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Protease-activated "prodrugs" for cancer chemotherapy

(plasminogen activator/plasmin/fibrinolysis/antineoplastic agents/peptides)

PHILIP L. CARL, PRASUN K. CHAKRAVARTY, JOHN A. KATZENELLENBOGEN, AND MICHAEL J. WEBER

Departments of Chemistry and Microbiology, University of Illinois, Urbana, Illinois 61801

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ABSTRACT Many types of malignant cells and human tumors display increased concentrations of the protease plasminogen activator that converts plasminogen to the highly active protease, plasmin. Because plasmin rapidly cleaves various low molecular weight compounds coupled to appropriate peptide specifiers, we hypothesized that coupling of such peptide specifiers to anticancer drugs might create "prodrugs" which would be locally activated by tumor-associated plasmin and consequently would be less toxic to normal cells. To provide an initial test of this concept we have synthesized peptidyl prodrugs of the structure D-Val-Leu-Lys-X in which the peptidyl portion has been designed to allow the prodrug to serve as an excellent plasmin substrate and X is an anticancer drug—either the glutamine analog (*αS*,5*S*) *α*-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) or the alkylating agent *N,N*-bis(2-chloroethyl)-*p*-phenylenediamine (phenylenediamine mustard). Treatment of these prodrugs with plasmin generated the free peptide and the free drug, demonstrating that these prodrugs are plasmin substrates. The prodrugs and free drugs were tested in an *in vitro* system against either normal chicken embryo fibroblasts, which display a low level of plasminogen activator, or their virally transformed counterparts, which produce high levels of plasminogen activator. In each case the peptidyl prodrugs displayed at least a 5-fold increase in selectivity for the transformed cells compared to the free drug. The greater selectivity of action of the peptidyl prodrugs against transformed cell cultures suggests that these or similar prodrugs that are substrates for tumor-associated proteases may show increased therapeutic effectiveness in the treatment of tumors that produce sufficiently increased amounts of plasminogen activator.

A persistent problem in cancer chemotherapy is the low therapeutic index of most anticancer drugs—i.e., the concentrations needed to kill tumor cells are close to those that produce severe toxicity to the host. One often-proposed approach to overcoming this problem has been to design anticancer "prodrugs" which are inactive until locally activated by some tumor-associated enzyme (1–5). Although this approach has heretofore shown little success, this may be due to incomplete characterization of tumor-associated enzymes and their substrate specificity as well as to the admittedly severe difficulty that no enzyme unique to tumor cells is known, so that the prodrugs are probably activated by some normal cells as well as tumor cells.

One characteristic of many cancer cells—including cells transformed *in vitro* by viral (6–9), chemical (10), or physical (11) agents and cell lines established from various human neoplasms (12–16)—is an increased production of the serine protease plasminogen activator. This enzyme cleaves the serum zymogen plasminogen to generate the serine protease plasmin. The plasmin production associated with this increased plasminogen activator activity of many tumors offers an attractive possibility for activation of anticancer prodrugs because large amounts of plasmin are produced by small amounts of activator, the substrate specificity of plasmin and other serine proteases has been extensively investigated (17–19), and plasmin activity is normally tightly regulated by hormonal controls on plasminogen activator (20–23) and by protease inhibitors that rapidly inactivate free plasmin (24), helping to ensure that tumor-associated plasmin activity is highly localized.

We show here that two anticancer agents with distinct modes of action can be converted to inactive peptidyl prodrugs that can be reactivated by the plasmin generated in transformed cell cultures, thus leading to selective cytotoxicity *in vitro*.

MATERIALS AND METHODS

Chemicals. Amino acid derivatives were obtained from Bachem Fine Chemicals (Torrance, CA), Penninsula Laboratories (San Carlos, CA), and Sigma and were of L configuration unless stated otherwise. AT-125 (NSC-163501) was a gift from B. Naff (National Cancer Institute) and from Upjohn. Phenylenediamine mustard was synthesized as described (25). [^{3}H]Thymidine (5 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [^{3}H]aniline (150 mCi/mmol) were from Amersham. Sera and media were from GIBCO. Dog plasmin was the gift of W. Mangel (University of Illinois). Silica gel 60 plates and silica gel G powder were from Brinkmann.

Synthesis of Peptidyl[^{3}H]anilides. N^{α}-*t*-Butyloxycarbonyl(Boc)-N^{ϵ}-benzyloxycarbonyl(Cbz)-lysine was condensed with [^{3}H]aniline via a mixed anhydride reaction with isobutylchloroformate/triethylamine in *N,N*-dimethylformamide to yield N^{α}-Boc-N^{ϵ}-Cbz-Lys-anilide(**1**). Cbz-D-valine was coupled with leucine methyl ester via a *N,N*-dicyclohexylcarbodiimide condensation in *N,N*-dimethyformamide. The resulting dipeptide was saponified with 1 M NaOH to yield Cbz-D-Val-Leu-OH (**2**). This compound was condensed with *N*-Cbz-Lys-anilide (**3**) (obtained by treatment of **1** with 50% trifluoroacetic acid/CH_2Cl_2) via a mixed anhydride reaction with isobutylchloroformate/triethylamine in *N,N*-dimethylformamide to yield N^{α}-Cbz-D-Val-Leu-N^{ϵ}-Cbz-Lys-anilide (**4**). Hydrogenation of **4** over 5% Pd/C in methanol yielded D-Val-Leu-Lys-anilide. The synthesis of D-Ala-Ala-Lys-anilide proceeded along similar lines. Cbz-D-alanine was coupled with alanine methyl ester to yield, after saponification, Cbz-D-Ala-Ala-OH (**5**). Condensation of **5** with **3** yielded Cbz-D-Ala-Ala-N^{ϵ}-Cbz-Lys-anilide. Finally, catalytic hydrogenation yielded D-Ala-Ala-Lys-anilide. Key intermediates and final products were purified by chromatography or recrystallization and were characterized by comparison with the chromatographic behavior of nonradioactive counterparts synthesized in a similar manner. The nonradioactive compounds had been characterized by microanalysis and NMR.

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Abbreviations: Boc, *t*-butyloxycarbonyl; Cbz, benzyloxycarbonyl.

Table 1. Kinetic constants of peptidyl anilides

	K_m, mM	V_{max}, nmol/hr/mg cell protein
D-Ala-Ala-Lys-anilide	1.6	110
D-Val-Leu-Lys-anilide	2.5	970

Synthesis of Peptidyl Prodrugs. The tripeptide Boc-D-Val-Leu-N^{ϵ}-Boc-Lys-OH (**6**) was synthesized as follows. Boc-D-valine was condensed with leucine methyl ester via *N,N*-dicyclohexylcarbodiimide to yield, after saponification, Boc-D-Val-Leu-OH (**7**). This was coupled with N^{ϵ}-Boc-Lys-methyl ester (26) via a mixed anhydride reaction with isobutylchloroformate/triethylamine to yield **6** after purification by silica gel column chromatography and saponification. This was treated with either AT-125 or phenylenediamine mustard via the usual mixed anhydride procedure to yield the protected prodrugs. Final removal of amino protecting groups was effected in either case with 50% trifluoroacetic acid in CH_2Cl_2.

Cell Culture. Preparation of normal and Rous sarcoma virus-transformed chicken embryo fibroblasts was as described (27). Plasminogen-free chicken serum was obtained by chromatography of normal serum on lysine-Sepharose by a modification of the procedure described by Deutsch and Mertz (28).

For drug testing and [^{3}H]anilide experiments, the following protocol was used. Secondary or tertiary cell cultures were trypsinized with a 0.05% solution of crystalline trypsin and plated on either 35-mm tissue culture dishes or 16-mm cluster dishes (Costar) at 1.5–2.5 × 10^5 cells per ml for the transformed cells and 1.0–2.0 × 10^5 cells per ml for the normal cells. Because fewer of the transformed cells attach and grow, this provides equivalent numbers of normal and transformed cells in the dish at the time of the experiment. Cells were initially plated in Dulbecco's modified Eagle's medium supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. After 1 day in culture the cells were changed to Dulbecco's medium supplemented only with 5% chicken serum. Cells were used for experimentation 48 hr after plating. To minimize the effects of day-to-day variability between cell cultures, in all the experiments presented, the effects of drugs and prodrugs on normal and transformed cells were always compared at the same time.

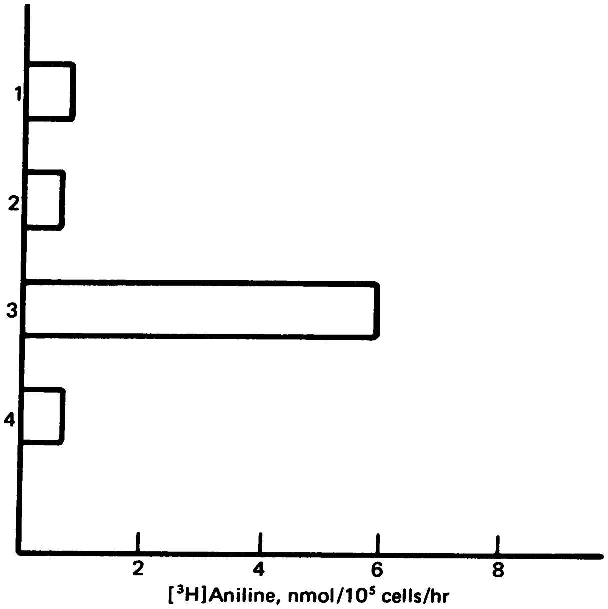

FIG. 1. Cleavage of D-Val-Leu-Lys-[^{3}H]anilide by: 1, normal cells; 2, normal cells in plasminogen-free medium; 3, Rous virus-transformed cells; and 4, transformed cells in plasminogen-free medium (cells were chicken embryo fibroblasts). D-Val-Leu-Lys-[^{3}H]-anilide (3 mCi/mmol) was present in the culture dishes at a final concentration of 0.5 mM for 5 hr. The reaction was terminated and the amount of free [^{3}H]aniline was determined. Hydrolysis due to serum alone was approximately equal to that in normal cell cultures and has not been subtracted from the data.

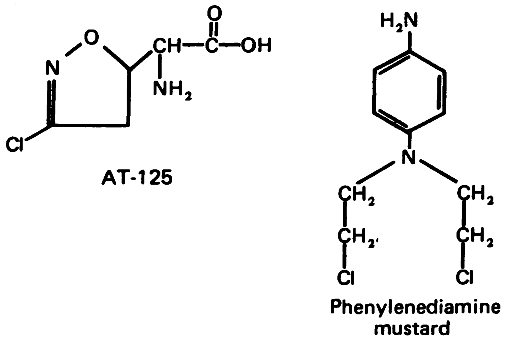

FIG. 2. Structure of AT-125 and phenylenediamine mustard. Peptidyl prodrugs are synthesized from these drugs by acylation of the primary amino groups with Boc-D-Val-Leu-N^{ϵ}-Boc-Lys-OH followed by acidolytic removal of the amine-protecting groups.

Kinetics. Rates of cleavage of peptidyl anilides were obtained by modificiation of our published method (29). Peptidyl anilides were dissolved in water and added at concentrations between 5 nM and 5 mM to the culture medium of cells growing in microtiter wells. After 3–5 hr the proteolytic cleavage was stopped by the addition of *p*-nitrophenyl *p*′-guanidinobenzoate, and 0.5 ml of the solution was added to the top of a minicolumn containing 0.5 g of silica gel G. The column was eluted with 4 ml of methanol/water, 1:1 (vol/vol), into a vial containing 5 ml of a toluene-based scintillation fluid. In this procedure, the peptidyl anilide derivatives are retained on the column and the free aniline is eluted quantitatively.

RESULTS

Selection of a Peptide Specifier. The amino acid sequence of protease cleavage sites is conventionally denoted as $H_2N...P_3P_2P_1P_{1'}P_{2'}P_{3'}...COOH$ where the bond cleaved is between amino acids P_1 and $P_{1'}$ (30). Plasmin is a protease with specificity for arginine or lysine at P_1. Examination of the preferred sites for plasmin cleavage of the fibrinogen molecule shows that the preferred P_1 amino acid is lysine. In 14 of 19 known plasmin cleavage sites on the fibrinogen molecule, P_2 is a hydrophobic amino acid. P_3 shows no apparent specificity (R. Doolittle, personal communication). Thus, many tripeptides

Table 2. ED_{50}* of drugs and prodrugs for normal and transformed chicken embryo fibroblasts

	AT-125			Phenylenediamine mustard		
	Normal ED_{50}	Transformed ED_{50}	S.I.†	Normal ED_{50}	Transformed ED_{50}	S.I.†
Free drug	8	7	1	14	12	1
Prodrug	210	40	5	70	10	7

* ED_{50}, = dose (μM) producing 50% of the activity in untreated controls.

† S.I., selectivity index = (ED_{50} in normal cells/ED_{50} in transformed cells).

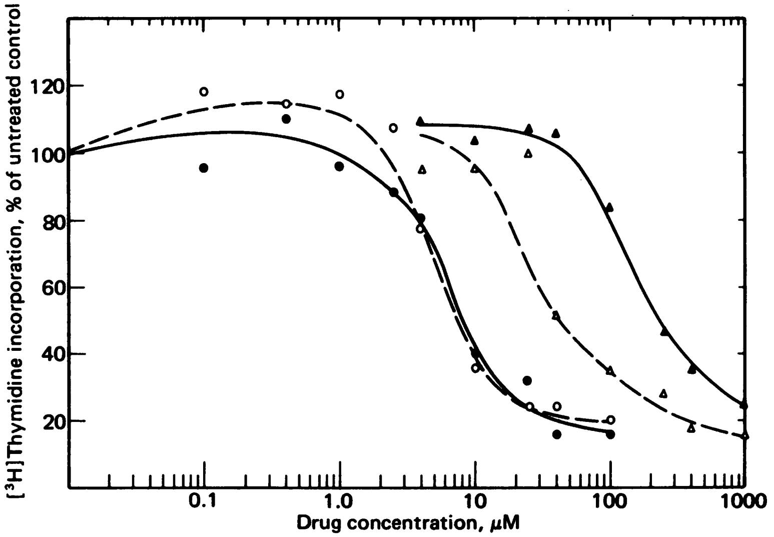

FIG. 3. Cytotoxicity of D-Val-Leu-Lys-AT-125 (▲, △) and AT-125 (●, ○) in normal and Rous virus-transformed chicken embryo fibroblasts. The drugs were present for 5 hr. [^{3}H]Thymidine was present for 30 min, at a final concentration of 1 μCi/ml; then, the incorporation of labeled material into DNA was measured as described (42). ●, ▲, Normal cells; ○, △, transformed cells. Data are the average of duplicate determinations from a single experiment except that the untreated (100%) values are the average of eight determinations.

of the sequence amino acid P_3-hydrophobic amino acid-lysine may be good specifiers for plasmin substrates, a deduction that has been confirmed by studies of the action of plasmin on low molecular weight substrates and the sensitivity of plasmin to synthetic inhibitors (31–35). In our initial evaluation we concentrated on two published sequences: D-Ala-Ala-Lys- and D-Val-Leu-Lys- (32–35).*

These two peptides were converted into [^{3}H]anilides with [^{3}H]aniline in order to assess their suitability as substrates for the plasmin generated in cultures of Rous sarcoma virus-transformed chicken embryo cells. The kinetic constants for the two substrates determined by a computer fit of the velocity-vs.-substrate data (36) are shown in Table 1. The K_m values of the substrates are not notably different, but the V_{max} for D-Val-Leu-Lys is almost 9 times that for D-Ala-Ala-Lys. In view of the marked superiority of D-Val-Leu-Lys-anilide as a substrate compared to D-Ala-Ala-Lys-anilide, the former substrate specifier was used in constructing the prodrugs.

Release of Aniline from Peptidyl Anilide Substrates by Normal and Transformed Cells. We next wanted to determine how D-Val-Leu-Lys-anilide was hydrolyzed by normal and transformed cell cultures. The Rous virus-transformed chicken embryo fibroblast system is particularly well suited for this study because the normal and transformed cells are closely matched and because the production of plasminogen activator by the transformed cells has been particularly well studied in this system (6). Transformed cells hydrolyzed about 7 times more substrate in 5 hr than did the normal cells; in the latter the rate of hydrolysis was at (or even slightly below) the control level of hydrolysis due to serum alone (Fig. 1). This difference was due to plasmin because the level of hydrolysis by transformed cells was similar to that by normal cells when both were grown in a plasminogen-free medium. It is important to note that in all the experiments we present, both normal and transformed cells were proliferating and were at nearly the same cell density, so that differences in the rates of hydrolysis of substrates or prodrugs are not due to differences in the cell concentration or their stage in the growth cycle.

Choice of Drug for Derivatization. Although plasmin and other serine proteases possess esterase as well as peptidase activity, ubiquitous esterases *in vivo* probably would nonspecifically activate ester-linked prodrugs, thus decreasing the selectivity of these agents. We therefore selected drugs that could be derivatized via amide linkage to an amino group on the drug. In addition, because it seemed probable that plasmin would cleave only primary amide linkages (37, 38), it was necessary to restrict our potential candidates to drugs having primary amino groups. Even among these drugs there was a further restriction because the prodrugs must be inactive until cleaved, which means that the peptide must be attached at a site and in a manner that inactivates the drug. Of several drugs that passed these criteria we have achieved success with two—AT-125, a glutamine analog that blocks CTP and GTP biosynthesis (39–41), and phenylenediamine mustard, an alkylating agent that is related to the simple nitrogen mustard already in clinical use (Fig. 2).

***In Vitro* Cytotoxicity Testing of Prodrugs.** Treatment of the prodrugs with plasmin released free drug and peptide (monitored by thin-layer chromatography), indicating that these prodrugs could be activated by the plasmin generated by transformed cells. To test this, the *in vitro* cytotoxicity of the prodrugs was determined by two types of tests. Because AT-125 rapidly inhibits DNA synthesis, effects of this agent and its prodrug derivative were assayed by inhibition of [^{3}H]thymidine incorporation after 5 hr of drug treatment (42) (Fig. 3). For the phenylenediamine mustard and its prodrug derivative, whose cytotoxic effects can take several generations to be manifested, the end point was cell number determined by a Coulter Counter 2 days after a 5-hr treatment with drug (Fig. 4). The doses at which the treated cells showed a level of thymidine incorporation or cell number 50% of that of the untreated control are presented in Table 2 along with the "selectivity index" of the drugs (the extent to which the drugs showed selective action against transformed cells). The prodrugs showed a 5- to 7-fold increase in selectivity compared to the free drugs. Addition of the plasmin inhibitor *p*-nitrophenyl *p*′-guanidinobenzoate at 2 μg/ml to transformed cultures protected the cells from the toxic effects of the phenylenediamine mustard prodrug (dose for 50% effect increased 9-fold) but had no effect on the toxicity of free phenylenediamine mustard (data not shown). Control experiments showed that the tripeptide D-Val-Leu-Lys at 1 mM had no effect on the cells (data not shown).

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* D-Amino acids were chosen for the amino terminus to help prevent degradation of the peptide specifiers by aminopeptidases (17). Although this also can be prevented by using a blocking group on the amino terminus, this would lower the aqueous solubility of the prodrugs.

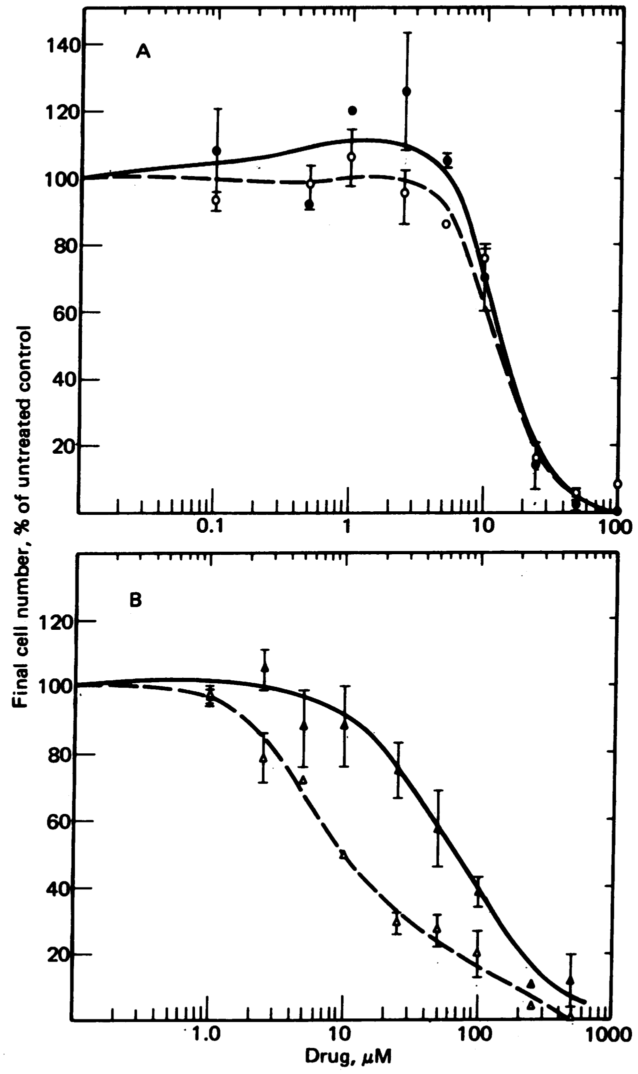

FIG. 4. Cytotoxicity of phenylenediamine mustard (*A*) and D-Val-Leu-Lys-phenylenediamine mustard (*B*) in normal (●, ▲) and Rous virus-transformed (○, △) chicken embryo fibroblasts. The drugs were present in cultures for 5 hr and then were removed; the cells washed with 1 mM Tris, pH 7.4/150 mM NaCl/1.0 mM EDTA, trypsinized with 0.05% crystalline trypsin, and then split 1:2 and replated in Dulbecco's modified Eagle's medium supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum. After 2 days of further growth the cell number was determined with a Coulter Counter. Values are the average and range of duplicate determinations, except the untreated samples were in quadruplicate.

DISCUSSION

We have presented evidence that two anticancer drugs that have widely different mechanisms of action [i.e., AT-125, an anti-metabolite (39–41) and phenylenediamine mustard, an alkylating agent] can be linked to a specifier peptide, D-Val-Leu-Lys, to create inactive prodrugs that are substrates for, and can be activated by, plasmin. Studies in cell culture show that, although neither of the underivatized free drugs shows selectivity for transformed cells *in vitro*, the prodrugs we have synthesized display a 5- to 7-fold increase in selective cytotoxicity against the transformed cells. Based on the relative cytotoxicity of the prodrugs compared to the free drugs, we estimate that nearly all of the peptidyl phenylenediamine mustard and about 20% of the peptidyl AT-125 are converted to the active form after 5 hr of exposure to the transformed cell cultures. The relatively lesser activation of the peptidyl AT-125 may reflect the lower inherent reactivity of the aliphatic amide compared to the aromatic amide in the phenylenediamine mustard derivative as well as the possibility that plasmin, like trypsin (37, 38), cleaves bonds more slowly when the carboxyl group of the amino acid adjacent to the cleavage site is unmasked.

We think that plasmin-activated prodrugs of the type described here provide a reasonable prospect for improved therapeutic effectiveness in cancer chemotherapy, for the following reasons.

i. Because the generation of plasmin is due to a cascade phenomenon (i.e., small amounts of plasminogen activator generate large amounts of plasmin), large amounts of enzymatic activity are available for drug activation.

ii. Considerable "tuning" of the peptide specifier is possible, by varying its amino acid sequence to maximize proteolysis by plasmin and minimize hydrolysis by other enzymes (17–19, 31–35; Table 1).

iii. The activity of plasminogen activator in normal tissues, unlike that of the tumor-associated enzyme, appears to be tightly regulated, (20–23) and fibrinolysis is inherently a highly localized phenomenon due to fast-acting plasmin inhibitors in blood (24). Thus, plasmin-mediated drug activation may also be highly localized.

iv. Even though plasmin action is localized, plasminogen activation is extracellular. Thus, the prodrugs will not have to cross cell membranes in order to be activated.

v. There is a substantial (although still imperfect) correlation between increased fibrinolytic activity and malignancy (6–16).

vi. Drugs with a wide variety of pharmacological properties and modes of action can be converted into plasmin-activated prodrugs. Although we are aware that it is far more difficult to demonstrate an increase in drug selectivity *in vivo* than *in vitro* because factors of drug distribution, clearance, and activation at extratumor sites become important, we think that, for the reasons enumerated above, the improved selectivity we have demonstrated at the cellular level *in vitro* augurs well for the possibility of demonstrating improved selectivity *in vivo*.

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1. Connors, T. A. (1976) in *Progress in Drug Metabolism*, eds. Bridges, J. W. & Chasocaud, L. F. (Wiley, New York), pp. 41–75.
2. Connors, T. A. (1978) *Biochemie* **60**, 979–987.
3. Workman, P. & Double, J. A. (1978) *Biomedicine* **28**, 255–262.
4. Hebborn, P. & Danielli, J. F. (1958) *Biochem. Pharmacol.* **1**, 19–24.
5. Marquisee, M. J. & Kauer, J. C. (1978) *J. Med. Chem.* **21**, 1188–1194.
6. Unkeless, J., Tobia, A., Ossowski, L., Quigley, J. P., Rifkin, D. B. & Reich, E. (1973) *J. Exp. Med.* **137**, 85–111.
7. Ossowski, L., Unkeless, J. C., Tobia, A., Quigley, J. P., Rifkin, D. B. & Reich, E. (1973) *J. Exp. Med.* **137**, 112–126.
8. Howett, M. K., High, C. S. & Rapp, F. (1978) *Cancer Res.* **38**, 1075–1078.
9. Christman, J. K., Acs, G., Silagi, S. & Silverstein, S. C. (1975) in *Proteases and Biological Control*, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pp. 827–839.
10. Barrett, J. C., Crawford, B. D., Grady, D. L., Hester, L. D., Jones, P. A. & Benedict, W. F. (1977) *Cancer Res.* **37**, 3815–3823.
11. Hamilton, J., Vasalli, J. D. & Reich, E. (1976) *J. Exp. Med.* **144**, 1689–1694.

12. Rifkin, D. B., Loeb, Z. M., Moore, G. & Reich, E. (1974) *J. Exp. Med.* **139**, 1317–1328.
13. Laug, W. E., Jones, P. A. & Benedict, W. F. (1975) *J. Natl. Cancer Inst.* **54**, 173–178.
14. Tucker, W. S., Kirsich, W. M., Martinez-Hernandez & Fink, L. M. (1978) *Cancer Res.* **38**, 297–302.
15. Nagy, B., Ban, J. & Brdar, B. (1977) *Int. J. Cancer* **19**, 614–620.
16. Rohrlich, S. T. & Rifkin, D. B. (1979) in *Annual Reports in Medicinal Chemistry*, ed. Hess, H. (Academic, New York), pp. 229–239.
17. Claeson, G., Aurell, L., Karlsson & Frigerger, P. (1977) in *New Methods for the Analysis of Coagulation Using Chromogenic Substrates*, ed. Witt, I. (de Gruyter, New York), pp. 37–54.
18. Mattler, L. E. & Bang, N. U. (1977) *Thromb. Haemostas.* **38**, 776–792.
19. Bang, N. U. & Mattler, L. E. (1978) *Haemostasis* **7**, 98–104.
20. Weinstein, I. B., Wigler, M., Yamasaki, H., Lee, L., Fisher, P. B. & Mufson, R. A. (1978) in *Biological Markers of Neoplasia: Basic and Applied Aspects*, ed. Ruddon, R. W. (Elsevier, New York), pp. 451–471.
21. Seifert, S. C. & Gelehrter, T. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6130–6133.
22. Roblin, R. (1978) in *Biological Markers of Neoplasia: Basic and Applied Aspects*, ed. Ruddon, R. W. (Elsevier, New York) pp. 421–432.
23. Ossowski, L. & Vasalli, J. D. (1978) in *Biological Markers of Neoplasia: Basic and Applied Aspects*, ed. Ruddon, R. W. (Elsevier, New York), pp. 473–479.
24. Collen, D. & Wiman, B. (1978) in *Fibrinolysis: Current Fundamental and Clinical Concepts*, eds. Gaffney, P. J. & Balkuv-Ulutin, S. (Academic, New York), pp. 17–26.
25. Everett, J. L. & Ross, W. C. J. (1949) *J. Chem. Soc.*, 1972–1983.
26. Schwyzer, R. & Rittel, W. (1961) *Helv. Chim. Acta* **44**, 159–169.
27. Weber, M. J. (1973) *J. Biol. Chem.* **248**, 2978–2983.
28. Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095–1097.
29. Kohn, D. B., Weber, M. J., Carl, P. L., Katzenellenbogen, J. A. & Chakravarty, P. K. (1979) *Anal. Biochem.* **97**, 269–276.
30. Schlechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162.
31. Kettner, C. & Shaw, E. (1978) *Biochemistry* **17**, 4778–4784.
32. Friberger, P., Axelsson, G. & Korsan-Bengtsen, K. (1976) *Thromb. Diath. Haemorrh.* **34**, 321.
33. Clavin, S. A., Bobbitt, J. L., Shuman, R. T. & Smithwick, E., Jr. (1977) *Anal. Biochem.* **80**, 355–365.
34. Niewenhuizen, W., Wungaards, G. & Groenveld, E. (1977) *Anal. Biochem.* **83**, 143–148.
35. Bang, N. U. & Mattler, L. E. (1977) *Haemostasis* 38, 98–104.
36. Cleland, W. W. (1967) *Adv. Enzymol.* **29**, 1–32.
37. Smyth, D. G. (1967) *Methods Enzymol.* **11**, 214–231.
38. Keil, B. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), pp. 249–275.
39. Houchens, D. P., Ovejera, A. A., Sheridan, M. A., Johnson, R. K., Bogden, A. E. & Neil, G. L. (1979) *Cancer Treat. Rep.* **63**, 473–476.
40. Neil, G. L., Berger, A. E., McPartland, R. P., Grindey, G. B. & Bloch, A. (1979) *Cancer Res.* **39**, 852–856.
41. Neil, G. L., Berger, A. E., Bhuyan, B. K., Blowers, C. L. & Kuentzel, S. L. (1979) in *Advances in Enzyme Regulation*, ed. Weber, G. (Pergamon, New York), pp. 375–398.
42. Weber, M. J. & Rubin, H. (1971) *J. Cell. Physiol.* **77**, 157–168.