Papmate-activated "prodrugs" for cancer chemotherapy
(plasminogen activator/plasmin/fibrinolysis/antineoplastic agents/peptides)

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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 glutaminic acid (α2,5S) α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) or the alkyllating 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 peptidyI 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). [3H]Thymidine (5 Ci/mmol; 1 Ci = 3.7 × 1010 becquerels) and [3H]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[3H]anilides. Nα-t-Butoxyoxycarbonyl(Boc)-N'-benzoyloxycarbonyl(Cbz)-lysine was condensed with [3H]aniline via a mixed anhydride reaction with isobutylchloroformate/triethylamine in N,N-dimethylformamide to yield Nα-Boc-N'-Cbz-Lys-anilide(1). Cbz-D-valine was coupled with leucine methyl ester via a N,N-dicyclohexylcarbodiimide condensation in N,N-dimethylformamide. 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-aniline (3) (obtained by treatment of 1 with 50% trifluoroacetic acid/CH2Cl2) via a mixed anhydride reaction with isobutylchloroformate/triethylamine in N,N-dimethylformamide to yield Nα-Cbz-D-Val-Leu-N'-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-N'-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.

Abbreviations: Boc, t-butoxycarbonyl; Cbz, benzoyloxycarbonyl.

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Table 1. Kinetic constants of peptidyl anilides

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<th>$K_m$, mM</th>
<th>$V_{max}$, nmol/hr/mg cell protein</th>
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<tr>
<td>D-Ala-Ala-Lys-anilide</td>
<td>1.6</td>
<td>110</td>
</tr>
<tr>
<td>D-Val-Leu-Lys-anilide</td>
<td>2.5</td>
<td>970</td>
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Synthesis of Peptidyl Prodrugs. The tripeptide Boc-D-Val-Leu-$N'$-Boc-Lys-OH (6) was synthesized as follows. Boc-D-valine was condensed with leucine methyl ester via $N,N$-dicyclohexylcarbodimide to yield, after saponification, Boc-D-Val-Leu-$N'$-Boc-Lys-OH (7). This was coupled with $N'$-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$_3$Cl$_2$.

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]aniline 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 $\times 10^5$ cells per ml for the transformed cells and 1.0-2.0 $\times 10^5$ 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.

Kinetics. Rates of cleavage of peptidyl anilides were obtained by modification 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 C. 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 aniline 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 $H_3N\ldots P_3P_3P_1P_1P_2P_3\ldots COOH$ where the bond cleaved is between amino acids $P_1$ and $P_2$ (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

![Fig. 1. Cleavage of D-Val-Leu-Lys-$[{}^3$H]aniline 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/mmml) 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]anilide was determined. Hydrolysis due to serum alone was approximately equal to that in normal cell cultures and has not been subtracted from the data.](image)

![Fig. 2. Structure of AT-125 and phenylenediamine mustard.](image)

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

<table>
<thead>
<tr>
<th></th>
<th>AT-125</th>
<th>Phenylenediamine mustard</th>
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<tr>
<td></td>
<td>Normal</td>
<td>Transformed</td>
</tr>
<tr>
<td>Drug</td>
<td>ED$_{50}$</td>
<td>ED$_{50}$</td>
</tr>
<tr>
<td>Free drug</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Prodrug</td>
<td>210</td>
<td>40</td>
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* ED$_{50}$ = dose ($\mu$m) producing 50% of the activity in untreated controls.

* S.I., selectivity index = (ED$_{50}$ in normal cells/ED$_{50}$ in transformed cells).
of the sequence amino acid P$_2$-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 phenylendiamine 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 phenylendiamine 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 $\mu$g/ml to transformed cultures protected the cells from the toxic effects of the phenylendiamine mustard prodrug (dose for 50% effect increased 9-fold) but had no effect on the toxicity of free phenylendiamine 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 phenylendiamine 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 phenylendiamine 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 derivatized 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 phenylendiamine 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 lower activation of the peptidyl AT-125 may reflect the lower inherent reactivity of the aliphatic amide compared to the aromatic amide in the phenylendiamine 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.

1. 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.

2. 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).

3. 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.

4. 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.

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

6. 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 vitro than in vivo 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 augers well for the possibility of demonstrating improved selectivity in vivo.

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