
| Daunomycin | 1 | 3 | 0.1 | 1 |
| Mitomycin C | 10 | 100 | 3 | 10 |
| Streptonigrin | 3 | 10 | 1 | 3 |

MAC: Minimal active concentration (lowest concentration showing activity).
OAC: Optimal active concentration (lowest concentration showing the greatest activity).

toxicity assay, both agents were capable of causing repairable DNA damage, as manifested by the preferential inhibition of *E. coli* DNA repair mutant WP100 *uvrA recA* in comparison to wild-type strain WP2 at concentrations ranging from 10~100 $\mu$g/ml (Table 1). Other antitumor antibiotics such as daunomycin, mitomycin C and streptonigrin, but not bleomycin, also produced differential toxic effects in this system (Table 1 and Fig. 1). Results of the two colorimetric assays demonstrated that PD 114,759 and PD 115,028 were potent inducers of both cell filamentation and prophage $\lambda$ in response to DNA damage (Tables 2 and 3, respectively). The potency of PD 114,759 and PD 115,028 in both the chromotest and the BIA were comparable to that for other known antitumor antibiotics. In comparing the results of the aerobic and anaerobic chromotests, it was found that PD 114,759 and PD 115,028 retained their DNA reactivity under anaerobic conditions, as did mitomycin C, whereas bleomycin and streptonigrin were markedly less potent under anaerobic conditions (Table 2).

## Discussion

FRY *et al.*[4] reported that PD 114,759 caused detrimental DNA effects on cultured L1210 cells based on the ability of the compound to cause significant single-strand DNA breakage (alkaline elution assays). The data obtained with the *E. coli* assays demonstrate further that PD 114,759 and its isomer, PD 115,028, are DNA-reactive compounds. The veractamycins appear to possess DNA-modifying properties found in other effective antitumor antibiotics, such as bleomycin, daunomycin, mitomycin C and streptonigrin[5~8]. Comparisons of the results for all these compounds thus may aid in elucidating the mechanism of action of PD 114,759 and PD 115,028.

In the *E. coli* assay for repairable DNA damage, PD 114,759 and PD 115,028 showed preferential toxicity toward DNA repair mutant WP100 (mean zone size differences between strains WP100 and WP2 exceeded 3 mm) at concentrations higher than that for mitomycin C and streptonigrin, and lower than that for daunomycin (Fig. 1). However, PD 114,759 and PD 115,028 appeared to show less repairable DNA-damaging effects than the latter 3 compounds when one takes into account the magnitude of the dose-response relationship, *i.e.*, the differential toxicity of the veractamycins varied from 3~4 mm over the range of concentrations tested, whereas the differential toxicity of daunomycin, mitomycin C and streptonigrin varied from 3~15 mm over a similar concentration range. It should be cautioned that the variability in agar diffusion rates of the compounds may play a role in the validity of these potency comparisons, and that liquid suspension (treat-and-plate) assays may be more appropriate for comparing the DNA-reactive potencies of these agents. Still, the results indicate that PD 114,759 and PD 115,028 are only weakly/moderately effective in causing DNA lesions in *E. coli* WP100 *uvrA recA* in comparison to wild-type strain WP2. Similarly, *E. coli* WP100 showed no increased sensitivity to bleomycin relative to strain WP2; the inability of bleomycin to preferentially kill *E. coli* DNA repair mutants relative to repair-proficient strains has been observed by other investigators[9~11]. It has been hypothesized that bleomycin may be producing concentrated DNA damage in a confined, specific region of the *E. coli* genome, and that such DNA damage is essentially non-repairable even in the DNA repair-proficient strains[11~13]. Because PD 114,759 and PD 115,028 show relatively weak preferential killing of the DNA repair mutant in this study, it may be hypothesized that the veractamycins produce DNA lesions in *E. coli* that are only partially repairable by excision repair and post-replication repair mechanisms.

The results of the tests for induction of prophage and of cell filamentation, two SOS functions of *E. coli*[14,15], provided further insight into the mechanism of DNA reactivity of the veractamycins. PD 114,759 and PD 115,028 were potent inducers of prophage $\lambda$ (BIA) and cell filamentation (chromotest), as were the four above-named antitumor antibiotics. These findings indicate that the veractamycins are capable of interfering with bacterial DNA replication. In the BIA, PD 114,759 and PD 115,028 were capable of inducing prophage both in *E. coli* strains BR513 and BR339, whereas bleomycin was active only against strain BR513. This provides evidence that the specific mechanism of DNA reactivity of the veractamycins markedly differs from that of bleomycin, despite similar bleomycin and veractamycin effects in the *E. coli* WP2/WP100 DNA repair assay. FRY *et al.*[4] also found significant differences in the DNA-reactive effects of bleomycin as compared to PD 114,759 in alkaline elution and other biochemical assays.

The most important insight into the mechanism of DNA reactivity, however, comes from the comparison of the *E. coli* chromotest results for the plates incubated aerobically and anaerobically. In these tests, PD 114,759 and PD 115,028 most resembled mitomycin C, in that all 3 compounds showed equivalent activity for anaerobic vs. aerobic incubation. In contrast, bleomycin and streptonigrin were 30~100-fold less active when plates were incubated anaerobically as compared to the aerobically-incubated plates. Both bleomycin and streptonigrin require oxygen to exert their DNA-reactive effects, whereas mitomycin C acts as a bioreductive alkylating agent[13,16~22]. This suggests that the specific mechanism of DNA reactivity by PD 114,759 and PD 115,028 is a reductive, process that does not require the presence of oxygen. It can further be hypothesized that, like mitomycin C, PD 114,759 and PD 115,028 may be active against hypoxic tumor cells. *In vitro* cytotoxicity tests on oxic vs. hypoxic cells are necessary to confirm this hypothesis.

In summary, PD 114,759 and PD 115,028 are capable of causing repairable DNA damage and the induction of SOS functions in *E. coli*. The veractamycins appear to have their own unique DNA-modifying properties, although they share properties common to other important DNA-reactive antitumor antibiotics. The results suggest that PD 114,759 and PD 115,028 cause fairly concentrated DNA damage in the microbial genome, and that the mechanism by which the DNA is damaged appears to involve bioreduction rather than oxidation. It is hoped that the observations made using the microbial systems are applicable to the interactions of the veractamycins with mammalian tumor cell DNA.

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