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# Characterisation of the Antitrypanosomal Activity of Peptidyl α-Aminoalkyl Phosphonate Diphenyl Esters

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**ABSTRACT.** Two groups of irreversible serine peptidase inhibitors, peptidyl chloromethyl ketones and peptidyl phosphonate diphenyl esters, were examined for antitrypanosomal activity against the bloodstream form of *Trypanosoma brucei brucei*. Both peptidyl chloromethyl ketones and peptidyl phosphonate diphenyl esters inhibited trypsin-like peptidases of the parasites and exhibited antitrypanosomal activity at micromolar concentrations. In live *T. b. brucei,* labelled analogues of both of these groups of inhibitors primarily targeted an 80-kDa peptidase, possibly a serine oligopeptidase known as oligopeptidase B. In an *in vivo* mouse model of infection, one of these inhibitors, carbobenzyloxyglycyl-4-amidinophenylglycine phosphonate diphenyl ester, was curative at 5 mg $kg^{-1}$ $day^{-1}$ but appeared toxic at higher doses. There was no significant correlation between the inhibitory potency (as evaluated against purified *T. b. brucei* oligopeptidase B) and the *in vitro* antitrypanosomal efficacy of either group of inhibitors, suggesting that these inhibitors were acting on multiple targets within the parasites, or had different cell permeability properties. These findings suggest that serine peptidases may represent novel chemotherapeutic targets in African trypanosomes. BIOCHEM PHARMACOL **60**;10:1497–1504, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** *Trypanosoma brucei*; oligopeptidase B; peptidyl phosphonate diphenyl ester; chemotherapy; serine peptidase inhibitor

African trypanosomes of the genus *Trypanosoma* are protozoan parasites that cause widespread disease in livestock (notably cattle) and humans, collectively referred to as African trypanosomiasis. *Trypanosoma brucei brucei,* together with *T. congolense* and *T. vivax,* are the etiological agents of bovine African trypanosomiasis (*nagana*). As a result of this disease, 10 million square kilometers of sub-Saharan Africa remain inhospitable for livestock production [1], and *nagana* is estimated to cost Africa US $5 billion annually [2]. The related trypanosomes *T. b. rhodesiense* and *T. b. gambiense* cause acute and chronic human African sleeping sickness, respectively. Africa is currently experiencing a significant resurgence in the incidence of these two forms of this disease [3]. Not only has the estimated number of new cases in the Democratic Republic of the Congo been claimed to be higher than the previous all-time high for the epidemics in the 1930s, but the mortality related to human African trypanosomiasis in some areas is estimated to be the same as that caused by AIDS [4].

Although four drugs are currently in routine use for the treatment of human African trypanosomiasis, only one of the agents, DL-α-difluoromethylornithine (Ornidyl®), is effective in treating advanced stages of the disease, and even this drug is effective only against *T. b. gambiense* [5]. Additionally, these treatments are costly and have considerable unpleasant side-effects [6]. This, together with recent reports of drug resistance by African trypanosomes [7, 8], underscores the importance of developing new and improved therapeutic strategies. Curiously, the mechanisms of the antitrypanosomal activity of most of these drugs are generally not understood (reviewed in Refs. 6 and 9).

During the course of our studies on the peptidases of African trypanosomes, we have identified, purified, and characterised a serine oligopeptidase (called OP-Tb,†† the

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†† *Abbreviations*: AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride; AMC, 7-amino-4-methylcoumarin; Boc, *t*-butoxycarbonyl; Cbz, $N^{\alpha}$-carbobenzyloxy; DCI, 3,4-dichloroisocoumarin; DMF, dimethylformamide;

*opdB* gene product) from *T. b. brucei* [10, 11] and a related peptidase from *T. congolense* [12]. OP-Tb is a cytosolic enzyme that appears to be an intracellular target for the active ingredients of a number of antitrypanosomal drugs currently in widespread use, including pentamidine isethionate (Pentacarinat®), suramin sodium (Germanin®), and possibly diminazene aceturate (Berenil®) [13]. This suggested to us that serine peptidase inhibitors may represent a class of lead compounds for the development of novel chemotherapeutic agents.

While the cysteine peptidases of African and South American trypanosomes have received considerable attention as potential targets for antitrypanosome chemotherapy [14–16], trypanosome serine peptidases have largely been ignored. Here, we report the antitrypanosomal activity of irreversible serine peptidase inhibitors, notably peptidyl α-aminoalkyl phosphonate diphenyl esters [peptidyl$^{P}(OPh)_2$]. Our results suggest that OP-Tb is a potential intracellular target of these inhibitors in trypanosomes.

## MATERIALS AND METHODS

### *Parasites and Enzymes*

*T. b. brucei* strain ILTat 1.1 was employed throughout. The history of this strain, originally isolated in Utembo, Kenya, is described in Ref. 17. Parasites were grown in adult male Wistar rats prior to passage in mice or were cultured as described previously [11]. For OP-Tb purification, parasites were purified from infected rat blood by a combination of Percoll® isopycnic gradient centrifugation [18] and anion-exchange chromatography on DEAE–cellulose [19]. OP-Tb was purified as described previously [11], and active enzyme concentration was determined with 4-methylumbelliferyl-*p*-guanidobenzoate [20]. Adult male Wistar rats and adult male BALB/c mice were obtained from the Biomedical Resource Center, University of Durban-Westville.

### *Specialised Reagents*

Peptidyl methyl ketones were obtained from the Sigma Chemical Co., Cambridge Research Biochemicals, or Bachem. Biotin–Arg$CH_2Cl$ was purchased from Biosyn. AEBSF was obtained from Boehringer Mannheim, and other general peptidase inhibitors were purchased from Sigma. Adipic acid monomethyl ester, 5-fluoresceinamine, EDC, and all common reagents and solvents were obtained from the Aldrich Chemical Co. Diphenyl (4-amidinophenyl)methanephosphonate dihydrochloride was synthesised as previously described [21].

EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fla-Adp-OH, *N*-(5-fluoresceinyl)-5-carbamoylpentanoic acid; Fla-Adp-Lys(Boc)-OH, $N^{\alpha}$-[(5-fluoresceinyl)-5-carbamoylpentanoyl]-ε-*t*-butyloxycarbonyllysine; Fla-Adp-Lys-(4-AmPhGly)$^{P}(OPh)_2$, diphenyl {$N^{\alpha}$-[*N*-(5-fluoresceinyl)-5-carbamoylpentanoyl]lysyl}amino(4-amidinophenyl)methanephosphonate; OP-Tb, oligopeptidase from *T. b. brucei;* PMSF, phenylmethanesulfonyl fluoride; PSG, phosphate-buffered saline containing glucose; Suc, succinyl; and Tos, toluene-*p*-sulfonyl.

**SYNTHESIS OF FLA-ADP-OH.** Adipic acid monomethylester (0.17 mL, 0.58 mmol) was dissolved in 2 mL DMF and kept at −10°. Thionyl chloride (0.10 mL, 1.4 mmol) was added and stirred for 10 min at −10°. A solution of 5-fluoresceinamine in 2 mL DMF was added and stirred overnight. The solvent was removed *in vacuo*. The residue was dissolved in EtOAc. The EtOAc layer was washed with 10% citric acid, water, and saturated NaCl. After drying with $Na_2SO_4$ and removal of the solvent, the residue was purified on a silica gel column eluted with $CHCl_3$:MeOH (7:1). Fractions with $R_f$ = 0.31 were collected and concentrated to give an orange oily residue. Ethyl ether was added to give Fla-Adp-OMe as a yellow solid: yield 29%; one spot on TLC ($R_f$ = 0.46, $CHCl_3$:MeOH:HOAc, 90:10:1); MS (FAB$^+$) *m/e* 490 (M + H).

Fla-Adp-OMe (95 mg, 0.19 mmol) was dissolved in 2 mL MeOH. A solution of 1 M NaOH (0.58 mL) was added, and the mixture was stirred at room temperature overnight. Most of the MeOH was removed under reduced pressure, and the aqueous solution was placed in an ice bath. Drops of diluted HCl were added with stirring until the mixture became just acidic (pH 2–3) and an orange solid came out. EtOAc was added to the orange suspension and washed with saturated NaCl. After drying with $Na_2SO_4$ and removal of the solvent, the residue was triturated with ethyl ether to give Fla-Adp-OH as a yellow solid: yield 91%; one spot on TLC ($R_f$ = 0.16, $CHCl_3$:MeOH:HOAc, 90:10:1); high-resolution FAB–MS, *m/e* (M + H) calculated 476.1345, found 476.1306. Anal. Calc. for $C_{26}H_{21}NO_8 \cdot 0.5H_2O$: C, 64.27; H, 4.58; N, 2.89. Found: C, 64.40; H, 4.94; N, 2.58.

**SYNTHESIS OF FLA-ADP-LYS(BOC)-OH.** ε-*t*-Butyloxycarbonyllysine methyl ester hydrochloride (65 mg, 210 μmol) was dissolved in 3 mL DMF. After neutralization by 1 equivalent of triethanolamine, Fla-Adp-OH (100 mg, 210 μmol) and 1 equivalent of EDC · HCl were added, and the mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, and the residue was dissolved in EtOAc. The EtOAc layer was washed with 10% citric acid and saturated NaCl. After drying with $Na_2SO_4$ and removal of the solvent, ethyl ether was added to give a yellow solid: yield 55%; one spot on TLC ($R_f$ = 0.33, $CHCl_3$:MeOH:HOAc, 90:10:1); MS (FAB$^+$) *m/e* 718 (M + H).

Fla-Adp-Lys(Boc)-OMe (50 mg, 70 μmol) was dissolved in 2 mL DMF. A solution of 1 M NaOH (0.35 mL) was added, and the mixture was stirred at room temperature for 6 hr. The solvent was removed *in vacuo*, and 10% citric acid was added to the residue in an ice bath until the mixture became acidic (pH 2–3). The desired product was extracted with EtOAc and washed with saturated NaCl. After drying with $Na_2SO_4$ and removal of the solvent, ethyl ether was added to give a yellow solid: yield 93%; MS (FAB$^+$) *m/e* 704 (M + H).

SYNTHESIS OF FLA-ADP-LYS-(4-AMPHGLY)$^{P}$(OPH)$_2$. Fla-Adp-Lys(Boc)-OH (50 mg, 70 μmol) and 2HCl · (4-AmPhGly)$^{P}$(OPh)$_2$ (32 mg, 70 μmol) were dissolved in 3 mL DMF followed by the addition of 1 equivalent of triethanolamine. HOBt (14 mg, 1.5 equivalents) and EDC · HCl (16 mg, 1.2 equivalents) were added, and the mixture was stirred at room temperature overnight. The solvent was removed *in vacuo,* and the residue was purified on a silica gel column eluted with $CHCl_3$:MeOH:HOAc (40:10:1). Fractions containing the desired product were collected and concentrated under reduced pressure. Ethyl ether was added to give a yellow solid: yield 47%; high-resolution FAB–MS, *m/e* (M + H) calculated 1067.3956, found 1067.3929.

Fla-Adp-Lys(Boc)-(4-AmPhGly)$^{P}$(OPh)$_2$ (30 mg, 27 mmol) was dissolved in 1 mL trifluoracetic acid. After 30 min of treatment at room temperature, the solvent was removed under reduced pressure. Ethyl ether was added to the residue to give a yellow solid: yield 77%; high-resolution FAB–MS, *m/e* (M + H) calculated 967.3472, found 967.3408.

### *Assays of Enzyme Inhibition*

OP-Tb activity was routinely measured against Cbz-Arg-Arg-AMC (5 μM) in 50 mM Tris–HCl, pH 8, at 37°. The effects of irreversible peptidase inhibitors were investigated by adding an aliquot of inhibitor (always in a final volume of 10 μL) to a buffered enzyme solution (140 μL, containing 50 ng OP-Tb in 50 mM Tris–HCl, pH 8.0, 37°) to initiate inactivation [22]. Peptidyl chloromethyl and diazomethyl ketone inhibitors were assayed at a final inhibitor concentration of 10 μM. The assay concentrations of peptidyl phosphonate diphenyl ester inhibitors ranged between 141 and 232 μM. Aliquots were removed at timed intervals, and residual activity ($v_t$) was determined against Cbz-Arg-Arg-AMC as described above. Pseudo-first-order inhibition rate constants ($k_{obs}$) were obtained from plots of $\ln v_t/v_0$ versus time, where $v_0$ represents the activity prior to the addition of inhibitor. Apparent second-order inhibition rate constants ($k_{ass}$) were obtained from the relationship $k_{ass} = k_{obs}/[I]$, where [I] represents the inhibitor concentration. The time required for the free enzyme concentration to decrease by 50% (half-life, $T_{1/2}$) is given by $T_{1/2} = 0.693/k_{ass}[I]$ [23].

### In Vitro *Antitrypanosomal Assays*

*In vitro* culture of *T. b. brucei* was undertaken in Minimal Essential Medium, supplemented with 0.3 g $L^{-1}$ L-glutamine, 0.25 mM cysteine, 0.01 mM bathocuproinedisulfonic acid, and 15% (v/v) fetal bovine serum. Cultures (2.5 mL) were maintained at 37° in a humidified atmosphere containing 5% (v/v) $CO_2$ [24]. To test the antitrypanosomal activity of general peptidase inhibitors, *T. b. brucei* was cultured as described above, and inhibitor stock solution (25 μL; 100 mM in DMSO) was added. Control cultures were incubated in the absence of the drug, but in the presence of 1% (v/v) DMSO. Cell numbers were determined manually with a hemocytometer (twice per culture dish) after a 24-hr incubation in the presence of the inhibitors. Trypanosomes were seeded at a density of $1 \times 10^5$ cells $mL^{-1,}$ and control cultures reached a density of $9.2 \times 10^5$ cells $mL^{-1}$ after 24 hr. To determine the antitrypanosomal efficacy of peptidyl chloromethyl ketones and peptidyl phosphonate diphenyl esters, these agents were added at various concentrations to a constant number of trypanosomes in culture, with the final DMSO concentration maintained at 1% (v/v) in experimental and control samples. Cell numbers were determined after 24 hr with a hemocytometer (twice per culture dish), and the data were analysed graphically by plotting cell numbers versus drug concentration. The effective concentrations that inhibited growth of trypanosome populations by 50% compared with controls ($EC_{50}$) were obtained from these plots.

### *Active-Site Labelling of Trypanosome Peptidases*

Parasites and purified OP-Tb were labelled with either biotin-$ArgCH_2Cl$ or Fla-Adp-Lys-(4-AmPhGly)$^{P}$(OPh)$_{2.}$ For active-site labelling with biotin-$ArgCH_2Cl$, purified OP-Tb (1 μg, 20 μL) was incubated (1 hr, 37°) in assay buffer (50 mM Tris–HCl, pH 8; 20 μL) with a 20-fold molar excess of biotin–$ArgCH_2Cl$ (0.25 nmol; 2.5 μL of a 100 μM stock solution in DMSO), after which non-reducing sample treatment buffer (20 μL; [25]) was added, and samples were boiled for 10 min. Additionally, *T. b. brucei* ($5 \times 10^6$ cells $mL^{-1}$) were cultured as described above in the presence of biotin-$ArgCH_2Cl$ (100 μM) for 24 hr. Cells were pelleted by centrifugation (3000 *g,* 10 min, 4°), resuspended in PSG [11], washed by three successive cycles of pelleting by centrifugation, and then resuspended in PSG. Cells were lysed by the addition of non-reducing sample treatment buffer (20 μL) and boiled for 10 min. Samples were subjected to Tris–Tricine SDS–PAGE and electroblotted onto nitrocellulose [25] in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid/10% (v/v) methanol, pH 11 [26]. Blots were developed with a Streptavidin®-alkaline phosphatase conjugate (1:50,000 dilution; Sigma) using 0.15 mg $mL^{-1}$ 5-bromo-3-chloro-indolylphosphate and 0.3 mg $mL^{-1}$ nitroblue tetrazolium, in 100 mM Tris–HCl, 5 mM $MgCl_{2,}$ pH 9.5, as substrates.

For active-site labelling with Fla-Adp-Lys-(4-AmPhGly)$^{P}$(OPh)$_{2,}$ purified OP-Tb (100 ng, 20 μL) was incubated (1 hr, 37°) in assay buffer (20 μL) with a molar excess of Fla-Adp-Lys-(4-AmPhGly)$^{P}$(OPh)$_2$ (58 μM final concentration, 2.5 μL of a 1 mM stock solution in DMSO), after which unbound probe was removed with a series of dilution/concentration cycles in 3-mL polysulfone concentrators (10-kDa cut-off, Millipore). Labelled OP-Tb was diluted in non-reducing sample treatment buffer (20 μL), boiled for 10 min, and subjected to Tris–Tricine SDS–PAGE. For labelling of live parasites, *T. b. brucei* ($5 \times 10^6$ cells $mL^{-1}$) were cultured in the presence of Fla-Adp-Lys-(4-AmPhGly)$^{P}$(OPh)$_2$ (100 μM, 2 hr), washed, and pel-

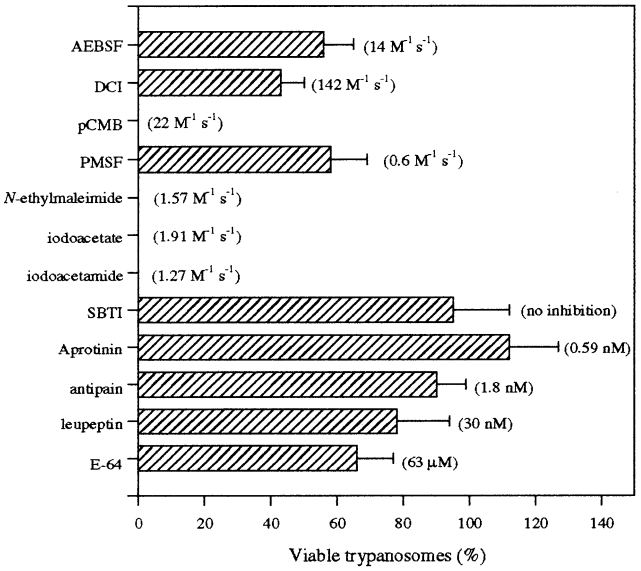


**FIG. 1. Trypanocidal activity of protease inhibitors. Bars indicate the fraction of viable cells after 24 hr relative to uninhibited controls (which contained an equivalent amount of the relevant inhibitor solvent). Numbers in parentheses indicate the $K_i$ of reversible inhibitors for OP-Tb inhibition (units of concentration, from Ref. 10) or the $k_{ass}$ of irreversible inhibitors for OP-Tb inhibition ($M^{-1}$ $sec^{-1}$, from Ref. 10). All inhibitors were used at 1 mM, with the exception of aprotinin (140 μM) and soybean trypsin inhibitor (200 μM). Bars represent mean percent (N = 3) ± SD.**

leted as described above. Cells were lysed in 0.1% (v/v) Triton X-100 in 100 mM Tris–HCl, pH 7.4, and subjected to three-phase partitioning as described previously [11]. The 10–50% $(NH_4)_2SO_4$ interfacial pellet was resuspended in non-reducing sample treatment buffer, boiled for 10 min, and resolved by Tris–Tricine SDS–PAGE. Fluorescent-labelled proteins were visualised on a Fotodyne Foto UV transilluminator (Fotodyne).

### In Vivo *Antitrypanosomal Assays*

The effect of Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ on experimental *T. b. brucei* infection was evaluated initially by injecting three groups of six BALB/c mice intraperitoneally with *T. b. brucei* ($6 \times 10^3$ parasites per mouse, in 100 μL PSG) and with Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ (either 1, 0.1, or 0.01 mg per mouse, in 25 μL DMSO). A control group of six mice received *T. b. brucei* ($6 \times 10^3$ cells per mouse, in 100 μL PSG) and DMSO (25 μL) alone (i.e. only trypanosomes and no inhibitors), and another six mice received PSG (100 μL) and DMSO (25 μL) alone (i.e. no trypanosomes and no inhibitors).

The effect of delayed, repeated administration of Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ on experimental infection was also examined. BALB/c mice were injected intraperitoneally with *T. b. brucei* ($6 \times 10^3$ cells per mouse, in 100 μL PSG). Three hours later, Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ in DMSO (25 μL) was administered intraperitoneally at three different concentrations (0.25, 0.1, or 0.005 mg per mouse; six mice per group). Thereafter, Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ in DMSO (25 μL) was administered daily at these three concentrations. Again, two sets of control groups were prepared as described above.

## RESULTS

### *Antitrypanosomal Activity of Peptidase Inhibitors*

A number of commercially available inhibitors of cysteine and serine peptidases were evaluated for activity against bloodstream-form *T. b. brucei* in a standard *in vitro* screen. With the exception of high molecular mass (protein) peptidase inhibitors, both classes of peptidase inhibitors were antitrypanosomal to varying degrees (Fig. 1). Of particular relevance to this study, three mechanism-based inhibitors of serine peptidases, without documented activity against cysteine peptidases, were able to reduce the number of viable parasites by 40–60% relative to controls over a 24-hr period. The antitrypanosomal efficacy of these three inhibitors, DCI, PMSF, and AEBSF, weakly correlated with their apparent second-order rate inhibition constant ($k_{ass}$) for OP-Tb (Fig. 1).

### *Kinetics of OP-Tb Inhibition by Irreversible Peptidase Inhibitors*

To further investigate the chemotherapeutic potential of OP-Tb inhibitors, three groups of peptidase inhibitors, peptidyl chloromethyl ketones (peptidyl-$CH_2Cl$), peptidyl diazomethyl ketones (peptidyl-$CHN_2$), and peptidyl phosphonates [peptidyl$^P(OPh)_2$], were first evaluated as inhibitors of purified OP-Tb. Members of all three groups of inhibitors, which contained basic amino acid residues in the $P_1$ position [arginine, lysine, or the arginine analogue 4-amidinophenylglycine (4-AmPhGly); subsite nomenclature of Ref. 27], exhibited inhibitory activity against OP-Tb (Tables 1 and 2). Peptidyl-$CH_2Cl$s and peptidyl-$CHN_2$s were approximately three orders of magnitude more potent as inhibitors of OP-Tb than were the inhibitors from the peptidyl$^P(OPh)_2$ group (Tables 1 and 2).

### *Antitrypanosomal Activity of Irreversible Peptidase Inhibitors*

Peptidyl-$CH_2Cl$, peptidyl-$CHN_2$, and peptidyl$^P(OPh)_2$ inhibitors of OP-Tb were also evaluated for antitrypanosomal activity against bloodstream-form *T. b. brucei* in a standard *in vitro* screen (Tables 1 and 2). Members of all three groups of inhibitors possessed antitrypanosomal activity, exhibiting $EC_{50}$ values in the lower micromolar range (27 to > 100 μM). Of the chloromethyl ketones, Tos-Lys$CH_2Cl$ (also called TLCK) was both the most potent inhibitor of OP-Tb ($k_{ass}$ = 5.23 ± 0.23 × $10^5$ $M^{-1}$ $sec^{-1}$) and the most effective ($EC_{50}$ = 27 ± 9 μM) in an *in vitro* antitrypanosomal assay. In contrast, Cbz-Glu-Gly-(4-AmPhGly)$^P(OPh)_2$ was the most potent OP-Tb inhibitor of the

**TABLE 1. Peptidyl chloromethyl and diazomethylketone inhibitors of OP-Tb**

| Inhibitor* | $k_{ass}$† ($\times 10^5 M^{-1} sec^{-1}$) | $T_{1/2}$‡ (sec) | $EC_{50}$§ ($\mu M$) |
|---|---|---|---|
| Tos-Lys$CH_2Cl$ | 5.23 ± 0.23 | 0.133 | 27 ± 9 |
| biotin-Arg$CH_2Cl$ | 4.45 ± 0.49 | 0.156 | 42 ± 7 |
| Boc-Val-Leu-Gly-Lys$CHN_2$ | 3.26 ± 0.36 | 0.212 | 38 ± 15 |
| Leu-Glu-Gly-Arg$CH_2Cl$ | 3.04 ± 0.27 | 0.227 | >100 |
| Cbz-Phe-Lys$CH_2Cl$ | 2.67 ± 0.08 | 0.260 | 32 ± 14 |
| Asp-Val-Phe-Lys$CH_2Cl$ | 2.47 ± 0.16 | 0.281 | >100 |
| Asp-Val-Leu-Lys$CH_2Cl$ | 2.07 ± 0.44 | 0.257 | >100 |
| Asp-Phe-Pro-Arg$CH_2Cl$ | 1.27 ± 0.39 | 0.546 | 67 ± 27 |

* No inhibition was detected after a 30-min preincubation with Ac-Ala-Ala-Ala-Ala$CH_2Cl$; Cbz-Gly-Gly-Phe$CH_2Cl$; Cbz-Gly-Leu-Phe$CH_2Cl$; Cbz-Leu-Leu-Met$CHN_2$; biotin-Phe$CH_2Cl$; Tos-Phe$CH_2Cl$; Cbz-Ala-Ala$CHN_2$; Cbz-Ile-Leu$CHN_2$; Cbz-Phe-Ala$CH_2Cl$; Cbz-Phe-Ala$CHN_2$; Cbz-Phe-Gly-Tyr$CHN_2$; Cbz-Phe-Phe$CH_2Cl$; Cbz-Phe-Phe$CHN_2$; Cbz-Phe-Tyr(OBut)$CHN_2$; or MeoSuc-Ala-Ala-Pro-Val$CH_2Cl$.

† Data reflect the mean $k_{ass}$ ± SD (N = 3).

‡ $T_{1/2}$ determined at a 10 $\mu M$ inhibitor concentration.

§ Data reflect the mean $EC_{50}$ ± SD (N = 3).

peptidyl$^P(OPh)_2$ group ($k_{ass}$ = 442 ± 43 $M^{-1}$ $sec^{-1}$), although it did not exhibit any *in vitro* antitrypanosomal activity at the concentrations tested.

### *Active-Site Labelling of Trypanosome Peptidases*

OP-Tb yielded a single 80-kDa band (Fig. 2) when it was active-site labelled with either biotin-Arg$CH_2Cl$ or Fla-Adp-Lys-(4-AmPhGly)$^P(OPh)_2$, a peptide phosphonate analogue with an attached fluorescein moiety. In contrast, incubation of live parasites with biotin-Arg$CH_2Cl$ yielded a major band at approximately 80 kDa and an extremely faint band at approximately 45 kDa, whereas Fla-Adp-Lys-(4-AmPhGly)$^P(OPh)_2$ yielded two labelled proteins of approximately 80 and 30 kDa (Fig. 2). The 80-kDa protein exhibited electrophoretic migration identical to purified, labelled OP-Tb. Biotin-Arg$CH_2Cl$ labelling of crude lysates of *T. b. brucei* yielded three major bands (120, 80, and 68 kDa; Fig. 2).

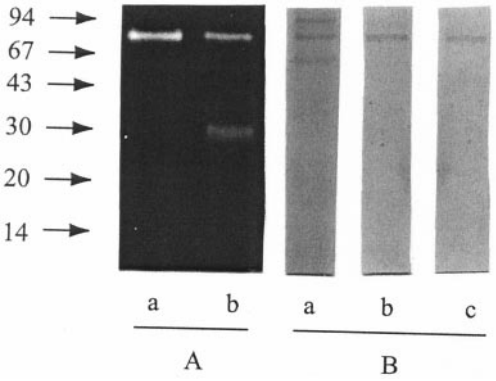


**FIG. 2. Identification of trypanosome peptidases targeted by irreversible peptidase inhibitors. (A) Labelling of purified OP-Tb (a) and live, cultured *T. b. brucei* (b) with Fla-Adp-Lys-(4-AmPhGly)$^P(OPh)_2$. (B) Labelling of (a) *T. b. brucei* lysates (100 $\mu g$), (b) purified OP-Tb (1 $\mu g$), and (c) live, cultured *T. b. brucei* with biotin-Arg$CH_2Cl$.**

### *Effect of Inhibitor Administration on Disease Progression in Mice*

Intraperitoneal administration of a single (total) dose of 1 or 0.1 mg per mouse of Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ increased the survival of mice that were challenged simultaneously with 6 × $10^3$ trypanosomes (Table 3). This compound completely cleared parasites from infected mice when administered daily at a dose of 5 mg per kg body mass (Table 3) after a single, initial inoculation of 6 × $10^3$ trypanosomes per mouse. At higher doses of this inhibitor (12.5 mg $kg^{-1}$ $day^{-1}$), 66% of the experimental group (N = 6) died by day 6 post-infection.

## DISCUSSION

The antitrypanosomal activity of cysteine peptidase inhibitors has been well documented in South American (for example, Ref. 15) and African (for example Ref. 16) trypanosomes. Indeed, the cysteine peptidase inhibitor E-64 exhibited antitrypanosomal activity in our *in vitro* screen (Fig. 1). Similarly, a number of thiol-reactive agents including iodoacetamide, iodoacetate, *N*-ethylmaleimide, and

**TABLE 2. Peptidyl phosphonate diphenyl ester inhibitors of OP-Tb**

| Inhibitor* | $k_{ass}$† ($M^{-1}sec^{-1}$) | $T_{1/2}$ (sec) | [I]‡ ($\mu M$) | $EC_{50}$§ ($\mu M$) |
|---|---|---|---|---|
| Cbz-Glu-Gly-(4-AmPhGly)$^P(OPh)_2$ | 442 ± 43 | 11 | 140 | >100 |
| Cbz-Gly-(4-AmPhGly)$^P(OPh)_2$ | 164 ± 27 | 30 | 140 | 47 ± 11 |
| Cbz-Ala-(4-AmPhGly)$^P(OPh)_2$ | 130 ± 19 | 23 | 230 | 51 ± 23 |
| Cbz-Lys-(4-AmPhGly)$^P(OPh)_2$ | 109 ± 8 | 45 | 141 | 62 ± 18 |
| Cbz-Pro-(4-AmPhGly)$^P(OPh)_2$ | 106 ± 27 | 29 | 229 | 72 ± 22 |
| Cbz-Phe-(4-AmPhGly)$^P(OPh)_2$ | 102 ± 12 | 31 | 221 | 42 ± 6 |
| Suc-(4-AmPhGly)$^P(OPh)_2$ | 12 ± 4 | 249 | 232 | >100 |

* No kinetic data were obtained for Fla-Adp-Lys-(4-AmPhGly)$^P(OPh)_2$ due to the intense fluorescence of this compound at the Cbz-Arg-Arg-AMC excitation and emmission wavelengths.

† Data reflect the mean $k_{ass}$ ± SD (N = 3).

‡ [I] = Inhibitor concentration at which $T_{1/2}$ was determined.

§ Data reflect the mean $EC_{50}$ ± SD (N = 3).

**TABLE 3. Effect of administration of Cbz-Gly-(4-AmPhGly)$^{P}$(OPh)$_2$ on the progression of trypanosomiasis in BALB/c mice**

| Treatment regimen and dose | Survival up to day 6 (number of mice surviving/ total number of mice treated) |
|---|---|
| Control 1 (+ parasites−inhibitor) | 0/6 |
| Control 2 (−parasites−inhibitor) | 6/6 |
| Coincident infection with parasites and administration of a single dose of inhibitor | |
| 1 mg* (±25 mg kg$^{-1}$) | 4/6 |
| 0.1 mg* (±2.5 mg kg$^{-1}$) | 5/6 |
| 0.01 mg (±0.25 mg kg$^{-1}$ | 0/6 |
| Single infection with parasites followed by daily administration of inhibitor, commencing 3 h after administration of the single parasite dose | |
| 12.5 mg kg$^{-1}$ day$^{-1}$ | 2/6 |
| 5 mg kg$^{-1}$ day$^{-1}$* | 5/6 |
| 0.1 mg kg$^{-1}$ day$^{-1}$ | 0/6 |

* All the surviving animals were still alive, and apparently free of parasites (as determined from a thick blood-film prepared from a tail-vein blood sample), 20 days post-infection.

*p*-chloromercuribenzoate were potently antitrypanosomal. Although these compounds are inhibitors of both trypanosomal cysteine [11] and serine [10] peptidases, they are highly reactive alkylating agents that would react with most sulfhydryl and some amino groups present within the trypanosome [28]. Therefore, it is highly unlikely that their antitrypanosomal activity is attributable to peptidase inhibition, but rather to the interference with intracellular thiol groups or trypanosome thiol metabolism (reviewed in Ref. 29). Our investigations with peptidase inhibitors also revealed that irreversible inhibitors of serine peptidases, in particular PMSF, AEBSF, and DCI, also possessed antitrypanosomal activity. The specific aims of this investigation were to determine the potential of irreversible serine peptidase inhibitors as antitrypanosomal agents, and to determine candidate intracellular targets of these inhibitors within African trypanosomes.

The peptidyl-$CH_2Cl$s are well-documented inhibitors of serine and cysteine peptidases [15, 16, 30, 31]. They covalently bind to the catalytic histidine and cysteine residues of serine and cysteine peptidases (reviewed in Ref. 31). The rates of inactivation of OP-Tb by peptidyl-$CH_2Cl$s were comparable to those observed for other trypsin-like serine peptidases, which also exhibited $k_{ass}$ values in the order of $10^5$ M$^{-1}$ sec$^{-1}$ [30]. Only peptidyl-$CH_2Cl$s with basic (arginine or lysine) residues in the $P_1$ position were inhibitory. These $P_1$ arginine- (or lysine-) containing peptidyl-$CH_2Cl$s were also antitrypanosomal at micromolar concentrations in *in vitro* culture. There was little correlation between the antitrypanosomal efficacy of these inhibitors and their rate of association with OP-Tb (Table 1), suggesting perhaps that if the antitrypanosomal effect was mediated, at least in part, through the inhibition of OP-Tb, these inhibitors were also acting on other targets within the trypanosome. This is not unreasonable, as these inhibitors also react with cysteine peptidases [31]. A lysosomal cysteine peptidase similar to mammalian cathepsin L, called trypanopain-Tb, has been described from *T. b. brucei* [11], and the antitrypanosomal activity of peptidyl chloromethyl and diazomethyl ketones, inhibitors of trypanopain-Tb, has been reported recently [16]. Inhibition of a cathepsin B-like 63-kDa cysteine peptidase from *T. b. brucei* by $P_1$-arginine chloromethyl ketones has also been described [32]. Another, alternate, target for these inhibitors would be the proteasome, which has also been described from *T. b. brucei* [33, 34]. However, when assessed by active-site blotting (Fig. 2), *T. b. brucei* cultured in the presence of biotin–Arg$CH_2Cl$ yielded a major single band corresponding to a molecular mass of ±80 kDa and a considerably weaker band at 45 kDa. This suggested to us that OP-Tb was a major intracellular target of $P_1$-arginine chloromethyl ketones.

Another possible cause for this disparity between enzyme inhibition and antitrypanosomal activity is variability in the cellular uptake and distribution of the inhibitors. It is likely that the different peptidyl-$CH_2Cl$ inhibitors have different cell-permeability or uptake properties. This is supported by our observation that, despite their similar abilities to inhibit OP-Tb, inhibitors containing negatively charged amino acid residues (i.e. aspartic or glutamic acid), in addition to the basic residues in $P_1$, had $EC_{50}$ values much greater than those observed for peptides lacking these residues (Table 1). This probably reflects the poor membrane permeability of these highly charged peptides. We are presently investigating the inhibitory and antitrypanosomal activity of these peptides after esterification or amidation of their acidic groups.

Because of the well-documented cross-reactivity of chloromethyl ketones with different classes of peptidases, and because of their comparatively short half-lives at physiological pH [31], we performed a similar study with class-specific inhibitors of serine peptidases that have enhanced physiological stability. Peptidyl$^{P}$(OPh)$_2$s represent one such class of inhibitors. These are class-specific inhibitors of serine

peptidases and have no documented activity against cysteine peptidases (reviewed in Ref. 35). We employed as inhibitors peptidyl$^{P}(OPh)_2$s containing the arginine analogue 4-amidinophenylglycine (4-AmPhGly) in $P_1$. These 4-AmPhGly derivatives are synthesised more easily and are more potent inhibitors than their arginine counterparts [21]. Furthermore, these inhibitors are extremely stable under physiological conditions (half-life $>$ 4 days at pH 7.5, and half-life $>$ 24 hr in plasma; [21]). Finally, they form extremely stable enzyme–inhibitor complexes, making them suitable for *in vivo* studies.

Compared with peptidyl-$CH_2$Cls, the peptidyl$^{P}(OPh)_2$s containing 4-AmPhGly in the $P_1$ position were considerably slower irreversible inhibitors of OP-Tb activity, which is consistent with their slower inhibition of other serine peptidases [35]. There was little difference between the $k_{ass}$ values between the various dipeptide inhibitors, irrespective of the residue in $P_2$. This is consistent with our observations that the $P_2$-binding site of OP-Tb can accommodate a wide range of amino acid side-chains [10]. However, a negatively charged group, such as that on the succinyl group, appears not to be so readily accommodated in the $P_2$-binding site. Lengthening of the peptide from a dipeptide to a tripeptide elevated the $k_{ass}$, perhaps suggesting that contact between the enzyme and peptide over the $P_3$–$P_1$ region is preferable to only $P_2$–$P_1$ interaction, and may help to "dock" the inhibitors for a longer period of time, or in a more favourable position, for phosphonylation of the active-site serine residue.

Despite the consistently lower $k_{ass}$ values for peptidyl-(4-AmPhGly)$^{P}(OPh)_2$s when compared with peptidyl-$CH_2$Cls, the peptidyl-$CH_2$Cls have comparable antitrypanosomal properties. This may be attributed to the greater stability of the peptidyl-(4-AmPhGly)$^{P}(OPh)_2$s at physiological pH when compared with peptidyl-$CH_2$Cls, which are unstable above pH 6 [31]. As with the peptidyl-$CH_2$Cls, no correlation was observed between the antitrypanosomal efficacy of peptidyl-(4-AmPhGly)$^{P}(OPh)_2$s and their rate of association with OP-Tb. This again suggested that these inhibitors had different cell-permeability properties, or that they were acting on multiple targets within trypanosomes. The latter suggestion is supported by our active-site labelling data. Culture of *T. b. brucei* in the presence of Fla-Adp-Lys-(4-AmPhGly)$^{P}(OPh)_2$ yielded two labelled proteins of approximately 30 and 80 kDa (Fig. 2). The 80-kDa protein exhibited electrophoretic migration identical to purified OP-Tb labelled with Fla-Adp-Lys-(4-AmPhGly)$^{P}(OPh)_2$, suggesting that OP-Tb was an important intracellular target of this inhibitor. A weaker band at 30 kDa may represent either a proteolytic degradation product of OP-Tb, or, more likely, another serine peptidase from *T. b. brucei* that has not yet been described. A serine peptidase of similar molecular mass (28 kDa) has been described from *Schistosoma mansoni* [36].

Studies in mice suggested that Cbz-Gly-(4-AmPhGly)$^{P}(OPh)_2$ had antitrypanosomal properties *in vivo*. This compound, when administered daily at a dose of 5 mg per kg body mass, was able to effect the complete clearance of parasites from infected mice (Table 3). All surviving animals were completely cleared of the infection, as determined by thick blood-films taken from the tail vein, and were still alive 20 days post-infection. At 50-fold lower concentrations, Cbz-Gly-(4-AmPhGly)$^{P}(OPh)_2$ had no observable effect on disease progression, and all experimental animals died at a time similar to that of the control animals that did not receive the inhibitor. Daily administration of higher quantities (12.5 mg per kg body mass) did not improve the proportion of mice that cleared infection, but rather led to the death of 66% of the experimental group. Due to the scarcity of material, the toxicity of these compounds in mice could not be investigated further. However, Cbz-Gly-(4-AmPhGly)$^{P}(OPh)_2$ is known to prolong the prothrombin time and the activated partial thromboplastin time [21], and it is possible that these compounds interfered with the haematological homeostasis of the mice, with lethal consequences.

Our results strongly suggest that the therapeutic potential of serine peptidase inhibitors warrants further study, especially as applied to African trypanosomiasis. Given the likelihood that OP-Tb is an intracellular target for these inhibitors, we are presently attempting to design alternative inhibitors with enhanced membrane permeability (which would facilitate crossing both the parasite plasma membrane and the blood–brain barrier) and improved specificity for oligopeptidase B. Unfortunately, the $P_4$–$P_1$ specificity of OP-Tb parallels that of many mammalian plasma serine peptidases, which seriously impedes the development of highly specific inhibitors with pharmacological potential. Nevertheless, an important difference between OP-Tb and these plasma peptidases is the acute sensitivity of OP-Tb to thiol-reactive agents [10], a property that is not exhibited by plasma serine peptidases. The development of novel chemotherapeutic agents that exploit this important characteristic of OP-Tb is currently underway in our laboratory. Furthermore, elucidation of the $P_1'$–$P_4'$ subsites of OP-Tb may reveal important differences in the P′ specificity of OP-Tb compared with host plasma peptidases, which may also be exploited in drug development. The identification of related enzymes in South American trypanosomes [37] and the malaria plasmodium [38] indicates that such work may also be extended to the development of new therapeutic approaches to the treatment of Chagas' disease and malaria.

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