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A NOVEL TARGETED DELIVERY SYSTEM UTILIZING A CEPHALOSPORIN-ONCOLYTIC PRODRUG ACTIVATED BY AN ANTIBODY β-LACTAMASE CONJUGATE FOR THE TREATMENT OF CANCER

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Abstract: Cephalosporins substituted at C-3' with oncolytic agents such as vinca alkaloids have been developed as prodrugs for the treatment of solid tumors. Conversion of prodrug to drug is mediated by an immunoconjugate consisting of a β-lactamase enzyme covalently attached to a monoclonal antibody Fab' fragment which is pre-localized at the tumor.

The targeted delivery of oncolytic agents to tumor cells using monoclonal antibody-drug conjugates has been an area of intense investigation.[1] Recently, a two-step approach was reported in which isotopes, drugs, or hapten modified drugs are localized to tumor targets by prelocalization of a bifunctional antibody (with specificity for both tumor and small molecule) followed by administration of the small molecule.[2] Subsequently, a two-step approach utilizing a monoclonal antibody-enzyme conjugate and a separately administered prodrug has been described.[3] The antibody-enzyme conjugate localizes on a tumor cell surface antigen and the prodrug, which is a substrate for the enzyme, is converted by the enzyme into the active cytotoxic agent at the tumor cell surface. The primary potential advantage of the latter approach, which we term Antibody Directed Catalysis (ADC), is that one can, in principle, deliver many drug molecules to the tumor site with each immunoconjugate molecule (Fig.1).

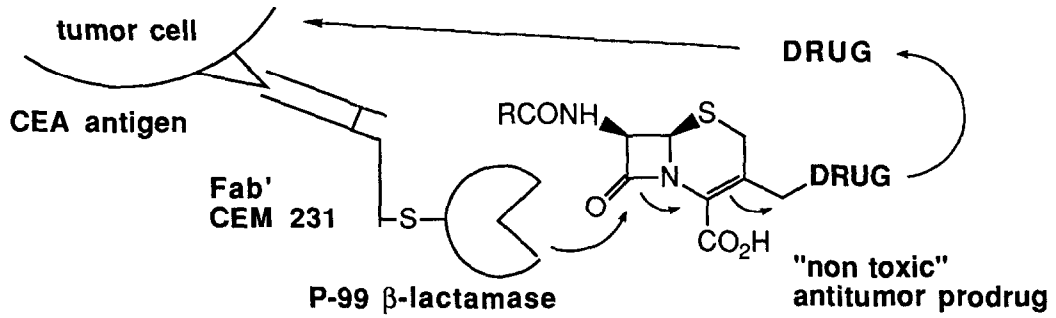


FIGURE 1

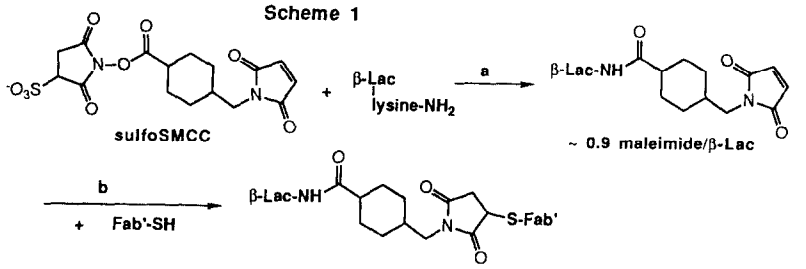
The use of an antibody-enzyme conjugate, in effect, magnifies the localized antibody concentration thereby lowering the amount of conjugate needed to deliver a toxic dose of drug. We describe here a system which consists of the Fab' fragment of monoclonal antibody CEM231[4] covalently bound to the P-99 β-lactamase enzyme isolated from *Enterobacter cloacae* (265A). The prodrug is derived from cytotoxic vinca alkaloid **LY233425** which has been attached to the C-3' position of the cephalosporin nucleus.

The choice of enzyme in this two-step approach obviously determines the make-up of the prodrug. The β-lactamase enzyme was chosen for several attractive properties. The enzyme's narrow specificity for its substrate (β-lactams) is well documented. It possesses good catalytic activity without metal ions or cofactors. Most importantly the enzyme is not endogenous to the mammalian system, and consequently is subject to minimal interference from inhibitors, substrates, or endogenous enzyme systems.

Substituted cephalosporins appeared to be excellent prodrugs because cephalosporins exhibit minimal inherent mammalian toxicity. By proper selection of the oncolytic agent attachment site one should significantly decrease the cytotoxity of the prodrug compared to the free drug. Secondly, the ability to control the release of the cytotoxic agent might be realized with cephalosporins. Pratt[5] and others have shown that when cephalosporins are hydrolyzed by a β-lactamase enzyme the C-3' substituent is expelled in accordance with its leaving group propensity. Proper selection of the linkage of cephalosporin to oncolytic agent should provide a releasable drug as well as a chemically stable prodrug.

Solid established tumors are the primary target of ADC. The antibody employed should be highly specific for the target antigen, while antigen density may be less important because of the catalytic nature of prodrug activation. The antigen system chosen for our studies was carcinoembryonic antigen (CEA). CEA is an oncofetal antigen that has been extensively utilized as a marker for human cancer.[6] Monoclonal antibody CEM231 has been shown to react with CEA.[4] The Fab' fragment of CEM231 was used to prepare the antibody-enzyme conjugate so that the molecular weight of

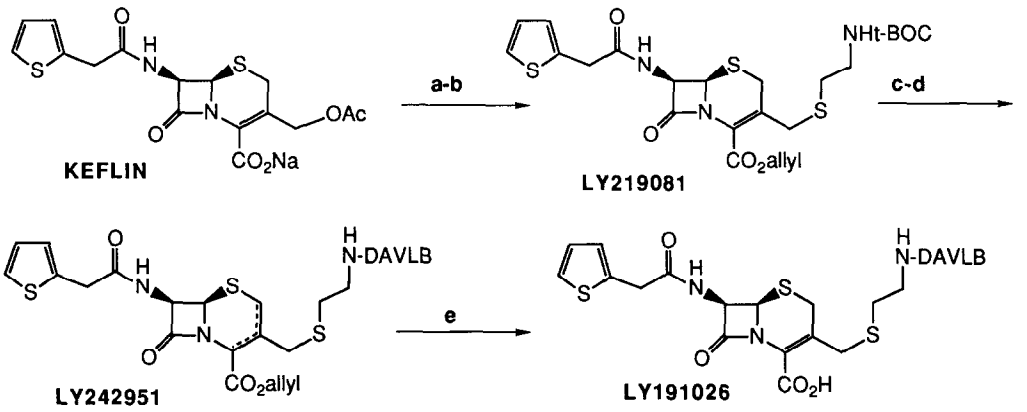
Scheme 1



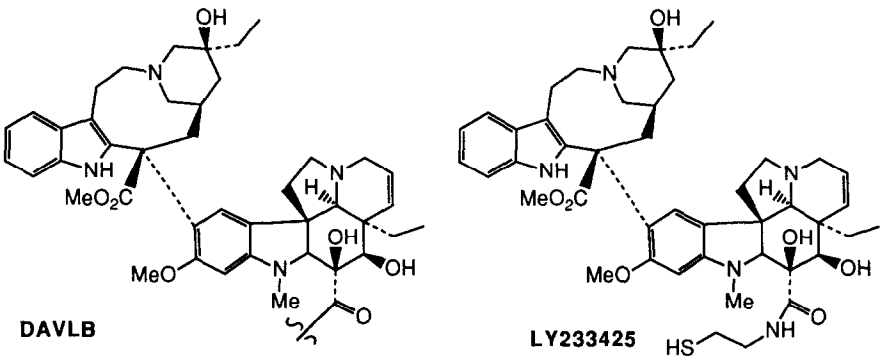
a) β-Lac, 10mg/ml; 2.0eq. sulfoSMCC; 50mM borate; 100mM NaCl, pH 8.4 0.01% NP40; 1hr, 22°C.
b) ~3mg/ml derivitized β-Lac; 1eq. Fab'/maleimide; 50mM citrate, 100mM NaCl, pH 6.4, 2hr, 22°C.

the construct (c.a. 90,000) would allow as rapid clearance as possible from the circulating serum following antigen localization. Scheme 1 outlines the preparation of the antibody-enzyme conjugate. The β-lactamase produced by *Enterobacter cloacae* strain 265A was affinity purified on an aminophenyl boronic acid resin.[7] Derivatization with SulfoSMCC was controlled to produce 0.8-0.9 maleimide/β-lactamase, as determined by cysteine/DTNB back titration. Derivatized β-lactamase was purified by passage over a desalting resin. Treatment with 1 mol Fab'/mol maleimide followed by an excess of N-ethyl maleimide produced a mixture of conjugated and non-conjugated proteins. The β-lactamase-Fab' conjugate was isolated by G-150 Sephadex superfine gel permeation chromatography. Subsequent studies showed that the β-lactamase-Fab' conjugate retained the same level of immunoreactivity and enzymatic activity as the parent proteins.[8]

Scheme 2



a) $HS(CH_2)_2NHt\text{-}BOC$, H_2O, KI; b) allyl bromide, DMF, $NaHCO_3$, $n\text{-}Bu_4NHSO_4$, 29% from Keflin;
c) TFA, CH_2Cl_2; d) DAVLB-N_3, CH_2Cl_2, N-methylmorpholine, 16% from LY219081;
e) Pd(0), Et_3SiH, HOAc, then chromatograph.



The vinca alkaloids are a well studied class of cytotoxic agents.[9] We decided to use the mercapto-substituted derivative of desacetylvinblasine (DAVLB) **LY233425** as the releasable portion of our prodrug. We expected, based upon cephalosporin literature,[10] that a sulfide moiety at the C-3' position of the cephalosporin would provide a reasonable leaving group while retaining chemical stability. Scheme 2 illustrates the synthesis of prodrug **LY191026**. Keflin was condensed with the N-*t*-BOC protected 2-aminoethanethiol under aqueous displacement conditions, then esterified with allyl bromide.[11] After workup the resulting oil was flash chromatographed with 1:1 ethyl acetate:hexanes (R_f=0.5) providing **LY219081** as a white solid.[12] The *t*-BOC protecting group was removed under acidic conditions using trifluoroacetic acid. The resulting amine salt was treated with desacetylvinblastine azide[9] (DAVLB-N_3)(0.7 equiv) in the presence of N-methylmorpholine to give the desired coupling product as a mixture of Δ-2:Δ-3 cephem olefin isomers. Palladium catalyzed removal of the allyl ester[13] gave the desired cephalosporin acid as a 3:2 mixture of Δ-2:Δ-3 olefin isomers. Preparative reverse phase chromatography (C-18 column; 30% acetonitrile, 0.2% formic acid, water) provided pure Δ-3 isomer, the target prodrug **LY191026**. Kinetic studies[8] of prodrug activation by the conjugate gave an encouraging $k_{cat}/K_M = \sim 400\ s^{-1}/\mu M$, with release of drug following the catalytic reaction despite the bulk of the C-3' substituent.

The ADC system was tested *in vitro* : CEA-bearing LS174T human colon carcinoma cells were exposed to the β-L-CEM 231 conjugate at 25 μg/ml for 1 hour at 37°C, 5% CO_2. The cells were then rinsed and resuspended in media containing **LY191026**. The rest of the procedure was carried out according to a standard procedure.[14] The results of this experiment are shown in Table 1.

Table 1
***In Vitro* Cytotoxicity (LS174T)**
Inhibitory Concentration$_{50}$ (μg/ml)

Exp #	Compound	Conjugate	6 Hour	48 Hour
1	LY191026	–	2.15	<0.001
2	LY191026	+	0.26	<0.001
3	LY233425	–	0.26	<0.001

A reproducible fivefold (mol/mol) difference in cytotoxicity between drug and prodrug was observed at early time points (entry 1 vs. 3). Pre-exposure of antigen-positive cells to conjugate reverses this difference, activating the prodrug (entry 1 vs. 2). These data demonstrate the ability of the ADC system to mediate antigen-dependent toxicity *in vitro*. Non-enzymatic cleavage of the β-lactam ring is a known phenomenon that could explain the time-dependent increase in potency of prodrug relative to drug. Furthermore, structure-activity relationships for vinblastine do not

preclude the possibility that **LY191026** has some intrinsic cytotoxic activity. We plan to test the ADC system with a variety of cephem-drug combinations as well as against solid tumors in mouse xenograft models. These studies will be reported in due course.

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References and Notes:

1. For a recent review on this subject : Koppel, G.A. *Bioconjugate Chem.* **1990**, *1*, 13.

2. Stickney, D.R.; Slater, J.B.; Kirk, G.A.; Ahlem, C.; Chang, C-H.; Frincke, J.M. *Antibody Immunoconjugates Radiopharm.* **1989**, *2*, 1. Corvalan, J.R.F.; Smith, W.; Gore, V.A.; Brandon, D.R. *Cancer Immunol. Immunother.* **1987**, *24*, 133.

3. Meyer, D.L.; Jungheim, L.N.; Shepherd, T.A.; Starling, J.J.; Hinson, N.A.; Mikolajczyk, S.D.; Parr Jr., T.R. Fifth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, Abstr#115, San Diego, **1990**. In the course of this work several publications have appeared which also describe a two-step approach to drug targeting: (a) Bagshawe, K.D. *Br.J.Cancer* **1989**, *60*, 275 and references therein. (b) Springer, C.J.; Antoniw, P.; Bagshawe, K.D.; Searle, F.; Bisset, G.M.F.; Jarman, M. *J.Med.Chem.* **1990**, *33*, 677. (c) Senter, P.D. *FASEB* **1990**, *4*, 188. (d) Senter, P.D.; Schreiber, G.J.; Hirschberg, D.L.; Ashe, S.A.; Hellstrom, K.E.; Hellstrom, I. *Cancer Res.* **1989**, *49*, 5789 and references therein. (e) Kuefner, U.; Lohrmann, U.; Montejano, Y.D.; Vitols, K.S.; Huennekens, F.M. *Biochem.* **1989**, *28*, 2288. (f) Senter, P.D.; Saulnier, M.G.; Schreiber, G.J.; Hirschberg, D.L.; Brown, J.P.; Hellstrom, I.; Hellstrom, K.E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4842.

4. Beidler, C.B.; Ludwig, J.R.; Cardenas, J.; Phelps, J.; Papworth, C.G.; Melcher, E.; Sierzega, M.; Myers, L.J.; Unger, B.W.; Fisher, M.; David, G.S. *J.Immunol.* **1988**, *141*, 4053.

5. Faraci, W.S.; Pratt, R.F. *Biochem.* **1986**, *25*, 2934. Faraci, W.S.; Pratt, R.F. *J. Amer . Chem. Soc.* **1986**, *108*, 5328. Mobashery, S.; Johnston, M. *J. Biol. Chem.* **1986**, *261*, 7879 and references therein.

6. Shively, J.E. *Crit.Rev.Oncol.Hematol.* **1985**, *2*, 355.

7. Cartwright, S.J.; Waley, S.G. *Biochem.* **1984**, *221*, 501.

8. Meyer, D.L.; Mikolajczyk, S.D.; Ahlem, C.D.; Parr Jr., T.R.; Jungheim, L.N.; Shepherd, T.A.; Starling, J.J.; Hinson, N.A.; ACS National Medicinal Chemistry Symposium, Abst#32, Austin, Texas, **1990**. Also see Eur. Pat. Appl. #382,411 A2.

9. Conrad, R.A.; Cullinan, G.J.; Gerzon, K.; Poore, G.A. *J.Med.Chem.* **1979**, *22*, 391. Magnus, P.; Stamford, A.; Ladlow, M. *J.Amer.Chem.Soc.* **1990**, *112*, 8210. For the preparation of desacetylvinblastine azide: U.S. Pat #4,203,898, example 5, column 20.

10. Boyd, D.B. "Chemistry and Biology of β-Lactam Antibiotics"; Morin, R.B.; Gorman, M.; Eds.; Academic Press: New York, 1982, Vol. 1, p 437.

11. For esterification of cephalosporins see: Mobashery, S.; Johnston, M. *J.Org.Chem.* **1986**, *51*, 4723.

12. Satisfactory spectral data were obtained for all new compounds. Structure and purity determinations were based upon TLC, HPLC, NMR, IR, UV and FAB exact mass measurements. In the NMR spectra of the prodrugs, due to the complexity of the vinca absorbances, assignment of every proton was not made but an overall maintenance of characteristic peaks was observed. Most importantly, in the NMR region between 5-6 ppm the vinca is generally void of absorbances which allowed for the identification of the β-lactam protons and Δ−2:Δ−3 olefin isomer ratios.
LY218081: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.20 (m,1), 6.92 (m,2), 5.90 (m,1,allyl), 5.75 (dd,1, J=5,9 Hz), 5.30 (m,2,allyl), 4.98 (d,1,J=5), 4.68 (d,2,allyl), 3.80 (s,2), 3.55 (q,2,J=12), 3.4-3.1 (m,4), 2.6 (t,2,J=5), 1.40 (s,9) Anal. Calcd for $C_{24}H_{31}N_3O_6S_3$: C, 52.06; H, 5.64; N, 7.59. Found: C, 52.13; H, 5.77; N, 7.36. IR (KBr): 1771, 1756, 1714, 1680 cm^{-1}. **LY191026**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.30 (s,1), 9.0 (d,1,J=9 Hz), 8.45 (m,1), 8.17 (brs,1), 7.9-7.6 (m,3), 7.31 (m,2), 7.20 (d,1,J=9), 7.0-6.8 (m,4), 6.39 (s,1), 6.14 (s,1), 5.7-5.5 (m,2), 5.40 (dd,1,J=5,9), 4.92 (d,1,J=5), 3.90 (s,1), 3.70 (s,2), 3.67 (s,3), 3.49 (s,3), 2.67 (s,3), 0.74 (t,3,J=7), 0.68 (t,3,J=7) remainder of the protons are several multiplets from 4.0-1.1. FAB exact mass calcd for $C_{59}H_{72}N_7O_{11}S_3$: 1150.4452. Found: 1150.4443. IR (KBr): 1768, 1764, 1648, 1630, 1616 cm^{-1}.

13. Jeffrey, P.D.; McCombie, S.W. *J.Org.Chem.* **1982**, *47*, 587. Dangles, O.; Guibe, F.; Balavoine, G.; Lavielle, S.; Marquet, A. *J.Org.Chem.* **1987**, *52*, 4984.

14. In vitro Cytotoxicity determination: Target cells (antigen positive or antigen negative) were resuspended in 75% leucine deficient EBSS-MEM (GIBCO, Grand Island, N.Y.) + 10% dialyzed fetal bovine serum (FBS) + gentamicin (GIBCO) at 100,000 cells/ml. Aliquots of this cell suspension, 0.2 ml, were seeded into 96 well plates and incubated overnight at 37°C, 5%CO_2. To the supernatants were added media or media containing the antibody-enzyme conjugate to a final concentration of 25 μg/ml. The supernatants were removed after 1 hr incubation, and the cells rinsed once with media. Media containing a substrate-cytotoxic agent at concentrations between 0.001 and 10 μg/ml were then added. The cells were incubated with the substrate-cytotoxic agent compound for a period of time ranging from 3 to 48 hr at 37°C, 5%CO_2. Supernatants were removed, cells washed, and media containing 4 μCi of ^{3}H leucine were added per well. The cells were incubated 18 hr with the labeled leucine, then harvested. Uptake of labeled leucine was determined by liquid scintillation counting. (All samples were run in quadruplicate.) Results are reported as the substrate-cytotoxic agent concentration at which leucine incorporation is reduced to 50% of the control value (ID_{50}).