Design of Antitumor Prodrugs: Substrates for Antibody Targeted Enzymes

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I. Introduction

Chemists have long sought the "magic bullet" for the treatment of cancer, a compound which would selectively kill cancerous cells without affecting healthy cells. Perhaps a more realistic objective is selective delivery of a cytotoxic agent to cancer cells, in other words, to maximize the concentration of the cytotoxic agent at the tumor while minimizing its concentration around healthy tissues.

The targeting of antitumor agents to tumor cells using monoclonal antibody (mAb)–drug conjugates (Figure 1) has received considerable attention in recent years. By utilizing the ability of the mAb to recognize and selectively bind to specific tumor associated antigens, a cytotoxic agent covalently bound to the mAb may exhibit both antitumor activity and decreased toxicity to nontargeted tissues (healthy cells not expressing the antigen). From the chemist's point of view there are two important issues to address with such an approach: stoichiometry, and the ability to control the timing of drug release. There are several facets of the stoichiometry issue which need to be considered. First, there are practical limitations on how many drug molecules one can attach to a mAb before it loses its ability to bind its target antigen. There are also practical limitations on dosing patients with large protein molecules. An added complication is the fact that some target antigens are heterogeneously expressed on solid tumors, both in terms of absolute numbers and cell to cell variation. In order to allow for release of the free drugs from the mAb the cytotoxic agents are often attached to the mAb via a semistable reversible linker such as a Schiff base. Release of the drug is thus controlled by an equilibrium process, therefore, free drug may be released prior to selective mAb–antigen binding on the tumor surface.

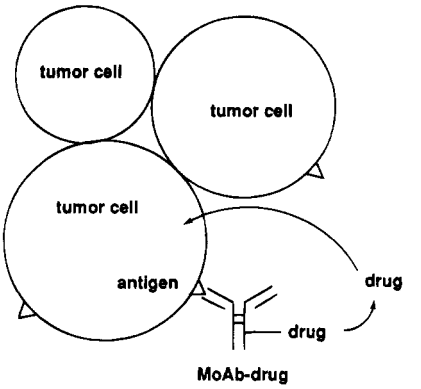

Figure 1. Antibody–drug conjugate.

An alternative to the scenario depicted in Figure 1 is a process involving antigen-mediated internalization of the entire mAb–drug immunoconjugate. This of course requires one to utilize an antibody which efficiently undergoes endocytosis. It remains to be seen whether or not a curative dose of any drug can be delivered to a patient via either type mAb–drug immunoconjugate. Excellent reviews of this area have been written by Koppel[1] and Hinman.[2]

An alternative two-step targeting approach has been reported wherein small molecules such as radiopharmaceuticals, cytotoxic agents, or hapten-modified cytotoxic agents are concentrated at tumor targets. This is achieved by prelocalization of a bifunctional antibody (a mAb with affinity for both a tumor antigen and a small molecule) followed by administration of the small molecule, which binds selectively to the targeted bifunctional antibody.[3-5]

We and others have developed another two-step approach which was based upon the insight gained from work on bifunctional antibodies as well as covalent mAb–cytotoxic agent constructs. This approach employs an enzyme covalently bound to a mAb which localizes on the targeted tumor cell surface. Subsequent administration of a prodrug, which is a substrate of the enzyme, allows for the specific enzyme-catalyzed release of the cytotoxic agent at the tumor site, as depicted in Figure 2. This approach was designed to address both the stoichiometry and controlled drug release issues presented by the covalent mAb–drug conjugates. We refer to this approach as antibody-directed catalysis or ADC, while Bagshawe has coined the phrase antibody-

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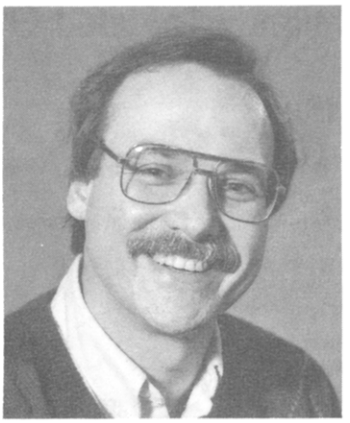
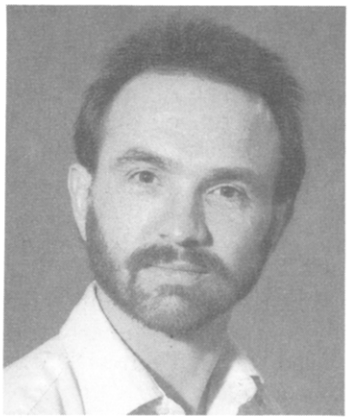


Louis N. Jungheim was born in Wisconsin (1953), grew up in Chicago, IL, and received his B.A. in chemistry from Northwestern University (1975) where he was also a member of the varsity baseball team. He received his Ph.D. from the University of Wisconsin—Madison in 1979 under the direction of Professor Barry M. Trost. This was followed by a postdoctoral appointment at the University of Rochester, where he worked in the laboratory of Professor Andrew S. Kende. In 1981 he joined the Infectious Disease Research group at Eli Lilly and Company (Indianapolis, IN) where he is currently a Senior Research Scientist. His research interests revolve around the design and synthesis of compounds which interact with serine and aspartyl proteases.



Timothy A. Shepherd was born in Cincinnati, OH (1958), and graduated from Miami University of Ohio with a B.A. in chemistry in 1980. He received his M.S. degree from the University of Cincinnati under the direction of Professor R. Marshall Wilson. He spent two years in the CNS Research Department at Bristol-Myers (Evansville, IN), and then in 1986 he joined the Infectious Disease Research group at Eli Lilly and Company (Indianapolis, IN), where he is currently an Associate Senior Organic Chemist. His current research interests involve the synthesis of antiviral agents.

directed enzyme prodrug therapy or ADEPT. We will use the term ADC throughout this review. The ADC approach has several possible advantages over the mAb–drug conjugate, or bifunctional antibody concepts. Due to the catalytic nature of the immunoconjugate one can, in principle, deliver many drug molecules to a tumor cell utilizing a single mAb–enzyme molecule. Thus lower immunoconjugate doses may be used and antigens present in relatively low copy number may be targeted. If properly designed the prodrug should be less cytotoxic than the parent drug, and thus higher doses of the prodrug relative to the parent drug may be tolerated. Furthermore, several different drugs may be delivered by using multiple prodrugs and a single antibody–enzyme construct.

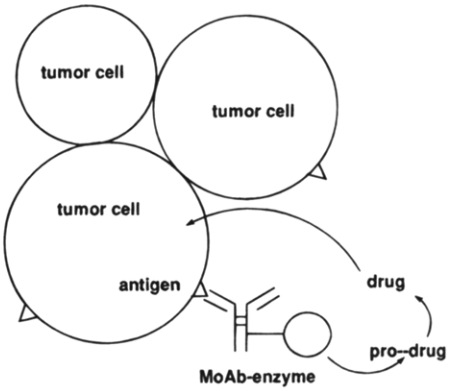


Figure 2. Antibody–directed catalysis.

In defining a construct for ADC, we considered the selection of the enzyme to be critical to the ultimate in vivo efficacy of this approach. Several attributes were considered desirable for the enzyme. First, the enzyme must catalyze a scission reaction of the covalent bond linking the cytotoxic agent to the enzyme's substrate moiety. It follows that high specificity for a single substrate moiety is preferable. Second, there should be no interference from endogenous inhibitors, endogenous substrates, or other enzymes in a mammalian system. In other words one wishes to minimize the possibility of a naturally occurring enzyme prematurely catalyzing release of drug elsewhere in the body. Furthermore, high catalytic activity without the need for cofactors should be beneficial in terms of the stoichiometry issue. Obviously the enzyme should be readily available, easily purified, and chemically stable.

When designing appropriate prodrugs for use in ADC one realizes that the choice of enzyme necessarily dictates the chemical makeup of the prodrug: the enzyme's substrate moiety attached covalently to a cytotoxic agent. Depending on the type of scission reaction catalyzed by the enzyme, additional "linking atoms" may be present between the enzyme's substrate moiety and the actual drug. As we shall see certain substrate moieties, such as the cephalosporin nucleus, are compatible with the use of many different cytotoxic agents. Judicious selection of the attachment site to the cytotoxic agent is an important design consideration. The chemist should ideally select a functional group on the free drug known to be critical for cytotoxicity as the place to attach the enzyme's substrate moiety. By so doing the prodrug obtained should be significantly less cytotoxic than the corresponding free drug. The covalent bond connecting the substrate moiety to the cytotoxic agent should be stable under physiological conditions to minimize nonspecific release of the free drug. Careful design should provide a chemically stable, relatively nontoxic prodrug which is a substrate for the targeted mAb–enzyme immunoconjugate, and which upon contact with the immunoconjugate rapidly releases the free cytotoxic agent.

One should keep in mind that the ultimate success of ADC is dependent upon the ability of the antibody portion of the immunoconjugate to selectively target solid tumors, display appropriate pharmacokinetics (unbound conjugate must be cleared from the bloodstream), and display low immunogenicity. Each

system described herein employs a unique antibody or antibody fragment which selectively binds to a specific tumor associated antigen. A recent review by Senter on the ADC concept focused primarily on the immunoconjugates, and critical factors for the ultimate success of ADC in humans such as immunogenicity, conjugate heterogeneity, and pharmacokinetics.[6]

With a properly functioning immunoconjugate in hand the chemist can design and synthesize appropriate prodrugs which are tested for cytotoxicity against human tumor cells in vitro, both in the presence and absence of a mAb–enzyme conjugate. Successfully designed prodrugs are significantly less cytotoxic than the corresponding free drugs in the absence of conjugate. A positive in vitro result is obtained when the combination, conjugate plus prodrug, is more cytotoxic than prodrug alone and ideally equipotent to the free drug. In a typical in vivo experiment a human tumor xenograft is established in nude mice, the animals are treated with mAb–enzyme conjugate, time is allowed for localization and clearance of unbound conjugate, and finally the prodrug is administered. Antitumor activity is monitored either by survival time or by actually measuring solid tumor mass. Appropriate control experiments can demonstrate that the free drug was released in an antigen-specific fashion by the conjugate bound to the tumor cell surface.

This review will concentrate on the chemistry of the prodrugs which have been developed for use in ADC approaches to cancer chemotherapy. We have chosen to organize our discussion via enzyme/cleavage reaction type rather than by different prodrugs of individual cytotoxic agents.

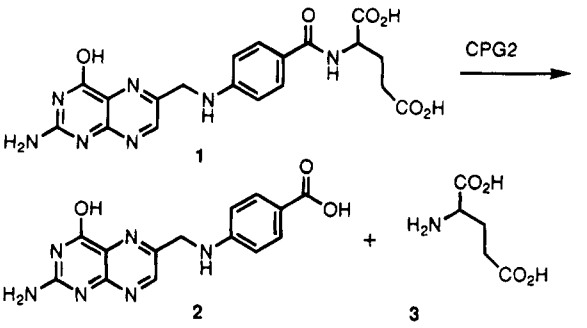
II. Peptidases

Several groups have developed ADC systems which employ peptidases, enzymes which cleave amide bonds. A wide variety of antitumor agents have been modified into prodrugs and impressive biological results have been observed.

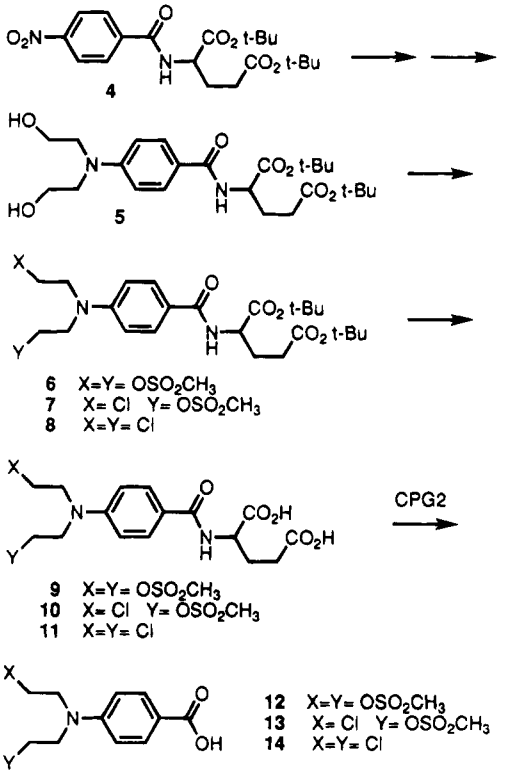
A. Carboxypeptidase G2

The first published report of an ADC system came from Bagshawe and co-workers at the Charing Cross Hospital in London.[7,8] They prepared an immunoconjugate which employed carboxypeptidase G2 (CPG2) as the catalyst. There is no known mammalian counterpart to CPG2, a metalloenzyme derived from *Pseudomonas* sp. whose normal function is to catalyze the conversion of reduced and nonreduced folates into pteroates and L-glutamic acid, e.g. **1** → **2** + **3**. In fact, Bagshawe originally intended to use the immunoconjugate alone in order to kill cancer cells via folate deprivation.[9]

Bagshawe developed three prodrugs **9**, **10**, and **11** of nitrogen mustard alkylating agents for use in ADC.[10] Each drug employed is a bifunctional alkylating agent in which the activating effect of the ionizable carboxyl group is masked by formation of an amide linkage to the enzyme's substrate moiety, glutamic acid (**3**). In the presence of their mAb–CPG2 conjugate the amide bond is cleaved, giving



rise to the free drugs **12**, **13**, and **14** and glutamic acid (**3**). The rate of amide bond cleavage is comparable to that reported for methotrexate, a folate analogue.[11] The prodrugs were synthesized from di-*tert*-butyl (4-nitrobenzoyl)-L-glutamate (**4**), which was reduced to the corresponding amine and condensed with ethylene oxide to give diol intermediate **5**. The diol was reacted with methanesulfonyl chloride/pyridine and depending upon the temperature employed, provided the bis-mesylate **6** (2 °C), monomesyl mono-chloride **7** (50 °C), or the bis-chloride **8** (80 °C). Hydrolysis of the *tert*-butyl ester protecting groups with TFA completed the synthesis of prodrugs **9**–**11**.



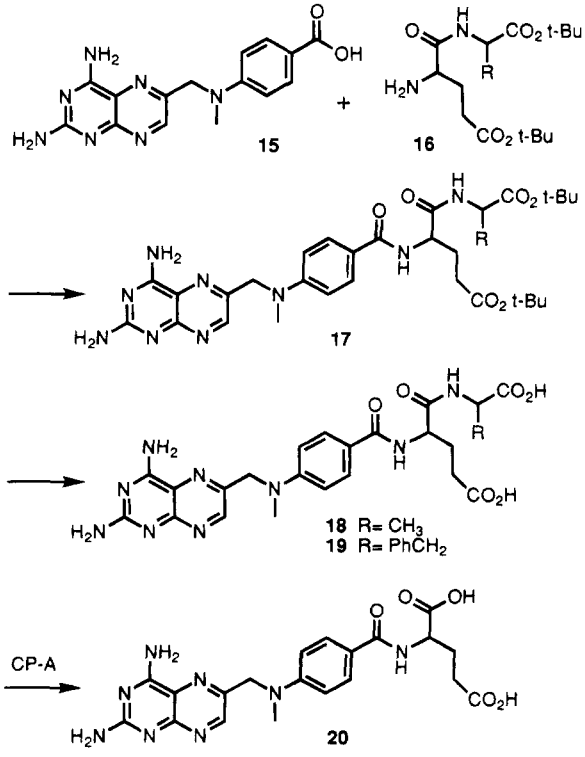
All three prodrugs were found to be relatively nontoxic when compared to their free drug counterparts and are chemically more stable as well.[10,12] The antitumor activity of this carboxypeptidase/nitrogen mustard ADC system has been demonstrated both in vitro[12] as well as in mouse xenograft models where regressions of LS174T human colon tumors have been observed.[11,13] The best antitumor activity was achieved with the mono-mesyl mono-chloride prodrug **10**, perhaps because there is a 17-fold difference (**13** > **10**) in alkylating ability between the free and pro-

drug, which may translate into enhanced tissue selectivity.[11,12] Prodrugs **9** and **11** are only 2- and 3.6-fold less reactive, as alkylating agents, than free drugs **12** and **14**. The combination of a mAb–CPG2 immunoconjugate and prodrug **10** is the only ADC system to date which has been advanced into human clinical trials.[14,15] The clinical trial also incorporates an additional step of using galactosylated SB 43 antibody, which rapidly clears nonlocalized mAb–CPG2 from the serum, thus minimizing non antigen-mediated drug release.

B. Carboxypeptidase A

Huennekens and co-workers at the Scripps Institute have reported on the synthesis and evaluation of methotrexate α-peptides **18** and **19** which are prodrugs of methotrexate (MTX) **(20)**.[16,17] The enzyme required to convert an MTX α-peptide into MTX is bovine pancreas carboxypeptidase A (CP-A). Unlike the CPG2 enzyme utilized by Bagshawe, CP-A cannot further hydrolyze MTX to the parent pteroic acid **15**. MTX is a drug commonly used in cancer chemotherapy, and α-peptide analogues such as **18** and **19** are known to be less cytotoxic than the parent MTX, presumably due to their reduced ability to penetrate cells.

The prodrugs were synthesized by coupling either L-Glu-α-L-Ala di-*tert*-butyl ester (**16**, R = CH_3) or L-Glu-α-L-Phe di-*tert*-butyl ester (**16**, R = CH_2Ph) with 4-amino-4-deoxy-10-methylpteroic acid **(15)** followed by acid-catalyzed removal of the *tert*-butyl esters to give **18** and **19** as the pure L-L diastereomers.[18] An earlier synthesis which involved coupling a protected form of MTX with alanine or phenylalanine led to racemization at the glutamate chiral center.[17] These D–L diastereomers were not converted into D-MTX by CP-A.



The L-L-MTX-Ala prodrug **18**, when incubated with a mAb–CP-A conjugate, was slowly converted into MTX **(20)**. Unfortunately the specific activity of the enzyme for hydrolysis of **18** was only about 1% of that observed for the hydrolysis of hippuryl L-phenylalanine, a natural substrate for CP-A. Prodrug **18** was found to be about 170 times less cytotoxic than MTX when tested in vitro against UCLA-P3 human lung adenocarcinoma cells.[19] The combination of a mAb–CP-A conjugate and prodrug **18** produced a marked reduction in cell growth, however, the ADC treatment was still 28 times less cytotoxic than free MTX, presumably because the prodrug is a relatively poor enzyme substrate.

These results led to the design of the L-L-MTX-Phe prodrug **19**.[16] Reaction rates for the conversion of **19** to **20** have not been reported. The in vitro cytotoxicity, against UCLA-P3 human lung adenocarcinoma cells, of the combination CP-A conjugate/prodrug **19** was essentially equivalent to the parent drug MTX. No in vivo experiments with this combination have been reported, however, the in vitro results demonstrate the potential of a MTX-based prodrug in ADC.

C. Penicillin V/G Amidase

Kerr and co-workers at Bristol-Myers Squibb have designed prodrugs to be used in conjunction with penicillin V amidase (PVA).[20] PVA, which is obtained from *Fusarium oxysporum*, is commercially employed in the synthesis of 6-aminopenicillanic acid from penicillin V. They selected the anticancer agents doxorubicin **(22)** and melphalan **(24)** for study because they contain free amino groups which are readily acylated and the *N*-acyl analogues are often less cytotoxic than the parent amines. The prodrugs **21** and **23** were prepared by coupling doxorubicin and melphalan with *p*-hydroxyphenoxy acetic acid, which is the enzyme's substrate moiety.

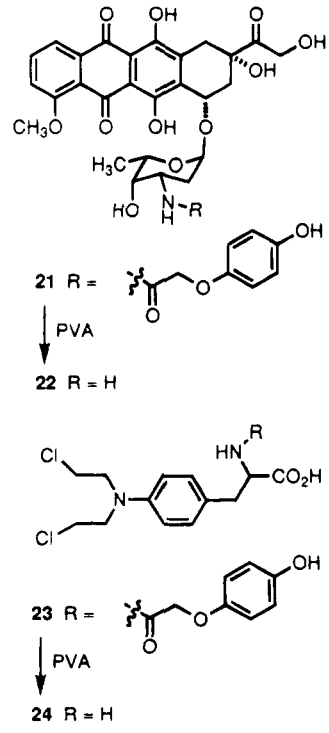
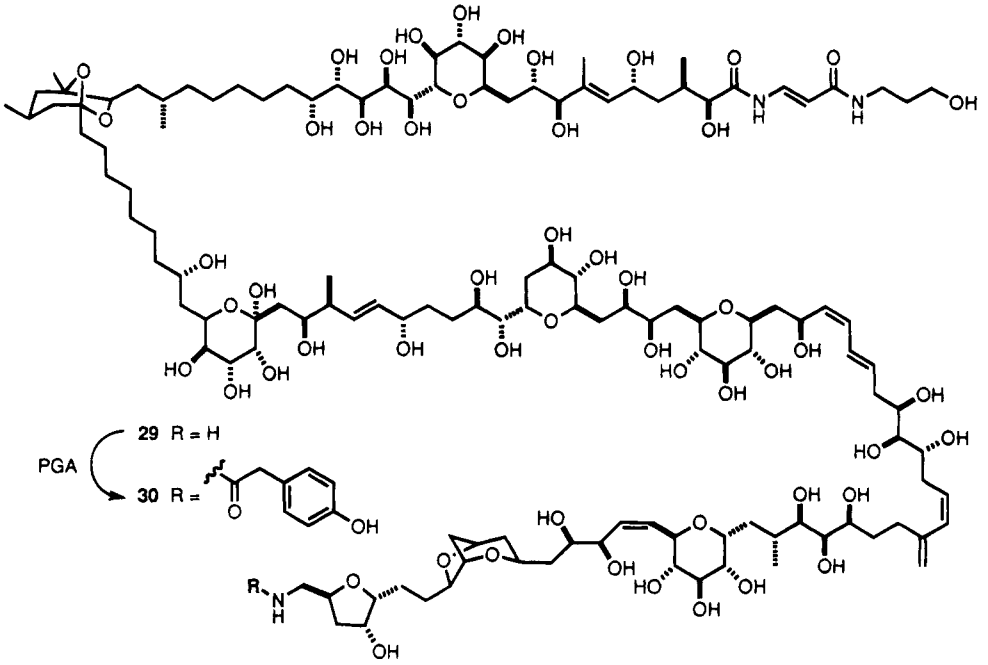


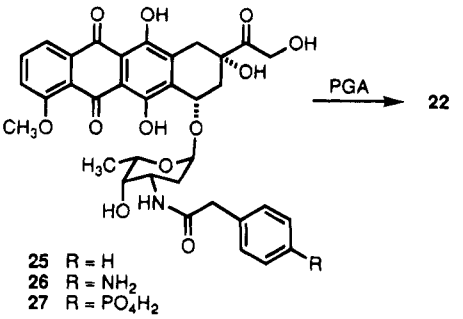
Chart 1



PVA catalyzed conversion of the prodrugs into the corresponding free drugs; however, **21** was converted to doxorubicin 25 times faster than **23** was hydrolyzed to melphalan. The in vitro results obtained with both prodrugs were rather disappointing. Prodrug **21** was 80 times less cytotoxic than doxorubicin against H2981 lung adenocarcinoma cells, and when a mAb–PVA conjugate was prelocalized on the cells the difference was only reduced to 12.5-fold, perhaps due to incomplete conversion to free drug. Prodrug **23** was also much less toxic than its parent, melphalan. Unfortunately, the presence of a mAb–PVA conjugate did not enhance the toxicity of **23**. Apparently PVA converts **23** to **24** much too slowly to achieve a cytotoxic concentration of free drug.

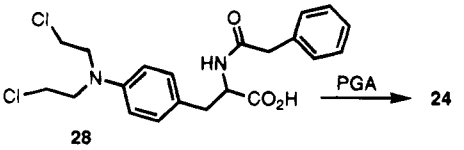
Vrudhula and co-workers at Bristol-Myers Squibb have worked with a related enzyme, penicillin G amidase (PGA),[21] which is obtained from *Escherichia coli*. The enzyme cleaves the phenylacetamide on penicillin G, thus phenylacetamides of doxorubicin and melphalan were employed as prodrugs. (*N*-Phenylacetamido)doxorubicin **25** was not soluble in aqueous solutions. This prompted the synthesis of the more water-soluble amino-substituted analogue **26**, which was shown to be a substrate for PGA. Phosphate analogue **27** did not release free doxorubicin upon treatment with PGA. Incubation of **27** with PGA and an alkaline phosphatase (AP) resulted in the apparent removal of the phosphate moiety followed by amide bond hydrolysis giving rise to free doxorubicin.

All three prodrugs **25**–**27** are significantly less cytotoxic against H2981 lung adenocarcinoma cells than doxorubicin especially the water-soluble analogues **26** and **27**. Prodrug **26** was more than 1000-times less cytotoxic than doxorubicin against H2981 lung adenocarcinoma cells, and when a mAb–PGA conjugate was prelocalized on the cells the difference was only reduced to 71-fold, perhaps due to incomplete conversion of **26** to free drug by the PGA. Prodrug **27** was also more than 1000-times less



cytotoxic than doxorubicin against H2981 lung adenocarcinoma cells. The combination of a mAb–PGA conjugate, AP which cleaves the phosphate moiety, and prodrug **27** resulted in cytotoxic activity equivalent to doxorubicin, thus establishing the utility of this system in vitro.

The phenylacetamido analogue of melphalan **28**, was found to be a much better substrate for PGA than any of the doxorubicin prodrugs **25**–**27**.[21] Prodrug **28** was about 20 times less cytotoxic than melphalan against H2981 lung adenocarcinoma cells. The combination of a mAb–PGA conjugate and prodrug **28** resulted in cytotoxic activity equivalent to melphalan, indicating the conjugate efficiently converted **28** into the parent drug. Unfortunately, no in vivo experiments have been reported with these promising ADC combinations of PGA plus prodrugs **27** or **28**.



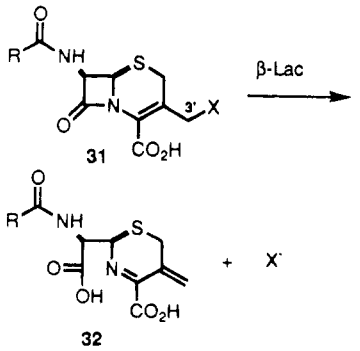
Bignami[22] and co-workers have prepared a prodrug of palytoxin (**29**, Chart 1), one of the most potent cytotoxins known. They synthesized *N*-[(4′-hydroxyphenyl)acetyl]palytoxin (**30**) and found it to

be approximately 1000-fold less cytotoxic than the parent drug **29** against a variety of tumor cell lines. When tested against H2981 lung adenocarcinoma cells, the combination of a mAb–PGA conjugate and prodrug **30** resulted in cytotoxic activity about 100 times greater than the prodrug **30** alone. This result may be due to slow or incomplete conversion of prodrug into drug at the cell surface. No in vivo data were reported. This system is different from the other ADC approaches in that palytoxin exerts its effect extracellularly, and thus it may be able to overcome the multidrug resistance phenotype.

D. β-Lactamases

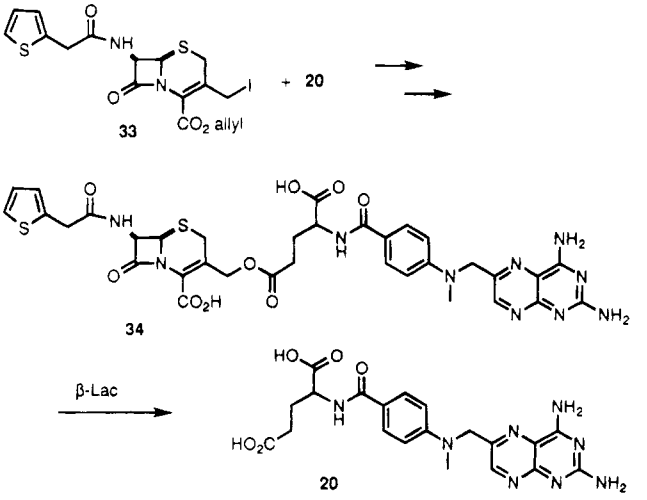
Exciting results for ADC have been obtained in vivo using mAb–β-lactamase conjugates and cephalosporins as the substrate moiety. β-Lactamase (β-Lac) enzymes are readily available in purified form, they are highly selective for β-lactam-containing substrates, and there are no mammalian enzymes whose function is to inactivate β-lactam antibiotics. As we shall see, utilization of a cephalosporin as the substrate moiety allows for the covalent attachment of a wide variety of potent antitumor agents to the 3-cephem nucleus, and β-Lac efficiently catalyzes release of these agents from the prodrugs. These highly desirable properties have prompted several groups to design cephalosporin-based prodrugs for use in ADC.

We at Lilly/Hybritech were the first to report on the development of an ADC system utilizing β-lactamase (β-Lac).[23–25] β-Lactamases are bacteria's natural defense mechanism against β-lactam antibiotics. Our work has been focused on using the P99 enzyme derived from *Enterobacter cloacae* 265A. Cephalosporins were chosen as prodrug candidates because the controlled release of a cytotoxic agent covalently attached to the C-3′ position might be realized. It is well known that when cephalosporins are hydrolyzed by a β-Lac enzyme, e.g. **31** → **32**, the C-3′ substituent is expelled in accordance with its leaving group propensity. This is a very important

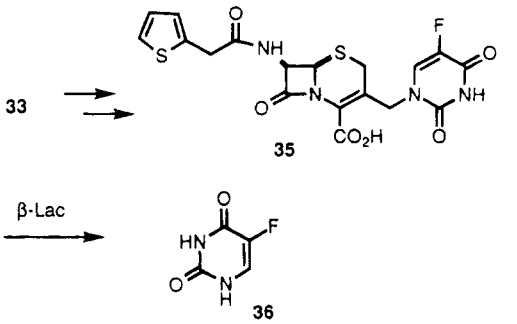


advantage for β-Lac-based ADC, as we have been able to covalently attach a wide variety of anticancer agents to the C-3′ position of the cephalosporin nucleus. Proper selection of the linkage, cephem to cytotoxic agent, provides a stable prodrug which possesses the ability to release the drug upon contact with the β-Lac. Judicious selection of the functional group used to attach the cytotoxic agent to the cephem should also provide a prodrug with significantly reduced cytotoxicity relative to the free drug. In addition, cephalosporins are known to exhibit minimal inherent mammalian toxicity.

Our initial attempts at developing a prodrug for ADC were based on the fact that the acetate ion is a good leaving group from cephems, e.g., X = OAc in the above equation. A potent cytotoxic agent which bears a free carboxylic acid moiety is methotrexate (MTX, **20**). We esterified the γ-carboxylic acid moiety of MTX with C-3′ iodocephem **33** and hydrolyzed the allyl ester to give the desired MTX-based prodrug **34**.[26] When a solution of **34** was treated with β-Lac free MTX was rapidly released, indicating prodrug **34** was a good substrate for the enzyme. Unfortunately the cytotoxicity of **34** could not be distinguished from that of free MTX, perhaps due to nonspecific hydrolysis of the ester moiety in tissue culture media.

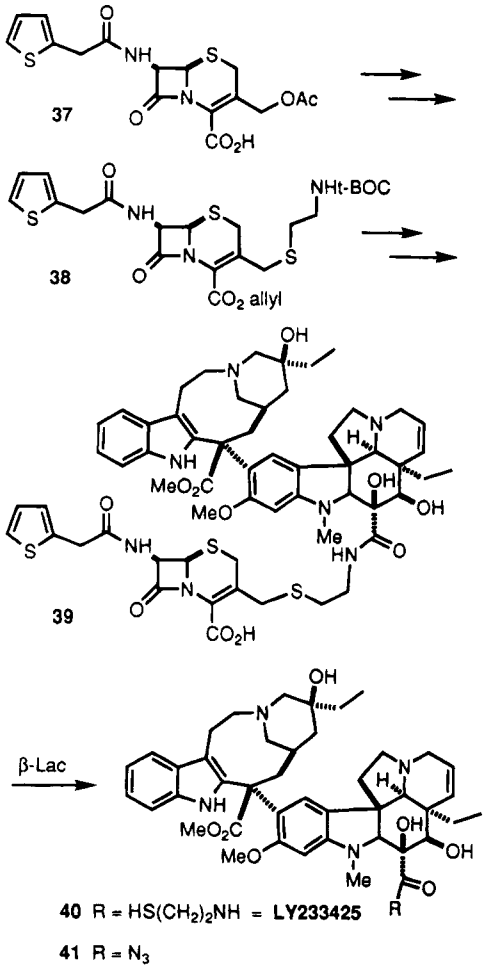


Another avenue we explored was a C-3′ amide-type prodrug. The antitumor agent we employed was 5-fluorouracil (**36**), which is often used in the treatment of colon cancer. Again the C-3′ iodocephem **33** was alkylated with 5-fluorouracil followed by ester hydrolysis to give the desired prodrug **35** along with some of the undesired Δ-2 cephem olefin isomer.[26] When a solution of **35** was treated with β-Lac free 5-fluorouracil was rapidly released, indicating prodrug **35** was a good substrate for the enzyme. However, the cytotoxicity of prodrug **35** was not significantly different from that of 5-flourouracil in vitro, thus making it difficult to assess the utility of prodrug **35** in ADC. The Δ-2 cephem olefin isomer was not a substrate for β-Lac.



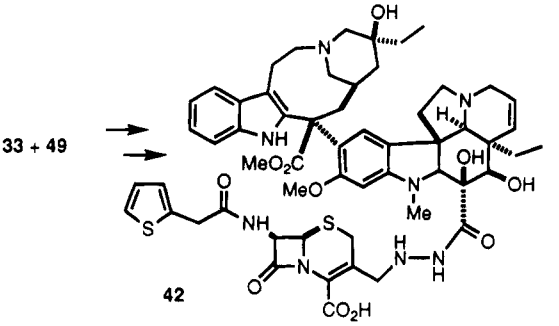
The vinca alkaloid-based prodrug **39** provided a clear demonstration of the ADC concept.[25] The drug

we used was LY233425 (**40**), a potent analogue of the widely used anticancer agent vinblastine. LY233425 contains a mercaptan, which, when attached to the cephem C-3′ position provided a prodrug with an alkyl thiol as a leaving group. Thus, cephalothin (**37**) was condensed with N-*t*-BOC protected 2-aminoethanethiol followed by esterification to give the cephem C-3′ sulfide **38**. Removal of the *t*-BOC group followed by reaction of the free amine with acyl azide **41** and allyl ester hydrolysis provided the desired prodrug **39**.



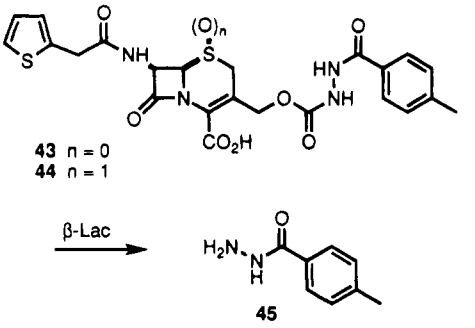
Kinetic studies of prodrug activation by β-Lac gave an encouraging k_{cat}/K_m of approximately 400 $s^{-1}/\mu M$ (k_{cat}/K_m for cephalothin **37** = 10 $s^{-1}/\mu M$). We were quite encouraged that free drug **40** was released from **39** despite the bulk of the C-3′ substituent and the fact that the leaving group was an alkyl rather than an aryl thiol. A reproducible 5-fold (molar basis) differential in cytotoxicity was observed between drug **40** and prodrug **39** when tested against LS174T human colorectal cancer cells. When incubated in the presence of a mAb–β-Lac conjugate the prodrug was equipotent with free drug **40**, a successful demonstration of the viability of our ADC system.

Another very potent vinca alkaloid that we investigated was desacetylvinblastine hydrazide (DAVLBHYD, **49**). We initially selected the directly attached C-3′ acylhydrazidocephem **42** as our target prodrug.[27] Once again the cephem C-3′ iodide **33** served as the starting material. The iodide was displaced with DAVLBHYD (**49**) to give the requisite N-alkylated product. Palladium(0)-catalyzed removal of the allyl ester completed the synthesis of prodrug candidate **42**.



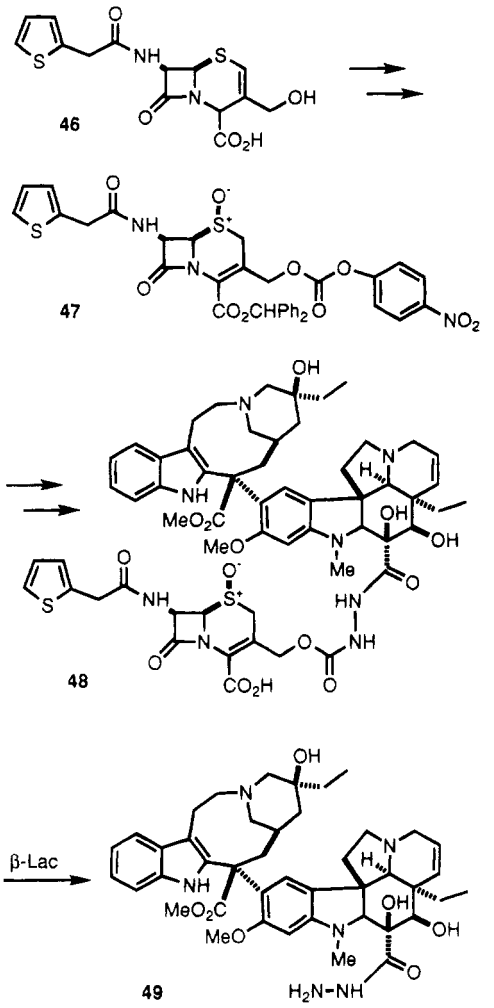
The β-lactam moiety in prodrug **42** was rapidly hydrolyzed by β-Lac; however, release of the free DAVLBHYD (**49**) was disappointingly slow. This finding motivated us to design a better leaving group which would ultimately allow for the attachment and more efficient release of free DAVLBHYD from the cephem nucleus.

A carbamoyloxy group on the C-3′ position of a cephalosporin is known to be a good leaving group. We proposed that an azacarbamate derived from DAVLBHYD's hydrazide moiety, e.g. **48**, might also be a good leaving group upon lactam hydrolysis, and that rapid decarboxylation would follow, under physiological conditions, to release free DAVLBHYD. Considering the cost and limited supply of DAVLBHYD we chose to first focus on the synthesis of a model compound **43**, which substituted tolyl hydrazide in place of DAVLBHYD.



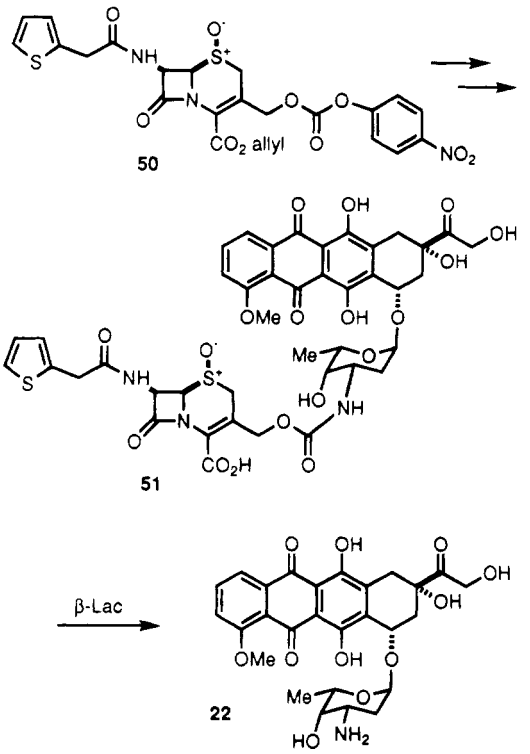
The cephem sulfoxide analogue **44** was also obtained, as an artifact of the synthetic approach developed for the preparation of these novel acylhydrazido-substituted cephems. Incubation of both model compounds **43** and **44** with the mAb–β-Lac immunoconjugate provided interesting results. Both compounds proved to be excellent substrates for β-Lac and rapidly released a molar equivalent of tolyl hydrazide upon cleavage of the β-lactam bond. Surprisingly, the sulfoxide **44** was a better substrate than the parent cephalosporin **43**.[27,28] Qualitatively the sulfoxide exhibited superior solution stability as well. Furthermore, the presence of the sulfoxide moiety in the synthetic intermediates precludes double-bond migration to the undesired Δ-2 cephem olefin isomer, which caused considerable problems in the synthesis of prodrugs **35** and **39**. These findings prompted us to select cephalosporin sulfoxide analogue **48** (LY266070) as our target prodrug.

The key step in the synthesis of cephem–DAVLBHYD prodrug **48** was the proposed N-acylation of **49** by the *p*-nitrophenyl carbonate moiety in intermediate **47**. Carbonate **47** was prepared in several steps from the readily available 3′-hydroxycephem **46**. DAVLBHYD (**49**) readily displaced the *p*-nitrophenyl carbonate moiety in **47**, and acid-catalyzed hydrolysis of the benzhydryl ester completed the synthesis of prodrug **48**.[27]



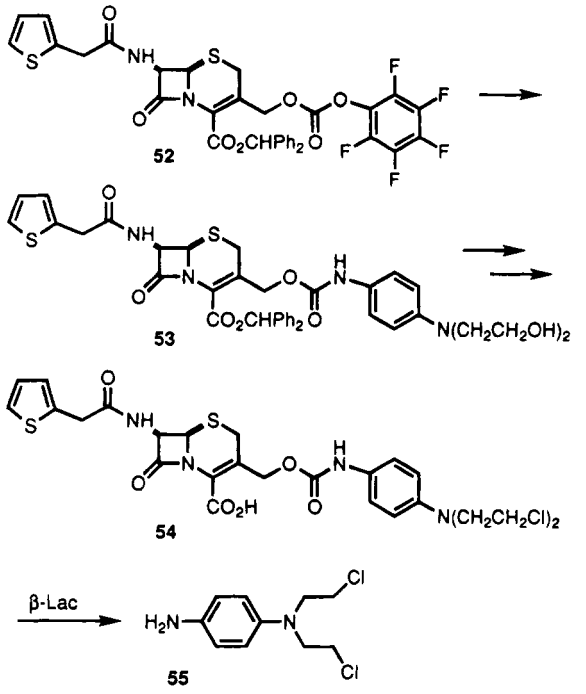
Prodrug **48** was found to be an excellent substrate for the mAb–β-Lac immunoconjugate (k_{cat}, 1700 s^{-1}; K_M, 160 μM) and concomitant release of free DAVLBHYD was observed by HPLC.[28] Differential in vitro cytotoxicity of prodrug **48** versus free drug **49** was measured using the LS174T human colorectal tumor cell line. A reproducible 5-fold (molar basis) differential in cytotoxicity was observed between DAVLBHYD and the less toxic prodrug **48** at short incubation times. Preexposure of antigen positive tumor cells to the antibody-enzyme immunoconjugate prior to administration of the prodrug reversed this differential, indicating the immunoconjugate catalyzes release of free DAVLBHYD in vitro. Mouse xenograft studies employing the antibody–enzyme immunoconjugate with prodrug **48** caused regression of established tumors, and this combination was significantly more active than either the prodrug **48** or free drug **49** administered alone.[29] These data clearly demonstrated the ability of our ADC system to mediate antigen-dependent cytotoxicity both in vitro and in vivo.

Another widely used anticancer agent is doxorubicin (**22**). We were attracted by the amino sugar moiety of doxorubicin as we had chemistry in hand to prepare a carboxamide type prodrug such as **51**. Furthermore, the amino group is required for potency as amide analogues are usually significantly less cytotoxic. In practice, the *p*-nitrophenoxy moiety in intermediate **50** was readily displaced by the amino group of doxorubicin. Removal of the allyl ester provided the desired prodrug **51** in good yield.[30]



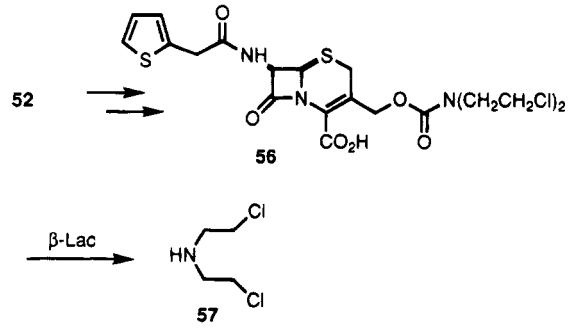
Prodrug **51** is a good substrate for β-Lac, and free doxorubicin (**22**) is rapidly released in the presence of the enzyme. As expected, this N-acylated analogue was less cytotoxic than free drug against LS174T human colorectal cancer cells. The combination of a mAb–β-Lac conjugate and prodrug **51** resulted in cytotoxic activity equivalent to doxorubicin in vitro, thus establishing **51** as a viable candidate for use in ADC. In vivo experiments demonstrated that this ADC system utilizing prodrug **51** was able to achieve antigen-mediated tumor suppression against LS174T colon and OVCAR-3 ovarian human tumor xenografts.[31]

Alexander and co-workers at Celltech[32] have also described a cephalosporin-based ADC system. They have prepared a series of cephem C-3′ carbamates, e.g. **54** and **56**, which release nitrogen mustard DNA alkylating agents **55** and **57** in the presence of β-Lac. Key to the synthesis of prodrug **54** was the displacement of the pentafluorophenyl carbonate moiety in **52**, giving rise to diethanolamine analogue **53**. The authors commented that if they employed a *p*-nitrophenyl carbonate, significant amounts of the undesired Δ-2 cephem olefin isomer were obtained. Diol **53** was converted to the corresponding bis-chloro analogue and ester hydrolysis completed the synthe-



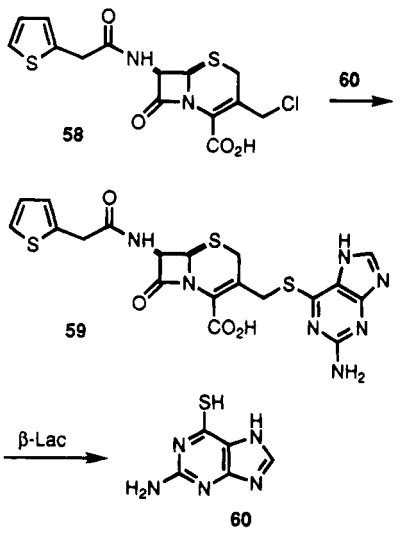
sis of prodrug **54**. Cephem **54** was found to be a good substrate for the P99 β-Lac (k_{cat} = 181 s^{-1}, K_m = 142 μM) and free drug **55** was rapidly released.

A simplified analogue, **56**, was prepared via an analogous route. Prodrug **56** was also found to be a good substrate for the P99 β-Lac (k_{cat} = 510 s^{-1}, K_m = 108 μM) and free drug **57** was rapidly released. Unfortunately, no in vitro or in vivo data for these prodrugs have been reported although it has been suggested that the nitrogen mustard prodrugs are less cytotoxic than their free drug counterparts.[33]

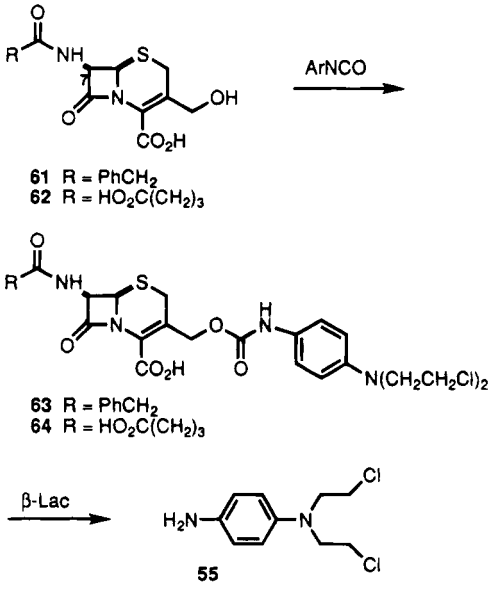


The Celltech group has also reported a thioguanine–cephalosporin prodrug **59**.[33] It was prepared by the S-alkylation of thioguanine (**60**) with chlorocephem **58**. Prodrug **59** was reported to be a substrate for β-lac and release of thioguanine was observed; however, kinetic parameters were not reported. Prodrug **59** was found to be 13 times less cytotoxic than thioguanine. Neither in vitro nor in vivo evaluation of prodrug **59** was reported.

Researchers at Bristol-Myers Squibb have also reported efforts to develop a cephalosporin-based ADC system. They prepared the nitrogen mustard-containing prodrug **63** which is identical to Celltech's prodrug **54** except for the C-7 acylamino side chain. The isocyanate of nitrogen mustard **55** was condensed with the C-3′ hydroxy cephem **61** to provide the desired prodrug **63**.[34] Prodrug **63** was hydrolyzed



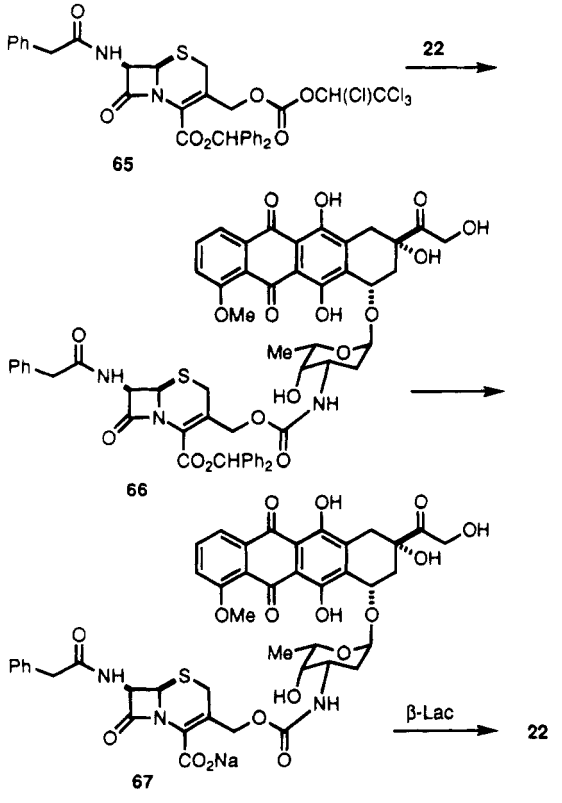
by the β-lactamase isolated from *Bacillus cereus*, a zinc metalloenzyme, and the half-life for release of free drug **55** was determined to be 10 min (enzyme 0.3 μg/mL, substrate 50 μM).[34] This enzyme had a K_m of 6.2 μM and a k_{cat} of 63 s^{-1}.[35] When tested against the H2981 human lung adenocarcinoma cell line prodrug **63** was significantly less cytotoxic (IC_{50} > 30 μM) than free drug **55** (IC_{50} = 1.5 μM). The cytotoxic activity of **63** on H2981 cells that were pretreated with an appropriate mAb–β-Lac conjugate was equal to that of free drug **55,** thus establishing the utility of prodrug **63** in ADC. In vivo evaluation of **63** has been hampered by severe tail necrosis following iv injection of this compound.[35]



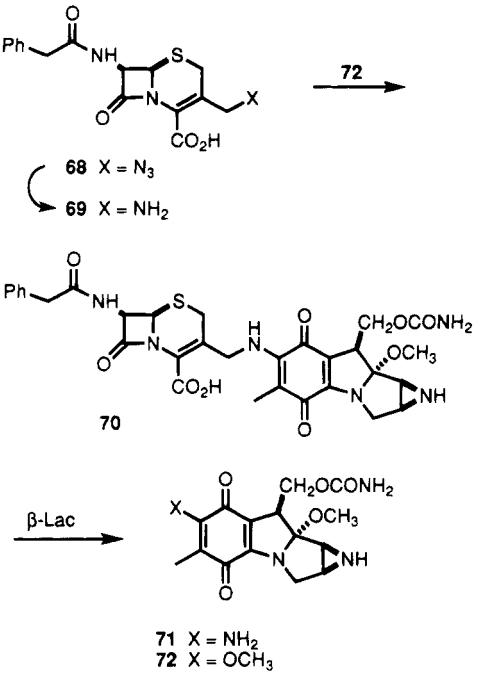
The problems encountered upon iv treatment of animals with **63** prompted the synthesis of a more water-soluble prodrug, **64**, via an analogous route starting with cephem **62**.[35] Prodrug **64** was also found to be a good substrate for the *B. cereus* enzyme (K_m = 31.1 μM , k_{cat} = 116 s^{-1}). When tested against the H2981 human lung adenocarcinoma cell line prodrug **64** was also significantly less cytotoxic (IC_{50} = 25–40 μM) than free drug **55** (IC_{50} = 1.5 μM). The cytotoxic activity of **64** on H2981 cells that were pretreated with an appropriate mAb–β-Lac conju-

gate (IC_{50} = 3.2 μM) was similar to that of free drug **55**. Appropriate control experiments were performed to show that the prodrug was activated in an antigen-specific manner. Mouse xenograft studies employing an antibody–enzyme immunoconjugate and prodrug **64** suppressed the growth of established H2981 tumors and this combination was more active than either the prodrug **64** or free drug **55** administered alone.[35] These data established the superiority of the ADC delivery system relative to systemic administration of a free nitrogen mustard alkylating agent.

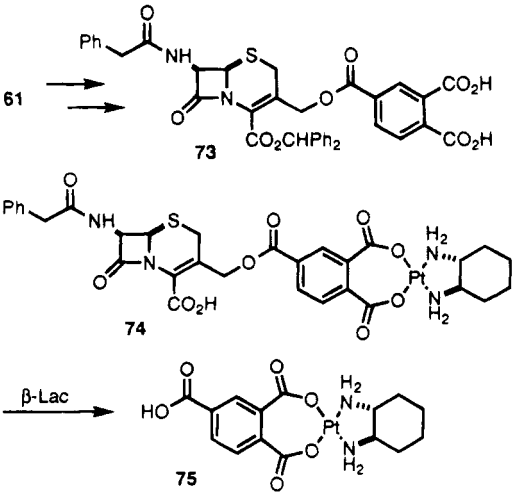
The Bristol-Myers Squibb group has also prepared a doxorubicin (**22**)-derived prodrug[36] which is nearly identical to our own compound **51**. The only differences are that the parent cephalosporin was utilized rather than the sulfoxide form and a phenyl group was substituted for the thiophene moiety in the side chain. Doxorubicin was N-acylated by the activated tetrachlorocarbonate **65** to give carboxamide **66** which was deesterified under carefully controlled conditions (the glycosidic linkage is very acid sensitive)[30,36] to give the desired prodrug **67**. Cephalosporin **67** proved to be an excellent substrate for the *B. cereus* β-lactamase, and concomitant release of free doxorubicin was observed. The enzyme kinetics measured allowed the authors to predict the level of cytotoxicity that might be achieved using this ADC system. They estimate that one-half of a lethal dose can be delivered per minute to a tumor cell, assuming that 10% of the targeted antigens on the tumor cell surface are bound to a mAb–β-Lac conjugate, and that each conjugate releases 1000 molecules of free doxorubicin per minute. No other biological evaluation of prodrug **67** was reported.



The Bristol group also explored the possibility of delivering mitomycin C (**71**) via prodrug **70**.[37] Mitomycin A (**72**) was condensed with the C-3′ amino cephem **69**, which was prepared by reduction of the corresponding azide **68**. Prodrug **70** was reported to be a substrate for β-Lac and release of free mitomycin C was observed by TLC. No in vitro or in vivo activity for prodrug **70** were reported.



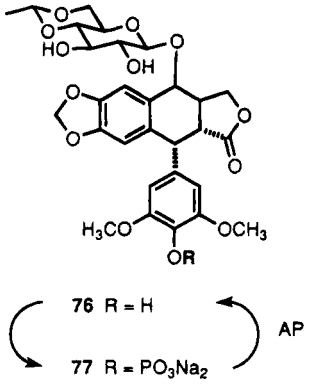
Finally, Hanessian[38] has reported the synthesis of a carboplatinum-based prodrug **74**. The drug he selected was DACCP (**75**) which is a potent antitumor agent. Once again the C-3′ hydroxy cephem **61** served as a starting material, which was converted to the diacid **73**. The benzhydryl ester was hydrolyzed to the corresponding triacid, followed by conversion to a monopotassium salt, and reaction with (*trans*-1,2-diaminocyclohexane)platinum(II) dinitrate to afford the desired prodrug **74**. The ester linkage in **74** was found to be stable in a carbonate buffer solution for more than 2 h. Addition of the P99 β-Lac to a buffered solution of **74** resulted in the rapid release of DACCP (**75**) which was observed by NMR. Evaluation of the antitumor activity of prodrug **74** was not reported.



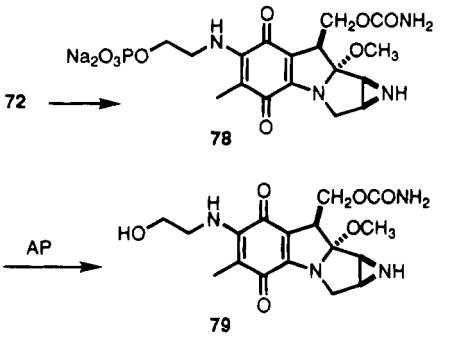
III. Alkaline Phosphatase

Some of the earliest reported work on ADC was done utilizing alkaline phosphatase (AP), a mammalian enzyme which liberates phosphate ions from various organic phosphates. Senter and co-workers at Bristol-Myers Squibb have prepared and studied a series of phosphate prodrugs which are substrates for mAb–AP immunoconjugates. The requisite prodrugs are chemically quite simple, phosphate analogues, and as charged species should have a more difficult time penetrating cellular membranes. Thus, they may also be less cytotoxic.

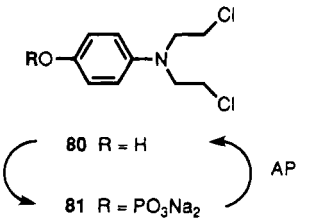
The first prodrug the Bristol group reported was etoposide phosphate (**77**), an analogue of etoposide (**76**).[39-41] Etoposide was treated with phosphoryl chloride followed by basic hydrolysis to provide the prodrug. Prodrug **77** (1.0 mM) was rapidly hydrolyzed by AP (calf intestinal, 1.0 μg/mL) and released free drug **76** with a half-life of 8 min.[40] When tested against the H3347 human colon carcinoma cell line the prodrug **77** was at least 100-fold less cytotoxic than free drug **76**. The combination of an appropriate mAb–AP conjugate and prodrug **76** was equipotent with etoposide (**76**) against H3347 cells.[39] More importantly, therapy experiments in a mouse xenograft model showed impressive results. A strong antitumor response was observed when mice bearing H3347 tumors were treated with the ADC combination which included prodrug **77**, with 6 out of 16 animals showing regression of the tumors.[39] It was interesting to note that the prodrug alone was more efficacious (growth suppression was observed) than the free drug alone in these studies, perhaps because they were able to utilize the former at a higher concentration due to its reduced cytotoxicity relative to etoposide (**76**).



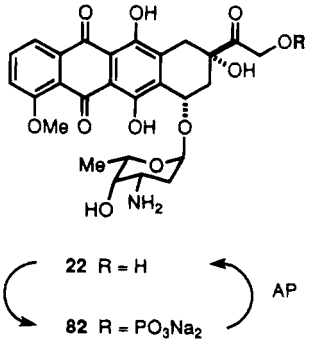
A mitomycin-based prodrug **78** was also studied. Condensation of 2-aminoethyl dihydrogen phosphate with mitomycin A (**72**) provided the desired phosphate analogue **78**. When tested against the H2891 lung adenocarcinoma cell line, prodrug **78** was approximately 100-fold less cytotoxic than free drug **79**, which itself is as potent as mitomycin C (**71**). The combination of an appropriate mAb–AP conjugate and mitomycin-based prodrug **78** was equipotent with the mitomycin analogue **79** against H2891 cells.[40] Therapy experiments[40] were performed in vivo on nude mice containing H2981 tumor xenografts. Tumor regressions were observed in 3 of 6 animals treated with a mAb–AP conjugate plus prodrug **78**, and this ADC combination was more efficacious than either prodrug **78** alone or the free drug **79**.



The Bristol group has also used a nitrogen mustard-based prodrug **81** in combination with alkaline phosphatase.[42] Again the requisite compound was prepared by reacting the drug, **80**, with phosphoryl chloride followed by alkaline hydrolysis to the desired phosphate analogue **81**. Prodrug **81** (0.1 mg/mL) was rapidly hydrolyzed by AP (calf intestinal, 20 μg/mL) and was quantitatively converted into free drug **80** within 10 min. Nitrogen mustard **80** exhibited significant cytotoxic effects against the H2891 lung adenocarcinoma cell line, whereas the prodrug was not cytotoxic. The ADC combination of prodrug **81** and a mAb–AP conjugate was nearly as active as free drug **80** in vitro. In vivo the ADC combination was more active against H2891 tumors than free drug alone; however, only tumor growth suppression was observed.



Finally, doxorubicin phosphate (**82**) has been reported to be rapidly converted into free doxorubicin (**22**) by AP.[41] No further evaluation of this prodrug has been published.



Clearly, an ADC approach employing AP has demonstrated impressive results in animal models with several different prodrugs. However, phosphatases are abundantly present in mammalian serum and other tissues and nonspecific release of free drug at sites other than the tumor will almost

certainly limit this particular combination. As summarized by Haisma et al.,[43] "For antibody–enzyme-mediated chemotherapy phosphorylated prodrugs do not appear to be the first choice because of rapid activation in serum and other tissues".

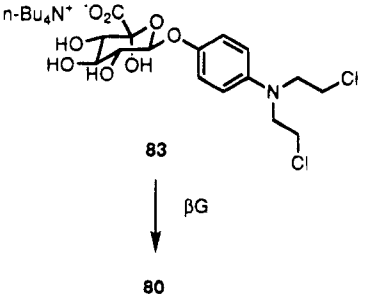
IV. Glycosidases

Two different ADC systems have been described which employ the combination of a glycosidase and sugar-based prodrugs which depend on the enzyme's ability to cleave the glycosidic linkage between the cytotoxic agent and the sugar.

A. β-Glucuronidase

Roffler and co-workers[44,45] are working on the development of an ADC system which utilizes β-glucuronidase (βG, derived from *Escherichia coli*), an enzyme which catalyzes the cleavage of the glycosidic linkage in glucuronic acid analogues. They also chose a nitrogen mustard analogue, **80**, as their cytotoxic agent. Thus, the prodrug they required was glucuronide **83**. Isolated β-glucuronidase was shown to readily convert prodrug **83** into free drug **80**.[44] Against COLO 205 human colon cancer cells the prodrug was about 150-fold less cytotoxic than the free drug. When the COLO 205 cells were preincubated with an appropriate mAb–βG conjugate and then treated with prodrug **83** the cytotoxic effect was nearly equal to the free drug alone.[44] This ADC combination was also found to be equipotent to free drug against AS-30D rat hepatoma cells.[45] No in vivo studies were reported.

There are two potential drawbacks to this system. First, βG is an enzyme which is also found in human serum as well as other tissues, thus selective activation of prodrug may be difficult to achieve in vivo. Second, endogenous "detoxification enzymes" such as ADPGT are capable of reglycosylating the free drug, in essence converting it back into the less cytotoxic prodrug form.[45]



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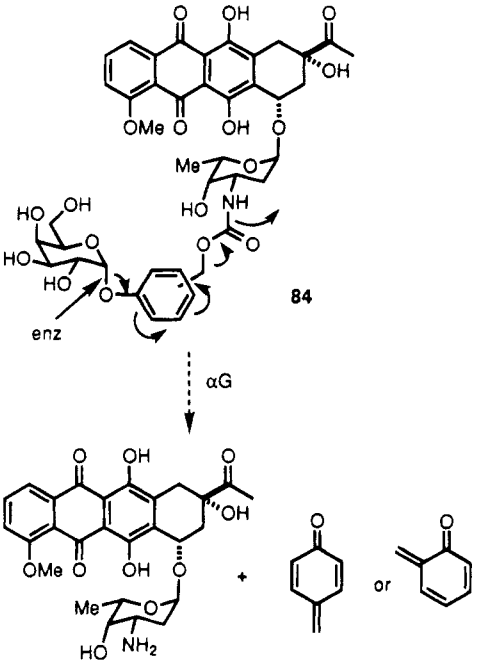
B. α-Galactosidase

A group of French workers[46] have described the synthesis of two daunorubicin-derived prodrugs **84**, both the *ortho*- and *para*-substituted analogues were prepared. These compounds make use of a novel self-immolative spacer placed between the drug and the sugar moiety. They speculate that the enzyme α-D-galactosidase (αG) should be able to cleave the glycosidic linkage in **84**, which would ultimately result in the liberation of daunorubicin **85**, a potent cytotoxin, along with a quinone methide and a mole of CO_2. Prodrug **84** was found to be at least 50-fold less cytotoxic against L1210 murine leukemia cells than daunorubicin (**85**). No other data were reported.

The feasibility of their proposed drug release mechanism is currently under study. The authors commented that αG-catalyzed hydrolysis had failed on several previously prepared prodrugs wherein the sugar had been directly attached to the anthracycline via one of the hydroxyl substituents or the amino sugar.



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V. Cytosine Deaminase

Cytosine deaminase (CDase, isolated from Bakers' yeast) is an enzyme capable of converting cytosine into uracil. Senter and co-workers[47] have taken advantage of this catalytic activity by utilizing CDase to convert the antifungal agent 5-fluorocytosine (**86**) into the often utilized antitumor agent 5-fluorouracil (**36**). Against H2891 lung adenocarcinoma cells prodrug **86** was at least 10 000-fold less cytotoxic than free drug **36**. When H2891 cells were treated with a mAb–CDase conjugate followed by prodrug **86** the antitumor activity observed was equal to the free drug **36**, thus drug was being formed in a specific manner at the tumor cell surface. The authors indicated that in vivo experiments were being pursued with this ADC combination. While promising in vitro results were obtained, this system is obviously restricted to the use of a single drug, 5-fluorouracil.

86

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VI. Nitroreductase

Knox and co-workers[48] have described an ADC system which would employ the nitroreductase en-

zyme isolated from *Escherichia coli*. The prodrug, 5-(aziridin-1-yl)-2,4-dinitrobenzamide, is reduced by this enzyme giving rise to 5-(aziridin-1-yl)-4-(hydroxyamino)-2-nitrobenzamide which is subsequently acetylated to give the cytotoxic alkylating agent 5-(aziridin-1-yl)-4-(acetylamino)-2-nitrobenzamide. Unfortunately, the use of a nitroreductase enzyme in ADC also requires the presence of a cofactor such as NADH. Neither in vitro nor in vivo evaluation of this system was reported.

VII. ADC Using a Bifunctional Antibody

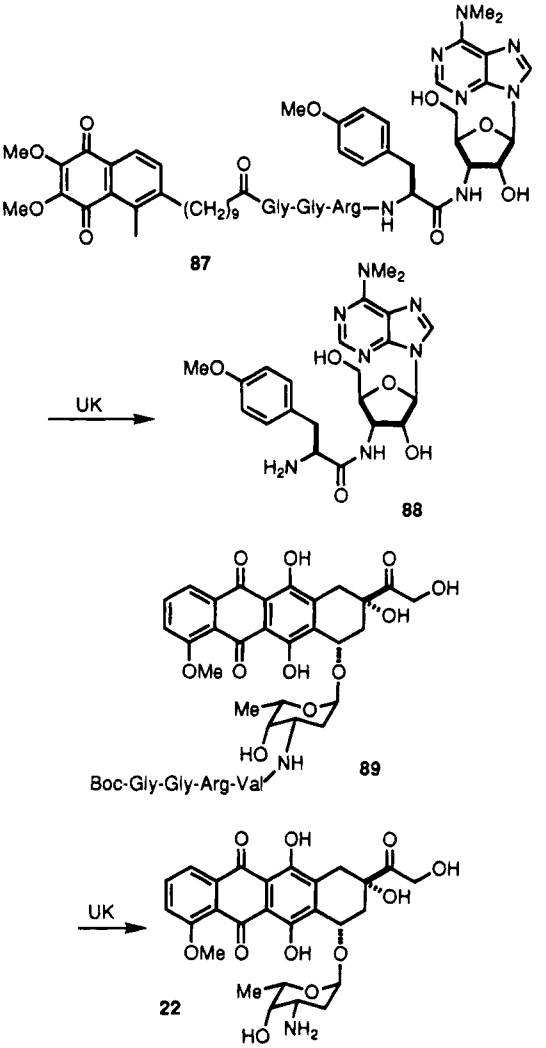
There are at least two groups attempting to develop a modified ADC system which utilizes a bifunctional antibody. Instead of preparing a conjugate wherein the enzyme is covalently attached to the mAb, they make use of a mAb which binds simultaneously to both the tumor cell-specific antigen as well as an epitope on the enzyme. Thus the enzyme is attached to the antibody via a recognition mechanism rather than a covalent bond. Of course this adds considerable complexity to the mAb preparation aspects of the ADC problem. The enzymes employed, urokinase and alkaline phosphatase, are found in the human system, thus the hope is to concentrate these endogenous proteins at the tumor site. By using an endogenous enzyme the issue of the potential immunogenicity of the enzyme is overcome, however, one also runs the risk of nonspecific prodrug activation at sites other than the tumor. This problem has already been observed with alkaline phosphatase based ADC (vide supra).

A. Urokinase

The Takeda group[49] has filed a patent application which describes their efforts to develop an ADC system using urokinase (UK), a peptidase found in human urine. They synthesized a series of tripeptide analogue prodrugs of puromycin (**88**) and doxorubicin (**22**) and found them to be less cytotoxic than the parent drugs against A431 human epidermoid carcinoma cells. Urokinase catalyzed the proteolytic cleavage of the prodrugs which released free drugs puromycin (**88**) or doxorubicin (**22**). Two prodrugs were selected for further in vitro evaluation, **87** which is a puromycin analogue and doxorubicin analogue **89**. The cytotoxicity of **87** and **89** against A431 cells was not reported, nor was the activity of the parent drugs puromycin (**88**) or doxorubicin (**22**). When A431 cells were pretreated with an appropriate bifunctional antibody followed by addition of UK and then either prodrug **87** or **89** cytotoxic activity was observed; however, in neither experiment was an IC_{50} achieved under the concentrations tested.

B. Alkaline Phosphatase

Pfreundschuh[50] has also worked with mitomycin analogue **78**. This prodrug was 100-fold less cytotoxic against Hodgkin's derived cell line L540 than free drug **79**. When L540 cells were pretreated with an appropriate bifunctional antibody followed by addition of AP and then prodrug **78** cytotoxic activity equivalent to the free drug was observed supporting the notion that the mAb was concentrating AP on the cell surface and this in turn was catalyzing release of toxic concentrations of drug.



VIII. Summary

In this review we have seen that a number of ADC systems have been studied which are able to mediate antigen-dependent cytotoxicity both in vitro and in vivo. A wide variety of enzymes have been employed and a significant number of prodrugs, which perform as designed, have been synthesized. Exciting antitumor activity has been observed in vivo in at least three instances, using carboxypeptidase G2, alkaline phosphatase, and β-lactamase. The cephem/β-lactamase-based ADC appears to be effective with a wider variety of cytotoxic agents than the other systems reported to date. Considering the arsenal of prodrugs described herein it seems that the continued development of the ADC concept will be more dependent upon the successful production of highly selective and nonimmunogenic mAb–enzyme conjugates, than on the limitations of synthetic chemistry.

We are confident that the exciting results with ADC in the laboratory will translate into clinical efficacy. It is apparent that early clinical attempts will make use of commonly employed antitumor agents, thus maximizing our ability to access what positive benefit might be derived from an ADC delivery system. The ultimate goal it appears would

be a nontoxic prodrug which is converted to a potent cytotoxic agent by a catalytic human antibody which is completely selective for the targeted tumor.

IX. Acknowledgments

We thank all the members of the multidiciplinary team responsible for the development of the ADC concept at Lilly/Hybritech, especially Dr. Damon Meyer, Dr. Jake Starling, Kevin Law, and Steve Mikolajczyk, as well as Dr. Homer Pearce for his support of the ADC program.

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