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# COMPOUNDS THAT INHIBIT CHYMOTRYPSIN AND CELL REPLICATION

BARRY GOZ* and CHANDANA GANGULI†

Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, NC 27514, U.S.A.

MARYSABEL TROCONIS‡, STEVEN WYRICK and KHALID S. ISHAQ

Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27514, U.S.A.

and

JOHN A. KATZENELLENBOGEN

School of Chemical Sciences, University of Illinois, Urbana, IL 61801, U.S.A.

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**Abstract**—Several compounds have been tested for their ability to inhibit bovine pancreatic α-chymotrypsin ($K_i$) and their ability to inhibit cell replication ($IC_{50}$). There is good agreement over three orders of magnitude between the $K_i$ and the $IC_{50}$ values of these compounds. The data support the hypothesis that a cellular, chymotrypsin-like activity is necessary for cell replication.

A few years ago there was debate in the literature as to whether the compound L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) inhibited cell replication by inhibiting a chymotrypsin-like activity in cells. The initial reports [1, 2] attributed the inhibition of replication to inhibition of chymotrypsin-like activity but other investigators produced evidence that TPCK was also an inhibitor of protein synthesis and that this latter activity could account for the replication block [3–5].

More recently an extensive group of compounds that inhibit chymotrypsin has been synthesized. These compounds have a wide range of potencies in this regard and differ from TPCK in mechanism of enzyme inhibition. One type, the phenyl ethane boronic acid compounds, are believed to form a complex with the active site of chymotrypsin that resembles the transition states for the enzyme's catalysis of substrates [6]. The other type, the haloenol lactones, are enzyme-activated irreversible chymotrypsin inhibitors [7]. These are designed to act as suicide substrates in which the most effective compounds have properties that approach the ideal of stoichiometric inactivation. With these compounds, we have reexamined the issue of whether a chymotrypsin-like activity in cells may affect cell replication.

* Author to whom correspondence should be sent.

† Present address: The North Carolina School of Science and Mathematics, 1912 West Club Blvd. Durham, NC 27705.

‡ Present address: Organic Chemistry-Department of Chemistry, Pharmacy Faculty, Central University of Venezuela, Caracas, Venezuela.

## METHODS

*Cell culture and* $IC_{50}$ *determination.* The Lewis lung mouse tumor cell line, obtained from Ms. Elizabeth A. Dulmadge and Dr. Lee J. Wilkoff of the Southern Research Institute, Birmingham, AL, was used for testing [8]. The cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, non-essential amino acids, and double the vitamin concentration. Cells were transferred or removed from monolayers for counting by a 1-min incubation with 0.02% sodium ethylenediamine tetracetate in phosphate-buffered saline.

Flasks were seeded at a density of $1 \times 10^3$ cells/cm$^2$, and after about 24 hr the compounds to be tested were added. All of the test compounds were dissolved in dimethyl sulfoxide (DMSO) at concentrations that would give a final DMSO concentration of 1%. Control flasks contained 1% DMSO. Control cell replication was followed until approximately three doublings had occurred, and then all of the treated cells were counted. The compound concentration for 50% inhibition of replication ($IC_{50}$) was determined graphically from these results.

$K_i$ *determination.* The $K_i$ values of the compounds were determined with bovine pancreatic α-chymotrypsin (salt free, 3X crystallized, 58 units/mg protein) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate (Sigma Chemical Co., St. Louis, MO).

The assay mixture contained 40 mM Tris–HCl (pH 7.8), 50 mM $CaCl_2$, BTEE at concentrations of 0.125 to 0.34 mM and 0.068 units/ml of chymotrypsin. The increase in absorbance of 256 nm with time was followed. The reference cuvette contained all of the above except the enzyme. The inhibitors were

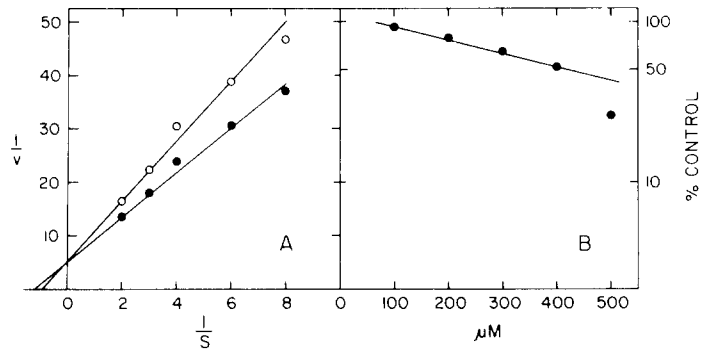


Fig. 1. Double-reciprocal and replication inhibition plots for determination of ortho-chloro-phenyl ethane boronic acid $K_i$ for chymotrypsin and $IC_{50}$ for Lewis lung cells. (A) Double-reciprocal plot for the unhibited (●) and inhibited (153 μM) reaction (○). The reciprocals of the activity ($1/v$) and the substrate concentration ($1/S$) were plotted. The values are the average of two separate determinations. (B) $IC_{50}$ plot. The ordinate is the log of the percent of control cell number after the control cells have doubled three times. The abscissa is the inhibitor concentration. Three separate determinations are averaged.

dissolved in DMSO at a concentration that yielded a final DMSO concentration of 3% in the cuvette. This concentration of DMSO alone did not alter chymotrypsin activity. The $K_i$ was determined by a double-reciprocal plot of the reaction velocities and substrate concentrations to determine the slopes of the uninhibited ($a$) and inhibited reactions ($i$). $K_i$ was calculated from $(I)a \cdot (i-a)^{-1}$ where $I$ is the inhibitor concentration [9].

## RESULTS

Representative plots for $K_i$ and $IC_{50}$ determination are depicted in Fig. 1A and 1B respectively. Table 1 tabulates the $K_i$ values of the various compounds for inhibition of bovine pancreatic α-chymotrypsin as well as the $IC_{50}$ values of some of these compounds. These compounds differ widely in their potency as chymotrypsin inhibitors from the most potent, *R*-1-carbobenzoxy-alanyl-2-phenyl ethane boronic acid (K4) (0.32 μM) [10] to the least potent *p*-methoxy phenyl ethane boronic acid (*p*-methoxy-PEBA) (14 mM). Some structure–activity relations emerged from these studies with regard to the phenyl ethane boronic acid (PEBA) series. PEBA had a $K_i$ of 481 μM. Substitution at the para position of the phenyl ring decreased potency probably related to the bulk of the substitution. Para-methyl PEBA was similar to PEBA in potency but the bulkier substitutions *p*-ethyl, *p*-isopropyl and *p*-methoxy increased the $K_i$ values to 1.8, 3.9 and 14 mM respectively. A methoxy group at the meta position seemed to, if anything, increase the potency. Substitution of the halogens chlorine or fluorine at the para position definitely increased the inhibitory potency and, in

Table 1. $K_i$ and $IC_{50}$ values for boronic acids and a haloenol lactone inhibitor of bovine pancreatic chymotrypsin

| Compound | $K_i$ | $IC_{50}$ |
|---|---|---|
| Phenyl ethane boronic acid (PEBA) | 481 μM | 773 μM |
| *p*-Chloro-PEBA | 52 μM | 140 μM |
| *o*-Chloro-PEBA | 93 μM | 425 μM |
| *p*-Fluoro-PEBA | 142 μM | 575 μM |
| *p*-Methyl-PEBA | 469 μM | ND |
| *p*-Ethyl-PEBA | 1.8 mM | ND |
| *p*-Isopropyl-PEBA | 3.9 mM | ND |
| *p*-Methoxy-PEBA | 14 mM | ND |
| *o*-Methoxy-PEBA | 625 μM | ND |
| *m*-Methoxy-PEBA | 340 μM | ND |
| Phenyl propane boronic acid | 1.6 mM | 1.1 mM |
| Naphthyl ethane boronic acid | 18.8 μM | ND |
| Cyclohexyl ethane boronic acid | 2.4 mM | 530 μM |
| *R*-1-Carbobenzoxy-alanyl-2-phenyl ethane boronic acid (K4) | 0.32 μM | 0.24 μM |
| 3-(1-Naphthyl)-5-(E)-(bromomethylidene) tetrahydrofuranone (K2) | 17.3 μM | 44.3 μM |

The $K_i$ and $IC_{50}$ values are each the average of three separate experiments. ND, not determined.

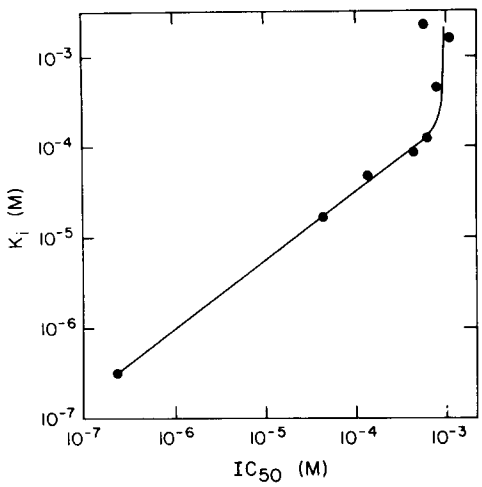


Fig. 2. Comparison of $K_i$ and $IC_{50}$ values of selected compounds. Each of the points was determined by at least three measurements of both the $K_i$ and the $IC_{50}$ and plotted on log scales for both parameters.

fact, ortho–chloro substitution also increased the potency. Possibly these halogen substitutions act by affecting the aromatic or electronic properties of the phenyl ring. The poor inhibitory activity of cyclohexyl ethane boronic acid agrees with this assessment as does the greater potency of napthyl ethane boronic acid. The distance between the phenyl group and the boron atoms seems important also for chymotrypsin inhibition since phenyl propane boronic acid was about three times less potent than PEBA. The potent inhibition by K4 was likely due to the greater complexity of structure and resemblance to the natural peptide substrates for chymotrypsin.

The $IC_{50}$ values also exhibited a wide range of potencies (0.24 μM to 1.1 mM). These values (Table 1) along with $K_i$ values are compared in Fig. 2, a log, log plot. What is most striking about Fig. 2 is that from K4 to *p*-F-PEBA the points appear to describe a straight line function. The points span a range of $K_i$ and $IC_{50}$ values of about three orders of magnitude. Furthermore, one of those points is 3-(1-naphthyl)-5-(E)-(bromomethylidene)tetrahydrofuranone (K2), a haloenol lactone that inhibits chymotrypsin by suicide inhibition [7] rather than as a transition state analog postulated for the boronic acid compounds [6, 11].

Beyond *p*-F-PEBA ($K_i$ = 142 μM) the linear relation ceased and in spite of increasingly poorer $K_i$ values (481 μM to 2.4 mM) the $IC_{50}$ concentrations did not increase proportionately. The inhibitory effect was not peculiar to the mouse Lewis lung cells. We observed similar inhibition of BHK (baby hamster kidney cells), BHK/SV28 cells (an SV40 transformed line of BHK cells) and KB cells (a human nasopharyngeal cancer) (Fig. 3). The inhibition of replication by the boronic acid compounds was fully reversible. BHK/SV28 cells treated for 24 hr with, for example, K4 (0.6 μM) and then assayed for viability by colony-forming ability were equal in viability (48% viable) to untreated control cells (47% viable). In this experiment, the control cells had increased in density from $4.7 \times 10^2$ cells/cm$^2$ to $2.3 \times 10^3$ cells/cm$^2$, while the density of the treated cells in the presence of K4 was unchanged (i.e. replication was inhibited completely) at $5.0 \times 10^2$ cells/cm$^2$.

The effect of 0.6 μM K4 on protein synthesis in BHK/SV28 cells was determined in three experiments (Table 2); K4 caused no inhibition of protein synthesis whether measured 1, 5 or 21 hr after addition. When protein synthesis (cpm) was normalized to a per $10^4$ cells basis, there was an apparent stimulation by K4 treatment which may reflect the cells continuing protein synthesis and enlarging without dividing. In the second experiment, 0.5 μg/ml cycloheximide was included as a control and, as can be seen, protein synthesis was inhibited. These findings are similar to those reported for another chymotrypsin inhibitor, chymostatin. Chymostatin has an $IC_{50}$ of 0.25 μM for inhibition of chymotrypsin [12], yet it does not inhibit protein synthesis of cultured mouse epidermal cells at 500 μM [13] or of isolated rat hepatocytes at 400 μM [14] although in the latter case it was reported that long term cell viability was affected, perhaps analogous to our observations.

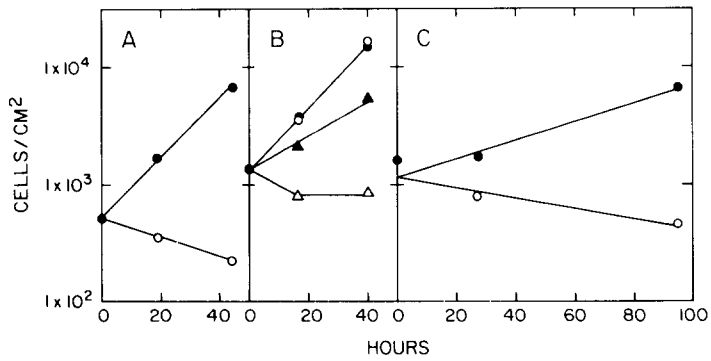


Fig. 3. *R*-1-Carbobenzoxy-alanyl-2-phenyl ethane boronic acid (K4) inhibition of cell replication. Cells were cultured and counted as described under Methods. (A) Baby hamster kidney cells (BHK) grown with no inhibitor (●) or with 0.8 μM K4 (○). (B) SV40 virus transformed BHK cells (BHK/SV28) grown with no inhibitor (●) or with 0.1 μM (○), 0.4 μM (▲) or 1.0 μM (△) K4. (C) Human KB cells (a nasopharyngeal carcinoma) grown with no inhibitor (●) or 0.6 μM K4 (○).

Table 2. Effect of *R*-1-carbobenzoxy-alanyl-2-phenyl ethane boronic acid (K4) on protein synthesis

| Treatment | Labeling period | cells/cm$^2$ | cpm/flask | cpm/$10^4$ cells | % Control |
|---|---|---|---|---|---|
| Experiment 1 | | | | | |
| Control | 1–2 hr | $1.2 \times 10^3$ | 3424 | 1038 | |
| 0.6 $\mu$M K4 | 1–2 hr | $8.9 \times 10^2$ | 3369 | 1531 | 147 |
| Control | 21–22 hr | $3.1 \times 10^3$ | 4205 | 539 | |
| 0.6 $\mu$M K4 | 21–22 hr | $1.1 \times 10^3$ | 2242 | 801 | 149 |
| Experiment 2 | | | | | |
| Control | 1–2 hr | $9.4 \times 10^2$ | 1035 | 431 | |
| 0.6 $\mu$M K4 | 1–2 hr | $8.1 \times 10^2$ | 998 | 499 | 116 |
| 0.5 $\mu$g/ml Cycloheximide | 1–2 hr | $9.7 \times 10^2$ | 638 | 266 | 62 |
| Experiment 3 | | | | | |
| Control | 5–6 hr | $2.2 \times 10^3$ | 178 | 32 | |
| 0.6 $\mu$M K4 | 5–6 hr | $1.4 \times 10^3$ | 178 | 51 | 159 |

The labeling period indicates the 1-hr interval after the addition of K4 (or cycloheximide) in which protein synthesis was measured by incorporation of radioactive amino acids into a trichloroacetic acid-insoluble fraction. In experiments 1 and 2, a $^{14}$C-labeled amino acid mixture was used at 0.36 and 0.12 $\mu$Ci/ml medium respectively. In experiment 3, [$^{14}$C]leucine was used at 0.4 $\mu$Ci/ml (1 $\mu$Ci/$\mu$mole). Both radioactive labels were from New England Nuclear Corp., Boston, MA. Duplicate flasks were used for each determination. At the end of the labeling period, the cells were harvested and precipitated with ice-cold 5% trichloroacetic acid, and the precipitates were collected by filtration on Whatman GF/A glass microfibre discs. The discs were washed three times with 5% trichloroacetic acid, once with 95% ethanol, dried, and counted by liquid scintillation.

## DISCUSSION

These data support the hypothesis that inhibition of a chymotrypsin-like activity in cells causes inhibition of cell replication. The range of $K_i$ values (three orders of magnitude) over which the $K_i$–$IC_{50}$ agreement holds is striking as is the fact that the relation appears to be held for two different types of inhibitors, boronic acids and a haloenol lactone. The loss of agreement between the two parameters with the poor chymotrypsin inhibitors is not surprising. It is not unreasonable to postulate that the poorer chymotrypsin inhibitors are less selective and that they inhibit cell replication by affecting other targets in addition to, or instead of, chymotrypsin-like activity.

Various reports in the literature describe chymotrypsin-like activity in cells. Indirect evidence comes from several studies with isolated hepatocytes. In these studies, the chymotrypsin inhibitor chymostatin or its analogs were shown to block protein degradation [14–17]. Chymotrypsin activities in these studies were shown to be located within and outside the lysosomes. A chymotrypsin-like activity has been found associated with the chromosomes of *Drosophila melanogaster* [18] and rat liver chromatin [19]. Hagiwara *et al.* [20] reported chymotrypsin-like activity associated with chromatin in various normal and tumor tissues of the rat. Of particular relevance to the data reported here is that Hagiwara *et al.* found that the chymotrypsin-like activity was significantly higher in rapidly proliferating cancer cells than in normal cells and, within normal cells, low growth fraction tissue, such as brain, the proteolytic activity was much lower than in, for example, thymus, which would be expected to have a greater fraction of dividing cells. Carter *et al.* [19] have demonstrated that some of the boronic acid compounds also inhibit protease activity in isolated rat liver chromatin. Their data indicated that PEBA is a more potent inhibitor of the chromatin protease than phenyl propane boronic acid similar to our results with chymotrypsin, but *p*-methyl-PEBA was much less inhibitory than PEBA. This latter finding differs from our data in which PEBA and *p*-methyl-PEBA have similar $K_i$ values.

Taken together the data support the hypothesis that the more potent and likely more selective inhibitors of chymotrypsin inhibit cell replication by inhibiting a chymotrypsin-like activity in cells. From the literature, a possible intracellular location of this activity is chromatin.

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