7-But-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one
is a novel competitive and selective inhibitor of dipeptidyl peptidase IV with an
antihyperglycemic activity

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Running title: Antidiabetic effects of a novel dipeptidyl peptidase IV inhibitor

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Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; C.I., confidence interval; DPP, dipeptidyl peptidase; E3024, 3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4H-imidazo[4,5-d]pyridazin-4-one tosylate; ER-319711, 7-but-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one; GLP-1, glucagon-like peptide-1; IC₅₀, 50% inhibitory concentration; K579, (S)-1-[4-methyl-1-(2-pyrimidinyl)-4-piperidylamino]acetyl-2-pyrrolidinecarbonitile; Lys[Z(NO₂)] pyrrolidide, 4-nitrobenzyl[(5S)-5-amino-6-oxo-6-(1-pyrrolidinyl)hexyl]carbamate; OGTT, oral glucose tolerance test; P32/98, di-[(2S,3S)-2-amino-3-methyl-pentanoic-1,3-thiazolidide] fumarate; S.E.M., standard error of the mean

Recommended section: Endocrine and Diabetes
Abstract

7-But-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one (ER-319711) is a novel dipeptidyl peptidase (DPP)-IV inhibitor discovered in our laboratories. In this study, we have characterized this DPP-IV inhibitor \textit{in vitro} and \textit{in vivo} as an antidiabetic agent. ER-319711-15 (the trifluoroacetate salt form of ER-319711) inhibited human DPP-IV with a 50% inhibitory concentration (IC\textsubscript{50}) value of 0.089 $\mu$mol/L, while its IC\textsubscript{50} values towards human DPP8 and DPP9 were $>$100 $\mu$mol/L. Inhibition kinetic pattern analysis indicated that ER-319711-15 inhibited DPP-IV in a competitive manner. ER-319711-15 (1 mg/kg) reduced glucose excursion in an oral glucose tolerance test (OGTT) using Zucker \textit{fa/fa} rats, with significant increases in plasma insulin and active glucagon-like peptide-1 levels. In an OGTT using mice fed a high-fat diet in which ER-319711-15 (0.1 to 10 mg/kg) was orally administered at 0 hr, and glucose was loaded at 0 and 5 hr, this compound improved glucose tolerance dose-dependently at both 0 and 5 hr glucose loading. Next, we compared efficacy of ER-319711-15, E3024 (a competitive DPP-IV inhibitor having an imidazopyridazinone structure) or vildagliptin (a slow-binding and long-acting DPP-IV inhibitor) at the same dose (10 mg/kg) in the same procedures. At the first glucose challenge, all compounds lowered area under the curve (AUC) values of delta blood glucose between 0 and 2 hr significantly to the same degree. At the second glucose load, the AUC values between 5 and 7 hr were significantly decreased by ER-319711-15 and vildagliptin, but not by E3024. Therefore, ER-319711 might be a potent, competitive and selective DPP-IV inhibitor with an antihyperglycemic activity.
Introduction

Dipeptidyl peptidase (DPP)-IV degrades active glucagon-like peptide-1 (GLP-1 (7-36)amide and GLP-1(7-37)), which is an incretin released from L cells in the intestine after meal intake that enhances insulin secretion in a glucose-dependent manner. GLP-1 has an antidiabetic action in patients with type 2 diabetes (Gutniak et al., 1994; Nauck et al., 1993). DPP-IV cleaves GLP-1 rapidly so the latter’s half-life is only 1-2 min. Accordingly, the prevention of GLP-1 inactivation by DPP-IV inhibition is currently being actively explored as a novel approach to the treatment of type 2 diabetes (Deacon et al., 2004). DPP-IV inhibition leads to blood glucose-lowering effects in animal models of diabetes (Pederson et al., 1998; Reimer et al., 2002; Burkey et al., 2005), and in patients with type 2 diabetes (Ahrén et al., 2002; 2005).

7-But-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one (ER-319711) is a novel DPP-IV inhibitor discovered in our laboratories. Demuth et al. (2005) categorized DPP-IV inhibitors based on their mode of inhibition and structures as follows: reversible product analog inhibitors (e.g. P32/98 (Sorbera, et al., 2001)), covalently modifying product analog inhibitors (e.g. vildagliptin (LAF237) (Villhauer et al., 2003)), and reversible non-peptidic heterocyclic inhibitors (e.g. sitagliptin (MK-0431) (Kim et al., 2005)). ER-319711 belongs to the third group, and is a novel, 8-oxo-purine derivative.

In this study, we have characterized the inhibition mode for DPP-IV and enzyme selectivity towards DPP-IV, DPP8 and DPP9 of the newly discovered DPP-IV inhibitor, and investigated its antihyperglycemic activity using Zucker falfa rats and mice fed a high-fat diet. The high-fat diet-fed mouse model considered to be a robust model for impaired glucose tolerance and early type 2 diabetes (Winzell and Ahrén, 2004), both of which are targets of DPP-IV inhibitors.
Materials and Methods

Chemicals. The trifluoroacetate salt form of ER-319711 (ER-319711-15), E3024 (3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4H-imidazo[4,5-d]pyridazin-4-one tosylate) and vildagliptin (1-[[3-hydroxy-1-adamantyl]amino]acetyl]-2-cyano-(S)-pyrrolidine) were synthesized in our laboratories. 4-Nitrobenzyl[(5S)-5-amino-6-oxo-6-(1-pyrrolidinyl)hexyl]carbamate (Lys[Z(NO2)] pyrrolidide) was purchased from Bachem AG (Bubendorf, Switzerland), which is a non-selective DPP-IV/DPP8/DPP9 inhibitor ((Lankas et al., 2005), used as a reference. The chemical structures of these compounds are shown in Figure 1.

Inhibitory effects towards DPP-IV, DPP8 and DPP9. Purified human recombinant DPP-IV was purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant human DPP8 and DPP9 were expressed by baculovirus and purified. An enzyme was mixed with ER-319711-15 or Lys[Z(NO2)] pyrrolidide in an assay buffer (10 mM phosphate-buffered saline, 0.1% bovine serum albumin; pH 7.4). The enzyme reaction was started by an addition of 2 mM glycyl-L-proline \( p \)-nitroanilide tosylate (Peptide Institute, Inc., Osaka, Japan), and the changing of absorbance at 405 nm was monitored for 20 min with a microplate spectrophotometer (SpectraMax; Molecular Devices, CA). Three separate experiments were performed, and means of 50% inhibitory concentration (IC\(_{50}\)) values and 95% confidence interval (95% C.I.) were calculated.

Inhibitory kinetic analysis. Recombinant human DPP-IV activity was measured at varied concentrations of ER-319711-15 (0.1, 1, 10 and 100 \( \mu \)mol/L). For each concentration, measurements were conducted in the presence of varied concentrations...
of glyceryl-L-proline p-nitroaniline tosylate (0.006, 0.024, 0.10, 0.39, 1.56, 6.25, 25 and 100 mmol/L). Three separate experiments were performed in triplicate. The inhibitory pattern was evaluated by the Eadie Hofstee plot using a curve fitting program (GraphPad Software, Inc., San Diego, CA).

**Animals.** Male Crlj:ZUC-Lepr<sup>fa</sup> (Zucker fa/fa) rats and male C57BL/6NCrlCrlj mice were purchased from Charles River Japan (Tokyo, Japan). The rats and mice were provided with a commercial diet (MF, Oriental Yeast, Tokyo, Japan) and water *ad libitum* and were kept under conventional conditions of controlled temperature, humidity and lighting (22 ± 2°C, 55 ± 5% and a 12-hr light/dark cycle with lights on at 07:00 a.m.). All procedures were conducted according to the Eisai Animal Care Committee’s guideline.

**Plasma insulin and active GLP-1 levels in an oral glucose tolerance test (OGTT) using Zucker fa/fa rats.** ER-319711-15 (1 mg/kg) or vehicle (0.5% methyl cellulose (MC)) was orally administered to overnight-fasted Zucker fa/fa rats 0.5 hr prior to oral glucose load (2 g/kg). Blood samples were drawn from the tail vein -0.5, 0, 0.5, 1, 2 and 3 hr after the glucose load. In addition, about 250 µL of blood samples were collected with heparinized capillary tubes at 0, 0.5, 1 and 2 hr. After centrifugation, supernatants were assayed for plasma insulin and active GLP-1 levels. Plasma insulin levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Kanagawa, Japan) and rat insulin as a standard with the microplate spectrophotometer. Plasma active GLP-1 levels were determined using an ELISA kit (GLP-1 (7-36) Active ELISA Kit; Linco Research, Inc., St. Charles, MO).

Mice were fed a high-fat diet (D12492 Rodent Diet with 60 kcal% fat) for four weeks from 11 weeks of age, and 42 mice were selected based on absolute body weight and randomly divided into seven groups. ER-319711-15 (0.1, 0.3, 1, 3 and 10 mg/kg), vildagliptin (10 mg/kg) as a positive control, or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice at the same time of oral glucose administration (2 g/kg) (0 hr). After five hours, glucose was orally administered again. Blood samples were collected from the tail vein 0, 0.5, 1, 2, 5, 5.5, 6 and 7 hr after the compound treatment.

OGTT using mice fed a high-fat diet: comparison of efficacy between E3024, ER-319711-15 and vildagliptin. Mice were fed a high-fat diet (D12492 Rodent Diet with 60 kcal% fat; Research Diets, Inc., NJ) for four weeks from 11 weeks of age, and 24 mice were selected based on body weight and randomly divided into four groups. E3024 (10 mg/kg), ER-319711-15 (10 mg/kg), vildagliptin (10 mg/kg), or vehicle (0.5% MC) alone was orally administered to overnight-fasted mice at the same time of oral glucose administration (2 g/kg) (0 hr). After five hours, glucose was orally administered again. Blood samples were collected from the tail vein 0, 0.5, 1, 2, 5, 5.5, 6 and 7 hr after the compound treatment.

Blood glucose determination. Blood samples (10 µL) were collected from the tail vein and mixed with 140 µL of 0.6 mol/L perchloric acid. After centrifugation, the supernatants were assayed for glucose using an enzymatic assay kit (Glucose CII-test WAKO, Wako Pure Chemicals, Osaka Japan) with the microplate spectrophotometer.
Statistical analysis. Data are expressed as the mean ± standard error of the mean (S.E.M.). To determine the integrated glucose response to the glucose challenge, the area under the curve (AUC) of delta blood glucose after the glucose load was calculated using a trapezoidal rule (between 0 and 2 hr, and between 5 and 7 hr). Differences in the AUC values of delta blood glucose in an OGTT using mice between the vehicle- and E3024-, ER-319711- and vildagliptin-treated groups were determined by one-way analysis of variance (ANOVA), followed by Turkey type multiple comparison test. Differences in the AUC values of delta blood glucose in an OGTT using mice between the groups treated with vehicle or ER-319711 at several doses were determined by one-way ANOVA, followed by Dunnett type multiple comparison test. Difference in the AUC between the vehicle- and vildagliptin-treated groups was determined by unpaired Student’s t-test. The dose-responsiveness was evaluated using regression analysis.

In the study using Zucker fa/fa rats, differences in the AUC values of delta blood glucose between 0 and 3 hr and delta plasma insulin levels at 0.5 hr (that is, the differences between 0 and 0.5 hr) of the vehicle- and ER-319711-treated groups were analyzed using unpaired Student’s t-test. Difference of delta plasma active GLP-1 levels at 0.5 hr of the vehicle- and ER-319711-treated groups were determined using Mann-Whitney’s U-test.

A probability (p) value <0.05 (two-sided) was considered statistically significant. Statistical analyses were performed using an SAS software package version 8.1 (SAS Institute Japan Ltd., Tokyo, Japan).
Results

Table 1 summarizes IC\textsubscript{50} and 95% CI values of ER-319711-15 and Lys[Z(NO\textsubscript{2})] pyrrolidide towards DPP-IV, DPP8 and DPP9, and ratios of IC\textsubscript{50} values towards DPP8 or DPP9 to those towards DPP-IV. The mean IC\textsubscript{50} value of ER-319711-15 towards DPP-IV was 0.087 µmol/L, but no inhibitory activity was observed for DPP8 or DPP9 at up to 100 µmol/L. Lys[Z(NO\textsubscript{2})] pyrrolidide had an inhibitory effect towards DPP-IV with an IC\textsubscript{50} value of 0.214 µmol/L. But this inhibitor also had strong inhibitory activities towards both DPP8 and DPP9 with IC\textsubscript{50} values of 0.013 µmol/L and 0.064 µmol/L, respectively. Its IC\textsubscript{50} values towards DPP8 and DPP9 were more potent than towards DPP-IV by 16-fold and 3-fold, respectively (Table 1). To elucidate DPP-IV inhibition kinetic pattern of ER-319711, we conducted inhibition kinetics analysis with human recombinant DPP-IV. As illustrated in Figure 2, ER-319711-15 showed a competitive inhibition pattern well fitted to the Eadie Hofstee plot.

In the OGTT using Zucker \textit{fa/fa} rats, administration of ER-319711-15 (1 mg/kg) produced a significant decrease in glucose excursion (Fig. 3A), resulting in a significant decrease in the AUC values of delta blood glucose (Fig. 3B). We also determined plasma insulin and active GLP-1 levels. A clear elevation of plasma insulin concentrations was observed 0.5 hr after glucose administration (Fig. 3C). Delta plasma insulin levels at 0.5 hr were significantly higher in the ER-319711-treated rats than in the vehicle-treated rats (Fig. 3D). In the same way, a peak of plasma active GLP-1 levels was seen at 0.5 hr in the ER-319711 group, while no peak was detected in the vehicle group (Fig. 3E). Figure 5F indicates delta plasma active GLP-1 levels at 0.5 hr, showing a significant increase in active GLP-1 levels by the ER-319711-15 treatment (Fig. 3F).

We performed an OGTT in which we administered ER-319711-15 (0.1, 0.3, 1, 3
and 10 mg/kg) or vildagliptin (10 mg/kg) to mice fed a high-fat diet, and glucose was loaded at 0 and 5 hr (Figs. 4A and 4B). At the first glucose challenge, ER-319711-15 caused significant decreases in AUC values of delta blood glucose from 1 mg/kg. The AUC values of 3 to 10 mg/kg ER-319711-15 were similar to that of 10 mg/kg vildagliptin. At the second glucose load, a significant decrease in the AUC value from 3 mg/kg ER-319711-15 was observed, and efficacy of 10 mg/kg ER-319711-15 and 10 mg/kg vildagliptin was almost identical. Results of regression analysis indicated that ER-319711-15 lowered the AUC in a dose-dependent manner at both 0 hr and 5 hr glucose administration.

In an OGTT using mice fed a high-fat diet, we examined the efficacy of antihyperglycemic effects between ER-319711-15 (10 mg/kg), E3024 (10 mg/kg) and vildagliptin (10 mg/kg) (Figs. 5A and 5B). At simultaneous administration of a compound and glucose, ER319711-15, E3024 and vildagliptin lowered glucose excursions in almost the same degree. At 5-hr post glucose load, ER-319711-15 and vildagliptin reduced the AUC significantly, but a decrease in the AUC by E3024 was not significant.
Discussion

GLP-1, which is secreted in nutrient-dependent manner, stimulates glucose-dependent insulin secretion and regulates glycemia. However, half-life of active GLP-1 is very short due to degradation by DPP-IV, and GLP-1’s actions do not last long. Then, development of DPP-IV inhibitors has been active worldwide, which is expected to control blood glucose levels by enhancement of GLP-1’s action for the treatment of diabetes. In a course of research of novel DPP-IV inhibitors in our laboratories, we discovered a novel series of 8-oxo-purine derived DPP-IV inhibitors. In this study, we reported in vitro and in vivo characteristics of a representative of 8-oxo-derivative compounds, ER-319711-15.

Kinetics study indicated that ER-319711-15 inhibited DPP-IV competitively. In an OGTT using Zucker fa/fa rats, we confirmed that ER-319711-15 improved glucose tolerance, accompanied with increases in both plasma insulin and intact GLP-1 levels. These are characteristic actions of DPP-IV inhibitors. In addition, ER-319711-15 ameliorated glucose tolerance in mice fed a high-fat diet and this effect was dose-dependent.

A recent focus of research of DPP-IV inhibitors is on long-acting inhibitors, aiming at less dosing frequency. Vildagliptin, which is a slow-binding DPP-IV inhibitor, was dosed to patients with type 2 diabetes once daily, then improving glycemic control (Ristic et al., 2005). Following vildagliptin, K579 ((S)-1-[4-methyl-1-(2-pyrimidinyl)-4-piperidylamino]acetyl-2-pyrrolidinecarbonitrile) (Takasaki et al., 2004) and saxagliptin (BMS-477118) (Augeri et al., 2005) have been reported to be long-acting DPP-IV inhibitors, both of which also show slow-binding inhibition. In this study, we investigated efficacy of ER-319711-15, with comparison with E3024 and vildagliptin, in 0- and 5-hr postdose OGTT using mice fed a high-fat
diet, at the same dose of 10 mg/kg. E3024 is a selective and competitive DPP-IV inhibitor with an imidazopyridazinone structure discovered in our laboratories (Yasuda et al., in press). In an OGTT with a simultaneous administration of the compound and glucose (0 hr), all compounds showed drastic decreases in blood glucose to the same degree. In a 5-hr postdose OGTT, we observed an equal, significant glucose-lowering effect between 10 mg/kg ER-319711-15 and 10 mg/kg vildagliptin. E3024, however, did not manifest significant decrease in AUC values. Thus, ER-319711-15 might be a longer active competitive DPP-IV inhibition than E3024.

DPP-IV is a member of a family of serine peptidases including quiescent cell proline dipeptidase, DPP8 and DPP9. Acute and chronic administration of a DPP-IV inhibitor with DPP8/9 inhibition activity caused thrombocytopenia and splenomegaly in rats, and bloody diarrhea in dogs (Lankas et al., 2005). A selective DPP8/9 inhibitor caused the same signs, while no toxicity was observed in rats or dogs treated with a selective DPP-IV inhibitor, suggesting that inhibition of DPP8/9 leads to profound toxicity in preclinical studies. Lankas et al. (2005) suggested that preclinical assessment of clinical DPP-IV inhibitor candidates for DPP8/9 inhibition might be important. Our study showed that ER-319711-15 had an inhibitory activity for DPP-IV, but no activity for DPP8 or DPP9 at up to 100 µmol/L. On the other hand, Lys[Z(NO₂)] pyrrolidide inhibited not only DPP-IV, but also DPP8 and DPP9 strongly, as reported previously (Lankas et al., 2005). Accordingly, ER-319711-15 is a specific inhibitor to DPP-IV, and has a low risk of DPP8/9-related toxicity.

In summary, ER-319711-15 is a competitive and selective DPP-IV inhibitor with an 8-oxo-purine structure. Its glucose-lowering effect lasts longer than E3024’s. Thus, ER-319711 might be a novel potent DPP-IV inhibitor with antihyperglycemic activity.
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References


incretin hormone glucagon-like peptide 1 abolishes postprandial glycemia in NIDDM. *Diabetes Care* **17:**1039-1044.


Legends for Figures

Figure 1. Chemical structures of ER-319711 (7-but-2-ynyl-9-(6-methoxy-pyridin-3-yl)-6-piperazin-1-yl-7,9-dihydro-purin-8-one) (A), E3024 (3-but-2-ynyl-5-methyl-2-piperazin-1-yl-3,5-dihydro-4H-imidazo[4,5-d]pyridazine-4-one tosylate) (B), and 4-nitrobenzyl[(5S)-5-amino-6-oxo-6-(1-pyrrolidinyl)hexyl]carbamate (Lys[Z(NO2)] pyrrolidide) (C).

Figure 2. Inhibition kinetics of dipeptidyl peptidase IV by ER-319711-15 (the trifluoroacetate salt form of ER-319711). Different concentrations of ER-319711-15 (0, 0.03, 0.1, 0.3, 1 and 3 µmol/L) were incubated in the presence of various concentrations of glycyl-L-proline p-nitroanilide tosylate (0.006, 0.024, 0.10, 0.39, 1.56, 6.25, 25 and 100 mmol/L). Initial rates of the reaction were measured, and the results were expressed as the Eadie-Hofstee plot. Data represent the mean ± S.E.M. of three separate experiments performed in triplicate.

Figure 3. Effects of ER-319711 on blood glucose, plasma insulin and active glucagon-like peptide-1 (GLP-1) levels in an oral glucose tolerance test in Zucker fa/fa rats. ER-319711-15 (the trifluoroacetate salt form of ER-319711; 1 mg/kg) or vehicle was orally administered 0.5 hr prior to glucose load (2 g/kg). Changes of blood glucose (A) and area under the curve (AUC) values of delta blood glucose between 0 and 3 hr (B). Changes of plasma insulin levels (C) and delta plasma insulin levels between 0 and 0.5 hr (D). Changes of plasma active GLP-1 levels (E) and delta plasma active GLP-1 levels between
Figure 4. Changes of blood glucose levels (A) and area under the curve (AUC) values of delta blood glucose between 0 and 2 hr, and between 5 and 7 hr (B) in an oral glucose tolerance test in mice fed a high-fat diet. ER-319711-15 (the trifluoroacetate salt form of ER-319711; 0.1, 0.3, 1, 3 and 10mg/kg), vildagliptin (10 mg/kg) or vehicle was orally administered at 0 hr with glucose (2 g/kg). At 5 hr, glucose (2 g/kg) was orally loaded again. Values are expressed as the mean ± S.E.M. *, $p<0.05$ vs. vehicle group by the Dunnett multiple comparison test. #, $p<0.05$ vs. vehicle group by Student’s $t$-test.

Figure 5. Changes of blood glucose levels (A) and area under the curve (AUC) values of delta blood glucose between 0 and 2 hr, and between 5 and 7 hr (B) in an oral glucose tolerance test in mice fed a high-fat diet. E3024 (10 mg/kg), ER-319711-15 (the trifluoroacetate salt form of ER-319711; 10mg/kg), vildagliptin (10 mg/kg) or vehicle was orally administered at 0 hr with glucose (2 g/kg). At 5 hr, glucose (2 g/kg) was orally loaded again. Values are expressed as the mean ± S.E.M. *, $p<0.05$ vs. vehicle group by the Turkey multiple comparison test.
Table 1. Inhibitory concentrations (IC$_{50}$) and 95% confidence intervals (95% C.I.) of ER-319711-15 and 4-nitrobenzyl[(5S)-5-amino-6-oxo-6-(1-pyrrolidinyl)hexyl]carbamate (Lys[Z(NO$_2$)] pyrrolidide) towards dipeptidyl peptidase (DPP)-IV, DPP8 and DPP9.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>IC$_{50}$ ($\mu$mol/L)</th>
<th></th>
<th>DPP8/DPP-IV</th>
<th>DPP9/DPP-IV</th>
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<tr>
<td></td>
<td></td>
<td>DPP-IV</td>
<td>DPP8</td>
<td>DPP9</td>
</tr>
<tr>
<td>ER-319711-15</td>
<td>0.087 (0.068 - 0.112)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>-</td>
</tr>
<tr>
<td>Lys[Z(NO$_2$)] pyrrolidide</td>
<td>0.214 (0.197 - 0.232)</td>
<td>0.0130 (0.0088 - 0.0178)</td>
<td>0.064 (0.044 - 0.092)</td>
<td>0.061</td>
</tr>
</tbody>
</table>

Data are means of IC$_{50}$ values determined from the results of three independent experiments. Values in parentheses are 95% C.I.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5