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SHORT COMMUNICATION

# Intracellularly Biorecognizable Derivatives of 5-Fluorouracil

## IMPLICATIONS FOR SITE-SPECIFIC DELIVERY IN THE HUMAN CONDITION

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**ABSTRACT.** The release of 5-fluorouracil from polymer-based conjugates can be influenced by the type of linkages used to bind the drug to the polymer carrier. The use of specific oligopeptide sequences designed to be biorecognizable by intracellular enzymes is a promising approach for increasing the site-specific release of 5-fluorouracil from polymer-based conjugates. In this study, we investigated the biorecognizability of specific oligopeptide sequences linking 5-fluorouracil to a water-soluble copolymer carrier based on *N*-(2-hydroxypropyl)methacrylamide by human cathepsin B (EC 3.4.22.1), cathepsin H (EC 3.4.22.6), and a homogenate of the human colon adenocarcinoma cell line SW 480. The cathepsins were chosen based on the hypothesis that they were two principal lysosomal enzymes responsible for the release of 5-fluorouracil from these conjugates. Our results support this hypothesis; however, these two enzymes may not be the only lysosomal enzymes responsible for the release kinetics observed. While the results for cathepsin B corresponded well to our hypothesis, the cleavage via cathepsin H was lower than predicted, suggesting the presence of additional lysosomal enzymes with catalytic activity toward these 5-fluorouracil derivatives. BIOCHEM PHARMACOL 52;6:957–962, 1996.

**KEY WORDS.** biorecognition; site-specific release; 5-fluorouracil; polymer carriers; cathepsin B; cathepsin H

The targeted delivery of anticancer compounds to cancer cells is an interdisciplinary field of study that incorporates the expertise of a large cross-section of scientific areas including but not limited to chemistry, biochemistry, pharmacology, pharmaceutics, and medicine. The goal of targeting anticancer agents to cancer cells is to increase the therapeutic efficacy and to decrease the nonspecific toxicity of the drugs. Achieving this goal could result in anticancer treatments with a greater cure rate. One promising approach for targetable drug delivery is the use of water-soluble polymer-based carriers [1]. In fact, targetable polymer-based carriers of doxorubicin are currently in Phase I/II clinical trials in the United Kingdom.

A critical requirement for the targeted delivery of antimetabolite anticancer compounds is the site-specific release of the drug within the target cell. Failure to release the drug from the polymer carrier has been shown to result in a therapeutically inactive conjugate [2]. In addition, the release of the drug from the polymer carrier, for example via chemical hydrolysis in the bloodstream, prior to reaching the target cell population will result in a decreased therapeutic potential since the carrier "warhead" will be lost before the target site is reached.

Approaches to the delivery of the anticancer compound, 5-FU§, using polymer-based carriers, have historically released the drug from the carrier by the process of chemical hydrolysis [3–6]. The site-specific release of the free drug from these conjugates was not maximized because a significant percentage of the 5-FU could be released into the bloodstream before reaching the desired site of release. A more rational approach to the effective site-specific delivery of 5-FU is through the use of oligopeptide sequences to link the drug to the polymer carrier [7–9]. These oligopeptide sequences are specifically designed to be stable in the blood circulation. They are also designed to release the drug within the target cell. The intracellular release is achieved via the biorecognition of the oligopeptide sequence and its subsequent enzymatically catalyzed cleavage by lysosomal enzymes present with the lysosomal compartment of the cell. Since the entry of polymer conjugates into cells is limited to the process of endocytosis, and following endocytosis the conjugate ultimately resides within the lysosomal compartment of the cell, this approach to the site-specific delivery of 5-FU is very encouraging.

The release of 5-FU from copolymers based on HPMA via rat liver lysosomal compartments (isolated as tritosomes) has been reported [7]. While the results demonstrated quantitative release of 5-FU from the polymer, either as a dipeptide derivative of 5-FU (that is biologically

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§ *Abbreviations:* 5-FU, 5-fluorouracil; CBZ, carbobenzyloxy; HPMA, *N*-[2-hydroxypropyl]methacrylamide; Leu-NAp, leucyl *p*-nitroanilide; NAp, *p*-nitroanilide; OAc, acetyl; and OMe, methyl ester.

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convertible to free 5-FU) or as free 5-FU, the effect of human lysosomal enzymes on the conjugates is unknown but is required if the conjugates are to proceed to clinical trials. To this end, we studied the biorecognition of these 5-FU conjugates by two human lysosomal enzymes, cathepsin B (EC 3.4.22.1) and cathepsin H (EC 3.4.22.6), and also by a homogenate of the human adenocarcinoma colon cancer cell line SW 480.

The 5-FU derivatives studied are shown in Fig. 1. Cathepsins B and H were chosen as model lysosomal enzymes based on the hypothesized release mechanism of 5-FU from the polymer conjugates. The hypothesis statement is as follows: the catalytic activity of cathepsin B [10, 11] releases a derivative of 5-FU from the polymer and the aminopeptidase activity of cathepsin H [12] releases free 5-FU from the derivative, according to Fig. 1B.

## MATERIALS AND METHODS

### *Materials*

Human cathepsin B and cathepsin H were purchased from Calbiochem, La Jolla, CA. All 5-FU derivatives and polymers were synthesized as previously reported [7]. Briefly, an α-substituted glycine dipeptide derivative of 5-FU was synthesized by first reacting a diprotected dipeptide containing serine (either CBZ-Ala-Ser-OMe or CBZ-Leu-Ser-OMe) with lead tetraacetate. This reaction converts the serine residue into an acylated glycine residue, creating an acylated (OAc) intermediate [either CBZ-Ala-Gly-(OAc)-OMe or CBZ-Leu-Gly-(OAc)-OMe]. Reaction of this intermediate with 5-FU replaces the OAc group with 5-FU. The protecting groups were removed by standard procedures. Polymers containing 5-FU were synthesized by polymer-analogous reaction between an activated polymer precursor and the dipeptide derivative of 5-FU. The activated parts of the polymer precursor consisted of oligopeptide side chains terminated with a *p*-nitrophenyl ester. The colon adenocarcinoma cell line SW 480 was purchased from the American Type Culture Collection, Rockville, MD, and cultured at 37° under a 5% $CO_2$ atmosphere in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution.

### *Enzyme Activity Assay*

The activities of cathepsin B and cathepsin H were determined spectrophotometrically and are expressed in units. The units were converted to molar concentrations assuming all protein present in the preparation was active enzyme. One unit of enzyme is defined as the amount that will hydrolyze 1 μmol of NAp from its substrate per minute.

Cathepsin B activity was assayed using the substrate Bz-Phe-Val-Arg-NAp. The formation of free NAp was followed spectrophotometrically at 410 nm ($\varepsilon$ = 8600 $M^{-1}$). The assay was carried out over 10 min at 37° in 1 mL volumes containing 0.86 mL phosphate buffer (88 mM $KH_2PO_4$, 12 mM $Na_2HPO_4$, 1.33 mM EDTA, pH 6.0), 0.02 mL cysteine stock solution (18.18 mg/mL buffer) to make the final concentration 3 mM, 0.02 mL Bz-Phe-Val-Arg-NAp (11.2 mM in DMSO), and 0.1 mL of cathepsin B stock solution in buffer.

Cathepsin H activity was assayed using the substrate Leu-NAp. The formation of free NAp was followed spectrophotometrically at 410 nm ($\varepsilon$ = 8600 $M^{-1}$). The assay was carried out over 10 min at 37° in 1 mL volumes containing 0.86 mL of phosphate buffer (37.5 mM $KH_2PO_4$, 37.5 mM $Na_2HPO_4$, 1 mM EDTA, pH 6.8), 0.02 mL cysteine stock solution (18.18 mg/mL buffer) to make the final concentration 3 mM, 0.02 mL Leu-NAp (24 mM in DMSO), and 0.1 mL of cathepsin H stock solution in buffer.

### *HPLC Analysis*

5-FU and low molecular weight derivatives containing 5-FU were analyzed and quantitated by HPLC using a Rainin system equipped with a Microsorb $C_{18}$ analytical column. The mobile phase was 0.1% acetic acid at a flow rate of 1.0 mL/min. The compounds were detected at 254 nm and quantitated according to standard curves of the compounds. All samples were filtered through a 0.2 μm Gelman Acrodisc LC 13 PVDF filter prior to analysis.

The analysis of the release of 5-FU derivatives and free 5-FU from compound **1** and from the polymers **2** through **5a** was carried out as previously reported [7] and analyzed by HPLC.

### *Homogenate of SW 480 Cells*

The SW 480 cells were trypsinized and suspended in 10 mL of RPMI-1640. The cells, $1 \times 10^7$ total, were centrifuged at 1000 *g* for 10 min, and the supernatant was removed. Then the cells were washed by suspension in PBS (pH 7.4) and centrifuged at 1000 *g* for 10 min. The supernatant was again removed and replaced with 1 mL of sterile deionized water to lyse the cells. Further cell lysis was completed by placing the cells in an ultrasound bath for 10 min. The enzyme activity of the homogenate was standardized using the cathepsin B and cathepsin H assays described above, but which also included 0.02 mL of a Triton X-100 solution (10% in buffer) to disrupt any remaining cell or lysosomal compartment membranes.

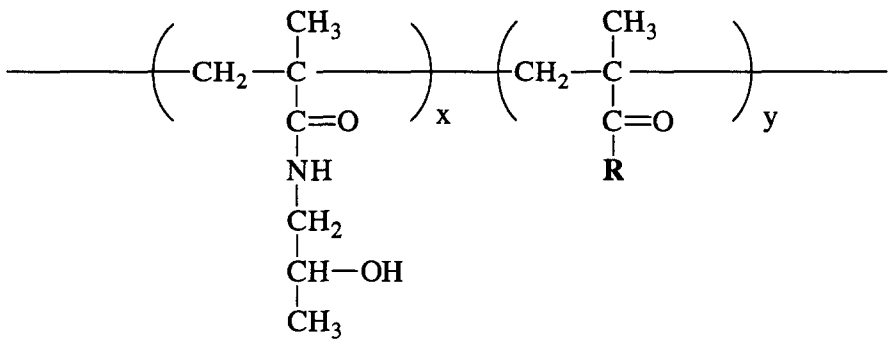
### *Release of 5-FU from 1 via Cathepsin H*

The release of 5-FU from **1** after 24 hr of incubation was determined by incubating **1** in 1 mL containing 0.8 mM compound **1**, 3 mM cysteine, and $2.53 \times 10^{-3}$ units of cathepsin H ($4.52 \times 10^{-7}$ M), all in pH 6.8 buffer (37.5 mM $KH_2PO_4$, 37.5 mM $Na_2HPO_4$, 1 mM EDTA).

### *Release of 5-FU from 1 via SW 480 Cell Homogenate*

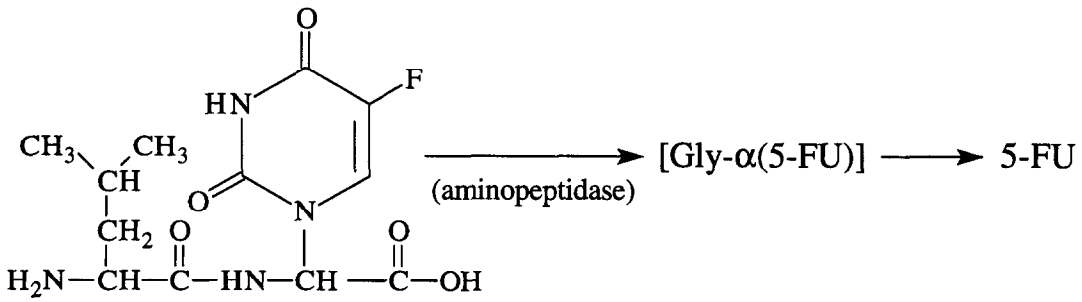
The release of 5-FU from **1** after 24 hr of incubation was determined in 1 mL volumes containing 0.8 mM compound **1**, 3 mM cysteine, 0.1 mL lysed SW 480 cells (equivalent to $9.74 \times 10^{-4}$ units of endopeptidase activity; however, note

**A**



| Number | R |
|---|---|
| 2 | —Gly—Phe—Ala—Gly—α(5-FU) |
| 3 | —Gly—Phe—Leu—Gly—α(5-FU) |
| 4 | —Gly—Phe—Leu—Gly—Ala— Gly— α(5-FU) |
| 5 | —Gly—Phe—Leu—Gly—Leu— Gly—α(5-FU) |
| 5a | —Gly—Phe—Leu—Gly—Leu— Gly—α(5-FU),(L) |

**B**




1
(Leu-Gly-α(5-FU))

**FIG. 1. (A) Structures of compounds 2–5a. (B) Structure of compound 1 and its enzymatically catalyzed degradation mechanism. The α carbon of the substituted glycine residue is in either the L or D configuration. Removal of the penultimate amino acid results in an unstable 5-FU derivative that degrades spontaneously to form free 5-FU. Previous studies [7] have shown that only the L-configuration undergoes this enzymatically catalyzed degradation. All compounds contain a racemic mixture unless otherwise indicated.**

that the homogenate showed no detectable aminopeptidase activity over the 10 min of the assay), and 0.02 mL of a Triton X-100 solution (10% in buffer), all in pH 6.0 buffer (88 mM $KH_2PO_4$, 12 mM $Na_2HPO_4$, 1.33 mM EDTA). All samples were run in triplicate.

### *Release of 5-FU Derivatives from Polymers 2, 3, 4, and 5 via Cathepsin B*

The release of 5-FU derivatives from polymers 2 through 5 after 24 hr of incubation was determined in 1 mL volumes containing the amount of each polymer that corresponded to 1 mM 5-FU (i.e. 2, 7.6 mg; 3, 9.4 mg; 4, 5.4 mg; 5, 5.8 mg), 3 mM cysteine, and $1.53 \times 10^{-3}$ units of cathepsin B ($3.64 \times 10^{-10}$ M), all in pH 6.0 buffer (88 mM $KH_2PO_4$, 12 mM $Na_2HPO_4$, 1.33 mM EDTA).

### *Release of 1 from Polymer 5a via Cathepsin B*

The release of 1 from polymer 5a over 24 hr was determined in 0.5 mL volumes containing 6.4 mg of polymer 5a

(equivalent to a final concentration of 1 mM 5-FU), 3 mM cysteine, and $2.88 \times 10^{-3}$ units of cathepsin B ($6.85 \times 10^{-10}$ M), all in pH 6.0 buffer (88 mM $KH_2PO_4$, 12 mM $Na_2HPO_4$, 1.33 mM EDTA).

### *Michaelis–Menten Kinetic Parameter Determination*

The $K_m$, $V_{max}$, and $k_{cat}$ constants for the release of 1 from polymer **5a** were determined by incubating polymer **5a** in 1 mL volumes containing concentrations of 5-FU ranging from $1 \times 10^{-3}$ to $4 \times 10^{-5}$ M and $5.76 \times 10^{-3}$ units of cathepsin B ($1.37 \times 10^{-9}$ M) all in the pH 6.0 buffer described above. All samples were run in triplicate, and the Michaelis–Menten parameters were determined by Lineweaver–Burk plot analysis.

### *Determination of Kinetic Rate Constant for the Release of* 1 *from* 5a

The data for the release of **1** from **5a** (see Fig. 2) were treated as a non-reversible pseudo-first-order reaction of the conversion of A to B. The data were fit into the analytical solution to the differential equation for the formation of B [i.e. $dB/dt = k[A]$, and $\ln[(A_o\text{-}B)/A_o] = kt$] and graphed as $\ln[(A_o\text{-}B)/A_o]$ vs $t$, and then fit linearly by the method of least squares to obtain the value of the slope of the line (i.e. $-k$).

### *Release of* 1 *and* 5-FU *from Polymer* 5a *via Cathepsin B and Cathepsin H*

The release of **1** and 5-FU from polymer **5a** after 24 hr of incubation was measured in a manner identical to that used to determine the release of **1** from polymer **5a** via cathepsin B except that $1.01 \times 10^{-2}$ units of cathepsin H ($3.61 \times 10^{-6}$ M; note: activity was assayed in pH 6.0 buffer) was also added.

## RESULTS AND DISCUSSION

The results of the incubation of 5-FU-containing compounds **1** through **5a** with various enzymes are summarized in Table 1. The numbers in parentheses are the results reported in Ref. 7 for the same compounds using a mixture of rat liver lysosomal enzymes isolated as tritosomes. They are included to allow easy comparison of the two sets of experiments.

The experiments conducted with **1** are perhaps the most intriguing. Incubation of **1** with cathepsin H resulted in a very low (1.14%) conversion of **1** to free 5-FU, compared with the conversion using rat liver tritosomes (50.6%), even though each experiment contained the same units of aminopeptidase activity toward the Leu-NAp substrate. From these results it appears that human cathepsin H has a lower activity toward **1** than rat cathepsin H. This explanation, however, is inconsistent with the literature that compares the homology of cathepsins between species. For example, cathepsins isolated from mouse [13], chicken [14, 15], rat [16–18], and human [19] sources have shown excellent conservation of amino acid sequences and activities toward a variety of substrates. Therefore, the large difference between the activities of rat and human cathepsin H toward **1** warranted further investigation.

In an effort to describe more adequately the characteristics of **1** in the human situation, we incubated **1** with a homogenate of the human colon cancer carcinoma cell line SW 480. The enzyme activity of the homogenate was characterized using the cathepsin B and H assays described. The assays indicated the presence of apparent endopeptidase activity; however, the homogenate showed no aminopeptidase activity toward the Leu-NAp substrate. Incubation of **1** with the cell line homogenate resulted in what would seem to be a modest conversion of **1** to 5-FU (12.8%). This conversion is, in fact, highly remarkable since the homogenate possessed no aminopeptidase assay activity toward the Leu-NAp assay substrate. This result strongly suggests that there is another enzyme(s) present in cells that is capable of converting **1** to 5-FU, but has no activity toward the aminopeptidase assay substrate, Leu-NAp. Moreover, judging from the tritosome experiments reported in Ref. 7, this enzyme(s) very likely exists within the lysosomal compartment.

The results from the incubation of polymers **2** and **3** with human cathepsin B produced no 5-FU or 5-FU derivatives. These results correspond well with those from the incubation of these polymers with tritosomes, and were not studied further.

The incubation of polymers **4** and **5** with cathepsin B did produce some interesting results. Incubation of polymer **4**

**TABLE 1. Results of the incubation of 5-FU-containing compounds 1 through 5a with various enzymes**

| Cmpd. | Enzyme(s) | mM Produced/24 hr | Mean % Release |
|---|---|---|---|
| 1 | Cathepsin H | $9.19 \times 10^{-3} \pm 4.71 \times 10^{-4}$* | 1.14 (50.6)† |
| 1 | SW 480 homogenate | $1.02 \times 10^{-1} \pm 4.93 \times 10^{-3}$ | 12.8 |
| 2 | Cathepsin B | 0 | 0 (0) |
| 3 | Cathepsin B | 0 | 0 (0) |
| 4 | Cathepsin B | 0 | 0 (63) |
| 5 | Cathepsin B | 0.682 | 68.2 (100) |
| 5a | Cathepsin B | $1.04 \pm 2.03 \times 10^{-2}$ | 100 (100) |

* Values are means ± SD, N = 3.

† Numbers in parentheses reflect results reported in Ref. 7 for the same compounds using rat liver tritosomes.

with cathepsin B produced no 5-FU or 5-FU-containing derivatives, whereas its incubation with tritosomes released 63% of the polymer-bound 5-FU only as a dipeptide derivative. This result was unexplained given the known X-ray crystalline structure of cathepsin B [20]. The $S'_1$ subsite in the cathepsin B subsite should readily accept an Ala residue. It is possible that the Ala side chain is not large enough to provide adequate interaction to facilitate cleavage. Since the incubation of polymer **4** with tritosomes produced 5-FU only as a dipeptide derivative and not as the free drug, it was not studied in further detail. The incubation of polymer **5** with cathepsin B resulted in a 68.2% cleavage of 5-FU (in the form of **1**) from the polymer. While this was not as quantitative as the 100% release achieved from tritosomes, it was certainly promising enough to justify closer investigation in the form of polymer **5a**.

The result of the incubation of polymer **5a** with cathepsin B over 24 hr is shown in Fig. 2. The curve corresponds well to that resulting from the incubation of **5a** with tritosomes with the exception that only compound **1** and no free 5-FU was produced. Since cathepsin B acts as an apparent endopeptidase [10], no free 5-FU was expected to be produced, and therefore the results agree with our original hypothesis. The Michaelis–Menten kinetic constants and the kinetic rate constant, $k$, associated with the release of **1** from **5a** via the apparent endopeptidase activity of cathepsin B were: $V_{max} = 3.73 \times 10^{-2} \pm 1.31 \times 10^{-3}$ mmol/L · min, $K_m = 8.12 \times 10^{-2} \pm 4.04 \times 10^{-3}$ mM, $k_{cat} = 0.459$ min$^{-1}$, and $k = 0.168$ hr$^{-1}$. The kinetic rate constant, $k$, resulting from the same experiments conducted with tritosomes [7] was 1.24 hr$^{-1}$. While the kinetic rate constant reported previously for tritosomes is an order of magnitude higher than that resulting from the same experiment using cathepsin B, the important overall release percentage (100%) remains after 24 hr.

Finally, the incubation of **5a** with an artificial mixture of cathepsins B and H for 24 hr produced promising results. While the concentration of cathepsin B used in the incubation mixture was the same as that used to produce Fig. 2, the amount of cathepsin H used was 8 times that used for the incubation of **1** with just cathepsin H. This increase in cathepsin H concentration was executed in order to assess whether an increase in cathepsin H concentration would correspond as predicted to an increase in the conversion of compound **1** to 5-FU. There was indeed an increase in the conversion of **1** to 5-FU (from 1.14 to 4.98%). While the increase in conversion was not directly proportional to the increase in the enzyme concentration, the experiment demonstrated that cathepsin H has specific activity toward **1**. The most probable reason for the disproportional increase for the conversion of **1** to 5-FU (4.4 times) with the increase in cathepsin H concentration (8 times) is that the concentration of **1** is zero at the start of the experiment and increases only as **1** is released from the polymer by cathepsin B, whereas in the experiment containing only **1** and cathepsin H, the concentration of **1** is at a maximum (0.8 mM) at the start of the experiment.

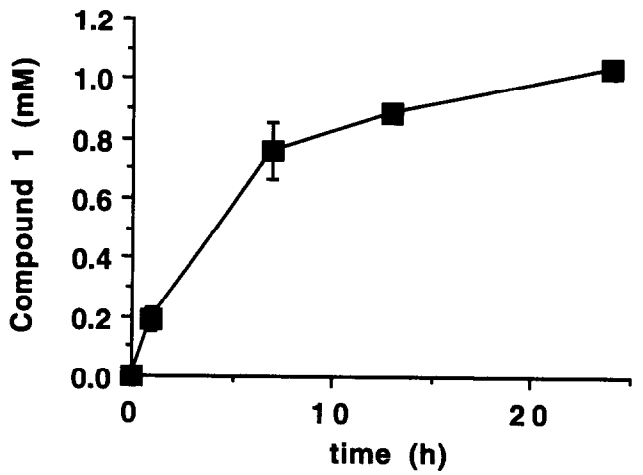



**FIG. 2. Release of compound 1 from polymer 5a via cathepsin B activity. Values are means ± SD, N = 3.**

In conclusion, the hypothesis that the lysosomal enzyme catalyzed release of 5-FU from the described 5-FU derivatives proceeds in two steps, first via apparent endopeptidase activity (i.e. cathepsin B) and second via aminopeptidase activity (i.e. cathepsin H), is supported by the experimental evidence. However, the experimental evidence also indicates the presence of additional lysosomal enzymes that also have catalytic activity toward these compounds. The identification of other lysosomal enzymes capable of cleaving these 5-FU substrates continues to be a focus of investigation in our laboratory.

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