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Identification of potentially cytotoxic lesions induced by UVA photoactivation of DNA 4-thiothymidine in human cells

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ABSTRACT

Photochemotherapy—in which a photosensitizing drug is combined with ultraviolet or visible radiation—has proven therapeutic effectiveness. Existing approaches have drawbacks, however, and there is a clinical need to develop alternatives offering improved target cell selectivity. DNA substitution by 4-thiothymidine (S4TdR) sensitizes cells to killing by ultraviolet A (UVA) radiation. Here, we demonstrate that UVA photoactivation of DNA S4TdR does not generate reactive oxygen or cause direct DNA breakage and is only minimally mutagenic. In an organotypic human skin model, UVA penetration is sufficiently robust to kill S4TdR-photosensitized epidermal cells. We have investigated the DNA lesions responsible for toxicity. Although thymidine is the predominant UVA photoproduct of S4TdR in dilute solution, more complex lesions are formed when S4TdR-containing oligonucleotides are irradiated. One of these, a thietane/S5-(6-4)T:T, is structurally related to the (6-4) pyrimidine:pyrimidone [(6-4) Py:Py] photoproducts induced by UVB/C radiation. These lesions are detectable in DNA from S4TdR/UVA-treated cells and are excised from DNA more efficiently by keratinocytes than by leukaemia cells. UVA irradiation also induces DNA interstrand crosslinking of S4TdR-containing duplex oligonucleotides. Cells defective in repairing (6-4) Py:Py DNA adducts or processing DNA crosslinks are extremely sensitive to S4TdR/UVA indicating that these lesions contribute significantly to S4TdR/UVA cytotoxicity.

INTRODUCTION

Photochemotherapy combines ultraviolet or visible radiation with photosensitizing drugs to produce cytotoxic effects which neither drug nor radiation can achieve alone. Psoralen plus UVA (320–400 nm) radiation (PUVA), for the treatment of cutaneous T-cell lymphoma and psoriasis, and photodynamic therapy (PDT) in which tetrapyrroles are activated by light to treat external and internal malignancies (1–3) are established photochemotherapies. Although they are highly effective, these approaches have drawbacks. Long-term use of PUVA is associated with an increased risk of skin cancer (4,5). PDT is not completely selective for the tumour tissue and can be very painful. Hence, further research into alternative approaches, preferably involving lower radiation doses and/or improved selectivity is warranted.

The deliberate induction of DNA damage underlies many successful therapeutic strategies. The canonical DNA bases are damaged when they absorb ultraviolet radiation (UVR) in the UVC and UVB spectral regions (100–320 nm), but have no significant absorption at UVA wavelengths (320–400 nm). Thus, DNA is largely insensitive to direct UVA-induced photochemical damage and skin is about 1000 times less sensitive to UVA than to UVB both at the molecular and clinical levels (6). The thiopurine 6-thioguanine (6-TG) and the thiopyrimidine 4-thiothymine (S4T) are examples of base analogs that are UVA chromophores with absorbance maxima being ~340 nm. Both are incorporated efficiently into DNA of dividing cells where they exhibit a profound synergistic cytotoxicity with UVA radiation (7–9).

4-Thiothymidine (S4TdR) is metabolized via the thymidine kinase (TK)-mediated pyrimidine nucleoside salvage pathway (8). TK is strongly up-regulated during DNA replication (10) and is more active in rapidly dividing...
cells (11). This property can be exploited for therapeutic advantage, as exemplified by trifluorothymidine [recently reviewed (12)]. Unlike trifluorothymidine, S4TdR itself is not detectably toxic or mutagenic in cultured human cells (8). In combination with low dose UVA, however, it causes significant toxicity in rapidly dividing cells and UVA sensitization factors of ~100-fold are easily achievable (8,9). This effect is largely independent of p53 status (9). These two properties: selective sensitization of rapidly dividing cells and p53 independence are key properties for a treatment aimed at cancers in which p53 is often mutated or absent.

The mechanism by which DNA S4TdR increases cellular UVA sensitivity is yet to be elucidated. The UVA energy absorbed by thiobases in DNA can cause DNA damage by Type I photosensitization, or it may be transferred to molecular oxygen to generate reactive oxygen species (ROS) in a Type II photosensitization reaction. ROS cause damage to DNA and proteins, and Type II photosensitization is the predominant mechanism by which the DNA thiopurine 6-TG exerts its photochemical effects (7,13,14).

Nucleotide excision repair (NER)-defective xeroderma pigmentosum (XP) cells are particularly sensitive to the combination of S4TdR and UVA (8). This indicates that the treatment produces potentially lethal DNA lesions that are normally removed by NER. This DNA repair pathway efficiently removes the (6-4) pyrimidine:pyrimidone [(6-4) Py:Py] intrasstrand DNA crosslinks induced by UVC and UVB radiation. A thietane photoproduct that is formed in a UVA-activated reaction between S4T with an adjacent thymine, and which resembles a (6-4) Py:Py DNA photoproduct, is a candidate for this potentially lethal NER substrate (15,16).

In the study reported here, UVA penetration of in vitro reconstituted skin is shown to be sufficiently robust to selectively kill dividing epidermal cells that contain DNA S4TdR. We demonstrate that DNA S4TdR photosensitization involves a predominantly Type I photosensitization and does not generate significant levels of ROS. The combination of S4TdR and UVA is only weakly mutagenic in mammalian cultured cells. DNA S4TdR photoproducts are identified and include a DNA thietane that is repaired by NER.

MATERIALS AND METHODS

Cell culture

Human HaCaT, XP12RO and MRC5VA and all Chinese hamster cell lines were grown in DMEM containing 10% fetal calf serum. The human T lymphoblast cell line CCRF-CEM from acute lymphoblastic leukaemia was grown in RPMI + 10% fetal calf serum. CHO irs1 (XRS6 mutant) and irs1/XRCC2 (XRC2XMR1 complemented irs1) cells (17) were provided by Dr John Thacker and CHO XR56 (Ku80 mutant) and its Ku80 parent K1 by Dr Mark O’Driscoll. The ERCC4(XPF) mutant CHO cell line UV41, ERCC5(XPG) mutant UV135 and their parental counterpart AA8 have been described (18,19).

Organotypic skin cultures on de-epidermalized dermis

Organotypic skin cultures were prepared as described (20). Briefly, glycerol-preserved γ-irradiated skin (Euro Skin Bank, Beverwijk, Holland) was washed extensively in PBS and incubated in antibiotic-containing PBS at 37°C for ~10 days. The epidermis was then removed using forceps, and de-epidermalized dermis (DED) was cut into small squares and placed in culture dishes with the papillary dermal surface on the underside. Steel rings were placed on top of the dermis and normal human dermal fibroblasts were added into the rings on the reticular dermal surface. After incubation overnight, the de-epidermalized dermis was inverted, the rings were replaced and normal human keratinocytes were seeded inside the rings onto the dermis. After 2 days, the dermis was raised to the air–liquid interface in the same orientation, by placing the composites on steel grids for 14 days. The medium was replaced every 3 days. At Days 6 and 7, medium supplemented with S4TdR was added for 3 days and the rafts were irradiated. After 24 h, they were fixed in 10% formalin and embedded in paraffin. Deparaffinized sections were stained with Haematoxylin and Eosin for histologic examination.

Drug treatment

S4TdR prepared according to Xu et al. (21) was obtained from Glen Research (Sterling, VA, USA). It was dissolved in deionized water, filter-sterilized and stored at ~20°C. All S4TdR treatments were for 48 h in medium containing 10% FCS which had been dialysed extensively (DFCS) through a 2 kDa exclusion membrane Spectra/Por (Spectrum Laboratories; Medicell International, Ltd.). In the mutagenicity assay, N-methyl-N-nitrosourea (MNU) (Sigma Aldrich, UK) was dissolved in dimethylsulphoxide (DMSO) and added to the medium.

Irradiation

UVA irradiation of oligonucleotides was at 100 W/m² with a UVH 250 W iron bulb with a low range cut-off at 320 nm (UVLight Technologies, Birmingham, UK). For irradiation of cells, the UVA source was a UVASpot lamp (Dr. Hönle AG UV Technology, Gräfelfing/München, Germany), emitting broadband UVA and UVB radiation. A thietane photoproduct that is formed in a UVA-activated reaction between S4T with an adjacent thymine, and which resembles a (6-4) Py:Py DNA photoproduct, is a candidate for this potentially lethal NER substrate (15,16).

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MATERIALS AND METHODS

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Bentham spectroradiometer (Bentham Instruments, Ltd., Reading, UK) calibrated against national UK standards.

Ionizing radiation (3.0 Gy/min) was from a $^{137}$Cs γ-radiation source with a Nordion GC-1000 S v2.09 cell irradiator (Ottawa, ON, Canada).

**Cell survival**

Irradiated cells were seeded in 48-well plates (5000 cells/well). Viability was determined 5 days later by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. For clonal survival, cells were plated in 6-well plates (300–500 cells/well). Colonies were stained and counted 10 days later. Data are based on triplicate samples from at least two experiments.

**Detection of ROS**

Cells were grown in the presence or absence of S$^4$TdR for 48 h, washed twice with PBS and incubated with 5μM CM-H2DCFDA (Invitrogen, Paisley, Scotland) for 20 min at 37°C. After additional washing with PBS, cells were UVA irradiated in PBS/CaMg. Fluorescence was analysed by flow cytometry on a Beckton Dickinson (BD) Canto II machine.

**APRT mutation assay**

Mutation Frequency. The Adenine phosphoribosyl-transferase (APRT) Mutation frequency was determined using CHO-D422 (22,23). Cells were maintained in HAT medium to eliminate spontaneous mutants. They were then grown for 48 h in a medium supplemented with 10% DFCS and 100μM S$^4$TdR and irradiated. After a further 7 days growth, $10^6$ cells were plated per 10 cm dish in medium containing 0.4 mM 8-azaadenine. The number of resistant colonies was determined after a further 10 days' growth. At least $1.5 \times 10^{7}$ cells were plated per experiment.

**Immunofluorescence**

HaCaT cells grown on culture slides (BD Falcon, San Jose, CA) in 100μM S$^4$TdR were irradiated with 10 kJ/m² UVA. Control cells were irradiated with 10 Gy γ-rays. Irradiated cells were rinsed with PBS and returned to normal medium for 4 h. They were then fixed and processed for detection of phosphorylated γH2AX as described by Brem et al. (24). For the detection of CPD and (6-4) photoproducts, irradiated HaCaT cells were fixed immediately after UV irradiation. Photoproducts were detected using antibodies specific for thymine dimers (clone TDM-2) or (6-4) Py:Py photoproducts (clone 64 M-2) from CosmoBio Co., Ltd (Tokyo, Japan) according to the company’s recommendations.

** Comet assay**

Alkaline comet assays were performed using the Single Cell Gel Electrophoresis Assay kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer’s protocol.

**RESULTS**

S$^4$TdR/UVA-induced cytotoxicity in an organotypic skin model

The powerful synergistic toxicity of S$^4$TdR and UVA was tested in an organotypic 3D skin model. Reconstituted skin (raft) cultures comprising human normal primary fibroblasts and keratinocytes were treated with S$^4$TdR by maintaining the rafts in growth medium containing the photosensitizer. The rafts were then exposed to UVA doses of ~100 kJ/m² (this is approximately one-fifth of a minimal erythema dose for human skin) (27) and paraffin sections were examined for the presence of apoptotic ‘sunburn’ cells (SBC). Figure 1 shows that the rafts that had received either UVA (Figure 1B) or S$^4$Tdr (Figure 1C) alone contained only apparently healthy cells and there was no sign of apoptosis. SBC were present only in rafts treated with the combination of drug and radiation (Figure 1D) and characteristic markers such as pyknosis (chromatin condensation, leading to enhanced nuclear colouration) and karyolysis (chromatin degradation leading to fading of nucleus colouration) were clearly
visible in the proliferating layers down to the basement membrane at the epidermal–dermal junction. Notably, there was no sign of cell damage in the upper non-proliferating layers of the epidermis. These data demonstrate that sufficient UVA penetrates through to the proliferating target cell population to activate DNA S4TdR and that S4TdR-containing cells in the epidermis are susceptible to killing by relatively low doses of UVA.

**Mutagenicity**

Although the toxicity of the S4TdR/UVA combination is well established (8,9), its potential mutagenicity has not been investigated in detail. We examined mutation to 8-azaadenine-resistance in the APRT hemizygous Chinese hamster ovary (CHO-D422) cell line. D442 cells incorporate S4TdR extremely efficiently (Supplementary Figure S1A) and therefore, provide a sensitive indicator of S4TdR/UVA mutagenicity. Cells were exposed to S4TdR and UVA alone or in combination and mutant colonies were scored after 10–12 days. Figure 2 shows that the most extreme condition (100 μM S4TdR plus 1 kJ/m² UVA), which reduced cell survival to ~1% (Supplementary Figure S1B) induced a modest 2.4-fold increase in mutation frequency. Less toxic combinations increased the mutation frequency by ≤2-fold. Neither S4TdR, nor UVA alone was measurably mutagenic at these doses. In contrast, in the absence of S4TdR treatment, a UVA dose of 500 kJ/m² induced a similar 2- to 4-fold increase in mutation frequency. This value is comparable with published results with the same cell line (28) or other CHO clones (29). Methylnitrosourea, an
acknowledged mutagen included as a positive control, induced a 25-fold increase in mutation frequency. We conclude that, the mutagenic effect of S4TdT/UVA is weak and is similar to that of high-dose UVA.

ROS

High UVA doses generate intracellular ROS. In addition, the thiopurines are Type II UVA photosensitizers and DNA 6-TG is a potent source of ROS following UVA activation (7). To investigate whether photoactivation of DNA thiopyrimidines might also produce ROS, human HaCaT keratinocytes were treated with S4TdT for 48 h to replace ~0.1% of DNA thymidine. They were then irradiated with a range of UVA doses and ROS production was measured using the ROS-activated fluorescent probe CM-H2DCFDA. Flow cytometry analysis (summarized in Figure 3A) confirmed that UVA irradiation alone generated ROS in a dose-dependent manner. The presence of DNA S4TdT did not, however, significantly ($P > 0.05$) increase the levels of ROS produced by UVA indicating that it is not acting principally as a Type II UVA photosensitizer.

DNA damage

Single-strand breaks, oxidized DNA guanine and cyclobutane pyrimidine dimers. The alkaline comet assay (30) was used to determine whether DNA S4TdT photoactivation leads to DNA strand breakage or oxidative DNA damage. A modest dose of UVA, 50 kJ/m², caused just detectable DNA single-strand breakage in HaCaT cells. The presence of DNA S4TdT did not detectably influence strand breakage at this UVA dose, whereas a UVA dose of 200 kJ/m² caused a substantial increase (Figure 3B).

The alkaline comet assay combined with recombinant human OGG-1 DNA glycosylase (hOGG-1) treatment as a probe for oxidative DNA damage indicated that UVA doses of 50 and 200 kJ/m² generated measurable DNA 8-oxoguanine in HaCaT cells. The presence of S4TdT did not alter the hOGG-1 sensitivity of DNA following irradiation with 50 kJ/m² UVA (Figure 3B). Thus, in agreement with the inability of DNA S4TdT to serve as a source of UVA-generated ROS, it does not detectably increase UVA-induced DNA guanine oxidation.

In parallel assays, inclusion of the cyclobutane pyrimidine dimer (CPD)-specific T4 endonuclease V (T4N5) (31) introduced significant DNA breakage in cells treated with 50 kJ/m² or 200 kJ/m² UVA. S4TdT treatment did not increase T4N5 susceptibility of DNA from cells exposed to 50 kJ/m² UVA (Figure 3B). These data confirm the introduction of CPD by UVA (32) and indicate further that DNA substitution by S4TdT does not measurably increase their formation.

In summary, UVA radiation induces DNA single-strand breaks, oxidized guanine and CPD in HaCaT cells. Despite acting as a UVA sensitizer for killing by UVA, DNA S4TdT does not detectably increase the yield of any of these DNA photoproducts even under conditions that are supralethal and result in <1% cell survival (Supplementary Figure S1B). These findings are consistent with the inability of DNA S4TdT to increase the yield of UVA-induced ROS in these cells and suggest that alternative DNA lesions underlie its cytotoxic potential.

Double-strand breaks. The accumulation of foci of phosphorylated histone H2AX (γH2AX) is a hallmark of the cellular response to DSB (33). HaCaT cells that had been treated with S4TdT (100 μM, 48 h) were UVA irradiated and γH2AX was detected by immunocytochemistry. After 4 h of irradiation, most S4TdT-treated cells displayed a pan-nuclear staining (Figure 4) that is consistent with inhibited replication. In contrast, following gamma irradiation (10 Gy) discrete focal γH2AX staining which reflects the presence of DSB was observed in all cells. Neither UVA nor S4TdT alone induced γH2AX staining. These findings suggest that shortly after UVA irradiation, Double-strand breaks (DSBs) are generated in, at most, a small minority of S4TdT-treated cells. They are also consistent with previous observations that
DNA $S^4$TdT photoactivation induces a severe inhibition of DNA replication (Montaner, B. and Karran, P., unpublished data).

$S^4$TdT photoproducts

$S^4$TdT in dilute solution. We examined the stability of the free thiodeoxynucleoside to UVA irradiation. An aqueous $S^4$TdT solution (0.1 mM) was irradiated and the products were separated by reverse phase HPLC. UVA induced a dose-dependent disappearance of $S^4$TdT (Figure 5) with the concurrent appearance of three photoproducts. Mass spectrometry identified thymidine as the predominant species. The remaining photoproducts comprised small amounts of dimeric $S^4$TdT (T$^SS^4$T) and thymidine sulphenate (T$^SO$) in approximately equal yields. The nature of dimeric photoproduct is not yet completely defined. Its UV absorbance spectrum has a maximum at 306 nm which is close to that of $S^4$-methylthymidine ($\lambda_{max}$ 314 nm) and $S^4$-hydroxyethylthiothymidine ($\lambda_{max}$ 308 nm). For this reason, we tentatively assign a T$^SS^4$T rather than disulphide-linked (T$^SO$T) structure. The identification of thymidine sulphenate was based upon high resolution MS analysis (275.06) of the isolated peak and its unusual UV absorption ($\lambda_{max}$ 357 nm).

Thus, in aqueous solution, the most favoured reaction of photoactivated $S^4$TdT is with water to generate thymidine. Since $S^4$T base pairs with A, this reaction generates a canonical base pair in DNA. For this reason, it will not contribute significantly to the cytotoxicity of $S^4$TdT/UVA. These findings suggest that reactions with the surrounding DNA bases or DNA-interacting proteins might be important in the formation of potentially lethal DNA photolesions in treated cells.

$S^4$TdT in oligonucleotides. A series of synthetic oligonucleotides that contained a single $S^4$TdT (Table 1) were used to examine the effects of UVA irradiation on DNA $S^4$TdT in different sequence contexts. Oligonucleotides were irradiated in either single- or double-stranded form and digested to nucleosides which were separated by RP-HPLC. Column eluates were monitored at 335 nm.

![Figure 4. γ-H2AX staining. HaCaT cells grown in the presence of 100 μM $S^4$TdT for 48 h were irradiated with 10 kJ/m² UVA as shown. Control cells grown in the absence of $S^4$TdT were treated with 10 Gy of gamma irradiation. In each case, γ-H2AX was visualized 4 h later. Note the pan-nuclear γ-H2AX staining induced by $S^4$TdT/UVA treatment which differs from the discrete focal staining following gamma irradiation. DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI).](http://nar.oxfordjournals.org/)

![Figure 5. UVA photostability of $S^4$TdT in dilute solution. A 0.1 mM aqueous solution of $S^4$TdT was irradiated with UVA as shown. Samples were analysed by HPLC. Products were detected by A$260$ or A$335$ and quantified. Further confirmation of T$^SO$ and T$^SS^4$T was obtained by mass spectrometry analysis.](http://nar.oxfordjournals.org/)

Table 1. Sequences of the $S^4$T-containing synthetic oligonucleotides used to examine the effects of UVA irradiation on DNA $S^4$TdT in different sequence contexts

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5'$-GAATCAGCC$S^4$TGCA$T$ACATACAGAG-3'</td>
<td>$S^4$TG</td>
</tr>
<tr>
<td>GAATACGCT$S^4$TGCACATACAGAG</td>
<td>$TS^4$TG</td>
</tr>
<tr>
<td>GAATACGG$S^4$TGCACATACAGAG</td>
<td>$GST^T$</td>
</tr>
<tr>
<td>GAATACGG$S^4$TGCACATACAGAG</td>
<td>$GSTC$</td>
</tr>
<tr>
<td>GAATACGG$S^4$TGCACATACAGAG</td>
<td>$GSTT$</td>
</tr>
<tr>
<td>GAATACGG$S^4$TGCACATACAGAG</td>
<td>$GStG$</td>
</tr>
<tr>
<td>GAATACGG$S^4$TGCACATACAGAG</td>
<td>$GSTA$</td>
</tr>
</tbody>
</table>

$S^4$T = 4-thiothymin. Abbreviations used in the text for the sequences are given in the Code column.

($S^4$TdT), 260 nm (normal nucleosides) and by fluorescence (Ex 320 nm; Em 370 nm).

Figure 6A and B shows representative HPLC profiles for the digests of the duplex oligonucleotide in which the TS$^4$TG oligonucleotide is annealed to a complementary sequence.
strand to generate an S₄T:A pair flanked by a 5’T and a 3’G (Table 1). A UVA dose of 10 kJ/m² reduced the yield of S₄TdR (elution time 26 min) by >60% and this coincided with the appearance of a fluorescent product eluting at 19 min (Figure 6B). This photoproduct also absorbed at 315 nm. The destruction of S₄TdR and the formation of the fluorescent/A₃₁₅ absorbing material were linearly related to UVA dose/C₁₀ kJ/m² (Figure 6C–E). UVA irradiation of the single-stranded T₄S₄T₅ oligonucleotide generated the same 19-min product in a similarly dose-dependent fashion in approximately similar yields (data not shown).

S₄T was more UVA resistant in the closely related G₅S₄T₅ duplex in which it is paired to A and flanked by 5’G:C and a 3’T:A base pair (Table 1). A dose of 10 kJ/m² destroyed only ~10% of the S₄TdR (Figure 7A). Higher UVA doses were more effective and only 30% of the starting S₄TdR remained after 100 kJ/m². No photoproducts were detectable by fluorescence (Figure 7B), A₃₃₅ or A₂₆₀ (data not shown). In particular, there was no concomitant increase in recovery of TdR, confirming that the photochemistry of DNA S₄TdR differs from that of the free thionucleoside. The photosensitivity of S₄TdR in single- or double-stranded G₅S₄T₅ was typical of oligonucleotides in which S₄T was not immediately 3’- to T. These included CS₄TG indicating that a 5’C cannot substitute for T to confer enhanced UVA sensitivity. Comparison between T₄S₄T₅ and other duplex oligonucleotides (CS₄TG & GS₄TT) is presented in Figure 7C.

Based on these findings, we conclude that S₄TdR in single- or double-stranded DNA is susceptible to destruction by UVA. It is atypically photosensitive when placed 3’- but not 5’T. In the former context, we observed a photoproduct with fluorescence and absorbance properties consistent with those reported for thio (6-4)T:T, (S₅-(6-4)T:T) (16), which is analogous to the (6-4) Py:Py UVC/UVB DNA photoproduct. The likely identity of the S₅-(6-4)T:T was confirmed by ELISA assays. Figure 7D shows that the UVA-irradiated T₄S₄T₅ oligonucleotide was recognized by the antibody against (6-4) Py:Py and gave a strong ELISA signal. In contrast, irradiated GS₄TT gave only background signals. In the absence of
a 5'T, the destruction of S4TdR requires higher UVA doses and is not associated with photoproducts with detectable UVA or UVC absorbance or fluorescence under our standard conditions. Although TdR is the predominant UVA photoproduct after high-dose irradiation of S4TdR in aqueous solution, we did not see an equivalent conversion to TdR in irradiated oligonucleotides.

### DNA interstrand crosslinks

When duplex S4TdR oligonucleotides were UVA irradiated and analysed by denaturing PAGE, a radioactive photoproduct that migrated with the characteristics of a cross-linked duplex was observed. Interstrand crosslinks (ICL) formation was UVA dose-dependent. The efficiencies of crosslinking with the GS^TTA, GS^TT and TS^TG duplexes were not widely different, suggesting that the generation of ICL is largely independent of the immediate sequence context of the S4TdR (Figure 8). Crosslinking was dependent on the presence of the complementary strand and UVA irradiation did not induce a change in migration of single-stranded S4TdR-containing oligonucleotides (Figure 8, middle panel).

### DNA repair

In order to investigate the potential toxicity of S4TdR DNA photoproducts, we examined the S4TdR/UVA sensitivity of a number of cell lines with known DNA repair defects. The previously reported hypersensitivity of NER-defective XPA cells was confirmed (Supplementary Figure S2).

**DNA DSB rejoining.** DNA S4TdR photoproducts inhibit replication (Montaner, B. and Karran, P., unpublished data). Homologous recombinational repair (HR) is one of the predominant pathways by which lesions that impair replication are processed. At comparable levels of DNA S4TdR (~0.3% replacement of TdR), HR-defective xrc2 hamster cells were extremely sensitive to UVA. Whereas >80% of xrc2^− cells survived a UVA dose of 0.1 kJ/m², the same dose reduced survival of the HR-defective cells to around 1% (Figure 9A). In the absence of S4TdR pre-treatment, neither the HR-proficient nor HR-defective cells were affected by these very low UVA doses. To address DSBs that arise outside of S phase, we examined cells defective in the non-homologous end joining (NHEJ) pathway. Neither the NHEJ-defective
(Ku80 mutant) xrs6, nor the repair-proficient parental K1 hamster cells exhibited any S\textsuperscript{4}TdR-related sensitivity over the UVA dose range that was extremely toxic to the HR-defective cells (data not shown). The xrs6 cells incorporated S\textsuperscript{4}TdR poorly, however, and in several experiments replaced ≤0.03% of DNA TdR compared with the 0.3% for the parental CHO K1 cells. Owing to this, we are unable to draw firm conclusions about the contribution of NHEJ to S\textsuperscript{4}TdR/UVA resistance. Taken together with the findings from the γH2AX immunocytochemistry, however, the data indicate that DNA lesion(s) that block replication and thereby generate DSB that require HR for repair are a major contributor to the lethal effects of S\textsuperscript{4}TdR/UVA.

**DNA crosslink processing.** The HR pathway is part of the complex system that processes ICL. The XPF:ERCC1 endonuclease is also involved and XPF-deficient cells are

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**Figure 8.** DNA interstrand photocrosslinking in S\textsuperscript{4}TdR-containing duplex oligonucleotides. The GS\textsuperscript{4}TA, GS\textsuperscript{4}TT and TS\textsuperscript{4}TG oligonucleotides were annealed to their [\textsuperscript{32}P]-labelled complementary strands and irradiated with the UVA doses shown. A [\textsuperscript{32}P]-labelled unannealed GS\textsuperscript{4}TT strand was also irradiated with 50 kJ/m\textsuperscript{2} UVA (far left lane of central panel). Products were separated by denaturing PAGE and detected by autoradiography.

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**Figure 9.** S\textsuperscript{4}TdR/UVA sensitivity in DNA repair-defective cell lines. (A) Homologous recombination. xrc2-deficient CHO irs1 cells and their XRCC2 complemented counterparts were grown for 48 h in 100 μM S\textsuperscript{4}TdR and UVA irradiated at the doses indicated. Survival was determined by clonal assay. (B) XPF and XPG. CHO UV135 (XPG) and CHO UV41 (XPF) cells were treated as in A. Survival was determined by clonal assay. (C) Fanconi A. FancA-defective and FancA-corrected MEFs were treated as in A. Survival was determined by clonal assay.
extremely sensitive to DNA interstrand crosslinking agents. CHO UV41 cells are defective in the hamster homolog of XPF. Figure 9B shows that 0.1 kJ/m² UVA reduced the survival of S⁴TdR-treated UV41 cells to ~0.1%. At comparable levels of DNA S⁴TdR (0.2–0.3%), the parental AA8 cells were essentially insensitive to UVA at doses up to five times higher. The UV135 XPG mutant CHO cells exhibited an intermediate sensitivity compatible with their NER defect (Figure 9B). Mouse embryonic fibroblasts defective in the FancA protein, a member of the Fanconi anaemia (FA) pathway core complex, were also more sensitive to S⁴TdR/UVA than their FancA-proficient counterparts (Figure 9C). The pattern of S⁴TdR/UVA sensitivity among these various mutant cell lines indicates that ICL are a significant contributor to toxicity.

DNA S⁵-(6-4)T:T and excision repair
We examined whether the S⁵-(6-4)T:T lesion was detectable in DNA from S⁴TdR/UVA-treated cells and whether it was subject to excision repair. HaCaT cells in which S⁴TdR replaced between 0.1% and 0.2% of DNA TdR were irradiated with UVA and DNA was extracted immediately or ~24 h after irradiation. DNA digests were analysed by HPLC with fluorescence detection. Figure 10A shows that a photoproduct with the characteristic fluorescence and elution time (19 min) of the S⁵-(6-4)T:T lesion was formed in cellular DNA. At 20 kJ/m² UVA, 30–40% of the DNA S⁴TdR was destroyed. The fluorescent photoproduct was easily detectable at doses as low as 5 kJ/m² and formation was linear with UVA dose up to 20 kJ/m², the highest dose examined (Supplementary Figure S3A). At similar levels of DNA S⁴TdR (0.2%), the UVA dose dependency of DNA S⁴TdR destruction and of S⁵-(6-4)T:T formation in human CCRF-CEM cells was comparable with that of HaCaT cells (Supplementary Figure S3B).

HaCaT cells removed S⁵-(6-4)T:T lesions. Figure 10B shows that they were excised with a half-life of between 6 h and 12 h. By 24 h, the fluorescent product was barely detectable in DNA from cells treated with 5 kJ/m² or 10 kJ/m² UVA. The removal of UVC-induced DNA

Figure 10. Induction and repair of the thietane/S⁵-(6-4)T:T photoproduct in HaCaT cells. (A) Formation of S⁵-(6-4)T:T. HaCaT cells that had been grown for 48 h in 300 μM S⁴TdR were irradiated with 10 kJ/m² UVA. DNA extracted immediately after irradiation was digested to nucleosides that were separated by HPLC with fluorescence (Ex 320 nm; Em 370 nm) detection. The arrow indicates the elution time of the thietane/S⁵-(6-4)T:T photoproduct. The fluorescence scale is the same for both traces. (B) Excision of the thietane/S⁵-(6-4)T:T photoproduct. HaCaT cells were treated with S⁴TdR (300 μM/48 h) and irradiated with UVA: 5 (filled grey square), 10 (open triangle) or 20 (filled black circle) kJ/m². XPA cells treated with 100 μM/48 h were irradiated with 5 kJ/m² (filled black triangle). DNA was extracted, digested to nucleosides and analysed by HPLC. The thietane/S⁵-(6-4)T:T photoproduct was detected by its fluorescence at 370 nm. Thietane/S⁵-(6-4)T:T levels are expressed by dividing the total fluorescent signal (mV) by the number of pmol of TdR determined by A₂₆₀ in the same sample. Inset: Excision of UVC-induced (6-4) Py:Py photoproducts. At the times indicated, DNA was extracted from HaCaT cells irradiated with 30 J/m² UVC and analysed by ELISA assay. (C) The thietane/S⁵-(6-4)T:T photoproduct equilibrium (38,39). The thietane species with the closed four-membered ring predominates and comprises ~75% of the total. For a normal (6-4) Py:Py photoproduct, the corresponding ring-closed oxetane structure is unstable and essentially all of this product is in the ring-open form.
(6-4) Py:Py photoproducts was considerably faster and they were excised with a half-life of <1 h (Figure 10B, insert). S4-TdR-(6-4):T:T repair was significantly less efficient in CCRF-CEM cells and they removed around half of the initial photoproducts in 24 h (Supplementary Figure S3C). The S4-(6-4):T:T lesions persisted at unchanged levels for 24 h in XPA-defective XP12RO cells (Figure 10B) confirming that repair of this photoproduct is by NER.

The high sensitivity of the fluorescence detection renders S4-(6-4):T:T photoproducts easily detectable in HPLC eluates. These rare DNA adducts were not, however, detectable in HaCaT cells by immunocytochemical staining (Supplementary Figure S4) or by ELISA (data not shown) using antibodies against UVC-induced DNA (6-4) Py:Py lesions.

**DISCUSSION**

The powerful synergistic toxicity of S4TdR and UVA in cultured cells is well established (8,9). Its recently reported effectiveness in inhibiting tumour growth in a rat bladder carcinoma model (34) indicates that it is a promising photochemotherapeutic option. The most attractive application for S4TdR would be for hyperproliferative skin conditions that are readily accessible to UVA irradiation. Possible limiting factors for effectiveness in this context are penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduces many of the three-dimensional aspects of skin (20). In this model, modest radiation doses delivered sufficient UVA to the lower levels of the epidermis to kill S4TdR-sensitized cells. Recent evidence from our laboratory shows that physiological doses of UVA induce CPD in human skin (35). Importantly, in our raft experiments, the penetrating UVA was not detectably toxic without the photosensitizer. The rat bladder carcinoma study provided indications that effective penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduces many of the three-dimensional aspects of skin (20). In this model, modest radiation doses delivered sufficient UVA to the lower levels of the epidermis to kill S4TdR-sensitized cells. Recent evidence from our laboratory shows that physiological doses of UVA induce CPD in human skin (35). Importantly, in our raft experiments, the penetrating UVA was not detectably toxic without the photosensitizer. The rat bladder carcinoma study provided indications that effective penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduces many of the three-dimensional aspects of skin (20). In this model, modest radiation doses delivered sufficient UVA to the lower levels of the epidermis to kill S4TdR-sensitized cells. Recent evidence from our laboratory shows that physiological doses of UVA induce CPD in human skin (35). Importantly, in our raft experiments, the penetrating UVA was not detectably toxic without the photosensitizer. The rat bladder carcinoma study provided indications that effective penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduces many of the three-dimensional aspects of skin (20). In this model, modest radiation doses delivered sufficient UVA to the lower levels of the epidermis to kill S4TdR-sensitized cells. Recent evidence from our laboratory shows that physiological doses of UVA induce CPD in human skin (35). Importantly, in our raft experiments, the penetrating UVA was not detectably toxic without the photosensitizer. The rat bladder carcinoma study provided indications that effective penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduces many of the three-dimensional aspects of skin (20). In this model, modest radiation doses delivered sufficient UVA to the lower levels of the epidermis to kill S4TdR-sensitized cells. Recent evidence from our laboratory shows that physiological doses of UVA induce CPD in human skin (35). Importantly, in our raft experiments, the penetrating UVA was not detectably toxic without the photosensitizer. The rat bladder carcinoma study provided indications that effective penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduces many of the three-dimensional aspects of skin (20). In this model, modest radiation doses delivered sufficient UVA to the lower levels of the epidermis to kill S4TdR-sensitized cells. Recent evidence from our laboratory shows that physiological doses of UVA induce CPD in human skin (35). Importantly, in our raft experiments, the penetrating UVA was not detectably toxic without the photosensitizer. The rat bladder carcinoma study provided indications that effective penetration of UVA through the epidermis and accumulation of DNA S4TdR in the proliferating target cells. As a first step in establishing the possible efficacy of S4TdR/UVA, we used reconstituted skin raft cultures, a model which reproduc...
Rapid repair of (6-4) Py:Py lesions reflects the significant DNA distortion they create. The relative persistence of S5^-(6-4)T:T suggests that it may cause less DNA distortion. This, in turn, may reflect the greater stability of the ring-closed, thietane form (38,39). Despite obvious structural similarities between S5^-(6-4)T:T and (6-4) T:T—they are identical except for the replacement of the single O4 atom of the 3'-thymine by S in the former—there are significant differences in behaviour (16,38,39). In particular, although, both photoproducts are formed via a four-membered closed ring intermediate, the ring-closed oxetane isomer of the (6-4) T:T is unstable and is immediately converted to the open ring form. The thietane is more stable and exists as an equilibrium mixture in which the ring-closed thietane is favoured over S5^-(6-4)T:T (38,39) (Figure 10C). It is possible that, although the structural similarities between (6-4) T:T and thietane permits recognition of the latter in the (6-4) Py:Py ELISA assay, the rotational constraints imposed by the four-membered ring mitigate DNA distortion by the thietane and impede its recognition by NER. We note, however, that in order to induce detectable levels of thietane/S5^-(6-4)T:T photoproducts, it was necessary to expose cells to combinations of S4TdR and UVA that cause significant cell death. It is therefore, possible that the one or more of the other DNA S4TdR photoproducts, such as ICL, interferes with the processing of thietane/S5^-(6-4)T:T lesions by NER. These possibilities are currently under investigation.

This study confirmed the extreme sensitivity of XP12RO cells. These findings indicate that NER repairs potentially lethal S4TdR/UVA DNA damage. The sequence dependency of DNA thietane/S4^-(6-4)T:T formation suggests that these lesions may comprise a relatively minor fraction of the total DNA photoproducts. Despite this, they contribute significantly to the lethal effects of S4TdR/UVA. The NER system is particularly efficient in keratinocytes (41,42) and the superior repair of thietane/S5^-(6-4)T:T photoproducts, it was necessary to expose cells to combinations of S4TdR and UVA that cause significant cell death. It is therefore, possible that the one or more of the other DNA S4TdR photoproducts, such as ICL, interferes with the processing of thietane/S5^-(6-4)T:T lesions by NER. These possibilities are currently under investigation.

In summary, UVA photoactivation of S4TdR—a highly efficient cytotoxic combination in cultured cells—has properties that suggest it might be an effective highly targeted treatment for hyperproliferative skin conditions. Sufficient UVA penetrates reconstituted skin and skin in vivo to generate cytotoxic DNA lesions in the lower levels of the epidermis. Its low mutagenicity suggests that the combination is a potentially effective and safe therapeutic option. The DNA lesions generated by these photosensitized reactions differ from those produced by high-dose UVA alone and thietane/S5^-(6-4)T:T photoproducts and ICLs are both likely contributors to the cytotoxicity of S4TdR/UVA.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figures S1–S4.

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REFERENCES


