T_2 Phage Sensitization by Linear and Angular Furocoumarins

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Z. Naturforsch. **34 c,** 811 – 814 (1979); received June 7, 1979

T_2 Phage sensitization, Angular Furocoumarins, DNA Cross-Links

T_2 bacteriophage sensitization has been studied using two furocoumarins capable of linking covalently to DNA to the same extent but producing different damages, psoralen and 4,5′-dimethylangelicin. Psoralen is a well-known linear furocoumarin capable of inducing in DNA both monoadducts and cross-links; 4,5′-dimethylangelicin is a new angular compound known as a pure monofunctional reagent. In the sensitization of T_2 mature virions both drugs proved very active, yielding survival curves practically superimposable; on the contrary, in the experiments with the T_2 vegetative form, *i. e.* its DNA inside the host, 4,5′-dimethylangelicin resulted much less effective, resembling the picture observed in the inactivation of the host bacteria. This result did not appear related to an enhancement of DNA repair by a Weigle effect. The different killing activity of 4,5′-dimethylangelicin can be explained supposing that this drug is capable of inducing cross-links in T_2 DNA inside the virus core, in which it exists in a very folded form, but not in the same DNA after injection into the host bacteria.

Furocoumarins are a group of photosensitizing drugs which by irradiation with long wave ultraviolet light photoreact with pyrimidine bases of DNA forming covalent linkages between the drug and the macromolecule. Furocoumarins, having a linear molecular structure, are bifunctional reagents, inducing two different kinds of photoproducts: monoadducts, arising from the reaction between one base molecule, and diadducts from two base molecules and one furocoumarin molecule, giving inter-strand cross-links [1]. Furocoumarins, having an angular molecular structure, are monofunctional reagents with DNA forming, for geometrical reasons, only monoadducts [2]. The biological consequences and the repair mechanisms of these two different damages have been widely studied [3 – 7]. Cross-links show a high killing effect in both bacterial [8] and mammalian cell [9] systems; skin-photosensitization also appears clearly related to cross-links [10]. On the other hand, monoadducts show a lower killing capacity [5] but seem responsible for two other furocoumarin effects, the mutagenic activity [11], and the ability of inducing lambda prophage [12].

Furocoumarin photosensitization has also been widely studied on bacterial [13] and mammalian [14] viruses; except in the case of some RNA containing viruses [15], a high inactivation capacity has been observed. Little is known at present about the effect on viruses of monofunctional furocoumarins, because generally linear compounds have been used.

In this paper we report on some experiments performed with two furocoumarins having different properties, psoralen and 4,5′-dimethylangelicin. Psoralen is a well-known linear furocoumarin, a very active bifunctional reagent [1]; 4,5′-dimethylangelicin is a recently studied angular furocoumarin, able to link covalently to DNA to the same extent as psoralen both *in vitro* and *in vivo,* behaving as a pure monofunctional reactive [16]. Therefore, carrying out parallel experiments using these two drugs, it is possible to obtain samples of cells or viruses having the same number of furocoumarin moieties linked to DNA with or without cross-links.

In such a manner we have studied some aspects of T_2 bacteriophage sensitization.

Materials and Methods

Psoralen was extracted from the leaves of *Ficus carica* [17]; 4,5′-dimethylangelicin was prepared by chemical synthesis which will be described elsewhere. *E. coli* B_{48}, a wild-type strain, was grown in Brain-heart infusion (Difco Laboratories, Michigan, USA) and collected in log phase by rapid filtration on Millipore dishes HAWP 02500; 0.45 μm. Its colony-forming ability was determined on agar plates prepared with the same medium. T_2 bacteriophage was grown in *E. coli* B_{48} cultures using Brain-heart infusion; virus titers were determined by the agar

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0341-0382/79/0900-0811 $ 01.00/0

two-layer method [18] using the same medium and the same host as indicator bacteria.

For the sensitization experiments, suspensions (5 ml aliquots) of bacteria (10^8 cells/ml) or of bacteriophages (10^{10} particles/ml), prepared in physiological saline solution containing 4 μg/ml of the studied furocoumarin, were poured into Petri dishes (5 cm in diameter), cooled on ice, and irradiated by a Philips HPW 125 lamp, provided with a Philips filter and emitting at almost 365 nm; the irradiation intensity incident on the whole sample was 2.02×10^{16} quanta/sec.

In the experiments with the vegetative form of T_2 phage, the Benzer method [19] with few modifications was used; *E. coli* cells were starved by incubating an hour in 0.1 M phosphate buffer, pH 7, containing 0.1 M NaCl and 10^{-4} M $MgSO_4$. Bacteria were infected with T_2 phages at a multiplicity of 0.5, incubated further for 10 min in buffer, collected and washed with the same medium to remove the unadsorbed virus particles. In these conditions only phage adsorption and injection of its DNA into the host occur, but no virus growth or expression [20]; this fact is very important because virus growth produces modifications in the sensitivity of the T_2 vegetative form, as tested with short-wave ultraviolet light or X rays [20]. The infected bacteria were then irradiated and the number of the infective centers was determined by plating them on agar dishes prepared as above for the virus particle counting.

Results

Sensitization of E. coli B_{48}, T_2 host

The sensitivity of *E. coli* B_{48} cells, used as T_2 host, was assayed; bacteria were irradiated for different times in the presence of 4,5′-dimethylangelicin or of psoralen, and the colony-forming ability was determined. As shown in Fig. 1, the results are very close to those already obtained with other *E. coli* strains [16]; the surviving fraction was affected by both drugs, but even though the damage afforded to DNA was of the same extent the survival curves resulted very different. The well-known lethal effect of psoralen cross-links is clearly evident.

Sensitization of mature T_2 virions

Samples of T_2 phages were irradiated for different times in the presence of the drugs and the plaque-forming units per ml were scored. As shown in Fig. 2, T_2 phage is dramatically inactivated by furocoumarin photosensitization; in this case, however, the survival curves thus obtained are practically superimposable. This experiment was carried out several times and no significant difference in the killing effect of the two drugs was ever observed.

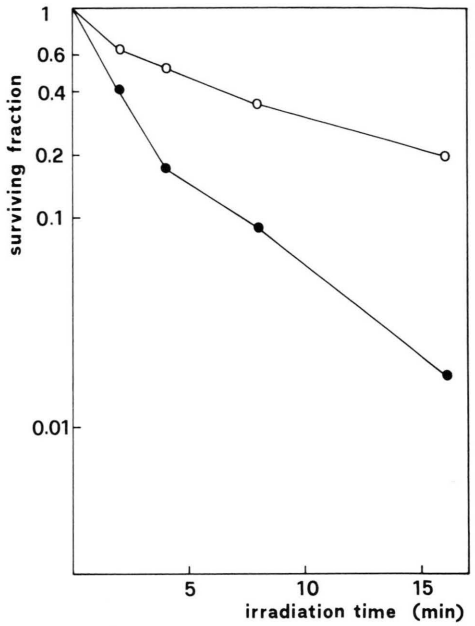

Fig. 1. *E. coli* B_{48} inactivation by psoralen (●) and 4,5′-dimethylangelicin (○) photosensitization. Bacteria were irradiated in the presence of 4 μg/ml of the drug and their colony-forming ability was assayed.

Sensitization of T_2 vegetative form

E. coli cells, starved in buffer, were infected with T_2 virions in the presence of salts to allow adsorption and DNA injection; after this, the furocoumarin was added and the infected cells were irradiated for different times. The number of infective centers was then determined; these results too, are reported in Fig. 2. While psoralen appears as effective on T_2 vegetative form, *i. e.* its DNA inside the host cell, as on the mature virion, 4,5′-dimethylangelicin shown clearly a lower activity, giving practically the same picture observed with bacteria.

T_2 inactivation after plating on sensitized hosts

Because in the above experiments phage DNA was irradiated together with its host, an induction of DNA repair by a Weigle effect [21] might be possible. To check this assumption, samples of T_2 virions

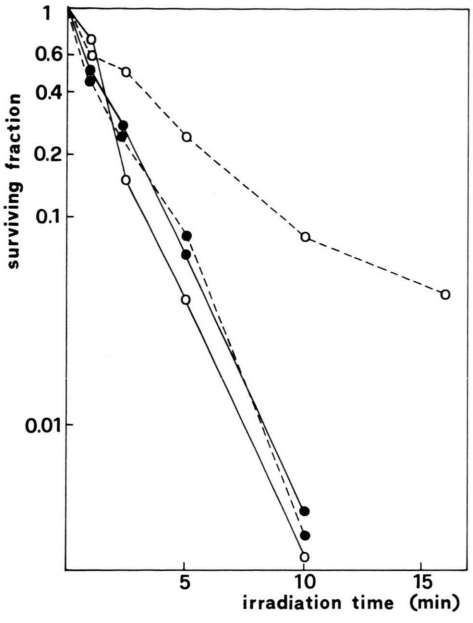

Fig. 2. T_2 inactivation by psoralen (●) and 4,5′-dimethylangelicin (○) photosensitization. Experiments with mature virions (solid lines): T_2 phages were irradiated in the presence of 4 μg/ml of the drug and the plaque-forming units were scored on plates prepared using *E. coli* B_{48} as indicator bacteria.
Experiments with the phage vegetative form (dotted lines): host bacteria *E. coli* B_{48} were starved in buffer and then infected with T_2 phages at a MOI of 0.5 in the presence of salts to allow only adsorption and DNA injection. Bacteria were then washed and submitted to furocoumarin sensitization as above. The number of infective centers was then scored by plating the so treated bacteria on plates prepared as above.

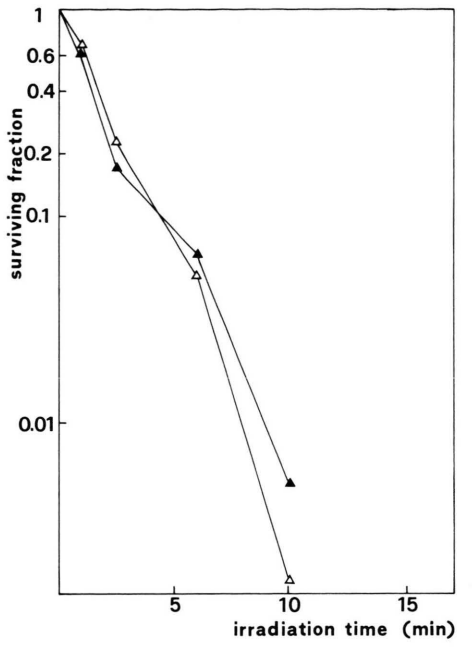

Fig. 3. T_2 inactivation by 4,5′-dimethylangelicin photosensitization after plating on sensitized hosts. T_2 virions were irradiated in the presence of 4 μg/ml of 4,5′-dimethylangelicin and then plated on dishes prepared with *E. coli* B_{48} cells previously sensitized by this drug, using the same concentration and the same UV radiation dose (▲). As a control the same samples of irradiated phages were plated on dishes prepared with untreated indicator bacteria (△).

sensitized by 4,5′-dimethylangelicin were plated on dishes prepared using indicator bacteria irradiated with the same u. v. dose in the presence of the same drug; as a control, a parallel experiment with untreated bacteria was carried out. As shown in Fig. 3, no significant difference in T_2 surviving was observed.

Discussion

In producing the biological effects of furocoumarin sensitization DNA repair plays a very important role; monoadducts are very easily removed by error-free excision repair, while cross-links are repaired by different pathways, by genetic recombination [4] or by an error-prone SOS repair [7]. In fact, even in wild-type bacteria, unless only a small number of cross-links is repaired, an inactive DNA is produced, yielding cell death [5].

The results obtained with *E. coli* B_{48} are in good agreement with these findings; on the contrary, in the inactivation of the T_2 mature virions the survival curves obtained with psoralen and 4,5′-dimethylangelicin have resulted superimposable, monoadducts and cross-links appearing strangely to have the same lethality. To explain this result, we have carried out some experiments with the vegetative form of T_2 phage, *i. e.* its DNA inside the host. Bacteria were infected with T_2 phage in experimental conditions which allow DNA injection into the host, but not virus growth or its expression [20]; therefore, the irradiation of thus infected bacteria permits the sensitization of the T_2 DNA within the host cell. When the bacteria so treated are plated on dishes prepared with full medium, phage growth starts and the number of infective centers, *i. e.* the phage surviving fraction, can be determined. In these experiments psoralen appeared of the same activity as that on mature virions, while 4,5′-dimethylangelicin resulted less effective, resembling the picture observed with the host bacteria.

This result might be due to an induction of DNA repair in the irradiated bacteria that might remove 4,5′-dimethylangelicin monoadducts more efficently than that of psoralen cross-links. As is known, when phages irradiated by short-wave u. v. light are plated on dishes prepared with host bacteria previously irradiated with small doses of the same light, an increase of the phage surviving fraction and of its mutation frequency is observed [21]. This effect, known as the Weigle reactivation, is related to an induction of an error-prone DNA repair.

We have performed analogous experiments in which both indicator bacteria and T_2 phage were sensitized by 4,5′-dimethylangelicin. In this experimental condition we did not observe an increase of the survival of the sensitized T_2 phage.

All these results can be explained assuming a different behaviour of phage DNA in the 4,5′-dimethylangelicin sensitization inside the virus core and inside the host bacterium. In fact, in the tailed bacteriophages, DNA is packed in a very folded form [22], different from the extended one showed in dilute solution or inside bacterial cells. Like linear furocoumarins, 4,5′-dimethylangelicin forms a complex with DNA in the dark by intercalation between two base pairs [16]. This preliminary intercalation and the furocoumarin angular structure hinder the possibility of the cross-link formation. However, 4,5′-dimethylangelicin might photoreact with T_2 DNA in different conditions, *e. g.*, without a preliminary intercalation, with two non-adjacent bases which are near in a very folded macromolecule; in this case the parameters of its angular structure might become less important. According to this hypothesis 4,5′-dimethylangelicin would act as a bifunctional furocoumarin, like psoralen, for T_2 DNA inside the virions, but not for the same DNA after injection into the host bacteria.

This idea is in agreement with the results obtained by Kittler and coworkers [23] with lambda phage, using xanthotoxin and angelicin; in particular they found evidences of an inhibition of DNA injection into the host attributed to cross-link formation between two non-adjacent bases. This damage might appear, therefore, very important in phage inactivation by furocoumarin sensitization; actually, if the damaged DNA cannot be injected into the host cell, it cannot reproduce itself and it cannot be repaired, because both these functions are carried out inside the host. Moreover, if a part of the so cross-linked DNA can penetrate into the host cell, it will be repaired by cellular enzymes but by means of an error-prone DNA repair, and therefore with a low chance of survival.

At present, new experiments are in progress to study by biochemical methods whether angular furocoumarins are able to induce cross-links in DNA inside the T_2 phage core.

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