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Preparation and characterization of biotinylated psoralen

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ABSTRACT

Biotinylated psoralen (BPsor), a psoralen derivative containing a biotin moiety attached via a long-chain, positively charged linker, has been synthesized and its interactions with DNA and avidin have been studied. As do other psoralen derivatives, BPsor photoreacts with DNA to form interstrand crosslinks. The biotin binds to streptavidin after the reaction of BPsor with DNA, and this property has been used to measure low levels of BPsor modified DNA by ELISA with streptavidin and biotinylated alkaline phosphatase. In addition, BPsor retains the biological activity of psoralen, as shown by its ability to inhibit lymphocyte proliferation at a level of 10 ng/ml.

INTRODUCTION

Psoralens are bifunctional photoreactive molecules which form covalent bonds with nucleic acids in the presence of near UV light. In a two-step reaction, they first intercalate into nucleic acids in the dark, then photoreact, at wavelengths of 320-400 nm, to produce monoadducts and interstrand crosslinks (1-4). Their ability to form crosslinks has made them useful probes in the study of nucleic acid structure and function.

The DNA lesions produced by psoralen photoreaction, the interstrand crosslinks in particular, inhibit DNA replication and cellular proliferation (1,5) Psoralen photochemotherapy is used clinically in the treatment of psoriasis (6), a hyperproliferative skin disorder, and cutaneous T-cell lymphoma (7). DNA repair, mutagenesis and carcinogenesis produced by psoralen damage have been studied in a wide range of systems.

Here we describe the synthesis of a biotinylated psoralen derivative, a reagent which combines the nucleic acid specificity of the psoralens with the detection sensitivity of the avidin-biotin system. The exceptionally high affinity of avidin for biotin ($K_d = 10^{-15}$) has provided the basis for the development of sensitive and specific detection systems (8-11). Typically, in a multi-step procedure, antigens are recognized by specific antibodies, which are then bound to a biotinylated anti-immunoglobulin. This then forms a tight

complex with avidin, either conjugated directly to a signal of some kind, such as a fluorescent dye or enzyme, or in turn bound to a biotinylated label. Alternatively, molecules that are themselves biotinylated can be recognized directly by avidin, omitting the antibody reactions, with their somewhat lower affinities.

Several methods of derivatizing nucleic acids with biotin for use as non-radioactive hybridization probes have been described. Among these are the enzymatic incorporation of biotinylated dUTP into DNA with DNA polymerase I (12,13) and of biotinylated ADP into RNA with T4 RNA ligase (14), the chemical addition of biotin at the 5' termini of oligonucleotides (15,16), and the photochemical attachment of an aryl azide derivative of biotin to DNA (17). Hybridizations with biotinylated nucleic acids have achieved detection sensitivities similar to those with ^{32}P labeled probes.

Following photoactivation, biotinylated psoralen binds to DNA, forming interstrand crosslinks, and inhibits the proliferation of lymphocytes, as do other psoralens. Its biotin moiety is available for avidin binding after reaction with DNA, as demonstrated by its detection by ELISA with a streptavidin-alkaline phosphatase system. A preliminary account of some of this work has already appeared (18). Cimino et al. (4) and Sheldon et al. (19,20) have also reported the synthesis of biotinylated psoralens containing different spacers between the psoralen and biotin moieties.

METHODS

Synthesis of biotinylated psoralen

Diamine psoralen was prepared from chloromethyltrimethyl psoralen and sym-dimethylethylene diamine (Aldrich) as described by Goldenberg et al. (21). Twelve mg of diamine psoralen were dissolved in 0.4 ml dimethylformamide and 15 mg of NHS biotin (Pierce) was added as a solid. The reaction proceeded at room temperature. Reaction progress was followed by TLC on silica in CH_2Cl_2:NH_3-saturated methanol (14:1). The R_f's of diamine psoralen, NHS biotin, and the reaction product were 0.25, 0 and 0.28. After 1 hour the reaction was complete, and the solvent was removed by rotoevaporation, leaving a yellow oil. All steps were carried out under subdued light, and vessels were covered with aluminum foil when possible.

The product was purified by flash chromatography on silica gel in the TLC solvent system (22). The reaction mixture did not dissolve directly in the running solvent, so the yellow oil was first taken up in 0.1 ml MeOH, and then 1.5 ml CH_2Cl_2 was added to the solution. The purified product was a single spot with blue fluorescence and R_f of 0.28 on TLC plates.

The flash column peak was analyzed by HPLC on a reverse phase column (Bondapak phenyl, Waters), in CH_3CN:0.05 M NH_4OAc 42.5:57.5. The purified biotinylated psoralen (BPsor) ran as a single sharp peak with a retention time of 5.5 min. In this system diamine psoralen and NHS biotin have retention times of 6.2 and 4.1 min respectively. The purified BPsor was further analyzed by fast atom bombardment (FAB) mass spectrometry in magic bullet matrix. The negative FAB spectra exhibited an ion at m/z 553, consistent with the proposed structure.

[^{3}H] labeled biotinylated psoralen was prepared from diamine psoralen and [^{3}H] NHS biotin (Amersham), and purified by preparative HPLC. The specific activity was 3.8×10^{11} cpm/mmol.

DNA crosslinking

Plasmid pBR322 DNA was linearized with HindIII, and the DNA purified by phenol extraction, followed by ethanol precipitation and resuspension in 10 mM Tris-1 mM EDTA at 0.1 mg/ml. Two μg of linear plasmid was mixed with 0 to 10 μl of 5.7 μM BPsor in a total volume of 12 μl. The samples were irradiated with 2 400 W mercury vapor lamps through a solution of 40% cobaltous nitrate (w/w), which transmitted light from 340 to 380 nm (23). The light intensity reaching the sample was approximately 0.1 W/cm^2 and irradiation was carried out for 10 min. The samples were then alkali denatured and run on a nondenaturing 1% agarose gel in Tris acetate-EDTA buffer, as described previously (24).

Detection of DNA modification by ELISA

Calf thymus DNA, at a concentration of 20 μg/ml (30 μM base pairs) was combined with 6 μM [^{3}H] BPsor in TE buffer. The sample was irradiated with near UV light, then phenol extracted and ethanol precipitated to remove unbound psoralen. The pellet was resuspended in phosphate buffered saline (PBS). The level of BPsor addition was 0.9%, or 1 psoralen/110 base pairs.

Control DNA samples were prepared by omitting BPsor. Unirradiated controls, prepared by incubating DNA with [^{3}H] BPsor in the dark, followed by phenol extraction and ethanol precipitation, did not incorporate any [^{3}H] BPsor. Manipulations with BPsor were conducted in subdued light, with fluorescent room lights turned off, and samples were covered with aluminum foil for storage. Mictrotiter plates were coated with the reacted DNA by adding samples, diluted into PBS, to the wells and drying the plates overnight in a 37°C room.

The plates were washed 3 times with 1X PBS-0.5% Tween 20, and 200 μl of 1% fetal calf serum in PBS-Tween was added to block the wells. After 1 hour at

37°C the solution was shaken off. 100 μl of 5 μg/ml streptavidin (BRL) was added per well, followed by 1 hour incubation at 37°C. The plates were washed 3 times with PBS-Tween, and 100 μl of 1 μg/ml biotinylated poly-alkaline phosphatase (BRL) was added, followed by a further incubation of 1 hour at 37°C. The plates were washed with PBS-Tween, then with 0.01 M diethanolamine, pH 8.6. 100 μl of alkaline phosphatase substrate (Sigma 104) in 1.0 M diethanolamine, pH 8.6, was added, and the plates were read at 405 nm after incubation at 37°C.

Lymphocyte proliferation assay

Psoralen-induced inhibition of lymphocyte stimulation was measured by the method of Scherer et al. (25), with modifications. Lymphocytes were isolated from 50 ml of whole blood by centrifugation on Ficoll Hypaque. They were washed twice in RPMI medium, then resuspended in PBS and adjusted to 10^6 cells/ml in PBS.

BPsor, in ethanol, was added to 4 ml of cells, to final concentrations of from 1 to 1000 ng/ml. The final ethanol concentration was 1%. Negative controls contained no drug, while positive controls contained 10 ng/ml

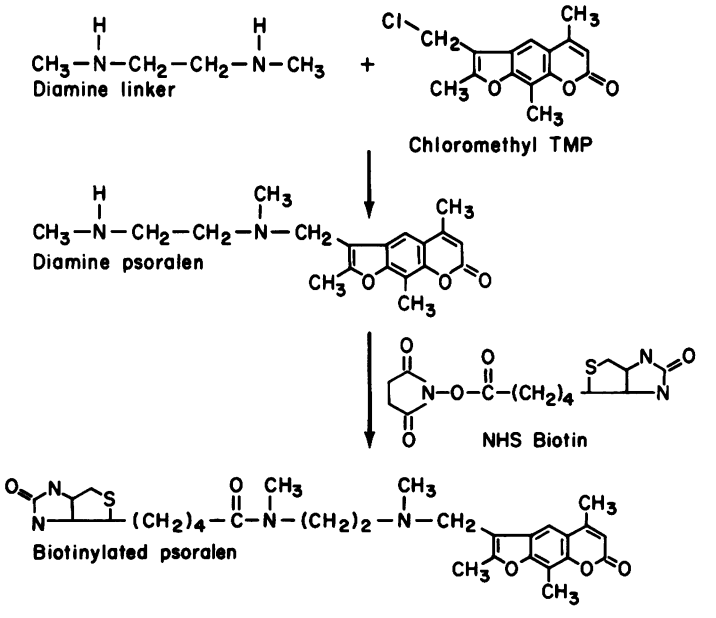

Fig. 1. Outline of biotinylated psoralen synthesis.

aminomethyltrimethylpsoralen (AMT), in ethanol. After 20 min for drug uptake, 200 1 of the cell suspension was added to wells in microtiter plates. Two dishes were prepared, each with 10 wells of each drug concentration. One was wrapped in aluminum foil and the second was irradiated with 3 J/cm^2 of near UV light. The microtiter plates were centrifuged at 1200 rpm for 7 min, then quickly inverted to discard the PBS. The lymphocytes in the wells were resuspended in 100 μl RPMI, then half the wells received 100 μl RPMI-20% fetal calf serum, while the other half received 100 μl RPMI-20% fetal calf serum-2% phytohemagglutinin (PHA). The dishes were incubated for 3 days at 37°C.

Lymphocyte proliferation was measured by adding 1 μCi [^{3}H] thymidine to each well on the third morning. After 6 hours the cells were collected with an automatic harvester onto filter paper and washed with 5% TCA and ethanol. The filters were counted in a scintillation counter, and the stimulation index was calculated as the ratio of [^{3}H] incorporation +PHA/ incorporation -PHA.

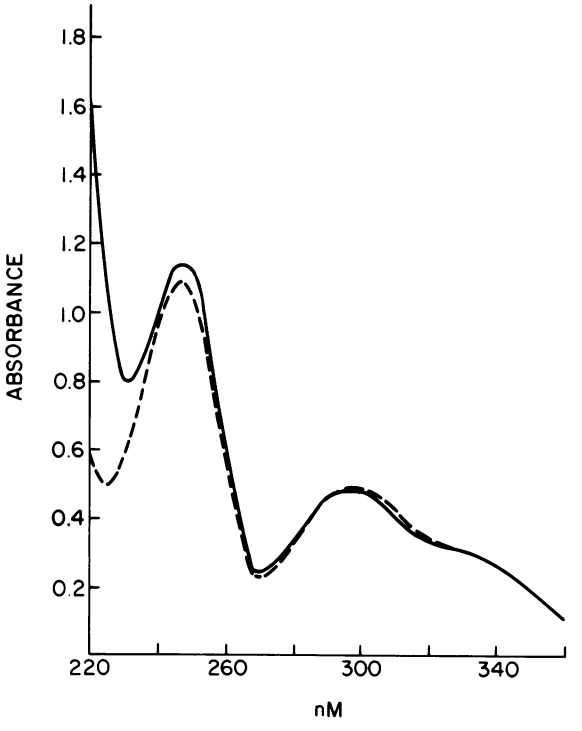

Fig. 2. Absorbance spectrum of BPsor. Dashed line, 43 μM AMT, solid line, 46 μM BPsor.

RESULTS

Synthesis of biotinylated psoralen

The biotinylated psoralen molecule (BPsor) consists of a psoralen moiety, connected to biotin by a positively charged linker. The positive charges confer good water solubility to the relatively insoluble psoralen part, and aid in binding to negatively charged nucleic acids.

The synthesis scheme is outlined in Fig. 1. Chloromethyl trimethylpsoralen was reacted with a diamine linker to form diamine psoralen. A slight excess of the N-hydroxy succinimide ester of biotin was added to the diamine psoralen. The product of this reaction is BPsor. It was isolated by flash chromatography on silica gel, and its purity was confirmed by HPLC using a reversed phase column. The UV absorbance spectrum is given in Fig. 2.

DNA crosslinking

Psoralens intercalate into DNA in the dark and form covalent bonds at their 3,4 and 4',5' double bonds with pyrimidines upon near UV irradiation. If both ends of the psoralen are reacted, the result is an interstrand DNA crosslink (3). The ability of the BPsor to form DNA crosslinks was tested by reacting linear double stranded plasmid DNA with this derivative and near UV light. The reacted DNA was alkali denatured and loaded onto a nondenaturing agarose

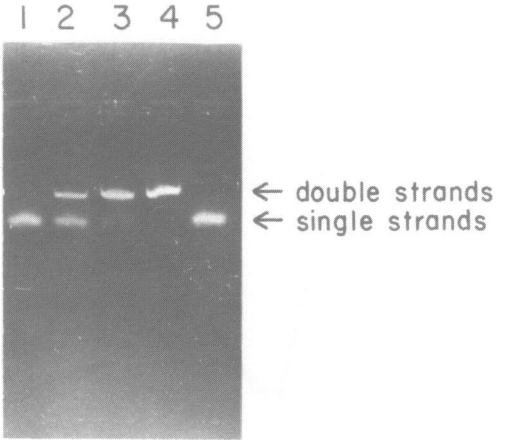

Fig. 3. DNA crosslinking by BPsor. Linear pBR322 DNA (0.2 μg) was irradiated with near UV in the presence of increasing amounts of BPsor. Samples were alkali denatured, then loaded onto a nondenaturing gel of 1% agarose in Tris-acetate buffer. Crosslinked DNA runs as the double-strand form, while non-crosslinked DNA runs as the single-strand form. Lane 1: no psoralen, lane 2: 6 ng, lane 3: 15 ng, lane 4: 30 ng, lane 5: 30 ng psoralen, but no irradiation.

gel. Crosslinked DNA immediately renatures in the neutral gel buffer and runs as the double stranded form, while non-crosslinked DNA remains single stranded and runs with greater mobility (24). Increasing amounts of BPsor resulted in increased levels of crosslinking after UV irradiation, while even the highest concentration of BPsor produced no crosslinking in the absence of light (Fig. 3).

ELISA detection of avidin binding to BPsor-reacted DNA

The availability of the biotin moiety to avidin binding after the reaction of BPsor with DNA was tested by ELISA with streptavidin and biotinylated alkaline phosphatase. The steps are outlined in Fig. 4. DNA was reacted with [^{3}H] BPsor and near UV light and, after removal of the noncovalently bound psoralen by phenol extraction, the level of addition was determined. Modified DNA, containing 0.5 to 1000 fmol added BPsor, was bound to microtiter plate wells. The plates were blocked with fetal calf serum and incubated, in succession, with streptavidin, biotinylated alkaline phosphatase, and phosphatase substrate. Alkaline phosphatase activity was assayed by absorbance at 405 nm.

The measured enzyme activity was proportional to the added BPsor and, after less than 2 hours of incubation, this assay was able to detect 2 fmol of bound BPsor, corresponding to 0.15 ng of DNA with 1% modification (Fig. 5). After overnight incubation 1 fmol could be detected above background (data not shown). DNA incubated in the dark with BPsor remained unmodified, as measured by [^{3}H] incorporation and streptavidin binding capacity. DNA irradiated with near UV light in the absence of BPsor also stayed at the background level of unreacted control DNA in the ELISA procedure.

Inhibition of lymphocyte proliferation

The biological effectiveness of BPsor was tested by assaying its ability to

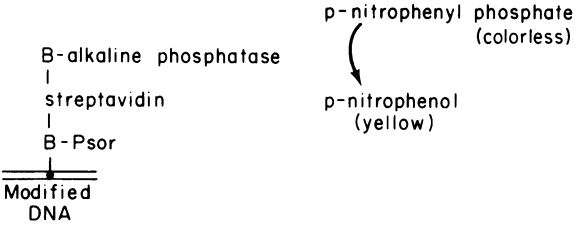

Fig. 4. Detection of BPsor modification of DNA by ELISA. BPsor-reacted DNA bound to microtiter dishes was incubated sequentially with streptavidin, biotinylated poly alkaline phosphatase, and phosphatase substrate. The phosphatase reaction was monitored by absorbance at 405 nm. B-, biotinyl residues.

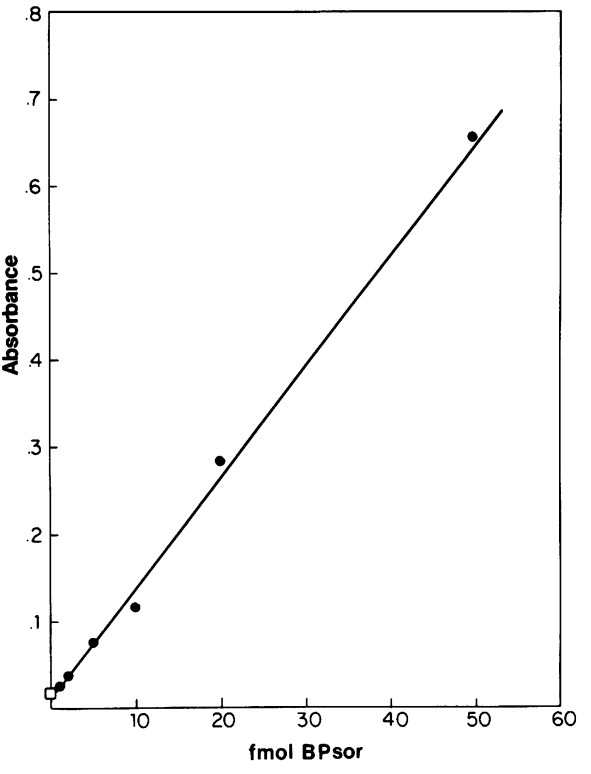

Fig. 5. ELISA of BPsor modified DNA. Calf thymus DNA was reacted with [^{3}H] BPsor to a modification level of 1 BPsor/110 base pairs. The DNA was diluted into PBS, bound to microtiter dishes, and the bound biotin was detected by the method outlined in Fig. 4. Alkaline phosphatase activity is shown, in absorbance units, after two hours incubation. Filled circles, treatment with BPsor + near UV light; open square, treatment with near UV light alone.

inhibit lymphocyte proliferation (25). Freshly prepared peripheral blood lymphocytes were incubated with BPsor and exposed to 3 J/cm^2 of near UV light. PHA was then added to the treated cells to stimulate their proliferation, and after 3 days their growth was assayed by measuring [^{3}H] thymidine incorporation. Proliferation is expressed as the stimulation index, the ratio of [^{3}H] incorporation into cells with and without PHA addition.

BPsor addition to lymphocytes at 1 μg/ml decreased the stimulation index by more than 99% after irradiation, but had no effect in the dark (Table 1). Lower levels of BPsor inhibited cell growth somewhat less, but even at 10 ng/ml there was an 85% decrease in stimulation. In comparison, previous investigations on the toxicity of various psoralen derivatives reported that

Table 1. Inhibition of lymphocyte proliferation

Treatment	Relative Stimulation Index -Light	+Light
None	1.00	1.00
10 ng/ml BPsor	0.94	0.16
100 ng/ml BPsor	N.D.	0.01
1000 ng/ml BPsor	1.01	0.01
10 ng/ml AMT	1.02	0.02

Peripheral blood lymphocytes were incubated with psoralen, at the indicated concentration, for 20 minutes, then distributed into microtiter dishes. The dishes were covered with foil (-light) or irradiated with 3 J/m^2 of near UV light (+light). Phytohemagglutinin (PHA) was added to half the wells and lymphocyte proliferation measured as [^{3}H] thymidine incorporation. The stimulation index was calculated as:

$$S.I. = \frac{[^3H] \text{ incorporation (+PHA)}}{[^3H] \text{ incorporation (-PHA)}}$$

diamine psoralen, the precursor of BPsor, had similar activity in this assay, while 8-methoxypsoralen (8-MOP), the clinically used derivative, had strong activity only above 100 ng/ml (26,27). AMT, the most active psoralen derivative tested, produces almost complete inhibition at 10 ng/ml.

DISCUSSION

A biotin-containing psoralen derivative, BPsor, has been synthesized in a simple two-step reaction. BPsor is a trifunctional nucleic acid- and avidin-binding reagent which combines the properties of psoralen, in its specific photoreaction with DNA, and of biotin, in its strong interaction with avidin. This reagent binds covalently to double-stranded DNA, in a near UV-dependent reaction, and produces interstrand crosslinks.

The biotin moiety of BPsor retains its affinity for avidin, even after photoaddition to DNA. Using an ELISA procedure with streptavidin and biotinylated alkaline phosphatase we were able to measure the amount of BPsor bound to DNA samples. Thus, the addition of a biotin group to psoralen permits the detection and isolation of psoralen adducts with the high sensitivity characteristic of the avidin-biotin interaction.

The technique may be extended to visualization of nucleic acids bound to membrane supports, by direct addition of BPsor or by hybridization with BPsor-derivatized probes. Like photobiotin, BPsor has the advantage of photochemical, rather than enzymatic, addition to DNA. It has the further advantage of high specificity for nucleic acids, rather than the broad reactivity of photobiotin, which also binds to proteins (17).

The specific binding affinity of psoralens for double-stranded regions of nucleic acids has been used to probe the structures of single-stranded nucleic acids, ribosomes, viruses and RNA splicing intermediates(4). Affinity chromatography of BPsor-reacted samples on avidin-agarose resins could be used for the isolation and mapping of psoralen crosslinked regions within complex structures.

Targeting of a biotinylated drug to specific cells through successive linkages to avidin and a biotinylated antibody was demonstrated by Urdal and Hakimori (28). They achieved killing of tumor cells with biotinylated neocarzinostatin bound to biotinylated anti-glycolipid antibodies, through an avidin bridge, although the derivatization with biotin decreased the biological activity of the drug.

BPsor could, in principle, be used to target psoralen to specific cells by attaching it to avidin and a biotinylated cell-specific carrier. The biotinylation of psoralen does not interfere with its biological activity in lymphocytes; treatment with BPsor at 10 ng/ml plus near UV light inhibits cellular proliferation. BPsor exhibits a potency comparable to that of its immediate precursor, diamine psoralen, and greater than that of the clinically used derivative, 8-MOP.

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