DOI: 10.1002/cmdc.200500084

T4 Phage Photoinactivation by Linear Furocoumarins and Angular Furoquinolinones

Frazia Bettio, Francarosa Baccichetti, Morena Simonato, Cristina Marzano, and Franco Bordin*[a]

Linear furocoumarins (psoralens) are active sensitizers that can damage numerous cell components such as nucleic acids,[1,2] proteins,[3] and lipids.[4,5] However, DNA is the main cellular target in which various lesions can be introduced: monofunctional adducts with pyrimidine bases (MA)[1,2] and bifunctional adducts, cross-links between two adjacent pyrimidine bases (interstrand cross-links; ISC),[6] and covalent linkages between DNA and proteins (DNA–protein cross-links; DPC).[7] Furocoumarins are widely used in photomedicine (PUVA therapy) for the treatment of various skin diseases[8] and in photopheresis to prevent rejection in organ transplantation.[9] Recently, furocoumarins have also been proposed as sterilizing agents for blood preparations[10,11] because they provide various advantages over conventional techniques; indeed, furocoumarins affect a wide spectrum of microorganisms, including viruses,[12] with a decreased capacity for affecting blood components. Whereas the mechanism of furocoumarin sensitization on mammalian cells, yeast, and bacteria has been extensively studied, only a few reports concerning the furocoumarin mechanism of virus inactivation have been published. The studies reported herein have addressed some aspects of this problem by using the simple and well-known T4 bacteriophage model; we checked the sensitivity of T4 in both its mature virion and vegetative forms. Three very active photosensitizing derivatives were chosen: a well-known linear furocoumarin, 4,5′,8-trimethylpsoralen (TMP)[13,14] and two angular furoquinolinones: 1,4,6,8-tetramethyl-2*H*-furo[2,3-h]quinolin-2-one (FQ)[15] and 4,6,8,9-tetramethyl-2*H*-furo[2,3-h]quinolin-2-one (HFQ)[16] (Figure 1). These compounds were selected for their different capacities for inducing various lesions in DNA, in particular bifunctional adducts, ISCs, and/or DPCs. Furocoumarins and their homologues form C_4-cycloadducts with pyrimidine bases (MA) through the double bonds at either the 3,4 position or on the furan group; adducts formed through the latter process can absorb light and thus further react with an another pyrimidine group to yield an ISC.[1,2,6] Alternatively, this second reaction can involve a protein to form a DPC.[7,16] All three derivatives chosen can form various MAs in DNA, but TMP is known to be capable of inducing numerous ISCs[13,14] and DPCs[17] as well. FQ and HFQ efficiently form DPCs, but not ISCs,[15,16] a behavior common to all furocoumarins with an angular molecular structure. In fact, FQ and HFQ are potentially bifunctional molecules, but for geometric reasons they behave as monofunctional reactive compounds when intercalated into DNA.[18] We initially studied the response of mature T4 virions and T4 in its vegetative form (phage DNA injected into recipient bacteria, blocked by starvation), that is, before and after the infection process, respectively. Under suitably selected mild experimental conditions, all compounds showed a strong killing activity toward T4 virions but not the vegetative form (Figure 2).

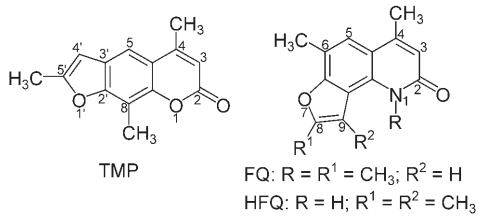

Figure 1. Photosensitizers tested. TMP: 4,5′,8-trimethylpsoralen; FQ: 1,4,6,8-tetramethyl-2*H*-furo[2,3-h]quinolin-2-one; HFQ: 4,6,8,9-tetramethyl-2*H*-furo-[2,3-h]quinolin-2-one.

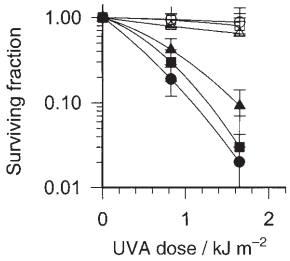

Figure 2. Photoinactivation of T4 mature virions and vegetative form by continuous irradiation in the presence of the tested compound (2 μM): TMP, ■ (mature), □ (vegetative); FQ, ● (mature), ○ (vegetative); HFQ, ▲ (mature), △ (vegetative). Error bars represent mean ±SD.

As furocoumarin derivatives induce bifunctional lesions through a two-step reaction, we carried out some experiments on T4 mature virions using the double irradiation method, a well-known procedure that is able to promote and discern the formation of bifunctional adducts.[14] For kinetic reasons, MAs are the main products formed with bifunctional compounds after a small dose of UVA. By washing out the unbound sensitizer molecules, only those covalently linked as MAs can absorb UVA light to react during the second irradiation step for the conversion of the monofunctional lesions into bifunctional adducts (which may be ISCs or DPCs).[6,16] As bifunctional lesions are known to be much more lethal than MAs, a decrease in survival is observed upon conversion of some MAs into ISCs (or DPCs) after the second step. In contrast, if MAs do not further photoreact, no changes are observed. Interestingly, with all three compounds the surviving fraction was extensively decreased by the second irradiation step (Figure 3). These results suggest that the lethal lesions formed in mature virions,

[a] *Dr. F. Bettio, Prof. F. Baccichetti, M. Simonato, Prof. C. Marzano, Prof. F. Bordin*
Department of Pharmaceutical Sciences
University of Padova
Via Marzolo 5, 35131, Padova (Italy)
Fax: (+39) 0468275366
E-mail: franco.bordin@unipd.it

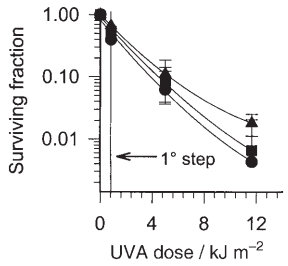

Figure 3. Photoinactivation of T4 phage virions by the double irradiation method. First step: 0.8 kJ m^{-2} in the presence of the tested compound (2 μM); the unbound drug molecules were washed out, and phages were further UVA irradiated. TMP, ■; FQ, ●; HFQ, ▲. Error bars represent mean ± SD.

but not in the vegetative forms, are bifunctional adducts, which could be ISCs or DPCs. At present, we do not have direct evidence which allows us to determine which of these two kinds of lesions is responsible for these results.

It is quite likely, however, that ISC formation does not sufficiently deform phage DNA (2 nm in diameter) to prevent its injection into recipient bacteria (the phage tail has an internal diameter of ≈7 nm[19]). On the other hand, it is reasonable to suppose that ISCs could be formed inside infected bacteria containing T4 phage in its vegetative forms. In both cases, ISCs can be reversed by bacterial DNA repair systems and/or they should induce their known lethality. Conversely, the formation of cross-links between phage DNA and proteins inside the viral core completely stops DNA injection into host bacterial cells, thus preventing infection; clearly, such an event would also hinder any repair of phage DNA. In fact, Kittler and co-workers,[20] in their studies of lambda phage sensitization with some linear and angular furocoumarins, observed a strong inhibition of viral DNA injection into recipient bacteria (more than 30%).

In this connection, a study carried out with TMP, 8-methoxypsoralen (8-MOP), and T7 bacteriophage[21] demonstrated that the formation of ISC alone does not completely explain the extent of phage survival, as determined by different experiments, including some carried out with the double irradiation protocol and on wild-type and excision-repair-defective bacteria. The authors concluded that during the second irradiation step (when bifunctional adducts are selectively formed), some lethal lesions other than ISCs are formed. Because, as mentioned above, TMP[17] and 8-MOP[7,16] can efficiently induce DPCs by a two-step photoreaction, we can speculate that these lesions could be DPCs.

In conclusion, our results confirm that furocoumarin sensitization is an effective sterilization tool. This phage inactivation mechanism, even if it should be further investigated and extended to animal viruses, appears to be connected to the capacity of furocoumarin to form bifunctional lesions capable of blocking the viral replication and infection process.

Experimental Section

Chemicals: 4,5′,8-trimethylpsoralen (TMP) was a kind gift from Professor Sergio Caffieri (Department of Pharmaceutical Sciences, University of Padova, Italy); 1,4,6,8-tetramethyl-2*H*-furo[2,3-h]quinolin-2-one (FQ) and 4,6,8,9-tetramethyl-2*H*-furo[2,3-h]quinolin-2-one (HFQ) were both prepared by chemical synthesis.[22] Compounds were dissolved in dimethylsulfoxide (DMSO, 4.5 mM), and the solutions were stored at −20 °C in the dark. Just before an experiment, a calculated amount of drug solution (observed in DMSO) was added to the phage medium up to a DMSO concentration of 0.5%. Similar quantities of DMSO were also added to the controls in every experiment. The phage phosphate buffer solution (0.1 M phosphate buffer, 0.1 M NaCl, 10^{-4} M $MgSO_4$, pH 7) was used for diluting phage suspensions. In all experiments, compounds were tested at a concentration of 2 μM.

Irradiation procedures: UVA irradiations were performed with Philips HPW 125 lamps (emission: $\lambda = 320$–400 nm, with a maximum at $\lambda = 365$ nm). The irradiation intensity, determined by a radiometer (model 97503, Cole–Parmer Instrument Co., Niles, IL, USA), was 0.9×10^{-6} W m^{-2}.

Infectivity test with T4 bacteriophage: T4 phage was obtained from the American Type Culture Collection; *E. coli* strain B48, a wild-type strain, was used as host bacteria. Phage growth and counting were both carried out according to procedures reported by Adams,[19] using the double layer agar method, nutrient broth agar, and *E. coli* B48 as host bacteria. Phage suspensions were diluted with phage phosphate buffer containing the compound to be tested at concentrations up to 10^{10} virus per mL; the samples were incubated for 15 min in the dark and then exposed to UVA light.

Experiments with the double irradiation protocol: In these experiments,[16] phage suspension containing the compound to be tested was exposed to an initial irradiation step with small a UVA dose; the unbound molecules of the sensitizer were washed out by filtering T4 virions with Nucleopore test tubes (Centricon Plus-20, Nucleopore Corp., Pleasanton, CA, USA) equipped with a filter ($0.5–10 \times 10^4$ Da cutoff) followed by centrifugation at 4000 rpm for 15 min. Phage particles, thus collected on the filter, were washed twice with a small amount of phage phosphate buffer, repeating the centrifugation step. The filter was then inverted and, by subjecting it to centrifugation for 5 min at 2000 rpm, phage particles were recovered in a conic support. A fraction of this phage suspension was submitted to virus titer determination, while the remaining was re-exposed to UVA light for excitation of only the covalently bound molecules of the sensitizer. For control experiments in which the first irradiation step was omitted, a significant decrease in survival by UVA exposure was never observed after the washing procedure (data not shown).

Experiments with T4 vegetative form: Bacteria were starved according to the methods reported by Benzer.[23] Under these experimental conditions, only phage absorption and injection of viral DNA into the recipient bacteria occur, but neither virus growth nor viral expression. Infection was always carried out at a multiplicity of 0.5; these T4-infected complexes were then exposed to UVA light. The starved bacteria were fed, and then the number of infective centers, that is, the number of bacteria releasing viruses, was determined.

Keywords: bifunctional lesions · furocoumarins · photochemistry · photosensitizers · T4 bacteriophage

[1] G. D. Cimino, H. B. Gamper, S. T. Isaacs, J. F. Hearst, *Annu. Rev. Biochem.* **1985**, *54*, 1151–1193.
[2] F. P. Gasparro, *Photochem. Photobiol.* **1996**, *63*, 553–557.

[3] L. M. Schmitt, S. Chimenti, F. P. Gasparro, *J. Photochem. Photobiol. B* **1995**, *27*, 101 – 107.
[4] Z. Zarebska, *J. Photochem. Photobiol. B* **1996**, *23*, 101 – 109.
[5] S. Frank, S. Caffieri, A. Raffaelli, D. Vedaldi, F. Dall'Acqua, *J. Photochem. Photobiol. B* **1998**, *44*, 39 – 44.
[6] J. W. Tessman, S. T. Isaacs, J. E. Hearst, *Biochemistry* **1985**, *24*, 1669 – 1976.
[7] F. Bordin, F. Carlassare, L. Busulini, F. Baccichetti, *Photochem. Photobiol.* **1993**, *58*, 13 – 136.
[8] D. Tran, Y. K. Kwok, C. L. Goh, *Photodermatol. Photoimmunol. Photomed.* **2001**, *17*, 164 – 167.
[9] F. Heshmati, *Transfusion and Apheresis Science* **2003**, *29*, 61 – 70.
[10] E. Ben-Hur, B. Horowitz, *Photochem. Photobiol.* **1995**, *62*, 383 – 388.
[11] F. Knutson, R. Alfonso, K. Dupuis, V. Mayaudon, L. Lin, L. Corash, C. F. Högman, *Vox Sang.* **2000**, *78*, 209 – 216.
[12] C. V. Hanson, *Blood Cells* **1992**, *18*, 7 – 25.
[13] M. Dardalhon, D. Averbeck, *Int. J. Radiat. Biol.* **1988**, *54*, 1007 – 1020.
[14] K. R. Kumaresan, M. Ramaswamy, A. T. Yeung, *Biochemistry* **1992**, *31*, 6774 – 6783.
[15] F. Bordin, C. Marzano, F. Carlassare, P. Rodighiero, A. Guiotto, S. Caffieri, F. Baccichetti, *J. Photochem. Photobiol. B* **1996**, *34*, 159 – 168.
[16] F. Bordin, F. Baccichetti, C. Marzano, F. Carlassare, G. Miolo, A. Chilin, A. Guiotto, *Photochem. Photobiol.* **2000**, *71*, 254 – 262.
[17] C. Marzano, E. Severin, F. Bordin, *J. Cell. Mol. Med.* **2001**, *2*, 171 – 177.
[18] F. Bordin, F. Dall'Acqua, A. Guiotto, *Pharmacol. Ther.* **1991**, *52*, 331 – 363.
[19] M. H. Adams, *Bacteriophages*, Interscience Publishers, New York, **1971**.
[20] L. Kittler, Z. Hradecna, J. Suhnel, *Biochim. Biophys. Acta* **1980**, *607*, 215 – 220.
[21] M. D. Mamet-Brately, B. Karska-Wysochi, *J. Photochem. Photobiol. B* **1997**, *38*, 48 – 53.
[22] P. Rodighiero, A. Guiotto, A. Chilin, F. Bordin, F. Baccichetti, F. Carlassare, D. Vedaldi, S. Caffieri, A. Pozzan, F. Dall'Acqua, *J. Med. Chem.* **1996**, *39*, 1293 – 1302.
[23] S. Benzer, *J. Bacteriol.* **1952**, *63*, 59 – 72.

Received: November 16, 2005
Revised: January 27, 2006
Published online on April 20, 2006

