Antibody-Catalyzed Prodrug Activation

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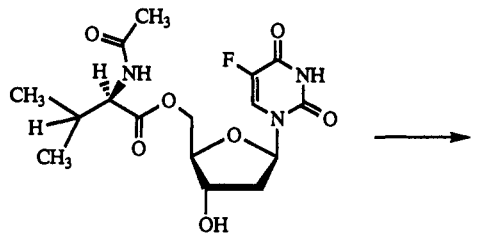
A major problem associated with cancer chemotherapy is the lack of selectivity of most cytotoxic drugs for tumor cells.[1] Consequently, considerable effort has focused on the development of prodrugs that are activated by either tumor-associated or tumor-localized enzymes such as naturally occurring proteases or antibody–protease conjugates.[2] Difficulties associated with this approach include the lack of tumor-specific enzymes, conjugate immunogenicity, and the widespread distribution of relatively nonselective enzymes capable of prematurely activating a prodrug.[2] Many of these problems might be avoided by the development of bispecific antibodies[3] derived from a tumor-specific antibody and a catalytic antibody.[4] The use of a catalytic antibody should both make possible the activation of prodrug via a reaction not catalyzed by endogenous enzymes[5,6] and minimize immunogenicity. To this end, we now report the generation of an antibody capable of catalyzing the conversion of a 5′-D-valyl ester of 5-fluorodeoxyuridine (**1**) to 5-fluorodeoxyuridine **2** (5-FdU) (Scheme 1).

The anticancer drug 5-FdU **2** is converted *in vivo* into 5-fluorodeoxyuridine 5′-monophosphate, which is a mechanism-based inhibitor of thymidylate synthetase.[7] Consequently, the 5′-D-Val ester of 5-FdU (**1**) should be significantly less toxic than 5-FdU since it cannot be converted to the 5′-monophosphate. Moreover, esterases do not, in general, hydrolyze β-branched D-Val esters to any appreciable degree.[8] In order to generate antibodies that catalyze the hydrolysis of **1**, antibodies were raised against hapten **3**, a transition-state analogue for the hydrolysis reaction. Hapten **3** was prepared by reacting dimethyl-[1-amino-2-(*R,S*)-methylpropyl]phosphonate (obtained by hydrogenolysis of the *N*-Cbz derivative)[9] with carbomethoxypropionyl chloride in pyridine followed by treatment with TMSBr in CH_2Cl_2.

Scheme 1



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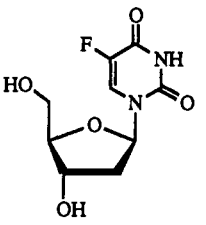

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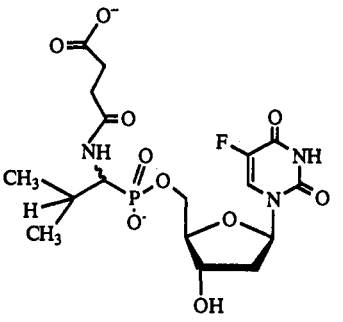

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Mitsunobu condensation[10] of the resulting phosphonic acid with 2′-deoxy-3′-*O*-(*tert*-butyldimethylsilyl)-5-fluorouridine afforded the protected form of **3** in 73% yield. Desilylation followed by saponification and ion-exchange chromatography on a Dowex-50 column afforded the bis-lithium salt **3**.

Hapten **3** was coupled to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) via the carboxylate group.[11] Monoclonal antibodies specific for hapten **3** were generated using standard methods[12] and purified from ascites by protein-A affinity chromatography followed by Mono-Q anion exchange chromatography.[12,13] Antibody 49.AG.659.12 was found to catalyze the hydrolysis of the ester **1** at a significant rate above that of the uncatalyzed reaction and characterized further. All kinetic parameters were measured in the presence of 5 μM antibody in 50 mM Tris, 25 mM NaCl, pH 8.0 at 37 °C by monitoring the hydrolysis of **1** to **2** using reverse-phase (C18) high-performance liquid chromatography (HPLC).[14] Under these conditions, antibody 49.AG.659.12 catalyzed the hydrolysis of **1** in a manner consistent with Michaelis–Menten kinetics. Computer fitting of the steady-state data to the Michaelis–Menten equation afforded a k_{cat} of 0.03 min^{-1} and a K_M of 218 μM. The observed first-order rate constant for the uncatalyzed hydrolysis of substrate **1** under the same condition was 3.1×10^{-5} min^{-1}. The antibody-catalyzed reaction was competitively inhibited by hapten **3** with a K_i value of 0.27 μM.[14]

Antibody 49.AG.659.12 was then tested for its ability to activate the prodrug **1** *in vitro*. The free drug 5-FdU **2** completely inhibited the growth of the *Escherichia coli* HB101 at a

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(11) Hapten **3** was coupled to the carrier proteins BSA and KLH using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) in PBS at pH = 6.5, giving epitope densities of 6 and 8, respectively.

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(14) A stock solution of **1** in EtOH was added to a solution of antibody in reaction buffer to give a final antibody concentration of 5 μM ($\epsilon_{1\ cm}^{0.1\%}$ = 1.37 with a molecular weight of 150 000 for IgG) and substrate concentrations between 0.1 and 5 mM. The inhibition assay was performed similarly with 0–10 μM **3**. The first-order rate constant (k_{uncat}) for the hydrolysis of **1** in the absence of antibody was measured under the same conditions. The products were analyzed by reverse-phase HPLC (Microsorb C18 and a gradient of 5–80% CH_3CH in H_2O over 15 min). The kinetic parameters were obtained by fitting the steady-state data into the Michaelis–Menten equation with the program Kaleidagraph on a Macintosh computer.

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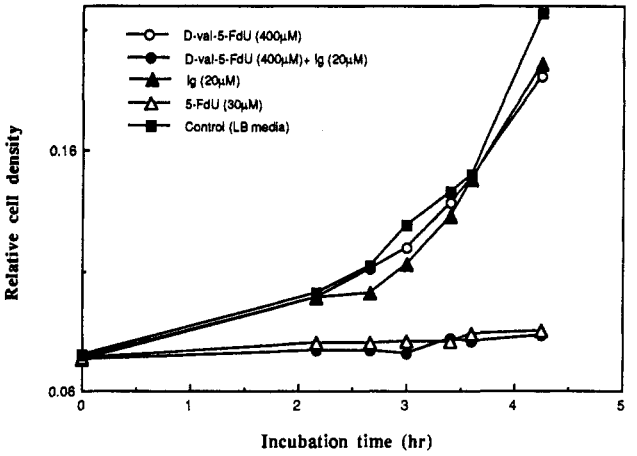

Figure 1. *In vitro* assay of prodrug activation by antibody 49.AG.659.12 using *E. coli* HB101.

concentration of 30 μM, whereas prodrug **1** did not affect the growth of the bacteria at a concentration of 400 μM[15] (Figure 1), consistent with the notion that the D-Val ester is a poor substrate for endogenous esterases. In the presence of the prodrug (400 μM) and antibody (20 μM), the growth of the bacteria was completely inhibited. The antibody alone (20 μM) did not affect the growth of the bacteria. These data demonstrate that antibody 40.AG.659.12 is capable of activating **1** *in vitro*, resulting in specific inhibition of bacterial cell growth. Incubation of prodrug **1** with calf serum does not result in any significant hydrolysis of **1** above the background rate,[16] suggesting that the bacterial studies described here may be applicable to tumor cell lines for which specific antibodies exist.

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Supplementary Material Available: Full experimental details for the syntheses of **1** and **3** (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(15) The assay of growth inhibition was carried out by adding 5 μL of an overnight culture of *E. coli* HB 101 in Luria–Bertani (LB) media to 995 μL of LB media (pH adjusted to 8.0) in the presence or absence of **1**, **2**, and the antibody. The relative cell density was determined at different time intervals by transferring 100 μL of the culture media to an immunoassay plate, which was then read by a plate reader (measurement wavelength, 405 nm; reference wavelength, 620 nm).

(16) Prodrug **1** (5 mM) was incubated in Dulbecco's modified Eagle's (DME) medium in the presence of 0%, 1%, 2%, 5%, and 10% calf serum at 37 °C in a CO_2 incubator. No difference in the rates of hydrolysis was detected within the error of measurement by reverse-phase HPLC.