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Development of a DNA Photoaffinity Probe for the Analysis of 8-OxodG-Binding Proteins in a Human Proteome

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Our genome is constantly damaged by exogenous and endogenous events.[1] Particularly problematic is oxidative DNA damage caused by the reaction of reactive oxygen species (ROS) with the genetic material.[2] The most common oxidative lesions are 8-oxodG and FaPy-dG.[3] Repair of both lesions is essential for the survival of cells; unrepaired lesions induce cell death or cause mutations.[3] Understanding of the chemistry that leads to the formation of these lesions has improved tremendously during the last decade[3a] and we now understand the principles of how these lesions are repaired.[1b] Several crystal structures provide detailed insight into the lesion-recognition and -repair processes.[4]

Despite these achievements, our understanding of the processes that occur inside the cell nucleus at a lesion site is still in its infancy. However, new quantitative mass spectrometry techniques are have recently been developed that revolutionize our ability to study cellular processes.[5] With the help of special trapping molecules (crosslinkers) that covalently connect the involved proteins to their targets it is possible to characterize the involved proteins. Here we report the development of the new photo-crosslinker **1** (Scheme 1) that, when incorporated in DNA,[6] is able to capture proteins involved in processing the main oxidative DNA lesion 8-oxodG.

We selected a diazirine unit as the photoreactive group responsible for forming the covalent bond with the protein.[7] The diazirine is small and upon irradiation at a comparatively long wavelength ($\lambda = 365$ nm) it forms a reactive carbene, which binds efficiently to proteins. The diazirine was attached to the 5-position of the nucleobase uridine so that it should not disturb the DNA duplex structure. This was important because we wanted to reduce binding of proteins that might only recognize a disturbed duplex structure. Finally we decided to link the diazirine to the nucleobase through a triethyleneglycol linker. This spacer molecule seemed the best choice for reducing unspecific binding to the probe itself. In addition, the chemical nature of the spacer reduced the chances of putative hydrophobic collapse of the reactive group with the DNA duplex.[8] The synthesis of the photoaffinity nucleoside **1** and of the corresponding phosphoramidite **2**, together with the prepared oligonucleotide probes (**P1**–**P8**) containing **1** and—for comparison—a series of other possible photoaffinity labels, is shown in Scheme 1.

The synthesis of **1** started with TBDMS protection of iododeoxyuridine (IdU, **3**), which provided TBDMS-IdU (**4**). Sonogashira coupling with the alkinole **5**[9] furnished the triethyleneglycol-substituted nucleoside **6**, which was coupled to the diazirine iodide **7**[10] to provide the nucleoside **8**. Deprotection furnished the target nucleoside **1**. Final conversion of **1** into the phosphoramidite **2** via the DMT-protected nucleoside **9** was accomplished by standard procedures.

Incorporation of **2** into oligonucleotides was possible through phosphoramidite chemistry. For the photoaffinity experiment we prepared DNA strands containing the diazirine trap next to a biotin (Bio) label for final affinity purification of crosslinked proteins and a fluorescein label (Fl) for detection. Finally an 8-oxodG lesion (*G*) was incorporated into the complementary counterstrand. Control strands lacking either the 8-oxodG lesion or the photo-crosslinker were prepared. All prepared DNA single strands were characterized by MALDI-TOF-MS and HPLC prior to hybridization. Finally they were hybridized to form the double-stranded DNA probes **P1**–**P8** (Supporting Information). Subsequent UV melting point studies confirmed that the oligonucleotides exist as undisturbed duplexes (Supporting Information).

We first analyzed the properties of crosslinker **1** within **P2** in comparison with probes (**P1**, **P3**–**7**) containing other photoaffinity labels.[7c] For this study we simply replaced the trap molecule **1** in **P2** either by a diazirine, which we attached to the nucleobase through a different spacer (**P1**),[11] with a benzophenone (**P3**, **P7**), or with nucleobases, which simply carry halogen atoms (**P4**–**P6**; Scheme 1).

For the study we used a special *E. coli* cell lysate prepared from *E. coli* cells that overproduce the *Lactococcus lactis* formamidopyrimidine glycosylase protein (*Ll*Fpg, MW = 33 kDa). This protein is one of the main repair enzymes that specifically recognizes and repairs 8-oxodG lesions.[4c] For the photoaffinity experiments we added the DNA probes (**P1**–**P8**) individually to the *E. coli* cell lysate in a special photoaffinity labeling buffer (Supporting Information) in an Eppendorf tube stored on ice (conditions: 1 μM DNA probe, 1 mg mL^{-1} total protein concentration). The solutions were irradiated with 365 nm light (2× 15 W) in the cases of the diazirine- and benzophenone-carrying probe duplexes and with 312 nm light (2×15 W) if a halogen-containing nucleobase was present as the crosslinking unit. The solutions were subsequently concentrated and analyzed by SDS-PAGE.

The results of the studies are depicted in Figure 1. All probes formed intensive crosslinks in the gel region where proteins with a weight between 30 kDa and 50 kDa are found. The observed band indeed represents the expected DNA-*Ll*Fpg

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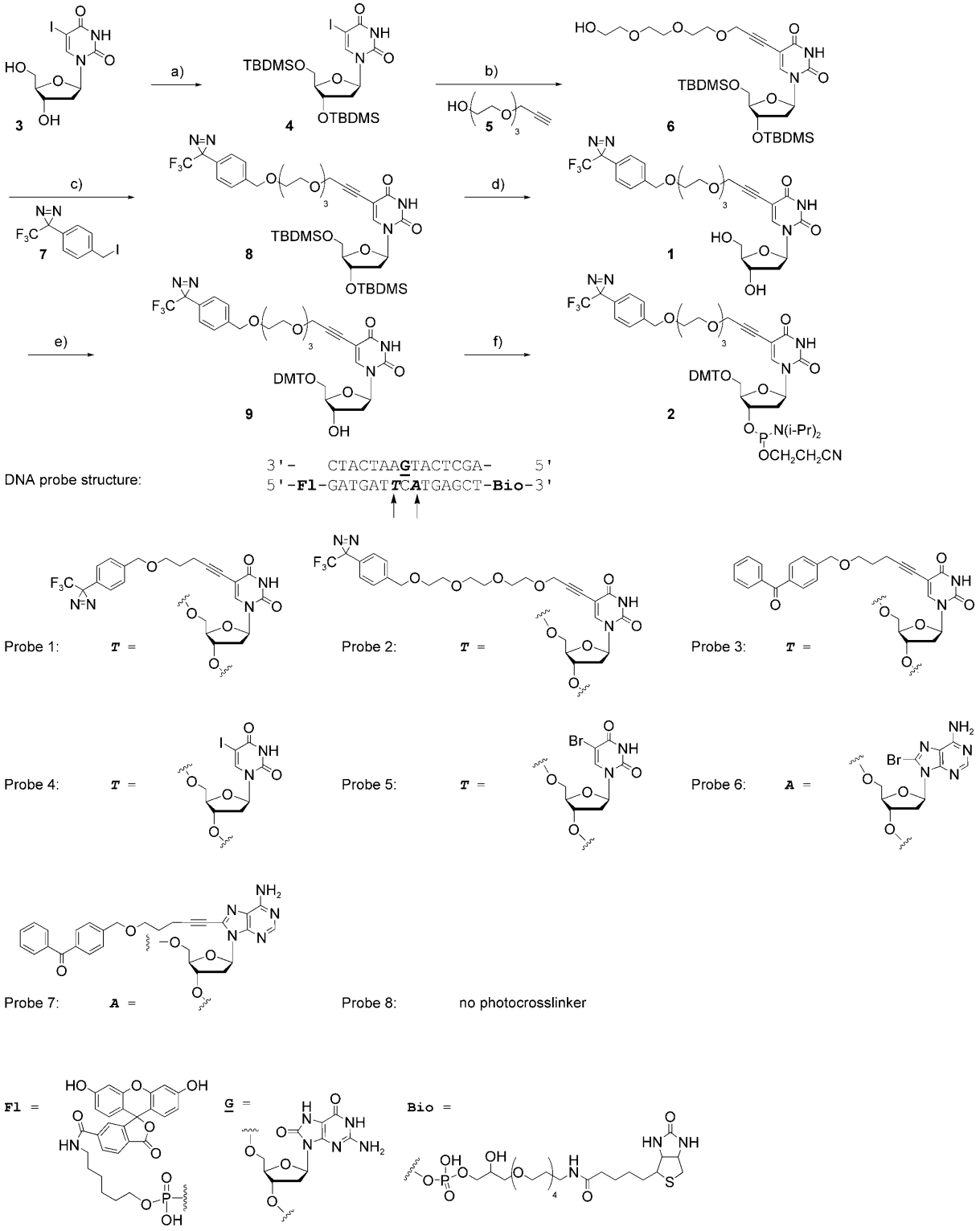

Scheme 1. Synthesis of the diazirine phosphoramidite **2** and of the DNA probes **P1**–**P8** used in this study. a) TMDMSCl, DMAP, DMF, RT, 4 h, 95%; b) **5**, Pd-$(Ph_3)_2Cl_2$, CuI, DMF, iPr_2NEt_3, RT, 12 h, 64%; c) **7**, NaH, THF, RT, 3.5 h, 67%; d) TBAF, THF, RT, 2 h, 100%; e) DMTCl, pyridine, RT→0 °C, 2 h, 40%; f) $(iPr)_2NO$-CH_2CH_2CN, diisopropylammonium tetrazolate, CH_2Cl_2, RT, 3 h, 55%.

crosslink (large box).[11] In view of the fact that the probe duplexes contain several other unnatural units (Fl, Bio, crosslinker) next to the 8-oxodG lesion, the observed selectivity is rather surprising. Despite this, the crosslinking efficiencies of the probes are clearly different. The most intense crosslinks were obtained with the probes **P2**, **P3**, **P5**, and **P7**. The results obtained with the control probes, which lacked 8-oxodG (**G** = dG) are also depicted in Figure 1 in the (−) lanes. In these cases

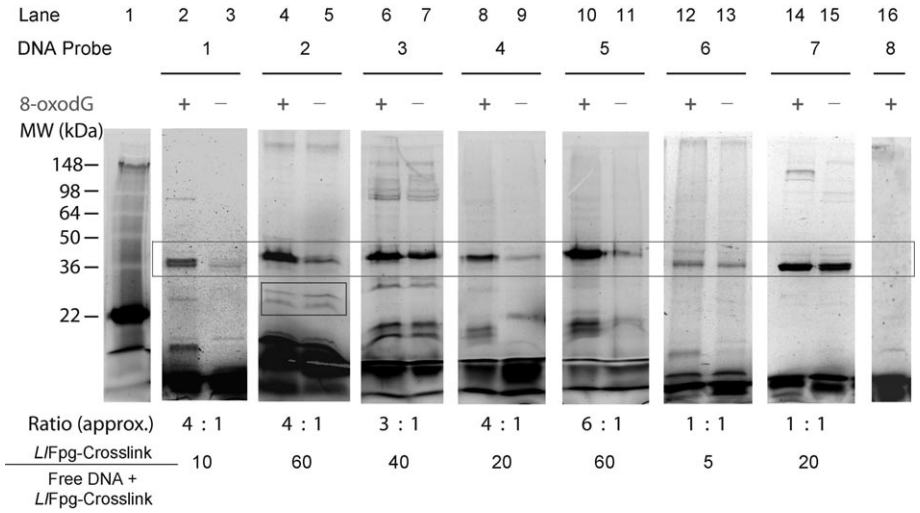

Figure 1. SDS-PAGE gels showing the results of the photo-crosslinking experiments with DNA probes **P1**–**P8** (fluorescence detection). Conditions: DNA probe (1 μM), 1 mg mL^{-1} protein concentration in photoaffinity labeling buffer (assay volume 100 μL; Supporting Information). +) Probe with 8-oxodG, −) without 8-oxodG. The ratio value describes the intensity difference with and without 8-oxodG in the probe. The yield value describes the amount of crosslink detected.

minor amounts of crosslinks are formed with other proteins in the same mass range, or some unspecific binding of the FPG protein to undamaged DNA might be detected. Comparison of the (+) lanes with the (−) lanes shows that the benzophenone-substituted DNA probes **3** and **7** provide rather large unspecific signals. Particularly favorable are the signal-to-noise ratios for **P2** and **P5**. Direct comparison of these two probes show that **P2** is able to label a significantly larger number of proteins in a very interesting mass range (small box in Figure 1); the crosslinker **1** in **P2** is an excellent trapping device. It should be noted that the DNA control strand **P8**, which contains no photo-crosslinker, gave no DNA–protein crosslink (lane 16); this indicates that protein association with the trap-unit itself is indeed a minor issue and that only the photocrosslinker moieties are able to induce DNA-protein crosslink formation. If the experiments were performed with a heat-denatured protein lysate, again no crosslinks were found (data not shown); this shows that only properly folded proteins are recognized by the DNA probes. Particularly noteworthy is the large difference observed between probes **1** and **2**; this demonstrates that the nature of the spacer unit has a tremendous effect on the photo-crosslinking yield and efficiency.

With these result in hand we next started to use **P2** to identify proteins that would bind specifically and directly to 8-oxodG in a human HCT-116 cell nuclear lysate. The nuclear extract (nuclear proteome) was chosen because it should contain the repair protein in an enriched state. The experiment was performed by incubation of **P2** (1 nmol) with the nuclear lysate (2 mg) for 30 min. The solution was subsequently irradiated for 30 min (1 μM DNA concentration). A small amount of the assay solution was removed, concentrated, and analyzed by analytical SDS-PAGE (Figure 2, lanes 2–6). The formation of crosslinks is clearly evident (lane 2). Crosslinks were also obtained, however, with the control DNA strand containing no 8-oxodG lesion (lane 3). Control experiments performed without a DNA probe (lane 4) or with heat-denatured cell lysate (lanes 5 and 6) again revealed no bands. For protein identification, we added avidin-agarose-beads to the remaining bigger part of the irradiated samples and isolated the formed crosslinks with the aid of the biotin unit. The beads were washed several times and the protein material was cleaved from the beads by heating the beads in buffer (95 °C, 5 min; Supporting Information). Analysis of the solution by gel electrophoresis (lanes 7–10) again showed crosslink formation with the DNA probe containing 8-oxodG (lane 8) but also to the

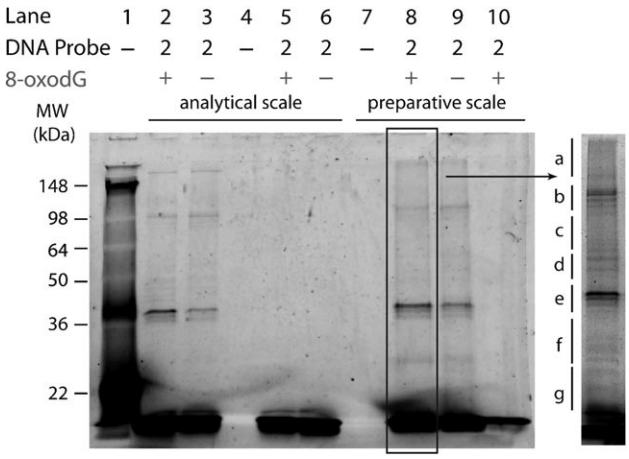

Figure 2. SDS-PAGE analysis of the photo-crosslinking experiments: analytical (lanes 2–6) and preparative (lanes 7–10) photoaffinity labeling experiments with DNA probe **2**, containing 8-oxo-dG (lanes 2, 5, 8, 10), and with undamaged controls (lanes 3, 6, 9) with use of a human HCT-116 cell nuclear lysate (lanes 5, 6, 10: heat controls).

probe lacking 8-oxodG (lane 9). Again, the control experiment with heat-denatured cell lysate provided no crosslink (lane 10).

We next prepared slices of the preparative gel (areas a–g). The gel slices were washed several times, the proteins were individually digested in-gel with trypsin, and the peptide mixtures were subsequently analyzed by nano-HPLC-ESI-MS/MS. The obtained data set was corrected by subtraction of the data obtained in a similar experiment performed in the absence of any probe (background subtraction, lane 7).

The corrected MS-data sets were bioinformatically compared to the human protein database with the help of the *BioWorks3.3.1.* program with use of appropriate filters (Supporting Information). We first discovered that the peptide fragments

obtained from lane 8 and from control lane 9 were surprisingly different (for a complete list see the Supporting Information). We found proteins that were present in both lanes, due to unspecific binding, and in addition a set of proteins that were only present in lane 8 and hence specifically associated with 8-oxodG. The observed specific proteins are compiled in Table 1.

Table 1. Proteins in human HCT-116 cell nuclear lysate that were photo-crosslinked to the DNA probe **2**. The values are the output of the *BioWorks3.3.1.* software. The sequence coverage values were found to be between 10 and 50%. P-value: artifact probability; NCBI-ID: Identification number in the NCBI database. MS/MS spectra of some selected peptides from these proteins are shown in the Supporting Information.

Gel area	Protein	*P* value	NCBI-ID	$N^{[a]}$	M_W [kDa]
a, d	PARP-1	5.1×10^{-11}	gi|130781	5	113
d	APEX nuclease	2.3×10^{-11}	gi|219474	4	36
d, e	uracil DNA glycosylase	1.0×10^{-12}	gi|35053	8	35
e	HMG-1	4.9×10^{-8}	gi|968888	10	33
e	hOGG1 isoforms	6.8×10^{-12}	gi|8670532	10	36–45

[a] Number of peptides found

Gel area e in lane 8 contains the crosslinked human protein hOGG1. This protein is a known 8-oxodG repair protein.[12] The finding that this protein provides such a strong signal shows that the method, and in particular **P2** with the new crosslinker **1**, provides important and meaningful results. hOGG1 was not detected in the gel slice (e) obtained from lane 9. This shows that the photoaffinity trap is able to specifically capture proteins associated with the lesion.

Other interesting proteins that are specifically associated with the 8-oxodG are the *high mobility group 1 proteins* (HMG-1). They recognize aberrant structures such as DNA kinks.[13] HMG proteins have previously been recognized to be involved in the binding of cisplatin lesions.[6c] Here we have shown that the rather small structural changes introduced by the 8-oxodG lesion are obviously sufficient to induce statistically relevant binding of the HMG proteins as well.

Furthermore, in the gel areas d and e we detected statistically relevant binding of uracil glycosylase, which is a major repair glycosylase, and of the APEX nuclease. This result is interesting because the APEX protein is part of the cellular response to oxidative stress and protects cells from the genotoxic and cytotoxic effect of oxidizing agents.[14]

MS analysis of the protein digest of gel area a provided another surprise. The PARP protein also shows statistically relevant selectivity for the 8-oxodG lesion and in this study it was not found in the experiment performed with DNA probes lacking 8-oxodG. This protein associates with DNA breaks and with large aberrant DNA structures such as those induced by cisplatin.[6c] The protein binds to the lesioned site and starts to polymerize NAD. This is thought to be a signal that recruits repair factors to the damaged site.[15] Our experiments now show that this protein may also be involved in recognition of small non-helix-disturbing lesions such as 8-oxodG. This observation is supported by a recent in vitro study in which binding of PARP to DNA duplexes containing small oxidative lesions was indeed observed.[16]

Other proteins that are not listed in Table 1 were found to bind to the 8-oxodG-containing and -noncontaining DNA with similar affinities. These unspecific proteins include, for example, the FUSE-binding protein (*P* value 3.0×10^{-13}, eight peptides),[17] the ATP-dependant DNA helicase II (*P* value 2.5×10^{-11}, 12 peptides), and the elongation factor 2b (*P* value 9.2×10^{-6}, 13 peptides). All these proteins most likely bind in general to DNA or to other structures present in the DNA probes, such as the photoaffinity trap, the fluorescence tag, or the biotin label.

In summary, we have developed a new DNA photo-crosslinker capable of efficient crosslinking of DNA-binding proteins to DNA in photoaffinity labeling experiments. The crosslinker shows a good selectivity profile. With DNA duplexes containing an 8-oxodG lesion next to the crosslinker it was possible to identify a set of proteins in the human nuclear proteome that are involved in processing the lesion. As well as proteins such as hOGG1 that are known to recognize the lesion, other interesting proteins such as PARP or the APEX nuclease were also found to be involved in lesion processing. We believe that this photo-crosslinker, in combination with other DNA lesions and mass spectrometry methods,[18] should allow scientists to characterize proteins that are specifically involved in DNA repair processes in detail.

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