The construction and *in vitro* testing of photo-activatable cancer targeting folated anti-CD3 conjugates

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Abstract

The construction and *in vitro* testing of a photo-activatable anti-tumour immuno-regulatory antibody is described. In this 'cloaked' folated anti-CD3 antibody conjugate, the folate portion of the conjugate is free to bind to folate receptor expressing cancer cells, whilst the anti-CD3 activity is effectively rendered inert by a coating of photo-labile 2-nitrobenzyl groups. On irradiation with UV-A light the activity of the anti-CD3 antibody is restored, not only when it is required, but more importantly, only where it is required. The conjugate can then attract killer T-cells to the surface of the tumour cells and kill them. Unirradiated normal tissues, to which the conjugate has been targeted by specific and non-specific binding, remain unharmed. We believe that these 'photo-switchable' conjugates could be used to markedly improve the targeting of the immune response to folate receptor (FR) expressing ovarian and breast cancers whilst minimising the side effects in the rest of the body.
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It has long been considered that bringing activated T-cells into direct contact with cancer cells offers a potent way of killing them [1–6]. Of the many bispecific antibodies that have been created to do this, the majority are composed of two antibody binding sites, one site targets the tumour and the other targets a T-cell [1–6]. A promising and simpler alternative strategy have used bispecific antibodies in which folate is conjugated to the anti-T-cell antibody [2,7–9]. This takes advantage of the fact that many tumour cells overexpress folate receptors (FRs) on their surface and will therefore bind the conjugate [8,10]. However in common with all bispecific antibodies containing an active T-cell binding site, peripheral T-cell binding will occur. This not only prevents the conjugate from getting to the tumour but can also lead to cytokine storms and T-cell depletion [3,9,11]. Furthermore, normal tissues, such as the kidney and lungs, which also express FR [10] can be targeted leading to potentially harmful side effects.

Photo-activatable folated-anti-T-cell antibodies, in which the anti-T-cell activity is only restored when and where it is required, following irradiation with UV light, could be used to overcome these problems. We had already shown that anti-human CD3 (T-cell targeting) antibodies could be reversibly inhibited with a photocleavable 1-(2-nitrophenyl)ethanol (NPE) coating [12], but the challenge lay in creating a folated-antibody conjugate in which the folate residues were still accessible when the inhibiting NPE coating was present. Folation of the anti-CD3 antibody could also damage the antibody or impede the cloaking–uncloaking process.

This report describes the making and testing of such highly specific photo-activatable folated anti-human T-cell conjugates prior to their use in extensive animal trials. The anti-human CD3 antibody, UCHT1, was selected for study as we had already demonstrated that its activity could be reversibly inhibited by a coating of NPE groups [12]. This

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antibody then had folate conjugated to it to enable the conjugate to bind to folate receptor (FR) expressing cancer cells. After demonstrating that the folated-UCHT1 antibody could still bind to T-cells, the T-cell binding activity of the folated-UCHT1 conjugate was then reversibly inhibited using an NPE coating [12–14]. The NPE-coated-folated-UCHT1 conjugate can bind to FR expressing cancer cells both before and after irradiation, whilst T-cell binding only occurs after irradiation with UV-A light.

Experimental

Antibodies and cell lines. The CD3+ T-cell line H9 was obtained from ECACC. The UCHT1 secreting hybridoma (IgG2a subclass) was obtained from Cancer Research UK. The FR expressing murine ovarian cell line M5076 [15] was kindly supplied by Dr. G.A. Turner.

Coupling of folate residues to the UCHT1 anti-human CD3 antibody. Ten milligrams of folic acid was first dissolved in 1 ml dry DMSO. Its acid groups were then converted to *N*-hydroxysuccinimide (NHS) esters by the addition of 6 mg NHS (in 500 μl DMF) followed by 5 mg of dicyclohexylcarbodiimide (DCC, in 500 μl DMF). This 2 ml mixture was gently shaken for 6 h at room temperature then aliquots (30–75 μl) of the mixture were added per ml to UCHT1 (1.0–1.6 mg/ml) dissolved in 0.1 M sodium bicarbonate. The NHS esters react with the antibody amine residues to form antibody–folate complexes. After an o/n incubation (gently shaken) the antibody–folate mixtures were dialysed three times against 5l of 25 mM phosphate buffer, pH 7.5, containing 0.9% NaCl to remove uncoupled folate molecules. Aggregates were removed by centrifugation at 13,000 rpm for 10 min in a MSE Micro Centaur microfuge and the absorbance (OD at 280 nm) and protein concentration of the clear supernatant (against BCA standard) measured. On subtraction of the known OD 280 nm of uncoupled antibody (OD 1.37 at 1 mg/ml) and knowing the OD 280 nm of 0.1 mg/ml folate to be approx. 4.4, the amount of folate and hence the number of residues per antibody molecule could be estimated.

Coating of the folated-UCHT1 with 2-nitrobenzyl groups. Eleven milligrams of NPE was dissolved in 250 μl of dioxan. To this 6 μl pyridine and 8 μl diphosgene were added. An immediate white precipitate of NPE-chloroformate was obtained. After a further 10 min the excess reagents were removed by evaporation in a flow of nitrogen and 1.0 ml fresh dioxan was added. Aliquots (5–20 μl) of this 1000 μl white crystalline suspension were then added to 1 ml aliquots of folated-UCHT1 (0.5–1.0 mg/ml) dissolved in 0.1 M sodium bicarbonate. Following o/n incubation (gently shaken in the dark) the antibody–NPE mixture was dialysed three times against 5l of 0.9% NaCl/25 mM phosphate buffer, pH 7.5, to remove uncoupled NPE molecules. Aggregates were removed by centrifugation at 13,000 rpm for 10 min in a microfuge and the absorbance (OD at 280 nm) and protein concentration of the clear supernatant (against BCA standard) measured. On subtraction of the known OD 280 nm of the uncoated folated antibody and knowing the OD 280 nm of 50 μg/ml NPE to be approx. 1.0, the amount of NPE and hence the number of residues per UCHT1 molecule could be determined.

T-cell binding assay. The human T-cell line, H9, was grown in suspension in RPMI-1640 media supplemented with 10 ml FCS and 1 ml Penicillin/Streptomycin per 100 ml bottle. The cells were maintained by splitting them at a 1–10 ratio into fresh medium every 4–5 days. To perform an assay a flask of cells (10 ml) was centrifuged for 8 min at 500g and the cells were resuspended at 10^6 cells/ml in fresh medium. Ten microliter aliquots of the diluted antibody conjugates (all diluted to 0.05 mg/ml) were added to 250 μl aliquots of the cell suspension and were left to bind for 30 min at 4 °C. The cells were then washed three times with 1 ml PBS followed by centrifugation for 2 min at 2000 rpm. They were then resuspended in 200 μl of a second layer goat anti-mouse FITC antibody (5 μl/ml in PBS). After a further 30 min incubation the cells were again washed three times, resuspended in 500 μl PBS, and their fluorescence (5000 gated cells) was measured using a Becton–Dickinson flow cytometer.

Cancer cell binding assay. The mouse cancer cell line M5076 was grown up in suspension in folate restricted (25% folate containing media, 75% folate free media) DMEM media containing 10% FCS in order to get it to up-regulate FR on its surface. To perform an assay the cells were collected by centrifugation for 5 min at 500g and were resuspended at approx 1.5×10^6 cells/ml in fresh medium. Ten microliter aliquots of the diluted antibodies (all diluted to 0.05 mg/ml) were added to 250 μl aliquots of the cell suspension and were left to bind for 30 min at 4 °C. The cells were then washed three times with 1 ml PBS followed by centrifugation for 2 min at 2000 rpm. The cells were then resuspended in 200 μl of a second layer goat anti-mouse FITC antibody (5 μl/ml in PBS). After a further 30 min incubation they were again washed three times, resuspended in 500 μl PBS and their fluorescence (5000 cells) was measured using a Becton–Dickinson flow cytometer.

ELISA for antibody bound folate determination. The amount of folate coupled to our antibody conjugates was measured in an 'in house' selective ELISA procedure. Absorbances given by pre-diluted samples (1/400) were compared to those given by replicate aliquots of 50 μl of folate standards.

Electrophoresis. Samples were separated by native electrophoresis (no reducing agent or detergent) in 8% polyacrylamide gels using a discontinuous buffer system. Bovine serum albumin (BSA) was used as a standard.

Results

In initial experiments we attempted to directly couple activated folate–NHS esters to NPE coated (fully inactivated) UCHT1 which had between 30 and 40 NPE residues on each antibody [12]. This was found to be impossible, probably due to the NPE and folate both binding via the same amine residues on the antibody. UCHT1 molecules were then only partially coated with NPE (approx. 20 NPE residues per antibody molecule), and hence only partially inactivated [12], prior to the addition of NHS-activated folate esters. The final products contained around 12–15 residues of folate per antibody molecule. Whereas the binding to T-cells of this final product was fully inhibited, very little activity (less than 5%) could be regained on UV-irradiation. The product also tended to precipitate fairly rapidly on storage. In all further experiments the folate was therefore coupled to the UCHT1 antibody prior to the antibody being inactivated with an NPE coating.

Coupling of folic acid to UCHT1

In initial experiments 50 μl of folate–NHS esters was added per ml (up to 5 ml) of UCHT1. The number of folate residues that coupled to the antibody depended on the initial antibody concentration. If higher concentrations (1.5–1.6 mg/ml) of the antibody were used the final yield of folated-UCHT1 was approx 1.1 mg/ml and there were between 1.9 and 3.6 folate residues bound to each UCHT1 molecule. If lower concentrations, 0.6 mg/ml, were used up to 12 residues of folate could be coupled with a final yield of 0.5 mg/ml. These more heavily folated preparations were however found to be liable to precipitation on storage.

After numerous couplings, it was found that if 30 μl of folate–NHS was added per ml of UCHT1 (at 1 mg/ml) then coupling was very reproducible with apparently

3.0–3.3 folate residues per UCHT1 molecule. If however dialysis was continued for an extra 3–4 days (5–6 extra changes of buffer) this value reduced to 1.4–1.7 folate residues per UCHT1 molecule. These figures are an average value, therefore, native electrophoresis in 8% polyacrylamide gels was carried out in an attempt to determine the heterogeneity of the UCHT1 folation. No detergent was used so the antibody migrates according to its natural charge. When folate residues couple to the antibody's amine groups the antibody's net negative charge increases so it migrates faster through the gel. This effect can clearly be seen in Fig. 1 where two folated conjugates are compared to unfolated UCHT1. The folated samples migrate much faster and as a much more diffuse band due to the heterogeneity of coupling. The folate–UCHT1 conjugate (3.6 res) migrates faster than the folate–UCHT1 conjugate (1.9 res), exactly as would be predicted, with no unconjugated UCHT1 being present in either sample. This was a good confirmation of the accuracy of our spectral data.

We were also able to confirm that the antibody was folated using an ELISA. The two UCHT1–folate conjugates which gave values of folate of 5.1 μg/mg UCHT1 (1.9 res) and 9.7 μg/mg UCHT1 (3.6 res) by spectrophotometry (and were separated by electrophoresis) had values of 16 and 53.6 μg/mg in the ELISA. The higher values are almost certainly due to the fact that the anti-folate antibody used in the assay will bind with a higher avidity to the antibody–f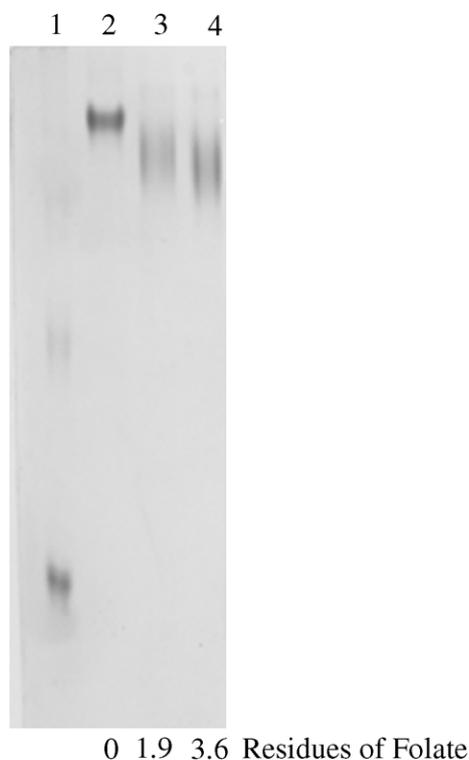olate conjugates than to free folate. All folate antibodies have to be raised against protein–folate conjugates as free folate is an essential vitamin and is not immunogenic.

Coating of folated-UCHT1 with NPE

When the UCHT1–folate conjugates were coated with NPE, two factors proved to be critically important, the UCHT1–folate concentration and the dioxan in which the NPE was dissolved. The dioxan had to be very fresh, if the same bottle was used over several weeks then the number of coupled NPE residues gradually declined. In our first experiments 5, 10 and 15 μl of NPE-chloroformate were added to 1 ml aliquots of folated-UCHT1 (1 mg/ml). The addition of 5 μl NPE-chloroformate coated the folated antibody with around 20 NPE residues per antibody but this did not fully inhibit the anti-CD3 activity. When 10 μl of NPE-chloroformate was added to 1 ml aliquots of conjugate, final yields initially varied from 0.2 to 0.4 mg/ml with between 25 and 50 residues of NPE on each folated-UCHT1 molecule. The yield tended to decrease as the number of coupled NPE groups increased, however the anti-CD3 activity was nearly always fully inhibited (see below). If 15 μl of NPE-chloroformate was added then approximately 60 NPE residues coupled to each antibody but yields decreased to 0.07–0.10 mg/ml. We now routinely add the equivalent of 10 μl of the NPE-chloroformate suspension [see Discussion] per ml of UCHT1–folate (at 0.75 mg/ml in bicarbonate). This gives a more reproducible final yield of approximately 0.25 mg/ml (33%) with around 45 NPE residues coating each folated antibody molecule.

Anti-CD3 biological activity of the UCHT1 conjugates

The ability of the various UCHT1 conjugates to bind to the CD3 expressing H9 T-cell line was measured using flow cytometry [12]. As irradiation was needed to re-activate the NPE-coated conjugates it was first necessary to demonstrate that UV-irradiation did not damage uncoated UCHT1. Fig. 2 demonstrates that UV-A irradiation has very little effect on the binding of UCHT1 to the H9 cells.

The ability of a folated-UCHT1 and NPE-coated-folated-UCHT1 to bind to the T-cells was then measured. These samples contained 3.6 folate residues per UCHT1 molecule and 48 NPE residues per each NPE-coated antibody molecule. The final concentration of the NPE-coated UCHT1–folate conjugate was 0.36 mg/ml. Fig. 3 shows that the binding of the UCHT1 antibody reduced to approximately half of its original activity when it was folated. When this conjugate was coated with NPE its activity reduced to virtually background levels, but after UV-irradiation the conjugate regained approx 25% of its binding capability. This degree of reactivation is however acceptable given that in the actual application of these conjugates the most important parameter is the degree of inactivation achieved. The mean values of the fluorescence for each of the peaks being 5, 64, 31, 6 and 18, respectively. We have now achieved
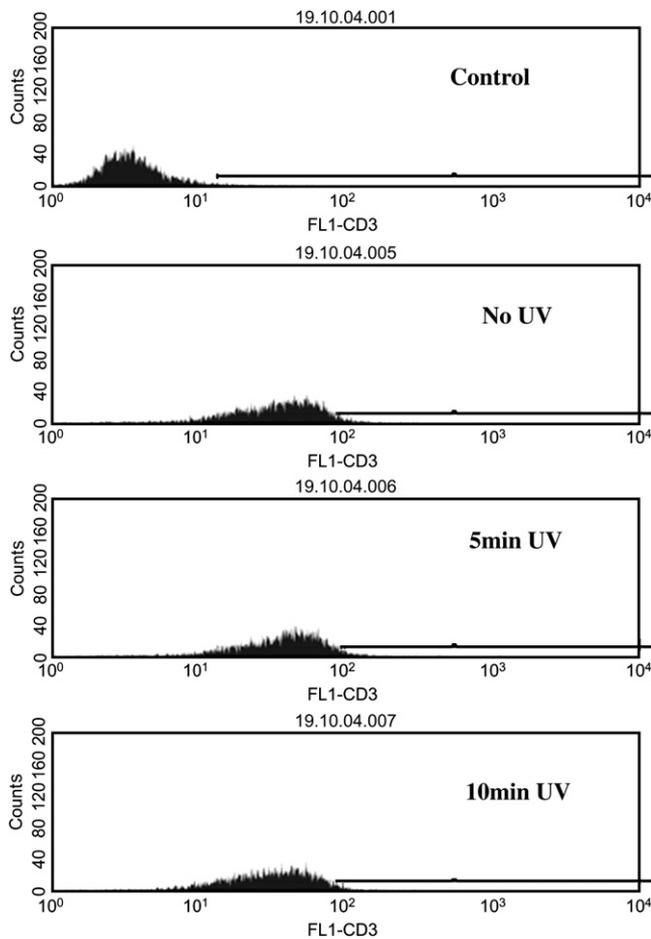

Fig. 1. The folation of UCHT1 as shown by electrophoresis in an 8% polyacrylamide gel. Lane one contains BSA, lane 2 UCHT1, lanes 3 and 4 Fol-UCHT1.

Fig. 2. The effect of UV-irradiation on the T-cell binding capability of UCHT1.

Fig. 3. Flow cytometry showing binding of various folate–UCHT1 conjugates to T-cells.

similar results on over 40 occasions using both UCHT1 and OKT3 [12] a second anti-human CD3 antibody.

Binding of the UCHT1-Fol and NPE–UCHT1-Fol conjugates to cancer cells

The folated-antibody conjugate had to be able to bind to cancer cells both before and after the NPE coating, as well as after UV-irradiation. To check this, we used a murine ovarian tumour cell line, M5076, in a flow cytometry binding assay. The M5076 cells were continuously grown in folate restricted DMEM at 25% normal folate levels to induce them to express FR on the cells. At lower concentrations of folate the cells died quite rapidly. Fig. 4 shows the binding of UCHT1 and two UCHT–folate conjugates to the M5076 cells (13 days after they had been switched to folate restricted media) compared to an unrelated control antibody. The mean values of the fluorescence peaks were 11, 14, 34 and 29 for the Control IgG, UCHT1, Fol-UCHT1 (1.9 res) and Fol-UCHT1 (3.6 res) samples, respectively. In another assay, 8 weeks later, the fluorescence peaks were 18, 30, 56 and 53 probably reflecting increased numbers of FR on the cells.
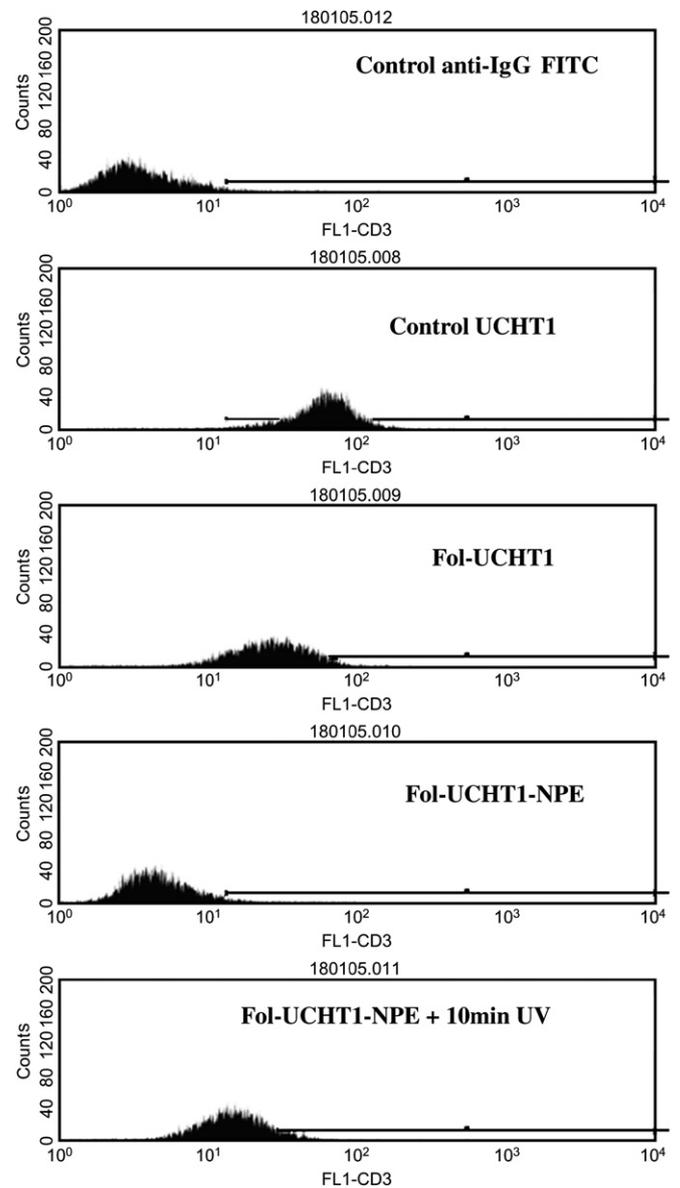
When the folated-UCHT1 conjugates were coated with NPE they were still able to bind to the M5076 cells. Indeed binding to the M5076 cells appeared to increase. In two separate assays using the folate–UCHT1 (3.6 res) sample the mean fluorescence of the peaks were: (i) 25, 32 and 53 and (ii) 29, 47 and 68 for the UCHT1, UCHT–folate and NPE–UCHT1–folate samples, respectively. This increase in binding has been a consistent finding with numerous NPE-coated folate–UCHT1 constructs. On treatment with UV light the values reduced back to the uncoated UCHT1–folate values. Surface folate was also still accessible in the folate ELISA. The UCHT1–folate, NPE–UCHT1–folate and NPE–UCHT1–folate (+UV light) samples had apparent folate values of 43, 44 and 39 µg/mg UCHT1. The folated-UCHT1 conjugates could therefore still bind to the FR expressing cancer cells even
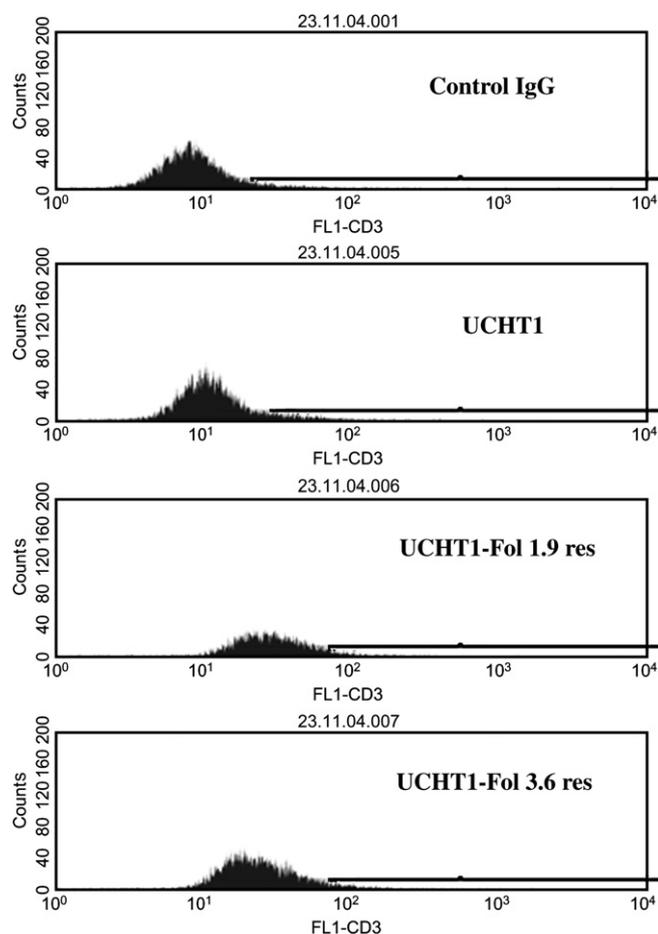
Fig. 4. Flow cytometry showing the binding of two UCHT1–folate conjugates to M5076 cells.

Table 1
The binding of a batch of UCHT1–folate conjugates to T-cells and M5076 cancer as measured by flow cytometry

Antibody conjugate	Mean T-cell fluorescence		Mean cancer cell binding
IgG control	2.0	2.2	20
UCHT1	134	111	28
UCHT1–folate	93	nd	nd
NPE–UCHT1–folate	8	6	68
NPE–UCHT1–folate + UV	35	32	46

This set of conjugates had 9 coupled folate residues and 33 coupled NPE residues to each UCHT1 molecule. Two T-cell binding assays, carried out several weeks apart, are included. nd, not determined.

Table 2
The binding of a second batch of UCHT1–folate conjugate to T-cells and cancer cells as measured by flow cytometry

Antibody conjugate	Mean T-cell fluorescence		Mean cancer cell binding
IgG control	2	2	28
UCHT1	98	163	36
UCHT1–folate	64	110	57
NPE–UCHT1–folate	4.7	6.8	76
NPE–UCHT1–folate + UV	25	46	nd

This set of conjugates had 2.4 coupled folate residues and 44 coupled NPE residues to each UCHT1 molecule. Two T-cell binding assays are included. nd, not determined.

after they were coated with NPE to inhibit their anti-CD3 binding activity.

Other NPE–UCHT1–folate conjugates

The T-cell and M5076 binding capabilities of 2 further NPE–UCHT1–folate constructs are given in Tables 1 and 2 below. We intend to use these, and other, conjugates to study their effects on the growth of the M5076 tumour in C57BL6 transgenic mice which express the human CD3 molecule on their T-cells [16].

Discussion

The above results demonstrate that folated-UCHT1 conjugates can still bind to CD3+ human T-cells although the activity of the UCHT1 antibody is reduced to approximately two-thirds of its original activity. The folated-UCHT1 conjugates can then be reversibly inhibited using an NPE coat to generate photo-activatable cancer targeting conjugates. These conjugates can bind to FR expressing cancer cells both before and after irradiation, whilst T-cell binding only occurs after irradiation with UV-A light. Final yields were between 0.1 and 0.4 mg/ml depending on the extent the conjugates were coated with NPE. Fewer coupled NPE residues gave higher yields but on some occasions this did not fully deactivate the UCHT1 antibody. This is the least reproducible step in the synthesis, and probably reflects the fact that the NPE-chloroformate was more a suspension in the dioxan rather than completely dissolved in it. We are now resuspending the chloroformate in 3 ml of dioxan and adding three times the volume (30 µl) per ml of folated-antibody (at 0.75 mg/ml). This, combined with the use of fresh dioxan in each experiment, has resulted in much more reproducible NPE coatings which completely inhibit the anti-CD3 activity of the conjugate. We have not carried out further characterisations to determine exactly where the 40–50 NPE residues bind to the antibody. As the Fc portion of the antibody is also coated [14], it would be very difficult to make a detailed estimation of how much coating is actually on the binding site. The most important factors are that there is a sufficient coating of NPE to effectively cloak the folated antibody, that this inhibition can be reversed by irradiation with UV-light, and that the procedure is reproducible.

We are currently testing the effects of these conjugates, both irradiated and unirradiated, on the growth of the M5076 tumour in a human CD3ε transgenic mouse model. These C57BL6 transgenic mice express human CD3 on their T-cell surface [16] and therefore can be used to directly study the effects of anti-human CD3 targeting conjugates which could be used to treat patients. We believe that these 'photo-switchable' conjugates could be used to greatly improve the targeting of the immune response to human tumours whilst minimising side effects in the rest of the body.

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