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NOVEL TRITYL LINKED DRUG IMMUNOCONJUGATES FOR CANCER THERAPY

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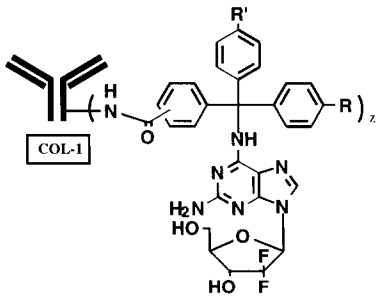
Abstract: Trityl linkers were utilized in the preparation of acid labile nucleoside monoclonal antibody conjugates, COL1-N6-Trityl-207702 (1a-f). Conjugation led to high levels of drug incorporation into the MoAb with retention of good immunoreactivity. A strong correlation was found between the cytotoxicity of the constructs and substituents R and R' on the aromatic rings of the trityl linker.

A major objective of cancer chemotherapy is to destroy malignant cells, while minimizing damage to normal cells. Although various antitumor agents have been found effective against certain tumors, there is still a great need for oncolytics which kill cancer cells more efficiently and selectively. For instance, doxorubicin is widely administered for the treatment of haematological malignancies and solid tumors,[1] however, its use is dose limited due to its cardiac toxicity and myelosuppression.[2] With the discovery of lymphocyte hybridoma technology by Kohler and Milstein[3] in the mid 1970's, which allowed the production of unlimited quantities of monoclonal antibodies, researchers gained a new tool to devise novel methods of delivering cytotoxic agents to target sites. For example, the administration of a drug linked to a monoclonal antibody (MoAb), that reacts with cell surface, tumor-associated antigens, offers an attractive approach to "selective" chemotherapy.[4] Drug immunoconjugates are composed of three distinct entities: (i) the monoclonal antibody for targeting, (ii) the cytotoxic drug and (iii) the linker which attaches the drug to the antibody. To maximize chemotherapeutic value, the drug conjugates must retain good immunoreactivity, possess potent antitumor activity and display minimal systemic toxicity. Early efforts in the field focused on the optimization of the monoclonal antibody and drug entities in search of an effective construct.[5] More recent studies have demonstrated that the linker, by facilitating the timely release of drug at the target site,[6] plays an equally critical role in the overall biological function of drug conjugates.

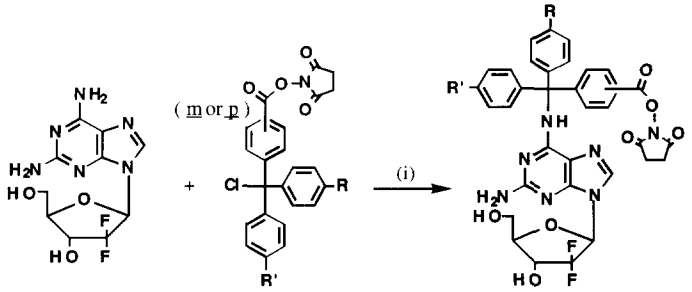


(1)

MoAb (N6-Trityl-207702)$_z$

Herein, we report the use of acid-labile trityl groups as versatile linkers in monoclonal antibody drug conjugates. It is known that the pH of human tumors averages 0.8 units lower than that of the surrounding normal tissues, mainly due to the anaerobic glycolysis of carbohydrates by malignant tumor cells.[7] Thus, the

conditions in the tumor would facilitate site specific release of the drug. The ability to introduce substituents on the aromatic rings of the trityl group offered an ideal opportunity to electronically tune the dissociation of drug to a rate which would complement the targeting properties of the antibody. Furthermore, the incorporation of an activated ester on the linker would allow standard attachment to the protein, *via* formation of a stable amide bond to the epsilon amino group of lysine residues on the antibody.[8] To demonstrate these features of trityl linkers, MoAb (N6-Trityl-207702)$_Z$ conjugates (1) were synthesised in which the drug is represented by LY207702 (2),[9] a potent nucleoside antitumor antimetabolite and the MoAb (13) by a non-internalizing, murine monoclonal antibody, COL1.[10]



(2) (3) (4)

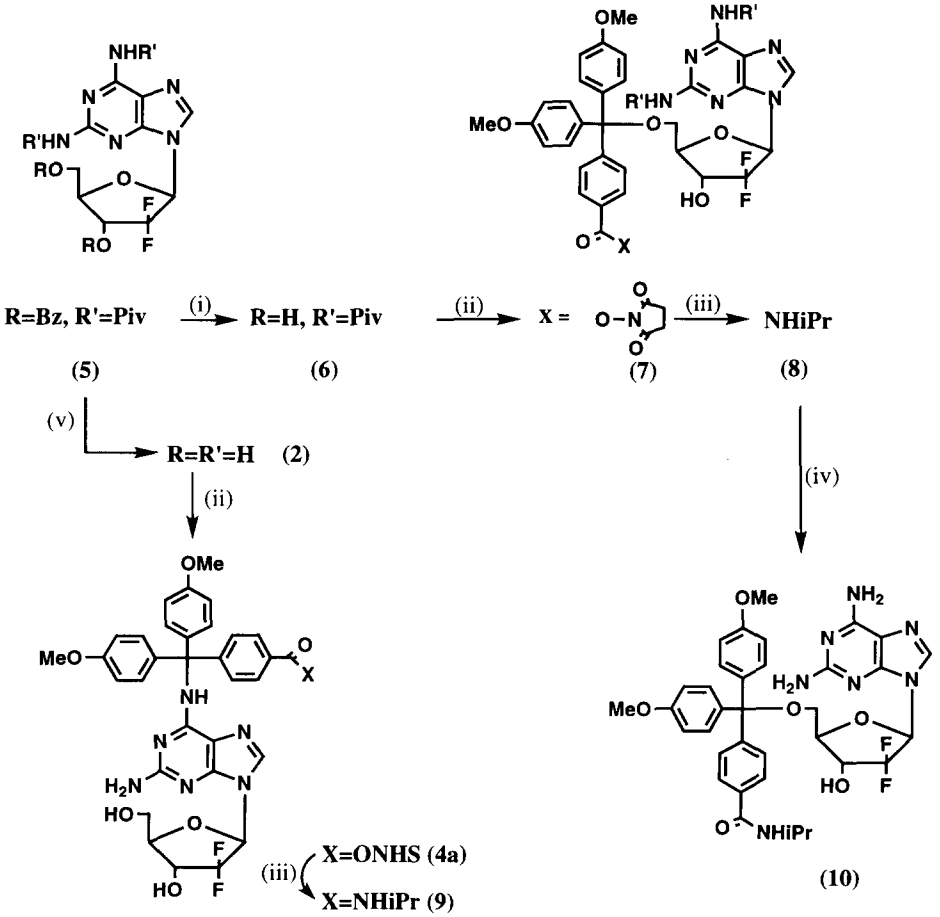
(i) nBu_4NClO_4, 2,4,6-collidine, DMF:CH_2Cl_2, r.t.

Table 1: Preparation of Tritylated LY207702 derivatives

	R	R'	Yield (%) (3)	(4)
(a) p-DMT	OMe	OMe	49	93
(b) p-MMeT	OMe	Me	30	84
(c) p-MMT	OMe	H	36	74
(d) p-MeT	Me	H	74	56
(e) p-T	H	H	39	54
(f) m-DMT	OMe	OMe	27	77

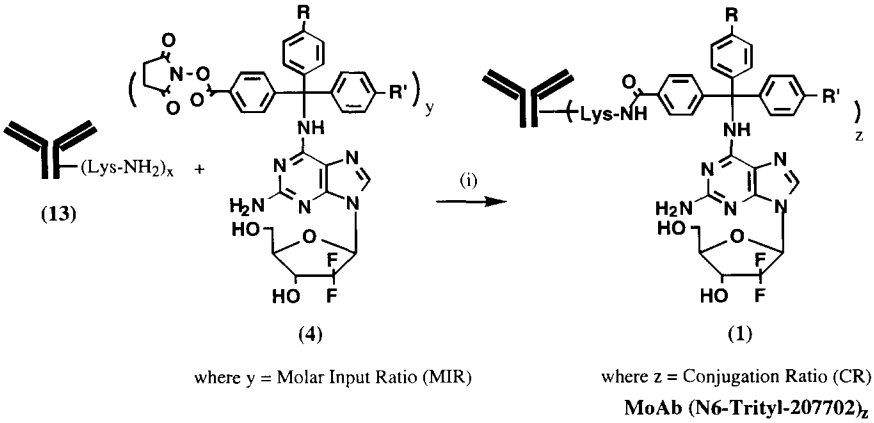
Trityl chlorides (3a-f) were synthesized according to the procedure previously described by Glidea.[11] The methodology was extended to prepare a range of trityl derivatives (**D**i**M**ethoxy**T**rityl, **M**ethoxy**M**ethyl**T**rityl, **M**ono**M**ethoxy**T**rityl, **M**ethyl**T**rityl, **T**rityl) in which the substituents on the aromatic ring were systematically varied in a manner to allow study of the electronic effects of acid-mediated dissociation of the drug. In the case of trityl chlorides (3b,c,d), where R≠R', the reagent was used as a racemic mixture. Accordingly, LY207702 (2) was alkylated[12] with 1.1 equivalents of trityl perchlorate, generated *in situ* from the chloride (3) and $nBuClO_4$, to provide a 54-93% yield of the desired mono-tritylated product (4)[13] as stable solids (Table 1). As expected, alkylated products (4b,c,d) were obtained as mixtures of unseparable diastereoisomers. The presence of the N-hydroxysuccinimide ester group in the product was evident from inspection of ^{1}H NMR spectrum which showed a singlet resonance at d 2.87 ppm. Furthermore, treament of active ester (4) with isopropylamine led to the corresponding isopropylamide with the concomitant formation of N-hydroxysuccinimide.[14] The determination of N6 regioselective alkylation was based on the knowledge that

Scheme 1



(i) KO^{t}Bu, THF, r.t. (ii) TrCl(3), see Table 1 (iii) iPrNH$_2$, CH_2Cl_2, r.t.
(iv) NaOMe (3eq), MeOH, reflux (v) NaOMe (6eq), MeOH, reflux

the N2 amino group in purine base was far less nucleophilic than the N6 amino group and that tritylation of the secondary 2'OH was significantly slower than the primary 5'OH of the ribose sugar.[15] However, in order to distinguish between N6 and 5'OH regioisomers, amides (9) and (10) were prepared. Thus, protected nucleoside (5)[16] was debenzoylated with KOtBu to give diol (6) which was then selectively tritylated at the 5'OH with trityl chloride (3a) to provide derivative (7) as the sole product. Subsequent conversion of (7) to the corresponding isopropylamide, (8), followed by deprotection of N2 and N6 pivalolyl groups using NaOMe led to amide (10) (Scheme 1). Comparison of amides (9), obtained by reacting ester (4a) with $iPrNH_2$, and (10) by tlc and ^1H NMR[17] clearly indicated that the nucleosides were different, leading to the conclusion that amide (9) and therefore active ester (4a) resulted from N6 tritylation of purine nucleoside (2). Thus, regioselective N6 mono-tritylation under these alkylating conditions proved to be a particularly useful reaction which circumvents the need for prior protection of nucleoside (2).



(i) 0.1M Borate buffer pH ~8.6, MIR = 8, 7.5% DMF, r.t., 1h

The final step in the synthesis of drug conjugates (1a-f) was accomplished by reacting active ester (4a-f), at a molar input ratio (MIR) of 8, with anti-CEA antibody COL1 (13), in a pH 8.60 buffered solution for 1h at room temperature, followed by isolation of the product using a G-25 Sephadex desalting column.[18] The conjugation ratio (CR), antibody and drug concentrations and the protein yield of the sterile-filtered drug conjugates (1a-f) were determined by UV spectroscopy. The conjugation led to constructs (1a-f) with high percentage of drug (2) incorporation (56-94%) and yielded good protein recovery (Table 2). Furthermore, characterization on a size exclusion Superose 12 column indicated the conjugates (1a-f) consisted of 92-96% of the desired monomeric form, with the remainder being 3-6% low molecular weight (M.W.~$3X10^5$) and 1-2% high M.W.(>$1X10^6$) aggregates.[19] Furthermore, no free drug was detected in the conjugate preparations. Evaluation of the drug immunoconjugates (1a-f) in direct and competitive binding assays showed 80-90% immunoreactivity with the target CEA antigen compared to unconjugated COL1 (13) indicating that the antigen binding region of the antibody was relatively unaffected by the conjugation procedure. Antitumor activity of drug conjugates (1a-f) was assessed in an *in vitro* cytotoxicity assay and compared to free drug (2) and unconjugated antibody COL1 (13) (Table 2).

Table 2: Analytical and biological data for COL1-(N6-Trityl-207702)z Conjugates

	CR [1]	Protein [2] Yield (%)	IC_{50} [3] (ug/ml)
(2) LY207702	-	-	0.260
(1)COL1-N6-Trityl-207702			
(a) m-DMT*	6.44	52	0.352
(b) p -DMT	7.44	65	0.270
(c) p -MMeT	7.49	44	2.71
(d) p -MMT	5.73	61	4.94
(e) p -MeT	5.20	47	6.04
(f) p -T	4.44	50	10
(13) COL1	-	-	>330

(1) CR - Conjugation Ratio (= moles of drug/ mole of antibody for MIR- Molar Input Ratio=8) was determined by U.V. spectroscopy at drug λmax=254nm (*λmax=263nm)

(2) Determined by U.V. spectroscopy at λmax=279nm and where A_{280}=1.40 at 1.0mg/ml of protein

(3) Cytotoxicity assay was performed by incubating LS174T (CEA +ve) Human Colon Carcinoma cells with drug for 48h and measuring ^{3}H-Leucine uptake. IC_{50} is defined as the concentration of drug required to inhibit the incorporation of ^{3}H-Leucine to 50% of control uptake.

The above preliminary results show that the relative acid lability of the linkers,[14] which is dictated *via* the electronic stablisation of the intermediate trityl cation by substituents R and R' on the aromatic rings of the trityl group, correlates well with the potency of the conjugates (i.e. pDMT = mDMT>pMMeT>pMMT>pMeT>>pT). The controllable and predictable releasing features of trityl linkers should, therefore, allow one to couple the selective tumor targeting characteristics of a monoclonal antibody with the cytotoxic activity of an oncolytic in a synergistic manner to provide a more selective antitumor agent with an improved therapeutic index. Extensive *in vivo* studies are underway to identify triyl linked drug conjugates which exhibit both tumor selectivity and antitumor activity, the results of which will be reported in due course.

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