Inhibition of the Abl Protein-Tyrosine Kinase in Vitro and in Vivo by a 2-Phenylaminopyrimidine Derivative

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ABSTRACT

Oncogenic activation of Abl proteins due to structural modifications can occur as a result of viral transduction or chromosomal translocation. The tyrosine protein kinase activity of oncogenic Abl proteins is known to be essential for their transforming activity. Therefore, we have attempted to identify selective inhibitors of the Abl tyrosine protein kinase. Herein we describe an inhibitor (CGP 57148) of the Abl and platelet-derived growth factor (PDGF) receptor-tyrosine kinases from the 2-phenylaminopyrimidine class, which is highly active in vitro and in vivo. Submicromolar concentrations of the compound inhibited both v-Abl and PDGF receptor autophosphorylation and PDGF-induced c-fos mRNA expression selectively in intact cells. In contrast, ligand-induced growth factor receptor autophosphorylation in response to epidermal growth factor (EGF), insulin-like growth factor-I, and insulin showed no or weak inhibition by high concentrations of CGP 57148. c-fos mRNA expression induced by EGF, fibroblast growth factor, or phorbol ester was also insensitive to inhibition by CGP 57148. In antiproliferative assays, the compound was more than 30–100-fold more potent in inhibiting growth of v-abl-transformed PB-3c cells and v-sis-transformed BALB/c 3T3 cells relative to inhibition of EGF-dependent BALB/MK cells, interleukin-3-dependent FDC-P1 cells, and the T24 bladder carcinoma line. Furthermore, anchorage-independent growth of v-abl- and v-sis-transformed BALB/c 3T3 cells was inhibited potently by CGP 57148. When tested in vivo, CGP 57148 showed antitumor activity at tolerated doses against tumorigenic v-abl- and v-sis-transformed BALB/c 3T3 cells. In contrast, CGP 57148 had no antitumor activity when tested using src-transformed BALB/c 3T3 cells. These findings suggest that CGP 57148 may have therapeutic potential for the treatment of diseases that involve abnormal cellular proliferation induced by Abl protein-tyrosine kinase deregulation or PDGF receptor activation.

INTRODUCTION

The abl oncogene was isolated originally from the genome of the A-MuLV2 (1). This acutely transforming, replication-defective virus encodes a transforming protein (p160-abl) with tyrosine-specific protein kinase activity (2, 3). A-MuLV, which is able to transform fibroblasts in vitro and lymphoid cells in vitro and in vivo (4, 5), seems to have been formed by recombination between the Moloney murine leukemia virus and the murine cellular c-abl gene (6). The N-terminal modification found in v-abl, acquired during transduction by A-MuLV, results in deregulation of its protein-tyrosine kinase activity. There is considerable evidence that activated abl genes play an important role in the pathogenesis of specific human leukemias. CML is a hematological stem cell disorder characterized by excessive proliferation of the myeloid lineage (7). The hallmark of CML is the Philadelphia chromosome (8), which is detected in virtually all cases of CML and 20% of adult acute lymphoblastic leukemia. It is formed by a reciprocal translocation between chromosomes 9 and 22 ([t(9;22) (q34; q11)] (9–14). The molecular consequence of this translocation is the replacement of the first exon of c-abl with sequences from the Bcr gene, resulting in a Bcr-Abl fusion protein with enhanced tyrosine kinase activity (15–17). Recent results have demonstrated that Bcr-Abl expression also can induce a disease resembling CML in mice (18–21) and provides strong evidence that the Bcr-Abl protein is a major factor in the pathophysiology of CML.

Due to its relatively clear etiology, CML represents a disease in which therapy using a selective inhibitor of the Abl protein-tyrosine kinase would seem attractive. Inhibition of the Abl tyrosine kinase activity has been reported for the benzopyranones and benzothiopyranones (22) and the tyrphostin classes of compounds (23). However, the compounds showed either limited selectivity or potency at the cellular level. Recently, a series of protein-tyrosine kinase inhibitors of the 2-phenylaminopyrimidine class have been synthesized. CGP 53716 has been characterized as a potent inhibitor of the PDGF receptor tyrosine kinase (24). Although this compound inhibited the v-Abl protein-tyrosine kinase in vitro, it showed preferential inhibition of the PDGF signal transduction pathway at the cellular level (24). Optimization of this compound class for inhibition of the v-Abl kinase has led to the identification of CGP 57148. This derivative shows potent inhibition of the v-Abl kinase in vitro and in cells and inhibits tumor growth of v-abl-transformed BALB/c 3T3 cells. In addition, its activity against the PDGF receptor tyrosine kinase indicates that the compound could have therapeutic potential in the treatment of cancers involving deregulated Abl or PDGF receptor tyrosine kinase activity.

MATERIALS AND METHODS

Materials. CGP 57148 and its methane sulfonate salt (CGP 57148B) were synthesized by CIBA Pharmaceuticals Division, as will be described elsewhere.3 For in vitro and cellular assays, a stock concentration of 10 mM CGP 57148 was prepared in Me2SO and stored at −20°C. No significant difference in results could be seen between the two forms of CGP 57148. The form used in vivo experiments is indicated in the text and legends. All in vivo experiments were performed using CGP 57148B. Monoclonal antiphosphotyrosine antibody PY20 coupled to horseradish peroxidase was from ICN Radiochemicals (Irvine, CA). Monoclonal antibody FRP 5, which acts as a ligand agonist for p185ε-v-abl (25), was kindly provided by N. Hynes (Friedrich-Miescher Institute, Basel, Switzerland). Mouse monoclonal antibodies directed against the c-abl gene product were from Oncogene Science (Manhasset, NY).

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2 The abbreviations used are: A-MuLV, Abelson murine leukemia virus; CML, chronic myelogenous leukemia; PDGF, platelet-derived growth factor; IL, interleukin; EGF, epidermal growth factor; IGf, insulin-like growth factor; bFGF, basic fibroblast growth factor; TAC, treatment versus control; FCS, fetal calf serum.

3 J. Zimmermann, manuscript in preparation.
DMEM supplemented with 10% (v/v) FCS and RPMI 1640 medium containing 20% (v/v) FCS, respectively. Transformed NIH-527-src cells, which contain an activated c-src gene carrying a tyrosine-to-phenylalanine mutation at amino acid 527 of the coding sequence,4 were grown in DMEM supplemented with 10% (v/v) bovine calf serum. Other cell lines and reagents are as described (24).

Kinase Assays. Purification of protein kinases and in vitro enzyme assays were performed as described (24).

Western Blotting. PB-3c c1.15 and PB-3c c1.15 v-abl cells were incubated for 90 min with the indicated concentrations of drugs. Serum-starved MDA-MB 453 cells [incubation in culture medium containing 0.1% (v/v) FCS for 48 h] were incubated for 2 h with the compound prior to stimulation with monoclonal antibody FRP 5 (5 μg/ml) for 10 min. Other cell lines were treated as described (24). Equal amounts of protein of cell lysates were assayed by Western blotting using 4G10 antiphosphotyrosine antibodies (28). MDA-MB 53 cell lysates were analyzed by using the PY20 horseradish peroxidase conjugate. Bound antibodies were detected using Enhanced Chemiluminescence (Amersham).

Northern Blot Analysis. Isolation of total RNA and analysis by Northern blotting using a fos-specific probe were carried out as described (29). Loading and integrity of RNA were verified by ethidium bromide staining of the gel (data not shown).

Antiproliferative Assays. Cell growth inhibition was assayed using methylene blue staining or the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (30).

Soft Agar Growth. Cells were plated in duplicate in 6-cm dishes in 6 ml culture medium supplemented with 0.3% noble agar and the indicated concentrations of CGP 57148, overlaying a 0.8% agar layer. Plates were incubated for 8–14 days at 37°C, after which colonies were stained by adding 2 ml PBS containing 0.5 mg/ml nitro blue tetrazolium for 24 h. Colonies were counted using an Artek 880 colony counter (Dynatech Laboratories, Inc.).

In Vivo Experiments. In vivo antitumor activity was tested using AMuLV-transformed BALB/c 3T3 cells, v-sis-transfected BALB/c 3T3 cells, or NIH-527-src cells. One × 10⁶ cells were injected into the left flanks of six female BALB/c nude mice (Bolinholdsgard, Copenhagen, Denmark) per experimental set. Treatment was started 24 h after cell injection, and the compound was administered once daily for up to 30 consecutive days. Solutions of CGP 57148B were prepared in PBS daily prior to i.p. administration. In all experiments, tumor growth was followed by measuring perpendicular tumor diameters. Tumor volumes were calculated as described previously (31) using the formula \( V = \frac{1}{2} \times L \times D^2 \times \pi \), where \( L \) is the longest diameter, and \( D \) is the diameter at right angles to it.

RESULTS

In Vitro Selectivity of CGP 57148 for Inhibition of Protein Kinases: Identification of CGP 57148 as an Inhibitor of the v-Abl Kinase. CGP 57148, the structure of which is shown in Fig. 1, was assayed in vitro for inhibition of a panel of tyrosine and serine/threonine protein kinases and was found to inhibit the v-Abl tyrosine kinase potently, with an IC₅₀ value of 0.038 μM.5 CGP 57148 did not inhibit protein kinase C isotypes, or other serine/threonine kinases such as cAMP-dependent protein kinase, phosphorylase kinase, cdc2/cyclin B protein kinase, and protein kinases CK 1 and 2 (IC₅₀ >100 μM). When tested against different protein-tyrosine kinases, CGP 57148 did not inhibit the EGF receptor intracellular domain (IC₅₀ >100 μM) and showed no or weak inhibition of protein kinases of the src family (c-Src, c-Lyn, c-Fgr, and c-Lck).

Selectivity of CGP 57148 in Intact Cells. The effect of CGP 57148 on v-Abl kinase in intact cells was studied using AMuLV-transformed PB-3c mast cells. CGP 57148 inhibited v-Abl tyrosine autophosphorylation with an IC₅₀ value between 0.1 and 0.3 μM.

\[ \text{Fig. 1. Structure of CGP 57148.} \]

\[ \text{Fig. 2. Inhibition of v-Abl and PDGF receptor autophosphorylation by CGP 57148 in intact cells. PB-3c c1.15 cells (A and B, Lane 1) and AMuLV-transformed PB-3c c1.15 cells (A and B, Lanes 2–8) were incubated with control medium (A and B, Lanes 1 and 2) or the indicated concentrations of CGP 57148 (free base; A and B, Lanes 3–8) for 90 min. Confluent, quiescent BALB/c 3T3 cells (C) were incubated with control medium or CGP 57148 (free base) for 90 min prior to stimulation with PDGF BB (50 ng/ml) for 10 min. Whole-cell lysates were corrected for protein content and analyzed by Western blotting using antiphosphotyrosine antibodies (A and C) or anti-AbI antibodies (B). R, receptor.} \]
The compound was without effect on Abl protein levels, as determined by Western blotting using anti-Abl antibodies (Fig. 2B). Due to its structural similarity to CGP 53716 (24), CGP 57148 was tested further for inhibition of the PDGF receptor protein-tyrosine kinase activity. Treatment of cells with CGP 57148 caused a concentration-dependent inhibition of ligand-stimulated PDGF receptor tyrosine phosphorylation with an IC50 value of approximately 0.3 μM (Fig. 2C). Immunoblotting experiments with anti-PDGFR receptor antibodies showed that CGP 57148 did not affect overall levels of the PDGF receptor (data not shown). To define the specificity of CGP 57148, the effect of the compound on ligand-stimulated EGF receptor autophosphorylation in A431 cells was tested. The addition of EGF to serum-starved A431 cells stimulated EGF receptor autophosphorylation (Fig. 3A, Lane 2). Pretreatment of the cells with up to 100 μM CGP 57148 had no significant effect on ligand-induced EGF receptor autophosphorylation (Fig. 3A, Lanes 3 and 4). Similarly, p185erbB2 autophosphorylation in MDA-MB 453 cells was affected only weakly at 10 μM (Fig. 3B). No inhibition of ligand-induced autophosphorylation of either the β chain of the insulin receptor in Rat-1 cells (Fig. 3C) or the β chain of the IGFR-I receptor in NIH 3T3 (LiSNc4) cells (Fig. 3D) was observed with concentrations of CGP 57148 up to 100 μM. The potency and selectivity of CGP 57148 were also apparent in its effects on c-fos mRNA expression induced by different growth stimuli. The compound inhibited the induction of c-fos mRNA by PDGF strongly, with an IC50 value between 0.3 and 1 μM (Fig. 4, Lanes 3–7). The induction of c-fos mRNA expression by EGF, bFGF, and phorbol 12-myristate 13-acetate was not affected by the drug even when used at concentrations up to 100 μM (Fig. 4, Lanes 8–18).

**Antiproliferative Activity of CGP 57148.** CGP 57148 was tested for growth inhibition of EOF-dependent BALB/c MK cells and the H-ras-transformed T24 bladder carcinoma line using methylene blue staining. Inhibition of IL-3-dependent growth of FDC-Pl cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction as described previously (30). The compound showed only weak antiproliferative activity against these cell lines, with IC50 values of 12.7 μM, 8.4 μM, and 29.2 μM, respectively (Table 1).
Table 2 Inhibition of soft agar growth by CGP 57148

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control No. colonies</th>
<th>% inhibition</th>
<th>1 μM No. colonies</th>
<th>% inhibition</th>
<th>10 μM No. colonies</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c AMuLV</td>
<td>335 ± 22</td>
<td>0</td>
<td>97 ± 3</td>
<td>71</td>
<td>20 ± 0.5</td>
<td>94</td>
</tr>
<tr>
<td>BALB/c 3T3 v-sis</td>
<td>963 ± 25</td>
<td>0</td>
<td>152 ± 16</td>
<td>84</td>
<td>25 ± 0.5</td>
<td>97</td>
</tr>
</tbody>
</table>

Fig. 5. In vivo antitumor activity of CGP 57148B. In vivo antitumor activity was tested using AMuLV-transformed (A) and v-sis-transformed (B) BALB/c 3T3 cells. Treatment was started 24 h after cell injection. CGP 57148B was administered i.p. once daily for 30 days (A) or 27 days (B). ○, placebo control; □, 50 mg/kg; △, 12.5 mg/kg; Δ, 3.13 mg/kg.

DISCUSSION

Bcr-Abl expression is thought to play an initiating role in the pathogenesis of CML. To test the hypothesis that selective inhibition of the Abl tyrosine kinase activity might be useful for chemotherapy of Philadelphia chromosome-positive leukemias, we have searched for selective protein-tyrosine kinase inhibitors from the 2-phenylaminopyrimidine class. We describe the biological profile of COP 57148, which inhibits the Abl tyrosine kinase potently and which, additionally, shows potent inhibition of the PDGF receptor tyrosine kinase.

Inhibitors of the 2-phenylaminopyrimidine class have been shown to act as competitive inhibitors of protein kinases with respect to ATP. Therefore, they are likely to bind in the ATP-binding cleft of the Abl and PDGF receptor enzymes. The selectivity of such inhibitors toward different classes of protein-tyrosine kinases, therefore, could be explained by interactions between the inhibitor and the amino acid residues lining the ATP-binding cleft. Recently, we have provided a rational basis for selectivity of ATP competitive inhibitors for the EGFR receptor kinase using a model of the kinase domain (32). Inspection of the residues that interact with ATP, as predicted from the X-ray crystal structure of the cyclic AMP-dependent protein kinase, reveals a large degree of similarity between the Abl and PDGF receptor kinases. However, a final understanding of the molecular basis of selectivity awaits the resolution of a crystal structure of an enzyme-drug complex.

CGP 57148 selectively inhibited the in vitro activity of the v-Abl protein-tyrosine kinase and showed preferential inhibition of v-Abl autophosphorylation in cells. We have examined the specificity of

6 T. Meyer and J. Zimmermann, unpublished results.
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CGP 57148 by analyzing its effects on signal transduction via different tyrosine kinase receptor-mediated pathways. Although the ligand-induced activation of the EGFR, BFGF, insulin, and IGF-1 receptor tyrosine kinases were not affected by CGP 57148, the PDGF pathway was sensitive to inhibition by the compound. The antiproliferative activity of CGP 57148 against both v-abl- and v-sis-transformed BALB/c 3T3 cells was compatible with the lack of inhibition of v-Ab1 and PDGF receptor autophosphorylation. In vivo antitumor efficacy against AM-Lv-transformed BALB/c 3T3 cells, which grow in response to autocrine PDGF production, CGP 57148 was well tolerated and showed similar antitumor activity. Our finding that CGP 57148 did not inhibit tumor growth of src-transformed NIH 3T3 cells is compatible with the lack of inhibition of Src kinase activity by CGP 57148 and demonstrates further that the compound is relatively selective for inhibition of Ab1- and PDGF activity. COP S7i48@ and demonstrates further that the compound is relatively selective for inhibition of Abl- and PDGF-driven tumor growth in vivo.

Cytogenetic studies established CML as the first human neoplasm to be associated consistently with a specific chromosomal abnormality, known as the Philadelphia chromosome. The Philadelphia translocation juxtaposes c-abl proto-oncogene sequences on chromosome 9 with the Bcr gene on chromosome 22 (33). This results in a Bcr-Abl protein, which has elevated tyrosine kinase activity relative to c-Ab1, has been shown to induce various hematopoietic malignancies in transgenic mice and mice reconstituted with Bcr-abl retrovirus-infected bone marrow (18, 19, 21, 34).

PDGF has been suggested to be a mediator of pathologic cell growth, e.g., carcinogenesis, as well as nonmalignant proliferative disorders, such as atherosclerosis and fibrosis (for review, see Ref. 35).

The reported findings with CGP 57148 suggest that it may be a development candidate for use in the treatment of Philadelphia chromosome-positive leukemias. Additional potential applications for CGP 57148 may include proliferative disorders that involve abnormal PDGF receptor activation.

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REFERENCES


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